

NOVEL APPROACHES FOR CAPTURING AND RECOVERING HUMAN NOROVIRUS
BY PORCINE GASTRIC MUCINS

by

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(Under the Direction of Jennifer L. Cannon)

ABSTRACT

Human norovirus is the primary cause of non-bacterial gastroenteritis world-wide. Porcine gastric mucins (PGM) contain histo-blood group antigens that are capable of binding multiple genotypes of HuNoV, unlike monoclonal antibodies (mAB) which are less cross-reactive. The purpose of this study was two-fold: 1) To determine the limit of detection (LOD) for PGM- and mAB- conjugated magnetic bead-based recovery assays in the context of elution buffers commonly used in Food Virology; 2) To determine if the addition PGM to existing low-cost recovery methods could increase virus recovery. When magnetic beads were coated with PGM, assay detection limits were lower than with mAB-coated beads for all buffer matrices tested. Neutral pH buffers were most compatible, providing a low LOD with minimal non-specific binding. Although repeatability was poor for the novel PGM assays, results warrant future exploration and are an important step in developing cost-effective, rapid and reliable method for norovirus detection.

INDEX WORDS: Norovirus, Porcine Gastric Mucins, Saliva, Histo-blood group antigens, Magnetic Beads, Immunomagnetic separation, Norwalk Virus

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DEDICATION

I would like to dedicate this thesis to my late husband, Kristopher Langley. I miss you. I wouldn't be who I am today without having known you. To my remarkable son, Willard Langley; I have never loved anything as much as I love you. To my father, Will Hedrick; thanks for hanging in there with me. Sorry it took me so long to get it together. Finally, to James Godino; I love you more than words can say. You have been such a source of strength for me and such a bright light in my life. Thank you for all that you are to me.

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CHAPTER 1

INTRODUCTION

Norovirus is the leading cause of gastroenteritis and is responsible for 90% of non-bacterial gastroenteritis cases and 50% of all gastroenteritis cases, world-wide¹. It is responsible for over 21 million cases and 800 deaths annually.¹ A variety of food-types have been associated with outbreaks of norovirus, including leafy greens and other produce, oysters and other shellfish that are often consumed raw or undercooked, and prepared, ready-to-eat foods. Because these foods are typically consumed without a cooking step, they are more likely to cause illness if contaminated prior to consumption.

There is a need for rapid and reliable detection methodologies for human norovirus. Typically, during an outbreak, a retrospective epidemiologic study is conducted to determine the cause of the outbreak; however, the implicated food itself is rarely tested. This type of surveillance is limited to the memories of the person infected and tends to include a high amount of error. Although current methods for norovirus detection do exist, they are limited by their ability to detect multiple genotypes of norovirus, sensitivity, reproducibility, and high costs.

Commonly, a four-step process is used to detect norovirus in foods: viral elution, concentration, RNA extraction, and detection by real-time RT-PCR. The efficiency of the elution process is critical. Elution releases viral particles from a surface allowing them to be further processed. It is crucial that an effective elution buffer be selected. Properties including pH, ionic strength, and protein concentration can all contribute to the effectiveness of the buffer and how it interacts with the viral particle. Buffers containing proteins or basic amino acids and

have a high pH (above 9.0) are used because the buffer proteins compete with the virus particles for binding with the food surface, thus forcing the viral particles (which are more negatively charged) off of the surface. Buffers with high ionic strength can change the charges of the virus and the food surface, thereby disrupting electrostatic forces between the two. A low pH buffer will change the charge on the virus particle and may repulse the virus into solution.

Magnetic separation using functionalized superparamagnetic beads can be used for both concentration and purification; however, the method is limited by its high cost and requirements for a small sample size. Immunomagnetic separation methods are based on the attachment of small magnetizable particles (beads) to virions via antibodies, carbohydrates, or ligands like those found in porcine gastric mucins (PGMs) or saliva. Limitations to using mAB exist because of its lack of cross-reactivity among norovirus genotypes. This can be circumvented by using the more broadly-reactive histo-blood group antigens (HBGAs). HBGAs, contained within PGMs or saliva, are capable of binding multiple genotypes and even genogroups of norovirus, whereas monoclonal antibodies (mAB) cannot. The addition of HBGA-containing ligands to existing viral recovery methods could enhance the capture of virions and provide a higher through-put and cheaper alternative to magnetic bead separation assays.

Other viral concentration methods include polyethylene glycol (PEG) precipitation, ultracentrifugation, and ultra-filtration; none of which aid in viral purification that is often needed to remove food debris or other chemicals and can interfere with RT-PCR. PEG precipitation is the most commonly used method for viral concentration and has a very high repeatability between experiments. Filtration can be used to separate the virus particle from a particular matrix and the size of the filter can be adjusted to meet those goals. The objectives of this study were to compare the performance of HBGA- or mAB-functionalized supermagnetic

beads and to determine the lower limit of detection of each assay. Also, the addition of HBGA-containing ligands to existing viral recovery methods were evaluated for their ability to enhance the capture of virions, thereby, providing a higher through-put and cheaper alternative to magnetic bead separation assays.

Monoclonal antibody, NV3901, is able to recognize a common epitope in GI Norwalk-like virus.² Saliva from a secretor-positive individual contains histo-blood group antigens that have been previously shown to bind norovirus.³ Porcine gastric mucins have been shown to contain type A, H-type 1, and Lewis b antigens.⁴ Since these antigens are also found in some secretor-positive humans, this makes them an excellent model for simulating binding proficiency within the GI tract. PGM has a broader specificity than NV3901 and can target multiple genotypes of virus, making it more useful in an outbreak scenario where the source strain may not be known. HBGA-conjugated magnetic beads have previously been used to recover norovirus from both food and water samples.³

The purpose of this study was two-fold: 1) To determine the limit of detection for PGM- and mAB- conjugated magnetic beads in the context of a variety of buffer matrices commonly used in food virology; and 2) to determine if the addition PGM to existing low-cost recovery methods could increase virus recovery. The success of these assays will determine future directions of the study of norovirus capture and recovery methods

CHAPTER 2

LITERATURE REVIEW

Overview

Norovirus is a non-enveloped, positive sense, single-stranded RNA virus and is characterized by cup-like structures on the capsid surface, which are characteristic of all viruses in the family *Caliciviridae*.⁵ A prototype strain, discovered in 1968 by Adler and Zickl in the town of Norwalk, Ohio, was originally referred to as “winter vomiting disease”. It resulted in a 50% attack rate of students and teachers at the local elementary school and a 32% attack rate for those in direct contact with infected individuals.⁶ In 1972, suspected viral particles collected from the outbreak were observed using immune electron microscopy (IEM) and were discovered to be approximately 27-nm in size.⁷ These pathogens would become known as the Norwalk virus. The use of IEM allowed for visualization of the viral-antibody complex and would eventually lead to discovery of Norwalk-like viruses and other small round structured viruses (SRSVs), such as the Hawaii virus in 1977 and the Snow Mountain virus in 1982.^{8; 9; 10} Antigenic diversity of Norwalk-like and other SRSVs observed through IEM originally led scientists to believe that they were not related to each other; however, the refinement of the PCR technique by Dr. Kary Mullis in 1993 ultimately allowed scientists to amplify and sequence genetic material found in these virus samples.¹¹ This new ability to determine genetic differences and similarities between samples resulted in taxonomic classification of norovirus and sapovirus.¹²

Norovirus is a major cause of epidemic gastroenteritis. It is a genus within the *Caliciviridae* family, which also includes sapovirus, lagovirus, vesivirus and the newly

discovered nebovirus. Human norovirus (HuNoV) is responsible for 95% of non-bacterial gastroenteritis and 50% of all gastroenteritis outbreaks in the world.¹³ In the U.S. alone, it is estimated that there are 23 million cases per year, resulting in roughly 50,000 hospitalizations and an estimated 300 deaths.¹³ Recent diagnostic studies have estimated that in developing countries, over 1 million hospitalizations and 200,000 deaths have occurred annually.¹⁴ Norovirus can be spread from person-to-person, through contaminated food or water, aerosolized vomitus, and fomites. According to the CDC, norovirus is the most common cause of foodborne outbreaks.¹⁵ It has a high rate of infectivity and is extremely stable in the environment. Resistance to common disinfectants, as well as its ability to cause incapacitating disease has led to its classification as a Category B biodefense agent. All age groups are susceptible to infection and secondary infections occur frequently.¹⁶ Norovirus outbreaks typically occur in schools, hospitals, nursing homes, military settings, or any other semi-closed communities.^{17; 18; 19} Nosocomial norovirus outbreaks commonly cause closures of hospital wards.²⁰ Norovirus is referred to as the “winter vomiting disease” because most outbreaks occur during the winter season.²¹ Norovirus particles have a high rate of infectivity²² and the ability to survive in the environment.^{23; 24; 25} These characteristics as well as prolonged shedding from both symptomatic and asymptomatic individuals have led to the extensive nature of norovirus outbreaks.^{26; 27; 28}

Infection with norovirus is rapid (24-48 hour incubation period), with symptoms occurring within 12-72 hours in immunocompetent adults.²⁹ Symptoms include a low-grade fever, vomiting and diarrhea, nausea, abdominal cramps, and general malaise. Symptoms typically resolve within a few days; however, in infants and children, the duration of infection can be prolonged (up to 6 weeks) and symptoms more severe.^{30; 31; 32} Outbreaks often occur in nursing homes and the elderly are at a significantly higher risk for severe infection and even

death.^{33; 34} Recovery is usually complete with no long-term effects. There have also been several documented cases of immunocompromised and suppressed individuals who have had symptoms that have lasted over 2 years.^{35; 36; 37; 38; 39} The infectivity of norovirus is dose-dependent and has been shown to have a 10% probability of infection with a dose of 1000 particles, with the likelihood of infection increasing as the viral load increases²²; however, as little as 10 particles may be sufficient in causing infection.^{40; 41} Viral shedding can begin prior to presentation of symptoms and may continue for two weeks or more after recovery; an estimated 10^4 - 10^{12} genomic copies are shed per gram of feces.^{42; 26} Immunity mechanisms are unclear and conflicting reports suggest both short-term, strain-specific immunity and long-term protection from reinfection.^{43; 44} HuNoV studies have been limited because virus culture is not yet possible.

