A Comparison of Centrifugal Forces to Reduce the Inhibitory Effects of Food Matrixes on Reverse Transcriptase Polymerase Chain Reaction for the Detection of Food Borne Viruses

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A Comparison of Centrifugal Forces to Reduce the Inhibitory Effects of Food Matrixes on Reverse Transcriptase Polymerase Chain Reaction for the Detection of Food Borne Viruses

by

Kristina Kim Carter

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science in Public Health Department of Environmental and Occupational Health College of Public Health University of South Florida

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Keywords: norovirus, poliovirus, foodborne illness, matrix inhibitors, RT-PCR

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A Comparison of Centrifugal Forces To Reduce The Inhibitory Effects Of Food Matrixes On Reverse Transcriptase Polymerase Chain Reaction For The Detection of Food Borne Viruses

Kristina Carter

ABSTRACT

The CDC estimated that foodborne infections resulted in approximately 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths per year in the United States (Mead, 1999). There are over 200 known diseases caused by viruses, bacteria, parasites, toxins, metals, or prions that can be transmitted through food. Of these illnesses caused by foodborne disease, the CDC estimates that 38.6 million cases are from identifiable pathogens and 30.9 million of these cases are caused by viruses. Hence, approximately 80% of foodborne illnesses of known etiology result from viral transmission (Mead, 1999). Viral gastrointestinal illness may be caused by virus families such as: enterovirus, rotavirus, calicivirus, astrovirus, or norovirus. These viruses are highly contagious and are spread through the fecal-oral route; transmission vehicles include contaminated food or beverages, infected food handlers, fomites or close contact with an infected individual (FDA Bad Bug Book, 2003).

Until recently, there have been few studies concentrating on viruses found in or on foods. There are several technical difficulties that hinder progress in detecting viral agents from foods. One of these problems is the presence of matrix inhibitors. Substances responsible for matrix inhibition include humic acid, polysaccharides,
myoglobins, metal ions, glycogen, and lipids (Monpoeho, 2001). These substances in foods produce smearing of the RT-PCR amplicon bands on agarose gels. Several methods to reduce inhibitory compounds utilize multiple toxic reagents in the procedure. In this study, varying centrifugal forces were tested at different steps of the virus extraction/concentration procedure to reduce matrix inhibitory effects for molecular detection of norovirus and poliovirus seeded onto food surfaces. This method incorporates the rapid detection capabilities of RT-PCR with the ability to reduce or eliminate matrix inhibitors present in food, by altering the centrifugal force.

Results for both viruses showed that band intensity decreased as the viral concentration decreased and no one method was superior for all food matrices. This investigation showed that matrix specific modifications to the basic protocol are required to efficiently extract viruses from the surface of foods. Each food should be assessed to determine modifications to the standard method that would be optimal for viral concentration and extraction.
Introduction

Crop production in the United States has dramatically decreased over the last few decades. In fact, the majority of the food consumed in the United States today is imported. Since food imports have sharply increased, the threat of importation of pathogenic organisms is an added concern to the food safety industry. There are over 200 known diseases caused by bacteria, parasites, toxins, metals or prions transmittable through food. These diseases range in severity from mild gastroenteritis to life-threatening neurological, hepatic and renal syndromes. Researchers from the Division of Bacterial and Mycotic Diseases at the Centers for Disease Control (CDC) estimated that the annual number of foodborne illnesses rose from 6.5 million in 1987 to 13.8 million in 1997 (Mead, 1999). The CDC estimates that foodborne infections caused 76-million illnesses, 325,000 hospitalizations, and 5,000 deaths per year in the United States (Mead, 1999). Of these only 38.6 million cases were from identifiable pathogens; 30.9 million of these cases were caused by viruses. Hence, approximately 50% of foodborne illnesses are from known etiology and of that, 80% of all foodborne illnesses result from known viral transmission such as, norovirus with approximately 23 million cases annually (Mead, 1999).

Surveillance of foodborne illness is complicated by three factors: under-reporting, pathogens transmitted through food may also be transmitted via person-to-person, and the fact that there are foodborne pathogens or agents transmitting disease that have not been identified (Mead, 1999). Under-reporting occurs when individuals with a
mild case of a foodborne illness do not go to the hospital or seek professional attention. As a result, the number of reported cases does not accurately represent the number of cases that actually occur in a community. This is also due to bias in reporting; more severe illnesses and large group outbreaks are more likely to be reported compared to those resulting in only mild diarrheal illnesses. Person-to-person transmission also complicates surveillance. Routes of contact are often untraceable, especially when considering travel and transportation between states and countries that can be accomplished in less than a day. The most notable difficulty regarding surveillance for agents of foodborne illnesses is that many of the pathogens of concern today (e.g., Campylobacter, E. coli O157:H7, Listeria) were not detectable until recently (Mead, 1999). Although many pathogens have been identified and detected from food, there are still many pathogens that remain unknown, yet may pose a threat to food safety.

A thorough assessment of the health hazards associated with foodborne viruses is complicated by the multitude of variables that exist in a viral-food relationship. These variables include understanding the virus’ binding and adaptation capabilities to a variety of food sources. Viruses are sub-cellular units containing genetic material in the form of DNA or RNA; they reproduce inside the living cells they invade (Potter, 1973). Many viruses harbored by foods cause acute gastroenteritis (AGE) with symptoms that include upper gastrointestinal upset followed by diarrhea for a one-to-four day duration (Goodgame, 2001). Although there are bacteria and protozoa capable of causing the same clinical symptoms, nearly 75% of all cases with these symptoms are caused by viruses (Mead, 1999). Unlike illness due to bacteria, viral illnesses rarely show symptoms other than diarrhea and vomiting. Viral gastrointestinal illness may be caused
by members of a number of virus families, such as enterovirus, rotavirus, calicivirus, astrovirus, or norovirus. These viruses are highly contagious and are spread through the fecal-oral route; transmission vehicles may include contaminated food or beverages, infected food handlers, fomites, or close contact with an infected person.

Until recently, there have been few studies that concentrated on viruses found in or on foods. The National Conference of Food Protection, sponsored by the American Public Health Association identified many technical difficulties other than surveillance methods that hinder the progress of identifying viral agents from foods. Among these there are less than adequate foodborne virus detection methods as well as incubation periods of viruses ranging from one to thirty days; both impair a conclusive relationship between the food and the agent (Potter, 1973). Incubation periods can vary from one (norovirus) to 30 days (Hepatitis A virus). Thus it is unlikely that an infected individual will be able to recall the reservoir of infection and the likelihood of obtaining the contaminated food source for testing is reduced. Additionally, there is a lack of detection methods for viruses in foods; to date many of the proposed methods using PCR have not been useful with a large variety of food items, other than bivalve mollusks (Schwab, 2000).

Noroviruses account for nearly 75% of all agents known to cause foodborne illness increasing the necessity for efficient detection methods (Mead, 1999). Unlike poliovirus, which grows in cell culture and is often used as a model for other viruses, detection of norovirus was limited because there was no cell culture or animal models that supported the growth of this virus (Goodgame, 2001). Since norovirus does not have the ability to grow in conventional cell culture, other detection methods have been
explored including Electron Microscopy (EM) and recently Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). RT-PCR focuses on the detection of RNA segments unique to the virus. Detection of norovirus from clinical samples can be completed within one to several working days. This rapid turn-around time has been effective in improving public health response to outbreaks of AGE.

The primary problem associated with PCR methods for the detection of viruses from foods and human waste is inhibition of nucleic acid amplification by matrix factors. Inhibitory substances responsible for matrix inhibition include humic acid, polysaccharids, myoglobins, metal ions, glycogen, and lipids (Monpoeho et. al., 2001). These inhibitory substances in foods can cause smearing of the amplicon bands on agarose gels reducing test sensitivity and decreasing detection capabilities. Methods, requiring multiple toxic reagents, have been attempted to reduce the levels of inhibitory compounds present for the detection of viruses from foods. In this study, variation of centrifuge speeds will be tested at different steps of the standard detection procedure used at the State of Florida DOH Tampa Laboratory in an attempt to reduce the inhibitory effect of the food matrix on the detection of poliovirus and norovirus from food surfaces.
Literature Review

Historically, viruses were mainly detected from water sources. This was of significant public health interest because a large number of viruses are excreted in human feces and urine, and at low concentrations these viruses can cause illness when ingested (Pinto, 1994).

Researchers found more than 70 viruses detectable in human feces. In addition to being found in sewage; many of these viruses were also identified from “fresh water” sources, such as, rivers, lakes, and estuaries. The viruses present in fresh water were due to the vast amount of fecal matter deposited into these sources. Human fecal matter and urine remain the most notable viral contamination sources (Berg, 1976).

Enteroviruses have been detected in all water environments including public water supply. Stringent water quality requirements have been successful in eliminating enterovirus from potable water sources, such as tap-water. However, the spread of viruses can occur through a variety of other methods, such as swimming pools, other recreational water sources, and food. Several sources, such as well water, still remain unfiltered and untreated and may be susceptible to contamination. Advancement in detection and purification technology has allowed effective removal of viruses from most potable water sources.

Despite the success of viral detection methods with water, these methods have been inefficient at recovering viruses from foods. In order to detect a virus from any
source whether water, sewage, sludge, or food, the virus must be eluted from the source and concentrated. This is necessary because enteroviruses, and other virus, such as norovirus, often exist at low levels in contaminated water or food. Concentration methods to detect viruses from water have focused on viruses having a net negative charge near the neutral pH (Berg, 1976). These methods include physical adsorption, precipitation, filtration phase partitioning, sedimentation under centrifugal force, and organic flocculation (Berg, 1976).

Physical Adsorption involves the virus binding to cellulose or fiberglass filters as water is passed through them. This method, as well as other membrane filter methods (ex. Zetapor filter), is based on the ability of the virus to reversibly adsorb to reactive surfaces due to electrochemical interaction. As water is passed through the filter, viruses are adsorbed to the filter surface by a number of possible mechanisms: hydrophobic bonding between non-polar aliphatic and aromatic groups on the surfaces of both the viruses and the filter, hydrogen bonding between polar groups on the surfaces of the viruses and the filters and salt bridge bonding between negatively charged groups on the surface of the viruses and the filters by adsorbed cations obtained from the solution (Berg, 1976). A study by Wallis and Melnick, in 1967, used the filter adsorption method to concentrate seeded viruses from raw sewage. The study implemented a fiberglass filter as a pre-filter and used a cellulose membrane as a second filter. They attempted to recover poliovirus from one gallon of raw sewage each day over a seven month period of time. The results from Wallis and Melnick’s experiment showed a 92% recovery of poliovirus and the ability to concentrate poliovirus by 50-fold from experimentally contaminated sewage.
Moore et. al., (1970) employed Wallis and Melnick’s method for recovery of seeded poliovirus from varying volumes of raw sewage, ranging from 0.25 liters to 4 liters. Moore’s recovery of seeded poliovirus from sewage average 30%, much lower than Wallis and Melenick’s of 92%.

Metcalf et. al., (1974) employed the use of portable filter concentrators that contained fiberglass cartridges. They used two types of filters: fiberglass cartridge dept filters and fiberglass asbestos epoxy filter discs. These filters were used in series to detect poliovirus from 95 liters of experimentally contaminated coastal water. Metcalf had 56-71% recovery of seeded poliovirus from experimentally contaminated coastal water.

The use of filter adsorption and concentration is the standard method for reclaimed waste water effluents and finished water, which do not require clarification prior to adsorption. The application of these methods to food requires modifications to overcome several limitations: suspended matter in the food eluent tends to clog the filter, organic material, also known as membrane-coating components (MCC,) interferes with adsorption by competing for sites on the filter and viruses that are bound to suspended matter may be washed off during a later step of the procedure (Berg, 1976). It is apparent from these studies that the use of filters for adsorption and concentration of viruses is effective for water and wastewater however, additions to the method are necessary for application to detect viruses from solid/semisolid foods.

The precipitation method for viral concentration involves the virus either precipitated (coagulated) by or adsorbed to various materials other than filters, such as, polyvalent cation salts, insoluble polyelectrolytes, or minerals. Effective adsorption
requires controlled pH and ionic conditions, because electrostatic forces are involved in the adsorption. The basis for this method involves an electrochemical interaction between charged groups on the virus protein coat and on the surface of the coagulant or adsorbent material. The most common polyvalent cation salts used are: aluminum hydroxide, aluminum phosphate, calcium phosphate, lime, ferric hydroxide, and protamine sulfate (Berg, 1976).

Wallis and Melnick (1967) studied the concentration of animal viruses using aluminum phosphate, aluminum hydroxide, and calcium phosphate precipitates, all at a pH of 6.0. They found aluminum hydroxide and calcium phosphate efficiently precipitate-out enteroviruses and adenoviruses, but did not precipitate-out reoviruses. Aluminum phosphate did not precipitate any of the viruses. Wallis used aluminum hydroxide to concentrate small amounts of seeded poliovirus from one liter of saline solution. Using this method recovery of poliovirus averaged about 82%.

Lal and Lund, in 1974, assessed precipitation of viruses using lime, aluminum sulfate, and ferric chloride. They experimentally contaminated 400ml of tap-water with the coxsackievirus B3 and attempted to precipitate the virus using lime, aluminum sulfate, and ferric chloride. Recovery of coxsackievirus was slightly higher than 50% for all three precipitants.

