Inactivation and Mechanism of Electron Beam Irradiation and Sodium Hypochlorite Sanitizers against a Human Norovirus Surrogate

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the degree of Doctor of Philosophy in the Graduate School of The Ohio State University

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2012

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ABSTRACT

Human norovirus remains the most prevalent foodborne pathogen, resulting in 58% of all foodborne illnesses in the United States, annually. Due to lack of successful cultivation techniques for this virus, research on intervention strategies and disinfection practices to combat this pathogen is still largely underreported. The research performed in this dissertation determined the efficacy of electron beam (e-beam) irradiation and sodium hypochlorite sanitizers at inactivating a human norovirus surrogate (murine norovirus 1, MNV-1) and compared the rates of inactivation against that of an enveloped virus (vesicular stomatitis virus, VSV). This research also attempted to determine the mechanism of viral inactivation for e-beam and sodium hypochlorite.

In Chapter 2, we evaluated the efficacy of e-beam at inactivating MNV-1 inoculated to liquid model systems (phosphate buffered saline, PBS; Dulbecco's Modified Eagle Medium, DMEM) and fresh produce (shredded cabbage, cut strawberries). MNV-1 proved to be resistant to irradiation in both liquid and food samples. In PBS and DMEM, a dose of 2 kGy provided a less than 1 log reduction of MNV-1. At doses of 4, 6, 8, 10, and 12 kGy, viral reduction in PBS ranged from 2.37 to 6.40 logs, and 1.40 to 3.59 logs in DMEM. At 4 kGy (the maximum irradiation dose approved by the FDA for fresh produce), MNV-1 inoculated to shredded cabbage only experienced a 1 log reduction, and less than 1 log reduction in cut strawberries. Even at 12 kGy, MNV-1 titers were reduced by 3 and 2 logs in cabbage and strawberries,

respectively. These results suggest that complex liquid media and the food matrix may protect MNV-1 from irradiation, and that viruses tend to be more resistant to irradiation than bacteria due to their small size and highly stable viral capsid. E-beam does not appear to be a feasible processing technology to inactivate foodborne viruses in food products.

Chapter 3 compares e-beam's ability to inactivate the nonenveloped MNV-1 versus the enveloped VSV inoculated into PBS and DMEM. Samples were treated with e-beam doses of 0, 4, 8, 16, 24, and 30 kGy. We also attempted to determine e-beam's mechanism of viral inactivation using transmission electron microscopy (TEM), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting, and reverse transcription polymerase chain reaction (RT-PCR). The mechanism of viral inactivation has been demonstrated with gamma irradiation, but no published study to date has evaluated if e-beam would have a similar mechanism. MNV-1 required 24 kGy in PBS and 30 kGy in DMEM for complete inactivation, while VSV was completely inactivated using 16 kGy for both media. TEM analysis demonstrated that increasing doses of e-beam disrupted the structure of the virions. SDS-PAGE and Western blotting analysis found that irradiation can also degrade viral proteins, though these proteins can remain antigenic in the presence of specific antibodies. Finally, using RT-PCR, irradiation was found to also degrade viral genomic RNA. As expected, the mechanism of inactivation of e-beam is similar to that of gamma irradiation.

Chapter 4 compared the rates of inactivation of MNV-1 and VSV subjected to varying concentrations of sodium hypochlorite (5, 10, 20, 50, 100, 200, 400, 800, and 1,000 ppm) and exposure times (0, 0.5, 1.0, 5.0, 10, and 30 minutes). We also attempted

to determine hypochlorite's mechanism of viral inactivation. As expected, MNV-1 was much more resistant to chlorine, being able to withstand 400 ppm of chlorine for up to 10 minutes (0.46 log PFU/ml of virus remaining). VSV was much more susceptible to chlorine, requiring treatment of at least 10 ppm for 10 minutes for complete viral inactivation. Purified MNV-1 treated with 200 ppm hypochlorite for 1.0, 5.0, and 10 minutes exhibited slight damage to the viral capsid protein, but no physical damage to the virus particle or degradation of the viral RNA was observed. Purified VSV treated at 10 ppm for 1.0, 5.0, and 10 minutes did not exhibit any significant changes in virion structure, viral proteins, or viral RNA. Sodium hypochlorite sanitizer concentrations used in the food industry may not be sufficient to reduce contaminating norovirus to safe levels (since less than 10 particles is sufficient to cause illness). Also, the sanitizer's mechanism of viral inactivation remains inconclusive.

DEDICATION

Dedicated to the

Sanglay family,

Derek O'Konek,

and our faithful four-legged companion, Gizmo.

Thank you for all your love and support!

ACKNOWLEDGEMENTS

First, I would like to thank Ken Lee for serving as my advisor. You have been an inspiration to me during my time as your doctoral student. I have learned so much from you and I look forward to working with you in the future. I would also like to thank Jianrong Li, for all his expertise and advice in virology, as well as giving me the opportunity to work in his laboratory. I would also like to thank V.M. Balasubramaniam and Jiyoung Lee, for their service on my committee and for all that I have learned in their classes. Finally, I would like to thank Ken, Jianrong, and Melvin Pascall for all their guidance and work with my postdoctoral fellowship grant. Without all of your combined expertise, I would not be where I am today.

For research support, there are many that I would like to thank. I would like to thank Dr. Roberto Uribe and Haliru Omosun (Kent State University) for all their time and assistance with my e-beam research. Also, many thanks to Di Cao (The Ohio State University) for assistance with statistical analyses. Thank you to Edward Calomeni (The Ohio State University) for your assistance with electron microscopy. Another thank you to Ahmad Tahajod (Sandridge Foods) for providing me with cabbage samples for the e-beam research. I would also like to thank all the members of Jianrong Li's laboratory (most notably, Yu Zhang, Yuanmei Ma, Fangfei Lou, Erin DiCaprio, Yongwei Wei, Yue Duan, and Ashley Predmore) for all of their assistance with virus and cell culture, as well

as their friendship. A special thank you goes to Erin DiCaprio and Ashley Predmore for helping me out in the lab with the last bit of my mechanistic studies.

I would also like to thank my past and present lab mates and friends here in the Food Science and Technology Department. To all those who have graduated (Andrew Wassinger, Setsuko Kamotani, Elizabeth Grasso, Alex Suter, Ryan Riddle, Lizanel Feliciano), thank you so much for being such good friends and colleagues. I hope your careers are going well, and I miss you dearly. To all those still here (Fangfei Lou, Yu Zhang, Yuanmei Ma, Allison Atnip, Gregory Sigurdson, Kyra Jones, Erin DiCaprio, Ashley Predmore), I am glad that you will still be around while I am here for my post-doc!

Finally, I would like to thank all of my friends and family outside of The Ohio State University. To my family, thank you for all your love and support during all my years in school. To all my friends, thank you so much for being who you are and you all bring something special to my life. And last, but not least, thank you to my partner Derek O'Konek and our dog Gizmo for all your love and support during my good days and bad. To everyone, all the support you have given me these past 5 years at The Ohio State University helped me to achieve my goals, and will continue to inspire me for years to come.

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Sanglay, G.C., Li, J., Uribe, R.M., and Lee, K. 2011. Electron beam inactivation of a norovirus surrogate in fresh produce and model systems. J. Food Prot. 74(7):1155-1160.

Field of Study

Major Field: Food Science & Technology

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ABBREVIATIONS

AI – Avian influenza
BHK – Baby hamster kidney cell line
CDC – Centers for Disease Control and Prevention
cDNA – Complementary DNA
CPE – Cytopathic effects
DMEM – Dulbecco's Modified Eagle Medium
DNA – Deoxyribonucleic acid
FAO – Food and Agricultural Organization of the United Nations
FBS – Fetal bovine serum
FCV – Feline calicivirus
FDA – Food and Drug Administration
FMD – Foot and mouth disease
FUT2 – Galactoside 2-alpha-L-fucosyltransferase 2 gene
HAV – Hepatitis A Virus
HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HEV – Hepatitis E Virus
HGBA – Histo-blood group antigen
IAEA – International Atomic Energy Agency
IFIC – International Food Information Council
JEFCI – Joint FAO/IAEA/WHO Expert Committee on the Wholesomeness of Irradiated Food
Kb – Kilobase
kGy – Kilogray
kW – Kilowatt
mA - Milliamp
MEM – Earle's Balanced Salts medium
MeV – Mega electronvolt
MMWR – Morbidity and Mortality Weekly Report
MNV-1 – Murine norovirus 1
MOI – Multiplicity of infection
ORF – Open reading frame
PBS – Phosphate buffered saline
PFU – Plaque forming units
Ppm – parts per million
RAW 264.7 – A mouse macrophage cell line
RNA – Ribonucleic acid
Rpm – Rotations per minute
RT-PCR – Reverse transcriptase polymerase chain reaction

SARS – Severe acute respiratory syndrome
SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SRSV – Small round structured virus
TBE – Tick-borne encephalitis
TEM – Transmission electron microscopy
Vero – An African Green Monkey kidney cell line
VSV – Vesicular stomatitis virus
WHO – World Health Organization
WNV – West Nile virus

CHAPTER 1

LITERATURE REVIEW

1.1. Foodborne Viruses

The major causative agents of foodborne illness include bacteria, viruses, fungi, parasites, prions, toxins, and metals (Mead et al., 1999). In the United States, it is estimated that between 6 and 81 million illnesses occur annually due to foodborne disease, with up to 325,000 hospitalizations and 9,000 deaths each year (Mead et al., 1999; Koopmans et al., 2002). Within these figures, approximately 30 million of these cases are caused by viral pathogens, with over 9 million (67.2%) of these cases being related to foods (Mead et al., 1999). As of 2011, estimates suggest that 5.5 million (59%) of all foodborne illnesses in the United States were caused by viruses, with norovirus being the most prevalent cause (Scallan et al., 2011).

Viruses are defined as "infectious, obligate, intracellular parasites" (Flint et al., 2004a). They are small particles, ranging in size from 15 to 400 nm, and cause many diseases in plants, animals, humans, as well as being capable of infecting bacteria. These particles consist of a protein capsid that encloses the viral genome (either DNA or RNA), and may or may not possess a lipid envelope. Viruses need a specific host organism in order to replicate, and cannot multiply outside of their host (Vasickova et al., 2005). Transmission of viruses can be accomplished in several different ways: aerosol or

airborne transmission, direct contact with an infected individual, fecal contamination (food, water, soil), exposure to virus-infected blood, contact with contaminated fomites, exposure to infected animals, sexual intercourse, or animal vectors (Vasickova et al., 2005; Koopmans and Duizer, 2004).

In foods, viruses cannot replicate but will remain present in the food matrix. Koopmans and Duizer (2004) describe the general features of foodborne viruses: 1) “only a few particles are required to produce illness,” 2) “high numbers of viral particles are shed in the stools of infected persons,” 3) “viruses need specific living cells in order to replicate and therefore cannot do so in food or water,” and 4) “foodborne viruses are typically quite stable outside the host and are acid-resistant.” Viral contamination of foods can occur via contact with human feces, water contaminated with feces or vomit, contact with fecally soiled materials, contact with vomit, aerosols, infected food handlers, or contact with environments where infected people were present (Koopmans and Duizer, 2004).

Viral pathogens associated with foods tend to be enteric viruses, which infect the human gastrointestinal tract, are excreted in feces or vomit, and can be transmitted person-to-person via the fecal-oral route (Greening, 2006). Enteric viruses can fall into three categories: 1) gastroenteritis, 2) enterically transmitted hepatitis, and 3) viruses that infect the human intestines but are capable of migrating to other organs (Greening, 2006; Koopmans and Duizer, 2004). Of the enteric viruses, noroviruses, hepatitis A virus (HAV), rotaviruses, and astroviruses are associated with approximately 80% of all foodborne illnesses in the United States (Greening, 2006; Mead et al., 1999).

1.1.1. Norovirus

Previously known as Norwalk-like viruses (NLVs) and small round structured viruses (SRSVs), noroviruses are members of Caliciviridae family (Greening, 2006; Jay et al., 2005b). The first norovirus, known as Norwalk virus, was associated with an outbreak in 1968 that occurred at a school in Norwalk, Ohio. Since no bacterial agent could be identified, the illness was termed "acute nonbacterial gastroenteritis" and "winter vomiting disease" until the virus was discovered in 1972 by Albert Kapikian using immune electron microscopy (Greening, 2006; Vasickova et al., 2005; Bresee et al., 2002). The suspected reservoir for the virus was water, but was never proven (Jay et al., 2005b). Since its discovery, noroviruses have been attributed to 66.6% (9.2 million cases) of total foodborne illness cases, resulting in 32.9% (20,000) of hospitalizations and 6.9% (approximately 124) deaths each year in the United States (Mead et al., 1999). As of 2011, it is estimated that noroviruses account for 5.4 million cases of illness, 14,663 hospitalizations, and 149 deaths in the United States, annually (Scallan et al., 2011). Death occurs in susceptible populations (elderly, immunocompromised, or the very young) due to dehydration (CDC, 2009a). Worldwide, estimates of norovirus illness are not currently available (CIDRAP, 2006). However, noroviruses are estimated to cause at least 900,000 cases of gastroenteritis in children in industrialized nations, and 1.1 million cases and 218,000 deaths in developing countries (Patel et al., 2008; Koo et al., 2010).

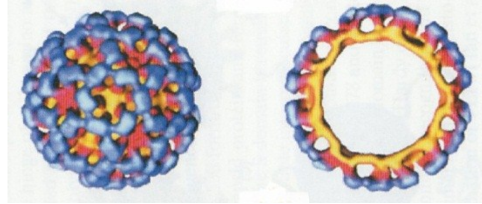


Figure 1. Three-dimensional structure of the human norovirus (Buesa and Rodriguez-Diaz, 2006).

Noroviruses are nonenveloped viruses, possessing an icosahedral capsid that contains a single strand of positive-sense RNA as its genome (Figure 1). Noroviruses were previously classified as SRSVs because of their small size (28-35 nm) and round shape under electron microscopy (Bresee et al., 2002). However, unlike other members of the Caliciviridae family, noroviruses do not possess the characteristic "cup-shaped" calyces on their capsid, instead appearing as "fuzzy" or "ragged" by direct electron microscopy (Greening, 2006; Bresee et al., 2002). Figure 2 illustrates the RNA genome, which is approximately 7.5-7.7 kb in length, and possesses three open reading frames (ORFs) (Wobus et al., 2006; Widdowson and Vinjé, 2008). ORF1 encodes for a large non-structural polyprotein, which encodes smaller nonstructural proteins such as p48, NTPase, p22, VPg (believed to initiate translation), $3CL^{Pro}$ (proteinase that inhibits host cell translation), and RDRP (RNA dependent RNA polymerase) (Widdowson and Vinjé, 2008; Buesa and Rodríguez-Díaz, 2006). ORF2 encodes the major capsid protein, VP1, while ORF3 encodes the minor capsid protein, VP2 (Buesa and Rodríguez-Díaz, 2006).

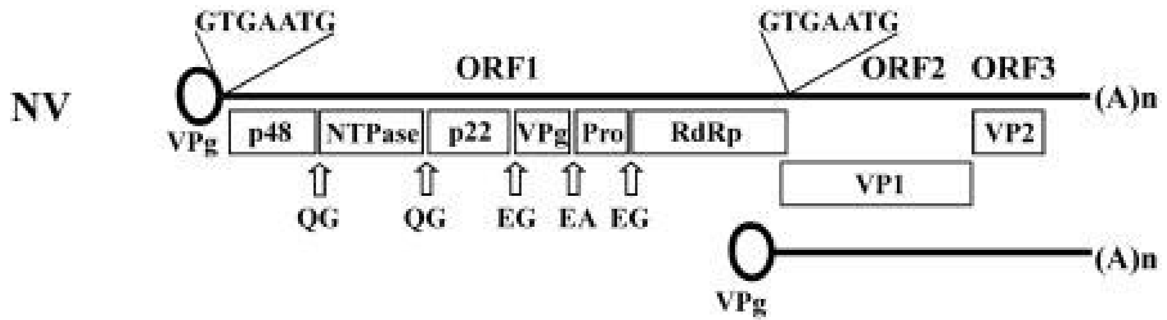


Figure 2. Human norovirus genome (Wobus et al., 2006).

Norovirus particles are highly infectious, and it is estimated that as low as 10-100 virus particles are sufficient to cause infection (Greening, 2006; Vasickova et al., 2005). Once inside the body, the incubation period for norovirus can be 1-3 days, but as rapidly as 12-24 hours for symptoms develop (Koopmans et al., 2002; Bresee et al., 2002). Common symptoms of norovirus gastroenteritis include can include projectile vomiting, non-bloody diarrhea, low-grade fever, and nausea (Koopmans et al., 2002; Greening, 2006; Vasickova et al., 2005). The illness is acute, self-limiting and generally lasts for 12-60 hours but fecal shedding of virus and viral antigen can last for days or even weeks after the illness subsides (Bresee et al., 2002; Greening, 2006). Peak viral shedding occurs within 2-5 days post-infection, with up to 10^{11} particles per gram of feces (Hall et al., 2011). The young and elderly populations are of particular concern because dehydration is a common complication, which can be combated with rehydration therapy (Greening, 2006).

The mechanism by which the norovirus infects humans is currently not well understood. It is known that noroviruses infect the mature enterocytes in the small intestines (Greening, 2006; Buesa and Rodríguez-Díaz, 2006). Studies have demonstrated that biopsies taken from infected volunteers showed inflammation, lesions

in the intestinal epithelium, abnormally shaped villi, and vacuolization of cells (Koopmans et al., 2002; Greening, 2006; Buesa and Rodríguez-Díaz, 2006). Once these cells are infected, they are unable to absorb fats and sugars, which are believed to cause delayed gastric emptying, nausea, and vomiting (Greening, 2006). Also, not much is understood about immunity against noroviruses. It is believed that the viral capsid is highly antigenic, and immunity is short-lived (Koopmans et al., 2002). Parrino et al. (1977) report that volunteers developed short-term immunity, but were susceptible to the same virus strain up to 3 years later (as cited by Bresee et al., 2002). Bresee et al. (2002) state that noroviruses are highly variable in their genomes, immunity is based on strain specificity, and that individuals are capable of being reinfected with the same strain.

Noroviruses have been demonstrated to have a binding preference to histo-blood group antigens (HGBAs). Tian et al (2006) demonstrated that norovirus will bind to group A-like antigens in the gastrointestinal tract of oysters. Bu et al (2008) state that HGBAs consist of carbohydrates that is frequently found on the cells of the digestive and respiratory tracts, on red blood cells, and float freely in numerous bodily fluids. Also, people with blood-type O have been shown to be more susceptible to norovirus infection, due to the *FUT2* gene, which encodes for an H-type carbohydrate on the surface of epithelial cells and mucosal secretions, to which the norovirus can bind to (Lindesmith et al., 2003). Karst (2010) states that individuals with a wild-type *FUT2* gene (about 80% of the population) are susceptible to norovirus infection, while those with a "null *FUT2* allele" (about 20% of the population) are resistant.

The most common mode of transmission is direct contact via an infected food handler that did not practice proper hygiene (Koopmans et al., 2002). The virus can also

be transmitted via contaminated irrigation or recreational water, the fecal-oral route, nasopharyngeal solutions, fomites, or by aerosols generated by vomiting or diarrhea (Li et al., 2011; Koopmans et al., 2002; Greening, 2006). Infection tends to occur in crowded, enclosed environments such as cruise ships, schools, daycare centers, nursing homes restaurants, and hospitals (Wobus et al., 2006; CDC, 2009a). Marks et al (2000) reported that at a United Kingdom restaurant, one guest experienced projectile vomiting and 91% of the people sitting at the same table became infected, while up to 25% of the people in the restaurant in the surrounding illness also came down with illness. Aside from bioaerosols, the virus is most commonly associated with shellfish, water and ice, fresh produce, ready-to-eat deli meats, sandwiches, and baked products (Greening, 2006).

1.1.2. Hepatitis A (HAV)

Hepatitis A virus (genus Hepatovirus) is a member of the Picornaviridae family, which includes viruses such as poliovirus, rhinovirus, foot-and-mouth disease virus, and Aichivirus (Flint et al., 2004b). HAV has a single stranded, positive-sense RNA genome, is 27-32 nm in diameter, nonenveloped, and possesses a icosahedral capsid symmetry (Greening, 2006). The HAV genome consists of a VPg (small polypeptide) at the 5’ end, contains one open reading frame (ORF) that consists of a polyprotein that encodes for both structural and nonstructural proteins (Aggarwal and Naik, 2008). The polyprotein has a P1 region that encodes the structural proteins (VP1, VP2, VP3, and VP4), while the P2 and P3 regions encode nonstructural proteins that are required for viral replication (Buesa and Rodríguez-Díaz, 2006). HAV strains can be divided into seven genotypes,

with genotypes I, II, III, and VII associated with human illness, and IV, V, and VI being simian in origin (Aggarwal and Naik, 2008).

It is estimated that HAV accounts for 5% (approximately 83,000 cases) of all foodborne outbreaks in the United States (Mead et al., 1999). Annually, 22% of adults with the illness are hospitalized, and approximately 100 people die (Atreya, 2004). However, it is important to note that these are estimates, and it is believed that the actual occurrence of illness is assumed to be three to ten times that of reported cases (Greening, 2006; Mead et al., 1999). Finelli and Bell (2008) report that in 2002-2004, the most frequently reported risk factors for contracting the illness were international travel (13.2%), household or sexual contact with an infected person (12.8%), children or employees of day care centers (10%), and intravenous drug use (9.4%). HAV infection tends to be uncommon in the United States, but is common in developing countries where a majority of people become infected during childhood (Koopmans et al., 2002). HAV tends to be associated in environments with poor sanitary conditions and low socioeconomic status (Aggarwal and Naik, 2008).

HAV can be transmitted to humans through multiple routes. The main mode of transmission is the fecal-oral route, but infection can occur from direct contact between people (household or sexual contact), or contaminated food and water (Finelli and Bell, 2008; Vasickova et al., 2005; Greening, 2006). Foods associated with HAV contamination can include raw or partially cooked shellfish that concentrate virus particles in their tissues, or fruits and vegetables (commonly raspberries, blueberries, strawberries, lettuce, and green onions) contaminated by water or food handlers (Greening, 2006).

HAV is very stable in the environment. HAV can survive in the environment for up to a month or longer when associated with organic debris (Aggarwal and Naik, 2008; Koopmans et al., 2002; McCaustland et al., 1982; Lemon et al., 1992). The virus can also survive on the hands and fomites for extended periods of time, thus potentially increasing the rate of transmission (Mbithi et al., 1992; Aggarwal and Naik, 2008). HAV is resistant to free chlorine in tap water (0.5-1.0 mg chlorine/L), but can be inactivated by chlorine greater than 2.0 mg/L and by heating foods greater than 85°C for 1 minute (Koopmans et al., 2002).

Currently, HAV pathogenicity is not well understood (Koopmans et al., 2002). HAV is known to infect the epithelial cells of the intestinal tract, where the virus replicates and is transported to the liver via the bloodstream (Greening, 2006; Koopmans et al., 2002). The hepatocytes of the liver become infected and are lysed by cytotoxic T-cells, which releases viral particles into the bile duct and are eventually excreted in the feces (Greening, 2006; Koopmans et al., 2002).

HAV either produces asymptomatic or symptomatic infection. The incubation period for the virus can last anywhere from 15-50 days prior to the onset of symptoms (Finelli and Bell, 2008). Initial symptoms can abruptly appear, and this includes fever, headache, nausea, vomiting, occasional diarrhea, fatigue, anorexia, dark urine, light-colored stools, jaundice, and abdominal pain (Finelli and Bell, 2008; Greening, 2006). Approximately 1 to 2 weeks after initial symptoms, jaundice and viremia occur, with HAV being present in the bloodstream and feces (Greening, 2006). Illness and debilitation can last for 2-6 months, with some individuals experiencing relapses or prolonged symptoms (Greening, 2006; Finelli and Bell, 2008). HAV does not cause

chronic infection, nor does it cause chronic liver disease (Koopmans et al., 2002; Finelli and Bell, 2008).

Even though HAV illness is self-limiting, there are no available treatments to cure the disease. People may feel sick for several months, but there is no permanent damage to the liver (CDC, 2009b). Most people will recover fully, while liver failure and death tends to occur in people over 50 years old but this is very rare (CDC, 2009b). Hospitalization is not common or required, with physicians recommending rest, proper nutrition, and fluid intake (CDC, 2009b). Finelli and Bell (2008) state that since 1996, the ACIP (Advisory Committee on Immunization Practices) recommends hepatitis A vaccination of children in susceptible populations such as American Indian, Alaska Natives, certain Hispanic groups, and migrant communities where the incidence of HAV illness is high. Currently, it is recommended that children in susceptible communities, adults that work in countries or environments with high HAV risk, homosexual men, illegal drug users, adults that work with non-human primates, adults that work with HAV in laboratories, or people with chronic liver disease, receive the vaccine (Finelli and Bell, 2008). At this time, there are three available vaccines for HAV. Havrix (produced by GlaxoSmithKline, Inc.) and Veqta (produced by Merck and Co.) are both inactivated vaccines that have been shown to produce 95-100% protection against the virus (Atreya, 2004). Another vaccine, Twinrix (produced by GlaxoSmithKline, Inc.) provides protection against hepatitis A and B for up to 4 years (Atreya, 2004; CDC, 2001; GlaxoSmithKline, 2012).

1.1.3. Rotavirus

Rotaviruses are members of the Reoviridae family, and are another important cause of foodborne illness. These viruses are estimated to cause approximately 3.9 million cases of foodborne illness each year in the United States, resulting in 50,000 hospitalizations, and resulting in few deaths (20-40 per year) (Mead et al., 1999). Rotaviruses are probably the most common cause of gastroenteritis in infants and young children, and it is estimated that almost all children will experience illness during the first 5 years of life (Payne et al., 2008). Rotaviruses can cause illness not only in humans, but also in various animals such as birds, primates, mice, cattle, pigs, dogs, cats, and horses (Atreya, 2004). Greening (2006) reports that there are five rotavirus species (A-E), with A, B, and C being most often associated with human illness.

Rotaviruses range in size from 60-80 nm in diameter, and their name comes from the Latin word for "wheel," thus indicating its wheel-like shape (Greening, 2006). The viruses are nonenveloped, possess a linear double-stranded RNA genome, and have an icosahedral capsid symmetry (Greening, 2006; Atreya, 2004). The genome is much more complex than the noroviruses or HAV. Rotaviruses possess a segmented genome that codes for 6 nonstructural proteins (NSP1-NSP6), and 6 structural proteins (VP1, VP2, VP3, VP4, VP6, VP7) (Buesa and Rodríguez-Díaz, 2006). Vasickova et al. (2005) state that the segmentation of the genome allows for genetic reassortment, which results in multiple antigenic types. Buesa and Rodríguez-Díaz (2006) report that mutation during viral replication is highly error-prone, with the mutation rate estimated to be greater than 5×10^{-5} per nucleotide per replication.

The virus has a complex capsid which consists of a double-layered protein shell and an inner core (Greening, 2006). The outer shell is encoded by VP4 (P protein) and VP7 (calcium-binding glycoprotein), and are known to be important for viral infectivity and serotyping (Greening, 2006; Payne et al., 2008; Buesa and Rodríguez-Díaz, 2006). VP6 encodes for the inner core and the group-specific antigen, and is believed to be important for the host to develop protective immunity (Greening, 2006). Another important aspect of the rotavirus genome is the region (NSP4) that codes for an enterotoxin. NSP4 is a nonstructural glycoprotein that induces diarrhea by inhibiting the sodium dependent glucose transporter (SGLT-1), as well as altering the host cell actin filaments and cytoskeleton (Buesa and Rodríguez-Díaz, 2006).