There has been difficulty ascertaining the total prevalence of norovirus in populations, particularly in developing countries. The acute nature of norovirus infection leads to a lack of reporting to health officials. There is also a lack of standardization among national and international diagnostic and surveillance networks. Significant improvements have been made in diagnostic assays, thus, increasing the recognition of the overall burden of norovirus disease rates. Several databases provide a place to aggregate data and allow a way to pin-point the source of an outbreak. The Food-Borne Viruses of Europe (FBVE) net allows surveillance for viral gastroenteritis as well as Hepatitis A and E.⁴⁵ NoroNet was developed in 2009 as an extension to FBVE and not only serves as an epidemiological surveillance tool, but also has clinical, diagnostic, and sequencing databases. CaliciNet was launched in March 2009 by the CDC and includes 20 certified state and local laboratories.⁴⁶ CaliciNet aims to improve norovirus surveillance standardization and also includes a database for sequencing data and outbreak information. This database allows for rapid identification of norovirus strains and can

lead to identification of multi-state and multi-source outbreaks. All 50 states have labs that can test for norovirus, although some do not have the capacity for genotyping.⁴⁶

Genetic features

Norovirus can be genetically classified into 5 genogroups (GI-GV). Average genome size is approximately 7.5 kb in length, containing 3 open reading frames (ORFs). Further classification into genotypes is made based on amino acid identities in major structural protein (VP1) region of the genome.⁴⁷ Strains within a genotype share greater than 85% amino acid (aa) identity, while strains of different genogroups share approximately 51-56% aa identity.⁴⁷ Genogroups I, II, and IV infect humans.⁴⁰ Porcine norovirus are the most closely related animal norovirus to HuNoV strains, sharing 65-71% aa identity with 9 HuNoV strains in genogroup II.⁴⁸ Genogroup III infects bovines and genogroup V was isolated in mice in 2003.^{49; 50} A GIV.2 variant that shared 69.3-70.1% aa identity with GIV.1 HuNoV was discovered in a lion in 2006 and in 2008, a canine norovirus was discovered that shared 90.1% amino acid identity with the GIV.2 lion strain.^{51; 52} Genogroups I and II show the most genetic diversity of the human genogroups, with 8 and 17 genotypes, respectively;⁵³ although, genogroup II, genotype 4 (GII.4) is the most common cause of infection in humans.^{40; 54; 55} It has been shown through molecular epidemiology studies, that the GII.4 variant is responsible for 70% of norovirus outbreaks.^{56; 57} It is possible that GII.4 may be more environmentally stable, transmissible, and/or virulent; however, its dominance in generating illness is not clearly understood.⁵⁸

The norovirus genome is linear, bound by a viral genome-linked protein (VPg) at the 5' end and is poly-adenylated at the 3' end. VPg is the largest of the non-structural proteins and is crucial for initial translation and viral infectivity and poly-adenylation provides over-all stability to the virion.⁵³ A viral 3C-like protease (3CL^{pro}) co- and post-translationally processes non-

structural proteins contained within ORF1 and releases 6 products: p48, NTPase, p22, VPg, 3CLpro, and RNA-dependent RNA polymerase (RdRp), that are encoded, respectively, from N-terminus to C-terminus.⁵⁹ The length and amino acid content of these products vary among genogroups. p48 appears to disrupt intracellular protein trafficking by forming a complex with the SNARE protein, VAP-A.⁶⁰ NTPase binds and hydrolyzes ATP.⁵⁹ Not much is known about the function of p22, but research suggests it has a role in the disassembly of the Golgi apparatus during NoV replication, as well as causing a disruption in cellular trafficking.⁶¹

The major capsid protein (VP1) is encoded by ORF2; ORF3 encodes the minor structural protein (VP2).⁵⁹ VP1 and VP2 are translated from a Vpg-linked sub-genomic RNA transcript that includes both ORF1 and ORF2. One norovirus particle consists of 90 dimers of VP1 and 1-2 copies of VP2.⁵⁹ VP1 contains a shell (S) domain and a protruding (P) domain that are linked by a flexible hinge.^{59; 62} The P domain can be further described as having two subdomains: P1 and P2.^{62; 63} The P2 region contains a receptor binding region and is important in determining host susceptibility.^{64; 65} Neutralizing antibodies can also recognize the P2 region, and therefore, mutation frequency is high in this region.^{66; 67} The S domain contains an 8-stranded antiparallel β -sandwich that forms the icosahedral shell which protects the genomic RNA.⁶² Localized conformational changes occur in the S domain and these interactions are maintained between the opposing S domains in dimers.⁶²

Expression of VLPs using baculovirus as a vector in insect cells can lead to the production of recombinant virus proteins.^{68; 69} Using this method, virus-like particles (VLPs) can be made by expressing VP1 and VP2 in insect cells. The expression of only the recombinant S domains of VP1 leads to a self-assembly into smooth VLPs roughly 30 nm in diameter.⁷⁰ Although VLP assembly is not dependent on the presence of VP2,⁷⁰ its absence leads to

decreased size and stability of the particle.⁷¹ VLPs are similar morphologically and antigenically to native norovirus virions and can be used to further laboratory studies.¹ VLPs have proven useful in gathering data on the capsid structure and functional aspects of their full-genome counterparts. VP2 is much smaller than VP1 and sequences can vary between genotypes. VP2 has been shown to be essential for the infectivity of the virion in feline calicivirus.⁷²

Histo-blood group antigens (HBGAs) are complex carbohydrate structures at found at the end of glycan chains. Roughly a century ago, ABO-specific antigens and antibodies were seen on human red blood cells. This discovery was made by Karl Landsteiner and the antigens were renamed to “Histo-blood group antigens”. HBGAs can be found on a number of cells in the body including red blood cells, but also for the majority of people, they can be displayed on epithelial cells found in the gastrointestinal and respiratory tracts.^{73; 74; 75} They can also be found in saliva and breast milk of some individuals. HBGAs are formed when monosaccharides are added sequentially to the terminal end of a glycolipid or glycoprotein. The make-up and orientation of these monosaccharides are the result of allelic variants of three genes: *FUT3*, *FUT2* and *ABO*, which ultimately determine the Lewis, Secretor, and ABO phenotype of an individual, respectively.⁷³

Norovirus recognizes α 1,2-linked fucose residues.⁶⁴ In order for an individual to express these residues on their intestinal epithelial cells, that individual must have *FUT2* gene. The *FUT2* gene encodes an enzyme called fucosyl-transferase that promotes fucosylation of the oligosaccharide chain.⁷⁶ Those with the *FUT2* gene are known as “secretors” and make up about 80% of the population. This demographic is susceptible to the prototype Norwalk virus (GI.1) infection whereas, the remaining 20% (those who possess a mutated *FUT2* gene and do not produce the enzyme) are known as “non-secretors” and are resistant to infection by some

norovirus genotypes.⁷⁷ Because HBGAs have been associated with infection by GI.1 and GII.4 norovirus, it has been deduced that they are important and likely necessary for infection, but their role as receptors or co-receptors is not completely understood.^{77; 78; 79; 80}

A series of saliva-based assays has revealed 8 HBGA binding patterns and additional tests using synthetic oligosaccharides have revealed additional binding patterns;^{81; 82} however, more research is needed to determine the relevance of these results. The binding patterns can be divided into two groups: those that bind A/B antigens and those that bind Lewis antigens.⁸³ Strains that bind A/B do not bind Lewis-type and the reverse is also true. It is possible that this mutual exclusivity might indicate a co-evolution of the virus with the host; however, there has been an emergence of distinct norovirus binding patterns that appear to be strain-specific.^{82; 84; 85} Two strains, VA115 and DSV, do not bind any HBGAs when tested.⁸² This might indicate that other molecules in the body are responsible for their binding and entry into the cell. Because of the diversity of HBGAs as well as the genetic variability of strains, there is likely a norovirus strain capable of infecting nearly every allelic variant in the population.⁸⁶ It is probable that the variation of HBGA phenotypes in the population indicate an mechanism of the body to ward off infection, whereas, the diversity of strains might indicate a strategy of norovirus to overcome these mechanisms.⁸⁶ This highlights the highly adaptive nature of norovirus and demonstrates the evolution of both the human norovirus and its host.

Immunity

The mechanisms of immunity to norovirus infection are unclear. Differing responses in the population, as well as fleeting immunity have complicated our understanding. In early human challenge studies, patients were administered a homologous virus inoculum of filtered stool from a norovirus outbreak and two infection patterns emerged.^{43; 87} The first group of

volunteers showed total resistance to primary and secondary infection. The second group showed primary and secondary infection susceptibility; however, in order for secondary infection to occur, a six-month time interval had to elapse between challenges, suggesting short-term immunity.^{43; 87} Because norovirus strains are so different, pre-existing antibodies in the blood from previous infections do not necessarily aid in the prevention of a new infection. Some antibodies are neutralizing to a certain strain, but not to others, so if a person gets infected with a strain that cannot be neutralized by the antibodies already present, infection will occur. This explains how those who have large quantities of antibodies present in their blood can still get sick.^{43; 77; 87; 88; 89; 90}

Development of herd immunity is also possible at the population level^{77; 91; 92} and is supported by the fluctuation of GII.4 between dominance and dormancy in the world population.⁹³ During the period of time when GII.4 is not dominant, it is proposed that altering of the amino acids that comprise the P2 region occurs as the virus attempts to escape herd immunity in the population.^{94; 95} This slow change in viral structure, or antigenic drift, and can cause re-emergence of new strains into a population that is naïve to the new mutation.