It is obvious from these studies that viral concentration methods involving either precipitation by or adsorption to materials such as: aluminum hydroxide, aluminum phosphate, calcium phosphate, lime, ferric hydroxide, or protamine sulfate, appear to best suited for detecting viruses in small volumes of water containing large amounts of virus.
Since viruses can infect at very low concentrations these methods are not appropriate for real-life scenario of viral infections from food sources.

Ultrafiltration is a membrane filtration process, in which the solvent (water) and small membrane-permeating substances (microsolutes) are driven through a membrane by pressure while macrosolutes such as viruses are retained because they are too large to penetrate the membrane pores. Prior to the development of soluble membranes these systems were limited by clogging, resulting in difficulty passing the water or sewage through the filter limiting recovery and retention of viruses. Soluble membranes are those that can be dissolved along with their retained viruses in small volumes of non-toxic solvents. The most common soluble membrane used is aluminum alginate gel membrane containing lanthanum ions which is soluble in sodium citrate (Berg, 1976).

Gaertner (1967) used a soluble ultrafiltration membrane to concentrate ten liters of poliovirus seeded tap-water to a volume of 1.5ml. The efficiency of poliovirus recovery averaged 66%. He also attempted this procedure with sewage and had an efficiency of 25% recovery. Borneff (1970), also used soluble alginate membranes and reported a low 25% viral recovery from one liter volumes of water.

Soluble ultrafilters have successfully recovered viruses from small volumes of clean waters. A major limitation of this method is that the membrane collects all extraneous matter in the water along with the virus. This poses a problem when the membrane is dissolved, leaving virus and all the extraneous matter which was also bound to the membrane in solution (Berg, 1976). Soluble ultrafilters would not be optimal for viral detection from food because they do not remove extraneous matter that may cause inhibition in detection.
Organic flocculation is based on lowering of the pH of a Beef Extract solution from 7.5 to 3.5 (Safferman, 1988 and Berg, 1982). Flocculation of beef protein occurs as the pH is lowered; the virus binds to the proteins in the solution and is co-precipitated. Centrifugation results in a pellet containing the virus-protein flocculate (Katzenelson et. al., 1976).

The Department of Health Bureau of Laboratories, tested varying concentrations, 1.5%, 3%, and 10%, of buffered beef extract (BBE) for viral recovery from food surfaces seeded with norovirus and poliovirus. The results showed that 1.5% and 3% BBE were very similar in there abilities to concentrate the virus. 1.5% concentration was found to be to dilute for the sample matrix, reducing the effectiveness for viral recovery. Three percent BBE was chosen as the standard because it contained a higher concentration of proteins for the virus to bind to during flocculation (Stark, personal communication).

Traore et al. (1998), researched four methods of viral extraction on mussels with RT-PCR detection. The four methods included: borate buffer extraction, glycine solution extraction, saline beef extraction, and saline beef Freon extraction. Each of these extractions was performed as the first step of the procedure. The viruses (poliovirus, Hep. A, and astrovirus) were concentrated by lowering the pH to 3.5 with stirring for 30 minutes. Although, the results showed no significant difference between the four extraction methods, efficiency differed: borate buffer > glycine solution > saline beef Freon > saline beef.

Taku et al, (2001), looked at two different methods for concentration of the norovirus from food contact surfaces. The first method was organic flocculation of 3% beef extract eluate. The second, was the filter-adsorption-elution method performed in
two steps: the 3% beef extract eluate was passed through a double layer of 25 mm diameter MDS filter, at a flow rate of 1-2 ml/min/cm$^2$ filter area, followed by elution of the virus bound to the filter with 1 ml of 0.05 glycine buffer containing 3% beef extract eluent. The average viral recovery for the filter-adsorption-elution method was 8%, and the average recovery using organic flocculation was 55% higher than the filter-adsorption-elution method. The results from this study illustrate several advantages of organic flocculation over filtration as a method for viral concentration. Organic flocculation does not require precipitating the virus from particulate matter that might clog filters, and the sediment pellet is compact and can be reconstituted in a small volume of buffer.

Many of the previous techniques have relied on laborsome methods of virus concentration and detection; these methods are expensive, time consuming, technically difficult and lack sensitivity (Jaykus et al., 1996). Organic flocculation using beef extract has been extensively used as a method for the concentration and detection of viruses from water sources because it overcomes some of the difficulties complicating viral concentration: 1) the small size of the viruses (25-100 nm), 2) low numbers of virus present for concentration from water, sewage, sludge, or food, 3) variability in amounts or types of virus present in water, sewage or sludge, 4) the water or food quality being tested (physical, biological, chemical characteristics), and 5) clogging of filters by competition between extraneous matter and virus matter (Huang et al., 2000). Organic flocculation succeeds where other method have failed in its ability to precipitate virus from particulate matter that may clog filters; and organic flocculation has the advantage
of a sediment pellet which can be reconstituted in a small volume of buffer (Taku et.al., 2001).

Historically, enteroviruses, such as poliovirus, have been used as model systems in the detection of viruses from water, sludge, sewage, and bivalve mollusks. Poliovirus is a useful surrogate model for other viruses, because of its ability to proliferate in cell culture, and bind tightly to substrates such as foods. Polioviruses ability to grow in cell culture can be used to enumerate levels of viable virus present (Richards, 1999). These factors enable better evaluation of detection capabilities of viruses from water or food samples.

**Poliovirus**

Poliovirus is a member of the enterovirus subgroup of the *Picornaviridae* family. The virus is believed to be ancient, causing disease dating back to the second millennium, or around Egyptian times. Physical and chemical properties of poliovirus affect transmission and detection of the virus. The virus is a non-enveloped, single-stranded RNA virus 30 nm in size and spherical in shape. The genome size ranges from 7.2 kb to 8.5 kb and contains a single long open reading frame, divided into three regions. Polioviruses are very resilient, stable at ambient temperature, a pH of less than 3.0 and 70% ethanol. (Pallansch and Roos, 2001) Inactivation occurs at 42°C and by exposure to ultraviolet (UV) light. Free chlorine ions, strong acidity solution, and formaldehyde will also inactivate the virus (Ackerman et.al., 1970).

Poliovirus or poliomyelitis virus was named because it affects the marrow near the spinal cord. Reports of paralysis with fever, were first described in the mid-1800s. Beginning the 1900s scientists recognized the communicable nature of poliomyelitis and
the importance of asymptomatic infected individuals in transmission of the poliovirus. Scientists later proved that the virus initiates infection and spreads in the gastrointestinal tract with the virus entering through the mouth and multiplying in the throat and the intestines (Pallansch and Roos, 2001). After infection, there is an incubation period of 4-35 days prior to development of symptoms such as: fever, fatigue, headaches, vomiting, constipation, stiffness in the neck, and pain in the limbs (WHO, 2003). Once a person is infected with the poliovirus, virus is shed in the stool. Transmission occurs primarily through, fecal-oral contact and is greatest in areas where hygiene and sanitation were poor.

There are three stains of poliovirus, type I, II, and III. Immunity to the virus is type specific showing homotypic immunity. Infection with one type does not protect against an infection with the other two types. The Sabin live vaccine contains modified virus of all three serotypes; after vaccination, the virus is shed in the stool for up to six weeks, with high levels of shedding occurring during the first 1-2 weeks. Because the virus is shed in the stool after vaccination, individuals can become infected if they come into contact with the fecal matter from a recently vaccinated person. (WHO, 2003)

Infectious poliovirus and norovirus are shed in the stool, making them readily transmissible when sanitary practices are disregarded, with potential risk for large scale outbreaks. The most important difference between poliovirus and norovirus is that norovirus does not grow in cell culture making it difficult to enumerate levels of the virus present. The inability to grow in cell or animal culture models limited the ability of detection of norovirus until within the last decade.
**Norovirus**

Norovirus was first recognized in 1968, causing gastrointestinal illness in school children in Norwalk, Ohio (Adler, 1969). Norovirus (NV) previously known as Norwalk or Norwalk-like virus (NLV), is characterized as a small round structured virus (SRSV) in the *Caliciviridae* family. Noroviruses range from 27-32 nm in diameter and are a non-enveloped virus (Cliver, 2001; Greenberg and Matsui, 1992). Noroviruses are characterized by a highly structured capsid with a pattern of surface depressions, from which the name calicivirus was derived (Cliver, 2001). Sequencing analysis has shown that the norovirus genome is composed of three open reading frames: the longest frame contains sequences similar to the poliovirus, the second open reading frame encodes the norovirus structural protein, and the last frame is the smallest open reading frame and is of unknown significance (Greenberg and Matsui, 1992).

Norovirus is comprised of four genogroups (G1-G4) which are further divided into 20 clusters. These 20 clusters acquired their names from the place where they were first detected as the cause of an epidemic of diarrhea in adults. Southampton, Norwalk, Desert Shield, and Cruise Ship which are all grouped in G1; Snow Mountain, Mexico, White River, Lordsdale, Bristol, Camberwell, Hawaii are clusters grouped in G2; Sapparo, Parkville, Manchest, Houston, London are clusters grouped in G3; and the identification of G4 clusters is continuing (Goodgame, 2001). Most frequently detected stool specimens in Florida are G2 group viruses (Stark, personal communication).

Noroviruses are very resilient with characteristics that enable survival on a variety of food items (Goodgame, 2001). Norovirus is capable of withstanding freezing, heat up
to 60°C, exposure to chlorine at concentrations below 10ppm, acidic condition as low as pH of 2.7, alcohol and high sugar concentrations (Schwab, 2000; Chris 2003).

Transmission of norovirus can occur year-around, but winter outbreaks occur more frequently. Mounts et. al. (2000) conducted studies evaluating surveillance data from four different countries (Japan, Denmark, The Netherlands, and the United States) for a one year period of time and the plotted the monthly occurrence of norovirus cases. All outbreaks from each country were confirmed through laboratory diagnosis. All countries showed a consistent pattern of lower or sporadic cases during the warmer months, and a peak of gastroenteritis during the winter months. Studies have demonstrated cold weather predominance of norovirus, but the CDC reports outbreaks to be equally and some years slightly higher in the summer months compared to outbreaks in the winter.

Humans are the only known hosts of norovirus G1 and G2 strains (Cowden, 2002). Norovirus is a highly contagious virus with an infectious dose of only 10-100 viral particles. The virus is shed in the feces of an infected person and generally peaks one or two days after infection; shedding can continue for over two weeks after infection (Estes et. al., 2000). Transmission is primarily through the fecal-oral route, from person-to-person, by fomites, contact surfaces, and consumption of contaminated food. Because infection with this virus requires only a small number of virons, attack rates can be extremely high (Kapikian, 2000). Once infected, a person may experience symptoms of explosive diarrhea and vomiting, nausea, and low-grade fever. Symptoms appear 12-48 hours after infection and last for 12-60 hours (Estes et. al., 2000). Shedding of the virus in the stool continues for about 48 hours after symptoms conclude (Graham et. al., 1994).
Foodhandler’s should be limited in their food preparation to avoid contamination events. There is no antiviral medication effective against norovirus; treatment includes oral rehydration with fluid and electrolyte replacement.

Serological research has shown that preexisting antibodies to norovirus do not provide protection from subsequent infections; short term immunity can be achieved following repeat exposure to a homologous viral strain (Numata et. al., 1997). The waning immunity of norovirus maintains susceptibility to repeat infections throughout life (Balbus et. al., 2002). This factor is of major public health importance, since individuals can become repeatedly re-infected, increasing transmission to others.

Norovirus has only been detectable within the last decade, because they cannot be cultivated in the laboratory. Recent technological advances such as Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) allow us to identify noroviruses by detection of their genetic structure. Effectiveness of RT-PCR is dependent on removal of matrix inhibitors during the concentration and extraction steps of the procedure.

**RNA Virus Extraction**

The steps involved in the extraction of RNA are concentration of the virus, release of nucleic acids from the virus and concentration of nucleic acids for RT-PCR detection. Purification of nucleic acid can be done using enzymatic digestions, silica beads, glass particles, or magnetic beads that are used to bind the RNA during multiple wash and purification steps. The classic procedure of nucleic acid purification involves detergent-mediated lysis, proteinase treatment, extractions using organic solvents or ethanol precipitates which can increase the risk of transmission of nucleic acids from sample to sample (Boom et. al., 1990). Toxic compounds such as cetyltrimethylammonium bromide
or phenol-chloroform are sometimes employed in releasing genetic material from the virus. Concentration of nucleic acids is necessary for RT-PCR. The actual extraction of genetic material from viruses can be done through various methods.

An analysis and evaluation of five different nucleic acid extraction techniques for norovirus was performed on stool by Sair et al. (2002): 1) guanidinium isothiocyanate (GITC) extraction, 2) TRIzol Reagent extraction, which uses commercially available guanidium-phenol based solution, 3) QIAmp Viral RNA Mini Kit, based on a spin column method, 4) QIAshredder prior to use of the Viral RNA Mini Kit, and 5) TRIzol Reagent extraction combined with the QIAshredder. The effectiveness of the different RNA extraction techniques was compared by concentration and purification of the RNA recovered and by RT-PCR limit of detection using serial dilution. Results showed that the QIAmp Viral RNA spin column blocked the flow of the sample through the column, even after treatment with the QIAshredder. TRIzol extraction combined with the QIAshredders, produced significantly better RT-PCR results than the GITC method, but did not purify the RNA any better, smearing of the band on the gel was still visible. There was an added impediment with the TRIzol method compared to the GITC method; TRIzol extraction by itself was the unable to completely dissolve the viral RNA pellet. The best method out of the five described was the TRIzol extraction followed by QIAshredder. This method showed some inhibition of RT-PCR by stool components, and only allowed detection of the virus at $10^{-3}$ dilution. (Sair et. al. 2002).

