THE COMPARISON OF ANTIBODY- AND LIGAND- COUPLED MAGNETIC BEADS FOR THE CAPTURE, RECOVERY, AND CONCENTRATION OF NORWALK VIRUS-LIKE PARTICLES AND NATIVE GI.1 VIRUS IN BUFFER AND FOOD EXTRACT MATRICES

by

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(Under the Direction of Jennifer L. Cannon)

ABSTRACT

Norovirus is recognized as the principal cause of acute gastroenteritis in adults, causing over 90% of non-bacterial and an estimated 50% of all-cause epidemic gastroenteritis globally(Patel et al., 2008). There is a need for fast, accurate, reliable and cost-effective methods to detect norovirus in foods. The objective of this experiment was to evaluate the efficiency of HBGA ligand-coupled magnetic beads in capturing native GI.1 virus or norovirus virus-like particles (VLPs) in the context of common elution buffers and food residuals in order to determine their value as part of a norovirus detection technique. The data showed that the ligand coated magnetic bead capture method can be utilized for Norwalk VLP and native GI.1 virus, and also has the potential with further research and exploration to be a useful rapid method for the concentration and recovery of norovirus in both buffer and food matrices.

INDEX WORDS: Native GI.1, Norovirus, Magnetic Beads, Detection, Ligands, VLP

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DEDICATION

I dedicate this thesis to my mother, Camille Pettway, who constantly gave me the encouragement, love, prayers and support throughout this journey to complete my masters degree, never letting me forget that I can do any and all things through Christ which strengthens me. I love you Mommy.

I would also like to dedicate my research to my phenomenal Aunt, Charlene Brown, who was a great source of support and motivation to completing my goals and dreams. She believed in reaching for the moon, while being surrounded by the stars. I know she is watching me from heaven and is with me every day, helping to guide me to a successful future.

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CHAPTER 1

INTRODUCTION

Norovirus is recognized as the principal cause of acute gastroenteritis in adults, causing over 90% of non-bacterial and an estimated 50% of all-cause epidemic gastroenteritis globally(Patel et al., 2008). Norovirus has also been responsible for an estimated annual 64,000 episodes of diarrhea that required hospitalization and 200,000 deaths among children less than 5 years of age in developing countries(Patel et al., 2008). Sporadic illnesses are also common. For example, in Italy, a recent study attributed fourteen to forty-four percent of sporadic cases of pediatric gastroenteritis to norovirus (Ramirez et al., 2006). Norovirus is known to contaminate many different food groups, such as leafy greens, fresh produce and ready-to-eat products, such as deli-meats. These food categories are susceptible to norovirus contamination due to the high amount of handling during consumption and preparation, and also because these foods commonly lack a cooking step before consumption.

There is a need for fast, accurate, reliable and cost-effective methods to detect norovirus in foods. Commonly, during an outbreak, volunteers that are thought to have been exposed to the contaminant are asked various questions such as symptoms, foods eaten etc. (Verhoef, 2009). This type of surveillance is often used to identify which foods may be involved in a suspected foodborne outbreak, but seldom are foods tested during outbreak investigation. Reliable methods for food testing could improve the accuracy and speed in which an outbreak source is

identified, given the time requirements and error associated with surveys which are dependent on human memory and the extent of questions asked by those doing the investigation.

While methods for norovirus detection in foods currently exist, they are costly and time-consuming, especially if dealing with a high number of samples to be analyzed. The most specific and efficient noroviral detection methods generally involve a four step process: viral elution, concentration, RNA extraction, and detection by real-time RT PCR. The elution process releases virus particles from foods so that they can be concentrated and the RNA extracted. The efficiency of the elution procedure ultimately dictates how much of the target particles can be recovered from the food being sampled. One critical part of the elution process is selection of the elution buffer. Various buffer properties (pH, ionic strength, and presence of protein ingredients) affect virus elution, but can also affect virus structure (degradation of protein). Commonly, buffers are adjusted to a high pH (> 9) and/or high ionic strength (>50 mM) and often also include proteins which are negatively charged in basic elution buffers. Such buffers are designed to disrupt electrostatic and Van der Waals forces between the virus particles and the food, therefore, increasing viral particle elution. Common viral concentration methods include polyethylene glycol precipitation, ultracentrifugation, and ultra-filtration; none which aid in viral purification. Purification methods are often used prior to, during or after viral RNA extraction to remove food debris or other chemicals which can interfere with reverse transcriptase polymerase chain reaction (RT-PCR), the “gold standard” for norovirus detection.

One method that is used for both norovirus concentration and purification is magnetic separation using functionalized superparamagnetic beads. Various types of superparamagnetic beads (strepavidin, amine, tosylactivated) are commercially available and have a broad range of applications in areas ranging from cancer research to virus detection. Much of this research has involved the coupling of monoclonal antibody to the beads. While sensitivity and specificity can be high when using monoclonal antibodies, limitations were quickly discovered when using antibody-coupled magnetic beads to capture norovirus. This is because there are likely numerous serotypes of noroviruses based on previous studies that have demonstrated lack of cross-reactivity between genotypes,especially between genogroups and with new strains continuing to emerge, evolving with time. Trying to capture these targets using a specific monoclonal antibody-coupled magnetic bead, very often restricts the number of genotypes one can capture and recover.

To lift this restriction, in this study, histo-blood group antigen (HBGA)-coupled magnetic beads were investigated for their compatibility with common virus elution procedures. This work evaluates such procedures as part of a rapid method to detect noroviruses or virus-like particles (VLPs) in food. Since differing serotype of norovirus are known to recognize common HBGAs (Huang et al., 2005), it may be possible to use a heterogeneous mix of HBGAs for universal capture of all norovirus serotypes. Human saliva (from certain individuals known to secrete HBGAs in their saliva) (Tan and Jiang, 2005a) and Porcine Gastric Mucins (PGM) contain a heterogeneous mix of HBGAs. Therefore, magnetic beads coated with these ligands have the potential to increase the percentage of norovirus serotypes recovered with this method, when compared to coating the magnetic beads with monoclonal antibodies.

The overall objective of this experiment was to evaluate the efficiency of HBGA ligand-coupled magnetic beads in capturing norovirus or norovirus VLPs in the context of common elution buffers and food residuals in order to determine their value as part of a norovirus detection technique. Human Norwalk virus and Norwalk virus VLPs was used as a model virus in this proof-of-principle study outlined in the following specific aims.

1. Elution buffers, 1M NaCl PBS buffer, glycine buffer and sterile de-ionized water at neutral pH, were used to evaluate virus/VLP capture.

2. Elution buffers, glycine buffer at pH 9.5 and citrate buffer at pH 3.6, were used to disrupt virus charge and evaluate the effect of virus/VLP capture under these conditions.

3. Lastly, we evaluated the impact of food residuals (from iceberg lettuce and roast beef deli meat) on native virus or VLP recovery using the ligand-coated magnetic beads suspended in elution buffers which were most compatible with the ligand-coated magnetic beads.

All studies with ligands were compared to capture with monoclonal antibodies to determine if ligands could be used as an alternate to monoclonal antibodies for norovirus capture with magnetic beads. Capture of native GI.1 virus particles as well as their VLPs was performed for all three sets of experiments to determine if VLPs and native virus particles behave similarly in capture experiments.

If successful, the results of this study will ultimately be used as a foundation for incorporating ligand-coupled magnetic beads in a capture and recovery method when detecting human norovirus within a food matrix.

CHAPTER 2

LITERATURE REVIEW

Overview of Prominent Causes of Foodborne Illness

Many foodborne illnesses are prominent within the United States today. Some examples of bacteria that have recently gained media attention include Listeria *monocytogenes*, *Salmonella,* and *Escherichia coli O145* (*E.coli 0145*). An outbreak of *Listeria monocytogenes* in cantaloupes was associated with 30 deaths from 28 states, and 146 illnesses (Prevention, Diseases, and Diseases, 2011). In 2011, an outbreak involving ground turkey was associated with *Salmonella Heidelberg,* a multi-drug resistant strain causing136 illnesses in 34 states and one death (Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases, and Division of Foodborne, 2011). Additionally, a 2012 outbreak of *E. Coli 0145* was reported to include 15 infections in 6 states, 1 death, and 4 hospitalizations, but no source for the outbreaks has yet been identified (Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases, and Division of Foodborne, 2012). Most microorganisms differently affect populations, with the elderly, infants and young children, and immune-compromised persons being most severely. According to the CDC, norovirus is credited as the most common cause of epidemic gastroenteritis, causing an estimated 50% of epidemic gastroenteritis outbreaks globally (Patel, 2009). Norovirus outbreaks often take place in long-term care centers and other healthcare facilities, restaurants, catered events, cruise ships, schools and daycare centers (National Center for Immunization and Respiratory Diseases and Diseases,

2012). Norovirus is responsible for an estimated 58% of foodborne acute gastroenteritis illness and over 5 million illnesses each year in the US (Scallan E et al., 2011).

What is norovirus?

Norovirus, its counterparts, and their properties were a mystery until 1968. Dr. Albert Z. Kapikian's work on the discovery of a viral etiologic agent responsible for an outbreak of acute gastroenteritis in Norwalk, Ohio served as the foundation to reveal norovirus to the world. In this outbreak, 50% of exposed students and teachers developed gastrointestinal illness. This virus named, Norwalk virus, was later determined to be part of a large group of unculturable, but related viruses, named "Norwalk-like viruses" or "small round structured viruses". Subsets of these viruses are now known as noroviruses. Kapikian utilized a technique called "direct" or "particle" virology, in which the virus particle could be studied without cultivation in a human or animal host system. During this time, many scientists had been doing human volunteer challenge studies, in which bacteria-free stool filtrates or aerosolized throat washings were ingested by the volunteers (Jordan, 1953). These challenge studies proved that there was an infectious filtrate present, but it did not aid in revealing additional etiological knowledge. A breakthrough occurred when Dr. Kapikian applied a new technique called immune electron microscopy (IEM) to his work on the Norwalk outbreak. IEM facilitates the direct visualization of antibody-antigen interactions that can be seen after aggregation and coating of specific antibodies to targeted microorganisms (Kapikian, 2000). This technique had been previously used in application to tobacco mosaic virus (Kapikian, 2000). A rectal swab was obtained from a secondary case in the Norwalk outbreak. From this, a 2% filtrate of the sample was made and fed to volunteers to induce illness. The filtrate that was fed to volunteers was then combined with convalescent sera from an infected patient as the antibody source. Antibody interaction with the virus was then

visualized using IEM. Kapikian also developed a scale that was used to confirm serological response to the virus. The scale ranged from 4+ to 1+ (1+ showing the least amounts of antibody binding and therefore the lowest serological response; 4+ being the highest serological response with very heavy coating of antibody). Since this detection of Norwalk virus, IEM has also been used to discover Hepatitis A virus (Kapikian, 2000). Although immune electron microscopy was originally used as a method of detection, studies have shown this technique to not be specific enough to adequately characterize the many genogroups and strains of norovirus because many of them are not serologically cross-reactive.

Noroviruses are viruses in the family *Caliciviridae* which is composed of non-enveloped, icosahedral viruses that range from 27 to 40 nm (Flint et al., 2003).The genomes of viruses in this family range from 7.3 to 8.5 kb in length and are arranged into two or more major open reading frames (ORF), depending on the genus. Caliciviruses are characterized by cup-like structures on their capsid surface. Other genera in this family include sapovirus, lagovirus, and vesivirus.

Norovirus is a positive sense, icosohedral, non-enveloped, single-stranded RNA virus; with a genome is approximately 7.7 kb in length encoding three ORFs. Its genome is protein-linked at the 5' end and polyadenylated at the 3'end. ORF1 encodes the nonstructural proteins that are post-translationally processed by the viral 3C protease of ORF1 (Hardy, 2005). ORF2 encodes for the major capsid protein VP1 and ORF3 encodes the minor structural protein VP2 (Hardy, 2005).

Norovirus is transmitted by the fecal-oral route or through aerosolized vomitus containing virus particles. It takes as little as 10 viral particles to cause norovirus infection.

Common routes of infection include fecally contaminated fomites and person-to-person transmission. The incubation period of the virus can range from twenty four to forty-eight hours; however, some studies have shown onset of symptoms within twelve hours of exposure. Once infected the shedding of norovirus usually begins with onset of symptoms and may continue for two weeks or more after recovery. There are typically 10^6 -10^{12} particles of virus shed per gram of feces (Patel, 2009). Norovirus has a prominent affect on the population, no matter their demographic, sex, or age; however, the most affected are the elderly, young children, and those that already have medical illnesses (Centers for Disease Control and Prevention and National Center for Emerging and Zoonotic Infectious Diseases, 2011). Symptoms include vomiting, non-bloody watery diarrhea with abdominal cramps, nausea, and mild fever. Symptoms may last from twenty-four to seventy-two hours. Some people infected may even be asymptomatic. Norovirus often occurs in family or community-wide outbreaks (Glass et al., 2000).

There are six non-structural proteins that are encoded by ORF1. Some non-structural proteins contain hydrophobic regions near the C-terminus and are involved in the regulation of cell proliferation (Hardy, 2005). They are variable in length and amino acid sequence between genogroups. A lot of the proteins within the non structural section of norovirus have a variety of functions, for example, P41 contains NTPase which is responsible for the binding and hydrolization of ATP. VPg is perhaps the largest of the non structural proteins with a weight of 15 kDa. VPg also has a critical role in the norovirus infectivity and initial translation at the 3' end (Morillo, 2011). Vpg gives stability to the protein molecule and initiates translation (Morillo, 2011). P22 is another non-structural protein; however, there is not much data on its functions, except that it has a precursor role in the proteolytic pathway. $3CL^{pro}$ is a non-structural norovirus protease. The RdRp (RNA-dependent RNA polymerase) displays catalytic and structural

characteristics of other positive-strand RNA viruses, such as the finger, palm and thumb domains common to all polymerases (Hardy, 2005).

The norovirus virion consists of 90 dimers of VP1 and one or two copies of VP2 (Hardy, 2005). VP1 and VP2 are both translated from a protein-linked subgenomic RNA transcript that contains both ORF2 and ORF3 regions.

Norovirus structural proteins self-assemble into virus-like-particles (VLPs) with morphologic and antigenic properties similar to native noroviruses (Patel, 2009). VLPs can be used to further understand structural and functional aspects of VP1 domains and norovirus capsids in addition to host-cell interactions including cellular receptors for norovirus. VLPs are often the tool used to gather the most data on the norovirus protein and capsid to due norovirus being unculturable. VLPs are similar to native virus particles except that they do not contain RNA. VLPs are constructed from the insect-cell baculovirus expression system (Palomares, 2012). The process of designing a VLP includes growing of insect cells to a desired cell density, the infection of the insect cell culture with one or more recombinant baculoviruses that contain the targeted gene or genes, and lastly the expression of recombinant genes. In order to control and regulate protein expression the multiplicity and time of infection can be manipulated as well as the cell concentration during the time of infection (Palomares, 2012).

VP1 contains between 530-555 amino acids and ranges between 58-60 kDa in weight (Hardy, 2005). The S domain is located on the N-terminal end and contains 225 amino acids, which are crucial for icosohedral shape formation. The P domain contains the remaining (about 325) amino acids and is split into two domains P1 and P2. P1 forms the internal core and P2 is a subdomain that builds up the outer layer of its structure. P2 is actually a 127 amino acid insertion into the P1 domain. P2 is plays an integral role in the receptor binding process. The P

domain contains a hinge that will form a dimer that binds to histo-blood group antigens which are associated with cellular recognition (Tan and Jiang, 2005b). Dimerization and polymerization of the P domain increases the stability of the capsid, creating the characteristic protrusions that can be visualized by electron microscopy. VP2 is quite smaller than VP1, containing from 208-268 amino acids with weights ranging from 22-29 kDa. VP2 sequences vary between norovirus strains. Based on VP2 protein chemistry (basic at neutral pH), researchers believe VP2 may be responsible for binding RNA(Hardy, 2005). All of the roles of VP2 are not known, however it is believed that VP2 is essential for the production of infectious virus (Hardy, 2005).