Norovirus detection methods

As a prerequisite to the detection of norovirus, certain steps may be necessary to remove the virus from the sample matrix and to concentrate it once it has been separated. These steps often use pH and/or ionic manipulation to encourage virus elution or adsorption to a food or environmental surface. Other purification steps may include one or more of the following: pretreatment with enzymes that can degrade carbohydrates, extraction with organic solvents to aid in the removal of lipids, crude- and ultra- filtration to remove particulates, or magnetic separation of virus particles using synthetic histo-blood group antigens³ or porcine gastric

mucins^{96; 97; 98} conjugated to magnetic beads. Precipitation or ultracentrifugation can be used to concentrate the virus after separation. In virtually all cases, successful extraction and/or separation, purification, and concentration of viruses from foods includes a combination of two or more of these steps.⁹⁹

Concentration may be necessary to enhance molecular detection when the number of virus particles in the sample may be limited. The most common type of concentration method is the addition of poly-ethylene glycol (PEG). High molecular weight PEG can more rapidly precipitate small viral proteins when it is present in high concentrations.¹⁰⁰ Another method of viral concentration is ultracentrifugation. This involves centrifuging a sample at extremely high speeds ($> 80,000 \times g$). The target will form a pellet at the bottom of the tube and the supernatant can be removed. The success of ultracentrifugation relies on heavily on the prerequisite purification step that is necessary to remove any debris that may interfere with virus detection. Ultrafiltration can concentrate a sample based on size exclusion and also requires a thorough pre-purification step to remove debris that may block or puncture the filter. Charged membrane filters can be used to concentrate viruses, exploiting the native negative charge of the viruses at neutral pH.

Carbohydrate-, ligand-, or antibody- conjugated functionalized magnetic bead separation is becoming a more popular means by which to separate and concentrate viral particles. The type of magnetic bead will vary depending upon which coating will be used: Amine beads are functionalized with amine groups that easily bind glycoproteins like those found in porcine gastric mucins and other carbohydrates. Tosyl-activated beads have sulphonyl esters that encourage covalent bonding to antibodies. In order to maximize target recovery, it is necessary to select a coating that has a high binding affinity for the target molecule.

For molecular detection, RNA can be released from the viral capsid in one of two ways: heat release by heating a sample to 99°C for ≥ 1 minute or by applying guanidinium thiocyanate to denature viral proteins and a silica membrane or solid support to bind the subsequently released nucleic acids.¹⁰¹ While it is bound, the viral RNA is then washed with ethanol and can be eluted by either using TE buffer or nuclease-free water.

Nucleic acids can then be subjected to conventional reverse transcriptase polymerase chain reaction (RT-PCR) or to real-time (quantitative) (RT-qPCR). RT-PCR is an assay that involves the transcription of viral RNA into complementary DNA (cDNA) which is then amplified with norovirus-specific primers.¹⁰² In conventional RT-PCR, products are then subjected to agarose gel electrophoresis to provide a purely qualitative result. RT-qPCR incorporates a fluorescent probe that can be used to quantify the amount of nucleic acid in the sample in real-time.¹⁰³ Target-specific probes can be used to increase the sensitivity of the assay.¹⁰³ The emission of a fluorescent signal by these probes indicates the accumulation of amplicons above a certain cycle threshold value (CT), separating the signal from background noise. The probe is inserted into the amplicons; therefore, the intensity of this fluorescent signal directly corresponds to the quantity of amplicons. The earlier the threshold is crossed, the higher the quantity of RNA was in the original sample. The RT and the PCR steps can be done in either a one-step or two-step reaction. The single step reaction is faster since it includes the production of cDNA as well as the amplification of those products.¹⁰⁴ It also limits the handling of the sample, decreasing opportunities for sample contamination. RT-qPCR assays can be multiplexed (targeting one genomic region) or multiplexed (targeting two or more genomic regions). Multiplexing can hinder the sensitivity of an assay by requiring that certain reaction conditions be compromised to accommodate all primers and probes present in the reaction mix.

Alternate methods not based on nucleic acid detection include the Kaplan criteria, electron microscopy, and immunological assays. Characterization of norovirus outbreaks is typically performed by applying the Kaplan criteria. To be considered an outbreak caused by norovirus, the following criteria must be met; the mean illness duration between 12 to 60 hours, a 24- to 48- hour incubation period, and greater than 50% vomiting among those involved in the outbreak.¹⁰⁵ The Kaplan criteria were developed by studying outbreaks that occurred in the US between 1976-1980, and were validated on outbreaks occurring from 1998-2000, where the criteria were found to have a 99% specificity and 68% sensitivity.¹⁰⁵ The Kaplan criteria are not valid for hospital outbreaks because the mean duration of illness for hospital outbreaks tends to exceed that listed in the criteria (>72 hours);¹⁰⁶ however; they can be used as an initial diagnostic tool for cases that are eventually confirmed with laboratory testing.

Norovirus was first visualized in 1972 by electron microscopy (EM).⁷ EM allows for visualization of the virus particle, as well as any other pathogens that may be present, but it has a low sensitivity ($\sim 10^5$ - 10^6 particles).¹⁰⁷ In addition to its low sensitivity, EM is not commonly used because it requires specially trained staff as well as access to an EM facility.

Immunological tests detect norovirus by using specific antibodies, but are limited by the antigenic diversity of norovirus.¹⁰⁸ Norovirus-specific Enzyme-Linked ImmunoSorbent Assays (ELISA) use a range of antibodies that are broadly reactive with multiple genotypes of viruses. Although, these cannot make the distinction between infectious and non-infectious particles, they can act as a useful preliminary detection tool. Because they do not detect all types of norovirus and have a high limit of detection ($>10^5$)¹⁰⁹, they can provide false negatives.^{110; 111} Because of these previously mentioned limitations, it can only be used as a preliminary detection tool and not a means to diagnose.

Norovirus is a global problem that causes illness in millions of people annually including children and the elderly. Current detection methods are limited in sensitivity or are too expensive and /or time consuming to perform. Rapid and reliable testing methods that are cost-effective and time-efficient are thus needed.

CHAPTER 3

MATERIALS AND METHODS

Preparation of Norwalk virus-positive stool suspension

A (GI.1) Norwalk virus-positive stool sample was acquired from an ill patient that was part of a human challenge study conducted at Baylor College of Medicine by Dr. Robert Atmar. A stool suspension was made by placing a pea-size amount of stool into a 1.5 ml tube containing 750 μ l of Phosphate Buffered Saline (PBS), providing a ~10-20% stool suspension. After vortexing the sample until thoroughly mixed (about 1 min), the sample was then centrifuged at 13,000 rpm for 10 minutes. The supernatant was distributed into 10 μ l-aliquots before storage at -80°C. A fresh aliquot was used for each experiment to avoid variation due to multiple cycles of freeze/thawing of the virus stock.

Determination of the native GI.1 stool suspension virus titer

Five different methods were used to determine the virus stock concentrations. Serial dilutions (10-fold) were made of previously extracted GI.1 stool suspension in nuclease-free water. A conventional RT-PCR targeting region C, using GI specific primers (GISKF and GISKR)¹¹², was performed using a Qiagen One-step RT-PCR kit (Valencia, CA) using an Applied Biosystems Veriti® 96-Well Fast Thermal Cycler (Life Technologies; Grand Island, NY) under the following conditions: (i) RT for 30 min at 42 °C, (ii) Denaturation for 15 min at 95 °C, (iii) 40 cycles of 30 s at 95 °C, 30 s at 50 °C, and 30 s at 72 °C, (iv) final extension for 10 min at 72 °C. Once complete, the amplified product was run electrophoresed on a 2% Seakem ME gel (Lonza; Basel, Switzerland) at 105V for 90 minutes. Each dilution series was performed in triplicate,

each including a negative control comprised of master mix and RNase-free water only. The last visible band in the dilution series was used to back-calculate the estimated concentration of viral RNA per ml of stool suspension.

In addition to the conventional assay, an end-point real-time RT-PCR method and two quantitative real-time (RT-qPCR) methods were used to calculate virus stock concentration. All real-time assays were performed using an Applied Biosystems Step-One Real-time PCR System (Life Technologies: Grand Rapids, NY) under the following conditions: (i) RT for 30 min at 45 °C, (ii) Denaturation for 15 min at 95 °C, (iii) 50 cycles of 10 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C. GI-specific primers and probes were used (Cog 1F/1R and Ring 1C, respectively)¹¹³ in a QuantiTect Probe Kit (Qiagen; Valencia, CA). Viral RNA concentrations in each sample were determined using the ABI software and inclusion of a standard curve made with 10-fold serial dilutions of either a GI.3b transcript, or a GI.1 transcript, both 3kb in length and obtained from the Gastroenteritis and Respiratory Virus Laboratory Branch at the CDC (a gift from Jan Vinjé) that included partial coverage of ORF1 and ORF3, and complete coverage of ORF2. The sample quantities were reported as log genome copies per milliliter.