Boom et. al. (1990), used a method based on the combination of guanidinium thicyanate (GuSCN) and silica particles to extract and then purify nucleic acids from virus matrices. In the presence of high concentrations of GuSCN, nucleic acids will bind
to silica particles. Viruses (ssDNA, dsDNA, and ssRNA) and bacterial nucleic acids were detected from serum, urine, stool, and foods, all yielded a viral recovery of greater than 50%.

Boom’s method proved effective for nucleic acid extraction and purification because: 1) the method is sensitive, reproducible, rapid, simple, and does not require specialized equipment, 2) the risks for personnel with regard to pathogen and hazardous materials is minimal, 3) the chance of transmission of nucleic acid from sample to sample was minimal (Boom et. al., 1990). This extraction technique does not require the use of additional hazardous compounds such as Freon, and it decreases the level of RT-PCR inhibitors which are co-extracted from complex food matrices (Shieh et. al., 1999).

**Detection Methods for Viruses**

Historically cell culture has been the standard for detection of viruses. However there are several limitations with this method: the process of cell culture is slow, taking days or even weeks to confirm specific strains of the virus, it is expensive and some viruses, specifically norovirus, do not proliferate in cell culture (Traore et. al., 1998; Kapikpan et. al., 2000; Estes et. al., 2000). Viral outbreaks require rapid detection techniques such as reverse transcriptase polymerase chain reaction (RT-PCR) for immediate public health response and to limit transmission and spreading of the outbreak.

Reverse transcriptase (RT) is an enzyme that functions as a RNA-dependent DNA polymerase. RT performs two functions: it builds DNA strands based on an RNA template, and allows amplification of RNA by coupling reverse transcription and PCR. This technique is commonly used for the detection of RNA viruses such as poliovirus and norovirus (Moe et. al., 1994 and Monpoeho et. al., 2001).
PCR is the amplification of regions of DNA by annealing specific primers to single stranded DNA (ssDNA) and rebuilding the double stranded molecule using polymerase enzyme. PCR involves a repetitive series of cycles, usually between 30 to 40 cycles, which consist of denaturation, annealing, and extension to create the sample DNA exponentially. There are three basic steps in PCR: denaturation, annealing, and extension. Denaturation of the RT product occurs at 94°C and involves the unwinding of the double stranded DNA (dsDNA), formally RNA virus, to ssDNA. Annealing, which typically occurs at 50-70°C is where the single stranded primers bind to their complementary base on the ssDNA. Extension which occurs at 72°C is when the polymerase enzyme interacts with the primer/ssDNA complex and rebuilds dsDNA molecules. This results in two new helixes in place of the first, each helix is composed of one of the original strand plus the newly assembled complementary strand. As the cycling continues, the number of dsDNA copies doubles with each cycle. Each cycle takes only one to three minutes; in 45 minutes PCR can amplify a single copy of target DNA to thousands of copies in a 30-40 cycle reaction.

(http://people.ku.edu/~jbrown/pcr.html)

Several methods have been proposed in the literature for the elution and concentration of viruses from water, but proposed methods for viral detection from foods has been limited by food matrixes. Of these methods, organic flocculation is effective for elution and concentration of viruses from food surfaces. Following flocculation to concentrate the virus from food, extracting the nucleic acids is necessary. The virus is suspended in lysis solution to release the nucleic acids from within the virus. Lastly, nucleic acids are purified and concentrated in preparation for RT-PCR. RT-PCR cycles
to produce replications of a specific target region of RNA. This complete procedure: flocculation, extraction, and RT-PCR has been used for detection of viruses from food; although, factors such as matrix inhibitors present in foods may reduce its effectiveness. Therefore, these techniques need to be fine-tuned to be robustly applicable for detection of viruses from a variety of foods.
**Objectives**

The purpose of this thesis project is to improve the standard RT-PCR-based method used at the State of Florida Department of Health Laboratory for detection of poliovirus and norovirus from foods, without using multiple toxic compounds to remove matrix inhibitors. Organic flocculation with 3% buffered beef extract eluent will be implemented for concentration and elution of the virus from foods. This thesis will compare multiple centrifuge speed at various steps of the procedure for increased viral recovery and reduction of matrix inhibitors. The concentration and elution steps will be followed by RNA extraction using the Boom method, and then RT-PCR for comparison.

This study hypothesis is that higher centrifugal forces at centrifuge steps one and three will settle particulate inhibitors out of solution and leave the virus; whereas at step two, higher forces will increase concentration of the virus. The objectives of this investigation are:

1. To develop a procedure to purify viruses from food matrices using varying centrifugal forces instead of toxic compounds.
2. To determine at which procedural step increasing centrifugal forces results in reduction of inhibitors for optimal detection of virus by RT-PCR.
3. To determine the most effective method for viral recovery from dark meats, light meats, and lunchmeats.
4. To determine the most effective method for the detection of low levels of virus (2,000-20,000 detectable units per 100µl of diluted virus) from surface contaminated foods.
Materials and Methods

Poliovirus Seed Stock Preparation

Poliovirus 3 (Sabin strain) was cultured in Buffalo Green Monkey Kidney (BGM) cells (American Type Culture Collection Manassas, VA) grown in Nunclon 25 cm² tissue culture flasks (Fisher #56340). The stock virus cell culture was diluted 1:10 in Earles Balanced Salt Solution (EBSS, Sigma #E6132), and 1 ml was inoculated into five 25 cm² tissue culture flasks containing confluent BGM cells. An additional flask was inoculated with 1ml of EBSS as a negative control. All the flasks were placed on a rocker platform (Bellco Biotechnology #7740-10000) at 37°C for one hour.

Nine milliliters of Eagles Minimal Essential Medium Earl’s Salts (EMEM, Sigma #M0643) with 5% fetal calf serum (FCS) was added to the flasks. The flasks were monitored for cell lysis each morning through a microscope. At 48 hours the cells were over 90% lysed and the bottles were frozen at -70°C.

The bottles were then rapidly thawed in a 37°C water bath, frozen at -70°C and thawed again. The contents from the tissue culture bottle were transferred to sterile 15mL polypropylene Falcon tubes (Fisher #352196) and centrifuged at 4536x g for 10 min. in a Beckman J6B centrifuge with Beckman 5.2 swing bucket rotor (Beckman Coulter Inc. Palo Alto, CA). One hundred twenty five microliters of the supernatant was pipetted into 96 sterile, tapered, 500ul centrifuge tubes (Fisher #05-669-25) and frozen at -70°C. Ampoules were quick-thawed immediately before use and were not refrozen.
Norovirus Seed Stock Preparation

In a bio-safety cabinet positive NV G2 Lordsdale Cluster stools from specimens associated with a single outbreak were pooled in a 50ml conical tube. Phosphate Buffer Solution (PBS) was added as needed to the pool to yield a final volume of 22ml. The pool was vortexed for five minutes, then homogenized with a handheld homogenizer for two minutes at 15,000 rpms. The pool was vortexed again for five minutes, and centrifuged at 2,000 x g for ten minutes in a Beckman J6-B centrifuge (5.2 swing-bucket rotor). The supernatant was transferred to eight 6ml ultracentrifuge tubes and centrifuged for 15 minutes in a Beckman L7 Ultracentrifuge (rotor Type 40) at 20,000 x g. The supernatant was then placed in 96, 125µL aliquots and frozen at -70°C.

Virus Dilution

Norovirus and poliovirus seed stock was thawed at 37°C and serially diluted to 10⁻⁷ in a bio-safety cabinet. Nine hundred milliliters of DNASE/RNASE free water (ICN Biomedical #821739) was pipetted into a 1.5mL sterile Eppendorf tube, and 100µL of the virus added, yielding 10⁻¹ dilution. Next, 100µL from the 10⁻¹ dilution was placed in 900µL of the water to yield the 10⁻² dilution. This step was repeated until the 10⁻⁷ dilution was completed for each virus.

Table 1: Stock Dilution Series

<table>
<thead>
<tr>
<th>Log</th>
<th>10⁻¹</th>
<th>10⁻²</th>
<th>10⁻³</th>
<th>10⁻⁴</th>
<th>10⁻⁵</th>
<th>10⁻⁶</th>
<th>10⁻⁷</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus</td>
<td>100µL</td>
<td>100µL</td>
<td>100µL</td>
<td>100µL</td>
<td>100µL</td>
<td>100µL</td>
<td>100µL</td>
</tr>
<tr>
<td>Water</td>
<td>900µL</td>
<td>900µL</td>
<td>900µL</td>
<td>900µL</td>
<td>900µL</td>
<td>900µL</td>
<td>900µL</td>
</tr>
</tbody>
</table>

Food Samples

All foods were obtained from a local supermarket prior to viral seeding. Pre-cut/pre-packaged Fresh Express Lettuce, three pounds of Boars Head Oven Gold Turkey Breast Lunchmeat and Publix Smoked Chicken Breast Lunchmeat, a 20lb Butterball
turkey, and two Tyson 9lb chickens were chosen for this experiment (Appendix A, Figure 52-55).

The two chickens were cooked at 350°F for approximately two hours. The turkey was thawed for five days in a refrigerator at 4°C, baked in at 350°F for approximately three hours. The meat was cut from the bone, divided into approximately 50g portions of light and dark meat, and frozen in a home refrigerator at 0°C until the next day. The meat was then packaged in a cooler for transport to the laboratory and stored in a walk-in freezer at -20°C. Prior to seeding of the virus, the meat was thawed for 48 hours at 4°C.

At the lab, all food products were weighed into 50 gram portions and placed in a sterile 600mL disposable beaker (Fisher #02-591-10F). 100µl of each dilution $10^3$-$10^6$ of each virus was seeded separately onto each individual food sample. The foods were held in a bio-safety cabinet at room temperature for two hours to simulate a cafeteria environment before being placed in a refrigerator overnight until processing.

**Decontamination**

Prior to processing the foods for viral recovery the countertop and all equipment was cleaned and decontaminated, to reduce the possibility of extraneous contamination. All countertops and all equipment to be used during processing was wiped down with Wescodyne, saturated with 10% household bleach for approximately one minute. Rnase/Dnase Away (Molecula BioProducts #7010) was sprayed on the equipment and countertops. Finally, all surfaces were flooded with 75% Ethanol solution. A prior experiment determined that this procedure is effective in sanitizing the surface of equipment and countertops that may have been contaminated with noroviruses.
Viral Flocculation and Concentration

Virus seeded specimens were removed from the refrigerator after 12 to 14 hours post-seeding and 200mL of 3% eluent, sufficient to cover the food sample, was added to each beaker. Eluent was composed of 30g/L of Bacto Beef Extract (BBE, Difco Catalogue #0115-17), 7.5g/L of glycine (Sigma #G8770) dissolved in reagent grade water and autoclaved for 30 min at 121°C, 15 psi. Tween 20 (Fisher #BP337-500) was added to achieve a 2% solution and the pH was adjusted to 7.5 with 5N NaOH. Beakers with specimens in eluent were shaken on a LED Orbital Shaker (Lab Line Instruments Inc. Melrose Park, IL) for 30 minutes at 140 rpm. The eluent was poured through a 42mm nylon Millipore mesh spacer (Micron Separation Inc #C32WP04200) into individual 250 mL disposable polypropylene conical tubes (Fisher #20-893B). Samples were centrifuged for 30 minutes according to treatment group. Supernatants were transferred to new 250mL conicals, and the solids were discarded. While stirring, the pH was adjusted drop-wise with 5M HCl to 3.5. Conicals were inverted (allowing the magnetic stir bar to spin freely), and stirred slowly for 30 minutes. The stir bar was removed and conicals were centrifuged for 20 minutes at their respective treatment speeds as described below. The supernatant was discarded and the pellet was saved. (Katzenelson et. al., 1976) Nine milliliters of 4M guanidine isothiocynate (GITC) lysis buffer (Biomerieux #84407) was added to lyse the virus and release nucleic acids into solution. Samples were then transferred into separate sterile 15mL polypropylene Falcon conical tubes (Fisher #352196), and stored at -70°C until processing.
Sample processing for recovery of virus

Each food sample was processed using five experimental treatments as illustrated in Figure 1. These methods compared different centrifuge forces applied at different steps of the virus concentration procedure.

The Normal treatment followed the standard protocol developed at the DOH Tampa Lab for viral extraction from food surfaces. (Appendix B)

For treatment “Centrifuge 1” the centrifuge force was increased from 4,536 x g to 6,840 x g prior to organic flocculation. The remainder of the process was left unchanged.

For treatment “Centrifuge 2” the centrifuge force was increased from 4,536 x g to 6,840 x g after flocculation. The remainder of the process was left unchanged.

For treatment “Centrifuge 3” the centrifuge force was increased from 4,536 x g to 6,840 x g after lysis of the flocculated sample, but prior to RNA extraction. The remainder of the RNA extraction procedure remained unchanged.

All of the above centrifuge methods were performed in the Beckman J-6B centrifuge with the Beckman 5.2 swing bucket rotor (Appendix C, Figure 56).