Norovirus reference strains

Currently, there are five recognized norovirus genogroups, which consist of at least 31 genotypes (Morillo, 2011). Three genotypes (GI, GII, and GIV), are known to infect humans. The GI genogroup has at least 8 genotypes and includes the GI.1 prototypical strain, Norwalk virus. GII genogroup has at least 17 genotypes and includes prototype strains such as Bristol, Lordsdale, Toronto, Mexico, Hawaii and Snow Mountain virus as well as genotypes that infect only swine. GIII strains have 2 genotypes which infected the bovine species and GV viruses infect mice. Since 2002, variants of the GII.4 genotype have been the most common cause of norovirus outbreaks in humans. Of the five genogroups, GI and GII show the most genetic diversity (Morillo, 2011).

Although, norovirus is a great problem in the United States, there are no standardized methods for detection of norovirus in foods in the United States. One reason for this is because human norovirus cannot be cultured outside of the human body, making it very difficult to work

with and perform experiments in a lab setting. Therefore, murine norovirus (MNV) or feline calicivirus is used as a surrogate because both viruses both have similar virus structure and genomic organization.

MNV is one of the most heavily researched norovirus surrogates because it is the only norovirus that can replicate both in cell culture. MNV was first isolated from severely immunocompromised mice. MNV has been used to study mechanisms of norovirus replication in both a natural host and tissue culture. MNV has also been used to study host immune responses following infection, the significance of defined mutations, and cellular and structural requirements for norovirus infection. MNV does not display every aspect of human norovirus and its infection, but the virus has some important aspects in common with its human counterpart. Some of these qualities include having the same size (28 to 35 nm in diameter), shape (icosohedral) and buoyant density (1.36 +/- 0.04 g/cm2) as human norovirus (Wobus, 2006). However, MNV has four ORFs (the 4th with an unknown function). MNV capsid proteins self-assemble into virus-like-particles when expressed in a baculovirus expression system, which is characteristic of noroviruses. Transmission of the virus in mice is similar to human norovirus as well, for example, MNV has the ability to be spread through the fecal/oral route and possibly through aerosolized droplets. Because of its similarities, MNV is often used to study mechanisms of replication and regulation of protein synthesis. Even with the MNV surrogate, development of a standardized method for detection has been slow.

Determining Norovirus Etiology in Foodborne Outbreaks

After a foodborne outbreak, epidemiologic surveillance is often used to determine the outbreak source. Retrospective surveillance is when the suspected audience that ate a common

meal or worked together in a specific place is contacted by phone or letter and asked a series of questions about the types of symptoms they are currently having or have had, and the types of foods they consumed over a specific time period. Retrospective surveillance provides possible linkage of symptoms, demographics, foods, and or places that could have contributed to an outbreak. Surveillance is a broad method of sourcing an outbreak for most microorganisms. After surveillance has been completed other techniques such as culturing or PCR are needed to confirm the outbreak etiology. Such data can be aggregated into surveillance systems. For example, the Food-Borne Viruses of Europe (FBVE) net is an epidemiological surveillance database for diagnostic data on outbreaks caused by agents of viral gastroenteritis, Hepatitis A virus and Hepatitis E virus (Duizer et al., 2008). NoroNet is also an epidemiological surveillance database which in addition to monitoring epidemiological data on outbreaks, containing clinical diagnostic information and sequence information. Epidemiologic survey information contained in this database may include information such as whether outbreaks are suspected to originate from foods (foodborne) or caused by person-to-person transmission (Verhoef, 2009). Surveillance can be a very useful tool. One study of FBVE net data, showed that foodborne outbreaks were found more often in households or restaurants than healthcare settings, and that genogroup GII.4 was the most common strain found in the non-foodborne outbreaks surveyed, mostly transmitted from person-to-person (Verhoef, 2009). In addition, study show typically norovirus outbreaks on cruise ships one season occurred more often in off-season (May-September) than the typical winter months (Verhoef, 2009). Perhaps the most significant part of this study conducted by Verhoef et al (2009), was that a mathematical model was created to estimate the likelihood of a norovirus outbreak being food related based exclusively on the genotype detected (Verhoef, 2009). This model can also retrospectively estimate the true

contribution of foods viral gastroenteritis. In addition, this model may contribute to consistent reporting and typing of outbreaks and improve overall surveillance data quality. This model was also used to calculate the true contribution of foodborne norovirus to general norovirus gastroenteritis outbreaks in Europe. Overall, surveillance methods can be used to categorize and monitor sources of current and potential norovirus outbreaks.

There are three important steps in the detection of food-borne viruses: viral elution, concentration, RNA extraction, and detection by real-time RT PCR. Virus extraction is a very critical part in this process, because without proper virus extraction there will be no virus particles to purify and detect. Virus extraction is performed to separate viral particles from the food matrix, concentrate them into a smaller volume and remove inhibitory compounds. Virus extraction is based on two main approaches which are acid adsorption-elution concentration and elution-concentration. These methods vary significantly depending upon the material or food one is extracting from. There are three main food categories: semi-solid foods such as fruits and vegetables, solid foods such as deli meats, and shellfish, and liquids. After the material from which virus is being extracted from is determined there are three possible ways of extracting the virus from the food. For example, extracting viruses from semi-solid foods often involves an acid adsorption elution-concentration method. The acid-adsorption-elution procedure depends first on an acidic buffer, causing the virus to become positively charged, and facilitating virus adsorption to the food surface. A neutral or basic buffer is then added to neutralize the buffer and negatively charge the virus to allow it to be eluted from the food and collected. The acid-adsorption method is used for foods that break apart easily (soft fruits, soups, bakery items) or items that may be internally contaminated (i.e. shellfish).

Elution-concentration methods are used on foods that are normally surface-contaminated and do not break apart with stomaching (i.e. lettuce, deli meats, fomites). There are various methods of virus elution from food surfaces. One of the more common methods is to use an alkaline buffer, pH between 9-10.5, containing (1-3% range) beef extract or 0.5 M glycine, which becomes negatively charged at basic pH. The beef extract proteins or amino acids will compete with the negatively charged virus particles adsorbed to the surface of the food and facilitate viral particle detachment from the food surface. The pH must be subsequently neutralized because the norovirus capsid is unstable at basic pHs

Some examples of buffers that have been used for elution are 50 mM Glycine with 0.1 M Tris-HCl and 3% beef extract and PBS with 1 M NaCl and 0.05% Tween 20. Glycine buffer with beef extract is a common elution buffer that is able to reduce non-specific virus adsorption to the food matrix during extraction. The high protein in the beef extract also aids in flocculation of the virus. 1M NaCl PBS has a high salt concentration which in turn has high ionic strength that will disrupt electrostatic interactions and between the virus and the contaminated surface or food and mask surface charges, exposing hydrophobic domains on the virus and as a result, allowing the virus to be eluted or released into the buffer matrix. Tween 20 is a detergent added as an additional measure for elution of the virus from the surface or food.

Virus Concentration

After the elution process, the next step in virus recovery is concentration. Concentration of the virus is important since viruses may be present in very low numbers on foodstuffs. There are several methods of viral concentration. The three most common methods are poly-ethylene glycol (PEG) precipitation, ultracentrifugation, and filtration (ultrafiltration or with charged membranes). PEG precipitation is based separation solubility. PEGs for protein precipitation are

usually classified by molecular weight and range from 4000 to 6000 Daltons (Sim et al., 2012). In virus detection, proteins are small and when using PEG, it takes high concentrations of larger MW PEGs to precipitate small proteins. Proteins are concentrated more readily when the proteins are present in high concentrations (Sim et al., 2012). PEG is the most common method to concentrate viruses from foods. PEG is used to increase the viral concentration to enhance molecular detection. PEG allows the precipitation of virus as neutral pH and high ionic concentrations (Stals et al., 2012). Ultracentrifugation is another method of concentration, involving centrifugation at high speeds (>80 K x g) in order for the target to pellet at the bottom of the centrifuge vessel and supernatant fluid to be discarded. In one previous study, ultracentrifugation yielded the highest recovery efficiencies of viruses contaminating lettuce and ham, whereas PEG precipitation recovered the highest yield of noroviruses from raspberries (Summa, Henrik von Bonsdorff, and Maunula, 2012). Average virus recoveries were 19%, 47% and 28% for lettuce, ham and raspberries, respectively. PEG precipitation had the highest repeatability between experiments (Summa et al., 2012). Ultracentrifugation often requires an extra step of purification before being executed to get rid of access debris and other components that may cause error in virus detection. Solvents that are used for purification include Freon 113, Vertrel XF, and chloroform: butanol. In addition to the need for an extra purification step, ultracentrifugation machinery is expensive. Ultrafiltration is a concentration method that is based on molecular weight and like ultracentrifugation the sample must be purified before filtering to prevent debris from blocking or rupturing the filter. Charged membrane filters can also be used in this concentration process, in which charge is used to separate the target from debris. Other methods of concentration that are less common, but slowly being integrated more into the mainstream of research are cationic separation and functionalized magnetic beads to recover

target viruses. Such methods can use a variety of types of magnetic beads and can be coated with a variety of ligands, carbohydrates and antibodies which interact with the surface of virus particles. Additives, such as pectinase, soya protein, beef extract and cat-floc are sometimes added to aid in virus recovery in combination with concentration methods from foods. Pectinase can prevent jelly formation when viruses are eluted from fruits and vegetables such a raspberries and tomatoes. A benefit of adding beef extracts is that it can help in flocculation of norovirus on during PEG precipitation. Sometimes a secondary concentration step is performed to further decrease the sample volume. PEG precipitation is often performed, but magnetic beads coated with antibodies or other ligands can be used. The advantage of using magnetic beads is that it is both a concentration and purification step, which is often necessary before or after RNA extraction.

Magnetic Bead Detection

Much research has been done in the area of norovirus recovery and concentration using various types of magnetic beads. The types of beads vary from those that are positively-charged, also called cationic beads, to those that are functionalized, including ligand-coated beads, or the more commonly used, antibody coated beads. The type of magnetic beads used varies based on whether it will be coated and the type of coating that will be applied. Cationic beads that bind target molecules by charge attraction. A coated or conjugated magnetic bead is usually coated with a ligand. A ligand is a material that is used for attachment; ligands can be a protein or carbohydrate. Examples of conjugated magnetic beads are amine magnetic beads, which are hydrophilic beads that have amine groups already attached to the surface. These beads can be used to bind glycoproteins and carbohydrates. Another type of bead is a tosyl-activated bead which has tosyl groups on the surface to facilitate the formation of covalent bonds with the target

molecule (such as antibodies) and bead surface. Binding efficiency of the target microorganism to the functionalized magnetic beads depends on the specific affinity of the microorganism to the bead surface coating. Affinity is the degree of attraction of one material to the other. When selecting the type of magnetic bead to use in an experiment the binding affinity of that bead, coating and target must be taken into consideration. Some materials have a greater affinity that others, for example antibodies have a high affinity to antigens due to the specific binding sites available for attachment and as a result have the potential to increase the percentage of target serotypes able to be recovered.

HBGAs as norovirus recognition ligands

Histo-blood group antigens (HBGA) are a family of carbohydrate moieties that bind noroviruses and are therefore ligands that can be used to coat magnetic beads for norovirus capture. HBGAs are terminal carbohydrate structures that are often linked to glycoproteins or glycolipids. They are present on red blood cells and the mucosal epithelium of the digestive and respiratory tracts of humans, or as free antigens in bodily fluids such as saliva, milk, intestinal contents and blood of some individuals (Tan and Jiang, 2005a). HBGA expression on gut mucosa is genetically determined. There are three major categories of HBGAs; Lewis, H type, and ABO, all which are capable of binding noroviruses in a strain-dependent pattern (Tan and Jiang, 2005a). Some individuals are known as "non-secretors". They have a mutated*FUT2* allele for the fucosyltransferase enzyme needed for expression of HBGAs in the gut and its secretions. As a result, they are not infected with GI.1 after challenge ((Huang et al., 2005)). Secretors on the other hand, encode a functional *FUT2* gene product and therefore are susceptible to Norwalk virus infection. Since 2002, eight receptor binding patterns of HGBA-norovirus interactions have been identified (Shirato, 2011). For Norwalk virus, the binding

region of the capsid is composed of eight amino acid residues that form between six and seven hydrogen bonds and a cation interaction with the H or A antigens (Tan and Jiang, 2011). The protruding domain of the viral capsid protein interacts with protein side-chains during HBGA recognition. These interactions follow a key in lock model; there is a structural or conformational fit between the binding surface and the HBGA molecules (Tan and Jiang, 2011).. However, viruses of genogroup I (such as Norwalk virus) have a different binding site for HBGA recognition when compared to that of genogroup II viruses (Tan and Jiang, 2011).

Noroviruses are highly adaptable human pathogens. They have a high mutation rate during replication of the RNA genome causing genetic and antigenic diversity among noroviruses. This partially explains the resistance of noroviruses to hosts immune responses (Tan and Jiang, 2005a). Studies have shown that norovirus has evolved with its human host (Tan and Jiang, 2005a). This quality sets norovirus apart from many other HBGA-recognizing pathogens. Some studies show that not only has norovirus evolved, but the host immunity has shaped this evolution, creating more diversity among noroviruses over time.

As stated earlier, human norovirus cannot be cultured within a laboratory, therefore many laboratory studies and experiments with norovirus-HBGA interactions have been performed with recombinant virus-like particles (VLPs). VLPs are capable of HBGA binding and therefore provide a vast amount of information about norovirus receptor binding. One of the more commonly used assays in the norovirus HBGA receptor studies is the saliva binding assay, due to its low cost and sensitivity. Saliva can contain HBGA depending on if the source is a secretor or non-secretor.

In addition to HBGAs being important for norovirus infection, they can be utilized as an alternative method to concentrate human norovirus from water and food samples (Tan and Jiang,

2011). One previous study used HBGAs in combination with magnetic beads as a rapid and effective tool for investigated suspected norovirus contamination of foods (Morton, 2009). In this study carbohydrate-coated magnetic beads were used to detect GII.4 norovirus strains as well as additional strains from GII and GI genogroups. This study also concluded that HBGA and norovirus interactions have the potential to advance food testing technologies if put in combination with other multiple rapid testing methodologies (Morton, 2009).

Porcine Gastric Mucins

Mucus is a slimy, viscous secretion that covers the some epithelial surfaces. It is made up of water, salts, immunoglobins, and secreted proteins. The most abundant macromolecules in mucus are mucins (Bansil, Stanley, and Lamont, 1995). Mucins are elongated, rod-shaped molecules that are composed of several structurally large, very viscous, glycoproteins composed of 75% carbohydrate and 25% amino acid (Bansil et al., 1995). The central core of a mucin is a liner polypeptide called an apomucin. The function of mucus in the body is to protect and lubricate the epithelium. For example, the mucus cells in the stomach secrete mucus that attaches to the epithelial cells in the stomach and forms a diffusion barrier between the lumen and the cell surface (Bansil et al., 1995). Properties of mucins include protease resistance, large water-holding capacity, and high charge density from sialic acid and sulfate residues. Mucins are negatively charged at a neutral pH. These properties and information are useful when using conjugated beads in combination with various elution buffers at different pH in order to utilize the charge of the target to enhance binding affinity. Porcine gastric mucins, or PGM, come from pig gastric mucosa. It is a complex secretion of the stomach consisting of approximately 95% water, 3% mucin glycoprotein and 2% other small molecules (Celli et al., 2005). Gastric mucin has a high molecular weight and a complex structure of polysaccharide chains on a protein core.

Gastric mucins are believed to be the protective agent in the stomach to prevent it from digesting itself in its highly acidic secreted juices. Porcine gastric mucins opposed to bovine or other mammalian species' mucin is often used in experiments due to the mucin's composition and physiology similarities to human stomachs (Celli et al., 2005). There is also a sequence similarity between porcine and human gastric mucins (Celli et al., 2005). Detailed properties and information about mucins is hard to obtain due to the complexity of the mucin oligosaccharides. PGMs consist of multiple carbohydrates, including HBGAs such as H type 1 and 2 in addition to an array of other carbohydrates. Like HBGA, PGM has also been known to bind norovirus and norovirus VLPs. One study showed that when using PGM-coated wells on an ELISA plate, norovirus VLPs could be captured just after fifteen minutes incubation, and the amount of capture increased with increasing incubation time (Tian et al., 2010). This study also showed that in an acidic buffer matrix (pH 3.0-4.2) recovery increased (Tian et al., 2010). These results were expected because the isoelectric point of GI ranges from 5.9-6.0 and for GII ranges from 5.5-6.9. At an acidic pH of 3.0-4.2 the virus would be positively charged. PGM's isoelectric point is between 2 and 3 and would be slightly negatively charged at these pHs.