The transcript was synthesized by inserting a 3 kb amplified DNA product into a TOP10 plasmid and cloning the plasmid in *E. coli* competent cells (Invitrogen). The plasmid was linearized with restriction enzymes and processed through a Mega Script kit (Ambion) to convert the product from dsDNA to ssRNA. The sample was then treated with DNase to remove any residual DNA. Finally, the virus stock concentration was determined by assaying and averaging the virus stock with each experimental replicate. The mean of the quantity in the inoculum was determined to be 2×10^5 genome copies per μl (or 2×10^8 genome copies per ml). Since this value was also

very similar to the values obtained by $\frac{3}{4}$ of the other methods, this value was considered the most accurate.

Preparation of monoclonal antibody (NV3901) and carbohydrate ligand coated magnetic beads

M-280 Tosylactivated Dynabeads (Life Technologies; Grand Island, NY; formerly Invitrogen), coated with sulphonyl esters that can bind with amino groups on antibodies, were vortexed for 1-2 minutes to re-suspend the magnetic beads into solution. A 165 μ l-portion of beads (5mg) of were placed in a 1.5 ml tube which was then placed on a Dynamag-2 magnet (Life Technologies; Grand Island, NY) until the beads were drawn completely to the magnet-facing side of the tube (referred to as pelleting) until the liquid became clear (about 1 min). The supernatant was removed and the beads were then washed by adding 1 ml of 0.1 M sodium borate buffer (pH 9.5) which was mixed by pipetting. The beads were again placed on the magnet, allowed to pellet, and the supernatant was removed. Monoclonal antibody (NV3901) at a concentration of 2.87 mg/ml specific to Norwalk virus GI.1 (a gift from Dr. Robert Atmar, Baylor College of Medicine, Houston, TX) was added to 115 μ l of 0.1 M PBS (pH 7.4). This ligand mixture was added to the beads. Ammonium sulfate (3 M) was then added and the bead-antibody mixture was incubated for 12-18 hours at 37°C with tilt and rotation using a Hula mixer (Invitrogen; Grand Island, NY) at 12° tilt and 5 rpm rotation. After incubation, the bead-antibody mixture was placed on a magnet, allowed to pellet completely, and the supernatant was removed. A 1 ml-portion of PBS (pH 7.4) containing 0.5% BSA was added and the beads were again incubated at 37°C with tilt and rotation for 1 hour. After incubation, the bead-antibody mixture was placed on a magnet and allowed to pellet completely. The supernatant was removed and washed two

times with 1 ml portions of PBS (pH 7.4) containing 0.1% (w/v) BSA. Prior to use, the coated beads were blocked overnight at 4°C with 5% Blotto made from dehydrated milk and PBS.

M-270 Amine-coated Dynabeads (Life Technologies; Grand Island, NY), chosen because amine-groups on the surface of the beads bind with carboxyl-groups of the ligand, were vortexed for 1-2 minutes to re-suspend them into solution. Approximately $1-2 \times 10^9$ magnetic beads per ml were transferred to a 1.5 ml tube. The beads were placed on a magnet and allowed to pellet for 4 minutes. The supernatant was aspirated and the beads were washed 2 times in 0.1 M 2-(*N*-morpholino) ethanesulfonic acid (MES) (pH 4.5-5). The beads were then re-suspended in 0.1 M MES without dilution. Type III Porcine Gastric Mucins (PGM), (Sigma, St. Louis, MO) were reconstituted with 0.1 M MES to a concentration of 10 mg/ml by heating in a 37°C water bath for 10 minutes with intermittent vortexing. The reconstituted PGM was then added to the beads in a ratio of 6 µl of PGM solution to 94 µl of beads. 15 mg of N-hydroxysuccinimide (NHS) and 10 mg of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was dissolved in 1 mL of cold de-ionized water immediately prior to use. The EDC/NHS solution was added to the beads in the amount of 50 µl per mg of ligand used, and the mixture was vortexed. The beads were incubated at room temperature for 2 hours on a Hula mixer. Hydroxylamine (10 mM) was added to quench the reaction and allowed to incubate for an additional 15 minutes with tilt and rotation. The beads were then washed with PBS containing 0.5% BSA three times before blocking overnight at 4°C in 5% Blotto prior to use.

Protein G Dynabeads (Life Technologies; Grand Island, NY), chosen to rule out the possibility that mAB NV3901 was being presented to the virus incorrectly with the tosylated bead. The assay was repeated with Dynabead Protein-g beads which bind ligands via their Fc-region (or tail), rather than the Fab region of the antibody. The beads were vortexed for >30

seconds to resuspend them in the vial. Portions of 50 μ L were transferred to a 1.5 mL Eppendorf tube. The beads were placed on a magnet and allowed to pellet for 4 minutes. The supernatant was aspirated and the beads were removed from the magnet. NV3901 (2.87 μ g/ml) was prepared in 200 μ L of PBS with 0.02% Tween-20 (Thermo Scientific; Waltham, MA) which was applied to re-suspend the beads. The bead-antibody mixture was incubated at room temperature on a Hula mixer for 10 minutes. The beads were then returned to the magnet and allowed to pellet for 4 minutes. The supernatant was aspirated and the beads were removed from the magnet and re-suspended in 200 μ L of PBS with 0.02% Tween-20. The beads were pre-blocked and, therefore, ready for immediate use according to the manufacturer's instructions.

Norwalk virus GI.1 capture by functionalized magnetic beads for determining the assay lower detection limit.

Twenty- μ l aliquots of each functionalized and pre-blocked bead type were made in 1.5 ml tubes. The beads were first washed 3 times with PBS, followed by addition of 1 ml of one of the following four buffers: 50 mM Glycine containing 0.1 M Tris-HCl and 3% beef extract (used at pH 7.0 or pH 9.5), PBS containing 1 M NaCl and 0.05% Tween 20, and Citrate buffer at pH 3.6. By changing the charge on the virus to a positive charge, citrate buffer at pH 3.6 increased viral binding to the negatively charged carbohydrates of porcine gastric mucin-coated magnetic beads.⁹⁷ Glycine buffer at pH 7.0 with 3% beef extract was chosen because its high protein concentration aids in viral elution¹¹⁴ and has been shown to reduce non-specific binding to various food matrices.⁹⁹ An increase to 9.5 creates a very negatively charged buffer which leads to competitive binding, thus, forcing virus particles off of a food surface. 1M NaCl PBS disrupts electrostatic interactions between a food surface and the viruses which are negatively charged at

a neutral pH. A 0.05% Tween 20 (a polysorbate surfactant) was added to aid in the disruption of hydrophobic forces between the virus and a food surface.

Serial dilutions (10-fold) of native GI.1 stool suspension were made and 1 μ L of each was added to individual bead/buffer-containing tubes, before incubating at 37°C for 1 hour with tilt and rotation. To determine non-specific binding, each type of functionalized bead was left uncoated and treated identically to the coated beads in each buffer matrix. Negative controls consisted of only the bead/buffer mixture and were not inoculated with virus. After incubation, unbound viruses in the supernatant were removed and the samples were washed 3 times with PBS. The beads were re-suspended in 50 μ L of PBS and placed in an Applied Biosystems Gene Amp PCR System 9700 thermocycler (Life Technologies; Grand Island, NY) at 99°C for 5 minutes to heat-release the viral RNA. Immediately after the heat-release step, the samples were placed on ice to limit denaturation of the RNA and then spun in a mini-centrifuge (Fisher Scientific; Pittsburgh, PA) for 3 minutes. The supernatant was removed and placed into a 1.5 mL tube and stored at -80°C until future testing.

Preparation of lysis buffer

Polyadenylic acid (25 mg) (Life Technologies; Grand Island, NY) was dissolved into 12.5 mL of nuclease free water. A 0.5X Tris-EDTA was made by adding 50 mL of 1X TE (Life Technologies; Grand Island, NY) to 50 mL nuclease free water. 120 g of Guanidine Thiocyanate (GuSCN) (Roche; Indianapolis, IN) was dissolved completely into 100 mL of 0.5X TE by heating the solution in a 56°C in a water bath. To this solution, 11 mL of 5 M NaCl (Life Technologies; Grand Island, NY) and 11 mL of 3 M NaOAc (Life Technologies; Grand Island, NY) was added. A polyadenylic acid solution was made by dissolving 25 mg Polyadenylic acid

in 12.5 mL of nuclease free water and 2.2 mL was also added to the lysis buffer. The lysis buffer requires protection from light and must be used before the 6 month expiration date.

Manual RNA extraction using a spin column

Samples were combined with equal portions of lysis buffer, vortexed for 5 seconds, and allowed to incubate at room temperature for 10 minutes. Molecular grade ethanol (100%) was then added with a ratio of two times the sample volume. Samples were vortexed for 5 seconds and transferred to an E.Z.N.A. Hibind RNA spin column (Omega Bio-tek; Norcross, GA), taking care not to touch the membrane located within the column. The spin columns were then centrifuged for 1 minute at 13,000 rpm in a bench top microcentrifuge and the filtrate was discarded. To each column, 500 μ L of 75% molecular grade ethanol was then added to wash the spin columns. After centrifugation and discarding the filtrate, a dry spin was conducted to remove any residual ethanol on the columns. The spin columns were placed in new 1.5 mL tubes and 50 μ L of RNase-free H₂O was added. The columns were centrifuged again at 13,000 rpm for 1 minute to elute the RNA from the columns. RNA was stored at -80°C until future use.

Concentration of Norwalk virus by mucins using alternatives to magnetic bead methods.