For treatment “Fixed” a fixed angle rotor (Appendix C, Figure 57), Beckman JLA 16.250, was used with a Beckman J6 Avainti centrifuge. The centrifuge force was increased from 4,536 x g to 38,400 x g, after organic flocculation, the remainder of the process was left unchanged.
Add of 200mL eluent

Stir sample in eluent 30 min, room temperature

Decant samples through filter into 250mL conicals

Normal 4536 xg
Centrifuge 1 6840 xg
Centrifuge 2 4536 xg
Centrifuge 3 4536 xg
Fixed 4536 xg

Supernant transferred to 250mL conical

Lower pH from 7.5 to 3.5

30 min slow stir FLOCCULATION
Discard supernants save pellet

Add 9mL of lysis buffer to dissolve pellet

Store samples in 15mL conicals at -70°C

Thaw specimens in 37°C water bath

Boom protocol for RNA isolation
RNA Extraction

Samples were removed from the freezer and thawed in a 37°C water bath for 15 minutes. All normal, centrifuge 1, centrifuge 2, and fixed treatment samples were centrifuged in the Beckman J6-B for 15 minutes at 5,200 x g. Centrifuge 3 samples were spun at 6,840 x g for 15 minutes in the Beckman J6-B. Supernatants from all samples were transferred to a new sterile, 15ml conicals and 50ul silica (NucliSens #284160) was added. Specimens were vortexed for 15 seconds then placed on a Dynal Rotamix (New Hyde Park, NY #RKDYNAL) for 10 minutes at 40 rpm, then vortexed again for 15 seconds. This ensures sufficient RNA-silica interaction. Specimens were centrifuged in J6-B at 4,536 xg for 15 minutes at room temperature. Supernatants were aspirated and 1 ml wash buffer (NucliSens #82944) was added. Specimens were vortexed and contents transferred to 1.5ml micro-centrifuge tubes with “screw top” caps. The remainder of the process followed the manufacturer’s protocol using the NucliSens Basic Kit (BioMerieux #28416). RNA products were stored at -70°C until amplification.

RT-PCR

Norovirus and Poliovirus amplification was performed in a Perkin Elmer 9700 thermocycler (Perkin Elmer Inc. Norwalk, CT #805S9083032). Specimens were prepared according to manufacturer’s instructions for the QIAGEN One-Step RT-PCR kit (Qiagen #210212). The RT-PCR mix contained: 12.575 µl of Rnase free water, 5 µl of 5X buffer, 1 µl of DNTPs, 0.15 µl of forward primer, 0.15 µl of reverse primer, 0.125 µl of Rnasin, and 1 µl of enzyme mix included in the kit. In a 96 well optical reaction plate
(PE Applied Biosystems #N801-0560), 20 µl of RT-PCR mix was combined with 5µl of sample RNA for a total volume of 25 µl.

The forward and reverse primers are specific for each virus. The primer sets for amplification of norovirus were the G2 Lordsdale Cluster capsid: primers C1 and C2. These primers amplify a 322-nucleotide segment from the 5’ non translated region of the norovirus genome (Sheih, 2002).

**NLV C1** 5’CCA-GAA-TGT-ACA-ATG-GTT-AT 3’
**NLV C2** 5’CAA-GAG-ACT-GTG-AAG-ACA-TCA-TC 3’

The primers used for the amplification of Sabin Polio 3 are: ENT 3 and ENT 4. These primers amplify a 196-nucleotide segment from the 5’ non-translated region of the poliovirus genome (Sheih, 2002).

**ENT 3** 5’ CTT-CCG-GCC-CCT-GAA-TG 3’
**ENT 4** 5’ ACC-GGA-TGG-CCA-ATC-CAA 3’

Thermocycler conditions for One-Step RT-PCR of norovirus and poliovirus are in table 2.

<table>
<thead>
<tr>
<th>RT</th>
<th>PCR:</th>
<th>40 Cycles</th>
<th>Extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>50°C 30 min</td>
<td>94°C</td>
<td>30s</td>
<td>72°C 7 min</td>
</tr>
<tr>
<td>95°C 15 min</td>
<td>58°C</td>
<td>30s</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>60s</td>
<td></td>
</tr>
</tbody>
</table>

Products were detected on a 2% agarose gels stained with ethidium bromide. The gels were composed of 0.87 grams of SeaKem LE Agarose (Cambrex #50004), 0.43g of NuSieve 3:1 argarose (Cambrex #50090), and 65 mL of 1X TAE buffer (Fisher #BP1332-4). The mixture was melted for one minute in household microwave and 5µl of ethidium bromide 1% solution (Fisher #BP1302-10) was added. The solution was poured
into a gel electrophoresis mold (Life Technologies Gaithersburg, MD #11068-012) and allowed to solidify for one hour. Ten micro-liters of sample were mixed with 2µl of 6x Blue/Orange dye (Promega #16241006 Madison, WI), and were loaded into the wells of the gel. A 120V current was passed through the gel for approximately 50 minutes.

The bands were visualized and photographed and peak intensity quantified using the Bio-Rad Gel Doc 2000.

**Band Intensity**

Band intensity was evaluated using the Quantity One 4.4.0 program (Bio-Rad Laboratories Hercules, CA #1708601). Each of the bands was framed. A sensitive level for band detection was selected and set at 2.626 and band width was selected and set at 5.12. The results were recorded in a Microsoft Excel spreadsheet.
Results

This study investigated the effect of different centrifugal procedures in order to determine which procedure would be most effective for concentration of intact virons eluted from food surfaces and to reduce matrix inhibitors for successful application of RT-PCR. Two centrifugal forces were assessed at three stages during the standard food-processing protocol (Appendix B) for concentration of two viruses (poliovirus 3 Sabin and norovirus G2 Lordsdale) inoculated on seven different food products at four different dilutions ($10^{-3} - 10^{-6}$). For this study, a high band intensity strength was interpreted as effective reduction in matrix inhibition; and a low band intensity strength was interpreted as not effective for reducing matrix inhibition. Band intensity strength from gel electrophoreses of RT-PCR amplicons were compared using SAS 8.02 © SAS Institute Inc. Cary, North Carolina, (2003-2004) with the assistance of Angela E. Butler. A total of 630 samples were processed and analyzed.

Gel Band Analysis

In order to perform a statistical analysis of the 630 samples a quantitative numerical value hat to be given to each sample. This was done using the gel dock apparatus in conjunction with Quantity One 4.4.0 software (Bio-Rad Laboratories, Hercules, CA). The bands in each lane were framed and a set sensitivity of 2.626 and width of 5.12 for each band was selected. Figure 2 is a picture of the gel for Dark Chicken Spiked with Poliovirus and treated with Centrifuge 1 treatment method. In Lanes six through twelve the band are very bright and blurry. Because of the smearing
between lanes and vertically they produce a lower quantitative value. The red line in the picture indicates the band width that the computer reads, because the sensitivity is set very low, 2.626, the computer reads only this line and anything above, below or on either side will reduce the band intensity reported by the computer. The numerical values determined by the computer are used for the ANOVA test and in the bar graphs for interpretation.

**Figure 2: Dark Chicken Centrifuge 1 Smeared Gel Picture**

Lane 1, Marker; Lane 2, Amp negative (115.985); Lane 3, BBE negative (114.788); Lane 4, Lysis negative (111.963); Lane 5, Dark Chicken (95.833); Lane 6, BBE $10^{-1}$ (149.275); Lane 7, Lysis $10^{-1}$ (149.275); Lane 8, BBE $10^{-2}$ (149.275); Lane 9, Lysis $10^{-2}$ (149.275); Lane 10, BBE $10^{-3}$ (149.275); Lane 11, Lysis $10^{-3}$ (152.833); Lane 12, Dark Chicken $10^{-3}$ (150.338); Lane 13, Marker; Lane 14, Amp positive (158.933).

**Figure 3** is a picture of the gel for Dark Chicken spiked with Poliovirus and treated with the Fixed treatment method. Again the red line indicates the width that the computer reads to yield a quantitative numerical value. This gel shows no smearing between lanes and minimal horizontal and vertical smearing; because of these factors and
the level the sensitivity selected, the band intensity is reported at a higher value (value of 255) by the computer.

Figure 3: Dark Chicken Fixed No Smearing Gel Picture

Lane 1, Marker; Lane 2, BBE $10^{-4}$ (255); Lane 3, Lysis $10^{-4}$ (255); Lane 4, Dark Chicken $10^{-4}$ (255); Lane 5, BBE $10^{-5}$ (255); Lane 6, Lysis $10^{-5}$ (255); Lane 7, Dark Chicken $10^{-5}$ (255); Lane 8, BBE $10^{-6}$ (97.833); Lane 9, Lysis $10^{-6}$ (102.755); Lane 10, Dark Chicken $10^{-6}$ (110.782); Lane 11, BBE $10^{-7}$ (255); Lane 12, Lysis $10^{-7}$ (255); Lane 13, Marker; Lane 14, Amplification positive (14.085).

**Endpoint Determination of Viruses**

This study also assessed recovery of four dilutions of virus seeded onto each of the foods. This was done to determine which treatment method would be optimal for viral recovery from foods at very low dilutions, a necessary objective, because virus infection can occur with as few as 10 viral particles.

Prior to this study we had determined the end point titers of both viruses by performing a serial 1:10 dilution out to $10^{-8}$ for norovirus and poliovirus. On hundred microliters of each viral dilution was then extracted and 5µl (out of 50µl) was then amplified using RT-PCR. Ten microliters (out of 25µl) of the amplicon was applied to an
agarose gel. The last detected band was the end point. By doing this we were able to determine the end point for our stock norovirus seed was $10^{-7}$ (Figure 4) and the end point for the poliovirus seed stock is $10^{-7}$ (Figure 5). The end point is defined as one RT-PCR unit. Therefore, $10^{-7}$ dilution of norovirus and poliovirus was 1RTPCR unit, however 1 RTPCR is only 4% of the original 100 µl of virus applied to the food; this is detectable when $2 \times 10^{2}$ virus RTPCR units are in the total extracted sample.

Table 3: RTPCR Units for each Dilution

<table>
<thead>
<tr>
<th>Dilution</th>
<th>$10^{0}$</th>
<th>$10^{-1}$</th>
<th>$10^{-2}$</th>
<th>$10^{-3}$</th>
<th>$10^{-4}$</th>
<th>$10^{-5}$</th>
<th>$10^{-6}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT PCR units</td>
<td>$2 \times 10^{8}$</td>
<td>$2 \times 10^{7}$</td>
<td>$2 \times 10^{6}$</td>
<td>$2 \times 10^{5}$</td>
<td>$2 \times 10^{4}$</td>
<td>$2 \times 10^{3}$</td>
<td>$2 \times 10^{2}$</td>
</tr>
</tbody>
</table>

Figure 4: Norovirus Endpoint Dilution Series

Lane 1, Marker; Lane 2, Amplification positive; Lane 3, $10^{-1}$; Lane 4, $10^{-2}$; Lane 5, $10^{-3}$; Lane 6, $10^{-4}$; Lane 7, $10^{-5}$; Lane 8, $10^{-6}$; Lane 9, $10^{-7}$; Lane 10, $10^{-8}$; Lane 11, Marker; Lane 12, Empty; Lane 13, Empty; Lane 14, Empty.
Figure 5: Poliovirus Endpoint Dilution Series

Lane 1, Amplification positive; Lane 2, Marker; Lane 3, $10^{-1}$; Lane 4, $10^{-2}$; Lane 5, $10^{-3}$; Lane 6, $10^{-4}$; Lane 7, $10^{-5}$; Lane 8, $10^{-6}$; Lane 9, $10^{-7}$; Lane 10, $10^{-8}$; Lane 11, Empty; Lane 12, Empty; Lane 13, Marker; Lane 14, Empty.

**Norovirus**

Five treatments were compared for concentration of norovirus (NV) from seven foods. Each sample was assessed by their band intensity strength (scale of 0-255). An Analysis of Variance (ANOVA) ($\alpha = 0.05$) and the Homogeneity of Covariance Test of Intensity (SAS Institute Inc.) was applied to the food band intensity averages to determine which of the five treatment methods significantly improved viral recovery and reduced matrix inhibition from foods. In order to determine which of the five treatments had a significant effect, an average of the results from each food spike was taken. The four dilutions for each food were averaged then analyzed using ANOVA and the Homogeneity of Covariance Test of Intensity to determine where there was significant differences among the treatment methods.
Individual Foods Compared by Treatment Methods

Dark Chicken

Band intensity strength showed no significant difference between the treatment methods for the reduction of matrix inhibitors for Dark Chicken. Although there were no statistically significant differences between the treatment methods, when the treatment methods were graphed (Figure 6) according to their band intensity averages, Centrifuge 3 treatment showed the highest band intensity average proving to be the most effective. Centrifuge 1 treatment method had the lowest band intensity average on the graph and proved to be the least effective for reduction of matrix inhibitors for Dark Chicken.

Figure 6: Comparison of Treatment Methods on the Reduction of Matrix Inhibitors from Dark Chicken for the Detection of Norovirus
**Light Chicken**

There were statistical significant differences among the various treatment methods applied to Light Chicken (p-value = 0.0326). The Normal method was less effective compared to the Centrifuge 3 and the Fixed treatment methods. Figure 7 shows the Normal method to have the lowest average band intensity strength and the Centrifuge 3 treatment method and the Fixed treatment methods to have the highest average band intensity strength. Therefore, the Fixed treatment method and the Centrifuge 3 treatment method were the most effective at reducing matrix inhibitors from Light Chicken and the Normal treatment method was the least effective method for reducing matrix inhibitors from Light Chicken.