Antibody-conjugated magnetic beads

Literature has shown that most successful conjugated magnetic bead work has been done with antibodies specific for the target microorganism. These antibodies can be either polyclonal or monoclonal in origin. Some advantages to using polyclonal antibodies are that they are inexpensive to make in comparison to monoclonal, they contain large amounts of antibodies that may be able to recognize multiple epitopes on a single target molecule thereby increasing the likelihood of recognizing the target molecule. However, this may be a disadvantage as well, because non-specific binding may cause background signals to be high. Other disadvantages

include unwanted cross-reactivity and batch to batch variability during production which may give inconsistent results from experiment to experiment. Monoclonal antibodies on the other hand are more expensive, but often give more reliable results due to their high specificity to bind to only the targeted organism. This quality, although it gives reliable results, may also be a disadvantage by the antibody being too specific, especially if there are multiple genotype variants in a sample and it is unknown what they are. This is important specifically when dealing with norovirus because there are several genotypes and strains that are each molecularly different and may not respond/bind to the particular epitopes of a monoclonal antibody.

Detection of norovirus by RT-PCR

PCR is a rapid procedure for enzymatic amplification of a specific segment of DNA *in vitro*. PCR can be used before applications such as cloning, engineering of DNA, and assays for the detecting the presence of pathogens, among an array of other applications. PCR, in essence, is generally when a segment of DNA is amplified. Two single-stranded oligonucleotide primers are added in excess to the amount of DNA. Then a series of denaturization, primer annealing and nucleotide extension cycles are performed to amplify the target. Reverse transcription PCR (RT-PCR) transforms the RNA into cDNA, therefore, allowing it to be amplified for sample detection. PCR is the most sensitive assay for detecting rare sequences. Disadvantages to PCR include the small volume requirement and the ease in which cross-contamination can occur. PCR assays include conventional and real-time formats. In norovirus work, the most common method of PCR is real-time due to its high numbers of advantages when interacting with the viral genome. Some of the advantages include increased assay sensitivity, lack of need to run gel electrophoresis, and real-time data collection through detection of a fluorescent signal generated

proportionally as the number of amplicons increases. Real-time can also be quantitative, with the use of a standard curve.

Conventional and real-time RT-PCR are common techniques used to identify norovirus presence in a sample. Some limiting factors of using these PCR techniques are low virus concentrations in fecal samples, inadequate RNA extraction, presence of inhibitors of RT-PCR, and need to use different primer sets to amplify the majority of norovirus genotypes Real time RT-PCR has been proven to have a higher sensitivity level in comparison to conventional RT-PCR, specifically with samples of low virus particle concentration and RT- PCR inhibitors (Kim, 2008). In order for RT-PCR to detect a noroviruses, it uses what is referred to as broadly reactive primers for both GI and GII genogroups (Kageyama, 2003). ORF 1 and ORF 2 in norovirus have conserved regions in the genome which is consistent within GI and GII genogroups.

Norovirus in Foods

Oysters can bioaccumulate or concentrate norovirus particles during filter feeding (Schultz et al., 2007). Oysters may become contaminated by being harvested in sewage-impacted waters. Shellfish extracts block the binding sites of noroviruses to HBGAs. Certain oysters express carbohydrates similar to human HBGAs in their digestive tissues ((Schultz et al., 2007)). Depuration processes (allowing oysters to filter in "clean" water after their harvest) do not consistently rid of norovirus. This makes them hazardous, especially when they are consumed raw. Virus identification is also difficult due to the presence of PCR inhibitors which can vary between oyster species and harvest areas (Schultz et al., 2007). The most common food source for norovirus infection is ready to eat foods such as salads and deli-meat sandwiches that require little to no cooking (Patel, 2009). Due to the low inoculum of norovirus required for infection

compared to the amount that is spread in vomit or feces, infected food handlers of these types of foods typically cause large outbreaks. Data from a study conducted from 1990-2005 determined norovirus to be the cause of the most outbreaks associated with fresh produce (DeWaal and Bhuiya, 2007). In this study, 25% of produce-associated outbreaks were from norovirus in lettuce and salad greens (DeWaal and Bhuiya, 2007). Raspberries have been associated with several norovirus infections due to them being irrigated with sewage-contaminated water or by infectious food handlers(Bresee et al., 2002). Norovirus detection in foods is lacking due to the limited assays available and the difficulty of retrospective surveillance to link outbreaks.

CHAPTER 3

MATERIALS AND METHODS

Preparation of stool suspensions containing Norwalk virus

A Norwalk virus-positive stool sample derived from a human challenge study with Norwalk virus was obtained from Dr. Robert Atmar, Baylor College of Medicine, Houston, TX. A pea-sized amount of stool was distributed into a 1.5 ml tube. 750 µl of PBS was added and vortexed until thoroughly mixed. The sample was centrifuged for 10 minutes at 13.3 x*g*. After centrifugation, the sample supernatant was distributed into 10µl and stored at -80°C.

Pretreatment of magnetic magnetic beads and with carbohydrate ligands or monoclonal antibodies

Superparamagnetic Dynabeads, amine-coated M-270 and tosyl-activated M-280, were obtained from Life Technologies (Grand Island, NY; formerly Invitrogen). After vortexing each vial of magnetic bead stock for 2-4 minutes to thoroughly re-suspend magnetic beads in solution, magnetic beads were transferred to 1.5ml microcentrifuge ubes for pretreatment and ligand attachment. Amine-coated magnetic beads (1-2 x 10^9 magnetic beads per ml) were washed two times with 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 4.5-5) using the Dynamag-2 Magnet (Life Technologies) and re-suspended in 1ml of 0.1 M MES. Porcine gastric mucins (PGM) Type III (Sigma, city, state) were diluted to a concentration of 10 mg per ml of MES and added to magnetic beads (6 µl PGM solution / 100 µl magnetic beads). 10 mg of

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was dissolved in 1 ml of cold deionized water and added to magnetic bead/ligand mixtures at 50 µl per mg of ligand. The bead/ligand mixture was incubated on a slow tilt (11 degree) at 7 ppm rotation using a Hula mixer (Invitrogen, Grand Island, NY) for 2 hr at room temperature. After the incubation, hydroxylamine (10 mM) was added to the bead/ligand mixture and incubated for an additional 15 min before magnetic beads were washed a total of 4 times using PBS containing 0.5% BSA (4 min per wash) and stored in 1 ml of PBS containing 0.1% BSA at 4° C. Saliva from a secretor positive individual (UGA archive sample) was centrifuged at 3,500 x *g* for 15 min before 200-µl aliquots were transferred into eight-well PCR strip tubes (Applied Biosystems, Carlsbad, CA) for heat treatment at 99°C for 5 min. Saliva was immediately transferred to ice before storage at -70°C. An identical procedure was followed for coating saliva on magnetic beads as described for PGM, except the starting concentration was 6 µl of saliva per 100 µl of amine-coated magnetic beads in 0.1 M MES. For each experiment, saliva aliquots were discarded after use to avoid any damaging effects of freeze/ thawing. Monoclonal antibody (NV3901), specific for GI.1 Norwalk virus, (a gift from Dr. Robert Atmar, Baylor College of Medicine, Houston, TX) was coated onto tosyl-activated magnetic beads per the manufacturer's protocol. Appropriate amount of magnetic beads based on the amount of reactions needed were distributed into a 1.5 ml microcentrifuge tube and washed three times using 0.1M sodium borate buffer. NV3901 was added to the washed magnetic beads to achieve a concentration of 20 mg/ml according to manufacturing table. 3M ammonium sulphate was added to the bead/antibody mixture and the mixture was incubated overnight at 37°C on a shaker plate. After incubation, the bead/antibody mixture was placed on a magnet, supernatant fluid was aspirated off, and 1 ml of PBS with 0.5% BSA was added. The bead/ligand mixture was incubated at 37°C for 1 hour on tilt and rotation.

After incubation, the bead/antibody mixture was washed twice using PBS with 0.1% BSA and stored in this solution at 4°C. Before use, NV3901-, PGM-, or saliva- coated magnetic beads were blocked overnight in phosphate buffered saline (PBS) and 5% Blotto (5% de-hydrated milk and PBS).

Norwalk VLP and native GI.1 virus capture by functionalized magnetic beads

Twenty-µl aliquots of each functionalized and blocked bead type were prepared in 1.5-ml microcentrifuge tubes, placed on Dynabead magnet, and washed three times in PBS. After washing, 1 ml of the appropriate buffer was added to each tube. Buffers used in these experiments were: 50 mM Glycine with 0.1 M Tris-HCl and 3% beef extract (referred to as "glycine buffer"), phosphate buffered saline (PBS) containing 1 M NaCl and 0.05% Tween 20 (referred to as "1 M NaCl PBS buffer"), sterilized deionized water, and citrate buffer. All buffers had an original pH of 7.0-7.5. Glycine buffer was experimented at pH 7.0 and pH 9.5. Citrate buffer was at pH 3.6. Some experiments included neutral pH buffers in the context of food matrices as described below. For some experiments, one hundred µl of, and Norwalk virus-like-particles (VLPs) (a gift from Dr. Robert Atmar, Baylor College of Medicine, Houston, TX) were distributed into each buffer/bead suspension. Negative controls for each bead/buffer combination containing no Norwalk VLPs were included for each experimental replicate so that a baseline OD values could be obtained when detecting VLP capture by ELISA. For other experiments, 1µl or 5 µl of native GI.1 stool suspension was inoculated into each tube. For these experiments, the negative controls contained only the bead/buffer mixture and no virus. Additional controls were included for which uncoated amine and tosyl-activated magnetic beads were inoculated with 1µl of 5 µl of native GI.1 stool suspension to monitor non-specific binding to the uncoated beads. All bead/buffer mixtures were incubated at 37°C for 1 hour on tilt and rotation. After

incubation, each bead/buffer mixture was placed on Dynabead magnet and washed three times using phosphate buffered saline (PBS, pH 7.4). The supernatant fluid aspirated after each wash was collected and stored at -80°C for future use.

Detection of VLP binding to magnetic beads by ELISA

After washing, NV3901 coated magnetic beads were inoculated with 100 µl of 0.0075% rabbit sera (a gift from Robert Atmar, Baylor College of Medicine, Houston, TX) produced after inoculation of rabbits with Norwalk virus VLPs, was a primary detection antibody. PGM- and saliva-coated magnetic beads were inoculated with 100 µl of 2 µg/ml of NV3901 as a primary detection antibody some experiments and used 0.0075% rabbit sera as the primary detection antibody for other experiments (as indicated in experimental results section). All magnetic beads were then incubated at 37°C for 1 hour using on tilt and rotation. After incubation, all magnetic beads were washed three times using PBS. After washing, NV3901-coated magnetic beads were inoculated with 100 µl of 1/2000 concentration of anti-rabbit IgG alkaline phosphatase antibody (Sigma-Aldrich Co., St. Louis, MO) produced in goats as the secondary detector. PGM- and saliva-coated magnetic beads were inoculated with 100 µl of 1/500 concentration of Anti-Mouse IgG antibody (Sigma-Aldrich Co.) produced in goat as the secondary detector. All magnetic beads were then incubated at 37°C for 1 hour on tilt and rotation. During this incubation, developer (SigmaFAST pNPP tablets, Sigma-Aldrich) was prepared and mixed on a vortex at the lowest speed for 30 minutes. After incubation, the magnetic beads were set on a Dynamagnet and washed three times using PBS. 200 µl of developer was added to each sample tube and vortexed to mix. High-binding ELISA plates (Co-Star) were previously blocked overnight with 5% Blotto (2.5 g of dried milk (Carnation) per 50 ml of PBS) and washed three times. The 200

µl liter developer/bead mixture was added to the first row of wells on the ELISA plate; 100 µl of developer was added to the remaining wells on the ELISA plate. One hundred µl of the bead/developer mixture from the first row of wells on the plate was taken and distributed to the next row; this process was repeated until the second to last row of wells making a two-fold dilution of the magnetic beads down the plate. The last row was left with only 100µl developer only. The ELISA plate was then placed into a spectrophotometer and optical density readings were taken after 5, 10, 20, and 30 minutes. Analysis of results from this experiment was performed as follows; the last row of optical density (OD) readings (plate negative control wells) on the ELISA plates were averaged and subtracted from each sample OD reading on the plate. All treatment OD values (those containing functionalized beads inoculated with VLP) were divided by the negative OD values (those containing functionalized beads without VLP) for their respective buffer, giving the positive/negative value, or P/N.

Detection of native Norwalk virus binding to functionalized magnetic beads

After magnetic beads were washed, 50µl of PBS was added to each tube. The PBS/bead mixtures were added to thin-walled PCR tubes and placed in a thermocycler for 5 min at 99° C. Immediately afterwards, the tubes placed on ice before centrifuging for 3 minutes. The supernatant containing the released viral RNA was collected, placed in a 1.5 ml tube and stored at -80°C. Real-time reverse transcriptase (RT) polymerase chain reaction (PCR) was performed on each sample using a Stratagene MX3005P and GI norovirus specific primers (Cog 1 F and R) and probes (Ring 1C) (Vega et al., 2011) in combination with a QuantiTect Probe kit (Qiagen, Valencia CA). Concentrations of viral RNA in each sample were determined by comparison to a standard curve consisting of 10-fold serially diluted GI.1 viral RNA obtained from the stool

suspension extracted using a viral RNA mini kit (Qiagen). Quantities of viral RNA in each sample were expressed as log genome copies/ml. Positive samples/total amount of samples tested were also reported from this experiment.

Preparation of Food Extracts

Two samples of 25 g of Iceberg lettuce and Heritage farm (Novi, MI) fresh roast beef were placed into sterile filtra stomacher bags (Fisherbrand, Pittsburgh, PA). Seventy five ml of either 50 mM Glycine with 0.1 M Tris-HCl (pH 9.5) and 3% beef extract or PBS with 1 M NaCl and 0.05% Tween 20 (pH 7.4) was added to the respective stomacher bag. The lettuce and roast beef samples with 50mM Glycine with 0.1 M Tris-HCl and 3% beef extract were rocked for 20 minutes at 200 rpm on a shaking platform. The liquid portion was aspirated into a glass beaker in which the pH was adjusted to 7.4. This neutral extract was dispersed into a 50 ml centrifuge tube. The lettuce and roast beef samples containing PBS with 1 M NaCl and 0.05% Tween 20 were placed in stomacher and homogenized for 2 minutes at 230 rpm. After stomaching, the liquid portion of the extract was dispersed into 50 ml centrifuge tubes. All extracts were centrifuged at 3500 x *g* for 15 minutes. After centrifugation, the extract was distributed into 1 ml amounts into 1.5 ml tubes.

Statistical Analysis

For each of three experimental replicates, duplicate samples were included for each treatment variable (n=6). For all samples, duplicate wells of were averaged after quantification by real-time RT-PCR or semi-quantitative analysis using the P/N valued calculated from the OD readings obtained after ELISA. Two data points were selected for each variable at VLP concentrations of 0.03 and 0.06 because those were the most consistently linear points without

significant variation. Data was analyzed by two-way analysis of variance (ANOVA) using SAS 9.2 software (Cary, NC) with buffer, bead type, experimental replicate and any interactions of these variables as factors used from which to draw conclusions. Difference between means were considered statistically significant at the $\alpha = 0.05$ level.