Muco-concentration by polyethylene glycol (PEG) Precipitation:

Porcine gastric mucins were hydrated overnight in milli-Q H₂O to a concentration of either 20 mg/mL (2% w/v) or 200 mg/mL (20% w/v). In a 1.5 mL tube, 100 μ L of PGM, heat-treated saliva or water (no mucin controls) was pipetted into 900 μ L of glycine buffer (pH 7.0). Approximately 10,000 genome copies of virus derived from a native GI.1 stool suspension was seeded into triplicate samples of each mucus suspension or control. Each sample was then mixed with 10% PEG 10,000 MW (Sigma Aldrich; St. Louis, MO) containing 0.3 mM NaCl (Sigma

Aldrich; St. Louis, MO) and incubated at room temperature for 4 hours on a hula mixer to dissolve the PEG and NaCl. Following incubation, the PEG was precipitated by centrifugation at 9,000 x g for 30 minutes. A 100 µL portion of the supernatant was retained for RNA extraction before discarding the remaining portion. The pellet was then re-suspended in 1 mL of deionized H₂O. A 100 µL portion of the re-suspended pellet was retained for RNA extraction.

Muco-concentration by particle membrane filtration:

Hydrated PGMs (2-20%), heat-treated saliva or water (no-mucus controls) were added to glycine buffer (pH 7) at ratios of 1:10. Approximately 10,000 genome copies of virus derived from a native GI.1 stool suspension was seeded into triplicate samples of each mucus suspension or control sample. Samples were incubated for 45 minutes using a hula mixer at room temperature to capture viruses in the network of mucins in the PGM and saliva samples. After incubation, the samples were very slowly (passage of 1 mL over 15 minutes) syringe-filtered using a 0.2 µm filter (VWR International North America, Radnor, PA). A 100 µL portion of the filtrate was retained for RNA extraction. Then, 500 µL of lysis buffer was applied to the filter and pushed through the filter slowly over the course of 10 minutes to extract viral RNA collecting on the membrane. An equal portion of 100% ethanol was applied to the syringe filter and collected with the expelled lysis buffer. A 100 µL portion of this sample (herein referred to as the “residue”) was retained for RNA extraction. In some experiments, 10% PEG 10,000 with 0.3 M NaCl was also included during virus-binding experiments using 20% PGM to see if the PEG could enhance virus retention on the membrane filters.

Determination of PBS inhibition during heat-release

Previous items in the literature suggest a possibility that PBS may inhibit RT-qPCR by binding Mg²⁺. For each of the three buffers tested (0.1 M PBS (pH 7.4) (Ambion; Grand Island,

NY), 50 mM Tris (pH 7.6) (Invitrogen; Grand Island, NY), and nuclease-free H₂O (Thermo Scientific; Logan, UT), 1 µL of GI.1 stool supernatant (2×10^7 genome copies/ml) was added to 49 µL of buffer in 1.5 mL tubes. RNA was heat-released at 99°C for 5 min. 10-fold serial dilutions were made and each was compared to a GI.1 standard curve in triplicate. A one log difference (indicated by a 3.3 change in CT value) indicated presence of inhibition, whereas differences in Ct values less than 3.3 were considered non-inhibitory.

Determination of PGM inhibition

To determine whether or not 20% or 2% (w/v) PGM caused inhibition of RT-PCR, RNA-extracts from these samples and samples from experiments containing 0% PGM (glycine buffer only) were examined. A GII NoV-specific monoplex RT-qPCR assay was set up except using 1 µL less H₂O per reaction in the master mix. Portions of 1 µL of each sample (20%, 2% or 0% PGM) were placed in each of duplicate wells. In addition, 1 µL of GII.4 RNA transcript obtained from the Gastroenteritis and Respiratory Virus Laboratory Branch at the CDC was also added to each well. Three positive controls (containing 1 µL of GII RNA transcript, and 1 µL of RNase-free water) and 1 negative control (water only) were included on each plate. Real-time RT-qPCR was performed using GII-specific primers and probes (Cog 2F and 2R, and Ring 2 (Cy5)). The CT values of PGM-containing samples and Glycine buffer only samples were compared to an average of the CT values obtained from the 3 GII-positive control samples to determine whether or not inhibition was present in the samples. A one log difference (indicated by a 3.3 change in CT value) indicated presence of inhibitory substances in the sample, whereas differences in Ct values less than 3.3 were considered non-inhibitory.

CHAPTER 4

RESULTS

Determination of the viral concentration in the inoculum

The concentration of HuNoV GI.1 in the stool suspension used as the inoculum for all experiments performed herein was determined by five different methods. First, real-time RT-PCR was performed on 10-fold serial dilutions of RNA derived from the stool suspension (Figure 1). The concentration of virus in the stock was estimated by end-point dilution to be $\sim 5 \times 10^8$ RT-PCRU / ml. Next, the RNA was quantified by real-time RT-qPCR using an in vitro-derived RNA transcript of either HuNoV GI.3 or GI.1 as a standard curve. By these methods, the virus stock concentration was determined to be 1.7×10^9 genome copies/ml using the GI.3 transcript or 6.2×10^8 genome copies/ml using the GI.1 transcript (Figure 2). Then, the virus stock concentration was estimated again by end-point titration, except this time by using a conventional RT-PCR assay and gel electrophoresis (Figure 3). Calculating back, the virus stock concentration was estimated to be $\sim 2 \times 10^8$ PCRU / ml. Interestingly, both of the end-point titration methods and the real-time RT-qPCR assay that used the GI.1 transcript as a standard were very similar. The concentration estimated based on the GI.3 standard curve was 0.4 logs greater than the concentration obtained using the GI.1 standard. This is most likely due to the preference of the assay primers for the GI.3 template over the GI.1 template. Lastly, the virus stock concentration was determined by assaying and averaging the virus stock with each experimental replicate. The mean of the quantity in the inoculum was determined to be 2×10^5

genome copies per μl (or 2×10^8 genome copies per ml). Since this value was also very similar to the values obtained by $\frac{3}{4}$ of the other methods, this value was considered the most accurate.

Recovery of GI.1 virus from common buffers using functionalized magnetic beads

The ability of porcine gastric mucin (PGM)- and monoclonal antibody (mAB)-coated magnetic beads to capture and recover HuNoV GI.1 from a variety of elution buffers that are commonly used in food virology was investigated in order to determine whether or not the ligand-coated magnetic beads could be used in combination with a food virology elution procedure. The assay limit of detection (LOD) was thus determined for each functionalized bead type in the context of each elution buffer. Also, ligand-coated beads were compared to uncoated beads to assure that the ligands were responsible for virus recovery and the virus was not binding non-specifically to the uncoated beads alone. Log genome copies of GI.1 virus recovered per ml from glycine buffer (pH 7.0) using PGM- and the NV3901 mAB-coated magnetic beads (Figure 4), ranged from 7.4 - 8.2 and 5.5 - 6.2, respectively, when a one μl undiluted inoculum (0 dilution) was used. When the inoculum was first diluted 10-fold (10x dilution), log genome copies of GI.1 virus ranged from 6.3 - 8.6 for PGM-coated beads and 0.0 - 6.4 log genome copies for mAB 3901-coated beads. Nearing the assay LOD when the virus stock was first diluted 100-fold (100x dilution), log genome copies of GI.1 recovered ranged from 0.0 - 8.0 and 0.0 - 0.0 for PGM- and 3901-coated magnetic beads, respectively. When uncoated amine beads (used for functionalizing beads with PGM) and tosyl-activated beads (used for functionalizing beads with mAB) were used, recovery of the undiluted GI.1 inoculum ranged from 5.1 - 5.8 and 0.0 - 6.1 log genome copies, respectively. However, when the uncoated beads were used to recover 10x or 100x diluted virus, no virus was detected.

Less GI.1 norovirus was recovered from a basic pH glycine buffer (pH 9.5) by PGM-coated and NV3901-coated beads (Figure 5), at all dilutions of the inoculum tested [0 or 10x dilutions (100x was not tested due to poor recovery when tested in Glycine 7.0)]. PGM-coated beads outperformed NV3901-coated beads, with recoveries ranging from 5.6 - 7.2 and 0.0 - 5.8 log genome copies / ml recovered when undiluted and 10x diluted inoculums were used. For NV3901-coated beads, recovery of the undiluted inoculum ranged from 0.0 - 5.2 log genome copies / ml but no virus could be detected when the 10x diluted inoculum was used. Only uncoated amine beads produced a virus recovery range of 4.1 - 6.7 log genome copies / ml when the undiluted stool suspension was used; all other dilutions yielded no virus recovery, similar to uncoated tosyl-activated at all inoculum concentrations tested.

In citrate buffer (pH 3.6) (Figure 6), PGM-coated beads provided a recovery range for GI.1 virus of 5.3 - 7.4 log genome copies / ml for the undiluted inoculum and 0.0 - 6.4 log genome copies / ml for the 10x diluted inoculum, while virus recovery using NV3901-coated beads ranged from 0.0 - 6.1 log genome copies / ml for the undiluted and 0.0 log genome copies / ml for the 10x diluted inoculum. Uncoated amine beads did not recover virus at any dilution. Uncoated tosyl beads recovered virus only when the undiluted inoculum was used (range 4.7 - 6.4 genome copies / ml), but the average number of viruses detected using the uncoated tosyl beads was greater than the average recovered using the NV3901-coated beads.