Figure 7: Comparison of Treatment Methods on the Reduction of Matrix Inhibitors from Light Chicken for the Detection of Norovirus
Lunchmeat Chicken

Lunchmeat Chicken also had no statistical significant difference between the treatment methods, but when the band intensity strengths were compared on a graphically, the results showed that one method was more effective over the other methods. Figure 8, compares the treatment methods by their average band intensity strength, showing Centrifuge 2 treatment method to be the most effective at reducing matrix inhibitors.

Figure 8: Comparison of Treatment Methods on the Reduction of Matrix Inhibitors from Lunchmeat Chicken for the Detection of Norovirus

![Bar Chart](chart.png)
Dark Turkey

For Dark Turkey, the results shows a statistical significant difference (p-value = 0.0003) between the Normal treatment method and Centrifuge 1, Centrifuge 2, and the Fixed treatment methods. There was also a significant difference between Centrifuge 3 treatment method and Centrifuge 1, Centrifuge 2, and the Fixed treatment methods.

There was no significant difference between the Normal treatment method and the Centrifuge 3 treatment method. Figure 9 shows the Normal treatment method and Centrifuge 3 treatment method to have the lowest average band intensity strength and these two treatment methods were the least effective for the reduction of matrix inhibitors from Dark Turkey. The graph also shows Centrifuge 1 to have the highest average band intensity strength and to be the most effective for reducing matrix inhibitors from Dark Turkey.

Figure 9: Comparison of Treatment Methods on the Reduction of Matrix Inhibitors from Dark Turkey for the Detection of Norovirus
Light Turkey

For Light Turkey, there was a statistical significant difference (p-value < 0.0001) between Centrifuge 2, Centrifuge 3, and the Fixed treatment methods compared to the Normal and Centrifuge 1 treatment method. Figure 10 shows Centrifuge 2, Centrifuge 3, and the Fixed treatment methods to have the highest average band intensity levels and to be the most effective at reduction of matrix inhibitors. The graph also shows the Normal treatment method to have the lowest average band intensity strength and is the least effect for reduction of matrix inhibitors.

Figure 10: Comparison of Treatment Methods on the Reduction of Matrix Inhibitors from Light Turkey for the Detection of Norovirus

![Bar chart showing treatment methods comparison](chart.png)
Lunchmeat Turkey

For Lunchmeat Turkey, there was a statistical significant difference (p-value <0.0001) between Centrifuge 3 treatment method and the other four treatment methods. Figure 11 compares the treatment methods for lunchmeat turkey by their average band intensity strength. It shows Centrifuge 3 treatment method to have the lowest average band intensity strength and to be the least effective for reduction of matrix inhibitors for Lunchmeat Turkey. The graph also depicts the other four treatment methods (Normal, Centrifuge 1, Centrifuge 2, and Fixed) to have much higher average band intensity levels and to be more effective at reducing matrix inhibitors from Lunchmeat Turkey.

Figure 11: Comparison of Treatment Methods on the Reduction of Matrix Inhibitors from Lunchmeat Turkey for the Detection of Norovirus
Lettuce

Lettuce also had no statistical difference between the treatment methods. The average band intensity strength for each treatment is compared in Figure 12. Although there were no statistical significant difference between the treatment methods the graph shows the Fixed treatment method to be the least effective with the lowest average band intensity strength and Centrifuge 1 treatment method to be the most effective with the highest average band intensity.

Figure 12: Comparison of Treatment Methods on the Reduction of Matrix Inhibitors from Lettuce for the Detection of Norovirus
A comparison of the different treatment methods overall foods was performed to determine which method would be optimal for reducing matrix inhibitors irregardless of the food product.

Overall, the Centrifuge 2 treatment method produced the highest band intensity levels irregardless of from which food norovirus (NV) was being concentrated (Figure 13). The Centrifuge 2 treatment method also had the least amount of variability in band intensity levels among the different foods showing its effectiveness at removing matrix inhibitors.

Figure 13: Comparison of Treatment Methods on the Effectiveness of Matrix Inhibitors
Treatment Methods Compared by Foods

Each treatment method was individual graphed and compared with every food. These graphs show which foods matrix inhibitors could be reduced with each treatment.

Normal Treatment Method

The Normal treatment method (Figure 14) produced that the highest band intensity level with Lunchmeat Turkey and the lowest band intensity levels with Dark Turkey and Light Turkey. This treatment method was most effective at reducing matrix inhibitors from Lunchmeat Turkey and was least effective at reducing matrix inhibitors from Dark Turkey and Light Turkey.

Figure 14: Comparison of Normal Treatment on Effectiveness of Matrix Inhibition and Virus Recovery on Foods Seeded with Norovirus
Centrifuge 1 Treatment Method

Figure 15 shows how effective the Centrifuge 1 treatment method was for each food. Highest levels of band intensity were for Dark Turkey, Lunchmeat Turkey, Lettuce, and Light Chicken. Lowest levels of band intensities for Centrifuge 1 were seen with Dark Chicken, Lunchmeat Chicken, and Light Turkey. The Centrifuge 1 treatment method was most effective at reducing matrix inhibitors from the following foods: Dark Turkey, Lunchmeat Turkey, Lettuce, and Light Chicken. This treatment method was least effective for reducing matrix inhibitors for Dark Chicken, Lunchmeat Chicken, and Light Turkey.

Figure 15: Comparison of Centrifuge 1 Treatment on Effectiveness of Matrix Inhibition and Virus Recovery on Foods Seeded with Norovirus
Centrifuge 2 Treatment Method

Centrifuge 2 treatment method proved to be very effective at reducing matrix inhibitors from all foods (Figure 16). All foods had band intensity levels greater than 150. Lettuce and Dark Chicken had the lowest band intensity level compared to the other five foods.

Figure 16: Comparison of Centrifuge 2 Treatment on Effectiveness of Matrix Inhibition and Virus Recovery on Foods Seeded with Norovirus
Centrifuge 3 Treatment Method

The Centrifuge 3 treatment method graph (Figure 17) shows high band intensity levels for Dark Chicken, Light Chicken, and Light Turkey. The graph also shows low band intensity levels for Dark Turkey and Lunchmeat Turkey. Thus, Centrifuge 3 treatment method to be most effective at reducing matrix inhibitors from Dark Chicken, Light Chicken, and Light Turkey; the method is least effective for Dark Turkey and Lunchmeat Turkey. For Lunchmeat Chicken and Lettuce, Centrifuge 3 treatment method had medium effectiveness at reducing matrix inhibitors (i.e. the band intensity levels were not low enough to say the method was ineffective for these foods and the band intensity levels were not high enough to conclude the method was effect for reducing matrix inhibitors from these foods).

Figure 17: Comparison of Centrifuge 3 Treatment on Effectiveness of Matrix Inhibition and Virus Recovery on Foods Seeded with Norovirus
Fixed Treatment Method

Figure 18, the Fixed treatment method, shows high band intensity levels for Dark Chicken, Light Chicken, Dark Turkey, Light Turkey, and Lunchmeat Turkey. Lunchmeat Chicken and Lettuce had low band intensity levels. The Fixed treatment method was most effective at reducing matrix inhibitors for Dark Chicken, Light Chicken, Dark Turkey, Light Turkey, and Lunchmeat Turkey. The Fixed treatment method was least effective at reducing matrix inhibitors for Lunchmeat Chicken and Lettuce.

Figure 18: Comparison of Fixed Treatment on Effectiveness of Matrix Inhibition and Virus Recovery on Foods Seeded with Norovirus
Treatment Methods Compared by Meat Products (Dark, Light, and Lunchmeat)

A comparison was made between the meat products (Dark, Light and Lunchmeat Chicken and Turkey) for each treatment. These products have a unique texture and composition that may result in smearing of band during gel electrophoresis. Comparing treatment methods for each of these products provide insight into which method would reduce matrix inhibitory effects for similar products.

Normal Treatment Method

Figure 19, the Normal treatment method, shows Lunchmeat Turkey to have highest band intensity average, greater than any of the other meat products. Both Dark and Light Turkey had the lowest band intensity average compared to their equivalent chicken products. This treatment method proved to be effect at reducing matrix inhibitors from Lunchmeat Turkey and Dark and Light Chicken. The Normal treatment method was least effect at reducing matrix inhibitors for Lunchmeat Chicken and Dark and Light Turkey.
Figure 19: Comparison of Normal Treatment Method on Elution and Reduction of Matrix Inhibitors for the Detection of Norovirus from Meat Surfaces
**Centrifuge 1 Treatment Method**

Figure 20, Centrifuge 1 treatment method, shows Dark Turkey and Lunchmeat Turkey have the highest band intensity averages when processed by this method. However, Dark Chicken and Light Turkey produced the lowest band intensity averages for this method. This method proved to be effective at reducing matrix inhibitors from Dark Turkey, Lunchmeat Turkey, and Light Chicken. The Centrifuge 1 treatment method was least effective at reducing matrix inhibitors for Dark Chicken, Light Turkey, and Lunchmeat Chicken.

Figure 20: Comparison of Centrifuge 1 Treatment Method on Elution and Reduction of Matrix Inhibitors for the Detection of Norovirus from Meat Surfaces
Centrifuge 2 Treatment Method

Centrifuge 2 treatment method produced the highest band intensity average for all the meats products when compared to the other methods. Figure 21, Centrifuge 2 treatment method, shows Dark Chicken had the lowest band intensity compared to the other meats, and the Lunchmeats (Chicken and Turkey) had a similar levels of band intensity. This method proved to be effective in reducing matrix inhibitors from all the meat products with band intensity levels equal to or greater than 150.

Figure 21: Comparison of Centrifuge 2 Treatment Method on Elution and Reduction of Matrix Inhibitors for the Detection of Norovirus from Meat Surfaces
Centrifuge 3 Treatment Method

Figure 22, Centrifuge 3 treatment method, showed high levels of band intensity for Dark Chicken, Light Chicken, and Light Turkey. The graph also shows low levels of band intensity for Dark Turkey, Lunchmeat Chicken and Lunchmeat Turkey. The Centrifuge 3 treatment method was not effective in reducing matrix inhibition for the detection of norovirus (NV) from Dark Turkey and both Lunchmeats (Chicken and Turkey). However, this method was effective at reducing matrix inhibitors for the Light meats and Dark Chicken.

Figure 22: Comparison of Centrifuge 3 Treatment Method on Elution and Reduction of Matrix Inhibitors for the Detection of Norovirus from Meat Surfaces
Fixed Treatment Method

The Fixed treatment method graph (Figure 23) showed high band intensities levels for both the Dark and Light meat products. The Lunchmeats showed a visible difference between the two products; Lunchmeat Turkey averaged higher band intensity levels by two-fold over that of Lunchmeat Chicken. The Fixed treatment method was effective at reducing matrix inhibitors for Dark Chicken and Turkey, Light Chicken and Turkey, and Lunchmeat Turkey. This method was least effective at reducing matrix inhibitors from Lunchmeat Chicken.

Figure 23: Comparison of Fixed Treatment Method on Elution and Reduction of Matrix Inhibitors for the Detection of Norovirus from Meat Surfaces

![Graph showing comparison of Fixed Treatment Method on elution and reduction of matrix inhibitors for different meats.](image-url)
Comparison of Treatment Methods on Foods at Lower Limits (10^{-5} & 10^{-6})

In order to fully evaluate viral contamination on foods it is necessary to simulate a real-life scenario, where foods are contaminated at very low levels, limiting detection. The capabilities for detection of each treatment on low virus levels for each food were compared by examining recovery of highly diluted virus (10^{-5} and 10^{-6} dilutions or 2,000-20,000 RTPCR units) used to spike foods.

Normal Treatment Method

Figure 24, the Normal method, shows high band intensity levels for Light Chicken 10^{-5}, Lunchmeat Turkey, and Lettuce 10^{-5}, but had no visible band at 10^{-6} dilution. Lunchmeat Turkey had higher band intensity levels near 200 (255 being the maximum), at both seeded dilutions. The Normal treatment method was effective at recovering norovirus (NV) from Lunchmeat Turkey at low dilutions.

Figure 24: Comparison of Normal Treatment Method on Eluting of Norovirus from Foods at Lower Dilutions (2,000-20,000 PCR units)
Centrifuge 1 Treatment Method

Figure 25, Centrifuge 1 treatment, shows high band intensity levels for Light Chicken, Dark Turkey, Lunchmeat Turkey, and Lettuce at both dilutions. This is an improvement in band intensity from the Normal treatment method. The graph also shows low band intensity levels for Dark Chicken at \(10^{-5}\) and near no recovery at \(10^{-6}\) dilutions. Lunchmeat Chicken had low band intensity levels for both dilutions, and Light Turkey showed a striking difference between \(10^{-5}\) dilution and \(10^{-6}\) (recovery of \(10^{-5}\) dilution was much higher than \(10^{-6}\)). The Centrifuge 1 treatment method was effective at recovering low levels of virus from Light Chicken, Dark Turkey, Lunchmeat Turkey and Lettuce. This method was least effective at recovery for Dark Chicken Lunchmeat Chicken, and Light Turkey \(10^{-6}\).