Rotaviruses are highly infectious, with fewer than 100 viral particles required to initiate illness (Payne et al., 2008). The virus undergoes an incubation period of 1-2 days, and symptoms can rapidly appear, which includes vomiting, watery diarrhea, fever, and abdominal pain (Greening, 2006). The illness generally lasts anywhere from 3-7 days, but serious complications can include high fever (greater than 102°F, or 39°C), severe dehydration, electrolyte imbalance, and death (Payne et al., 2008). The virus is shed in the feces in high titers (10^8 to 10^{11} particles/gram of feces) for 5-7 days after infection (Payne et al., 2008; Buesa and Rodríguez-Díaz, 2006; Vasickova et al., 2005). Greening (2006) states that short-lived immunity develops after infection, but repeat infections can occur with less severe symptoms than the primary infection.

Greening (2006) reports that rotaviruses are highly stable and can persist in the environment. The virus can survive for several months at refrigeration or freezing temperatures, but are susceptible to inactivation after multiple freeze-thaw cycles. They

are also resistant to drying, and can persist for several months on inanimate objects. Heat (greater than 50°C), extreme pH (less than 3.0 and greater than 10), disinfectants, and EDTA are capable of inactivating the virus.

Rotaviruses are primarily transmitted via the fecal-oral route, but transmission can also occur due to contaminated water, surfaces, person-to-person contact, or aerosols (Vasickova et al., 2005; Payne et al., 2008). Rotaviruses can be found in shellfish that bioconcentrate the virus in their tissues, but illness has not been linked to consumption of these products (Vasickova et al., 2005). Contamination of food products tends to occur with poor hygiene and food handling. Some commonly implicated foods include water, ice, salad, cold foods, fresh produce, or consumption of meat from an infected animal (Greening, 2006; Vasickova et al., 2005; Atreya, 2004).

In 1998, a rotavirus vaccine was developed by Wyeth-Ayerst, called Rotashield, and was approved by the FDA (Scott et al., 2009). Rotashield was a tetravalent, live, attenuated vaccine that contained three reassortant viruses that contained human genes that encoded VP4 or VP7, as well as a rhesus monkey strain that was genetically similar to human strains (Scott et al., 2009; Atreya, 2004). Prior to licensing, research trials found that the vaccine reduced the duration of rotavirus illness, as well as preventing infections (Scott et al., 2009). However, after licensing, reports of adverse reactions occurred. The adverse reaction, called intussusception, involved folding of the bowel within another bowel segment, resulting in bowel obstruction (Atreya, 2004; Scott et al., 2009). While the cause of intussusception was never identified, Rotashield was removed from the marketplace by its manufacturer less than a year after its introduction (Atreya, 2004).

In 2006, the FDA approved the use of RotaTeq, which is currently the only rotavirus vaccine licensed for use in the United States (FDA, 2006). The vaccine is produced by Merck & Co., Inc. (Payne et al., 2008; Merck, 2009a; Merck, 2009b). RotaTeq is a live, oral vaccine that consists of five reassortant rotavirus strains from both human and bovine sources that express capsid proteins of the five most common strains (G1, G2, G3, G4, and P8) associated with human illness (Payne et al., 2008). The vaccine consists of 2 ml of approximately 2.0-2.8 x 10^6 infectious units (IU) per dose, and is given in a series of three inoculations starting at 6-12 weeks of age, and two more subsequent doses at 4-10 week intervals (Merck, 2009b). RotaTeq has been shown to be effective, preventing 74% of rotavirus gastroenteritis and 98% of severe gastroenteritis in the United States and Finland (Payne et al., 2008; FDA, 2006). Infants who should not receive the vaccine include those with illness with fever (greater than 100.5°F, or 38.1°C), has a current gastrointestinal illness, chronic gastrointestinal problems, blood disorders, weakened immune system or close contact with family members with weakened immune systems, cancer, has a history of intussusceptions, or has not been gaining weight (FDA, 2006; Merck, 2009a). RotaTeq's most common side effects include diarrhea, vomiting, ear infection, runny nose, sore throat, wheezing and coughing (FDA, 2006). The more serious adverse effects, which only occurred in 2.4% of patients, include bronchiolitis, gastroenteritis, pneumonia, fever, urinary tract infection, and intussusception (Merck, 2009b). In 2007, the FDA reported that 28 cases of intussusceptions have been reported in the US, but it is not known if the vaccine truly caused the adverse event (FDA, 2007a). At this time, the FDA recommends health care

professionals to report cases of intussusceptions, and that parents closely monitor their children after vaccination for several weeks after the last dosage (FDA, 2007a).

1.1.4. Hepatitis E (HEV)

The hepatitis E virus was first discovered in 1983, after a waterborne outbreak of hepatitis that was not associated with HAV or hepatitis B (Greening, 2006; Atreya, 2004; Balayan, 1983). Due to structural morphology and genome characteristics, HEV was initially assigned to the Caliciviridae family, and was reclassified into the Togaviridae family because of similarities in enzymes (Greening, 2006). Van Regenmortel (2000) report that, under the International Committee on Taxonomy of Viruses, HEV is now classified under the Hepeviridae family, under the genus Hepevirus.

HEV is characterized by a 30-34 nm diameter particle, nonenveloped, icosahedral capsid symmetry, and is indistinguishable to HAV under electron microscopy (Buesa and Rodríguez-Díaz, 2006; Atreya, 2004). The genome consists of a linear, positive-sense, single-stranded RNA with 3 overlapping ORFs (ORF1, ORF2, and ORF3) (Buesa and Rodríguez-Díaz, 2006; Greening, 2006). ORF1 codes for the nonstructural proteins (a 5' methylated cap that may play a role in replication, a methyltransferase, protease, helicase, and RNA-dependent RNA polymerase), ORF2 codes for the capsid protein, and ORF3 codes for a protein that has been shown to bind to signal transduction proteins, but its purpose and functionality remains to be discovered (Buesa and Rodríguez-Díaz, 2006; Emerson and Purcell, 2003).

Research on HEV is currently hampered due to a lack of an effective culture system. Currently, HEV has not been successfully or repeatedly grown in cell culture, so

most information has been obtained from various recombination technologies (Emerson and Purcell, 2003). It is known that HEV replicates in the cytoplasm of liver hepatocytes, and symptoms can include jaundice, pain, fever, vomiting, nausea, and anorexia (Emerson and Purcell, 2003). The incubation period for the disease lasts from 22-60 days, and the illness is self-limiting (Greening, 2006). The illness lasts approximately 2 weeks, with generally no complications arising after illness. The mortality rate for the illness is very low (approximately 1%), but mortality can increase dramatically in pregnant women (17-30%) (Greening, 2006). It is currently not understood why the mortality rate becomes higher in pregnant women (Emerson and Purcell, 2003).

HEV is most prevalent in developing countries such as Mexico, Latin America, Africa, and Asia, but sporadic cases do emerge in North America, Europe, and Japan (Emerson and Purcell, 2003; Greening, 2006). The most common mode of transmission is the fecal-oral route, as well as fecally contaminated water and food (Atreya, 2004; Emerson and Purcell, 2003). Contaminated water tends to be the main reservoir in areas of poor sanitation, but HEV cases that occur in developed countries are often associated with travel to HEV-endemic areas (Greening, 2006). Zoonotic transmission from animals to humans is still not well understood, but research suggested that HEV transmission can occur. HEV has been isolated from a number of animals, but the mainly swine and deer (Greening, 2006). Outbreaks have been identified where humans have contracted the virus by direct contact with infected swine or deer, or consumption of raw or undercooked pork or deer meat (Greening, 2006).

1.1.5. Viruses of Potential Importance in Foods

Other viruses have been identified as potential emerging pathogens. These viruses either have been shown to be capable of being transmitted to food (although their incidence is rare), or these viruses are the causative agents of serious illness and therefore, should definitely be considered as potential pathogens (Vasickova et al., 2005). Several factors can contribute to the emergence of new pathogens and diseases. For example, human migration can contribute to the emergence of pathogens. The movement of people from one geographic location to another may introduce pathogens that were not present in the new environment (Sharma et al., 2003). Also, migration tends to lead to gatherings of populations that may have to cope with poor living conditions and hygiene, and the new environment that they live in may have difficulties adapting to the demands of the current food and water supply (Duizer and Koopmans, 2008). Globalization of food trade may also play a role. Global trade of foods has increased more than three times over the past two decades, and while microbiological criteria and testing for bacteria has been established, no methods have been established for the detection of viruses (Duizer and Koopmans, 2008). Foods can become contaminated prior to harvesting (primary contamination), or during harvest, processing, or by food handlers (secondary contamination) (Carter, 2005). Also, ecological factors may have an influence on the emergence of pathogens. Duizer and Koopmans (2008) state that a majority of emerging diseases are zoonoses, and animals are major contributors. Deforestation and invasion of humans into wildlife areas can force animals to move into new environments, thus increasing the possibility of spreading pathogens to the human populace (Duizer and Koopmans, 2008). Humans that work with animals can experience

viral transmission. For example, people that work with pigs have the potential to become infected with HEV, porcine noroviruses, and sapoviruses (Domingo and Vennema, 2008). Finally, pathogen-related factors can play a role in emerging pathogens. Viral replication is prone to errors and genetic exchange, which can lead to development of new strains or cross-species transmission (Duizer and Koopmans, 2008; Domingo and Vennema, 2008).

Duizer and Koopmans (2008) describe factors for foodborne and zoonotic foodborne transmission. Pathogenic foodborne viruses must infect humans through consumption of contaminated food products, multiply and infect the human host, be stable in the environment, and can resist food processing methods. These viruses can also be spread when infected food handlers, who can shed virus in very large numbers, contaminate the food product. Zoonotic foodborne transmission is a much rarer event. Direct transmission occurs when humans consume food animals infected with a zoonotic pathogen. On the other hand, indirect transmission involves infected animals fecally contaminating other foodstuffs with zoonotic pathogens.

Various authors describe various viruses to be considered as potentially significant in foods. Duizer and Koopmans (2008) identify Coronoaviridae (SARS), Flaviviridae (Tick-borne Encephalitis, West Nile Virus), and Orthomyxoviridae (Avian Influenza), as viral families with known foodborne transmission. Vascikova et al (2005) also identify Arenaviridae (Lassa virus), Bunyaviridae (Hantavirus), and Picornaviridae (Foot-and-Mouth disease, Aichi virus) as other potential foodborne viral pathogens.

Coronaviridae has the coronavirus, SARS, which causes severe acute respiratory syndrome (Greening, 2006). The virus is enveloped, has a positive-stranded RNA

genome, and is relatively large compared to other foodborne viruses (80-220 nm in diameter) (Greening, 2006; Duizer and Koopmans, 2008). Symptoms of illness can include severe respiratory symptoms, high fever, malaise, rash, muscle stiffness, headaches, and diarrhea (Duizer and Koopmans, 2008). Bats, civets, and raccoons are believed to be the reservoirs of the virus, and the virus is known to be transmitted via the respiratory route, but foodborne routes have not yet been identified (Duizer and Koopmans, 2008; Wang et al., 2006). The SARS virus has been shown to infect the respiratory and gastrointestinal tract, therefore foodborne transmission may occur by consumption of infected animals, or contamination of foodstuffs by infected food handlers (coughing, sneezing, fecal contamination) (Duizer and Koopmans, 2008). Although foodborne transmission of SARS has yet to be demonstrated, the potential still remains.

Flaviviridae contain arboviruses, which are enveloped positive-sense RNA viruses (Vasickova et al., 2005). In this family, Tick-borne encephalitis virus (TBE) and West Nile virus (WNV) both have arthropod vectors, and foodborne transmission has been demonstrated (Vasickova et al., 2005; Duizer and Koopmans, 2008). TBE is transmitted by ticks, and can result in disease in the nervous system, potentially resulting in neurological damage or death (Vasickova et al., 2005). However, various cases of foodborne transmission have occurred due to consumption of unpasteurized milk in several countries such as Slovakia and Russia (Vasickova et al., 2005). WNV is another arthropod transmitted arbovirus, usually transmitted by mosquitoes. Duizer and Koopmans (2008) report that the virus is capable of infecting many different types of animals, such as birds, hamsters, mice, horses, alligators, and humans. Foodborne

transmission has been shown to occur in animals that have consumed other infected animals, however foodborne transmission to humans has not been shown but can potentially occur.

Duizer and Koopmans (2008) also describe Orthomyxoviridae, specifically avian influenza (AI), as potentially emerging foodborne pathogens. AI strain H5N1 is highly pathogenic, and is an enveloped negative-stranded RNA virus. It is normally found in birds (specifically waterfowl), but is capable of spreading to mammals. AI is capable of spreading across multiple species (pigs, birds, humans, felines) and has been found in the edible tissues and eggs of birds. It is possible that AI can be transmitted via foods to humans, but a better understanding of AI replication in the host needs to be elucidated.

Vasickova et al (2005) describe Hantavirus, Foot-and-Mouth Disease virus (FMD), and Aichi virus as potential foodborne pathogens. Hantavirus is normally found in the feces, urine, or saliva of deer mice. They are the causative agents of Hantavirus pulmonary syndrome and "hemorrhagic fever with renal syndrome." Infection of humans can occur through cuts in the skin, or inhalation or consumption of mouse saliva, urine, or feces. FMD is a zoonotic pathogen that is capable of infecting cows and humans. It is characterized by malaise, fever, red lesions in the oral tissues, or blistering of the skin. It has been shown to be shed in cow's milk before symptoms manifest, and has also been found in fresh, partially cooked, or cured meat products. Although its incidence in humans is rare, the potential for causing foodborne illness has yet to be understood. Aichi virus (member of the family Picornaviridae, genus Kobuvirus) was identified in 1989, when it caused gastroenteritis in a man who had consumed oysters. People in

Japan or Southeast Asia tend to contract the illness after consumption of oyster meat, and possess antibodies against the virus.

1.1.6. Challenges with Viral Cultivation and Detection

Unlike bacteria, viruses are much more challenging to cultivate and detect, especially in foods. Some of the foodborne viruses have not been successfully cultured in the laboratory (human norovirus and HEV), while some grow slowly or yield low numbers after replication (HAV) (Greening, 2006). Another challenge is that viruses can be difficult to detect in foods. Viruses do not replicate in the food matrix and are usually present in very low numbers, thus making detection difficult. Also, food matrices tend to be complex, and may have inhibitory substances that can impede viral detection. Detection techniques that are currently available, such as RT-PCR (reverse transcriptase polymerase chain reaction), are generally performed in academic or government laboratories and require expensive equipment, as well as training and expertise to perform (Bresee et al., 2002).

Most foodborne viruses either have not been successfully cultured in the laboratory, or grow poorly in cell culture, with the exception of rotaviruses. Human noroviruses, which are the most prevalent form of foodborne illness, are an example of important viruses that have not been successfully cultured. Duizer et al. (2004a) attempted to cultivate the human norovirus in multiple cell lines using numerous cell culture supplements, but were not successful. Straub et al (2007) reported the first successful culture assay for human norovirus. The researchers grew cells in rotating wall vessel bioreactors, in order to create a 3-D model of the human intestinal tract. They

observed positive cytopathic effects (CPE) in cells that came into contact with viruses, but note that further research needs to be conducted in order to gain a more comprehensive understanding of norovirus replication. Chan et al. (2007) questioned the amount of actual virus replication in Straub's system, and suggested the use of "quantitative real-time PCR or semiquantitative endpoint dilution PCR" to determine the suitability of the method. Straub et al. (2007) argued that either PCR method would be incapable of distinguishing between infectious and noninfectious viral particles. Leung et al. (2010) found increasing viral RNA levels in *ex vivo* human duodenal tissue inoculated with strains of human norovirus. The researchers also report that they are screening other glandular epithelial cell lines for norovirus replication and their ability to produce cytopathic effects, which would lead to development of a successful plaque assay (Leung et al., 2010).

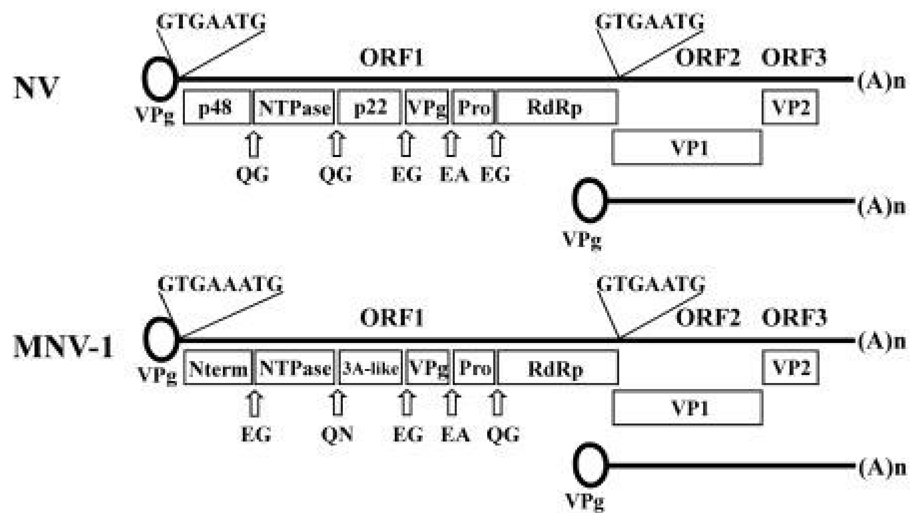


Figure 3. Genome comparison of human norovirus (NV) and murine norovirus (MNV-1) (Wobus et al., 2006).

Research on human noroviruses has been hampered because of this, so surrogate strains such as feline calicivirus (FCV) or murine norovirus (MNV) have been used. FCV was among the first surrogates used, due to its ability to be cultured in cell lines, as well as its genetic similarities to human noroviruses (Grove et al., 2006; Widdowson and Vinjé, 2008). FCV causes a wide variety of diseases in cats, such as conjunctivitis, ulcers, and limping syndrome (Grove et al., 2006). However, FCV might not be an appropriate surrogate because it is a respiratory pathogen in cats, and cannot survive at acidic pH values, which is important for enteric virus survival (Widdowson and Vinjé, 2008). MNV was first discovered in immunodeficient mice (Karst et al., 2003). MNV has been the only norovirus that has been successfully cultured to date (Wobus et al., 2006). Because of its ability to survive at lower pH, and its genetic similarity to human norovirus, MNV may be a more appropriate surrogate than FCV (Widdowson and Vinjé, 2008).

HAV is an example of a foodborne virus that can be difficult to culture in the laboratory. Wild-type strains of HAV cannot be cultured, therefore HAV needs to be adapted in order to successfully culture the virus (Pintó and Bosch, 2008). Even some the adapted HAV strains have been shown to either grow slowly in tissue culture, produce low viral yield, or establish persistent infection in cell lines with no visible CPE (Pintó and Bosch, 2008; Greening, 2006). This provides difficulties in propagating and detecting HAV in food samples.

Currently, there is a lack of sensitive methods and reliable methods to detect viruses in food products. Viruses can be difficult to detect in foods because they do not replicate in the food matrix, and are generally present in very low numbers, but are still

capable of causing illness (Pintó and Bosch, 2008). Also, the complexity of the food matrix provides difficulty because there may not be homogeneous distribution of the viral particles, and various food components can interfere with molecular detection techniques such as PCR (Vasickova et al., 2005; Goyal, 2006).

Originally, electron microscopy was the only method available to detect viruses, and this technique was insensitive and required training and expensive equipment (Bresee et al., 2002). Currently, PCR-based techniques are commonly used for viral detection because of their high sensitivity and specificity (Bresee et al., 2002). RT-PCR involves reverse transcription (using reverse transcriptase) of the viral RNA into complementary DNA (cDNA), and specific primers are used to amplify targeted portions of the genome (Atmar, 2006). RT-PCR can be coupled with multiplex or real-time PCR. In multiplex RT-PCR, several primers are used to amplify different portions of the genome, and can be used to detect multiple viruses in a single sample (Goyal, 2006; Atmar, 2006). In real-time RT-PCR, fluorescent-dye labeled probes can quantify the amount of viral DNA in a sample, and is less time consuming because gel electrophoresis is not used (Fong and Lipp, 2005). However, PCR-based methods are not capable of differentiating infectious virus particles from non-infectious particles (Goyal, 2006). Also, the food matrix, or contaminants during the PCR preparation procedures, may have inhibitory compounds that can interfere with detection. These inhibitory compounds can include clay, humic acid, glycogen, acidic polysaccharides, and tissues (Goyal, 2006). Genetic diversity of viruses can also play a role, due to mismatches between the target sequence and viral genome (Atmar, 2006).

1.1.7. Viral Outbreaks

Norovirus outbreaks are still the main cause of foodborne gastroenteritis outbreaks in the United States, accounting for approximately 67% of all foodborne illness cases (Mead et al., 1999). These outbreaks tend to be associated with shellfish, fresh produce, contaminated water, or infected food handlers. Because of its high infectivity, norovirus infection can spread rapidly through cruise ships, schools, resorts, institutions, camps, and other densely populated environments (Greening, 2006).

In 2006, the CDC determined that there had been an increase in the incidence of norovirus outbreaks, as compared to 2005. Given information from health departments across the United States, the CDC found that the number of norovirus outbreaks had increased, especially in long-term care facilities (MMWR, 2007). With response from 40 states, the CDC used data from 24 states that experienced at least five outbreaks, and found a total of 1,316 cases of norovirus illness from October to December 2006 (MMWR, 2007). Within these 24 states, 22 of them reported an increase in illness (18-800%) as compared to 2005 (MMWR, 2007). This increase in illness is believed to be attributed to 2 newly discovered strains (GII.4) of norovirus (MMWR, 2007; CIDRAP, 2007).

Shellfish tends to be one of the most commonly implicated foods in norovirus outbreaks, due to the organism's ability to concentrate viruses from the water column into their edible tissues. In 2007, the FDA advised consumers not to eat oysters harvested from San Antonio Bay (FDA, 2007c). These oysters were linked to 25 cases of illness that occurred at the Bull & Oyster Event in Maryland (FDA, 2007c). The oyster beds in San Antonio were closed down by the Texas Department of Health Services, while the

Rose Bay Oyster Company in North Carolina issued a voluntary recall because they had mislabeled their harvest location as "Galveston Bay" instead of "San Antonio Bay" (FDA, 2007c). In December of 2007, the FDA issued a warning to consumers, instructing them not to consume oysters harvested from the West Karako Bay area of Louisiana (FDA, 2007d). These oysters were linked to seven cases of illness in a restaurant in Chattanooga, Tennessee (FDA, 2007d).

Fresh produce is also commonly associated with norovirus outbreaks. Fresh fruits and vegetables undergo very little to no processing, so contamination can occur due to contaminated irrigation water or infected food handlers. From 1973 to 1997, noroviruses caused 9 outbreaks in fresh produce, with 6 outbreaks associated with salads, 2 outbreaks associated with lettuce, and 1 outbreak associated with mixed fruit (Sivapalasingam et al., 2004). From 1998 to 2000 in the United States, norovirus outbreaks in foods totaled 76, and of these cases salads were associated with 26% (20 cases) of outbreaks, and produce was associated with 17% (13 cases) (Widdowson et al., 2005a). In Finland, imported frozen raspberries (from Eastern European countries) used to make a raspberry dressing were responsible in a norovirus outbreak that sickened 108 employees at a large company (Ponka et al., 1999). None of the four kitchen staff that prepared the dressing were ill prior to the outbreak, but were among the first individuals to become sick after tasting the raspberry dressing (Ponka et al., 1999). The researchers concluded that the imported raspberries, contaminated by irrigation or rinsing water (prior to being frozen), were the source of norovirus (Ponka et al., 1999).

Norovirus outbreaks also occur in enclosed and crowded environments. Outbreaks can easily occur from aerosols from vomiting or diarrhea, direct person-to-

person contact, or from infected food handlers. Cruise ships tend to be very susceptible to norovirus outbreaks. In January 2009, a norovirus outbreak occurred on the cruise ship Celebrity Mercury which resulted in 150 passengers and 7 crew members becoming ill (CDC, 2009c). The following month, another outbreak occurred on the Zaandam of Holland America Line, with 74 passengers and 21 crew members experiencing symptoms of diarrhea and vomiting attributed to norovirus (CDC, 2009d). Isakbaeva et al (2005) reported that in 2002, a cruise ship on a 7-day voyage from Florida to the Caribbean experienced 84 passengers (out of 2,318) experienced gastroenteritis symptoms. Despite vigorous cleaning and sanitation, outbreaks still occurred two more times on the same ship (Isakbaeva et al., 2005).

Aside from cruise ships, norovirus outbreaks can occur in other environments. In 2012, a norovirus outbreak occurred at a Subway restaurant in Indiana (Food Safety News, 2012). Over a 3-day period, 90 residents became ill due to contamination from food handlers that continued to report to work despite displaying symptoms of norovirus infection (Food Safety News, 2012). In 2010, a norovirus outbreak occurred at Florida steakhouse where a group dinner was being held (Tellado et al., 2010). It was found that poor personal hygiene of the food handling staff, high fecal coliform counts in the meatloaf being served, cross-contamination of meat and fresh produce, and a possible asymptomatic carrier were responsible for this outbreak (Tellado et al., 2010). In 2005, over 27,000 evacuees were housed in a large "megashelter" at the Reliant Park Complex in Houston, Texas (Yee et al., 2007). A norovirus outbreak occurred where over 1,000 people became ill over a period of 11 days (Yee et al., 2007). Norovirus was identified as the causative agent, but several different strains were identified (Yee et al., 2007). At a

restaurant in England, one person at one table experienced a bout of vomiting due to norovirus (Marks et al., 2000). Two ceiling fans proximate to the ill person were shown to aid in spreading the aerosolized virus to other patrons of the restaurant (Figure 4; Marks et al., 2000). Persons sitting at the same table experienced a 91% attack rate, adjacent tables experienced a 56-71% attack rate, while the table farthest away from the ill person experienced a 25% attack rate of illness (Marks et al., 2000). In 2003, British troops in Iraq, as well as the hospital staff that treated them, suffered norovirus gastroenteritis from March to April (Bailey et al., 2005). The initial source of the outbreak was locally produced fresh foods (salads and fruit), but the prolonged spread and duration of the outbreak was believed to be direct person-to-person contact (especially with clinical workers) in the hospital (Bailey et al., 2005). Prolonged hospital stays of the troops were due to dehydration, exhaustion, and poor living conditions (Bailey et al., 2005). In 2003, a holiday resort in Italy also experienced a norovirus outbreak due to fecally contaminated groundwater and seawater leaking into the non-drinking and drinking water of the resort (Migliorati et al., 2008). The study had found that bathing in the sea, using cabin and shared toilets, and showers were all significant factors in the spread of the disease (Migliorati et al., 2008).