Recovery of GI.1 virus from 1 M NaCl buffer (Figure 7) by PGM-coated beads was 7.0 for all replicates using an undiluted inoculum and 7.2 - 7.4 log genome copies / ml for the 10x diluted inoculum. GI.1 virus recovery using NV3901-coated beads ranged from 0.0 - 5.5 log genome copies / ml for the undiluted inoculum, but was not detected when the 10x inoculum was used. No non-specific binding was detected with using uncoated amine beads at the undiluted

and 10x diluted inoculum levels, but the uncoated tosyl- beads recovered 7.2 - 7.4 log genome copies / ml from the undiluted inoculum and 7.0 - 7.4 log genome copies / ml from the 10x diluted inoculum.

The tosyl activated beads bind in the Fab region of the antibody and the protein G beads bind the Fc or “tail” region. Binding of the Fab region may cause steric hindrance that prohibits viral binding to the surface. To rule out the possibility that the NV3901 mAB was either not being presented in the correct orientation on the magnetic beads, or it was damaged in the process of conjugating it on the tosyl-activated beads; the assay was repeated using protein G coated magnetic beads. Unlike the coating process for tosyl-activated beads, mild buffering conditions were used to coat mAB in the correct orientation on the surface of protein G beads. PBS was used as the suspension buffer in these assays. Log genome copies of GI.1 virus / ml recovered by NV3901-coated protein G beads ranged from 4.6 - 6.3 for undiluted, and 0.0 - 6.6 for 10x diluted virus inoculum (Figure 8). There was no recovery of virus when the 100x dilution was used as the inoculum. Uncoated protein G beads provided high levels of non-specific virus binding, resulting in respective ranges of log genome copies of 4.7 - 6.8, 0.0 - 6.9, and 0.0 - 5.2 for undiluted, 10x diluted and 100x diluted virus inoculums, respectively. For comparison, NV3901 coated tosyl beads were also used to recover GI.1 virus from PBS. Log genome copy numbers recovered using these beads ranged from 0.0 - 6.6 and 5.7 - 7.5 for undiluted and 10x diluted virus stock, respectively, but no virus was recovered from NV-3901 coated tosyl beads where the 100x diluted inoculum was used. Uncoated tosyl beads again provided high levels of non-specific binding with log genomic copies of GI.1 recovery ranging from 6.3 - 8.1 for undiluted, 0.0 - 7.7 for 10x diluted, and 0.0 - 5.1 for the 100x diluted inoculum.

Inhibition of RT-qPCR by PBS

Concern over the inhibition of RT-qPCR due to the presence of PBS during heat-release of viral RNA was the motivation for testing for RT-qPCR inhibition when viral RNA heat-release was conducted in the presence of PBS, Tris buffer, or RNase-free water. Comparison of the quantified values of each of the buffer types to a standard curve demonstrated less than 1 log difference (< 3.3 CT) (PBS: 32.3, Tris: 31.5, and H₂O: 33.3), indicating similar performance in each buffer; however, Tris buffer provided the lowest average CT value, so future studies may use this buffer during the heat-release step instead of PBS.

Recovery of GI.1 Norovirus using Novel PGM- and Saliva- based Concentration Methods

Filtration- and precipitation- based methods are relatively inexpensive and commonly used in food virology to concentrate viruses from larger sample volumes. A set of experiments was conducted to test the hypothesis that specific norovirus-binding ligands found in PGM and saliva from a “secretor” individual can improve the capture and recovery of human norovirus if used in conjunction with other commonly used concentration methodologies. Ligand-containing samples were compared to controls which contained water in place of ligand and were termed glycine buffer only (GBO) controls. Recovery data are thus presented as the ratio (percentage) of viruses detected in samples containing ligands (from PGM or saliva) to GBO control samples (Table 2); therefore, only ratios greater 100% indicate an improvement due to the addition of the ligand.

Precipitation of GI.1 norovirus using ligand-containing xanthan gum gels

Xanthan gums (2%) containing 2% PGM or saliva were used to capture GI.1 norovirus by precipitation (Figure 9). The ratio (percentage) of viruses recovered with the addition of

ligand as compared to the no ligand controls ranged from 15% - 113% for the PGM-containing gel plugs and from 42 - 59% for saliva-containing gel plugs. All three experimental replicates resulted in recovery ratios near or less than 100%, indicating virus precipitation by xanthan gum-containing plugs was not improved by the addition of PGM or saliva.

Precipitation of GI.1 norovirus using polyethylene glycol and ligands from PGM or saliva

Polyethylene glycol (MW 10,000) 10% (w/v) precipitation was performed in the presence of 2% PGM or saliva and without the addition of ligand (control samples) (Figure 10). For samples containing PGM, the ratio of recovery due to the ligand ranged from 9 - 339%. Similarly, samples containing 2% saliva and precipitated by PEG resulted in a range of recovery ratios from 35 - 364%. Results were highly variable for this set of experiments; in replicate three, the addition of ligands increased the amount of virus that could be recovered by PEG precipitation. However, in replicate two, the opposite was observed; the addition of PGM and saliva appeared to decrease the amount of virus recovered by PEG precipitation.

Filtration of GI.1 norovirus using ligands from PGM or saliva

When used in conjunction with a 0.2 μm filter, 2% PGM provided a 67-183% recovery and 2% saliva yielded results that were more varied, ranging from 3-146% (Figure 11). PGM provided higher recovery than saliva in 2 of 3 replicates tested and the results were more consistent and reproducible.

Impact of increasing PGM concentration and combining recovery methods

To determine if an increase in PGM concentration was beneficial to viral recovery, the PGM concentration was increased from 2%-20%. The filter assay was retested with this higher concentration of PGM. In addition to increasing the concentration alone, the higher PGM

concentration was combined with 10% PEG 10,000 to determine if a combination of these two methods would promote recovery. Performing these assays in the presence of increased PGM concentration, yielded negative results for each of three replicates of each assay type (20% PGM with 10% PEG 10000 as well as 20% PGM with the 0.2 μm filter). Presence of RT-PCR inhibitors in the samples was investigated as a cause of the negative results. However, with the exception of one sample containing both 2% PGM and 10% PEG, RT-PCR inhibition was not a concern.

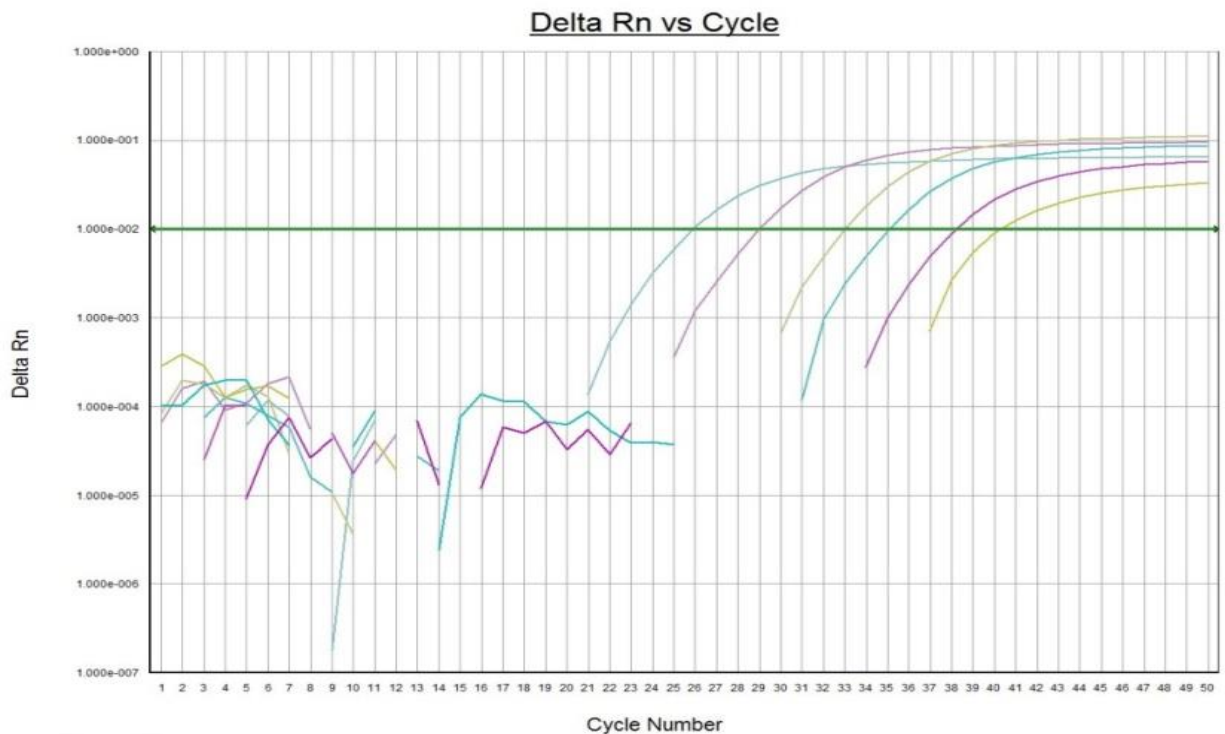


Figure 1: End-point titration and real-time RT-PCR amplification of RNA from the GI.1 positive stool suspension used in the experiments described herein. Ct (cycle threshold) values where the change in fluorescence (Delta Rn) crosses a threshold (separating signal from background noise) for the 10-fold serial diluted (10^{-1} to 10^{-7} from left to right) RNA is depicted.