Figure 25: Comparison of Centrifuge 1 Treatment Method on Eluting of Norovirus from Foods at Lower Dilutions (2,000-20,000 PCR units)
**Centrifuge 2 Treatment Method**

Figure 26, Centrifuge 2 treatment, shows higher band intensity levels overall for all food products at both dilutions. Foods with low band intensity levels with the Centrifuge 2 method were Dark Chicken $10^{-5}$, Dark Turkey $10^{-6}$, and Lettuce $10^{-6}$. The Centrifuge 2 treatment method showed more consistency for recovery of the virus at the lower dilutions then any of the other methods. This method was least effective for low level of viral recovery for Dark Chicken $10^{-5}$, Dark Turkey $10^{-6}$, and Lettuce $10^{-6}$.

Figure 26: Comparison of Centrifuge 2 Treatment Method on Eluting of Norovirus from Foods at Lower Dilutions (2,000- 20,000 PCR units)
**Centrifuge 3 Treatment Method**

Figure 27, Centrifuge 3 treatment, shows low band intensity levels for Dark Turkey and Lunchmeat Chicken at both dilutions and for Lettuce at $10^{-6}$. Lunchmeat Turkey at $10^{-6}$ also had low band intensity levels, although $10^{-5}$ was not substantially higher than $10^{-6}$. Light Chicken and Light Turkey had high levels of band intensity for both dilutions, proving Centrifuge 3 method to be effective for viral recovery from those meats at low dilutions.

Figure 27: Comparison of Centrifuge 3 Treatment Method on Eluting of Norovirus from Foods at Lower Dilutions (2,000-20,000 PCR units)
Fixed Treatment Method

Figure 28, the Fixed treatment, showed high band intensity levels at both dilutions for Light Chicken and Lunchmeat Turkey. Dark Turkey and Light Turkey at the $10^{-5}$ dilution also had high band intensity levels. Lettuce showed very poor band intensity levels at both dilutions with this treatment method. The Fixed treatment method was effective at recovering low levels of virus for Light Chicken and Lunchmeat Turkey, as well as, Dark and Light Turkey at $10^{-5}$ dilution. This method was least effective for Lettuce and Lunchmeat Chicken $10^{-6}$ dilution.

Figure 28: Comparison of Fixed Treatment Method on Eluting Norovirus from Foods at Lower Dilutions (2,000-20,000 PCR units)
**Poliovirus 3 Sabin**

Five treatments were compared for concentration of Poliovirus 3 Sabin (P3) from seven foods. Each sample was assessed by their band intensity strength (scale of 0-255). An Analysis of Variance (ANOVA) ($\alpha = 0.05$) and the Homogeneity of Covariance Test of Intensity (SAS Institute Inc.) was applied to the food band intensity averages to determine which of the five treatment methods had significantly improved viral recovery and reduced matrix inhibition from foods. In order to determine which of the five treatments had a significant effect, an average of the results from each spiked food was taken. The four dilutions for each food were averaged then analyzed using ANOVA and the Homogeneity of Covariance Test of Intensity to determine where there was significant differences among the treatment methods.

**Individual Foods Compared by Treatment Methods**

**Dark Chicken**

There were statistical significant differences among the various treatment methods applied to Dark Chicken ($p$-value = 0.0326). The Normal, Centrifuge 1, and the Fixed treatment methods were found to have the same effectiveness for the reduction of matrix inhibitions for detection of poliovirus from Dark Chicken. Centrifuge 2 and Centrifuge 3 treatment method were significantly less effective than the Normal, Centrifuge 1, and the Fixed treatment methods. Figure 29, shows Centrifuge 3 treatment, to have the lowest average band intensity strength and the Fixed, Normal and Centrifuge 1 treatment methods had the highest average band intensity strength. Therefore, the Fixed, Centrifuge 1, and the Normal treatment method were the most effective at
reducing matrix inhibitor for Dark Chicken and Centrifuge 3 treatment method was the least effective method for reducing matrix inhibitors from Dark Chicken.

Figure 29: Comparison of Treatment Methods on Reduction of Matrix Inhibitors for the Detection of Poliovirus from Dark Chicken
**Light Chicken**

The results showed no significant difference (p-value >0.50) between the treatment methods for reduction of matrix inhibitors for Light Chicken. Although there were no statistical significant differences between the treatment methods, Figure 30 shows Centrifuge 3 treatment to have the highest band intensity average followed by the Fixed and the Normal treatment methods. Centrifuge 1 treatment method had the lowest band intensity average and was the least effective at reducing matrix inhibitors from Light Chicken. Centrifuge 3, the Normal and the Fixed treatment method had the highest band intensity average thus, the most effective for reduction of matrix inhibitors from Light Chicken.

**Figure 30: Comparison of Treatment Methods on Reduction of Matrix Inhibitors for the Detection of Poliovirus from Light Chicken**
Lunchmeat Chicken

There were statistically significant differences among the various treatment methods applied to Lunchmeat Chicken (p-value = 0.0024). Centrifuge 3 treatment method was significantly different from the Normal, Centrifuge 1, Centrifuge 2, and the Fixed treatment methods. Figure 31 shows Centrifuge 3 and Centrifuge 2 treatment methods to have the lowest level of band intensity and the Normal, Centrifuge 1, and the Fixed treatment methods to have the highest band intensity levels. Thus, Centrifuge 3 and Centrifuge 2 treatment method was the least effective method for reduction of matrix inhibitors from Lunchmeat Chicken. The Fixed, Centrifuge 1, and the Normal treatment methods were the most effective for reducing matrix inhibitors from Lunchmeat Chicken.

Figure 31: Comparison of Treatment Methods on Reduction of Matrix Inhibitors for the Detection of Poliovirus from Lunchmeat Chicken
Dark Turkey

There were statistically significant differences (P-value = 0.0074) among the various treatment methods applied to Dark Turkey. Centrifuge 1 was significantly different from the Normal, Centrifuge 2, Centrifuge 3, and the Fixed treatment methods. Although there were no statistical differences between the Normal, Centrifuges 2, Centrifuge 3, and Fixed treatments, Figure 32 shows that Centrifuge 3 was the least effective, with the lowest average band intensity, among the four treatments. Centrifuge 1 was the least effective in reduction of matrix inhibitors for viral recovery and Centrifuge 2 was the most effective treatment for reduction of matrix inhibitors from Dark Turkey.

Figure 32: Comparison of Treatment Methods on Reduction of Matrix Inhibitors for the Detection of Poliovirus from Dark Turkey
**Light Turkey**

The results showed no significant difference (p-value >0.50) between the treatment methods for Light Turkey. Although there were no statistical significant differences between the treatment methods, Figure 33 shows the Normal and Centrifuge 1 treatment method to have the lowest band intensity average. Centrifuge 2, Centrifuge 3, and the Fixed treatment method had the highest band intensity average thus, the most effective for reduction of matrix inhibitors for Light Turkey. The Normal treatment method was the least effective for reduction of matrix inhibitors from Light Turkey.

Figure 33: Comparison of Treatment Methods on Reduction of Matrix Inhibitors for the Detection of Poliovirus from Light Turkey
**Lunchmeat Turkey**

There were statistically significant differences (p-value = 0.0001) among the various treatment methods for Lunchmeat Turkey. The Fixed treatment was significantly different from Centrifuge 1 and Centrifuge 2 treatments. The Fixed, Centrifuge 1, and Centrifuge 2 treatments were all significantly different from Centrifuge 3 treatment, which was significantly different than the Normal treatment. The Normal treatment was the least effective treatment, with the lowest average bane intensity, and the Fixed treatment was the most effective treatment, with the highest band intensity, for reducing matrix inhibitors from Lunchmeat Turkey (Figure 34). Centrifuge 1 and Centrifuge 2 treatment were equivalent in their effectiveness at reducing matrix inhibition for the detection of poliovirus from Lunchmeat Turkey.

Figure 34: Comparison of Treatment Methods on Reduction of Matrix Inhibitors for the Detection of Poliovirus from Lunchmeat Turkey
Lettuce

Lettuce had no statistical difference (p-value >0.50) between the treatment methods. Although there were no statistical significant differences between the treatment methods, Figure 35 shows the Normal treatment to have the lowest band intensity average. Centrifuge 2, Centrifuge 3, and the Fixed treatment method had the highest band intensity average thus, the most effective for reduction of matrix inhibitors from Lettuce.

Figure 35: Comparison of Treatment Methods on Reduction of Matrix Inhibitors for the Detection of Poliovirus from Lettuce
A comparison of the different treatment methods overall foods was performed to determine which method would be optimal for reducing matrix inhibitors irregardless of the food product.

Overall, the Fixed method produced the brightest band intensity levels irregardless of from which food poliovirus was being concentrated (Figure 36). This treatment method also had the least amount of variability in band intensity among the different foods showing its effectiveness at removing matrix inhibitors. The best method overall for reduction of matrix inhibition for the detection of P3 from any food is the Fixed method.

Figure 36: Comparison of Treatment Methods on Effectiveness of Matrix Inhibition
Treatment Methods Compared by Foods

Each treatment method was individual graphed and compared with every food. These graphs show which food had the best or the worst level of effective for reduction of matrix inhibitors for each treatment method.

Normal Treatment Method

The Normal treatment method (Figure 37) produced that the highest band intensity level with Dark Chicken and the lowest band intensity levels with Lunchmeat Turkey. The graph shows that Light Chicken, Lunchmeat Chicken, and Dark Turkey also had high band levels with this treatment method. The treatment method was most effective at reducing matrix inhibitors from Dark Chicken, Light Chicken, Lunchmeat Chicken, and Dark Turkey; and the least effective for reducing matrix inhibitors from Lunchmeat Turkey.

Figure 37: Comparison of Normal Treatment on Effectiveness of Matrix Inhibition and Virus Recovery on Foods Seeded with Poliovirus
Centrifuge 1 Treatment Method

Figure 38 shows how effective Centrifuge 1 treatment method was for each food. Highest levels of band intensity were for Dark Chicken, Lunchmeat Chicken, Lunchmeat Turkey and Lettuce. Lowest levels of band intensity for Centrifuge 1 were seen with Light Chicken, Dark Turkey and Light Turkey. The Centrifuge 1 treatment method was most effective at reducing matrix inhibitors from the following foods: Dark Chicken, Lunchmeat Chicken, Lunchmeat Turkey, and Lettuce. This treatment method was least effective for reducing matrix inhibitors for Light Chicken, Dark Turkey and Light Turkey.

Figure 38: Comparison of Centrifuge 1 Treatment on Effectiveness of Matrix Inhibition and Virus Recovery on Foods Seeded with Poliovirus
Centrifuge 2 Treatment Method

Figure 39, Centrifuge 2 treatment, shows Dark Turkey to have the highest band intensity level followed by Lettuce, Lunchmeat Turkey, and Light turkey. This method shows Lunchmeat Chicken, Dark Chicken, and Light Turkey to have the lowest band intensity level. The Centrifuge 2 treatment method was the most effective at reducing matrix inhibitors from Dark Turkey, Lettuce, Lunchmeat Turkey, and Light Turkey. This treatment method was the least effective at reducing matrix inhibitors from Lunchmeat Chicken, Dark Chicken, and Light Chicken.

Figure 39: Comparison of Centrifuge 2 Treatment on Effectiveness of Matrix Inhibition and Virus Recovery on Foods Seeded with Poliovirus
Centrifuge 3 Treatment Method

The Centrifuge 3 treatment method (Figure 40) shows high band intensity levels for Light Chicken and Lettuce and low band intensity levels for Dark Chicken and Lunchmeat Chicken. Dark Turkey, Light Turkey, and Lunchmeat Turkey fell in-between the high band intensity levels and the low band intensity levels. This method proved to be the most effective at reducing matrix inhibitors from Light Chicken and Lettuce; and least effective at reducing matrix inhibitors from Dark Chicken and Lunchmeat Chicken. The Centrifuge 3 treatment method had medium effectiveness at reducing matrix inhibitors for Dark Turkey, Light Turkey and Lunchmeat Turkey (i.e. the band intensity levels were not low enough to say the method was ineffective for these foods and the band intensity levels were not high enough to conclude the method was effective for reducing matrix inhibitors from these foods.)

Figure 40: Comparison of Centrifuge 3 Treatment on Effectiveness of Matrix Inhibition and Virus Recovery on Foods Seeded with Poliovirus
Fixed Treatment Method

Figure 41, the Fixed treatment, shows high band intensity levels (>150) for all food products. This treatment method proved to be the most effective at reducing matrix inhibitors from all foods. Light Chicken and Light Turkey had the lowest band intensity levels, but this method was still effective at reducing matrix inhibitors from those foods too.

Figure 41: Comparison of Fixed Treatment on Effectiveness of Matrix Inhibition and Virus Recovery on Foods Seeded with Poliovirus
Treatment Methods Compared by Meat Products (Dark, Light, and Lunchmeat)

A comparison was made between the meat products (Dark, Light and Lunchmeat Chicken and Turkey) for each treatment. These products have a unique texture and composition that may result in smearing of bands during gel electrophoresis. Comparing treatment methods for each of these products provides insight into which method would reduce matrix inhibitory effects for similar products.

Normal Treatment Method

Figure 42, the Normal treatment, shows the Chicken products to have higher band intensity levels compared to their counterpart Turkey. Lunchmeat Turkey had the lowest level of band intensity. This treatment method proved to be effective at reducing matrix inhibitors from Chicken products and for Dark Turkey. The Normal treatment method was least effective at reducing matrix inhibitors for Lunchmeat Turkey.