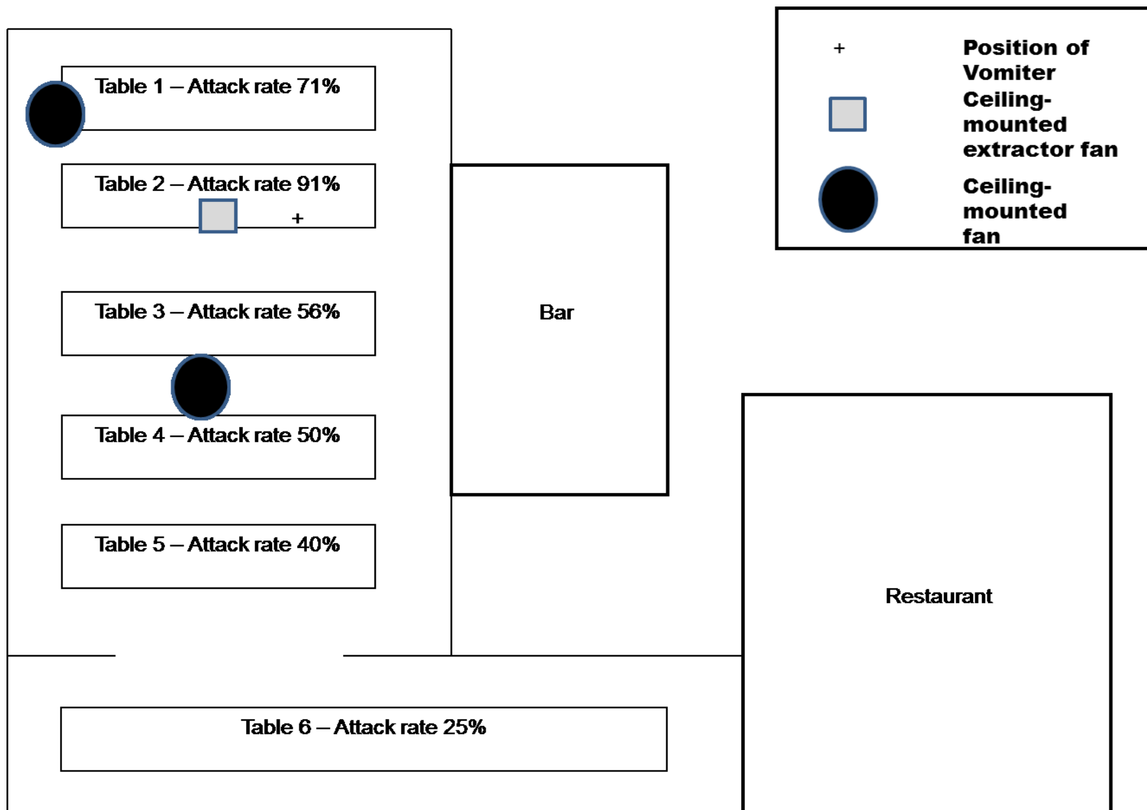


Figure 4. Layout of tables and attack rate of norovirus at a restaurant where a vomiting incident occurred (adapted from Marks et al., 2000).

Rotavirus outbreaks are not quite as prevalent as the noroviruses. Grove et al (2006) state that rotaviruses and astroviruses are less of a concern in food products, as compared to noroviruses and HAV. It is estimated that rotaviruses account for 3.9 million cases of illness each year, with 39,000 cases attributed to foods (Mead et al., 1999). Outbreaks, while not common, do occasionally occur. In 1997, a rotavirus outbreak occurred at the Memorial Sloan-Kettering Cancer Center in New York, where 8 children acquired the illness due to shared contact with infected toys (Rogers et al., 2000). In 2003, a severe outbreak of rotavirus occurred in Jamaica from June to July (CDC, 2003a). The country had reported an increase in hospital admissions in children

(less than 8 years old) experiencing acute gastroenteritis, which resulted in 12 deaths mainly attributed to diarrhea (CDC, 2003a). Australia had experienced one of the largest outbreaks of rotavirus gastroenteritis in 2001 (Kirkwood et al., 2004). The outbreak had occurred in several locations in central Australia due to an emerging serotype (G9P[8]), which resulted in 246 children becoming ill, and 137 of them being hospitalized (Kirkwood et al., 2004).

HAV and HEV outbreaks are even less prevalent than noroviruses or rotaviruses. HAV is estimated cause over 83,000 cases of illness in the United States annually, with approximately 4,100 cases being related to foods (Mead et al., 1999). In 2003, there was an outbreak of HAV at a restaurant in Monaca, Pennsylvania (CDC, 2003b). The outbreak was associated with green onions (used for salsas), harvested in Mexico, that may have been contaminated at either the growing, harvest, packing, or cooling stages (CDC, 2003b). Approximately 555 people became infected with HAV and 3 people died (CDC, 2003b). In 2004, 351 European people who had traveled from Egypt became infected with HAV, and the infection source was believed to be orange juice that was contaminated during the manufacturing processes (Frank et al., 2007). HEV outbreaks are rare in the United States, but are much more prevalent in developing countries. From March to December 2005, an HEV outbreak occurred in Hyderabad, India, where 1,611 cases of illness were reported (Sailaja et al., 2008). The source of the outbreak was water supply lines that were crossing through open sewage drains, but once these lines were fixed, the incidence of illness decreased (Sailaja et al., 2008). Li et al (2005) found that there was zoonotic transmission of HEV in wild boar meat in Japan. HEV may be found in boar, pork, or birds, and can be transmitted to humans. In this study, 10 people had

consumed wild boar meat, with only one 57-year old woman contacting the illness (Li et al., 2005). In 1994, an outbreak of HEV occurred in southwestern Vietnam, and contaminated river water (used for bathing and drinking) was the source of the virus (Corwin et al., 1996).

1.1.8. Food Virology Research Needs

Controlling foodborne viruses in food products and food service facilities need to be further examined. Foodborne viruses, with an emphasis on human noroviruses, tend to be of great significance due to their highly infectious nature. Viruses do not replicate in the food matrix, and are difficult to detect due to their low numbers and uneven distribution in a food sample. Viruses also tend to be difficult to work with because they either grow poorly in cell culture, or have not been successfully cultivated *in vitro*. Although surrogate viruses may be used to better understand viral replication and functions, research should focus on successful cultivation of the human noroviruses because the molecular biology of the virus, gene expression, pathogenesis, and sensitivity to food processing techniques has yet to be understood (Li et al., 2011). Viral detection in food samples is another issue. While PCR and other molecular biology techniques can be used in the laboratory setting, these tend to be time-consuming, require expensive equipment, and require extensive knowledge and training of the concepts. Research should focus on developing a highly sensitive and reliable technique that would be able to detect viruses in food products with minimal cost and training.

1.2. Food Irradiation

The use of ionizing radiation (also known as irradiation) has a wide variety of applications, but two of its most important uses involve sterilization of health care products and for the preservation of foods (Hansen and Shaffer, 2001). In the realm of food, irradiation is considered a "cold process" where very little to no heat is generated, thus maintaining nutritional quality and sensory characteristics (Fellows, 2000; Barbosa-Canovas et al., 1998). Some commercial operations irradiate the food, such as meats, in the frozen state. Jay et al. (2005a) define radiation as "the emission and propagation of energy through space or through a material medium." For food use, radiation can be generated using two sources: electromagnetic radiation (such as gamma radiation from an isotope source) where an atomic nucleus changes from an excited state to ground state thus emitting energy, or particle radiation where electrons (or β-particles) are accelerated to a high-energy state and are used directly on the food product (Fellows, 2000; Hansen and Shaffer, 2001).

In irradiation processing, the "degree of chemical and physical change produced when food is exposed to high energy irradiation is determined by the energy absorbed" (Barbosa-Canovas et al., 1998). This is known as the "absorbed dose" or just "dose" (Barbosa-Canovas et al., 1998). The unit of measurement for the dose is the kilogray (kGy), where 1 kGy equals 1000 grays (Gy), and 1 Gy equals 1 joule/kg (Hansen and Shaffer, 2001; Barbosa-Canovas et al., 1998).

Irradiation has a wide array of applications in foods. Ionizing radiation (at doses of 0.1 to 2 kGy) can be used to inhibit sprouting of potatoes, onions, or garlic, to kill

insects and larvae in grain or fruit products, or to control ripening of fruits and vegetables by inhibition of cell division and hormone production (Fellows, 2000). However, one of the most important uses of food irradiation is to reduce or eliminate microorganisms in order to prolong shelf-life of the product and to kill any pathogens that may be present. Regarding microorganisms, irradiation applications can fall into one of these categories: radurization, radicidation, and radappertization. Radurization involves using low-dose irradiation (0.75 to 2 kGy) in order to prolong shelf-life by destroying naturally occurring yeasts, molds, and non-spore forming bacteria (Jay et al., 2005a; Fellows, 2000). Radicidation, equivalent to pasteurization, involves using doses of 2.5 to 10 kGy to eliminate pathogenic microorganisms (non-spore forming bacteria and parasites such as *Trichinella spiralis* or tapeworms) other than viruses (Jay et al., 2005a; Fellows, 2000; Barbosa-Canovas et al., 1998). Radappertization, also considered radiation sterilization, uses doses of 10 to 50 kGy to eliminate virtually all viable microorganisms in the food product, and thus making it shelf-stable (Barbosa-Canovas et al., 1998).

1.2.1. Brief History of Food Irradiation

Experimentation with food irradiation began in the late 1800s, though practical food irradiation did not begin until the 1950s. From 1895-1896, W.K. von Roentgen reported the discovery of x-rays and Henri Becquerel first reported about radioactivity and this triggered research on the effect of radiation on biological organisms (Molins, 2001; Barbosa-Canovas et al., 1998). S.C. Prescott (1904) published his findings on the bactericidal effect of ionizing radiation from radium capsules on colon *Bacillus*, diphtheria *Bacillus*, and yeasts. In 1905, British scientists received a patent to use

ionizing radiation to kill bacteria in food products (Molins, 2001). However, the patent never came to fruition because of the limited availability of the radium used to generate the ionizing radiation (Diehl, 1995a). In 1921, B. Schwartz of the United States published the use of x-ray irradiation to kill the parasite, *Trichinella spiralis*, in contaminated raw pork (Molins, 2001; Barbosa-Canovas et al., 1998). While promising, the x-ray facilities at the time were not powerful enough to treat pork at commercial quantities (Diehl, 1995a). From the 1940s to the 1950s, research on food irradiation intensified because of the need to provide safe food for soldiers overseas, as well as the increased availability of artificial radioisotope sources (Barbosa-Canovas et al., 1998). From 1953 to 1960, the U.S. army supported research to develop irradiated meat products as a substitute for canned or frozen goods (Diehl, 1995a). In 1958, food irradiation was classified as an "additive" in the U.S. Food Additives Amendment of the Food, Drug, and Cosmetic Act (Molins, 2001). From 1961 to 1962, a large irradiation facility, equipped with a cobalt-60 source and a linear electron accelerator, was built at the U.S. Army Natick Laboratory in Natick, Massachusetts (Diehl, 1995a). In 1963, the FDA approved the use of irradiation to control insects in wheat and wheat flour (Barbosa-Canovas et al., 1998). Irradiation of bacon was also approved, but this was rescinded in 1968 due to poor data collection and experimental design flaws (Molins, 2001). In 1976, the Joint Food and Agricultural Organization of the United Nations (FAO), International Atomic Energy Agency (IAEA), and World Health Organization (WHO) Expert Committee on the Wholesomeness of Irradiated Food (JEFCI) recognized irradiation as a safe physical process (Molins, 2001). In 1980, JEFCI also declared that "irradiation of any food commodity up to an overall average dose of 10 kGy presents no toxicological

hazards…and introduces no special nutritional or microbiological problems.” (Molins, 2001). A few years later, Canada and the United States approved irradiation of pork to control *T. spiralis* in 1985 (Molins, 2001). Irradiation was later approved in the U.S. for disinfection and delay of maturation for spices and vegetables, respectively (1986), poultry (1990) and eggs (2000) to control *Salmonella*, meat products to control pathogens (1998), and fresh produce to prolong shelf-life and control pathogens (2008) (Molins, 2001; FDA, 2008). Currently, more than 40 countries (Table 1) have approved the use of food irradiation for many different types of food products (Barbosa-Canovas et al., 1998).

Table 1. Countries approving food irradiation (Barbosa-Canovas et al., 1998).

Algeria	Germany	Philippines
Argentina	Hungary	Poland
Bangladesh	India	Russia
Belgium	Indonesia	South Africa
Brazil	Iran	Spain
Bulgaria	Israel	Syria
Canada	Italy	Thailand
Chile	Ivory Coast	Ukraine
China	Japan	United Kingdom
Croatia	Korea	United States
Cuba	Mexico	Uruguay
Czech Republic	Netherlands	Vietnam
Denmark	New Zealand	Yugoslavia
Finland	Norway	
France	Pakistan	

1.2.2. Sources of Irradiation

Ionizing radiation for food use can be generated from the following sources: 1) gamma rays from either cobalt-60 (^{60}Co) or cesium-137 (^{137}Cs), 2) electron beams generated from machine sources, or 3) x-rays generated from machine sources (Diehl, 1995b). Typically gamma rays and electrons are used to irradiate foods. Figure 4

illustrates the different types of radiation sources, while Figure 5 demonstrates the interactions of the ionizing radiation types with matter.

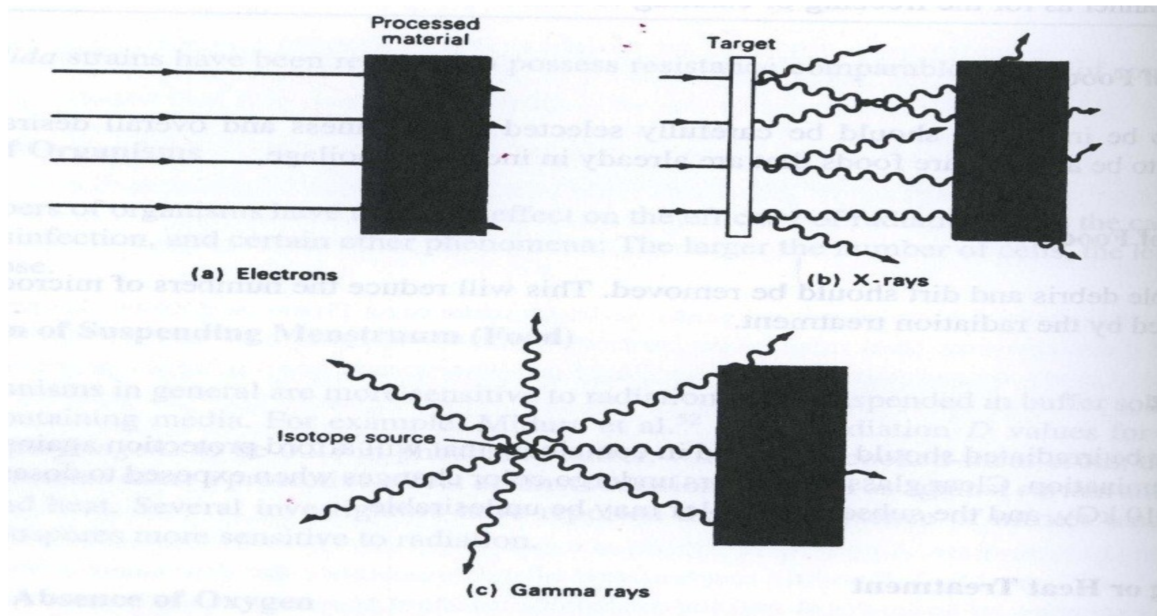


Figure 5. The three types of irradiation techniques for food processing. Electron beams (a), x-rays (b), and gamma rays (c). (Jay et al., 2005).

Gamma irradiation requires the use of radioisotopes such as ^{60}Co or ^{137}Cs. ^{60}Co is formed in a nuclear reactor when ^{59}Co pellets absorb an additional neutron by neutron bombardment (Hansen and Shaffer, 2001; Stewart. 2001). ^{137}Cs is also a nuclear reactor by-product that is formed nuclear fission of uranium, however very few reprocessing facilities in the world are capable of extracting this isotope, thus making ^{137}Cs not readily available (Hansen and Shaffer, 2001; Diehl, 1995b). Gamma radiation is emitted when these isotopes, which are already in an excited state after gaining an extra neutron, transition into a ground state (Hansen and Shaffer, 2001). As seen in Figures 5 and 6, gamma rays are emitted in all directions and are capable of completely penetrating a product. The gamma rays cannot be shut off, so when not in use, they must be shielded

in a large pool of water (Fellows, 2000). When a gamma source is in operation, the isotope is raised from the water and the product is conveyed into the irradiation chamber in a circular pattern, thus maximizing radiation exposure and ensuring a uniform dose (Fellows, 2000). Another limitation of gamma irradiation is that the ^{60}Co and ^{137}Cs radioisotopes have half-lives of 5.27 and 30.17 years, respectively (CDC, 2004a; CDC, 2004b). Radioactive half-life is defined as "the time required for a quantity of a radioisotope to decay by half" (CDC, 2004c).

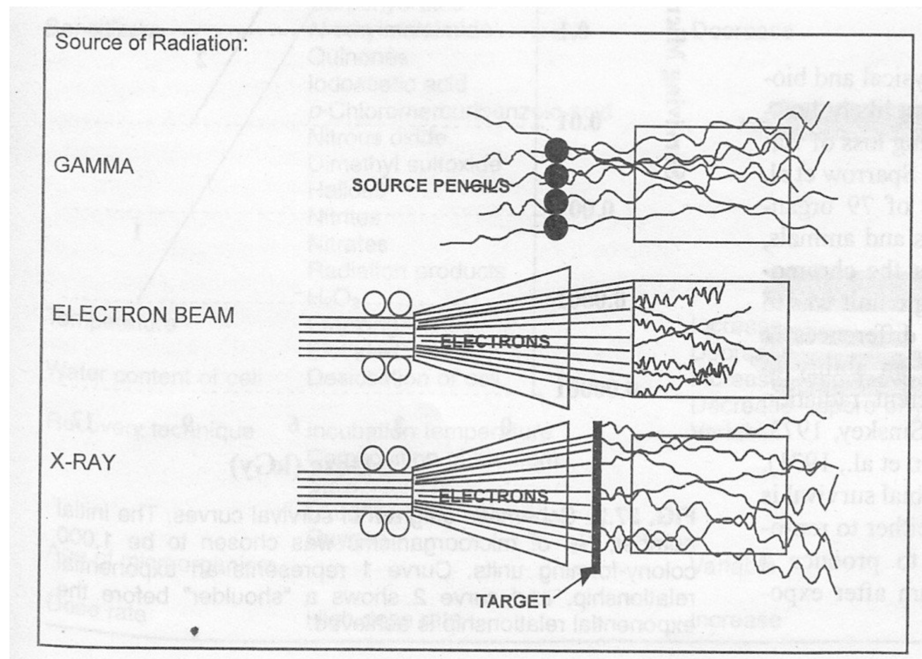


Figure 6. The interaction of ionizing radiation with matter. Ionizing radiation, whether from ^{60}Co source pencils or from an electron accelerator, cause a series of localized events during their passage or "track" through the material. Photons or excited electrons ionize the material, produce free radicals, and can excite adjacent atoms to form delta rays (provided that the electrons are of sufficient energy). Ionizing radiation never travels in a straight line through the material being treated (Hansen and Shaffer, 2001).

Aside from gamma radiation, electron beams (e-beams) generated from machine sources are the other most commonly used form of irradiation. From Figure 7, e-beams are generated from a high voltage generator that is inside a pressurized tank of sulfur hexafluoride gas (SF_6). Electrons are emitted from a heated cathode and are accelerated to high speeds (close to the speed of light) by a high-voltage electrostatic field in an evacuated accelerator tube (Diehl, 1995b; Fellows, 2000; Stewart, 2001). The emitted e-beam is deflected by a scanning magnet, which moves back and forth and directs the beam over the treatment area (Diehl, 1995b). The e-beam itself is only a few millimeters or centimeters in diameter, so scanning is required to direct the beam evenly over the targeted material (Diehl, 1995b). Unlike gamma irradiation, e-beams can be shut off completely when not in use, however e-beams have a limited penetration depth of 5 to 10 cm (Stewart, 2001).

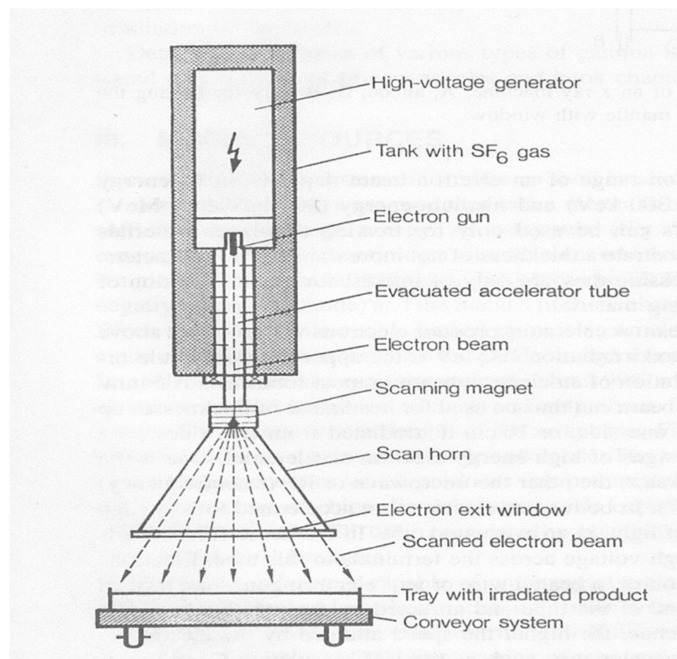


Figure 7. Diagram of an electron accelerator (Diehl, 1995b).

X-rays utilize the same electron accelerator as with e-beam. The only difference is that there is a water-cooled heavy metal target converter plate (such as copper or tungsten), located underneath the scan horn (Figure 8, Diehl, 1995b). When the accelerated electrons strike the converter plate, x-rays are produced (Fellows, 2000). The conversion from e-beam to x-ray allows greater penetration of the product, however the energy conversion is inefficient (ranges from 1 to 30%, depending on machine voltage and the type of converter plate used) and much of the energy is lost from heat from the converter plate (Hansen and Shaffer, 2001; Diehl, 1995b). Because of its inefficiency, x-rays have not found much use for the disinfection or sterilization of products, including food (Hansen and Shaffer, 2001).

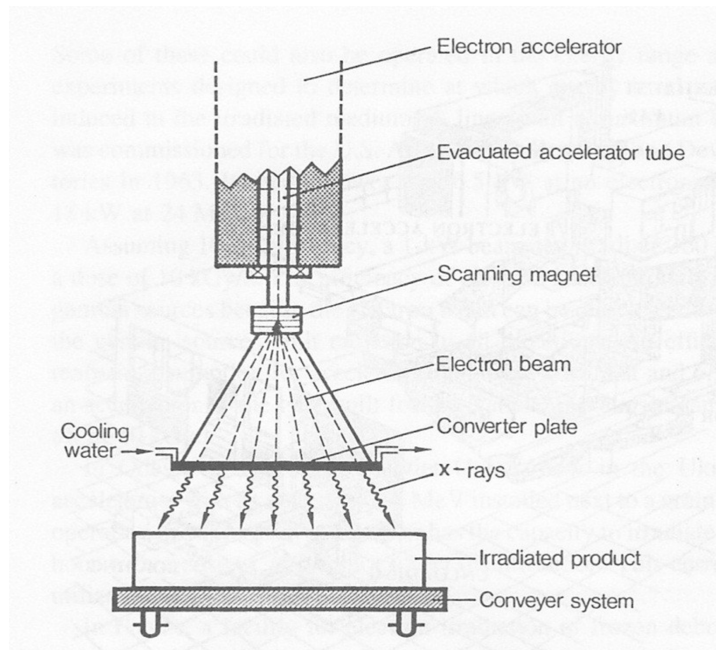
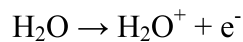


Figure 8. Electron accelerator with an x-ray converter plate (Diehl, 1995b).

1.2.3. Mechanism of Microbial Inactivation

With regard to microorganisms, ionizing radiation can cause either direct or indirect damage. The main direct effect of ionizing radiation on a microorganism is damage to the genetic material, whether it is RNA or DNA. Dickson (2001) states that photons or electrons will randomly strike the genetic material and cause breaks or lesions. Single-strand lesions may not be lethal or may cause a mutation, but multiple lesions would make the microorganism non-viable (Dickson, 2001). The indirect effect of ionizing radiation on microorganisms involves the interaction of the radiation on other atoms or molecules within the organism. Diehl (1995c) and Dickson (2001) both state that when water molecules are irradiated, they lose an electron to form:



$$H_2O \rightarrow H_2O^+ + e^-$$

These products will combine with each other or water molecules to form molecular hydrogen and oxygen, hydrogen peroxide (H_2O_2), hydroxyl radicals (^-OH), hydrogen radicals ($H\cdot$), and hydroperoxyl radicals ($HO_2\cdot$) (Fellows, 2000). Of these compounds, the most significant are the hydroxyl radicals and hydrogen peroxide, which will negatively affect the nucleic acids and break the bonds that hold them together (Dickson, 2001). Although the radicals are very short-lived (less than 10^{-5} seconds), they can be quite lethal to microorganisms (Fellows, 2000). Ionizing radiation can also affect membranes, enzymes, plasmids, or proteins that are vital to the microorganism targeted (Dickson, 2001).

1.2.4. Regulations

Table 2. Minimum and maximum doses of ionizing radiation allowed for treatment of specified food products (adapted from 21 CFR 179.26).

Use	Limitations
Control of *Trichinella spiralis* in pork	Minimum dose: 0.3 kGy Not to exceed 1 kGy
Control growth and maturation of fresh foods	Not to exceed 1 kGy
Insect disinfestation in food	Not to exceed 1 kGy
Microbial disinfection of dry or dehydrated enzyme preparations	Not to exceed 10 kGy
Microbial disinfection of spices	Not to exceed 30 kGy
Control of foodborne pathogens in fresh or frozen, uncooked poultry products	Not to exceed 3 kGy
Sterilization of frozen, packaged meats used for NASA programs	Minimum dose: 44 kGy
Control of foodborne pathogens in, and extension of shelf-life of, refrigerated or frozen, ground meat or meat byproducts	Not to exceed 4.5 kGy (refrigerated) Not to exceed 7.0 kGy (frozen)
Control of *Salmonella* in fresh shell eggs	Not to exceed 3 kGy
Control of microbial pathogens on seeds for sprouting	Not to exceed 8 kGy
Control of *Vibrio* and other foodborne microorganisms in fresh or frozen molluscan shellfish	Not to exceed 5.5 kGy
Control of foodborne pathogens and extension of shelf-life in fresh iceberg lettuce and fresh spinach	Not to exceed 4.0 kGy

The United States Code of Federal Regulations (CFR) define the sources of radiation that can be used for food processing, the minimum and maximum doses for various food products, labeling, and approved packaging materials (21 CFR 179.26; 21 CFR 179.45). For the treatment of foods, ionizing radiation must come from either gamma rays of ^{60}Co or ^{137}Cs, electrons generated from a machine source that does not exceed 10 million electron volts (10 MeV), x-rays generated from a machine source that

does not exceed 5 MeV, or x-rays generated from a machine source using tantalum or gold that does not exceed 7.5 MeV (21 CFR 179 Subpart B). Table 2 illustrates the specific use of ionizing radiation and the minimum and maximum doses allowed for food products, as specified in the CFR.

Products that are irradiated must be labeled in a specific manner, as specified by the CFR. Irradiated food products must have the Radura symbol (Figure 9) placed conspicuously on the package, followed by either "Treated with radiation" or "Treated by irradiation." (21 CFR 179.26). For irradiated, unpackaged food products, the Radura logo and one of the above statements must be displayed on the bulk container in plain view of the purchaser, on a counter sign or card next to the food product (21 CFR 179.26). Finally, irradiated foods that are shipped to a manufacturer or processing company for further processing must have on their invoices or bills of lading "Treated with irradiation – do not irradiate again" or "Treated by radiation – do not irradiate again." (21 CFR 179.26).