CHAPTER 4

RESULTS

Norwalk VLP capture in the context of common elution buffers

The efficacy of Norwalk VLPs capture and recovery using NV3901-, PGM-, and saliva-coated magnetic beads was compared in the context of common elution buffers used in virus recovery procedures, in order to determine whether ligand coated magnetic beads can be used as a capture system for these target organisms after elution procedures are performed with common elution buffers. P/N ratios for PGM-coated magnetic bead capture of Norwalk VLP ranged from 6-8.5, 2.4-2.8, 3.8-7.4 for glycine buffer (pH 7), 1M NaCl PBS buffer (pH 7) and sterile de-ionized water (pH 7), respectively (Figure 1). P/N ratios for saliva-coated magnetic bead capture of Norwalk VLP (Figure 2) ranged from 5.3-7.1, 5.1-5.7, 3.7-5.3 for glycine buffer, 1M NaCl PBS buffer and sterile de-ionized water, respectively. P/N ratios for NV3901-coated magnetic bead capture of Norwalk VLP (Figure 3) ranged from 14-29.5, 13-31.4, 15-29.8 in the context of glycine buffer, 1M NaCl PBS buffer and sterile de-ionized water, respectively. When suspended in the1M NaCl PBS buffer, PGM-coated magnetic beads were less efficient at VLP capture, but when suspended in glycine buffer; these PGM-coated magnetic beads resulted in the greatest level of Norwalk VLP capture (Table 1). Saliva- coated magnetic beads captured significantly more Norwalk VLP in the glycine buffer in comparison to water and 1M NaCl PBS buffers (Table 2). Overall, the experimental data show that there was no statistically significant difference due to the buffer used when comparing VLP capture using the NV3901-coated

magnetic beads ($p = < 0.05$). Based on P/N ratios, the NV3901-coated magnetic beads appeared to capture the most VLPs, regardless of the elution buffer used. However, there was a critical difference between experiments conducted with the NV3901-coated magnetic beads and the PGM- and saliva- coated magnetic beads. The primary detection antibody used in the ELISA experiments with NV3901-coated magnetic beads was anti-Norwalk virus VLP rabbit sera, whereas in the ELISA experiments with PGM- and saliva- coated magnetic beads, the primary detection antibody was NV3901, a monoclonal antibody produced in mice.

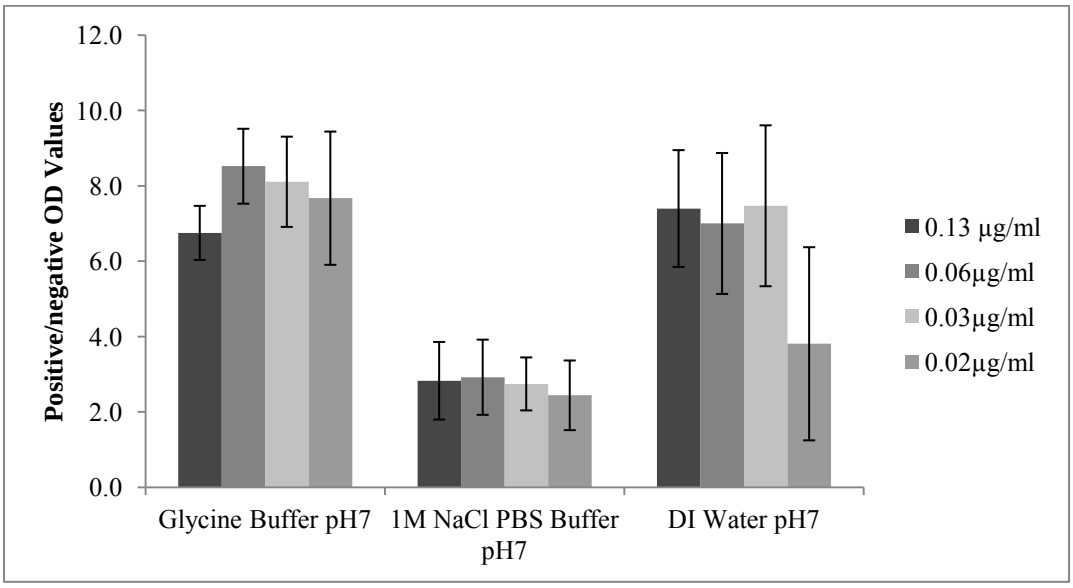

Figure 1-Capture of Norwalk virus virus-like particles using porcine gastric mucin-coated magnetic beads suspended in elution buffers at neutral pH. Legend indicates estimated virus-like particle concentration. Error bars represent the standard error of the means.

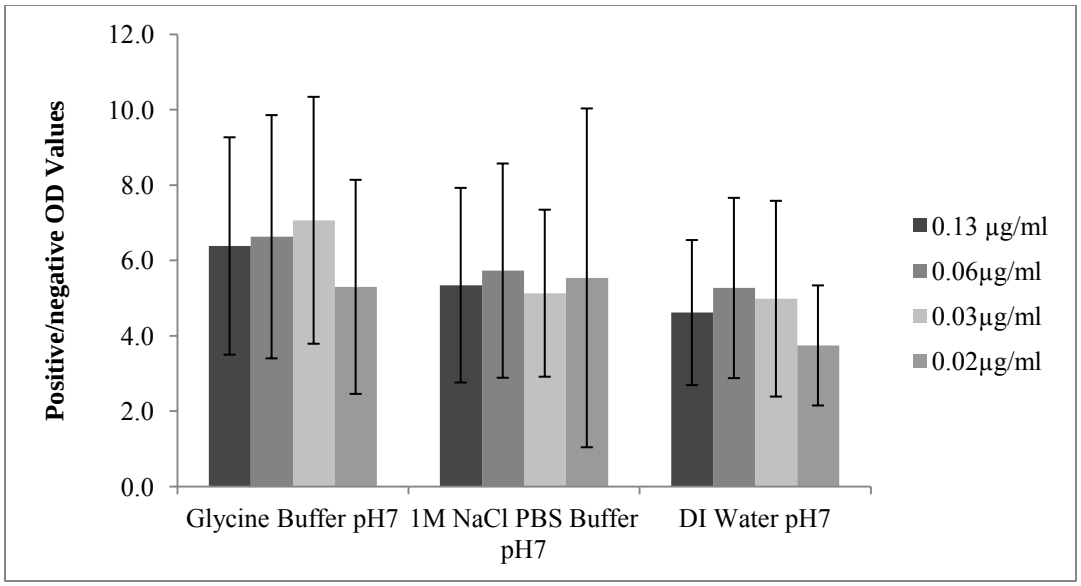

Figure 2-Capture of Norwalk virus-like particles using saliva-coated magnetic beads suspended in elution buffers at neutral pH. Legend indicates estimated virus-like-particles concentration. Error bars represent the standard error of the means.

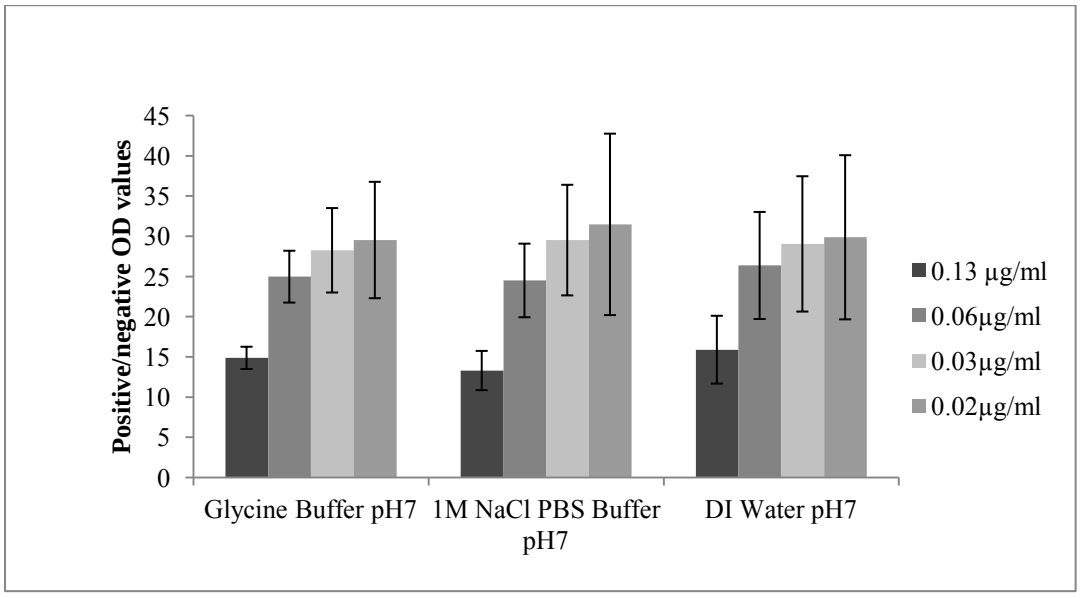

Figure 3-Capture of Norwalk virus-like particles using NV3901-coated magnetic beads suspended in elution buffers at neutral pH. Legend indicates virus-like particles concentration. Error bars represent the standard error of the means.

Table 1: Differences in mean positive/negative ratio determined for Norwalk VLP capture with PGM-coated magnetic beads in the context of different elution buffers. *****Letters that are different indicate statistically significant differences in the means at the α= 0.05 level**.**

Elution buffer	Least squared Means	Test of significance*
Glycine	8.3	A
1M NaCl PBS	2.8	C
water	7.2	B

Table 2: Differences in mean positive/negative ratio determined for Norwalk VLP capture with saliva-coated magnetic beads in the context of different elution buffers. *****Letters that are different indicate statistically significant differences in the means at the α= 0.05 level**.**

Elution Buffer	Least Squared Mean	Test of Significance*
Glycine	6.9	A
1M NaCl PBS	5.4	B
Water	5.1	B

VLP capture in the context of common elution buffers and using rabbit antisera as the primary detector antibody in the ELISA assay

To determine whether or not the apparent better performance of the NV3901-coated magnetic beads is real, or simply an artifact of the more sensitive detector antibody used in the ELISA assay, it was necessary to make a more direct comparison using rabbit antisera as the primary detector for all magnetic bead capture assays. Using rabbit sera as the primary detector in the ELISA increased the P/N ratios for the experiments performed with PGM- and saliva-coated magnetic beads (Figure 4). This is likely due to the increased number of VLP epitopes recognizable by the hyperimmune rabbit antisera. In the experiments with PGM-coated magnetic beads and using rabbit antisera as the primary detector, the P/N ratios increased to 8-16.5 which was approximately 2 -fold higher than P/N ratios determined after using NV3901 as the primary detection antibody. Similarly, saliva-coated magnetic beads produced higher P/N ratios after VLP capture, with values ranging from 14-25, which was approximately 3 fold higher than P/N

ratios determined after using NV3901 primary detection antibody. Although, using the rabbit antisera as the primary detector resulted in higher P/N ratio values for the PGM- and saliva-coated magnetic beads, the NV3901-coated magnetic beads still had the highest P/N values (Table 3). This indicates that the higher level of binding by the NV3901-coated magnetic beads is real. P/N ratios obtained during the PGM-coated magnetic bead studies resulted in the lowest P/N values of all the functionalized beads (Table 3). Taken together, there was no difference in the mean P/N ratios obtained for the glycine and 1M NaCl PBS elution buffers, but both were higher than the mean P/N ratio obtained using water (Table 4).It was also shown that there were significant differences between bead coating and buffer interaction (Table 5). Although there appeared to be a decrease in VLP binding to PGM-coated beads in the context of 1M NaCl PBS buffer as was shown using NV3901 as the primary detection antibody, this difference was not statistically significant when using rabbit antisera as the primary detection antibody (Table 5). Also, the glycine buffer did not outperform the other neutral pH buffers used during saliva binding assays as was previously shown.

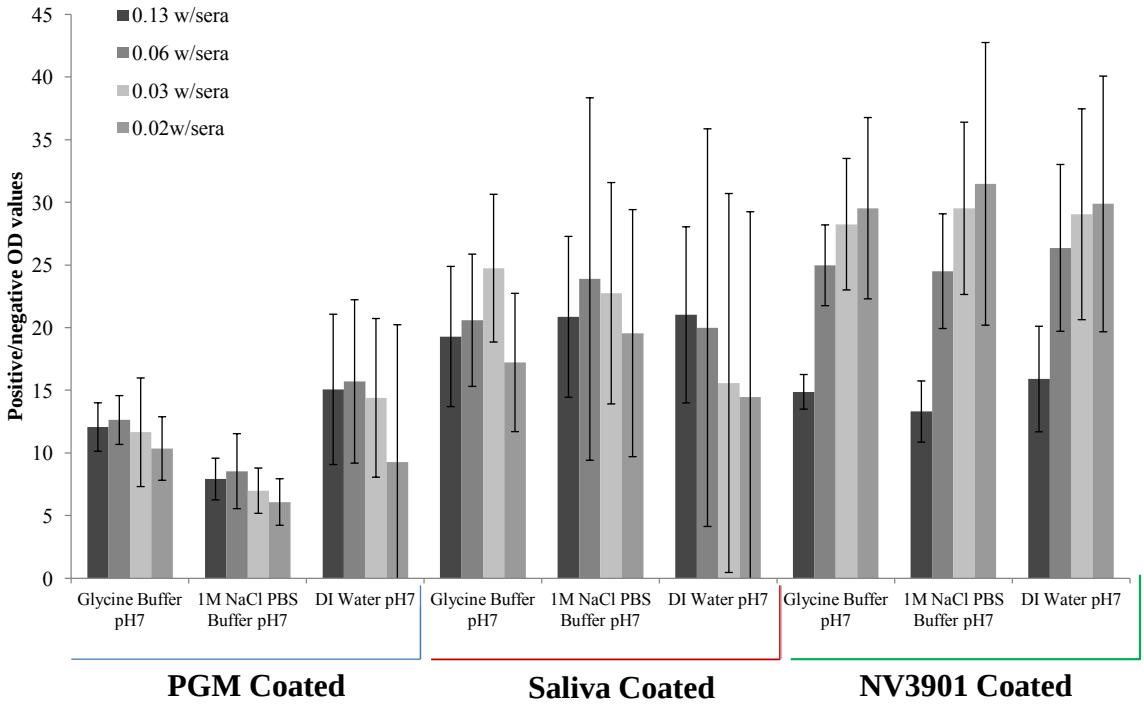

Figure 6: Comparison of porcine gastric mucins or saliva coated magnetic beads capture of Norwalk virus-like particles with NV3901 coated magnetic bead with either rabbit sera as the primary detection mechanism. Legend indicates VLP concentration (micrograms/ml). Error bars represent the standard error within the means.

Table 3: Differences in mean positive/negative ratio determined for Norwalk virus-like particles capture with all magnetic beads in the context of different bead coatings. *****Letters that are different indicate statistically significant differences in the means at the α= 0.05 level**.**

Bead Coating	Least squared Mean	Test of significance*
PGM	13.4	A
Saliva	22.0	B
NV3901	25.3	C

Table 4: Differences in mean positive/negative ratio determined for Norwalk virus-like particles capture with saliva-coated magnetic beads in the context of different bead coatings. *****Letters that are different indicate statistically significant differences in the means at the α= 0.05 level**.**

Bead Coating	Least squared Mean	Test of significance*
Glycine	21.7	A
1M NaCl	22.7	A
Water	16.4	B

Table 5: Differences in mean positive/negative ratio determined for Norwalk virus-like particles capture with all coated magnetic beads in the context of different bead coatings. *****Letters that are different indicate statistically significant differences in the means at the α= 0.05 level**.**

Bead Coating	**Elution Buffer**	**Least Square Mean**	**Test of Significance***
PGM	Glycine	13.8	A
	1M NaCl PBS	9.9	AC
	Water	16.5	AB
Saliva	Glycine	25.0	D
	1M NaCl PBS	23.3	D
	Water	17.8	AB
NV3901	Glycine	26.3	D
	1M NaCl PBS	34.9	E
	water	14.9	AB

Native Norwalk virus capture in the context of common elution buffers

To determine how well the functionalized magnetic beads interact with native Norwalk virus (GI.1), and compare this to their interaction with Norwalk VLPs, a stool suspension containing Norwalk virus was used as the inoculum and real-time RT-qPCR was used for

quantifying virus recovery. The average log genome copies/ml recovered using functionalized magnetic beads are demonstrated in Figure 5. This data indicate that the type of elution buffer the functionalized beads were suspended in did not impact virus capture ($p \leq 0.05$). For the NV3901-coated magnetic beads, there was a low amount of virus capture in all buffers compared to the ligand coated magnetic beads. This trend of less of virus capture for the NV3901-coated magnetic beads than for the ligand-coated magnetic beads differed from the VLP capture experiments. Although, virus and VLP capture for the NV3901- coated magnetic beads are not directly comparable, such trends are comparable. Such findings could be an indication that there is a difference in the structure of the virus compared to the Norwalk VLP, or significant blocking of the antibody recognition epitopes. As negative controls, uncoated amine and tosyl-activated beads were included to measure the amount of non-specific binding of virus to the beads themselves when Norwalk virus was included. The result showed non-specific binding of the amine beads while suspended in both the glycine buffer and water (Figure 5). For the tosyl-activated beads non-specific binding was shown in the 1M NaCl PBS buffer and the water. Statistical analysis showed that there was no significant difference between the bead coatings or the buffer suspensions ($p=0.776$). However, Figure 5 shows and the statistical analysis was only performed on data for those samples yielding positive results by real time RT-qPCR. In some cases, no signal could be detected by RT-qPCR. To account for this, the ratio of the number of positive samples over the total number of samples tested is indicated in Table 6. NV3901-coated magnetic beads recovered Norwalk virus in only 1/6 samples, regardless of the elution buffer used. This is in contrast the PGM- and saliva-coated magnetic beads. The results also revealed that uncoated amine and tosyl-activated beads adsorbed Norwalk virus non-specifically in some

control samples, indicating that to some degree, viruses adsorb to the beads themselves. Non-specific binding of both types of magnetic beads was detected with 2/3 buffer solutions.