Figure 42: Comparison of Elution of Poliovirus from Meats using the Normal Treatment Method
Centrifuge 1 Treatment Method

Figure 43, Centrifuge 1 treatment, shows Dark Chicken, Lunchmeat Chicken and Turkey to have the highest band intensity averages. The lowest band intensity levels for the Centrifuge 1 treatment were from Dark Turkey, and Light Chicken and Turkey. This method proved to be effective at reducing matrix inhibitors from Dark Chicken and both Lunchmeat products. The Centrifuge 1 treatment method was least effective at reducing matrix inhibitors for Dark Turkey, Light Chicken and Light Turkey.

Figure 43: Comparison of Elution of Poliovirus from Meats using the Centrifuge 1 Treatment Method
**Centrifuge 2 Treatment Method**

Centrifuge 2 treatment method produced the highest band intensity average for all Turkey products. Figure 44, Centrifuge 2 treatment, shows the Turkey products to have the highest band intensity levels. The Chicken products had lower band intensity levels, with Lunchmeat Chicken having the lowest out of all the Chicken products. This method proved to be effective at reducing matrix inhibitors from the Turkey products.

Figure 44: Comparison of Elution of Poliovirus from Meats using the Centrifuge 2 Treatment Method
Centrifuge 3 Treatment Method

Figure 45, Centrifuge 3 treatment, shows high band intensity levels for Light Chicken, Lunchmeat Turkey and Light Turkey, and low band intensity levels for Dark Chicken, Dark Turkey, and Lunchmeat Chicken. The Centrifuge 3 treatment method was the least effective for reducing matrix inhibitors from all of the meats; it produced the lowest band intensities levels compared to the other treatment methods. This method proved to be the most effective at reducing matrix inhibitors from Light Chicken.

Figure 45: Comparison of Elution of Poliovirus from Meats using the Centrifuge 3 Treatment Method
Fixed Treatment Method

The Fixed treatment method (Figure 46) shows consistency in the band intensity levels between the meat products. The method had the highest band intensity levels for Lunchmeat Turkey and the lowest band intensity level for Light Turkey. The Fixed treatment method was the most effective method for reducing matrix inhibitors from all the meat products, with band intensity levels approaching or greater than 200.

Figure 46: Comparison of Elution of Poliovirus from Meats using the Fixed Treatment Method
Comparison of Treatment Methods on Foods at Lower Limits (10^{-5} & 10^{-6})

In order to fully evaluate viral contamination on foods it is necessary to simulate a real-life scenario, where foods are often contaminated at very low levels, limiting detection capabilities. The capabilities for detection of each treatment on low virus levels for each food were compared by examining recovery of high diluted virus (10^{-5} and 10^{-6} dilutions) used to spike foods.

Normal Treatment Method

Figure 47, the Normal treatment, shows high band intensity levels at both dilutions (10^{-5} and 10^{-6}) for Dark Chicken, Light Chicken, Lunchmeat Chicken, and Dark Turkey. This method produced low band intensity levels for Lunchmeat Turkey at both dilutions, Light Turkey 10^{-5} dilution. For both Light Turkey and Lettuce at 10^{-6} the Normal treatment method had not visible bands (ie. virus seeded at that dilution was not detected in the concentrated eluent). The Normal treatment method proved to be effective at recovering poliovirus from Dark Chicken, Light Chicken, Lunchmeat Chicken, and Dark Turkey at both dilutions.
Figure 47: Comparison of the Effectiveness of the Normal Treatment Method for Elution of Virus from Foods Seeded with Low Concentration of Poliovirus (2,000-20,000 PCR units)
Centrifuge 1 Treatment Method

Figure 48, Centrifuge 1 treatment, shows high band intensity levels at both $10^{-5}$ and $10^{-6}$ dilutions for Dark Chicken, Lunchmeat Chicken, Lunchmeat Turkey, and Lettuce. Light Chicken, Dark Turkey, and Light Turkey had low band intensity levels at both dilutions. The Centrifuge 1 treatment method was effective at recovering low levels of poliovirus from Dark Chicken, Lunchmeat Chicken, Lunchmeat Turkey and Lettuce. This method proved ineffective at recovery low levels of poliovirus from Light Chicken, Dark Turkey and Light Turkey.

Figure 48: Comparison of the Effectiveness of the Centrifuge 1 Treatment Method for Elution of Virus from Foods Seeded with Low Concentration of Poliovirus (2,000-20,000 PCR units)
**Centrifuge 2 Treatment Method**

Figure 49, Centrifuge 2 treatment, shows high band intensity levels for the Turkey products and for Lettuce at both dilutions. Foods with low band intensity levels include all Chicken products. The Centrifuge 2 treatment method showed consistency in recovering low levels of poliovirus from Turkey and from Lettuce, but had reduced effectiveness in recovering poliovirus from Chicken.

Figure 49: Comparison of the Effectiveness of the Centrifuge 2 Treatment Method for Elution of Virus from Foods Seeded with Low Concentration of Poliovirus (2,000-20,000 PCR units)
**Centrifuge 3 Treatment Method**

Figure 50, Centrifuge 3 treatment, shows high band intensity levels for Light Chicken $10^{-5}$ and Lettuce $10^{-5}$. This method produced the lowest band intensity levels at both dilutions for Dark Chicken, Lunchmeat Chicken, and Dark Turkey. This method produced a striking difference between $10^{-5}$ and $10^{-6}$ dilutions for Light Chicken and Lettuce, thus it is effective for recovering from these food at $10^{-5}$, but had reduced effectiveness at recovering at $10^{-6}$ dilutions. The method showed it could recover poliovirus at low levels, although not very effectively, from Dark Chicken and from Lunchmeat Chicken.

Figure 50: Comparison of the Effectiveness of the Centrifuge 3 Treatment Method for Elution of Virus from Foods Seeded with Low Concentration of Poliovirus (2,000-20,000 PCR units)
Fixed Treatment Method

The Fixed treatment method (Figure 51) shows this method could effectively recover poliovirus inoculated at low levels from all foods. The Fixed treatment method resulted in good band intensity levels ($\geq 150$) for all foods at $10^{-5}$ dilution. Light Turkey and Lettuce samples showed a striking decrease in band intensity levels between $10^{-5}$ and $10^{-6}$ seeded dilution. This method proved effective at recovering poliovirus from all foods at $10^{-5}$ dilution.

Figure 51: Comparison of the Effectiveness of the Fixed Treatment Method for Elution of Virus from Foods Seeded with Low Concentration of Poliovirus (2,000-20,000 PCR units)
Discussion

In the last several years there has been an increase in acute gastrointestinal illness (AGI) outbreaks, mainly caused by norovirus, in the United States. Some of these outbreaks can be linked to food contamination from an infected food handler and affect students, day-care centers, nursing homes, military personal, and cruise ship passengers. Secondary spread, from person-to–person is commonly seen in close quarter environments, which increase the risk of transmission and infection. With nearly 80% of the population affected by foodborne viral illnesses each year, detection of specific virus from a food is of utmost public health importance (Mead, 1999). Development of viral detection methods from food is necessary in order to limit transmission to others through isolation or limitation of activities of those infected, and education of those infected on how to prevent transmission to other family members or co-workers. Multiple food types, in addition to shellfish, have been linked to viral outbreaks. In the 2000 Texas University cafeteria outbreak traced to norovirus contaminated lunchmeats, 125 students became ill and 20 were hospitalized (Schwab et. al., 2000). Methods used for the detection of viruses from food have been the same methods as those used for the detection of viruses from shellfish or environmental samples. These methods, with minor variations, have shown success in detecting viruses from foods (Leggitt and Jaykus, 2000). The detection of norovirus from foods has also been limited by their inability to grow in cell culture. Recent technological developments have enabled the detection of such previously non-detectable agents. The use of nucleic acid amplification methods
(RT-PCR) provides a rapid detection method for viruses, specifically norovirus, however, it has been limited by food matrix effects, which cause inhibition of amplification of the virus nucleic acids, and smearing of bands on agarose gel electrophoresis of the cDNA products of amplification.

This study compared varying centrifugal forces to reduce matrix inhibitors for RT-PCR detection of two viruses (norovirus and poliovirus) from seven foods. This study suggested that increasing the centrifugal force at particular steps of the standard/Normal protocol will effectively reduce matrix inhibitors.

A study by Lucore et. al. in 2000 proved that a reduction in centrifugal forces, at the second concentration spin, reduced the tendency to co-precipitate PCR inhibitors for the detection of foodborne bacteria. This step in the procedure followed immobilization of the bacteria with zirconium hydroxide. Our study proposed increasing centrifugal force to reduce matrix inhibition for the detection of virus and saw positive results.

Bacteria replicate on a food given the appropriate temperature and time. This allows for the presence of more bacteria colonies at the time of detection, then initially present when the food was contaminated. Viruses do not replicate on the food and therefore there are often fewer infectious virions present, compared to bacteria, when the food is washed prior to assay. Bacteria are much larger in size than viruses. Therefore, a lower centrifuge force may be adequate to concentrate bacteria but it is necessary to first trap viruses in a protein floc in order to concentrate them at moderate centrifugal force.

Our study also compared two meats (chicken and turkey), analyzed them by dark meat, light meat, and lunchmeat for each meat product and also analyzed the detection
limit of the five treatments methods, to determine which method would be optimal for
detecting low level of virus that may be present on foods.

The standard/Normal protocol involved washing the food with 3% Buffered Beef
Extract (BBE) eluent to remove the virus from the food surface, decanting and
centrifugation of the eluate at 4,536 xg to remove food solids and associated inhibitory
substances while leaving the virus in solution. The pH of the supernatant is lowered to
3.5, and it is slowly stirred for 30 minutes (flocculation of protein and adsorbed virus).
Another centrifugation performed at 4,536 xg pellets the flocculate, which is then
dissolved in lysis buffer. The lysis buffer releases viral RNA into solution, it is
centrifuged at 4,536 xg to remove solids prior to RNA extraction. The four other
methods (Centrifuge 1, Centrifuge 2, Centrifuge 3, and Fixed) involved increasing the
centrifugal force either at the first centrifuge step, the second centrifuge step or the third
centrifuge step, which is first step in the RNA extraction process. All of the methods
were effective in increasing recovery over the standard/Normal method, and our study
found a method that was superior for removal of matrix inhibitors and detection of virus
at low levels.

Lettuce

Lettuce is a leafy green vegetable containing plant carbohydrates that may cause
matrix inhibition (Jaykus et. al., 2000). These plant carbohydrates, such as cellulose and
pectin, cannot be easily broken down except with sulphuric acid and high heat; they are
composed of polymers of glucose synthesized in the chloroplast compartment of plant
cells as large, semi-crystalline, insoluble granules with a complex internal lamellar
structure (Campell and Reece, 2002). The smooth texture of the lettuce leaf leads
researchers to assume that virus particles can be easily washed from the surface for
detection. This may not be the case; instead, the virus may have become bound or
adsorbed to the lettuce leaf surface.

**Chicken**

The Centrifuge 2 treatment method with Dark Chicken also had reduced
effectiveness at removal of matrix inhibitors. Dark Chicken is a meat high in protein and
sodium (www.nutritiondata.org). Dark meats generally contain higher levels of
myoglobin, a protein, and higher levels of fat, that cause increased matrixes between the
food and the virus. The theory behind organic flocculation is that the lowering of the pH
to 3.5 will allow for the viral particles in solution to bind to the BBE proteins. With
meats that are high in protein, these proteins found in the food maybe also washed off
during the washing step and suspended in the eluent solution. When the pH is lowered,
the viral particles may coagulate with the protein particles from the meat and with the
BBE proteins and all flocculate. This may cause inhibition at the viral lysis step. If the
virus is bound to both the BBE protein and the proteins in the meat the virus may not be
exposed to the lysis buffer and thus, viral nucleic acids may not be released into solution.

**Turkey**

The Normal/standard treatment method was the least effective method for
extraction and detection of norovirus from spiked foods. This method was least effective
for removal of matrix inhibitors from Light and Dark Turkey meats. Turkey meat is high
in protein, and in phosphorus. The turkey used for this experiment was a Butterball
Turkey (Appendix A, Figure 57) which contained a seven percent solution of enhanced
juices. This solution was composed of water, salt, modified food starch, sodium
phosphate, and natural flavor. Both Light and Dark Turkey meats are low in sodium, but with the added salt in the solution the sodium level may be increased. The added sodium phosphate is used for thickening or gelling of a food product. This additive in addition to the natural myoglobins (high levels of protein) present may cause matrix inhibition by trapping the virus in a matrix and not exposing it to the lysis buffer.

**Lunchmeat Chicken**

Although the Lunchmeats did not show a high level of matrix inhibition for the Centrifuge 2 treatment method, their ability to cause matrix inhibition was seen in the Centrifuge 1 and Centrifuge 3 treatment methods for the detection of norovirus.

Lunchmeat Chicken, was Publix Smoked Chicken Breast Lunchmeat containing: chicken breast meat, water, salt, sodium phosphate, natural roast chicken flavor, (rendered chicken fat, flavor, safflower oil). Sodium phosphate is most likely a cause of viral inhibition during detection. This additive comes in several forms: monobasic, dibasic, and tribasic; each acid reacting; any of these forms of sodium phosphate can be used to thicken, stabilize, and gel a food product (Canadian Food Inspection Agency).