Figure 9. US FDA Radura symbol (left) and the international Radura symbol as specified by the Codex Alimentarius (right) (21 CFR 179.26; Ehlermann, 2009)

Packaging materials must be easily penetrated by the ionizing radiation, must reduce the risk of post-processing contamination, and must not undergo significant

chemical changes after irradiation (Fellows, 2000). Some packaging materials, when irradiated, may produce hydrocarbons or halogenated polymers which can contaminate the food product (Fellows, 2000). Also, packaging materials may start failing if they are treated above the maximum dose allowed. For example, glass can turn brown above 10 kGy, polyvinyl chloride will brown and produce hydrogen chloride above 100 kGy, and paper products and polypropylene can lose mechanical strength above 100 and 25 kGy, respectively (Fellows, 2000). To prevent this, the CFR specifies approved packaging materials for irradiation and the maximum dose allowed (Table 3).

Table 3. FDA-Approved Packaging Materials for Food Irradiation (21 CFR 179.45, 2012).

Material	Maximum Dose (kGy)
Kraft paper used for flour	0.5
Glassine paper	10
Wax-coated paperboard	10
Cellophane, nitrocellulose or vinylidene chloride coated	10
Polyolefin film	10
Polystyrene film	10
Vinylidene chloride-vinylchloride copolymer	10
Rubber hydrochloride	10
Nylon-11	10
Ethylene-vinyl acetate	30
Vegetable parchments	60
Polyethylene film	60
Polyethylene terephthalate	60
Nylon-6	60
Vinyl chloride-vinyl acetate film	60
Acrylonitrile copolymers	60

1.2.5. Advantages and Limitations of Irradiation

Whether ionizing radiations from gamma rays or e-beams are used, food irradiation has many advantages. Irradiation is essentially a nonthermal process, where there is minimal to no heating of the food, thus eliciting negligible sensory changes (Fellows, 2000). It is highly effective at inhibiting growth and maturation of fresh produce, as well as eliminating pests and pathogens such as insects, bacteria, and parasites (Molins, 2001; Farkas, 1998). Fresh foods may be preserved in a single operation, without the need for further chemical preservation (Fellows, 2000). Frozen and packaged foods can be treated, thus allowing for inactivation of pathogenic microorganisms, as well as preventing recontamination (Fellows, 2000; Diehl, 1995a). Irradiation causes minimal changes to the nutritional quality of foods, and is comparable to other methods of food preservation such as cooking (Fellows, 2000; Tauxe, 2001). Ionizing radiation does not have the power to affect the neutrons in a nucleus, thus causing it to be incapable of making food radioactive, and this has been proven numerous times (Diehl, 1995e; Farkas, 1998). Finally, irradiation is considered a safe, energy efficient, and environmentally safe process with low operating costs (Fellows, 2000; Farkas, 1998).

One of the main disadvantages of food irradiation is the capital costs, which consists of facility, irradiation source (^{60}Co or electron accelerator), and the hardware (totes, conveyors, control systems) (Kunstadt, 2001). Kunstadt (2001) reports that capital costs, not including land or warehouse costs, can range from 2.3 to 5.8 million dollars for a gamma or e-beam facility. Another major disadvantage is the public's negative perception about irradiation, due to fears of induced radioactivity (Fellows, 2000).

Negative perception about food irradiation may also come from the consumers' lack of knowledge on the subject (Farkas, 1998). Fox (2002) reports that a large majority of people are not familiar with food irradiation, and that only 48% of people sampled in a survey had even heard of the process. Also, not all foods are suitable for irradiation. These can include softening and discoloration of fresh produce, egg white proteins can denature and turn milky white, or development of off-flavors and odors in fatty and proteinaceous foods (Mahapatra et al., 2005; Tauxe, 2001). Microbiologically, there are also concerns that irradiation could be used to reduce high bacterial loads to make unacceptable food appear sellable, if spoilage microorganisms are killed but pathogenic ones are not, and if toxin producing bacteria are destroyed after contaminating the food with toxins (Fellows, 2000). Even though JEFCI concluded that irradiating foods at 10 kGy presented no toxicological, nutritional, or microbiological hazards, the acceptance of irradiation as a food technology has been hampered (Diehl, 1995a; Molins, 2001; Fellows, 2000). Despite the opposition, various consumer studies have suggested that consumers would be more inclined to purchase irradiated products if they were provided with information about irradiation (Fan et al., 2008; Fox, 2002; Nayga et al., 2004).

1.2.6. Irradiation and Foodborne Viruses

The effect of ionizing radiation on bacteria and parasites in food products has been widely researched and well documented. Prior to the 2000s, there has been a relative dearth of information on the effect of irradiation on foodborne viruses. Previously, information on foodborne viruses has been limited, due to unsuccessful cultivation of these pathogens in the laboratory. With the development of successful

cultivation methods and plaque assays, molecular techniques such as PCR, and with the discovery of closely related and cultivable viral surrogates, research on these important foodborne pathogens has progressed significantly.

Some of the first studies on irradiation and viruses occurred between 1969 and 1973. Heidelbaugh and Giron (1969) found that 6 kGy of gamma irradiation produced a 2-log reduction on poliovirus titers inoculated to fish fillets. Sullivan et al. (1971) tested the effect of gamma irradiation on 30 different non-foodborne viruses (including strains of adenoviruses, polioviruses, coxsackieviruses, echoviruses, herpes viruses, and influenza viruses). The researchers found that viruses suspended in distilled water were more susceptible to gamma radiation than those that were suspended in Eagle's minimum essential medium supplemented with 2% FBS (Sullivan et al., 1971). They also discovered that viral resistance to gamma radiation increased if the viruses were in a frozen state (Sullivan et al., 1971). The researchers reported D-values of the 30 different viruses, which ranged from 3.9 to 4.7 kGy (Sullivan et al., 1971). Sullivan et al. (1973) reported that gamma irradiation doses of 7.5, 7.1, and 6.8 kGy were required to achieve a 1-log reduction of coxsackievirus in ground beef frozen at -30°C, -60°C, and -90°C, respectively.

From 1990-2000, a couple of notable papers on foodborne viruses and irradiation were published. Mallett et al. (1991) reported a gamma irradiation D-value of 2 kGy for HAV, and 2.4 kGy for rotavirus SA11 in clams and oysters, and these doses did not negatively affect shellfish survival rates or sensory qualities. Bidawid et al. (2000) reported that doses of up to 1 kGy would only result in a 0.2 log reduction of HAV on lettuce and strawberries. The researchers also reported HAV D-values of 2.72 and 2.97

kGy on lettuce and strawberries, respectively (Bidawid et al., 2000). They concluded that higher doses of gamma irradiation, or irradiation with a combination of hurdles, would provide a greater level of viral inactivation (Bidawid et al., 2000).

After 2000, more research became available on irradiation of foodborne viruses, with the emergence of potential surrogates and better viral cultivation methods. Brahmakshatriya et al. (2009) used e-beam against avian influenza and reported D-values of 2.4 kGy in PBS, 1.6 kGy in egg whites, and 2.6 kGy in ground turkey. Jung et al. (2009) used gamma irradiation to treat poliovirus, as a model for human norovirus, in PBS, Eagle's MEM, and raw oysters. They reported D-values as 0.46 kGy for PBS, 2.84 kGy for MEM, and 2.94 kGy for oysters, and that radiation sensitivity was not affected by altering the pH or salt content of the samples (Jung et al., 2009). Feng et al. (2011) reported that only a 1.7 to 2.4 log reduction of MNV-1 in romaine lettuce, strawberries, and spinach treated with 5.6 kGy of gamma irradiation. The researchers also demonstrated that gamma irradiation was capable of disrupting viral genomic RNA and proteins by using SDS-PAGE, Western blotting, transmission electron microscopy, and RT-PCR (Feng et al., 2011). Sanglay et al. (2011) reported that 4 kGy of e-beam irradiation only provided less than 1 log reduction of MNV-1 inoculated into shredded cabbage and cut strawberries. Over 3 logs of virus still remained in both types of produce after e-beam treatment of 12 kGy, and the researchers reported discoloration and softening of the strawberries (Sanglay et al., 2011). Zhou et al. (2011) used e-beam to treat FCV inoculated into lettuce samples, and reported a D-value of 2.95 kGy. Espinosa et al. (2011) used e-beam to treat lettuce and spinach inoculated with poliovirus and rotavirus. The researchers reported D-values of rotavirus to be 1.29 and 1.03 kGy (in

spinach and lettuce, respectively), and poliovirus was 2.35 and 2.32 kGy (in spinach and lettuce, respectively) (Espinosa et al., 2011). Based on the research conducted by numerous studies, the results suggest that foodborne viruses are highly resistant to radiation, and that different types of viruses exhibit different sensitivities to irradiation.

1.3. Sodium Hypochlorite

To combat any spoilage or infectious microorganisms, the use of sanitizers, disinfectants, and sterilants are important tools to prevent microbial contamination and subsequent product loss or infection. The U.S. Environmental Protection Agency defines these three microbial treatments as:

- Sanitizer – an antimicrobial substance that "…reduces but not necessarily eliminate all the microorganisms on a treated surface. To be a registered sanitizer, the test results for a product must show a reduction of at least 99.9% in the number of each test microorganism over the parallel control." (EPA, 2012a)
- Disinfectant – an antimicrobial substance that "…destroys or irreversibly inactivates infectious or other undesirable organisms, but not necessarily their spores. EPA registers three types of disinfectant products based upon submitted efficacy data: limited, general or broad spectrum, and hospital disinfectant." (EPA, 2012a).
- Sterilant – an antimicrobial substance that "…destroys or eliminates all forms of bacteria, fungi, viruses, and their spores. Because spores are considered the most difficult form of a microorganism to destroy, EPA considers the term sporicide to be synonymous with 'sterilizer.'" (EPA, 2012a).

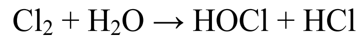
Of all the sanitizer and disinfectant compounds available (iodine compounds, peroxyacetic acid, hydrogen peroxide, ozone, quaternary ammonium compounds, glutaraldehyde), chlorine-based compounds are some of the most commonly used. Chlorine-based sanitizers and disinfectants can include liquid chlorine, chloramines, and chlorine dioxide, but the hypochlorites are among the most active and widely utilized (Marriott and Gravani, 2006; Gerba, 2009). Elemental chlorine is not found in a free state on earth, but exists in combination with sodium, potassium, calcium, and magnesium (Dychdala, 2001). The use of chlorine dates back to the early 19th century, where chlorinated lime was used for sewage treatment and decontamination of medical facilities (Dychdala, 2001). The microbial effect of chlorine was not demonstrated until 1881, when a German microbiologist (Robert Koch) reported that pure cultures of bacteria can be inactivated by hypochlorites (Dychdala, 2001). Another German scientist, Moritz Traube, demonstrated hypochlorite's ability to disinfect water in 1894 (Dychdala, 2001). Chlorine was also used to treat wounds, previously. During World War I, 0.45 to 0.50% sodium hypochlorite was used to disinfect open and infected wounds (Dychdala, 2001).

Though chlorine does not exist in a free state on earth, it can be generated by passing an electrical current through a saltwater solution (Eifert and Sanglay, 2002). The reaction is described below:

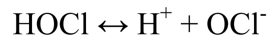
$$2\ NaCl + 2\ H_2O + \text{electricity} \rightarrow Cl_2 + 2\ NaOH + H_2$$

The resultant products are sodium hydroxide (NaOH), hydrogen gas (H_2) and gaseous chlorine (Cl_2) (Eifert and Sanglay, 2002). The gaseous Cl_2 can be dried, chilled,

pressurized, or converted into liquid for ease of transport (Eifert and Sanglay, 2002). When gaseous or liquid Cl_2 is added to water, the following reactions occur:

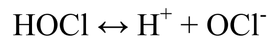


$$Cl_2 + H_2O \rightarrow HOCl + HCl$$



$$HOCl \leftrightarrow H^+ + OCl^-$$

Chlorine is hydrolyzed by water to form hypochlorous acid (HOCl) and hydrochloric acid (HCl) (Marriott and Gravani, 2006; Gerba, 2009). HOCl, which is the active antimicrobial form of chlorine, dissociates in water to form:



$$HOCl \leftrightarrow H^+ + OCl^-$$

At neutral or acidic pH, the concentration of HOCl is greater than that of the hypochlorite ion (OCl^-), and at alkaline pH, the concentration of OCl^- is greater (Gerba, 2009). While both compounds have disinfecting capabilities, HOCl is the most effective of all the chlorine fractions (Eifert and Sanglay, 2002). The presence of HOCl, OCl^-, and Cl_2, in the absence of nitrogenous compounds, is termed *free available chlorine* (Dychdala, 2001; Gerba, 2009). *Combined chlorine* is defined as when HOCl combines with ammonia and organic compounds to form monochloramines, dichloramines, and trichloramines (Gerba, 2009). The chloramines have disinfecting power, but are not as effective as HOCl (Gerba, 2009).

1.3.1. Effect against Microorganisms

The effect of chlorine compounds against microorganisms has been extensively studied, but the actual mechanism remains unclear. Gerba (2009) states that chlorine inactivation of bacteria may result from altered membrane permeability which results in cell leakage, hindrance of cell-associated membrane functions, inactivation of essential

enzymes or proteins, nucleic acid denaturation, or a combination of these events. Marriott and Gravani (2006) also suggest that chlorine may disrupt protein synthesis, cause “oxidative decarboxylation of amino acids to nitrites and aldehydes”, form lesions in the DNA, inhibit oxygen uptake, and possibly form toxic *N*-chloro compounds. Researchers have speculated that HOCl “liberates nascent oxygen”, which combines with components of the cell protoplasm and results in cellular death (Dychdala, 2001). Marriott and Gravani (2006) report that vegetative cells take in free available chlorine, which impairs transport of nutrients across the cell membrane and also makes the membrane more permeable, thus more prone to cellular leakage.

With regard to viruses, the chlorine inactivation mechanism still remains unclear. It is believed that chlorine will interact with either the viral genetic material or the capsid proteins (Gerba, 2009; Wigginton and Kohn, 2012). To date, research has shown degradation of viral genetic material and capsid protein modifications by chlorine, however what specific areas are degraded or what protein modifications have been made are still unknown (Wigginton and Kohn, 2012).

1.3.2. Advantages and Limitations of Sodium Hypochlorite

The use of sodium hypochlorite as a sanitizer or disinfect has many advantages. These advantages include:

- Being highly effective against bacteria, fungi, yeasts, and some viruses (Marriott and Gravani, 2006)

- Can be used in a wide variety of industries such as drinking and wastewater treatment, treating recreational water sources (public swimming pools, spas, hot tubs) (Dychdala, 2001)
- Sanitizing and disinfecting food handling and processing equipment (Marriott and Gravani, 2006; Dychdala, 2001)
- Treatment of environmentally-contaminated foods (Dychdala, 2001)
- Fast-acting, inexpensive, and readily available in granular or liquid form (Marriott and Gravani, 2006)
- Does not have to be rinsed off at concentrations of 200 ppm or less (Marriott and Gravani, 2006)

Marriott and Gravani (2006) also describe the disadvantages of using sodium hypochlorite. These can include:

- Highly corrosive to stainless steel and other metals
- Will deteriorate in the presence of heat (above 60°C), light, or organic soil
- Irritating to the skin and mucous membranes
- Decreased efficiency at higher pH values (greater than 8.0)

Also, enteric viruses and protozoan parasites tend to be more resistant to chlorination than bacteria (Gerba, 2009). Finally, the potential formation of carcinogenic trihalomethane (THM) compounds and disinfection by-products (DBPs) from drinking water chlorination can be of concern. THMs are formed when chlorine reacts with certain organic compounds (humic acids) to form compounds such as chloroform, bromodichloromethane, dibromochloromethane, and bromoform (Dychdala, 2001; Marriott and Gravani, 2006). Other DBPs, such as haloacetic acid, are also formed when

chlorine reacts with organic contaminants (Dychdala, 2001). The EPA (2012b) states that total THM levels are not to exceed 80 ppb and haloacetic acids are not to exceed 60 ppb in public water systems. To remove these contaminants from treated water, activated carbon columns or enhanced coagulation systems can be employed prior to chlorination (Dychdala, 2001).

1.3.3. Research with Sodium Hypochlorite and Foodborne Viruses

Although the exact mechanism of sodium hypochlorite against foodborne viruses is still to be determined, numerous studies have been conducted to learn how to control these important pathogens. Nuanualsuwan and Cliver (2003) found that treating poliovirus, HAV, and FCV with 1.20 to 1.25 mg/L of sodium hypochlorite not only inactivated the viruses' ability to bind to cells and antibodies, but also degraded viral RNA. Li et al. (2002) found that HAV lost infectivity after being treated with 10 to 20 mg/L of sodium hypochlorite after 30 minutes, and that the 5' nontranslated regions of the viral genome were the most susceptible to chlorine inactivation. Gulati et al. (2001) found that 200 to 800 ppm of chlorine only provided a 0.3 to 1.1 log reduction of FCV on food contact surfaces, and the same concentrations only provided a 0.0 to 1.0 log reduction of the virus inoculated onto strawberries and lettuce. Duizer et al. (2004b) used FCV and canine calicivirus to test various sodium hypochlorite solutions and found that the viruses were resistant to concentrations of less than 300 ppm sodium hypochlorite. Urakami et al. (2007) found that partially purified FCV lost infectivity by more than 4.6 logs after a 5 minute treatment with 300 ng/ml sodium hypochlorite. Belliot et al. (2008) reported that MNV-1 was susceptible to 0.26% (2,600 ppm) of sodium hypochlorite from

0.5 to 3 minutes, and that the viral capsid was denatured by the chlorine. Kitajama et al. (2010) reported a greater than 4 log reduction in MNV-1 in drinking water treated with 0.1 and 0.5 mg/L chlorine for 120 minutes and 30 seconds, respectively. However, there was not a significant difference in the viral RNA reduction rate between MNV-1 and human norovirus. D'Souza and Su (2010) obtained a greater than 6-log reduction of both MNV-1and FCV after treatment with 5,000 ppm sodium hypochlorite for 30 seconds and 1 minute. Nowak et al. (2011) reported that FCV experienced a 4 log reduction after treatment of 48 and 66 ppm sodium hypochlorite, however the three human norovirus strains that they used were 10 times more resistant to virolysis than FCV.

Clearly, virus susceptibility to chlorine varies among the different types of viruses, as well as the treatments they are subjected to. Until the human norovirus can successfully be cultured, we cannot fully understand how this virus will react to sanitizers or disinfectants. In the meantime, we must rely on the use of surrogate viruses and PCR techniques (which cannot distinguish between infectious and noninfectious virus particles) in order to predict how the human norovirus will survive under treatment conditions.

CHAPTER 2

ELECTRON BEAM INACTIVATION OF A NOROVIRUS SURROGATE IN FRESH PRODUCE AND MODEL SYSTEMS

Published In Journal of Food Protection (Accepted March 24, 2011)

2.1. Abstract

Norovirus remains the leading cause of foodborne illness. Currently, there is no effective intervention to eliminate viral contaminants in fresh produce. Murine norovirus 1 (MNV-1) was inoculated to either 100 ml of liquid or 100 g of food. The inactivation of a murine norovirus (MNV) by e-beam, or high energy electrons, at varying doses was measured in model systems (phosphate buffered saline, PBS; Dulbecco's Modified Eagle Medium; DMEM) or from fresh foods (shredded cabbage, diced strawberries). E-beam was applied at a current of 1.5 mA, with doses of 0, 2, 4, 6, 8, 10, and 12 kGy. The surviving viral titer was determined by plaque assays in RAW 264.7 cells. In PBS and DMEM, e-beam at 0 and 2 kGy provided less than 1 log reduction of virus. At doses of 4, 6, 8, 10, and 12 kGy, viral inactivation in PBS ranged from 2.37 to 6.40 logs, while in DMEM inactivation ranged from 1.40 to 3.59 logs. Irradiation of inoculated cabbage showed up to a 1 log reduction at 4 kGy, and less than 3 log reduction at 12 kGy. On strawberries, less than 1 log reduction occurred at doses up to 6 kGy, with a maximum

reduction of 2.21 logs at 12 kGy. These results suggest the food matrix may provide increased survival for viruses. In foods, noroviruses are difficult to inactivate due to the protective effect of the food matrix, their small size, and their highly stable viral capsid.

2.2. Introduction

Noroviruses remain the number one cause of foodborne illness, more so than any other bacterial, viral or protozoan pathogen. It is estimated that noroviruses cause more than 67% of all foodborne infections, but this is likely understated due to lack of reporting, culturing and detection methods (Koopmans and Duizer, 2004; Koopmans et al., 2002; Mead et al., 1999). Noroviruses are highly infectious, easily transmissible, resistant to environmental stress, and ubiquitous, thus contamination can occur anywhere in the food chain, from pre-harvest to point of service (Gerba and Kayed, 2003; Heidelbaugh and Giron, 1969; Seymour and Appleton, 2001; Widdowson et al., 2005b). Fresh produce becomes a significant vehicle in transmission of noroviruses because it undergoes minimal processing before consumption. Due to annual increases in consumption of fresh produce, the incidence of foodborne illnesses and fresh product recalls also increases (Fan et al., 2008).

E-beam irradiation maintains fresh quality as it is a non-thermal process that minimizes microbial contamination. Irradiation processes that commonly use e-beam or electromagnetic radiation are not new techniques, as they were first patented for food preservation in 1905 (Barbosa-Canovas et al., 1998; Molins, 2001). Food irradiation has regulatory approval in over 40 countries for many different food products. In the United States the FDA has approved doses of up to 4.0 kGy to control foodborne pathogens in

fresh iceberg lettuce and spinach (FDA, 2009; FDA, 2008). Irradiation has a wide variety of applications including insect disinfestation, inhibiting sprouting and senescence, extending shelf life, pasteurization, and sterilization. Ionizing radiation, either gamma or e-beam, inactivates microorganisms by causing random breaks in the nucleic acid, proteins, and key enzymes, or by the production of hydroxyl radicals and hydrogen peroxide by radiolysis of water (Dickson, 2001; Hansen and Shaffer, 2001; Stewart, 2001; Tauxe, 2001). E-beams are generated by passing electrons through high voltage electrostatic fields (Diehl, 1995b; Fellows, 2000). Compared to gamma radiation (which can penetrate completely through a product), e-beam has a limited penetration depth of 3-10 cm, depending on machine voltage and product density (Barbosa-Canovas et al., 1998; Diehl, 1995b). Some advantages over gamma radiation include: The beam can be shut off when not in use, electron beams are generated electrically rather than using a radioactive isotope (cobalt 60 or cesium 137), the beams can be focused directly onto the products allowing for a more controlled application of radiation, it is efficient for high throughput processing, and exposure times for a particular product will only be minutes as compared to several hours for gamma irradiation (Barbosa-Canovas et al., 1998; Diehl, 1995b; Fellows, 2000; Hansen and Shaffer, 2001; IFIC, 2002).

Irradiation has disadvantages, notably the public's negative perception of irradiated foods. There is no induced radioactivity in the food, but there is measurable vitamin and nutrient loss (but these losses are not significantly different from other traditional thermal processing methods), worker safety issues, and concern with using irradiation to conceal low-quality or spoiled products (Barbosa-Canovas et al., 1998; Fan et al., 2008; Fellows, 2000; Fox, 2002; Nayga et al., 2004). However, studies have

suggested that consumers are more willing to purchase irradiated products if they were provided with information about irradiation (Fan et al., 2008; Fox, 2002; Nayga et al., 2004).

Numerous studies have successfully demonstrated effective reductions of bacteria, such as *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* in food products, including fresh produce (Mintier and Foley, 2006; Neal et al., 2008; Schmidt et al., 2006). However, very few studies have examined how irradiation affects viruses in food products. The D_{10} values, using gamma irradiation, for hepatitis A virus (HAV) in lettuce and strawberries were 2.72 ± 0.05 and 2.97 ± 0.18 kGy, respectively (Bidawid et al., 2000). The D_{10} value for gamma irradiating HAV in clams and oysters was 2 kGy, while rotavirus required 2.4 kGy (Mallett et al., 1991). Poliovirus inoculated to fish fillets required a dose of 6 kGy to achieve a 2-log reduction (Heidelbaugh and Giron, 1969). Gamma irradiation doses of 7.5, 7.1, and 6.8 kGy were required to achieve a 1-log reduction of coxsackievirus in ground beef frozen at -30°C, -60°C, and -90°C, respectively (Sullivan et al., 1973). In liquid media, 30 different viruses had D_{10} values of 3.9 to 4.7 kGy, but the researchers found that viral resistance to irradiation increased if they were in a frozen state (Sullivan et al., 1971). However, none of the 30 viruses they used were related to foodborne outbreaks.

Currently no data are published on e-beam irradiation on human noroviruses or their surrogates. The objectives of this research were to: 1) determine the e-beam susceptibility of the murine norovirus (MNV) in liquid model systems and 2) determine the rate of inactivation of MNV inoculated onto fresh produce.

2.3. Materials and Methods

2.3.1. Cell culture

The RAW 264.7 cell line (mouse leukaemic monocyte macrophage cell line) was obtained from ATCC (American Type Culture Collection, Manassas, VA). Cultivation of cells was performed as described previously, with some slight modifications (Wobus et al., 2004). Cells were cultured in 150 cm^2 tissue culture flasks (Corning Inc., Corning, NY) containing high-glucose Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco-Invitrogen, Grand Island, NY), 25 mM HEPES buffer (Gibco-Invitrogen), and 2 mM Gluta-MAX-1 (Gibco-Invitrogen). Cells were grown at subcultivation ratios of 1:3, 1:6, or 1:10. Flasks were incubated at 37°C, 5% CO_2 for 24-72 h, until cells had reached at least 70% confluence.

2.3.2. Preparation of MNV-1

Murine norovirus 1 (MNV-1) was kindly provided by Dr. Herbert W. Virgin IV (Washington University School of Medicine, St. Louis, MO). The growth medium was removed from confluent flasks of RAW 264.7 cells and was infected with MNV at a multiplicity of infection (MOI) of 1. Flasks were incubated at 37°C, 5% CO_2 for 1 h with agitation every 15 minutes. DMEM supplemented with 2% FBS was added to the flask and was incubated at 37°C, 5% CO_2 for 24 h. When extensive cytopathic effects (CPE) were observed, flasks were subjected to three freeze-thaw cycles (-80°C, 37°C) to lyse cells and release virus particles. The cell and virus suspensions were dispensed into 50 ml conical centrifuge tubes (USA Scientific, Ocala, FL) and were centrifuged using an Allegra 6R centrifuge with a GH-3.8 swinging bucket rotor (Beckman Coulter, Brea, CA)

at 3000 rpm for 20 min to remove any remaining cellular debris. The supernatant fluid was collected and stored at -80°C until ready for use. The titer of virus stock was 8.35 $\pm$ 0.36 log PFU/ml.

2.3.3. Sample preparation and inoculation

For food samples, shredded cabbage was kindly provided by Ahmad Tahajod of Sandridge Foods (Medina, OH). Strawberries were purchased from a local grocery. Strawberries were washed with tap water, the hulls and leaves removed, and were cut into pieces no larger than 20 mm x 20 mm. 100 g of each food type were placed into individual 3 mil high barrier nylon/ethylene vinyl alcohol/copolymer/polyethylene bags (15.2 x 21.6 cm; Thompson Equipment and Supply, Cincinnati, OH).

For liquid samples, DMEM with no serum and 1X phosphate buffered saline (PBS; 0.85% NaCl, 0.12% Na_2HPO4, 0.022% NaH_2PO_4, pH 7.4) were used (Fisher Scientific, Hanover Park, IL). 100 ml of each solution was dispensed into separate polyethylene bags described above.

Samples were inoculated with 5 ml of thawed virus stock to provide a final titer approximately 7 log PFU/ml or g in the liquid or food, respectively. Pouches were gently swirled or mixed by hand, and then heat sealed using an AIE-200 Impulse Sealer at setting 4 (American International Electric, Whittier, CA). For liquid samples, air was pushed out of the bag as much as possible prior to sealing. Samples were stored at 4°C until ready for e-beam processing the following day.