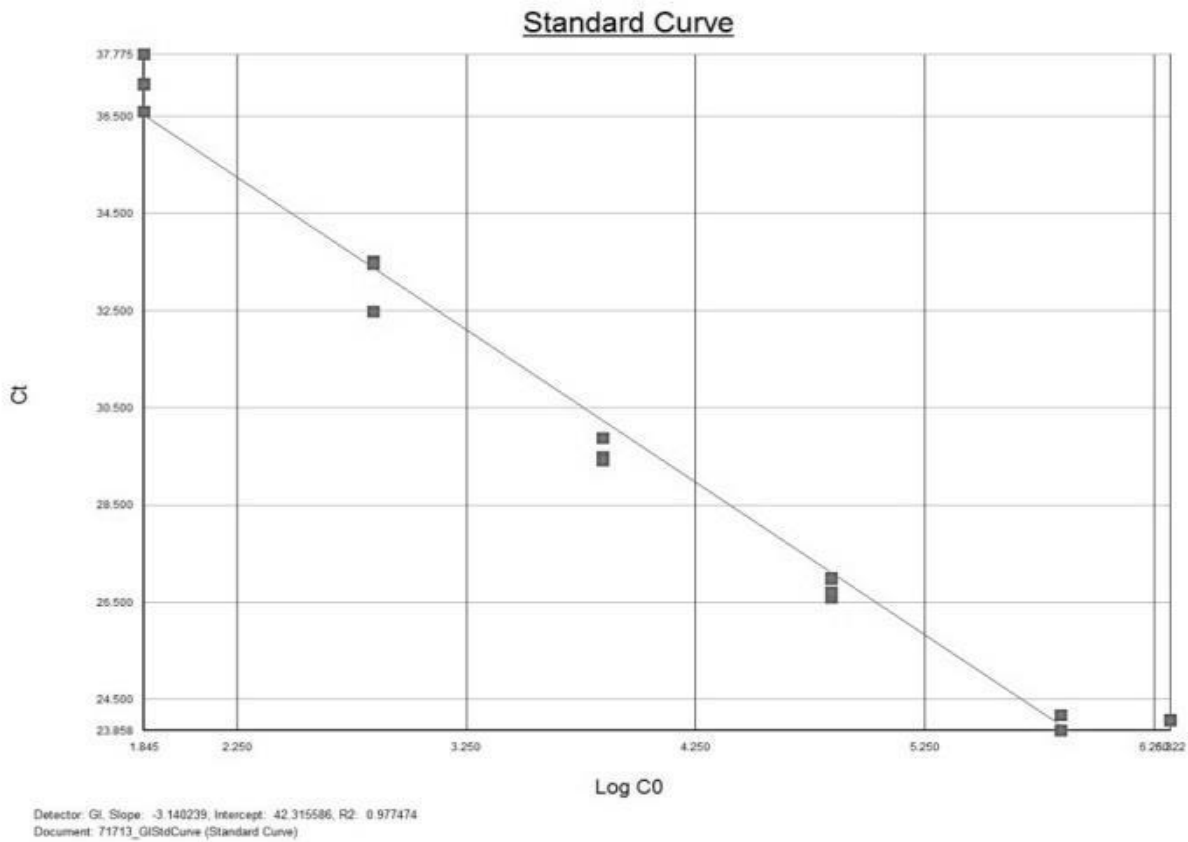


Figure 2: A standard curve generated by 10-fold serial dilution and real-time RT-PCR amplification of an in vitro-derived HuNoV GI.1 RNA transcript. Log concentrations of transcript, determined using a Nanodrop, were entered into the ABI software package and Ct (cycle threshold) values were determined after amplification. The equation for the line and R^2 value generated using the ABI software were $y = -3.1402x + 42.3156$ and 0.9774, respectively.



Figure 3: Gel image of endpoint titration performed on a 10-fold dilution series of RNA derived from the GI.1 stool suspension. Image shows three replicate experiments and well labels (from left to right) are 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , and 10^{-7} dilutions, as well as a negative control comprised only of master mix.

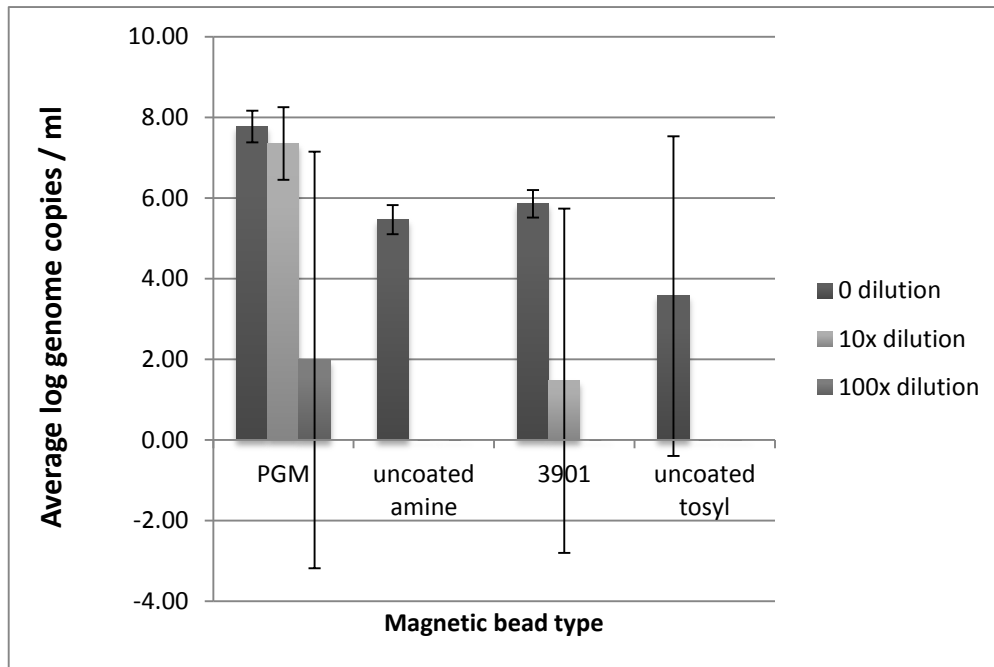


Figure 4: Capture of Norwalk virus (GI.1) using Amine- and Tosyl- conjugated superparamagnetic beads coated with PGM or NV3901, respectively, in a glycine buffer (7.0). Uncoated beads were used to determine non-specific binding. All values indicate log genome copies per ml. Legend indicates the dilution of the inoculum used in the assay. Error bars represent the standard deviation of the mean. The assay limit of detection was 2 log genomic copies/ mL for the magnetic bead experiment.

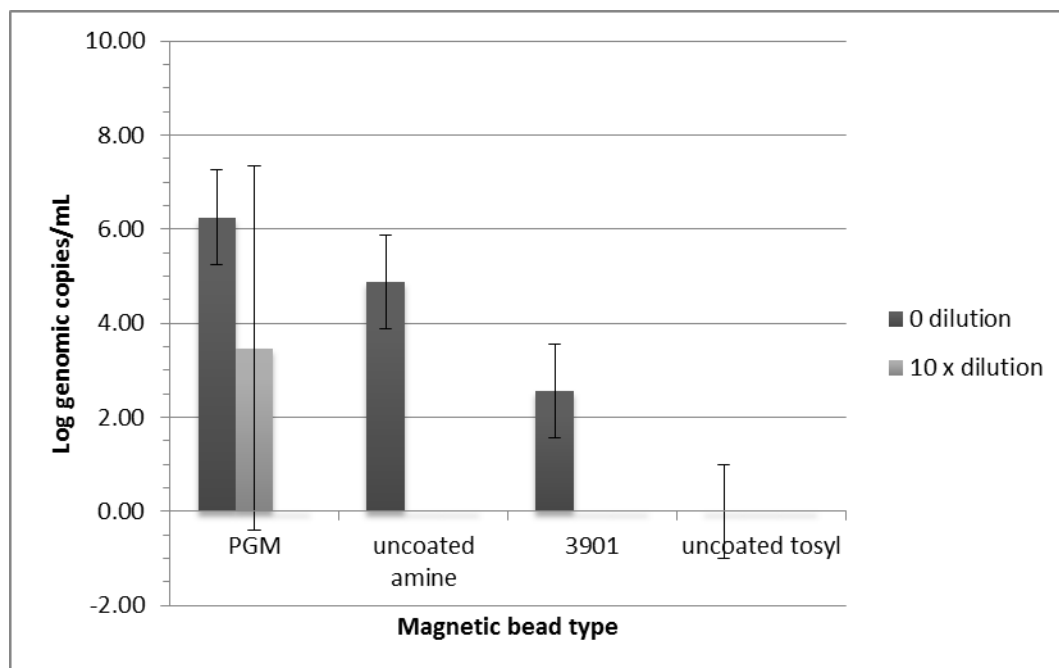


Figure 5: Capture of Norwalk virus (GI.1) from a basic a glycine buffer (pH 9.5) using amine- and tosyl- conjugated superparamagnetic beads coated with PGM or NV3901, respectively. Uncoated beads were used to determine non-specific binding. All values indicate log genome copies per ml recovered. Legend indicates the dilution of the virus inoculum used in the assay. Error bars represent the standard deviation of the mean. The assay limit of detection was 2 log genomic copies/ mL for the magnetic bead experiment.