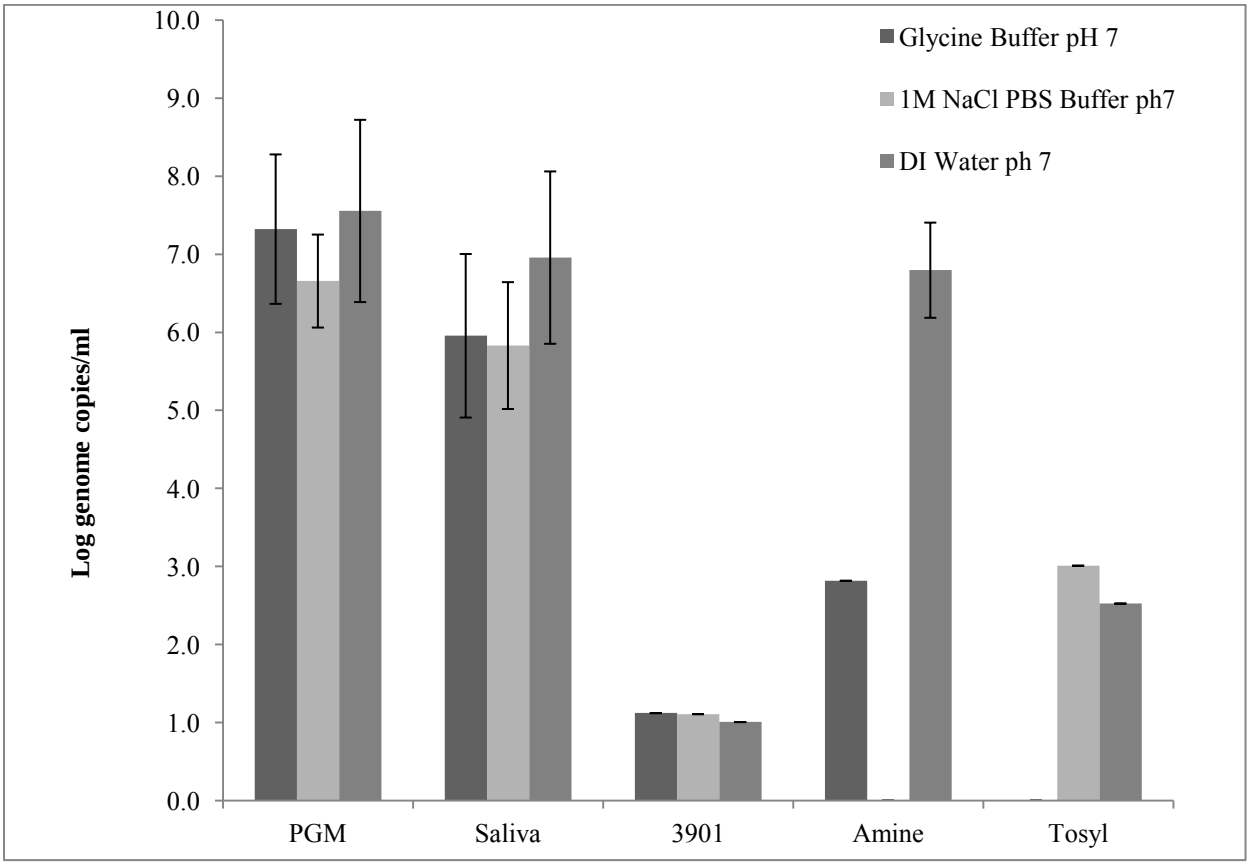

Figure 5- Capture of Norwalk virus (GI.1) using porcine gastric mucins-, saliva- or NV3901-coated magnetic beads suspended in common elution buffers. Uncoated amine and Tosyl-activated beads were included as non-functionalized negative controls to measure non-specific binding of Norwalk virus. Error bars represent the standard deviation for the means.

Table 6: The ratio of positive samples over the total number of samples tested per bead coating or type and buffer used during capture experiments with Norwalk native GI.1 virus. All buffers were pH 7

Bead Coating/Type	Buffer and pH	Positive samples/total number of samples
PGM	Glycine Buffer	6/6
	1M NaCl PBS	6/6
	DI Water	6/6
Saliva	Glycine Buffer	5/6
	1M NaCl PBS	5/6
	DI Water	6/6
NV3901	Glycine Buffer	1/6
	1M NaCl PBS	1/6
	DI Water	1/6
Amine	Glycine Buffer	1/2
	1M NaCl PBS	0/2
	DI Water	2/2
Tosyl	Glycine Buffer	0/2
	1M NaCl PBS	1/2
	DI Water pH 7	1/2

VLP capture while suspended in pH-adjusted buffers

A comparison of the capture, recovery and concentration of Norwalk VLPs using NV3901-, PGM-, and saliva-coated magnetic beads suspended in buffers of varying pH was conducted to determine the effect of pH on Norwalk VLP binding to ligand- and antibody-coated surfaces. Glycine buffer at pH 9.5 and citrate buffer at pH 3.6 were included and compared to sterile de-ionized water at pH 7. When using the pH adjusted buffers, a difference in VLP capture was seen when varying the pH. With the PGM-coated magnetic beads, basic glycine buffer and acidic citrate buffer both reduced VLP capture, with P/N values ranging from 1.22-1.39, 2.5-3.7, respectively, in comparison to glycine buffer at neutral pH. Statistical analysis

showed that there was a significant difference between the amount of Norwalk VLP captured within each buffer suspension using the PGM-coated beads (Table 7). Saliva-coated magnetic beads suspended in the basic glycine buffer had a lower P/N ratio, ranging from 1.61-1.86, for Norwalk VLP capture, in comparison to the neutral glycine buffer. Saliva-coated magnetic beads suspended in citrate buffer and water had similar P/N ratios at 3.68-3.80, 3.5-4.0, respectively, for Norwalk VLP capture (Table 8). With the NV3901-coated magnetic beads citrate and basic glycine buffers provided P/N ratios ranging from around 11-12 and 21-22 respectively, in comparison to pH 7 glycine buffer which provided P/N ratios ranging from 25-30. This indicates a lower amount of VLP capture was obtained with the acidic and basic buffers. Statistical analysis showed a significant difference between all buffer suspensions (Table 9). Saliva- and PGM- coated magnetic beads resulted in low P/N ratios in the citrate and basic glycine buffers, indicating that at these extremes in pH (3.6 and 9.5 respectively), the buffers may have acted as eluents, inhibiting Norwalk VLP binding to PGM and saliva. In these experiments, we were able to compare the impact of buffer pH on VLP binding for each functionalized bead type; however, we were not able to compare the response of the PGM- and saliva- coated magnetic beads to the NV3901-coated magnetic beads due to the different primary detection antibodies used. Experiments with PGM- and saliva-coated magnetic beads used NV3901 as the primary detection reagent, but experiments using NV3901-coated magnetic beads used rabbit sera as the primary detection reagent.

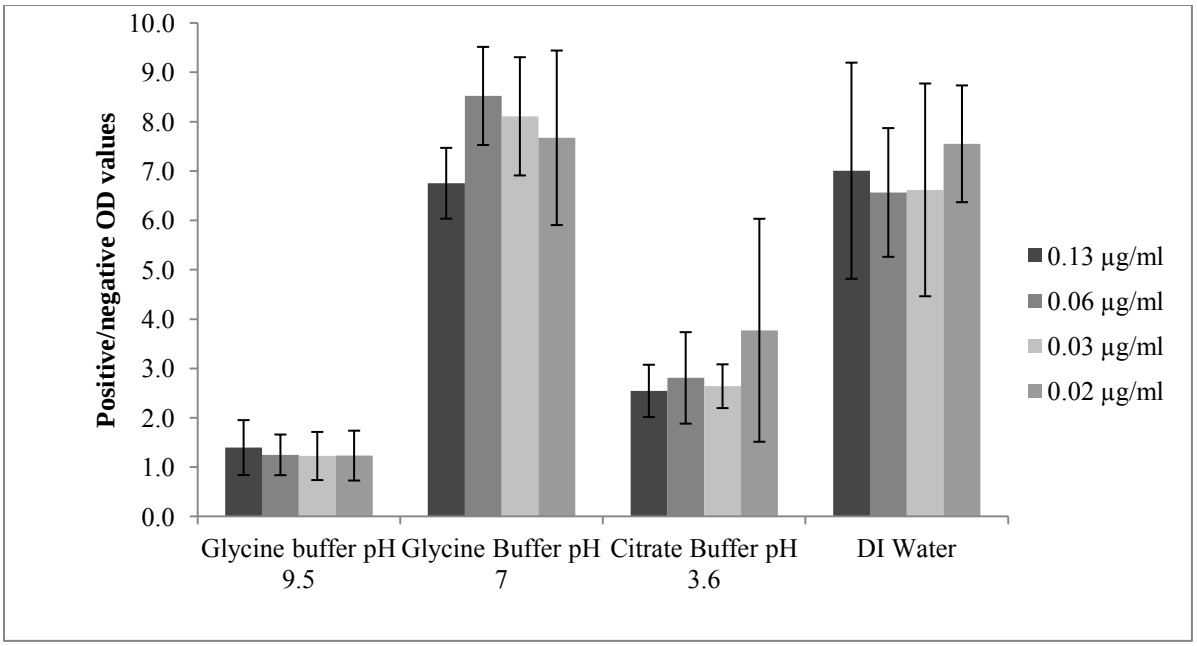

Figure 6- Capture of Norwalk virus-like particles (VLP) using porcine gastric mucins-coated magnetic beads suspended in elution buffers at basic, acidic and neutral pH. Legend indicates VLP concentration. Error bars represent the standard deviation of the means.

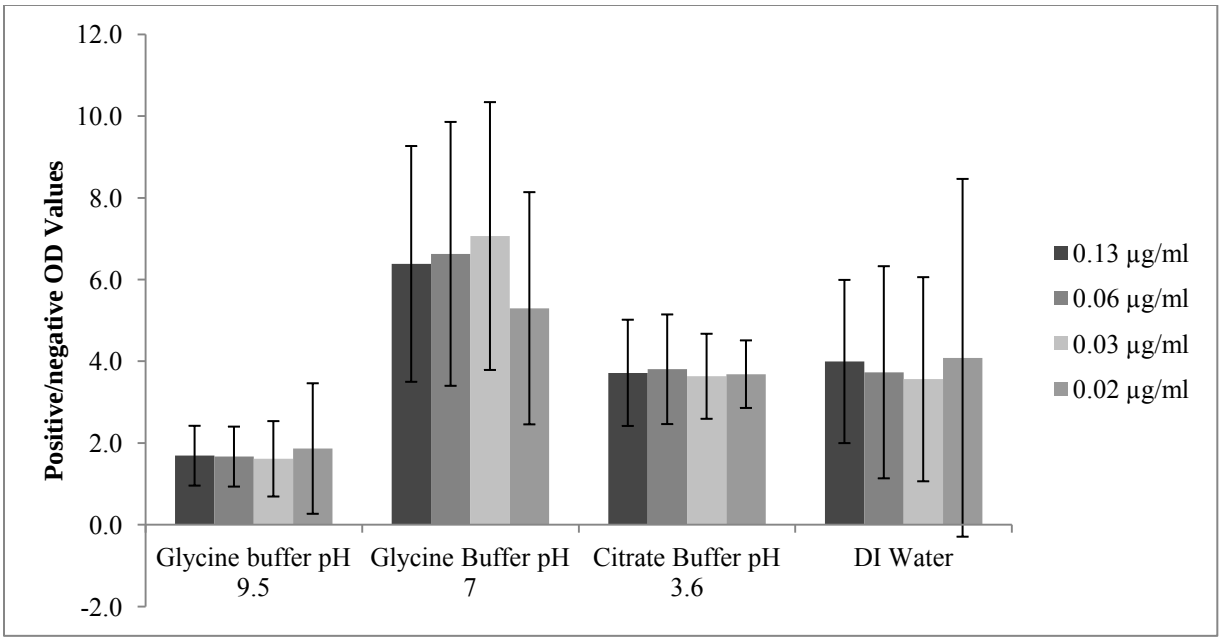

Figure 7-Capture of Norwalk virus-like particles (VLP) using saliva-coated magnetic beads suspended in elution buffers at basic, acidic and neutral pH. Legend indicates VLP concentration. Error bars represent the standard deviation of the means.

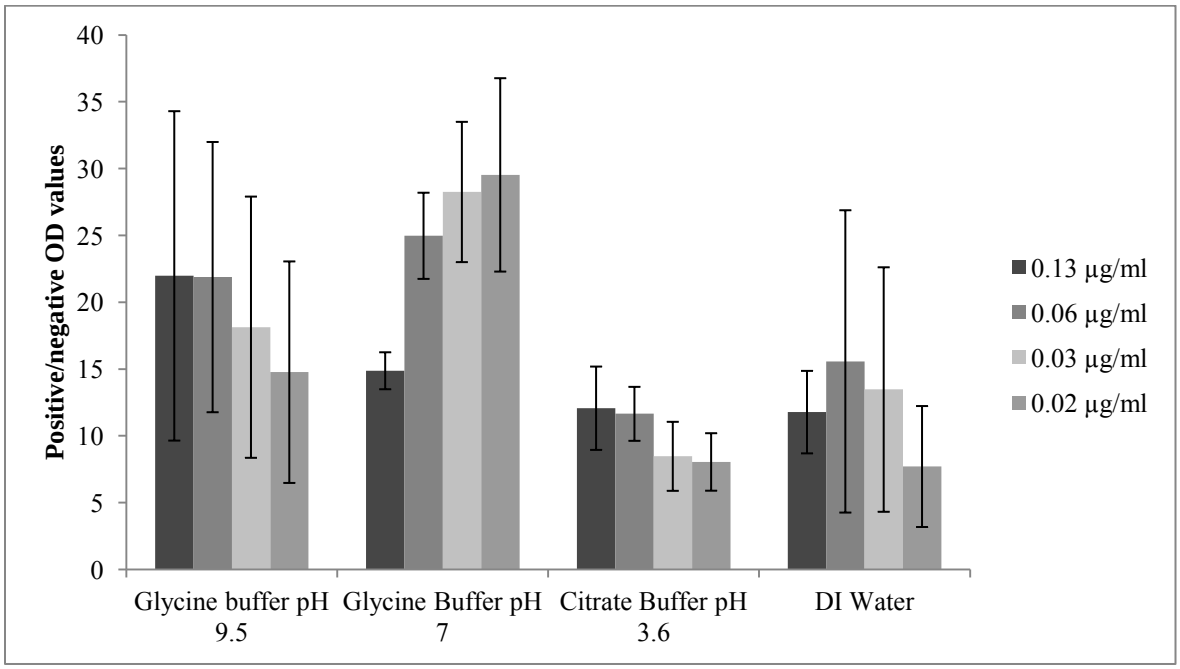

Figure 8-Capture of Norwalk virus-like particles (VLP) using NV3901-coated magnetic beads suspended in elution buffers at basic, acidic and neutral pH. Legend indicates VLP concentration. Error bars represent the standard deviation within the means.