2.3.4. E-beam Irradiation

Samples were transported in coolers to the NEO Beam facility in Middlefield, OH. The facility uses a Dynamitron electron beam accelerator (Radiation Dynamics Inc., Edgewood NY) with a 120 cm exit aperture at 100 Hz. Samples were treated with 0, 2, 4, 6, 8, 10, or 12 kGy under the following conditions: 5.0 MeV (voltage), 7.5 kW (output power), 1.5 mA (beam current). The accelerator has a 27 cart exposure system (120 x 180 cm trays; SI Handling Systems, Easton, PA) with speeds ranging from 6.38 to 1.22 m/min to vary dose by varying exposure time (Table 4). Sample pouches were gently flattened to a width of < 3 cm, secured to cardboard sheets, secured to the cart trays (Fig. 10). Initial and final sample temperatures were recorded using a thermocouple attached to a datalogger (OM-3001 Portable Datalogger, Omega Technologies Co., Stamford, CT) to measure temperature increases due to irradiation treatment (not shown).

Table 4. Conveyor speeds (v = m/min) used to achieve target irradiation dose.

Target Dose (kGy)	v (m/min)
2	6.83
4	3.57
6	2.44
8	1.83
10	1.46
12	1.22

Figure 10. Clockwise from top left, two packages of PBS, cabbage, strawberries, and DMEM were secured on a cardboard sheet to be treated with e-beam. Cardboard sheets were secured to the cart conveyor system. Alanine dosimeters were secured to the top and bottom of the packages (bottom row).

To measure the absorbed dose, four BioMax alanine dosimeter films (Eastman Kodak Co., Rochester, NY) were placed on the top and bottom of the pouches. After e-beam treatment, dosimeters were read using a Bruker e-scan electron spin spectrometer (Bruker BioSpin Corp., Billerica, MA). The absorbed dose in each sample was calculated as the average of the dose obtained at the top and bottom of the samples.

After e-beam treatment, samples were repackaged into coolers with frozen ice packs and transported back to The Ohio State University for microbial analysis. Samples were held at 4°C until testing.

2.3.5. Plaque assays

For liquid samples, pouches were aseptically opened in a biosafety hood and serially diluted. For solid food samples, pouches were aseptically opened and the entire contents of each pouch were transferred to a sterile filtered stomacher bag (Fisher Scientific). One hundred ml of sterile PBS was added to each bag (1:1, w/v), and samples were stomached for 2 min using an EasyMix paddle blender (240 rpm paddle speed; Microbiology International, Frederick, MD). Serial dilutions were performed using DMEM (no serum) blanks.

Confluent monolayers of RAW 264.7 cells were grown in 6-well CellBIND plates (Corning) containing DMEM with 10% FBS for 24 h at 37°C, 5% CO_2. The growth medium was removed, and 0.5 ml of each sample dilution was applied to the wells in duplicate. Plates were incubated for 1 h at 37°C, 5% CO_2, with agitation every 15 min to evenly disperse the virus and to allow virus to attach to cells. Each well was overlaid with 2 ml MEM (Earle's balanced salts) supplemented with 5% FBS, 1.6% sodium bicarbonate (7.5% w/v; Fisher), 0.5% penicillin-streptomycin (10,000 units of penicillin and 10,000 μg/ml of streptomycin in 0.85% saline; Gibco-Invitrogen), 2.5% HEPES, 1% glutamine, and 1.5% low melting point agarose (Gibco-Invitrogen). Plates were refrigerated (4°C) for at least 30 min or until the MEM overlay was solidified. Plates were then incubated at 37°C, 5% CO_2 for 48 h.

After incubation, each well was fixed with 2 ml of 10% formaldehyde (Fisher Scientific) in PBS for at least 2 h. The formaldehyde and overlay was removed, and each well was stained with 0.05% (w/v) crystal violet for at least 1 h to visualize plaques.

2.3.6. Statistical analyses

For each liquid or food type, two samples of each were prepared for each dose level. Plaque assays were performed in duplicate, and the experiments were repeated three times. Data were analyzed using the General Linear Model function and Tukey's pairwise comparison test in Minitab 16 (Minitab Inc., State College, PA). A p-value of less than 0.05 was considered statistically significant.

2.4. Results and Discussion

The effect of e-beam irradiation on inactivating MNV in liquid and fresh produce systems was measured. For liquids, PBS was selected as a minimal ingredient medium, while DMEM was a much more complex medium with sugars, salts, amino acids, buffers, and pH indicators. The MNV titers for our untreated PBS and DMEM samples (0 kGy) were 6.98 and 7.09 log PFU/ml, respectively (Table 5). After 2 kGy of treatment, a 1.12 log reduction was observed for PBS, and 0.85 logs for DMEM (Fig. 11). As doses increased from 4-12, MNV in PBS experienced a higher rate of inactivation (2.37 to 6.40 log reduction) than MNV in DMEM (1.4 to 3.59 log reduction. Even at 12 kGy, 0.89 log PFU/ml of virus remained in PBS and 3.64 log PFU/ml remained in DMEM. For PBS, there were significant differences in virus titer between each treatment dose from 0 to 12 ($p<0.05$). For DMEM, there were no significant

differences between 6-8 kGy and 10-12 kGy. Taken together, these results demonstrated that MNV was more easily inactivated in PBS than DMEM.

Table 5. MNV-1 titer (log PFU/ml or g ± standard deviation) remaining in food and liquid samples before and after e-beam treatment. Data are the means of three replicates. Means within columns with different lowercase letters are significantly different ($p < 0.05$).

Dose (kGy)	Log PFU/ml or g ± S.D.			
	PBS	DMEM	Cabbage	Strawberry
0	6.98 ± 0.16 a	7.09 ± 0.28 a	6.08 ± 1.53 a	5.37 ± 1.90 a
2	5.86 ± 0.19 b	6.24 ± 0.29 b	5.78 ± 0.17 b	5.25 ± 0.53 a
4	4.61 ± 0.99 c	5.69 ± 0.41 c	5.38 ± 0.31 c	5.00 ± 0.40 a
6	3.66 ± 0.81 d	5.16 ± 0.30 d	4.91 ± 0.23 d	4.43 ± 0.55 a
8	2.67 ± 0.35 e	4.62 ± 0.38 d	4.43 ± 0.15 e	3.81 ± 1.12 b
10	1.65 ± 0.65 f	3.98 ± 0.38 e	3.90 ± 0.14 f	3.50 ± 0.73 b
12	0.89 ± 0.64 g	3.64 ± 0.50 e	3.26 ± 0.73 g	3.16 ± 0.90 b

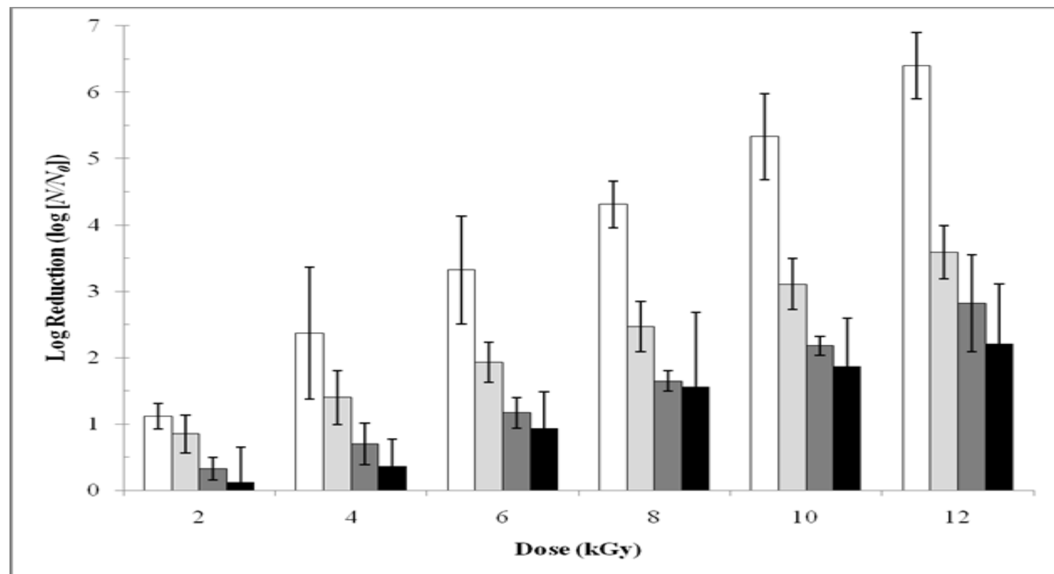


Figure 11. Log reduction of MNV-1 in food or liquid after e-beam treatment. Data are the means of three replicates. Error bars represent standard deviation. PBS (□), , DMEM (▢), cabbage (▩), and strawberry (■).

The rate of inactivation was significantly lower in fresh produce than in liquid media. The target inoculum level for shredded cabbage and cut strawberries was approximately 7 log PFU/g. As seen in Table 5, the amount of virus recovered from positive control samples (0 kGy) was 6.08 log PFU/g for cabbage, and 5.37 log PFU/g for strawberries. Even at 2-4 kGy, the FDA approved doses for fresh lettuce and spinach, log reductions were 0.33-0.70 for cabbage and 0.12-0.37 for strawberries. The doses that achieved a 1 log reduction of MNV were 6 kGy for cabbage (1.17 log reduction) and 8 kGy for strawberries (1.56 log reduction). Even at 12 kGy, there was only a 2.82 log reduction in cabbage and 2.21 log reduction in strawberries. There were significant differences ($p<0.05$) between virus titer in cabbage for all dose levels (Table 5). For strawberries, no significant differences were observed except for doses between 6 and 8 kGy. Therefore, these experiments demonstrated that MNV is much more difficult to inactivate in fresh produce than in liquid medium, suggesting the food matrix interfered with virus inactivation.

PBS is a clear liquid, while DMEM is a bright red solution. As expected, there was no color or turbidity change in PBS after e-beam treatment. However, DMEM faded after 2 kGy of treatment as shown in Fig. 3. At 2 kGy, the color faded from bright red to a dull pink-orange color likely due to acid production. As the e-beam dose increased, the color shifted to a pale yellow color. It is also noted that small gas bubbles formed in PBS and DMEM after e-beam treatment. When sugars are chemically modified by irradiation they produce acids and a mixture of gases including hydrogen, carbon dioxide, minute quantities of methane, and carbon monoxide (Stewart, 2001). DMEM contains phenol red, which changes from red to yellow as conditions become more acidic (Xiao et al.,

2002). At higher doses, more sugars become degraded, thus producing more acid (Stewart, 2001). The degradation of the sugars and production of acid may account for the color change in DMEM, as well as the gas bubble formation in both liquid samples.

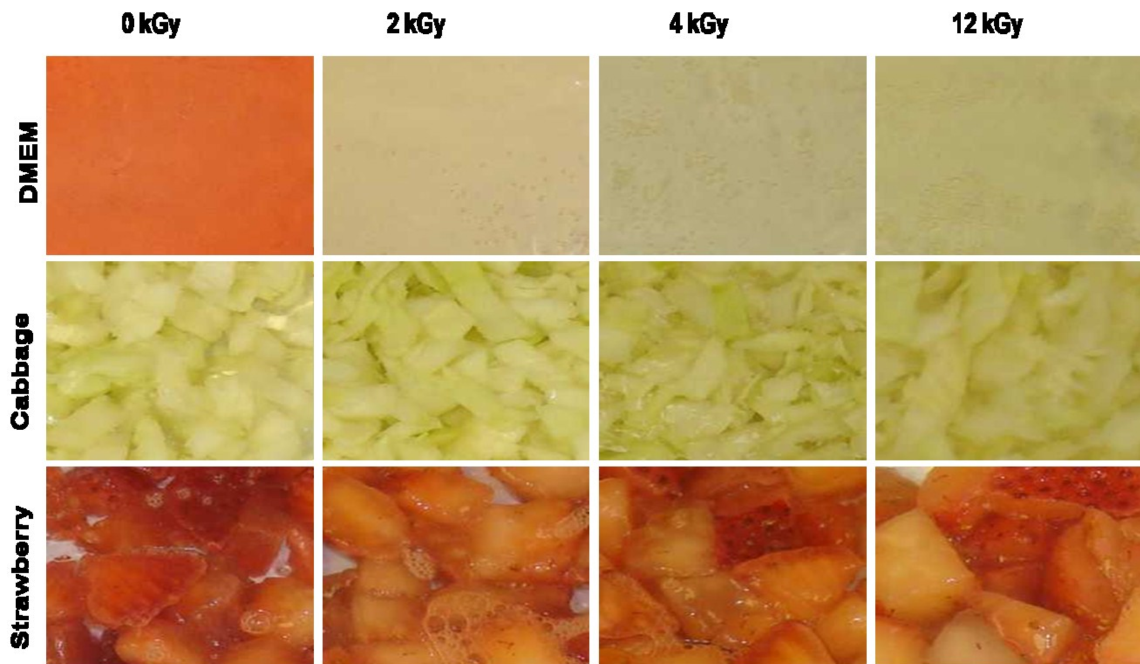


Figure 12. Appearance of DMEM, cabbage, and strawberries before and after e-beam treatment.

Irradiation of cabbage had no effect on its color or texture, even at doses greater than 4 kGy. Khattak et al. (2005) report that there were no significant differences in appearance, firmness, or flavor for cabbage treated with gamma radiation up to 3 kGy, though there were significant decreases in scores after 7-14 days of storage at 5°C. Bari et al. (2005) found that appearance, color, taste, and texture of cabbage treated at 1.0 kGy of gamma irradiation did not undergo significant changes. However for strawberries, as the dose level increased, there was a loss of color and texture (Fig. 12). The strawberries started fading and losing their characteristic red color (Fig. 12). The texture was also

affected, with strawberries becoming much softer at higher irradiation doses. The pigments in strawberries are anthocyanins that shift color due to conformational or covalent change. Thomas (2001) reports that doses up to 2.5 kGy does not affect sensory attributes in strawberries, however irreversible pigment loss can occur at higher doses. Irradiation can also affect textural properties. Irradiation can degrade cell wall polysaccharides, cellulose, and pectin, contributing to softening (Fan et al., 2008; Thomas, 2001).

These results show that more complex media or food may provide a protective effect for viruses against irradiation. PBS consists of water, sodium chloride (NaCl), sodium phosphate monobasic (NaH_2PO_4), and sodium phosphate dibasic (Na_2HPO_4). DMEM has complex organic ingredients such as 15 amino acids, 8 vitamins, 7 inorganic salts, sugars, buffers, and phenol red. These results are in agreement with Sullivan et al. (1973), who found D_{10} values at 0.5°C for coxsackievirus B-2 in distilled water (1.40 kGy) were much lower than those of Eagle minimal essential medium with 2% FBS (MEM; 4.5 kGy). Sullivan et al. (1971) also reported similar gamma irradiation D_{10} values in MEM medium for adenovirus, echovirus, poliovirus, herpes simplex virus, Newcastle disease virus, influenza, reovirus, and simian virus, ranging from 4.1-5.3 kGy. These results suggest that viral inactivation in liquid media depends upon the chemical composition (Sullivan et al., 1973; Sullivan et al., 1971). E-beam inactivates microorganisms by directly damaging genetic material (DNA or RNA) and proteins, or indirectly by producing hydroxyl radicals and hydrogen peroxide from radiolysis of water (Dickson, 2001; Hansen and Shaffer, 2001; Stewart, 2001; Tauxe, 2001).

Water is a very important factor in irradiation inactivation of microorganisms. It has been suggested that the effect of media during irradiation may be attributed to water activity (Dickson, 2001). Perhaps solutes or suspended ingredients may bind water preventing the radiolytic reactions that help destroy the virus, but this has yet to be determined. Huhtanen et al. (1989) observed D_{10} values of 0.35 (nutrient broth) and 0.77 kGy (ground chicken) for *L. monocytogenes* treated with gamma irradiation. Ley et al. (1963) found a higher D_{10} value for *Salmonella* Senftenburg in bone meal (0.56 kGy) than in buffer (0.13 kGy) treated with gamma irradiation. Also, viruses and their genetic material are much smaller than bacteria, thus making them more resistant as they are much smaller targets to hit with an accelerated electron (Grove et al., 2006). Finally, noroviruses possess a highly stable protein capsid with no lipid envelope, which affords them greater resistance to environmental assault.

In food it was much more difficult to recover and inactivate MNV from irradiated and non-irradiated food products. Currently, no reliable and effective method exists for recovering viruses from food products. The target inoculum level for cabbage and strawberries was 7 log PFU/g, however the actual amount of MNV recovered was 1-1.5 logs less than the target goal. Strawberries are more porous than cabbage, and also have exterior seeds. The cellular receptor for MNV has been recently identified. MNV binds to sialic acid receptors on mouse macrophages and dendritic cells, but many viruses have the ability to bind to carbohydrate moieties on host cell glycoproteins and glycolipids, which is an effective strategy to bind to target host cells (Taube et al., 2009). It is possible that cabbage and strawberries possess various sialic acid-like receptors such as carbohydrates and sugars. Perhaps MNV bound tightly to these molecules, thus making

recovery more difficult. After e-beam treatment, viral inactivation was minimal, with a maximum of 2.21-2.82 log reduction of MNV in strawberries and cabbage treated with 12 kGy, respectively. Bidawid et al. (2000) found that gamma irradiation doses of 2.72 and 2.97 kGy provided a 1-log reduction of HAV in lettuce and strawberries, respectively. Sullivan et al. (1973) reported gamma irradiation doses of 6.8-8.1 kGy to give a 1-log reduction of coxsackievirus B-2 in cooked and raw ground beef. Heidelbaugh and Giron (1969) reported a 2-log reduction of poliovirus in fish fillets after being treated with 6.0 kGy of gamma irradiation. No study has been conducted to investigate the destruction of the human norovirus or its surrogates by e-beam or by gamma irradiation. Similar to the results of the liquid study, the more complex the food matrix, the harder it is to inactivate MNV in food products. For example, at 4 kGy, only a 0.5 log reduction of virus was observed in cabbage, while DMEM and PBS experienced log reductions of 1.5 and 2.5, respectively. From these data it is clear the food matrix plays an important role in viral inactivation. Even though e-beam can be focused directly on the food, disadvantages include limited penetration depth due to product thickness or density, and inconsistent penetration as e-beams do not travel in a straight line through matter. Hansen and Shaffer (2001) state that ionizing radiation is not equally distributed, and that photons or electrons tend to follow a "track," rather than a straight line, during their passage. Thus adjacent areas within the same food may receive minimal exposure and intense exposure to e-beam radiation.

MNV was used as a surrogate because human noroviruses have yet to be successfully cultured in the laboratory. MNV may be a better surrogate than feline calicivirus (FCV) because FCV belongs to the *Vesivirus* family while MNV belongs to

the *Norovirus* family. MNV shares more genetic similarities to human noroviruses than FCV, is shed in the feces and follows a fecal-oral route of infection, and FCV cannot withstand acidic pH compared to MNV that is important for enteric virus infection (*4, 38, 39*).

2.5. Conclusion

The human norovirus surrogate investigated here, MNV, was highly resistant to e-beam. Our findings indicate the doses required to achieve a 1-log reduction in MNV titer is well beyond the current maximum dose allowed for lettuce and spinach by the FDA. If higher doses of radiation are used to inactivate viruses, losses in color, texture, and flavor are likely in fresh produce. E-beam is not an effective process for virus inactivation within foods, and other interventions or combination processes are needed for viral food safety.

CHAPTER 3

ELECTRON BEAM IRRADIATION EFFICIENCY AND MECHANISM OF INACTIVATION OF MURINE NOROVIRUS 1 (MNV-1) AND VESICULAR STOMATITIS VIRUS (VSV)

3.1. Abstract

Ionizing radiation, whether electron beams or gamma rays, is a nonthermal processing technique used to improve the microbial safety and shelf-life of many different food products. This technology has been shown to be highly effective against bacterial pathogens, but data on its effect against foodborne viruses has been limited until recent years. The mechanism of viral inactivation has been demonstrated with gamma irradiation, but no published study to date has disclosed if e-beam has a similar or different mechanism. In this study, murine norovirus (MNV-1, a nonenveloped human norovirus surrogate) was much more resistant to e-beam treatment than vesicular stomatitis virus (VSV, an enveloped virus). VSV was completely inactivated in phosphate buffered saline (PBS) and Dulbecco's Modified Eagle Medium (DMEM) after treatment of 16 kGy, but MNV-1 required doses of 24 to 30 kGy for complete inactivation. Using transmission electron microscopy, it was observed that e-beam negatively affected the structure of both viruses. Analysis of viral proteins by SDS-PAGE and Western blotting found that irradiation can also degrade viral proteins, though

these proteins can remain antigenic in the presence of specific antibodies. Finally, using RT-PCR, irradiation was found to also degrade viral genomic RNA. As expected, the mechanism of inactivation of e-beam is similar to that of gamma irradiation.

3.2. Introduction

Foodborne viruses, specifically human norovirus, remain an important pathogen in the realm of food safety. As of 2011, estimates suggest that 5.5 million (59%) of all foodborne illnesses in the United States were caused by viruses, with norovirus being the most prevalent cause (Scallan et al., 2011). Noroviruses are highly infectious, with less than 10 virus particles being sufficient to cause illness (Buesa and Rodriguez, 2006). Once inside the body, the incubation period for norovirus can be 1-3 days, but as rapidly as 12-24 hours for symptoms to develop (Koopmans et al., 2002; Bresee et al., 2002). Common symptoms of norovirus gastroenteritis can include projectile vomiting, non-bloody diarrhea, low-grade fever, and nausea (Koopmans et al., 2002; Greening, 2006; Vasickova et al., 2005). High titers of virus can be shed in the feces, with up to 10^8 viral particles per gram of stool (Koopmans and Duizer, 2004). The illness is acute, self-limiting and generally lasts for 12-60 hours but fecal shedding of virus can last for days or even weeks after the illness subsides making it highly infectious (Bresee et al., 2002; Greening, 2006).

The most common mode of transmission is direct contact via an infected food handler that did not practice proper hygiene (Koopmans et al., 2002). The virus can also be transmitted via contaminated irrigation or recreational water, the fecal-oral route, or by aerosols generated by vomiting or diarrhea (Koopmans et al., 2002; Greening, 2006). Infection tends to occur in enclosed environments such as cruise ships, schools, daycare

centers, nursing homes, restaurants, military barracks and hospitals (Wobus et al., 2006; CDC, 2009). Aside from bioaerosols, the virus is most commonly associated with shellfish, water and ice, fresh produce, ready-to-eat deli meats, sandwiches, and baked products (Greening, 2006).

Data on food irradiation effects on noroviruses were not available until the 2000s. Even though human noroviruses are currently non-cultivable in the laboratory, the use of closely related surrogates (such as murine norovirus and feline calicivirus) or the use of human norovirus virus-like particles (VLPs) may give some insight on how susceptible or resistant the virus is to irradiation. Recent research studies have examined the efficacy of gamma irradiation or electron beam (e-beam) irradiation on norovirus surrogates and human norovirus VLPs. Feng et al. (2011) demonstrated only a 1.7 to 2.4 log reduction of MNV-1 in fresh produce treated with 5.6 kGy of gamma irradiation, but also that irradiation can disrupt the virion structure and degrade the viral proteins and genomic RNA. Sanglay et al. (2011) observed that an e-beam dose of 4 kGy (which is the maximum dose allowed for fresh produce by the FDA) only provided a 1 log or less than 1 log reduction in cabbage and strawberries, respectively, and that the food matrix may provide a protective effect for the virus against irradiation. Zhou et al. (2011) also used e-beam irradiation and found the D_{10} value of FCV inoculated into lettuce to be 2.95 kGy.

Feng et al. (2011) provided the first insight into the mechanism of viral inactivation of gamma irradiation. Although e-beam is ionizing radiation like gamma, it does not come from an isotope source but rather, machine-accelerated electrons. E-beam has the advantage of being able to be shut off when not in use, the beams can be focused

directly onto the product allowing for a more controlled application of irradiation, and treatment times will be much shorter than gamma irradiation (Sanglay et al., 2011; Barbosa-Canovas et al., 1998; Diehl, 1995b; Fellows, 2000; Hansen and Shaffer, 2001). However, the main disadvantage of e-beam is that it has a limited penetration depth of 3 to 10 cm (Barbosa-Canovas et al., 1998; Diehl, 1995b). To date, no study has demonstrated the e-beam mechanism of viral inactivation, although it is presumed to be similar to gamma irradiation.

The objectives of this study were to: 1) compare MNV-1 (which is a nonenveloped virus) and vesicular stomatitis virus (VSV, as an example of an enveloped virus) susceptibility to e-beam irradiation from 0 to 30 kGy and 2) determine e-beam's mechanism of viral inactivation using transmission electron microscopy, SDS-PAGE, Western blotting, and real-time reverse transcriptase polymerase chain reaction (RT-PCR).

3.3. Materials and Methods

3.3.1. Cell Culture and Virus Stock

Murine norovirus 1 (MNV-1) was kindly provided by Dr. Herbert W. Virgin IV (Washington University School of Medicine, St. Louis, MO). MNV-1 was propagated in the RAW 264.7 cell line (mouse leukaemic monocyte macrophage cell line; American Type Culture Collection, Manassas, VA). Cells were cultured in 150 cm^2 tissue culture flasks (Corning Inc., Corning, NY) containing high-glucose Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco-Invitrogen, Grand Island, NY), 25 mM HEPES buffer (Gibco-Invitrogen), and 2 mM Gluta-MAX-1

(Gibco-Invitrogen). The growth medium was removed and cells were infected with MNV at a multiplicity of infection (MOI) of 1. Flasks were incubated at 37°C, 5% CO_2 for 1 h with agitation every 15 minutes. DMEM supplemented with 2% FBS was added to the flask and was incubated at 37°C, 5% CO_2 for 24 h. When extensive cytopathic effects (CPE) were observed, flasks were subjected to three freeze-thaw cycles (-80°C, 37°C) to lyse cells and release virus particles. The cell and virus suspensions were dispensed into 50 ml conical centrifuge tubes (USA Scientific, Ocala, FL) and were centrifuged using an Allegra 6R centrifuge with a GH-3.8 swinging bucket rotor (Beckman Coulter, Brea, CA) at 3,000 rpm for 20 min to remove any remaining cellular debris. The supernatant fluid was collected and stored at -80°C until ready for use.

Vesicular stomatitis virus (VSV; Indiana strain) was kindly provided by Dr. Sean Whelan (Harvard Medical School, Boston, MA). VSV was propagated in BHK-21 cells (baby hamster kidney cells; ATCC) using 150 cm^2 tissue culture flasks containing DMEM supplemented with 10% FBS, 25 mM HEPES buffer, and 2 mM Gluta-MAX-1. The growth medium was removed and cells were infected with VSV at an MOI of 0.01. Flasks were incubated at 37°C, 5% CO_2 for 1 h with agitation every 15 minutes. DMEM supplemented with 2% FBS was added to the flask and was incubated at 37°C, 5% CO_2 for 24 h. VSV was harvested by centrifugation at 3,000 rpm at 4°C for 20 minutes to remove any remaining cellular debris. The supernatant was collected and stored at -80°C.

3.3.2. Sample Preparation

100 µl of MNV-1 or 10 µl VSV were inoculated into either 900 µl of 1X phosphate buffered saline (PBS; 0.85% NaCl, 0.12% Na_2HPO_4, 0.022% NaH_2PO_4, pH

7.4) or DMEM (no serum). Samples were placed into Bitran S-Series polyethylene and SARANEX specimen bags (5.1 x 10.2 cm, 3 mil; Com-Pac International, Carbondale, IL) and were heat sealed using an AIE-200 impulse sealer at setting 4 (American International Electric, Whittier, CA). For pH measurement, 2 ml of PBS or DMEM were placed into separate specimen bags and heat sealed as described above. Samples were stored at 4°C until ready for transport, and experiments were carried out in triplicate.