Because it is also acid reacting; this compound may take on new forms under acid conditions, becoming thicker. Organic flocculation requires the lowering of the pH to an acidic level (3.5), which may have adverse effects on the virus binding to the BBE proteins in the eluent solution. Instead, the virus may be trapped in the sodium phosphate induced gel and not sediment as expected. When lysis buffer is added, sodium phosphate may be carried through to PCR. Additionally, the sites on the virus may be already bound to the sodium phosphate preventing binding to the BBE protein during
flocculation. Therefore, the virus is not trapped in the floc and not precipitated but instead is discarded with the supernatant.

**Lunchmeat Turkey**

Boars Head OvenGold Turkey Breast Lunchmeat is composed of turkey breast, water, salt, sodium phosphate and dextrose. The meat is coated with Dextrose, salt, honey, paprika, spices, onion, natural flavor, flavoring, extractives of paprika, and tumeric. This product was different from the lunchmeat chicken in that it contained Dextrose, a nutritive sweetener made from glucose and corn sugar. Both glucose and corn sugar are carbohydrates that may influence the virus binding to the protein. The virus could also be trapped in a coating of the carbohydrate and the protein preventing release when in lysis buffer and the whole component may be carried through PCR causing matrix inhibition.

**Detection of Low Levels of Virus**

This study specifically looked at the detection capabilities of each treatment method at $10^{-5}$ and $10^{-6}$ viral seed dilutions. The Centrifuge 2 treatment method proved to be the best method at eluting norovirus from foods at low seed virus applications. The Normal method was the least effective, with sporadic recovery; however, random pipetting effects may have caused this. The Fixed method was the second most effective method for removal of norovirus from the food. The Fixed method may not have been as effective as the Centrifuge 2 treatment method for the detection of low levels of norovirus. It was observed that in the experiment with the Fixed method, the re-suspension of the viral pellet in lysis buffer was incomplete. This may have been caused by the high centrifugal forces producing too compact a pellet. The viral pellet could not
be completely re-dissolved in lysis buffer and may have subsequently been lost during the RNA extraction procedure, reducing the apparent effectiveness of the Fixed method.

**Norovirus and Poliovirus**

Centrifuge 2 treatment appeared to be the most effective method for reduction of matrix inhibitors for the detection of norovirus in the limited samples tested. Band intensity for food products averaged >150 (Figure 14) from each of the seven foods. Nevertheless, this method was less effective at reducing matrix inhibitor for Lettuce and Dark Chicken.

Poliovirus was used as a model for other foodborne viruses, because of its capability to grow in cell culture allowing for exact numeration of the virus whereas other viruses (e.g. norovirus) lack this ability. It is a member of the picornavirus family and can be readily transmitted through food or water. Additionally, because poliovirus is well studied, the binding and behavior of this virus is well understood.

The Fixed method was found to be the most effective treatment method for the removal of matrix inhibitors for the detection of poliovirus. This method increased the second centrifuge force (spin) of the standard/normal protocol to 38,400 xg. It was hypothesized that the higher centrifugal forces would result in more effective viral concentration and removal of matrix inhibitors. The second centrifuge spin was used to form a viral-protein pellet, which would later be re-suspended in lysis buffer. A major draw-backs of this method was the equipment required; a high speed centrifuge with a fixed angle rotor, and proper containers that can withstand the centrifugal force (38,400 xg). Re-suspension of the pellet was more complicated with this method; the 9mL of lysis buffer barely covered the base of the container and the pellet was usually stuck to
the side of the container. The Fixed treatment method, as well as the Centrifuge 2 treatment method, which was the second most effective method for enhancing recovery of detectable virus, proved that an increased centrifuge force at the second spin was an effective measure for enhancing recovery of virus.

For the reduction of matrix inhibitors for the detection of poliovirus the Fixed treatment method was the best followed by the Centrifuge 2 treatment method. For the detection of Poliovirus at low levels these methods were switched; the Centrifuge 2 treatment method was the best and the Fixed treatment method followed; the Normal/standard method was near the bottom in ranking. Although there were no differences statistically between the Centrifuge 2 and the Fixed methods, there were visual difference in band intensity levels between the Centrifuge 2 treatment method and the Normal treatment method, as well as the Fixed treatment method and the Normal treatment method. This difference suggests that an increased centrifuge force after flocculation may reduce matrix inhibitors and increase detection capabilities at low levels.

Thus, this study found the increased centrifugal force during the second centrifuge spin may be effective at enhancing detection of eluted virus, removal of matrix inhibitors, and for detection of virus low levels. This spin pelleted the viral-protein flocculate from the eluate. At lower gravitational forces, as used in the Normal treatment method, the centrifuge spin may not have been able to completely precipitate the virus-protein flocculate from the solution therefore, leaving some virus in the solution that was discarded.
The differences between the treatments for poliovirus and norovirus were minimal. For both viruses it was demonstrated that detection levels could be increased and matrix inhibition from seven different food types could be reduced by an increase in centrifuge force after flocculation. Both poliovirus and norovirus are similar in their size (norovirus 27-32 nm and poliovirus 30 nm), both are non-enveloped viruses, and have similar environmental tolerances.

**Study Limitations**

There were several limitations to this study: a modification to the Normal/standard protocol occurred after processing of the first food product (Lettuce) and because of such a large number of samples (280 food samples) each experiment was performed without replication. During processing of the Lettuce samples, pieces of the lettuce leaf broke off in the eluate and were floating in the BBE solution. These pieces were then decanted with the eluate into a 250 mL conical for centrifugation and were not pelleted from the solution. They were inadvertently retained in the sample through virus lysis and may have caused smearing in the bands on the gel (Figure 52 and 53). The investigator changed the protocol to limit the possibility of food particle carry over, by pouring the eluate through a nylon mesh filter prior to flocculation. Virus does not bind to the filter which prevents food particles from passing through with the eluate. A reduction of smearing was observed on the gel when the nylon mesh filter was used with a second set of Lettuce samples (Figure 54 and 55). Thus it was decided to incorporate the use of the filter as part of the standard procedure.
Figure 52: Normal Treatment Method for Lettuce Without Nylon Mesh Spacer Top

Top: Lane 1, PCR marker; Lane 2, amplification negative; Lane 3, lysis negative; Lane 4, BBE negative; Lane 5, amplification positive; Lane 6, lysis positive; Lane 7, BBE $10^1$; Lane 8, Lysis $10^1$; Lane 9, BBE $10^2$; Lane 10 Lysis $10^2$; Lane 11, BBE $10^3$; Lane 12, lysis $10^3$; Lane 13, Lettuce $10^3$; Lane 14, PCR marker.

Figure 53: Normal Treatment Method for Lettuce Without Nylon Mesh Spacer Bottom

Bottom: Lane 1, PCR marker, Lane 2, amplification positive; Lane 3, BBE $10^4$; Lane 4, lysis $10^4$; Lane 5, Lettuce $10^4$; Lane 6, BBE $10^5$; Lane 7, lysis $10^5$; Lane 8, Lettuce $10^5$; Lane 9, BBE $10^6$; Lane 10, lysis $10^6$; Lane 11, Lettuce $10^6$; Lane 12, BBE $10^7$; Lane 13, lysis $10^7$; Lane 14, PCR marker
Figure 54: Normal Treatment Method for Lettuce With Nylon Mesh Spacer Top

Top: Lane1, PCR marker; Lane 2, amplification negative; Lane 3, lysis negative; Lane 4, BBE negative; Lane 5, Lettuce negative; Lane 6, BBE $10^{-1}$; Lane 7, lysis $10^{-1}$; Lane 8, BBE $10^{-2}$; Lane 9, lysis $10^{-2}$; Lane 10, BBE $10^{-3}$; Lane 11, lysis $10^{-3}$; Lane 12, Lettuce $10^{-3}$; Lane 13, PCR marker; Lane 14, amplification positive.

Figure 55: Normal Treatment Method for Lettuce With Nylon Mesh Spacer Bottom

Bottom: Lane 1, PCR marker; Lane 2, BBE $10^{-4}$; Lane 3, lysis $10^{-4}$; Lane 4, Lettuce $10^{-4}$; Lane 5, BBE $10^{-5}$; Lane 6, lysis $10^{-5}$; Lane 7, Lettuce $10^{-5}$; Lane 8, BBE $10^{-6}$; Lane 9, lysis $10^{-6}$; Lane 10, Lettuce $10^{-6}$; Lane 11, BBE $10^{-7}$; Lane 12, lysis $10^{-7}$; Lane 13, PCR markers; Lane 14, amplification positive.
The second limitation to our study was that only one food sample at each dilution for each treatment procedure was assayed. This made statistical analysis impossible. In order to overcome this, the dilutions ($10^{-3}$-$10^{-6}$) were used as repetitive measures for each food allowing for an Analysis of Variance to be performed. In order to provide a complete assessment of an optimal method for reduction of matrix inhibition, this study needs to be replicated.

If replications had been performed, I hypothesize that significant differences would be seen between each of the foods as well as between each of the treatment methods. Replication of this study would determine whether similarities found between poliovirus and norovirus in detection from food surfaces were real. The DOH State Laboratory in Tampa has implemented the use of a mesh filter and an increased centrifuge force after flocculation. This method has subsequently been used on outbreak associated food samples (data not presented) and appears to be effective at matrix inhibition reduction on a wide variety of food products from soy-sauce to salad dressings and breads.
Conclusion

Different food classes are known to produce varying degrees of inhibition of RTPCR amplification of viral genomes, requiring modification to any virus detection assay protocol. Lunchmeats generally have the greatest level of additives and processing among meat products. Turkey and chicken are comprised of two types of muscular tissue, white and dark; generally dark meats have higher levels of fat content and myoglobin proteins. Lettuce, previously has shown to consistently inhibit RT-PCR of eluted virus. It is hypothesized that the inhibition is due to the variety of plant carbohydrates. Prior to testing for viruses from any food source, an evaluation of the food components should be conducted to determine which steps in the procedure need adjustment. Our study suggested that the Centrifuge 2 treatment method would be the best overall method for removal of matrix inhibitors for the detection of foodborne viruses. This method did not require expensive high-speed centrifuge equipment or expensive bottles that are required for use at high gravitational forces.

In conclusion, this investigation suggested that there may be more that one specific method necessary to efficiently extract viruses from the surface of foods. Each food may require an individually analysis to determine if or when a higher centrifuge force is necessary for viral concentration and extraction. This study suggested that increasing centrifugal forces above the standard method may be necessarily for efficient viral recovery from foods; however, use of a nylon filter also had an effect on reduction of matrix inhibitors and improved viral recovery.
References


Figure 56: Lettuce Label

Figure 57: Turkey Label
Appendix A: (Continued)

Figure 58: Chicken Label

Figure 59: Lunchmeat Labels
## Appendix B: Food Processing Bench Sheet

### Food Processing Log

<table>
<thead>
<tr>
<th>Specimen:</th>
<th>Date:</th>
<th>Technician:</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th><strong>Procedure</strong></th>
<th><strong>Time</strong></th>
<th><strong>Comments</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Weigh 50gm of sample food on top loader balance.</td>
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<tr>
<td>2. Place the sample food in a 600mL sterile plastic beaker.</td>
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<tr>
<td>3. Place sample in the hood and spike with 100uL of virus. Allow to sit in hood for 2 hrs</td>
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<tr>
<td>4. Place in refrigerator over night.</td>
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<tr>
<td>5. Add 200mL of Buffered Beef Extract (BBE) enough to cover the sample, should be at a pH of 7.5</td>
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<tr>
<td>6. Place sample on table top orbital shaker at 120 rpms for 30 mins.</td>
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</tr>
<tr>
<td>7. Pour off solution through Millipore filter into sterile 250mL conical.</td>
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<td></td>
</tr>
</tbody>
</table>
| 8. Centrifuge: Normal xg for 30min  
Centrifuge 1 xg for 30 min  
Centrifuge 2 xg for 30 min  
Centrifuge 3 xg for 30 min  
Fixed xg for 30 min | | |
| 9. Transfer the supernatant to a 250mL sterile conical. Discard the solid. | | |
| 10. Add sterile magnetic stir bar and pH electrode into the eluate. | | |
| 11. Adjust the pH from 7.5 to 3.5 with 5N HCl. Do NOT allow the pH to drop below 3.5 | | |
| 12. Remove the electrode from the beaker and disinfect it (bleach, thio, water wash) | | |
| 13. Invert conical and allow stirring slowly for 30 minutes. **FLOCCULATION STEP** | | |
| 14. Remove the magnetic stir bar and Centrifuge: Normal xg for 20min  
Centrifuge 1 xg for 20mins  
Centrifuge 2 xg for 20mins  
Centrifuge 3 xg for 20 mins  
Fixed xg for 20 mins | | |
| 15. Discard the supernatant. SAVE the SOLID | | |
| 16. Add 9mL of NASBA Lysis buffer and magnetic stir bar. | | |
| 17. Allow to stir till pellet is completely dissolved | | |
| 18. Remove supernatant with 10mL pipet and transfer to sterile 15mL Falcon conical. | | |
| 19. Store in -70C freezer till extraction. | | |
Appendix C: Centrifuge Rotors

Figure 60: Beckman Swing Bucket Rotor
Appendix C: (Continued)

Figure 61: Beckman Fixed Angle Rotor