Table 7: Differences in mean positive/negative ratio determined for Norwalk virus-like particle capture with porcine gastric mucins-coated magnetic beads in the context of different bead coatings. *****Letters that are different indicate statistically significant differences in the means at the $\alpha = 0.05$ level**.**

Elution Buffer	pH	Least Squared Mean	Test of Significance*
Glycine	9.5	1.2	C
Citrate	3.6	2.7	B
water	7	6.6	A

Table 8: Differences in mean positive/negative ratio determined for Norwalk virus-like particle capture with saliva-coated magnetic beads in the context of different bead coatings. *****Letters that are different indicate statistically significant differences in the means at the $\alpha = 0.05$ level**.**

Elution Buffer	pH	Least Squared Mean	Test of Significance*
Glycine	9.5	1.6	C
Citrate	3.6	3.7	B
water	7	3.7	A

Table 9: Differences in mean positive/negative ratio determined for Norwalk virus-like particle capture with NV3901-coated magnetic beads in the context of different bead coatings. *****Letters that are different indicate statistically significant differences in the means at the $\alpha = 0.05$ level**.**

Elution Buffer	pH	Least Squared Mean	Test of Significance*
Glycine	9.5	20.0	A
Citrate	3.6	10.1	C
water	7	14.5	B

Native GI.1 capture while suspended in pH-adjusted buffers

The purpose of this experiment was to determine if native GI.1 capture by ligand- or antibody- coated magnetic beads was affected by the pH of the buffer in which it was suspended. Therefore, we performed an identical capture experiment as previously mentioned above, suspending the functionalized beads in glycine buffer at pH 9.5, citrate buffer at pH 3.6 and sterile de-ionized water at pH 7 as the buffer treatments, but using native GI.1 virus as the target and real-time RT-PCR as the detection method. The average log genome copies/ml from each positive duplicate sample and all three positive experimental replicates were graphed for

convenient comparison between the treatments (Figure 7). Overall, statistical analysis supported the trends observed with the Norwalk VLP experiments, showing a significant difference between the pH adjusted buffers and the neutral buffers (Table 10). The neutral pH buffers outperformed the acidic and basic pH buffers. Amine and tosyl-activated beads with no ligand or antibody coating were included to measure the amount of non-specific binding by native GI.1, and to determine whether or not nonspecific binding could be aided or inhibited by being suspended in the various buffers. The citrate buffer aided in non-specific binding for some virus capture in all bead types as opposed to the basic glycine buffer. NV3901-coated magnetic beads had the lowest virus capture in comparison to the PGM- and saliva- coated magnetic beads. The uncoated amine and tosyl-activated magnetic beads show non-specific binding in 3/3 buffer solutions. To further compare the beads, the ratio of the number of positive what over the total number of samples tested was included in Table 11. The table shows a lack of consistency in the amount of positives compared to the amount tested which could infer that the pH in the buffers could decrease stability/consistency in virus binding. PGM- coated magnetic beads showed consistently high in the ratios (positive/ number tested) when suspended in neutral buffers, whereas in both the basic and acidic buffer there were less samples that were. Saliva-coated magnetic beads showed an equal amount of positive samples in both the neutral and pH buffers. Alternatively, NV3901-coated magnetic beads showed an improvement in the number of samples that were positive when suspended in the acidic and basic pH buffers, in comparison to when suspended in the neutral pH buffer.

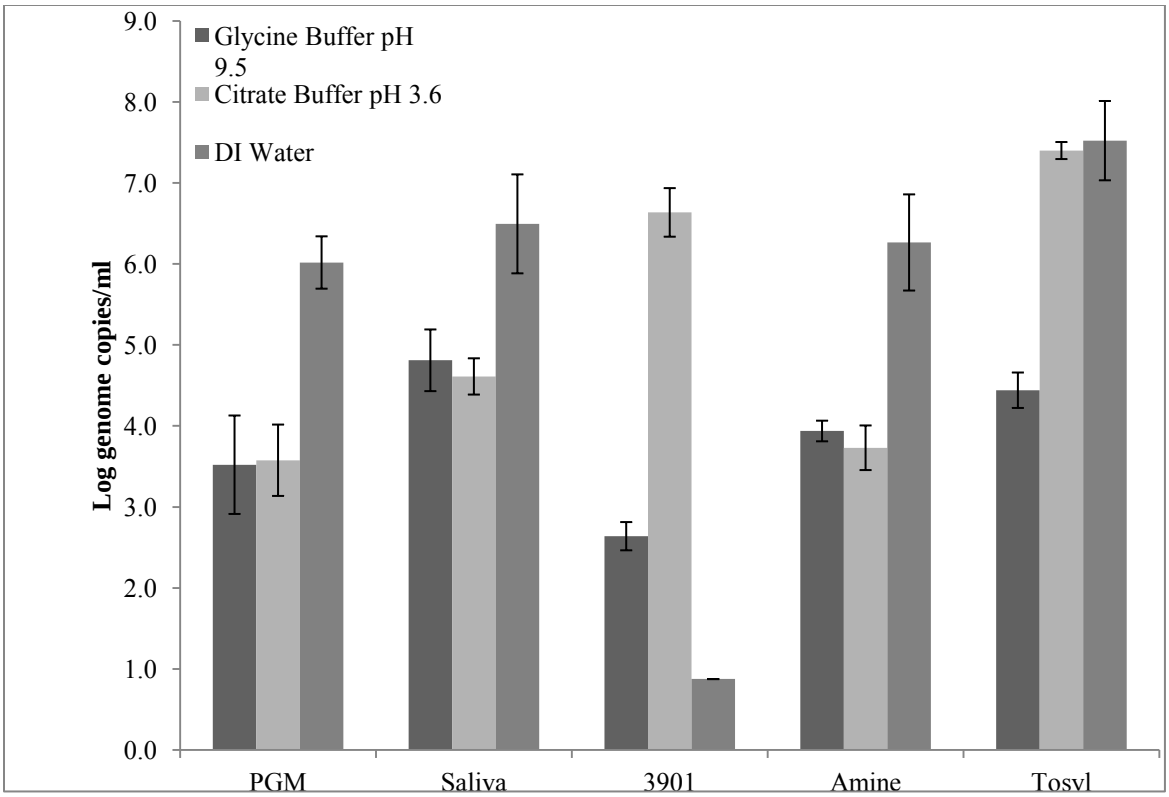

Figure 9- Capture of native virus (GI.1) using saliva, porcine gastric mucin, or NV3901 coated magnetic beads suspended in elution buffers. Amine and Tosyl labels are shown representing an uncoated bead. Legend indicates log genome copies/ml. Error bars represent the standard error within the means.

Table 10: Differences in mean positive/negative ratio determined for native GI.1 virus capture with all coated magnetic beads in the context of different bead coatings. *****Letters that are different indicate statistically significant differences in the means at the α= 0.05 level**.**

Bead Coating/ Type	Elution Buffer	pH	Least Squared Means	Test of Significance*
3901	Glycine	9.5	4.98	B
	Citrate	3.6	6.64	CD
	Water	7.0	4.78	B
Amine	Glycine	9.5	5.97	BCD
	Citrate	3.6	5.50	BCE
	Water	7.0	6.27	EFG
PGM	Glycine	9.5	5.08	B
	Citrate	3.6	5.10	B
	Water	7.0	6.02	CG
Saliva	Glycine	9.5	5.59	BG
	Citrate	3.6	5.39	B
	Water	7.0	6.49	EFG
Tosyl	Glycine	9.5	6.00	BCD
	Citrate	3.6	7.40	DE
	Water	7.0	7.52	A

Table 11: The ratio of positive samples over the total number of samples tested per bead coating or type and buffer pair during capture experiments with Norwalk virus.

Bead Type/Coating	Elution Buffer	pH	Positives/Total amount of samples tested
PGM	Glycine Buffer	9.5	4/6
	Citrate Buffer	3.6	4/6
	DI Water	7	6/6
Saliva	Glycine Buffer	9.5	5/6
	Citrate Buffer	3.6	5/6
	DI Water	7	6/6
NV3901	Glycine Buffer	9.5	3/6
	Citrate Buffer	3.6	6/6
	DI Water	7	1/6
Amine	Glycine Buffer	9.5	2/3
	Citrate Buffer	3.6	2/3
	DI Water	7	3/3
Tosyl-activated	Glycine Buffer	9.5	2/3
	Citrate Buffer	3.6	3/3
	DI Water	7	3/3

VLP Capture in the context of Food Matrices

The purpose of this experiment was to test the capture efficiency of Norwalk VLPs using ligand- and antibody- coated magnetic beads while suspended in a food matrix extract. The food matrices that were chosen were extracts from iceberg lettuce and roast beef deli meat. The buffers used for the Norwalk VLP capture were glycine buffer and 1M NaCl, both at pH 7. In the VLP capture experiments, rabbit sera was used as the primary detector for all bead types in the ELISA assay so that the results were directly comparable. Results indicate a P/N value of at least 10 for nearly all bead types (Figures 10-5.3). However, PGM-coated magnetic beads suspended in lettuce extract and 1M NaCl PBS had a significantly lower P/N value ranging from 4-6, in comparison to the other buffer/food extract combination (Table 11). Significant interactions between food extract, buffer and bead coating were found in almost every case (Table 11). For

PGM-coated magnetic bead capture, using glycine buffer on lettuce and using 1M NaCl PBS on beef did not contribute to significant difference in VLP capture. For saliva- coated magnetic bead capture, using glycine buffer on beef and using 1M NaCl PBS buffer on lettuce did not contribute to significant differences in Norwalk VLP capture. Statistical analysis showed that there was no significant difference between NV3901-coated magnetic bead capture when using glycine buffer on beef and when using 1M NaCl PBS buffer on lettuce. As shown in the previous experiments, the NV3901-coated magnetic beads had an overall higher P/N ratio when compared to the other ligand-coated magnetic beads, with P/N values ranging from 10-42, whereas PGM- and saliva-coated P/N values ranged from 4-12 and 8-14, respectively.

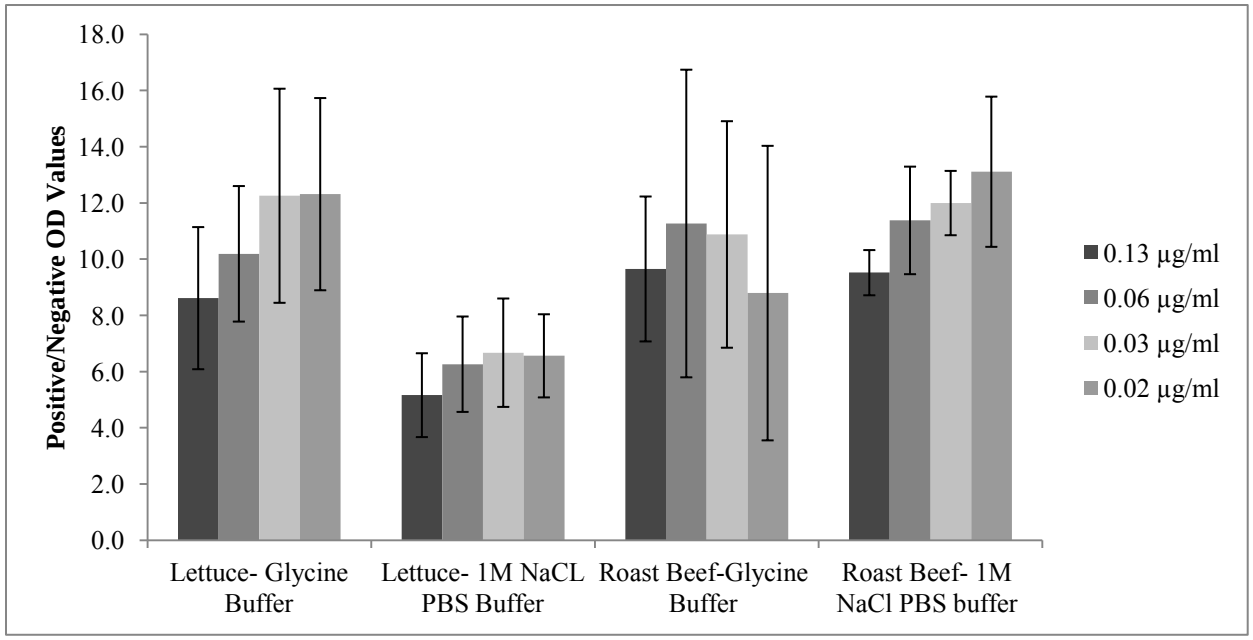

Figure 10: Capture of Norwalk virus-like particles (VLP) using porcine gastric mucin-coated magnetic beads suspended in neutral pH elution buffers and in the context of either iceberg lettuce or roast beef food residuals. Legend indicates the estimated VLP concentration used. Error bars indicate standard error of the means.

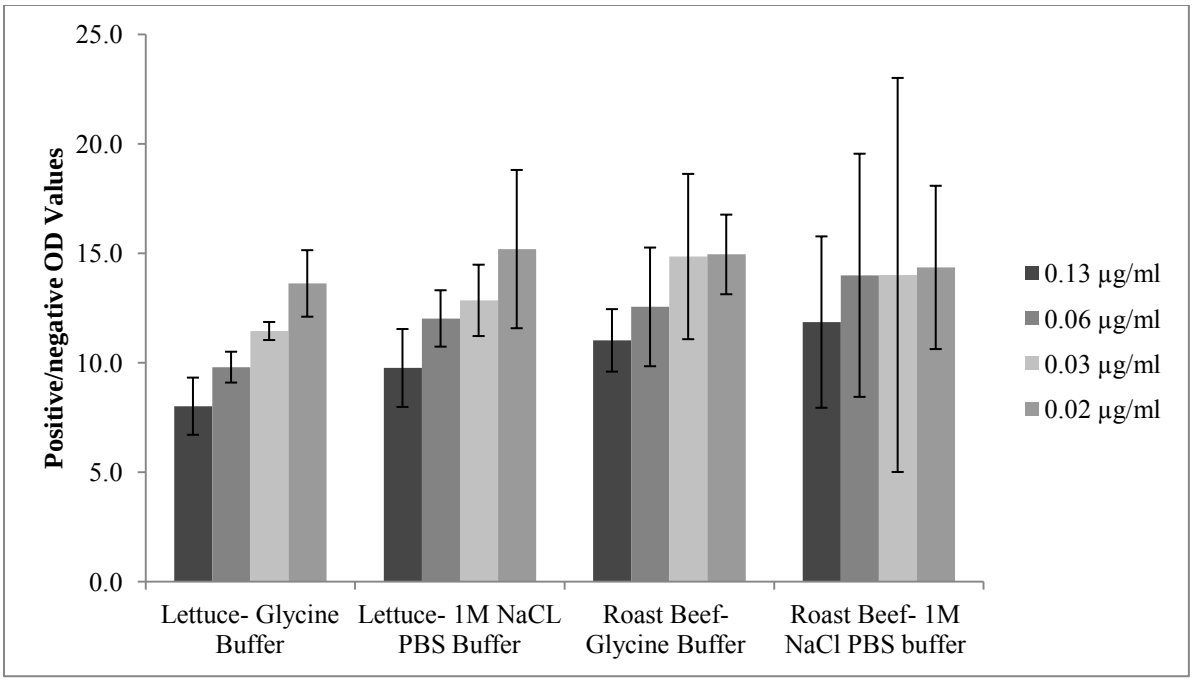

Figure 11: Capture of Norwalk virus-like particles (VLPs) using saliva-coated magnetic beads suspended in neutral pH elution buffers and in the context of either iceberg lettuce or roast beef food residuals. Legend indicates the estimated VLP concentration used. Error bars indicate standard error of the means.