For the mechanistic study (TEM, SDS-PAGE, Western blotting, and RT-PCR), 50 µl of purified MNV-1 or VSV was added to 50 µl of PBS. These samples were placed into sample bags and heat sealed as described above.

3.3.3. E-beam Irradiation

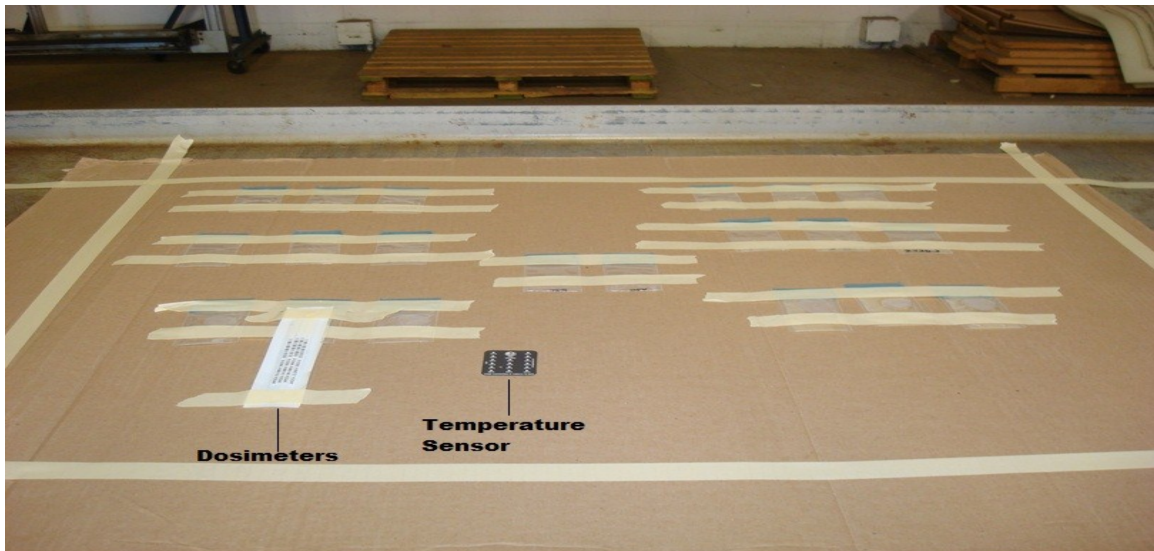


Figure 13. Sample arrangement for e-beam treatment. Four alanine dosimeters were placed above and below sample bags (bottom left). An irreversible temperature indicator (bottom center) was also used to monitor temperature changes.

E-beam irradiation was carried out as described previously (Sanglay et al., 2011). Samples were transported in coolers to the NEO Beam facility in Middlefield, OH. The facility uses a Dynamitron electron beam accelerator (Radiation Dynamics Inc., Edgewood NY) with a 120 cm exit aperture at 100 Hz. Samples were secured to cardboard sheets that were secured to the cart conveyor system (Figure 13). Samples were treated with 0, 4, 8, 16, 24, and 30 kGy under the following conditions: 4.5 MeV (voltage), 6.75 kW (output power), 5 mA (beam current). The accelerator has a 27 cart exposure system (120 x 180 cm trays; SI Handling Systems, Easton, PA) with speeds ranging from 10.5 to 1.7 m/min to vary dose by varying exposure time (Table 6). Cart speed was calculated using the following equation:

$$D = k\,(I / v)$$

D corresponds to the target dose (kGy), I is the beam current (mA), v is the speed (m/min), and k is an experimental constant that is determined by dosimetry data, which is approximately 8.22 kGy * m/(min*mA).

To ensure samples received the desired dose, four BioMax alanine dosimeter films (Eastman Kodak Co., Rochester, NY) were placed on the top and bottom of the bags, as described previously (Sanglay et al., 2011). Preliminary experiments (data not shown) indicated that regardless of sample placement on the cart, all areas received the same e-beam dose, within the uncertainty of the dosimeter (6%). After e-beam treatment, dosimeters were read using a Bruker e-scan electron spin spectrometer (Bruker BioSpin Corp., Billerica, MA). The absorbed dose in each sample was calculated as the average of the dose obtained at the top and bottom of the samples (not shown). To monitor

temperature, irreversible temperature indicators (GEX Corporation, Denver, CO) were also placed on the cardboard sheets.

Table 6. Conveyor speeds (v = m/min) and number of passes used to achieve target irradiation dose.

Target Dose (kGy)	Speed (m/min)	Number of Passes
4	10.5	1
8	5.2	1
16	2.6	1
24	1.7	1
30	2.7	2

After e-beam treatment, samples were repackaged into coolers with frozen ice packs and transported back to The Ohio State University for microbial and pH analysis. Samples were held at 4°C until testing.

3.3.4. Plaque Assays and pH Measurement

Sample bags were aseptically opened in a biosafety hood and were serially diluted using PBS blanks. Confluent monolayers of RAW 264.7 or Vero (African Green Monkey kidney epithelial cells; ATCC) cells were grown in 6-well CellBIND plates (Corning) containing DMEM with 10% FBS for 24 h at 37°C, 5% CO_2. The growth medium was removed, and 0.2 ml of each sample dilution was applied to the wells in duplicate. Plates were incubated for 1 h at 37°C, 5% CO_2, with agitation every 15 min to evenly disperse the virus and to allow virus to attach to cells. Each well was overlaid with 2 ml MEM (Earle's balanced salts) supplemented with 5% FBS, 1.6% sodium bicarbonate (7.5% w/v; Fisher), 0.5% penicillin-streptomycin (10,000 units of penicillin and 10,000 µg/ml of streptomycin in 0.85% saline; Gibco-Invitrogen), 2.5% HEPES, 1%

glutamine, and 1.5% low melting point agarose (Gibco-Invitrogen). Plates were refrigerated (4°C) for at least 30 min or until the MEM overlay was solidified. Plates were then incubated at 37°C, 5% CO_2 for 48 h.

After incubation, each well was fixed with 2 ml of 10% formaldehyde (Fisher Scientific) in PBS for at least 2 h. The formaldehyde and overlay was removed, and each well was stained with 0.05% (w/v) crystal violet for at least 1 h to visualize plaques.

pH measurement of uninoculated PBS and DMEM treated with e-beam was conducted with an Accumet AB15 pH meter (Fisher Scientific, Pittsburgh, PA).

3.3.5. Purification of MNV-1 and VSV

Purification of MNV-1 and VSV was performed as described previously (Feng et al., 2011). To generate large stocks of purified MNV-1, 20 confluent flasks of RAW 264.7 cells were infected with MNV-1 at an MOI of 0.01. Flasks were incubated at 37°C, 5% CO_2 for 1 h with agitation every 15 minutes. DMEM supplemented with 2% FBS was added to the flask and was incubated at 37°C, 5% CO_2 for 48 h. When extensive cytopathic effects (CPE) were observed, flasks were subjected to three freeze-thaw cycles (-80°C, 37°C) to lyse cells and release virus particles. The cell and virus suspensions were centrifuged using an Sorvall RC-5C Plus centrifuge (Kendro Lab Products, Newtown CT) with a Sorvall SS-34 rotor at 12,000 rpm for 10 min to remove any remaining cellular debris. The supernatant was collected and digested with DNase I (10 µg/ml) and $MgCl_2$ (5 mM) at room temperature for 1 h. DNase activity was halted by addition of 1% lauryl sarcosine in 10 mM of EDTA. The virus suspension was centrifuged in an Optima L-100 XP ultracentrifuge (Beckman Coulter, Fullerton, CA),

using a Ty 50.2 rotor, at 30,000 rpm for 6 h at 4°C. The supernatant was discarded, and the viral pellets were resuspended in 200 µl of PBS overnight at 4°C on ice. Viruses were further purified by ultracentrifugation in a sucrose gradient (7.5 to 45%) in a SW55 Ti swinging bucket rotor at 41,000 rpm for 6 h at 4°C. The virus pellets were resuspended in 100 µl of PBS overnight at 4°C on ice. The purified virus suspension was stored at -80°C.

To generate large stocks of VSV, 10 confluent flasks of BHK-21 cells were infected with VSV at an MOI of 0.01. Flasks were incubated at 37°C, 5% CO_2 for 1 h with agitation every 15 minutes. DMEM supplemented with 2% FBS was added to the flask and was incubated at 37°C, 5% CO_2 for 24 h. The cell and virus suspensions were centrifuged using an Allegra 6R centrifuge with a GH-3.8 swinging bucket rotor (Beckman Coulter) at 2,000 rpm for 5 min to remove any remaining cellular debris. The supernatant was collected and was ultracentrifuged at 21,000 rpm for 90 min at 4°C. The supernatant was discarded and the virus pellets were resuspended in 500 µl of NTE buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA) overnight at 4°C on ice. The virus was further purified by ultracentrifugation with a sucrose cushion (10% sucrose in NTE buffer) at 41,000 rpm for 1 h at 4°C. The supernatant was discarded and the pellet was resuspended in 300 µl NTE buffer overnight at 4°C on ice. The purified virus suspension was stored at -80°C.

To determine the virus titer of purified MNV-1 and VSV, plaque assays were performed as described above. The titer of purified MNV-1 was 10.22 $\pm$ 0.06 log PFU/ml, and VSV was 11.92 $\pm$ 0.24 log PFU/ml. Viral protein content was measured by

the Bradford assay, and the protein concentrations were 1 mg/ml for purified MNV-1 and 10 mg/ml for purified VSV.

3.3.6. Transmission Electron Microscopy (TEM)

Untreated and treated e-beam samples (using purified virus) were analyzed using TEM to see if there was physical damage to the virus particles. Briefly, 20 µl aliquots of sample were fixed on copper grids (Electron Microscopy Sciences, Hatfield, PA) and negatively stained with 1% ammonium molybdate. Fixed samples were analyzed using a FEI Tecnai G2 Spirit transmission electron microscope at 80 kV at the Microscopy and Imaging Facility at The Ohio State University. TEM pictures were taken using a MegaView III side-mounted charge-coupled-device (CCD) camera.

3.3.7. Analysis of Viral Proteins by SDS-PAGE

5 µl of untreated and e-beam treated purified virus suspensions were boiled in loading buffer (1% sodium dodecyl sulfate [SDS], 2.5% β-mercaptoethanol, 6.25 mM Tris-HCl [pH 6.8], and 5% glycerol) for 5 minutes. Samples were loaded onto a 12% polyacrylamide gel and analyzed using SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). Viral proteins on the gel were visualized using Coomassie blue staining.

3.3.8. Western Blotting

2 µl of untreated and e-beam treated purified virus suspensions were separated using 12% SDS-PAGE as described above. Separated MNV-1 and VSV viral proteins

were then transferred to a Hybond ECL nitrocellulose membrane (Amersham, Piscataway, NJ) in a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad, Hercules, CA). For MNV-1, the blot was probed with rabbit polyclonal MNV-1 antibody (kindly provided by Dr. Herbert Virgin) at a dilution of 1:5,000 in blocking buffer (5% skim milk). The blot was rinsed three times using PBS supplemented with 0.05% Tween 20 (PBST), and then probed with horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:5,000 in blocking buffer. For VSV, the blotting procedure is essentially the same as MNV-1, except mouse monocolonal anti-VSV glycoprotein primary antibody (Sigma-Aldrich, St. Louis, MO) and HRP-conjugated anti-mouse IgG secondary antibody were used at dilutions of 1:5,000. For both viruses, after treatment with secondary antibody, membranes were washed three times with PBST. The blots were then developed using SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Pittsburgh, PA) and exposed to Kodak BioMax MR film (Kodak, Rochester, NY).

3.3.9. Reverse Transcription PCR (RT-PCR)

MNV-1 or VSV genomic RNA, in both untreated and e-beam treated samples, were extracted using an RNeasy minikit (Qiagen, Valencia, CA) using the manufacturer's instructions. Reverse transcription polymerase chain reaction (RT-PCR) was performed using a One-Step RT-PCR kit (Qiagen). Two primers targeting the MNV-1 VP1 capsid gene, and two primers targeting the VSV nucleocapsid (N) gene were used, and are listed in Table 7. The 50μl RT-PCR reaction mixture consisted of 400 μM each deoxynucleoside triphosphate (dNTP), 0.6 μM of each primer, 4 μl of RNA template, 5

units of RNase inhibitor, and 2 µl of RT-PCR enzyme mix. The amplified PCR products were analyzed using 1% agarose gel electrophoresis.

Table 7. Primer sets used for MNV-1 and VSV RT-PCR (Feng et al., 2011).

MNV-1 Primers
5'-ATGAGGATGAGTGATGGCGC-3' (forward) 5'-TTATTGTTTGAGCATTCGGCC-3' (reverse)
VSV Primers
5'-ATGTCTGTTACAGTCAAGAG-3' (forward) 5'-TCATTTGTCAAATTCTGAC-3' (reverse)

3.3.10. Statistical Analyses

All experiments were performed in triplicate. Virus survival was expressed as the mean log plaque forming units (PFU) per milliliter $\pm$ standard deviation. Data were analyzed using the General Linear Model function and Tukey's pairwise comparison test in Minitab 16 (Minitab Inc., State College, PA). A p-value of less than 0.05 was considered statistically significant.

3.4. Results and Discussion

Table 8. E-beam inactivation of MNV-1 and VSV (log PFU/ml ± standard deviation) in aqueous solutions. Data are the means of three replicates. Means within columns with different lowercase letters are significantly different ($p < 0.05$).

Target Dose (kGy)	MNV-1 (log PFU/ml ± S.D.)		VSV (log PFU/ml ± S.D.)	
	PBS	DMEM	PBS	DMEM
0	7.20 ± 0.23 a	7.24 ± 0.12 a	5.44 ± 0.80 a	5.73 ± 0.57 a
4	5.62 ± 0.34 b	6.09 ± 0.30 b	0.63 ± 0.77 b	2.54 ± 0.55 b
8	3.75 ± 0.33 c	5.26 ± 0.12 c	0.12 ± 0.29 b	1.17 ± 0.43 c
16	0.45 ± 0.50 d	3.27 ± 0.18 d	ND	ND
24	ND	0.75 ± 0.59 e	ND	ND
30	ND	ND	ND	ND

ND – not detectable

As shown in Table 8, MNV-1 much more resistant to e-beam irradiation than VSV. After 4 kGy of e-beam, MNV-1 titer was reduced by approximately 1.5 logs in PBS, and by 1 log in DMEM. VSV experienced a 4.8 log reduction in PBS, and a 3.2 log reduction in DMEM. MNV-1 was still detected in PBS at 16 kGy and in DMEM at up to 24 kGy. VSV was not detectable at 16 kGy or higher doses. These results are in agreement with Feng et al. (2011), who observed MNV-1 being stable in aqueous solutions (water, PBS, and DMEM) after 5.6 kGy of gamma irradiation, while VSV suspended in water and PBS was highly susceptible. The researchers also observed that gamma-irradiated (5.6 kGy) VSV only experienced a 1.1 to 2.5 log and 1.3 to 2.1 log reductions in DMEM and DMEM plus 10% FBS, respectively (Feng et al., 2011). Sanglay et al. (2011) reported similar results where MNV-1 was more resistant to e-beam irradiation when suspended in DMEM versus PBS. At 12 kGy of e-beam treatment, the MNV-1 titer in PBS was 0.89 log PFU/ml, while DMEM was 3.64 log PFU/ml (Sanglay et al., 2011). Sullivan et al. (1971) reported similar findings where The D_{10} value for

coxsackievirus B-2 in Eagle's minimum essential medium treated with gamma irradiation to be 4.5 kGy, but in water the D_{10} value was much lower (1.40 kGy). Sullivan et al. (1973) reported similar D_{10} values for 30 different viruses in Eagle's minimum essential medium treated with gamma irradiation, and these values ranged from 4.1 to 5.3 kGy. Based on the inactivation data presented in this study, as well as what previous researchers reported, viral inactivation in liquid media by irradiation is dependent on the chemical composition of the media itself. If the liquid media contains more ingredients, these components may provide a protective effect for the viruses against irradiation.

Table 9. pH ± standard deviation values of PBS and DMEM before and after e-beam irradiation. Means within columns with different lowercase letters are significantly different ($p < 0.05$).

Dose (kGy)	pH ± SD (PBS)	pH ± SD (DMEM)
0	7.85 ± 0.04 a	7.45 ± 0.03 a
4	7.72 ± 0.10 ab	7.26 ± 0.12 ab
8	7.74 ± 0.03 ab	7.21 ± 0.11 ab
16	7.68 ± 0.05 ab	7.20 ± 0.10 ab
24	7.67 ± 0.05 ab	7.15 ± 0.14 b
30	7.62 ± 0.04 b	7.17 ± 0.12 b

Sanglay et al. (2011) reported a color change in DMEM after e-beam irradiation of up to 12 kGy. In their study, DMEM changed from a red color to pale yellow after irradiation, possibly due to irradiation degradation of sugars to produce acid which in turn, causes the phenol red indicator to change from red to yellow (Sanglay et al., 2011). To further investigate this, our study irradiated PBS and DMEM samples of up to 30 kGy and measured the pH of the solutions. In Table 9, PBS pH ranged from 7.85 to 7.62 after e-beam treatment from 0 to 30 kGy, while DMEM ranged from 7.45 to 7.17. PBS is colorless and therefore did not change color, while DMEM changed from red to pale

yellow at up to 16 kGy, then to a pale orange above 24 kGy (Figure 14). Statistically significant differences were observed between pH values of 0 and 30 kGy in PBS, and between 0, 24 and 30 kGy in DMEM ($p < 0.05$). Even though these differences are statistically significant, a change of 0.2-0.3 in pH is not that great of a change. Irradiation of the DMEM may not actually cause a significant drop in pH, and the color change may be due to degradation of the phenol red dye. Weber and Schuler (1952) report that the radiolysis of water produces hydroxyl radicals, which can react with phenol red and cause decolorization of the dye. Gupta and Hart (1971) report that when various sulfonephthalein dyes (phenol red, xylenol orange, thymol blue, cresol red, and p-benzoquinone) are treated with gamma irradiation, electrons can cause some decolorization of the dyes but the main reason is likely the reactivity of the hydroxyl radical. Based on the results obtain in this study, as well as the available literature, it appears that radiation decolorization of DMEM is not due to change in pH, but rather degradation of the phenol red dye.

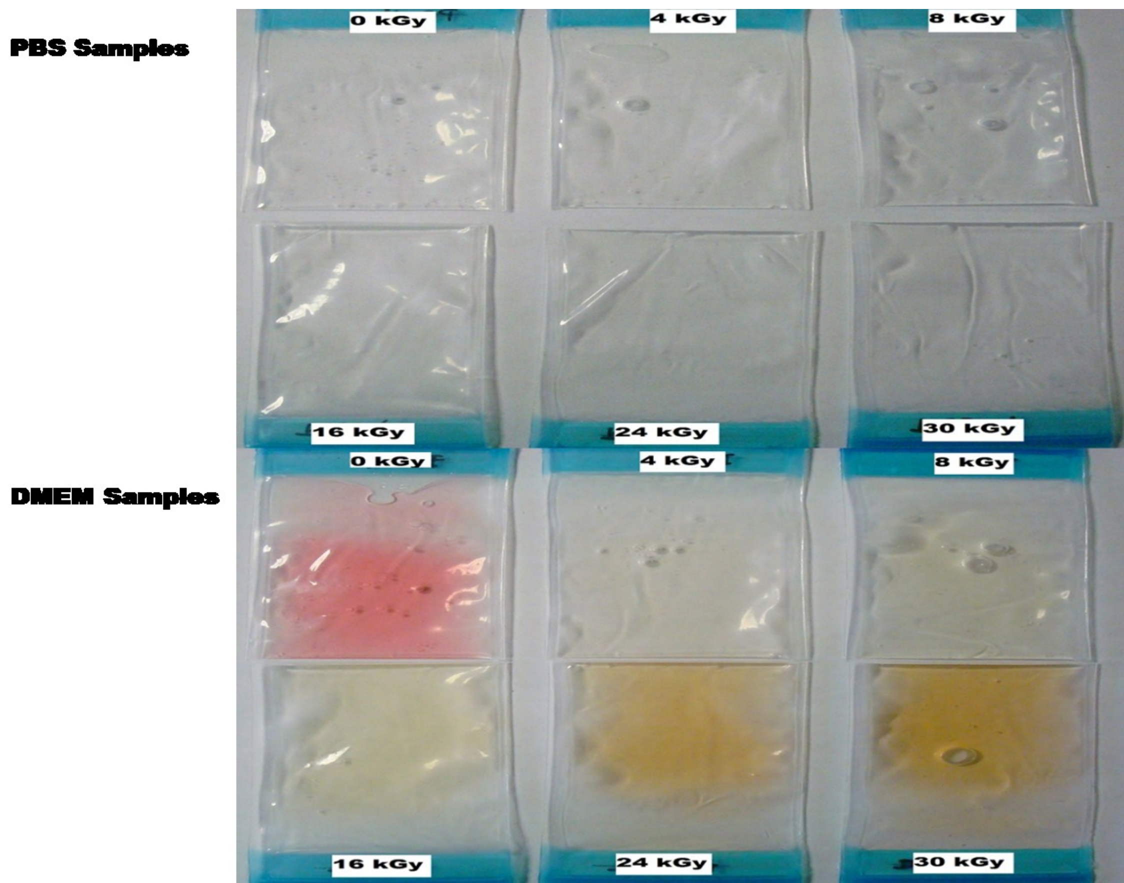


Figure 14. Appearance of PBS and DMEM before (0 kGy) and after e-beam treatment at five dose levels of 4, 8, 16, 24, and 30 kGy.

TEM images were captured and analyzed for physical damage to viral particles. In Figure 15, the undamaged MNV-1 virion ranges from 28 to 35 nm in diameter, with icosahedral capsid symmetry (Wobus et al., 2006). E-beam doses of 4 and 8 kGy do not appear to cause much damage to the particles, but the amount of viral particles appears to decrease. At 16 and 24 kGy, there are sufficiently less particles than the lower doses, and it appears that the size of the virions are diminishing. At 30 kGy, the MNV-1 capsid appears completely degraded by e-beam.

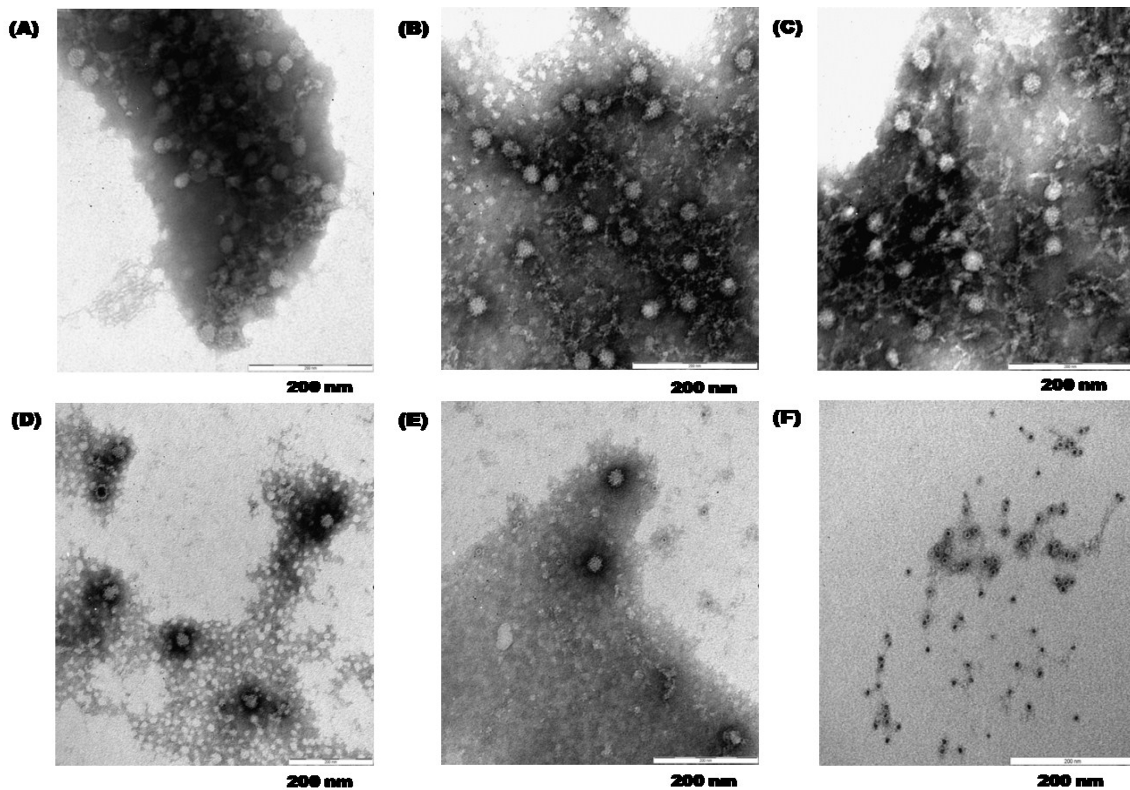


Figure 15. TEM photographs at 200 nm magnification of MNV-1 treated with 0 (A), 4 (B), 8 (C), 16 (D), 24 (E), and 30 kGy (F) of e-beam irradiation.

For VSV (Figure 16), the virion is bullet-shaped and is approximately 70 nm in diameter and 140 nm long. After 4 kGy of treatment, VSV particles start to lose their characteristic bullet shape, though some intact particles remain. It also appears that there is damage to the viral lipid envelope. As the dose of e-beam increases from 16 to 30 kGy, no intact virions are observed, and the damage to the viral particles appears to be substantial.

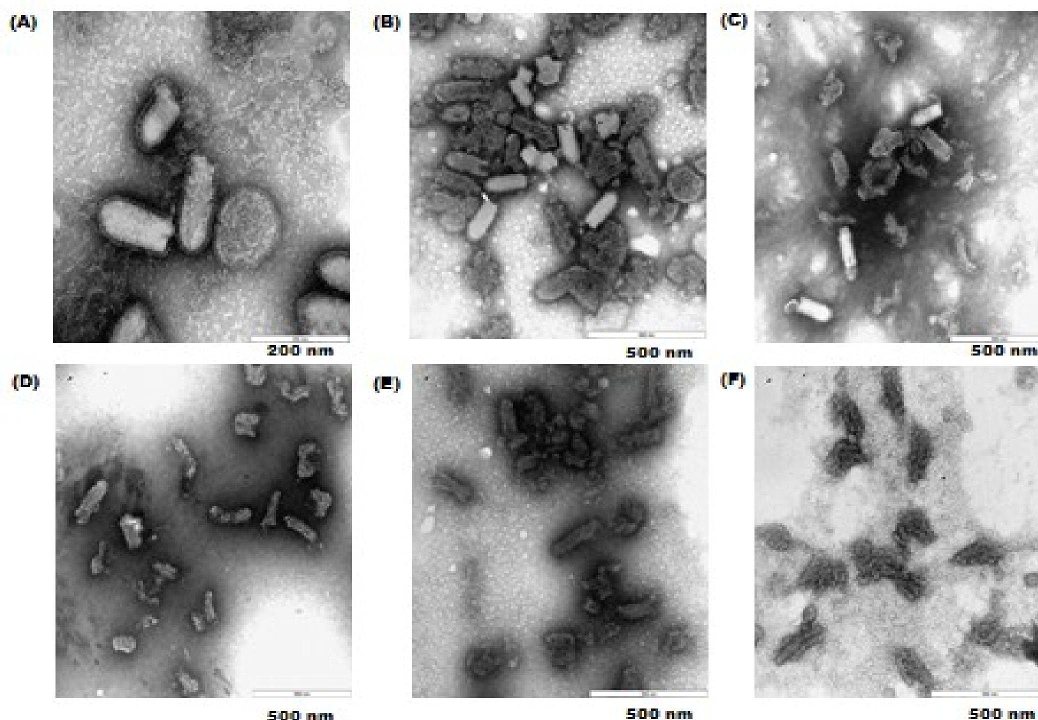


Figure 16. TEM photographs of VSV treated with 0 (A), 4 (B), 8 (C), 16 (D), 24 (E), and 30 kGy (F) of e-beam irradiation. 200 nm magnification was used to visualize the undamaged VSV particles. 500 nm magnification was used to visualize more damaged VSV particles within the field of vision.

To evaluate if e-beam degraded the viral proteins of MNV-1 and VSV, SDS-PAGE and Western blotting of untreated and e-beam treated samples were performed. For MNV-1 (Figure 17), the only protein that appeared on the SDS-PAGE gel was the VP1 major capsid protein, which is about 58.9 kDa in size (Wobus et al., 2006). The VP2 minor capsid protein did not appear on the gel. As the dosage of e-beam increased, the concentration of the VP1 protein diminished, with no protein detected at 30 kGy. For VSV, the G (glycoprotein) and L (large polymerase) proteins diminished as e-beam dosage increased, while the P (phosphoprotein), N (nucleocapsid), and M (matrix)

proteins slightly diminished. To confirm the presence of the MNV-1 VP1 capsid protein and VSV G glycoprotein, Western blots were performed (Figure 18). For both viruses, e-beam treated MNV-1 VP1 capsid protein and VSV G glycoproteins were still reactive with the antibodies. The MNV-1 VP1 capsid protein diminished with increasing e-beam treatment, while the VSV G glycoprotein decreased slightly. At higher doses of e-beam (≥ 16 kGy), larger dimer and trimer bands appeared on the VSV blot, indicating possible cross-linking or aggregation of the proteins.

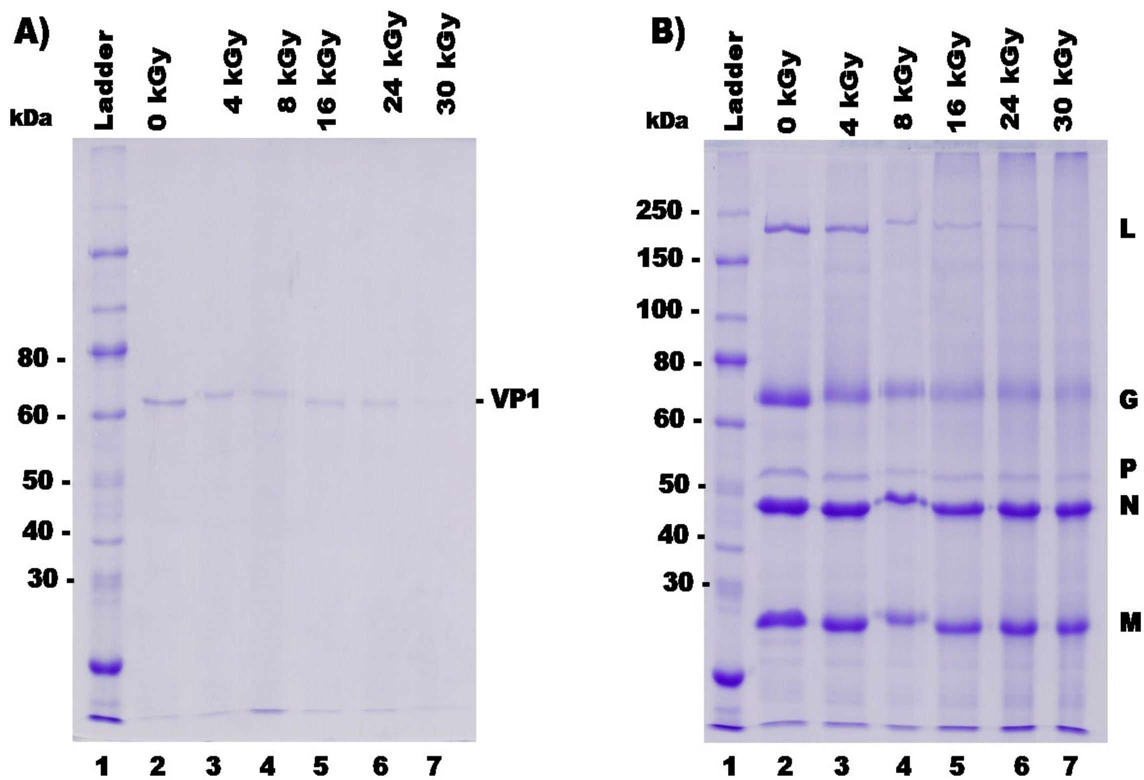


Figure 17. SDS-PAGE viral protein analysis of untreated (0 kGy) and e-beam treated (4 to 30 kGy) purified MNV-1 (A) and VSV (B).

Based on the results of the TEM, SDS-PAGE, and Western blots, it appears that e-beam treatment degrades viral proteins. These results are in agreement with Feng et al. (2011). The researchers found degradation of MNV-1 and VSV viral proteins by up to 22.4 kGy of gamma irradiation. They observed a decrease in the MNV-1 VP1 capsid protein after irradiation treatment, while the susceptibility of the 5 VSV viral proteins varied (Feng et al., 2011). Feng et al. (2011) also reported that, for their Western blot analysis, MNV-1 VP1 and VSV G glycoproteins diminished with increased gamma irradiation doses, but the viral proteins were still reactive with antibodies and may have retained their amino acid sequences. With regards to irradiation and its effects on proteins, amino acids vary in their susceptibility. Amino acids containing sulfur (cysteine, cystine, methionine) or aromatic compounds (tyrosine, phenylalanine) tend to be susceptible to irradiation because they act as scavengers and will react with hydroxyl radicals more easily than aliphatic (alanine, leucine, valine) amino acids (Stewart, 2001; Diehl, 1995c). Histidine is another example of an amino acid that is highly sensitive to radiation, where the amino acid undergoes a high rate of deamination (Stewart, 2001; Diehl, 1995c). Also, crosslinking or aggregation of proteins can occur during irradiation, which may involve disruption of secondary and tertiary structures and exposure of reactive groups (Stewart, 2001). This crosslinking or aggregation may account for the extra bands that formed in the Western blots for both MNV-1 and VSV.

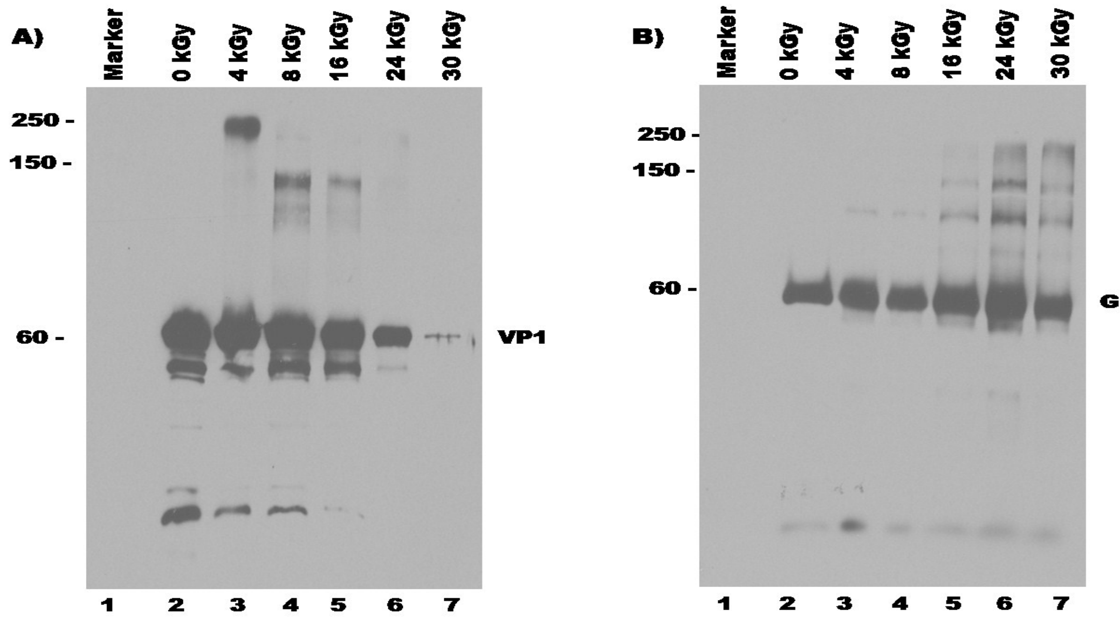


Figure 18. Western blot analysis of untreated (0 kGy) and e-beam treated (4 to 30 kGy) MNV-1 VP1 capsid protein (A) and VSV G glycoprotein (B).

Figure 19 illustrates the effect of e-beam irradiation on the genomic RNA of MNV-1 and VSV. Primers were designed to target the VP1 gene of MNV-1 and the nucleocapsid gene (N) of VSV. As the dose of e-beam increased, the strength of the PCR bands for both viruses decreased. No bands were detected for MNV-1 treated at 24 and 30 kGy, and for VSV at 30 kGy. Dickson (2001) states during irradiation, photons or electrons cause random breaks or lesions in the genetic material of the microorganism. If there are multiple lesions in the genetic material, the result would be lethal for the microorganism (Dickson, 2001). Also, the formation of hydroxyl radicals and hydrogen peroxide during radiolysis of water can react with bonds that bind the nucleic acids together (Dickson, 2001). Our results are also in agreement with Feng et al. (2011). The VP1 gene of MNV-1 was not detected in RT-PCR at doses above 20 kGy, thus

suggesting that the VP1 gene was degraded by irradiation (Feng et al., 2011). Also, our results were similar with regards to VSV, where the N gene was still detectable at 22.4 and 24 kGy, even though the virus was inactivated at 16 kGy. Feng et al. (2011) speculate that because VSV genomic RNA is housed within the N protein, this may have protected the genetic material from the effects of irradiation. This may account for why VSV was inactivated by 16 kGy of irradiation, but viral RNA was still detectable at higher doses.

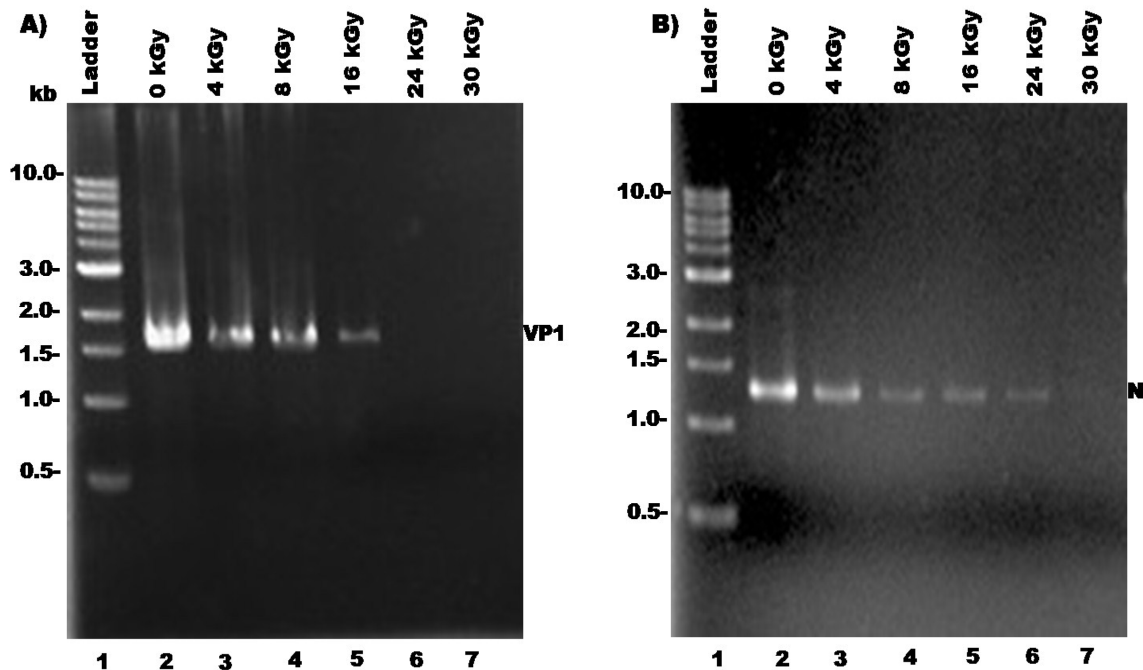


Figure 19. RT-PCR of MNV-1 (A) and VSV (B) before and after e-beam treatment.

3.5. Conclusion

The human norovirus surrogate, MNV-1, was much more resistant to e-beam treatment (requiring 24 kGy in PBS and 30 kGy in DMEM for complete inactivation) than VSV (complete inactivation at 16 kGy). Based on the mechanistic study, e-beam,

like gamma irradiation, disrupted virion structure and degraded viral proteins and genomic RNA. Viruses tend to be more resistant to ionizing radiation than bacteria, parasites, yeasts, or fungi due to their much smaller size and simple structure. However, it appears that the combination of disrupting the structure, viral proteins, and RNA are all essential in viral inactivation by irradiation.

CHAPTER 4

SODIUM HYPOCHLORITE SANITIZER INACTIVATION AND MECHANISM AGAINST A HUMAN NOROVIRUS SURROGATE (MURINE NOROVIRUS 1) AND COMPARISION TO AN ENVELOPED VIRUS (VESICULAR STOMATITIS VIRUS)

4.1. Abstract

This study evaluated the effectiveness of sodium hypochlorite solutions at sanitizer concentrations typically used in the food industry (5 to 1,000 ppm sodium hypochlorite) and at various exposure times (0 to 30 min) at inactivating the human norovirus surrogate, murine norovirus 1 (MNV-1). The inactivation of this virus was compared to that of an enveloped virus, vesicular stomatitis virus (VSV). MNV-1 was much more resistant to chlorine, being able to withstand up to 400 ppm of chlorine for up to 10 minutes, while VSV was completely inactivated at 20 ppm. This study also attempted to provide some mechanistic insight into viral inactivation by chlorine examining if the viral proteins and genetic material were degraded. Both MNV and VSV did not appear to have any significant changes in viral proteins or RNA after treatment with 200 and 10 ppm of chlorine, respectively, as seen with transmission electron microscopy, SDS-PAGE, and RT-PCR. It is suggested that chlorine inactivation of

viruses occurs due to degradation of the viral proteins or genetic material. However, based on the results of our study, the mechanism of chlorine inactivation of viruses remains inconclusive.

4.2. Introduction

Enteric viruses, specifically noroviruses, are important pathogens associated with food, water, and the environment. In the United States annually, noroviruses are estimated to cause 58% of all foodborne illness cases out of all foodborne pathogens (bacterial, viral, and parasitic), approximately 14,663 hospitalizations, and 149 deaths, though these numbers are likely underestimated due to underreporting, lack of sensitive detection methods, or asymptomatic infections (Scallan et al., 2011; Koopmans et al., 2002; Koopmans and Duizer, 2004). Noroviruses can be spread via contaminated shellfish or fresh produce, contaminated water, food handled by an infected food handler, or by aerosolization of vomitus (Koopmans et al., 2002; Hirneisen et al., 2010). Noroviruses are known for their high infectivity and low infectious dose (less than 10 particles may be sufficient to cause illness), environmental stability and persistence (capable of withstanding pH values of 3 or less, arid conditions, and refrigeration or freezing temperatures), and their resistance to disinfection (Li et al., 2011; Cliver, 2009; Buesa and Rodriguez, 2006; Greening, 2006). Unfortunately, research on human noroviruses has been hampered due to lack of successful cultivation techniques in the laboratory and also the lack of a small animal model to study pathogenesis and immunology (Li et al., 2011).

Chlorine compounds, especially sodium hypochlorite, are widely used sanitizers and disinfectants. However, noroviruses and their surrogates have been shown variable resistance to these compounds. Feline calicivirus (FCV) and canine calicivirus (CaCV), in aqueous suspension, were completely inactivated after 10 and 30 minutes of treatment with 3,000 ppm of sodium hypochlorite, but at 300 ppm FCV (less than 2 log reduction) was much more resistant than CaCV (greater than 3 log reduction) (Duizer et al., 2004b). D'Souza and Su (2010) found that high titers (approximately 7 log PFU/ml) of FCV and bacteriophage MS2 on formica coupons experienced a greater than 6 log reduction in titer after treatment with 5,000 ppm available chlorine for 0.5 and 1.0 min, but MNV-1 only experienced a 2.53 to 2.73 log reduction. Gulati et al. (2001) reported only a 0.3, 0.3, and 1.1 log reduction of FCV on stainless steel disks treated with 200, 400, and 800 ppm of sodium hypochlorite, respectively. Kim et al. (2012) reported a less than 1 log reduction of MNV-1 and FCV on stainless steel coupons treated for 5 min with 200 ppm hypochlorite, and that 1,000 ppm produced a greater than 1 log reduction for both viruses. Using partially purified FCV (to minimize organic contamination), Urakami et al. (2007) reported that the virus was sensitive to 0.3 mg/L of free chlorine, resulting in a 4.6 log reduction in infectivity after 5 min of treatment. Belliot et al. (2008) reported that MNV-1 titer (both log PFU/ml and log copy RNA/ml) was sensitive to 2,600 ppm of hypochlorite after treatment of 0.5, 1.0, and 3.0 min, and it was likely that the MNV-1 capsid was denatured by this concentration. The researchers also report that further studies should be conducted to see if the MNV-1 capsid can withstand lower concentrations of hypochlorite (Belliot et al., 2008). Nowak et al. (2011) reported that the RT-QPCR targets of three GII.4 norovirus isolates experienced a less than 0.01%

survival rate (equivalent to a 4 log reduction) after exposure to 600 ppm hypochlorite for 30 minutes under light soil conditions.

The mechanism of chlorine inactivation of viruses has yet to be discovered. It is believed that chlorine compounds will either denature viral capsid proteins or nucleic acids (Gerba, 2009). Wigginton and Kohn (2012) report that proposed viral inactivation mechanisms by chlorine vary greatly "and are often contradictory." Nuanualsuwan and Cliver (2003) found that the primary target of hypochlorite is the viral capsid, which was tested on HAV, FCV, and poliovirus. They also noted that poliovirus and FCV RNA was not detected after hypochlorite treatment using RT-PCR, but HAV RNA was still detectable (Nuanualsuwan and Cliver, 2003). The researchers suggest that hypochlorite is an effective agent, and that it may affect the viral capsid, genome, or both (Nuanualsuwan and Cliver, 2003). Li et al. (2002) report that 30 minutes of exposure to 10-20 mg/L of chlorine, the 5'NTR region of the HAV genome was sensitive to chlorine attack, but that HAV antigenicity was still present. To date, no studies are available giving mechanistic insight into chlorine inactivation of MNV-1.

The objectives of this study were to: 1) examine sodium hypochlorite sanitizer concentrations (10 to 1000 ppm) and its efficacy in inactivating MNV-1 and VSV in aqueous solution, and 2) use electron microscopy, SDS-PAGE, and RT-PCR to provide some insight on the viral inactivation mechanism by chlorine.

4.3. Materials and Methods

4.3.1. Cell Culture and Virus Stock

Murine norovirus 1 (MNV-1) was kindly provided by Dr. Herbert W. Virgin IV (Washington University School of Medicine, St. Louis, MO). MNV-1 was propagated in the RAW 264.7 cell line (mouse leukaemic monocyte macrophage cell line; American Type Culture Collection, Manassas, VA). Cells were cultured in 150 cm^2 tissue culture flasks (Corning Inc., Corning, NY) containing high-glucose Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco-Invitrogen, Grand Island, NY), 25 mM HEPES buffer (Gibco-Invitrogen), and 2 mM Gluta-MAX-1 (Gibco-Invitrogen). The growth medium was removed and cells were infected with MNV at a multiplicity of infection (MOI) of 1. Flasks were incubated at 37°C, 5% CO_2 for 1 h with agitation every 15 minutes. DMEM supplemented with 2% FBS was added to the flask and was incubated at 37°C, 5% CO_2 for 24 h. When extensive cytopathic effects (CPE) were observed, flasks were subjected to three freeze-thaw cycles (-80°C, 37°C) to lyse cells and release virus particles. The cell and virus suspensions were dispensed into 50 ml conical centrifuge tubes (USA Scientific, Ocala, FL) and were centrifuged using an Allegra 6R centrifuge with a GH-3.8 swinging bucket rotor (Beckman Coulter, Brea, CA) at 3,000 rpm for 20 min to remove any remaining cellular debris. The supernatant fluid was collected and stored at -80°C until ready for use. The MNV-1 titer was determined to be 8.24 $\pm$ 0.18 log PFU/ml.

Vesicular stomatitis virus (VSV; Indiana strain) was kindly provided by Dr. Sean Whelan (Harvard Medical School, Boston, MA). VSV was propagated in BHK-21 cells (baby hamster kidney cells; ATCC) using 150 cm^2 tissue culture flasks containing

DMEM supplemented with 10% FBS, 25 mM HEPES buffer, and 2 mM Gluta-MAX-1. The growth medium was removed and cells were infected with VSV at an MOI of 0.01. Flasks were incubated at 37°C, 5% CO_2 for 1 h with agitation every 15 minutes. DMEM supplemented with 2% FBS was added to the flask and was incubated at 37°C, 5% CO_2 for 24 h. VSV was harvested by centrifugation at 3,000 rpm at 4°C for 20 minutes to remove any remaining cellular debris. The supernatant was collected and stored at -80°C. The VSV titer was determined to be 8.93 $\pm$ 0.27 log PFU/ml.

4.3.2. Sodium Hypochlorite Test Solutions

Bleach (6% sodium hypochlorite) was purchased from a local retailer. 9.9 ml solutions of varying concentrations of sodium hypochlorite (20, 50, 100, 200, 400, 800, and 1000 ppm for MNV-1; 5, 10, and 20 ppm for VSV) were prepared in sterile distilled, deionized water. To ensure that the solutions were the correct concentration of sodium hypochlorite, a Chlorine Ultra HR Ion Specific Meter (HI 95771; Hanna Instruments, Inc., Woonsocket, RI) was used according to the manufacturer's instructions. For the neutralized chlorine control samples (0 m), hypochlorite solutions were neutralized with 0.25 M sodium thiosulfate (1:1, v/v) prior to addition of virus stock (Belliot et al., 2008). Either 1.1 ml of MNV-1 or 0.1 ml of VSV stock was added to the treatment solution. 1 ml aliquots were taken at each time point (0.5, 1.0, 5.0, 10, and 30 min) and were neutralized with 0.25 M sodium thiosulfate (1:1, v/v) to stop any hypochlorite activity. Neutralized samples were then serially diluted with DMEM (no serum) and applied to plaque assays.

4.3.3. Viral Plaque Assays

Confluent monolayers of RAW 264.7 or Vero (African Green Monkey kidney epithelial cells; ATCC) cells were grown in 6-well CellBIND plates (Corning) containing DMEM with 10% FBS for 24 h at 37°C, 5% CO_2. The growth medium was removed, and 0.2 ml of each sample dilution was applied to the wells in duplicate. Plates were incubated for 1 h at 37°C, 5% CO_2, with agitation every 15 min to evenly disperse the virus and to allow virus to attach to cells. Each well was overlaid with 2 ml MEM (Earle's balanced salts) supplemented with 5% FBS, 1.6% sodium bicarbonate (7.5% w/v; Fisher), 0.5% penicillin-streptomycin (10,000 units of penicillin and 10,000 µg/ml of streptomycin in 0.85% saline; Gibco-Invitrogen), 2.5% HEPES, 1% glutamine, and 1.5% low melting point agarose (Gibco-Invitrogen). Plates were refrigerated (4°C) for at least 30 min or until the MEM overlay was solidified. Plates were then incubated at 37°C, 5% CO_2 for 48 h.

After incubation, each well was fixed with 2 ml of 10% formaldehyde (Fisher Scientific) in PBS for at least 2 h. The formaldehyde and overlay was removed, and each well was stained with 0.05% (w/v) crystal violet for at least 1 h to visualize plaques.

4.3.4. Purification of MNV-1 and VSV

Purification of MNV-1 and VSV was performed as described previously (Feng et al., 2011). To generate large stocks of purified MNV-1, 20 confluent flasks of RAW 264.7 cells were infected with MNV-1 at an MOI of 0.01. Flasks were incubated at 37°C, 5% CO_2 for 1 h with agitation every 15 minutes. DMEM supplemented with 2% FBS was added to the flask and was incubated at 37°C, 5% CO_2 for 48 h. When

extensive cytopathic effects (CPE) were observed, flasks were subjected to three freeze-thaw cycles (-80°C, 37°C) to lyse cells and release virus particles. The cell and virus suspensions were centrifuged using an Sorvall RC-5C Plus centrifuge (Kendro Lab Products, Newtown CT) with a Sorvall SS-34 rotor at 12,000 rpm for 10 min to remove any remaining cellular debris. The supernatant was collected and digested with DNase I (10 µg/ml) and $MgCl_2$ (5 mM) at room temperature for 1 h. DNase activity was halted by addition of 1% lauryl sarcosine in 10 mM of EDTA. The virus suspension was centrifuged in an Optima L-100 XP ultracentrifuge (Beckman Coulter, Fullerton, CA), using a Ty 50.2 rotor, at 30,000 rpm for 6 h at 4°C. The supernatant was discarded, and the viral pellets were resuspended in 200 µl of PBS overnight at 4°C on ice. Viruses were further purified by ultracentrifugation in a sucrose gradient (7.5 to 45%) in a SW55 Ti swinging bucket rotor at 41,000 rpm for 6 h at 4°C. The virus pellets were resuspended in 100 µl of PBS overnight at 4°C on ice. The purified virus suspension was stored at -80°C.

To generate large stocks of VSV, 10 confluent flasks of BHK-21 cells were infected with VSV at an MOI of 0.01. Flasks were incubated at 37°C, 5% CO_2 for 1 h with agitation every 15 minutes. DMEM supplemented with 2% FBS was added to the flask and was incubated at 37°C, 5% CO_2 for 24 h. The cell and virus suspensions were centrifuged using an Allegra 6R centrifuge with a GH-3.8 swinging bucket rotor (Beckman Coulter) at 2,000 rpm for 5 min to remove any remaining cellular debris. The supernatant was collected and was ultracentrifuged at 21,000 rpm for 90 min at 4°C. The supernatant was discarded and the virus pellets were resuspended in 500 µl of NTE buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA) overnight at 4°C on ice. The virus

was further purified by ultracentrifugation with a sucrose cushion (10% sucrose in NTE buffer) at 41,000 rpm for 1 h at 4°C. The supernatant was discarded and the pellet was resuspended in 300 µl NTE buffer overnight at 4°C on ice. The purified virus suspension was stored at -80°C.

To determine the virus titer of purified MNV-1 and VSV, plaque assays were performed as described above. The titer of purified MNV-1 was 10.22 $\pm$ 0.06 log PFU/ml, and VSV was 11.92 $\pm$ 0.24 log PFU/ml. Viral protein content was measured by the Bradford assay, and the protein concentrations were 1 mg/ml for purified MNV-1 and 10 mg/ml for purified VSV.