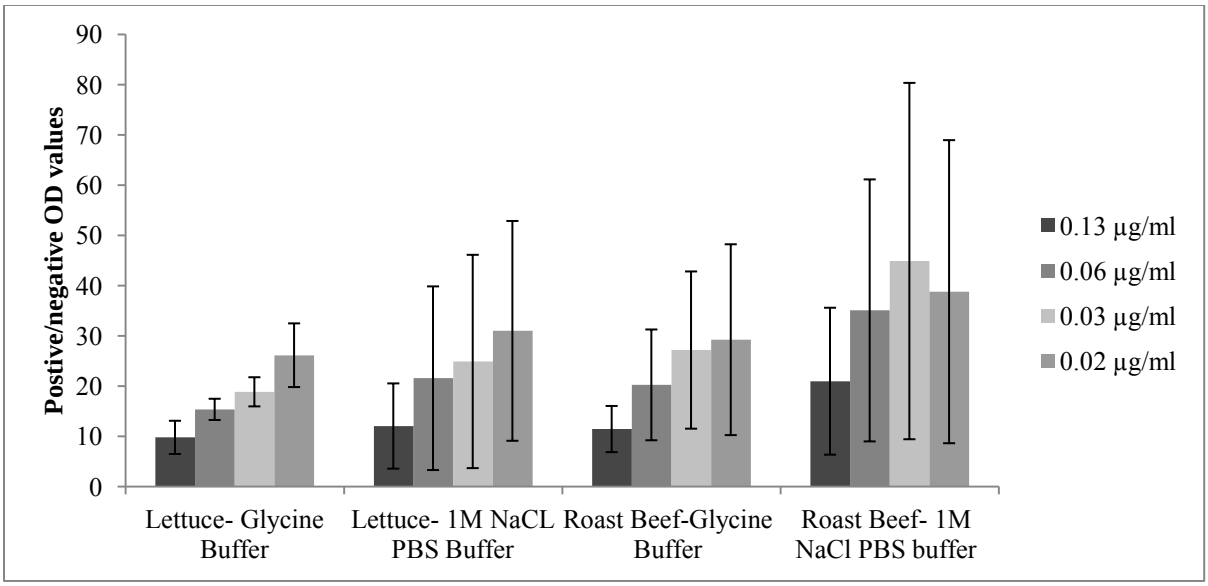

Figure 12: Capture of Norwalk virus-like particles (VLPs) using NV3901-coated magnetic beads suspended in neutral pH elution buffers and in the context of either iceberg lettuce or roast beef food residuals. Legend indicates the estimated VLP concentration used. Error bars indicate standard error of the means.

Table 12: Differences in mean positive/negative ratio determined for Norwalk virus-like particles capture with all coated magnetic beads in the context of different elution buffer and food extract combinations. *****Letters that are different indicate statistically significant differences in the means at the α= 0.05 level**.**

Bead Coating	Glycine Buffer	Food Extract	Least Squared Mean	Test of Significance with all beads*	Test of Significance within each bead coating*
NV3901	Glycine	Lettuce	17.1	F	C
		Beef	23.7	G	B
	1M NaCl PBS	Lettuce	23.2	G	B
		Beef	40.0	H	A
PGM	Glycine	Lettuce	11.2	BC	AB
		Beef	10.5	B	B
	1M NaCl PBS	Lettuce	8.6	A	C
		Beef	12.2	C	A
Saliva	Glycine	Lettuce	10.6	B	C
		Beef	13.7	D	B
	1M NaCl PBS	Lettuce	12.4	CDE	AB
		Beef	14.0	E	A

Native GI.1 Capture in the context of food matrices

In this experiment, the procedure of the Norwalk VLP capture experiments in the context of food extracts and elution buffers were repeated, except native GI.1 was used as the target for capture. In the native GI.1 capture experiments, samples were inoculated with 5µl of GI.1 from a stool suspension instead of 1µl. The inoculum was increased due to the probability that the introduction of food particles would inhibit virus capture and detection. We also increased the inocula because we took into account the In the previous experiments with neutral pH buffers and the 1 µl Norwalk virus inoculum, high CT values and a high amount of samples generating no CT value (negative results) were obtained after RT-qPCR. The inoculum was increased so that we could detect the virus to make more accurate comparisons between buffer and food

matrices. Log genome copies/ml of native GI.1 captured by all functionalized and uncoated beads are indicated in Figure 6.1. For the PGM- and saliva- coated magnetic beads, the log genome copies/ml of GI.1 captured were similar between the bead, buffer, and food extract suspensions, except for the PGM-coated magnetic beads suspended in 1M NaCl PBS, which was shown to be significantly different from the others (Table 12). However, for the NV3901-coated magnetic beads, as in the previous experiments with a stool inoculum, the log genome copies/ml of GI.1 captured was lower in comparison to the PGM- and saliva- coated magnetic beads. Statistical analysis proved these experimental observations to be correct. There were some significant differences between the buffer and bead coating type in this experiment (Table 12). Also, in these experiments, amine and tosyl-activated uncoated magnetic beads were tested for the amount of non-specific binding. Amine and tosyl-activated uncoated magnetic beads show non-specific binding in 3/3 buffer solutions. There is an overall high amount of non-specific binding in the uncoated magnetic beads which may be due to the proteins and carbohydrates the food matrix will introduce and/or the increased inoculums of 5 µl of native GI.1 virus. Amine uncoated magnetic beads showed a high amount of non-specific binding in all buffers except for the beef with 1M NaCl PBS buffer in comparison to the tosyl-activated uncoated magnetic beads. All beads and coatings showed 6/6 ratio of positive results except NV3901-coated magnetic beads suspended in the beef matrix and 1M NaCl PBS buffer and tosyl-activated uncoated magnetic beads in both the beef glycine and 1M NaCl PBS buffers (Table 13).

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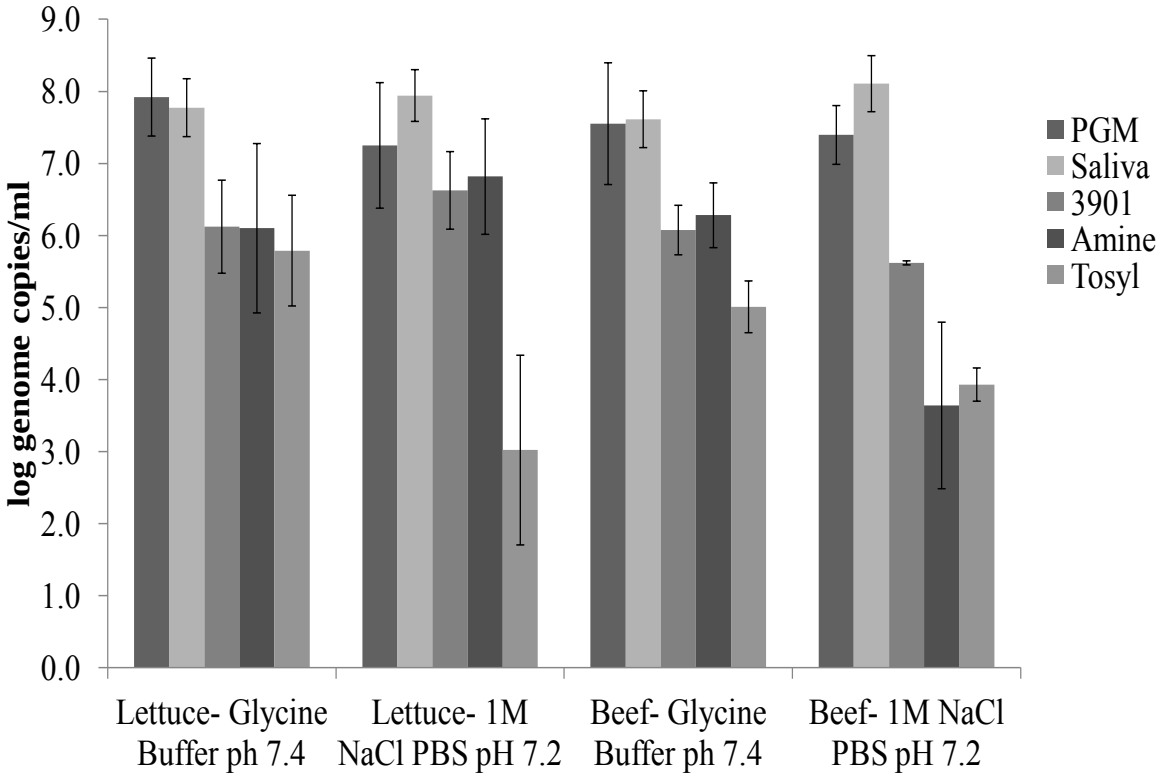

Figure 13: Capture of native virus (GI.1) using saliva, porcine gastric mucin-, or NV3901 coated magnetic beads suspended in neutral elution buffers with either lettuce or beef food extract. Amine and Tosyl labels are shown representing an uncoated bead. Legend indicates log genome copies/ml. Error bars represent the standard error within the means.

Table 13: Differences in mean positive/negative ratio determined for native GI.1 virus capture in the context of different bead coatings. *****Letters that are different indicate statistically significant differences in the means at the $\alpha = 0.05$ level**.**

Bead Coating	Least Square Means	Test of Significance*
NV 3901	6.31	B
Amine	6.10	B
PGM	7.53	A
Saliva	7.86	A
Tosyl	5.22	C

Table 14: The ratio of positive samples over the total number of samples tested per bead coating or type and buffer pair during capture experiments with Norwalk virus.

Bead Coating	Food Extract	Buffer	pH	Positve/ total amount of samples tested
PGM	Beef	Glycine	7.4	6/6
		1M NaCl PBS	7.2	6/6
	Lettuce	Glycine	7.4	6/6
		1M NaCl PBS	7.2	6/6
Saliva	Beef	Glycine	7.4	6/6
		1M NaCl PBS	7.2	6/6
	Lettuce	Glycine	7.4	6/6
		1M NaCl PBS	7.2	6/6
NV3901	Beef	Glycine	7.4	6/6
		1M NaCl PBS	7.2	5/6
	Lettuce	Glycine	7.4	6/6
		1M NaCl PBS	7.2	6/6
Amine	Beef	Glycine	7.4	3/3
		1M NaCl PBS	7.2	2/3
	Lettuce	Glycine	7.4	3/3
		1M NaCl PBS	7.2	3/3
Tosyl	Beef	Glycine	7.4	3/3
		1M NaCl PBS	7.2	2/3
	Lettuce	Glycine	7.4	3/3
		1M NaCl PBS	7.2	2/3

CHAPTER 5

DISCUSSION

The purpose of this work was to determine if ligands such as those contained in porcine gastric mucins and saliva, when attached to functionalized magnetic beads, are compatible with methodologies used for virus elution as part of a norovirus detection procedure. Binding experiments were performed with both Norwalk VLPs and native GI.1 virus particles and PGM- and saliva- binding studies were compared to binding studies using monoclonal antibodies specific for the target of capture. One advantage to using ligands instead of monoclonal antibodies is that they are have a broader specificity in the targets they capture, which is beneficial in an outbreak situation where the microorganism or specific genotype of the microorganism is unknown. Having less specificity can also be a disadvantage due the increased amount of debris and non-target microorganism that can bind to the ligand-coated magnetic bead surface.

In our study, it was found that there consistently was a decrease in target binding when suspended in 1M NaCl PBS in saliva- and PGM- coated beads which showed that high salt buffers may be too efficient at eluting due to the buffer not only eluting virus from surfaces, also eluting the virus from the ligand, causing the target not to bind, and therefore, not detected. This study also showed that native GI.1 binding showed no significant difference between bead coatings or the buffer suspensions ($p=0.776$), which means that the buffers at neutral pH do not have an effect on virus capture. Although statistical data showed no significant difference between virus capture in each bead coating, across all functionalized beads tested, the

experimental results showed neutral pH glycine buffer consistently had the highest P/N ratios and therefore the most VLP recovery.

We considered using rabbit sera as the primary detector reagent to increase the amount of Norwalk VLP that would be detected on the PGM- and saliva- coated beads due to sera having more epitopes for binding. Changing the primary detector to the rabbit sera did increase the P/N values for the PGM- and saliva- coated beads; however, the P/N values were still lower than the NV3901- coated magnetic beads.

The buffers in the experiments were chosen based on success in viral elution from foods and surfaces in similar experiments found during a literature search or because of their success in aiding in binding of virus to the ligand. Citrate buffer was chosen because of a previous study by Tian (2010) that showed by adding citrate-buffered saline at pH 3.6 norovirus binding was enhanced to PGM coupled magnetic beads(Tian et al., 2010). Distilled water was found to suitable for the elution of human norovirus from tomatoes and blueberries and glycine buffer (pH 9.5) improved the elution from human norovirus inoculated salad (Pan et al., 2012). Another study by Kim (2008) proved using RT-PCR that glycine buffer with 3% beef extract was the most effective for norovirus elution from various fruits due to its high protein concentration (Kim, 2008). Glycine buffer with beef extract is commonly used because it is able to reduce non-specific virus adsorption to the food matrix (Stals et al., 2012). The 1M NaCl PBS was chosen because the high salt masks charges and therefore causes disruption of electrostatic interactions that may exist between the viral particles and the ligand surface and should therefore discourage binding to surfaces. The1M NaCl PBS buffer also had the addition of Tween 20 which is a detergent suitable for elution, this ingredient should have also aided in the discouragement of viral attachment by masking hydrophobic areas of the virus that may have been exposed by the

high salt in the buffer. In general, the various neutral pH elution buffers did not have a significant effect on the amount of VLP capture within each bead type. The PGM/1M NaCl PBS combination did show a reduction in VLP capture, and according to statistical analysis, this was a significant reduction at the 0.05 level. This observation is consistent with a previous study (Tian et al., 2010) which also showed viral capture with PGM coated magnetic beads was decreased at high salt concentrations.

Changing the pH of the elution buffers to acidic or basic does not increase target capture. Our results showed, changing the pH of the elution buffers causes a reduction in capture to which was not seen in the Tian paper (Tian, Brandl, and Mandrell, 2005), where binding of G1 strains of human norovirus was increased when suspended in citrate buffer at a pH of 3.6. Tian (2005) also showed that in all strains tested, the recovery of RNA at low pH always was better than at neutral pH, which is contrary to the results from our study. Based on the pH and charges of the ligand and the virus, the virus should have been positively charged at the acidic pH of 3.6, whereas the PGM should have been negatively charged, based on its isoelectric point being between 2-3 and therefore should have been attracted to each other and resulted in a high recovery(Bansil and Turner, 2006). Similarly, in the basic pH buffers the previous study (Tian et al., 2010) showed that viral binding was minimal. Our results proved this to be correct. Some reasons for the disagreement in our results and previous studies are volumes of reagents that were used, and incubation time. Larger volumes were used in previous experiments (Tian et al., 2010) such as washing beads with 10ml of PBS compared to our 1ml of PBS and incubating beads with PGM for 30 minutes instead of the 2 hours in our experiment. There was also a shorter time of incubation between the beads?? and target, this paper used a 15 minute incubation which yielded the greatest results with viral RNA decreasing with increasing incubation times,

where as we used a constant incubation variable of 60 minutes in our study. Another contributor to differing results could be a difference in the viral source and/or storage conditions (number of freeze/thaws). Time, storage, and volume are major factors in the outcome of results because they are directly related to the amount of contact between the virus and elution buffer/extracts and, in the case of storage, the viability of the virus. Time was also proven to be an important factor by a previous study in which, suggests that virus capture by magnetic beads could be limited by the limited capacity of the bead itself or insufficient time for virus to be exposed to the magnetic beads ((Liangwen Pan, 2012; Pan et al., 2012).

Saliva from a secretor individual and porcine gastric mucins were chosen because they contain histo-blood group antigens. Some saliva samples contain histo-blood group antigens which have been linked to being receptors for the binding of norovirus in various environments. HBGA- conjugated magnetic beads have been used to capture and recover norovirus from water and food samples (Cannon and Vinje, 2008; Tian et al., 2010).

Porcine gastric mucins are a conglomerate of various carbohydrates that may be able to act as universal vehicle for attachment due to the many non-specific binding sites they offer and types of HBGAs. PGMs were used with magnetic beads for norovirus detection (Tian, Engelbrektson, and Mandrell, 2008). PEG methods in comparison to re-circulating affinity magnetic separation system (RCAMS) for concentrating VLPs shows a significant difference in favor of RCAMS method. This was shown by RCAMS having a .004 real-time PCR units (RTU) which was an adjusted concentration of 225 fold increase in comparison to no positive detected samples before using RCAMS (Tian, Yang, and Mandrell, 2011). The PEG method only showed a positive detection of 0.04 RTUs and an adjusted concentration of 72 fold increase in comparison to samples taken before PEG (Tian et al., 2011); these results prove that RCAMS is

a more sensitive and direct method of detection when directly compared to PEG. Even though the results shown in this study made strong conclusions, it should be noted that the authors used qualitative assessment which is not the most accurate especially when reporting a detection limit of an assay. Studies showed that when using RCAMS a proper recirculation time and flow rate is key to a successful concentration step. In this same study it was found that the pH of elution buffers should be adjusted to and acidic 3.6 to get the most viral recovery. RCAMs is said to be more sensitive than conventional methods for recovery such as PEG.