4.3.5. Transmission Electron Microscopy (TEM)

20 µl of purified MNV-1 was applied to either 20 µl of sterile water (0 m control) or 20 µl of hypochlorite treatment solution (200 ppm) and were treated for 1.0, 5.0, and 10 min. 10 µl of purified VSV was applied to 90 µl of sterile water (0 m control) or 90 µl of hypochlorite treatment solution (10 ppm) and were treated at the time points mentioned above. Samples were not neutralized with sodium thiosulfate to prevent salt crystal formation during fixing and staining for TEM. 20 µl aliquots of sample were removed at each time point, fixed on copper grids (Electron Microscopy Sciences, Hatfield, PA), and negatively stained with 1% ammonium molybdate. Samples were analyzed using a FEI Tecnai G2 Spirit transmission electron microscope at 80 kV at the Microscopy and Imaging Facility at The Ohio State University. TEM pictures were taken using a MegaView III side-mounted charge-coupled-device (CCD) camera.

4.3.6. SDS-PAGE Analysis of Viral Proteins

For the control sample, 35 µl of purified MNV-1 was added to 35 µl of sterile water, and 50 µl of purified VSV was added to 450 µl sterile water. For treatment samples, 160 µl of purified MNV-1 was added to 160 µl of treatment solution, and 100 µl of purified VSV was added to 900 µl of treatment solution (200 ppm for MNV-1, 10 ppm for VSV). At each time point (1.0, 5.0, and 10 m), a 100 µl aliquot was removed and neutralized with 10 µl of sodium thiosulfate.

10 µl of untreated and e-beam treated purified virus suspensions were boiled in loading buffer (1% sodium dodecyl sulfate [SDS], 2.5% β-mercaptoethanol, 6.25 mM Tris-HCl [pH 6.8], and 5% glycerol) for 5 minutes. Samples were loaded onto a 12% polyacrylamide gel and analyzed using SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). Viral proteins on the gel were visualized using Coomassie blue staining.

4.3.7. Viral RNA Extraction and Reverse Transcription PCR (RT-PCR)

From the samples in 4.3.6., 10 µl of the control or treated samples were used for RNA extraction. RNA was extracted using an RNeasy minikit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Reverse transcription polymerase chain reaction (RT-PCR) was performed using a One-Step RT-PCR kit (Qiagen). Two primers targeting the MNV-1 VP1 capsid gene, and two primers targeting the VSV nucleocapsid (N) gene were used, and are listed in Table 7. The 50µl RT-PCR reaction mixture consisted of 400 µM each deoxynucleoside triphosphate (dNTP), 0.6 µM of each primer,

4 μl of RNA template, 5 units of RNase inhibitor, and 2 μl of RT-PCR enzyme mix. The amplified PCR products were analyzed using 1% agarose gel electrophoresis.

4.3.8. Statistical Analyses

Plaque assays were conducted in duplicate and experiments were repeated three times. Results were reported as log PFU/ml ± standard deviation. Data were analyzed using the General Linear Model function and Tukey's pairwise comparison test in Minitab 16 (Minitab, Inc., State College, PA). Also, a two-way analysis of variance (ANOVA) was performed to determine if the effects of hypochlorite concentration (ppm), time (min), and their interaction were significant. A p-value of less than 0.05 was considered statistically significant.

4.4. Results and Discussion

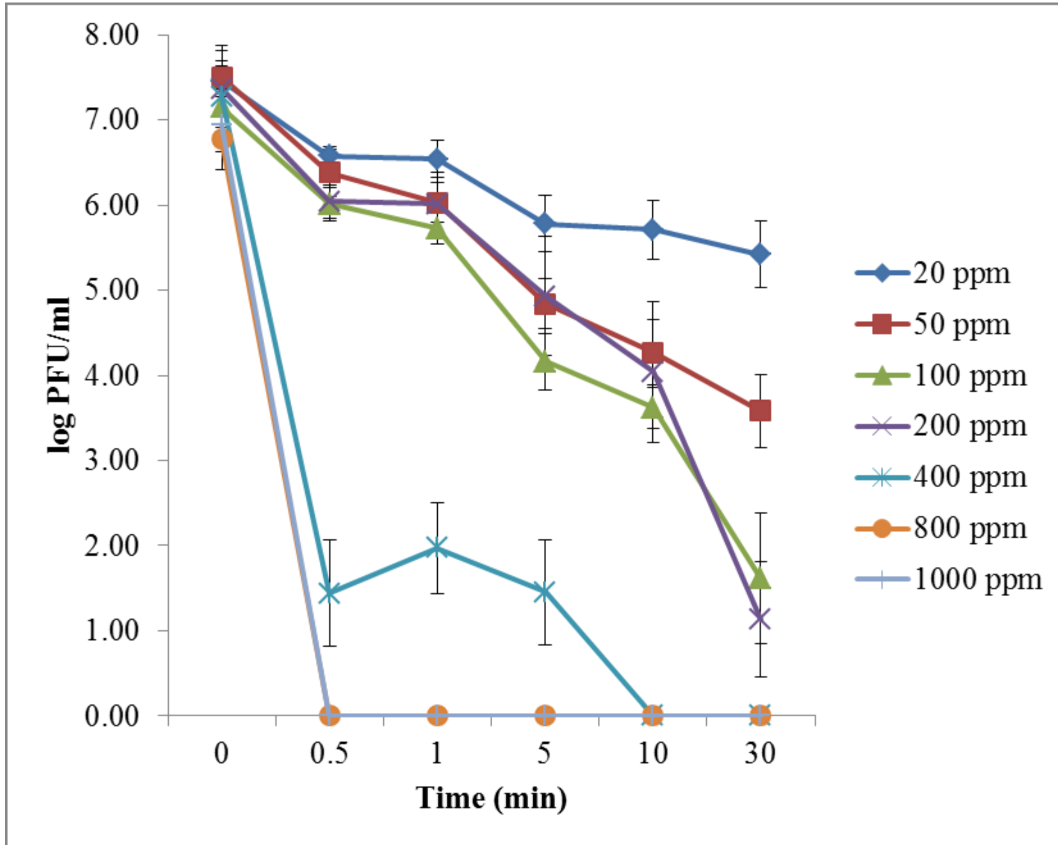


Figure 20. Sodium hypochlorite inactivation of MNV-1 in aqueous solutions. Data are the means of three replicates. Error bars represent standard deviation.

Figure 20 and Table 10 illustrate the MNV-1 and VSV titer, respectively, after being treated with varying concentrations of sodium hypochlorite and exposure times. MNV-1 was resistant to 20 and 50 ppm of chlorine, with about a 1 log reduction after treatment for 1.0 minute, and a 2 and 4 log reduction after 30 minutes, respectively. 100 and 200 ppm of chlorine resulted in almost a 1.5 log reduction after 1 minute, but over 1 log MNV-1 remained after 30 minutes of treatment. MNV-1 appeared to be sensitive to chlorine concentrations of 800 and 1000 ppm, since no virus was recovered. VSV was not completely inactivated with 5 ppm chlorine (over 2.5 log reduction in titer after 1.0

minute, and over 4.5 log reduction after 30 minutes), or 10 ppm after 5.0 minutes. VSV was much more sensitive to chlorine treatment, requiring at least 10 minutes of treatment at 10 ppm of chlorine for complete inactivation. The virus was not detected at any treatment time involving 20 ppm. Based on a two-way ANOVA, the effect of concentration (ppm), exposure time (min), and their interaction were found to be significant ($P < 0.05$). Also, no significant differences were observed between 0.5 and 1.0 min of treatment time for MNV-1 (20 to 200 ppm) and VSV (5 and 10 ppm).

Table 10. Sodium hypochlorite inactivation of VSV (log PFU/ml ± standard deviation) in aqueous solutions. Data are the mean of three replicates. Means within columns with different letters are significantly different ($p < 0.05$).

	Log PFU/ml ± S.D.		
Time (min)	5 ppm	10 ppm	20 ppm
0.0	6.97 ± 0.27 a	6.83 ± 0.24 a	7.18 ± 0.29
0.5	4.89 ± 0.49 b	1.82 ± 1.18 b	ND
1.0	4.33 ± 1.66 b	1.86 ± 0.96 b	ND
5.0	3.90 ± 1.51 bc	0.47 ± 0.41 c	ND
10.0	3.78 ± 1.44 bc	ND	ND
30.0	2.37 ± 1.98 c	ND	ND

ND – not detectable

The MNV-1 inactivation results here are similar to what other researchers reported in the literature. Baert et al. (2009a) found that washing shredded iceberg lettuce with 200 ppm of chlorine only yielded a 1 log reduction in MNV-1 inoculated onto the produce. Predmore and Li (2011) found that washing fresh strawberries with tap water only yielded a 0.8 log reduction of MNV-1, and that washing with 200 ppm chlorine provided an additional 1 log reduction. Gulati et al. (2001) observed only a 0.3 log reduction in FCV titer on stainless steel disks using 200 ppm chlorine, and observed

no reduction of FCV titer inoculated onto lettuce and strawberries washed with 200 ppm chlorine. Kim et al. (2012) reported a less than 1 log reduction of MNV-1 and FCV on stainless steel coupons treated for 5 min with 200 ppm hypochlorite. The results from these prior studies confirm that norovirus surrogates tend to be more resistant to 200 ppm of chlorine, whether on food contact surfaces or on fresh produce. To achieve a 2 to 3 log reduction, higher chlorine levels would be required, but this is not feasible for fresh produce (chlorine would be detrimental to sensory attributes) or for food contact surfaces (chlorine compounds are corrosive and concentrations above 200 ppm would require rinsing) (Baert et al., 2009b; Marriott and Gravani, 2006).

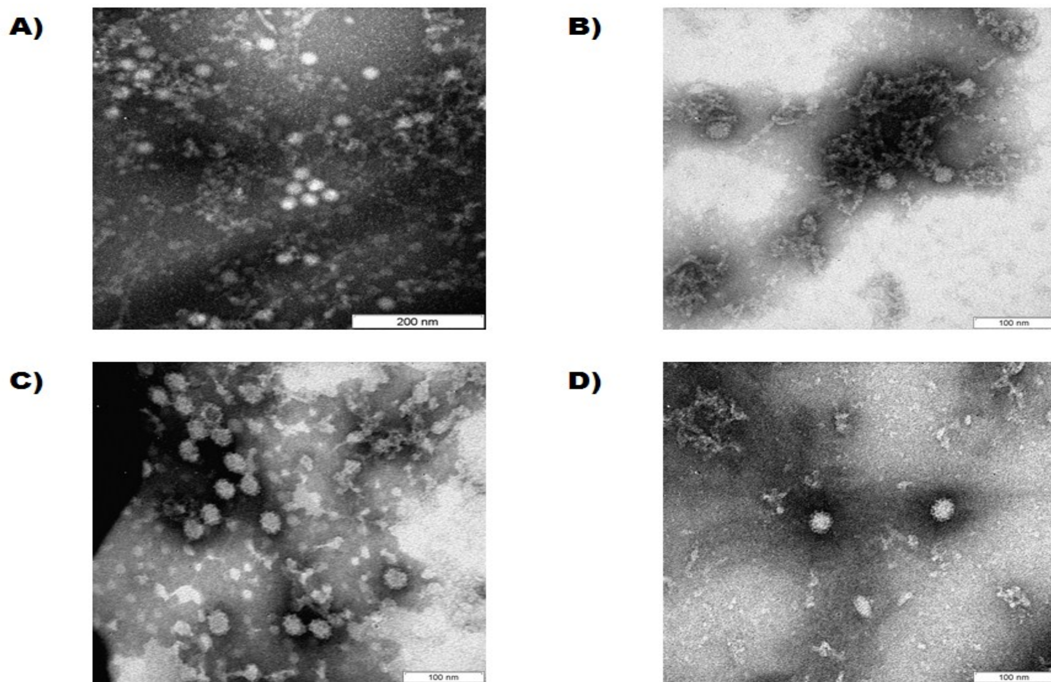


Figure 21. TEM photographs of MNV-1 treated with 200 ppm chlorine. A) Untreated control, B) 10 ppm, 1.0 min, C) 10 ppm, 5.0 min, D) 10 ppm, 10 min. Different magnification levels (200 nm for the control, 100 nm for the treated samples) were used to determine if morphological changes to the virus particles.

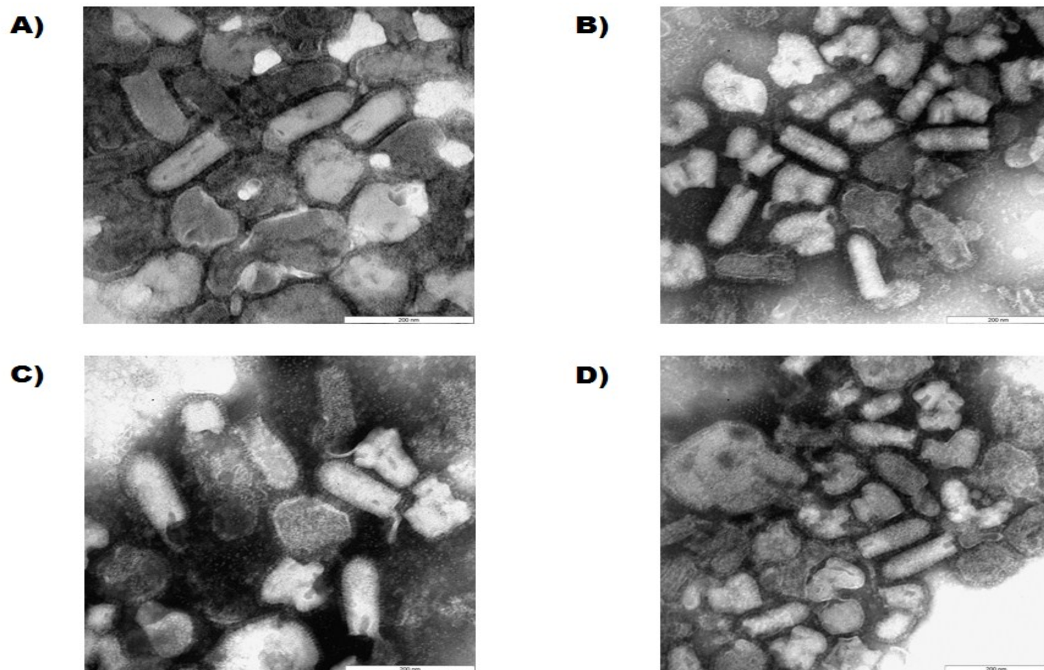


Figure 22. TEM photographs at 200 nm magnification of VSV treated with 10 ppm chlorine. A) Untreated control, B) 10 ppm, 1.0 min, C) 10 ppm, 5.0 min, D) 10 ppm, 10 min.

We attempted a mechanistic analysis to determine chlorine's mechanism of viral inactivation for both MNV-1 and VSV. TEM was performed to see if there was physical damage to the virus particles. Figures 20 and 21 depict TEM images of MNV-1 and VSV untreated and treated with 200 and 10 ppm chlorine, respectively, for 1.0, 5.0, and 10 min. Based on the images, it does not appear that there was any physical damage to the MNV-1 and VSV particles. For VSV, the glycoprotein spikes on the exterior of the virus appear to be intact. There are a number of non-bullet shaped or dark viral particles, but this may be due to the presence of defective interfering (DI) particles which form during VSV replication. These DI particles are generated either by using a high multiplicity of infection or repeated, undiluted passage of the virus (Keene et al., 1978).

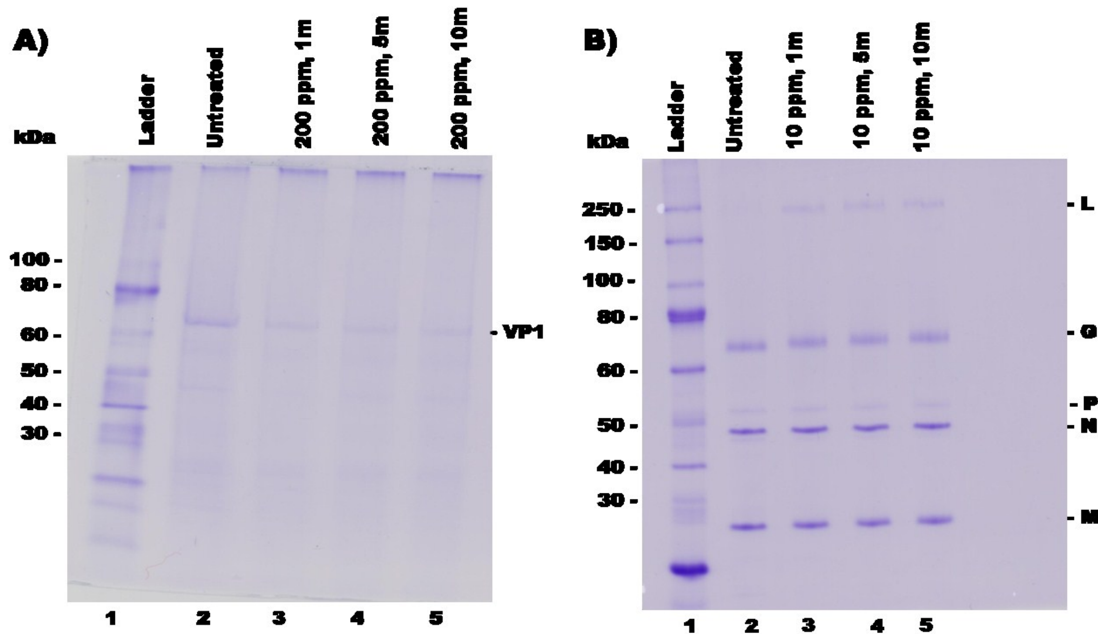


Figure 23. SDS-PAGE of MNV-1 (A) and VSV (B) treated with 200 and 10 ppm chlorine, respectively, for 1.0, 5.0, and 10 min.

To examine if there was changes in the viral proteins, SDS-PAGE of the untreated and chlorine treated viral particles were performed (Figure 22). Untreated and chlorine-treated MNV-1 (200 ppm for 1.0, 5.0, and 10 minutes) protein bands appeared, but were very faint. The purified protein concentration of MNV-1 is only 1 mg/ml (as compared to 10 mg/ml for VSV), and this may account for the differences in strengths of bands between the two viruses. For MNV-1, the band intensity decreases slightly when comparing the control and treated samples. This suggests that 200 ppm of chlorine may degrade MNV-1 viral capsid proteins, but not completely. For VSV, it appears that chlorine treatment, regardless of exposure time, did not significantly affect the viral proteins of VSV, since bands did not change in intensity during treatment. These appear consistent with the TEM images. However, VSV maintained its infectivity when exposed to 10 ppm of chlorine for up to 5.0 minutes (Table 11). If there were changes to

the viral proteins due to chlorine treatment, they were not detected by TEM or SDS-PAGE. Li et al. (2002) reported that HAV lost its ability to attach to host cell receptors but retained its antigenicity after exposure to 10 and 20 mg/L of chlorine for 30 minutes.

Nuanualsuwan and Cliver (2003) suggest that hypochlorite also has the capability of degrading viral RNA. To examine this, we conducted an RT-PCR analysis of MNV-1 and VSV treated with chlorine (Figure 23). For MNV-1, strong bands appeared for both the control and treated samples, thus suggesting that hypochlorite did not have an effect on the VP1 capsid gene of the virus. It has been noted that viral RNA can be resistant to environmental stressors, provided that the protein capsid remains intact (Nuanualsuwan and Cliver, 2003). Duizer et al. (2004b) reported that concentrations of greater than 300 ppm of sodium hypochlorite were effective in reducing the infectivity of animal caliciviruses (FCV and CaCV), however viral RNA was still detectable with real-time RT-PCR after chlorine treatment. The researchers also note that detection of viral RNA does not necessarily indicate the presence of infectious organisms (Duizer et al., 2004b). O'Brien and Newman (1979) also reported that poliovirus RNA was released and degraded into smaller fragments after being treated with 1 mg/L of free chlorine. However, the researchers also report that it was not known whether poliovirus inactivation by chlorine was the result of RNA release or if the virus capsid was degraded (O'Brien and Newman, 1979). VSV RNA appeared on both the untreated and 10 ppm treated samples, and it does not appear that the viral RNA was degraded by chlorine. It should be noted that VSV RNA is encapsidated by the nucleocapsid protein, and this may provide a protective effect for the RNA against disinfection practices (Feng et al., 2011). Li et al. (2002) reported that the 5'NTR region of HAV RNA was most susceptible to

chlorine treatment (10 to 20 mg/L for 30 minutes), but the coding region was resistant. The researchers stated that the 5'NTR region has a stem-loop structure on the 5' end, and this is required for protein signal identification (Li et al., 2002). They also state that the 5'NTR is also involved in viral RNA replication, translation of proteins, and virus particle composition, thus if this area was damaged by chlorine then HAV would be inactivated (Li et al., 2002). O'Brien and Newman (1979) also note that chlorine-inactivated poliovirus capsid proteins did not undergo a conformational change and retained the same isoelectric point as untreated viral particles. They found that although there was no change in the protein conformation, the inactivated virus particles were still able to attach to host cells, but did not cause infection (O'Brien and Newman, 1979). The results obtained in our study, as well as that of other research, indicate that the mechanism of chlorine inactivation of viruses is elusive, and it appears this may potentially vary between viral species.

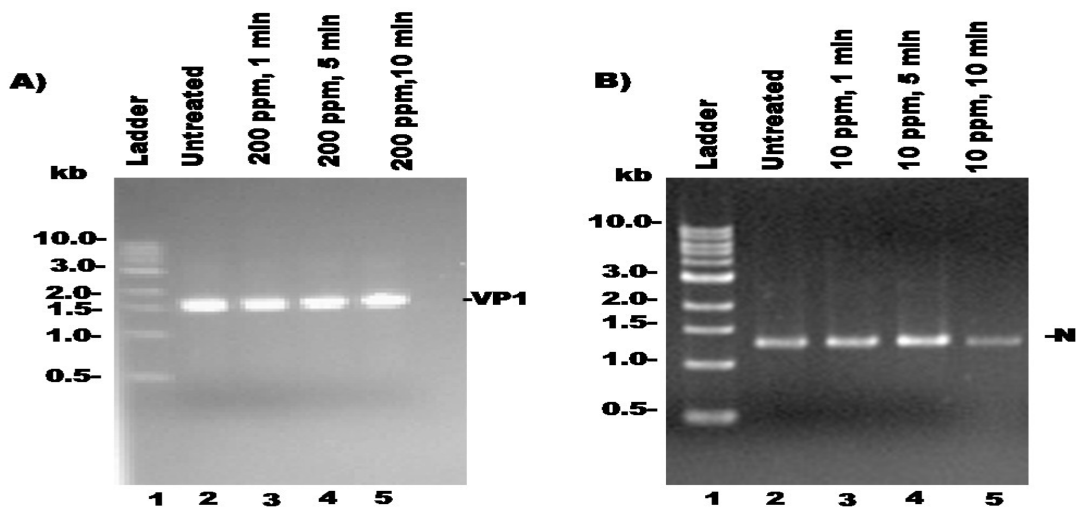


Figure 23. RT-PCR analysis of MNV-1 (A) and VSV (B) treated with chlorine (200 and 10 ppm, respectively) for 1.0, 5.0, and 10 min.

4.5. Conclusion

As expected, MNV-1 (a nonenveloped virus) was much more resistant to chlorine treatment than VSV (an enveloped virus). MNV-1 required chlorine concentrations above 400 ppm for complete viral inactivation, while VSV was completely inactivated at 20 ppm. The mechanism of chlorine inactivation remains inconclusive. For MNV-1, it does not appear that hypochlorite damages viral RNA, due to the protective effect of the viral capsid protein. It may be possible that the genes that code for the nonstructural proteins or minor capsid proteins may be susceptible to chlorine treatment, but this has yet to be determined. However, there may be degradation of the capsid protein after treatment with 200 ppm hypochlorite as observed with the SDS-PAGE analysis, which may play a role in viral inactivation. For VSV, there was no apparent damage to the viral particle, nor were there noticeable changes to the viral proteins or RNA. Even though chlorine did not affect the nucleocapsid gene segment of VSV RNA, perhaps chlorine affects the other genes that encode for the glycoproteins, matrix, phosphoproteins, or RNA synthesis.

CHAPTER 5

CONCLUSION AND FUTURE DIRECTIONS

Human noroviruses remain one of the most prevalent and important causes of foodborne gastroenteritis, more so than any other type of foodborne pathogen. Unfortunately, our knowledge of the biological properties of norovirus, as well as intervention strategies, is limited due to the inability to culture this virus in the laboratory. Therefore, we must rely on closely related surrogate viruses (most notably, murine norovirus, MNV-1) in order to predict how these resistant or susceptible these pathogens are to electron-beam (e-beam) irradiation and sodium hypochlorite sanitizers.

In Chapter 2, we evaluated the efficacy of e-beam (0 to 12 kGy) on inactivating MNV-1 in liquid model systems (phosphate buffered saline, PBS; Dulbecco's Modified Eagle medium, DMEM) and in fresh produce (shredded cabbage and cut strawberries. At doses of up to 12 kGy, MNV-1 was still present in both types of liquid media. It was also found that the more complex the liquid media, the more protection it afforded MNV-1 to e-beam irradiation. At irradiation doses of 4 kGy (approved for fresh produce by the FDA), e-beam irradiation only provided a 1 log or less reduction in MNV-1 inoculated onto both produce types. Even after 12 kGy of e-beam, MNV-1 still persisted in these food products. Irradiation doses above the FDA-approved limit of 4 kGy, would not be feasible for inactivating foodborne viruses in fresh produce, and would also result in

detrimental sensory characteristics (color loss, softening, degradation of vitamins and nutrients) in these food products. Further studies may examine the susceptibility of other human norovirus surrogates (Tulane virus, porcine sapovirus) or additional foodborne viruses (HAV, rotavirus) in different fresh produce products (green onions, raspberries) or aqueous systems (water, fruit juices).

In Chapter 3, we evaluated the efficacy of e-beam (0 to 30 kGy) at inactivating MNV-1 and VSV (an enveloped virus) in aqueous media (PBS and DMEM). We also examined e-beam's mechanism of viral inactivation. As expected, MNV-1 (a nonenveloped virus) was much more resistant to e-beam treatment, surviving in PBS at up to 16 kGy and DMEM at up to 24 kGy. VSV was much more susceptible, with complete inactivation at 16 kGy for both liquid samples. Based on our mechanistic studies, e-beam inactivated viruses by degrading the viral proteins and most importantly, the genetic material. Future research may include examining e-beam's effect and mechanism of inactivation against different types of foodborne viruses (rotavirus, which is a double-stranded RNA virus; adenovirus, which possesses double-stranded DNA; influenza virus, which is a potential enveloped foodborne virus). Further research could also utilize real-time RT-PCR to quantify human norovirus RNA before and after e-beam treatment.

Finally in Chapter 4, we evaluated sodium hypochlorite sanitizers (5 to 1,000 ppm chlorine) at inactivation of MNV-1 and VSV. VSV was susceptible to chlorine treatment, with complete inactivation of the virus at 20 ppm chlorine. MNV-1 was much more resistant, requiring concentrations above 400 ppm for complete inactivation. We also attempted to provide insight on the chlorine mechanism of viral inactivation,

however these results proved inconclusive. It has been suggested that viral inactivation by chlorine occurs by degrading viral proteins or RNA. Based on the results obtained with MNV-1 and VSV, we could not determine the exact mechanism of inactivation. Future work may include examining the susceptibility of other human norovirus surrogates (Tulane virus, porcine sapovirus) treated with the chlorine concentrations and exposure times used in this study. Using real-time RT-PCR, the susceptibility of human norovirus RNA to chlorine treatment may be further examined. Further research with chlorine's mechanism of viral inactivation may utilize different chlorine concentrations and exposure times (ranging from a low, medium, high, and extreme chlorine treatment up to 1,000 ppm) and their effects on the viral proteins or different segments of the RNA genome. Finally, Western blot analysis of untreated and chlorine-treated virus particles may be performed to see if viral proteins are still antigenic.

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