We were interested in testing food matrices because even though norovirus cannot replicate without a host, it is highly stable on a food surface, making it critical to detect and eliminate. Iceberg lettuce and roast beef deli meat was selected for testing due to 58.3% of food-borne disease outbreaks being associated with leafy greens (Herman, 2008) and ready to eat foods being associated with norovirus (Malek, 2009). Another reason for the selection of these foods is because the consumption of ready to eat and minimally-processed fruit and vegetables and associated human norovirus outbreaks have both increased rapidly in recent years (Herman, 2008).

A previous study, showed that food matrix has a great impact on virus recovery, with lower recovery of human norovirus in the context of produce (fruits, salads) food samples (Liangwen Pan, 2012; Pan et al., 2012). Buffers at pH 9-10.5 are used to elute the viral agents from food surfaces commonly because the alkaline pH allows the viral particles to detach from the food matrix (Stals et al., 2012). Conversely, acidic buffers, such as citrate buffer at pH 3.6, would cause viral particles to want to bind to the food surface; negatively affecting virus elution. Food particles themselves can also assist in target binding, but not necessarily to the desired ligand. Gandhi (2010) shows that VLPs of Norwalk virus should bind to romaine lettuce at a

higher level than other vegetables with non-HBGA binding sites, which means that Norwalk VLPs has the ability to bind to lettuce without any aid (Gandhi, Mandrell, and Tian, 2010). The data from Gandhi (2010) could also lead to the question of was Norwalk VLPs that were not bound to the ligand or antibody inhibited by the lettuce extract and bound to it instead. The food matrices used in this experiment did not greatly interfere with VLP capture. Results were comparable in the VLP capture experiments while suspended in food extracts and in buffers alone. Overall, the native GI.1 food extract suspension results were similar in all except the NV3901-coated magnetic beads in which virus capture increased when suspended in food extract compared to in a buffer matrix. There are a few possible explanations for this, one being that the food extract caused non-specific binding. For example, the virus particles could have gotten sequestered by a food carbohydrate or protein and the outer protein or carbohydrate layer and caused non-specific binding.

Another reason for our results could be due to the increased amount of inoculum of 5 microliters in the food extract experiments compared the 1 microliter inoculums in the buffer matrix experiments. We anticipated inhibition from the food extracts and therefore increased the inoculum so that comparisons could be made between the different beads, buffers and food matrices used. However, throughout all the treatments, beef and lettuce extract had a similar impact on virus and VLP capture for all bead types. This was not expected due to the increased protein and fat content in the beef extraction suspension. Our results could suggest that the concentration of meat proteins and carbohydrates present in the samples used in this experiment minimally affect virus capture. The increased inoculums in this experiment also confirmed what was found in a previous study that demonstrated that including a higher starting inoculum did not always result in a higher virus capture or recovery (Pan et al., 2012).

It was also found in our Norwalk virus capture experiments that the uncoated magnetic beads themselves can cause some non-specific binding of the native Norwalk virus. The degree of non-specific binding depended on the type of buffer in which the magnetic beads were suspended and the amount of virus that was inoculated into the sample. The inocula in the native virus experiments in a buffer matrix were inoculated with 1µl of native virus whereas the food buffer extracts were inoculated with 5µl of native virus, which could be a reason for the spike in non-specific binding on the uncoated beads. For example, in native virus capture experiments neutral pH buffers, acidic and basic pH buffers and in the presence of food extract, a lot of non-specific binding was detected on the uncoated beads. These results were consistently observed when suspended in water at a neutral pH and in all buffer matrix experiments with 1M NaCl PBS buffer except when the amine beads were suspended in neutral 1M NaCl PBS; no non-specific binding was observed in this treatment. The presence of non-specific binding means that the beads themselves cause target attachment or adsorption to the bead. For amine and tosyl-activated magnetic beads, in addition to bead/buffer combinations, functional groups found on the magnetic beads from the manufacturer may contribute to the non-specific binding. Initially seen as a disadvantage, one could look at these results as an advantage because this data could also lend to further research using an uncoated amine or tosyl-activated magnetic beads as alone, eliminating the need to use a ligand or antibody for coupling to the magnetic bead, saving time and money. Another experiment for future research could be diluting the virus out and using the coated magnetic beads to establish the lower limit of detection of the assay for each type of bead coating.

In all native GI.1 capture experiments; there was a considerably lower amount of capture when using NV3901 coated beads in comparison to the other ligands. The results from these

experiments suggest that native GI.1 used may have underwent structural changes of capsid proteins or that the proteins that interact with the antibody may have been masked or deleted due to mutations and as a result different from Norwalk VLP capture. Another contributor to this discrepancy between Norwalk VLP and native GI.1 virus could be fecal inhibitors that prevented binding or caused non-specific binding and resulted in a low capture of the target virus.

Overall, these experiments proved there is need for improvement to more effectively achieve the purpose of developing a rapid method for norovirus concentration and recovery in a food matrix. During real time RT- PCR quantification, an endpoint dilution method was used to determine initial virus quantities, this method gives an estimated initial quantities based on the highest positive sample dilution multiplied by the dilution factors in the experiment. Improvements to this study could be using a standard for quantification with a known starting concentration so that the log genome copies would be actual and not estimated this will make for more accurate and precise data. Another improvement could be to elute the virus off the bead surface before doing ELISA assays using chemical reagents (change in pH) to give more specific results, similarly to what was done in a previous study with PGM conjugated beads (Pan et al., 2012). Further research can also be done exploring other food matrices associated with norovirus such as small fruits like strawberries and raspberries because they have also been linked to norovirus outbreaks and they may behave differently in detection experiments based on their composition (Kim, 2008). More experiments and research optimizing and characterizing the conditions for the best recovery efficiency, concentration, and sensitivity of the method and assays (ELISA) need to be done to make a firm decision whether or not ligand coated magnetic beads can make for an accurate and reliable alternative to antibody coated magnetic beads in the context of buffer and food matrices. Some examples of variables to explore are the detection

limit of the coated-magnetic bead assay, to remove the beads before doing any detection assays, and /or to determine the appropriate amount of ligand that is most efficient for virus/VLP detection in magnetic bead capture. Another experiment that could be further explored is manipulated the elution buffer's concentration of ingredients to determine if that has an effect on the efficiency of coated magnetic bead capture of Norwalk VLP and/or native virus.

CHAPTER 6

CONCLUSIONS

This study is important because it prepares the foundation to establishing a novel rapid method for the concentration and recovery of norovirus in buffer and food matrices. This study showed that ligands such as PGM and saliva can be used to bind Norwalk VLPs and GI.1 virus particles. This study also showed that pH does affect the binding of VLP and virus to both ligands and antibody and is a variable that should be carefully considered and monitored throughout experiments. This method has proven that not only is rabbit antisera a more efficient detector of virus binding to ligands in comparison to NV3901 monoclonal antibody, but also allows for direct virus capture comparison to NV3901-coated magnetic beads. There is also a significant difference in the capture of Norwalk VLP particles and GI.1 virus particles, which is a very interesting finding and is something that should be further investigated. Ligand coated magnetic bead VLP and virus capture can be applied in food matrices, however the impact of food residuals on the amount of capture needs to be further investigated. In conclusion, the ligand coated magnetic bead capture method for Norwalk VLP and native GI.1 virus has the potential with further research and exploration to be a useful rapid method for the concentration and recovery of norovirus in both buffer and food matrices.

CHAPTER 7

REFERENCES

Bansil, R., Stanley, E. and Lamont, J.T., 1995. Mucin Biophysics. Annual Review Physiology 57, 635-657.

Bansil, R. and Turner, B.S., 2006. Mucin structure, aggregation, physiological functions and biomedical applications. Current Opinion in Colloid & Interface Science 11, 164-170.

Bresee, J., Widdowson, M., Monroe, S. and Glass, R., 2002. Foodborne viral gastroenteritis: challenges and opportunities. Clinical infectious diseases 35.

Cannon, J.L. and Vinje, J., 2008. Histo-blood group antigen assay for detecting noroviruses in water. Journal of Applied Microbiology, 6818-6819.

Celli, J., Gregor, B., Turner, B., Afdhal, N.H., Bansil, R. and Erramilli, S., 2005. Viscoelastic Properties and Dynamics of Porcine Gastric Mucin. Biomacromolecules 6, 1329-1333.

Centers for Disease Control and Prevention and National Center for Emerging and Zoonotic Infectious Diseases, 2011. Norovirus in Health Care Settings.

Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases and Division of Foodborne, W., and Environmental Diseases 2011. Investigation Update: Multistate Outbreak of Human Salmonella Heidelberg Infections Linked to Ground Turkey.

Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases and Division of Foodborne, W., and Environmental Diseases 2012. Multistate Outbreak of Shiga Toxin-producing Escherichia coli O145 Infections.

DeWaal, C. and Bhuiya, F., 2007. Outbreaks by the numbers: fruits and vegetables.

Duizer, E., Kroneman, A., Siebenga, J., Verhoef, L., Vennema, H., Koopmans, M. and network, t.F., 2008. TYPING DATABASE FOR NOROVIRUSES. Eurosurveillance 13.

Flint, S.J., Enquist, L.W., Racaniello, V.R. and Skalka, A.M., 2003. Principles of Virology: Molecular Biology, Pathogenesis, and Control of Animal Viruses.

Gandhi, K.M., Mandrell, R.E. and Tian, P., 2010. Binding of Virus-Like Particles of Norwalk Virus to Romaine Lettuce Veins. Applied and Environmental Microbiology 76, 7997-8003.

Glass, R.I., Noel, J., Ando, T., Fankhauser, R., Belliot, G., Mounts, A., Parashar, U.D., Bresee, J. and Monroe, S.S., 2000. The Epidemiology of Enteric Caliciviruses from Humans: A Reassessment Using New Diagnostics. The Journal Of Infectious Diseases.

Hardy, M.E., 2005. Norovirus protein structure and function. FEMS Microbiology Letters 253, 1-8.

Herman, K.M., Ayers, T.L., Lynch, M., 2008. Foodborne disease outbreaks associated with leafy greens, 1973-2006. International Conference Emerging Infectious Diseases.

Huang, P., Farkas, T., Zhong, W., Tan, M., Thornton, S., Morrow, A.L. and Jiang, X., 2005. Norovirus and Histo-Blood Group Antigens: Demonstration of a Wide Spectrum of Strain Specificities and Classification of Two Major Binding Groups among Multiple Binding Patterns. Journal of Virology 79, 6714-6722.

Jordan, W.S., Jr., 1953. A study of illness in a group of Cleveland families. VII. Transmission of acute non-bacterial gastroenteritis to volunteers: evidence for two different etiologic agents. The Journal of experimental medicine 98, 461.

Kageyama, T., 2003. Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. Journal of Clinical Microbiology 41, 1548.

Kapikian, A.Z., 2000. The discovery of the 27-nm Norwalk virus: an historic perspective. The Journal of infectious diseases 181, 295.

Kim, H.Y., 2008. Optimization of methods for detecting norovirus on various fruit. Journal of Virological Methods 153, 104.

Liangwen Pan, Q.Z., Xiang Li, Peng Tian, 2012. Detection of human norovirus in cherry tomatoes, blueberries and vegetable salad by using a receptor-binding capture and magnetic sequestration (RBCMS) method. Food Microbiology 30, 420-426.

Malek, M., 2009. Outbreak of norovirus infection among river rafters associated with packaged delicatessen meat, Grand Canyon, 2005. Clinical infectious diseases 48, 31.

Morillo, S.G., 2011. Norovirus: an overview. Revista da Associação Médica Brasileira 57, 462.

Morton, V., 2009. Detection of Noroviruses in Ready-To-Eat Foods by Using Carbohydrate-Coated Magnetic Beads. Applied and Environmental Microbiology 75, 4641.

National Center for Immunization and Respiratory Diseases and Diseases, D.o.V., 2012. Surveillance for Norovirus Outbreaks.

Palomares, L., 2012. Simultaneous expression of recombinant proteins in the insect cell-baculovirus system: Production of virus-like particles. Methods 56, 389.

Pan, L., Zhang, Q., Li, X. and Tian, P., 2012. Detection of human norovirus in cherry tomatoes, blueberries and vegetable salad by using a receptor-binding capture and magnetic sequestration (RBCMS) method. Food Microbiology 30, 420-426.

Patel, M.M., 2009. Noroviruses: a comprehensive review. Journal of Clinical Virology 44, 1.

Patel, M.M., Widdowson, M.-A., Glass, R.I., Akazawa, K., Vinje', J. and Parashar, U.D., 2008. Systematic Literature Review of Role of Noroviruses in Sporadic Gastroenteritis. Emerging Infectious Diseases 14.

Prevention, C.f.D.C.a., Diseases, N.C.f.E.a.Z.I. and Diseases, D.o.F.W.a.E., 2011. Multistate Outbreak of Listeriosis Linked to Whole Cantaloupes from Jensen Farms, Colorado.

Ramirez, S., De Grazia, S., Giammanco, G.M., Milici, M., Colomba, C., Ruggeri, F.M., Martella, V. and Arista, S., 2006. Detection of the norovirus variants GGII.4 hunter and GGIIb/hilversum in Italian children with gastroenteritis. Journal of Medical Virology 78, 1656-1662.

Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL and PM, G., 2011. Foodborne illness acquired in the United States--major pathogens. Emerging Infectious Diseases 17, 7-15.

Schultz, A., Saadbye, P., Hoorfar, J. and Norrung, B., 2007. Comparison of methods for detection of norovirus in oysters. International Journal of Food Microbiology 114, 352-356.

Shirato, H., 2011. Norovirus and Histo-Blood Group Antigens. Japan Journal of Infectious Disease 64, 95-103.

Sim, S.-L., He, T., Tscheliessnig, A., Mueller, M., Tan, R.B.H. and Jungbauer, A., 2012. Protein precipitation by polyethylene glycol: A generalized model based on hydrodynamic radius. Journal of Biotechnology 157, 315-319.

Stals, A., Baert, L., Van Coillie, E. and Uyttendaele, M., 2012. Extraction of food-borne viruses from food samples: A review. International Journal of Food Microbiology 153, 1-9.

Summa, M., Henrik von Bonsdorff, C. and Maunula, L., 2012. Evaluation of four virus recovery methods for detecting noroviruses on fresh lettuce, sliced ham, and frozen raspberries. Journal of Virological Methods.

Tan, M. and Jiang, X., 2005a. Norovirus and its histo-blood group antigen receptors: an answer to a historical puzzle. Trends in Microbiology 13, 285-293.

Tan, M. and Jiang, X., 2005b. The P Domain of Norovirus Capsid Protein Forms a Subviral Particle That Binds to Histo-Blood Group Antigen Receptors. Journal of Virology 79, 14017-14030.

Tan, M. and Jiang, X., 2011. Norovirus–host interaction: Multi-selections by human histo-blood group antigens. Trends in Microbiology 19, 382-388.

Tian, P., Brandl, M. and Mandrell, R., 2005. Porcine gastric mucin binds to recombinant norovirus particles and competitively inhibits their binding to histo-blood group antigens and Caco-2 cells. Letters in Applied Microbiology 41, 315-320.

Tian, P., Engelbrektson, A. and Mandrell, R., 2008. Two-Log Increase in Sensitivity for Detection of Norovirus in Complex Samples by Concentration with Porcine Gastric Mucin Conjugated to Magnetic Beads. Applied and Environmental Microbiology 74, 4271-4276.

Tian, P., Yang, D., Jiang, X., Zhong, W., Cannon, J.L., Burkhardt Iii, W., Woods, J.W., Hartman, G., Lindesmith, L., Baric, R.S. and Mandrell, R., 2010. Specificity and kinetics of norovirus binding to magnetic bead-conjugated histo-blood group antigens. Journal of Applied Microbiology.

Tian, P., Yang, D. and Mandrell, R., 2011. A simple method to recover Norovirus from fresh produce with large sample size by using histo-blood group antigen-conjugated to magnetic beads in a recirculating affinity magnetic separation system (RCAMS). International Journal of Food Microbiology 147, 223-227.

Vega, E., Barclay, L., Gregoricus, N., Williams, K., Lee, D. and Vinjé, J., 2011. Novel Surveillance Network for Norovirus Gastroenteritis Outbreaks, United States. Emerging Infectious Diseases 17.

Verhoef, L.P.B., 2009. Selection Tool for Foodborne Norovirus Outbreaks. Emerging Infectious Diseases 15, 31-38.

Wobus, C.E., 2006. Murine norovirus: a model system to study norovirus biology and pathogenesis. Journal of Virology 80, 5104.