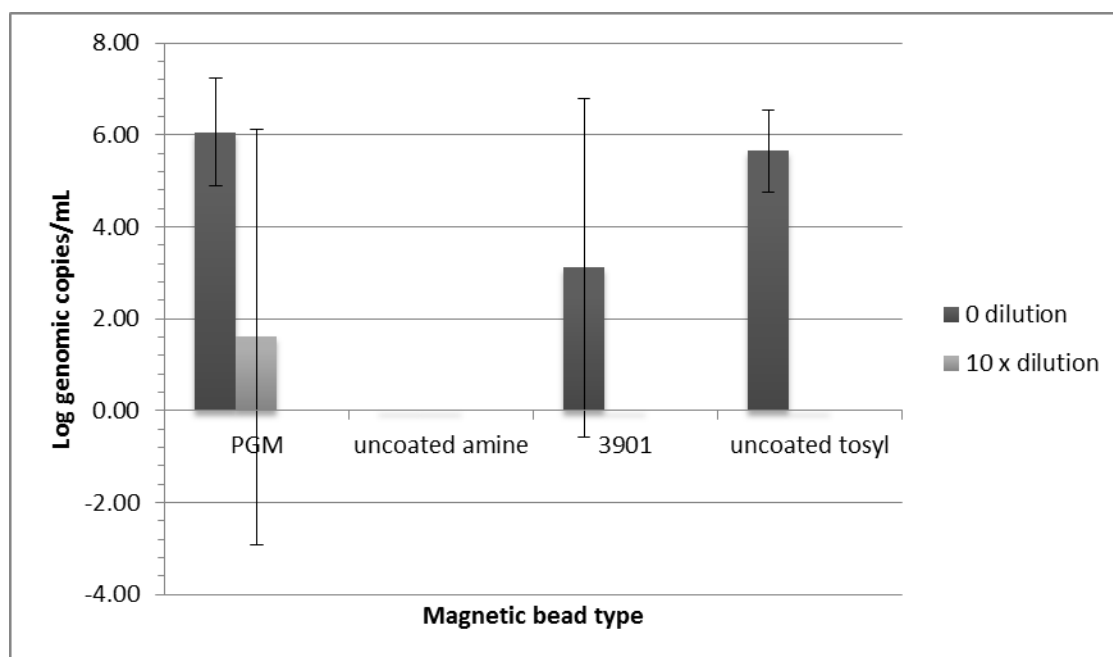


Figure 6: Capture of Norwalk virus (GI.1) from citrate buffer (pH 3.6) using amine- and tosyl-conjugated superparamagnetic beads coated with PGM or NV3901, respectively. Uncoated beads were used to determine non-specific binding. All values indicate log genome copies per ml recovered. Legend indicates the dilution of the inoculum used in the assay. The assay limit of detection was 2 log genomic copies/ mL for the magnetic bead experiment.

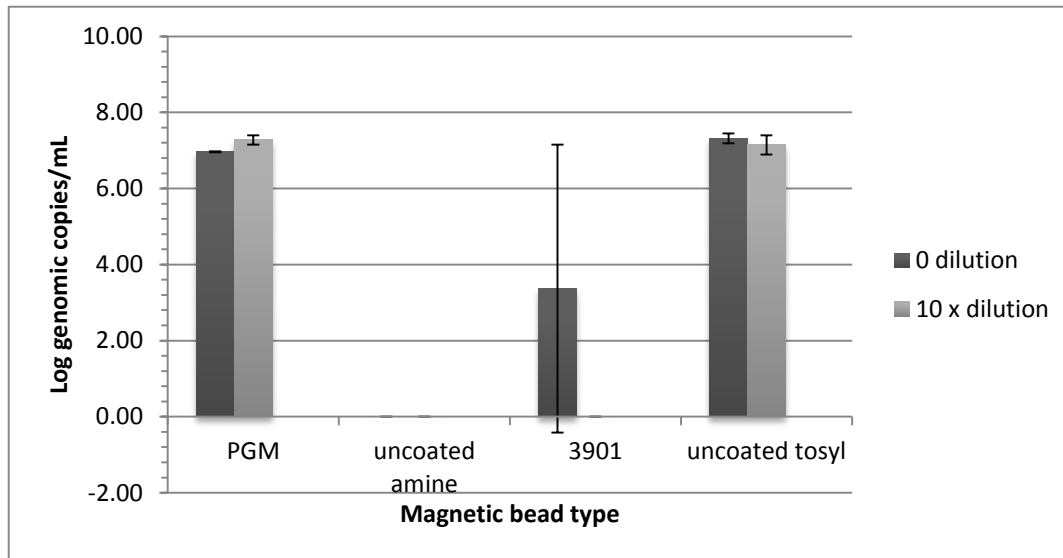


Figure 7: Capture of Norwalk virus (GI.1) from a 1 M NaCl buffer using amine- and tosyl-conjugated superparamagnetic beads coated with PGM or NV3901, respectively. Uncoated beads were used to determine non-specific binding. All values indicate log genome copies per ml recovered. Legend indicates the dilution of the inoculum used in the assay. The assay limit of detection was 2 log genomic copies/ mL for the magnetic bead experiment.

Table 1: Summary of GI.1 norovirus recovery from common buffers used in food virology using amine- and tosyl-conjugated bead coated with PGM or NV3901. Results are expressed as mean genome copies recovered, percent recovery, and a ratio of samples producing positive results over number the total number of samples tested for each experimental treatment.

Ligand-bead type	Buffer	Amount seeded (genome copies)	Mean recovery with ligand (genome copies)	% Recovery (95% CI) with ligand	Number positive /Number exp with ligand	Mean recovery non-specific (genome copies)	% Recovery (95% CI) non-specific	Number positive /Number exp non-specific
PGM/Amine	Glycine 7.0	200,000	15,420	38.6(0.72-76.4)	3/3	<100	1.8(0.4-3.2)	3/3
	Glycine 7.0	20,000	1092	272.9(-60.5-606.3)	3/3	<100	0	0/3
	Glycine 7.0	2,000	< 100	1479.3(-1420.1-4378.6)	1/3	<100	0	0/3
	Glycine 9.5	200,000	1038	2.6(-1.8-7.0)	3/3	348	8.7(-8.3-25.6)	1/3
	Glycine 9.5	20,000	< 100	1.7(-0.1-3.6)	2/3	<100	0	0/3
	Citrate	200,000	1709	4.3(-3.8-12.4)	3/3	<100	0	0/3
	Citrate	20,000	109	10.6(-10.2-31.5)	1/3	<100	0	0/3
	1 M NaCl	200,000	1833	4.6(4.4-4.7)	3/3	<100	0	0/3
	1 M NaCl	20,000	388	97.0(64.7-129.3)	3/3	<100	0	0/3
NV3901/tosyl	Glycine 7.0	200,000	161	.4(0.04-0.8)	3/3	150	2.9(-1.2-7.0)	2/3
	Glycine 7.0	20,000	<100	4.1(-4.0-12.2)	1/3	<100	0	0/3
	Glycine 7.0	2,000	< 100	0	0/3	<100	0	0/3
	Glycine 9.5	200,000	< 100	0.24(-0.02-0.07)	2/3	<100	0	0/3
	Glycine 9.5	20,000	< 100	0	0/3	<100	0	0/3
	Citrate	200,000	117	0.21(-0.2-0.6)	2/3	230	5.7(-2.0-13.5)	3/3
	Citrate	20,000	< 100	0	0/3	<100	0	0/3
	1 M NaCl	200,000	< 100	0.12(0.002-.024)	2/3	4272	106.8(68.9-144.7)	3/3
	1 M NaCl	20,000	< 100	0	0/3	316	790.9(217.8-1364.0)	3/3

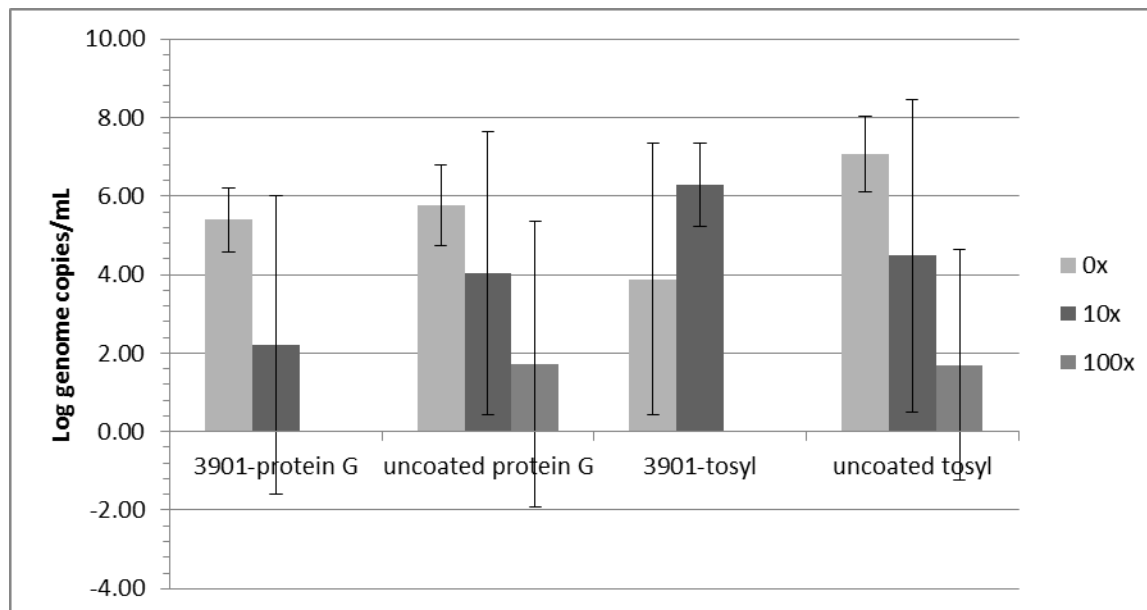


Figure 8: Capture of Norwalk virus (GI.1) from PBS using protein G- and tosyl- conjugated superparamagnetic beads coated with NV3901 mAB. Uncoated beads were used to determine non-specific binding. All values indicate log genome copies per ml recovered. Legend indicates the dilution of the inoculum used in the assay. Error bars represent the standard deviation of the mean. The assay limit of detection was 2 log genomic copies/ mL for the magnetic bead experiment.

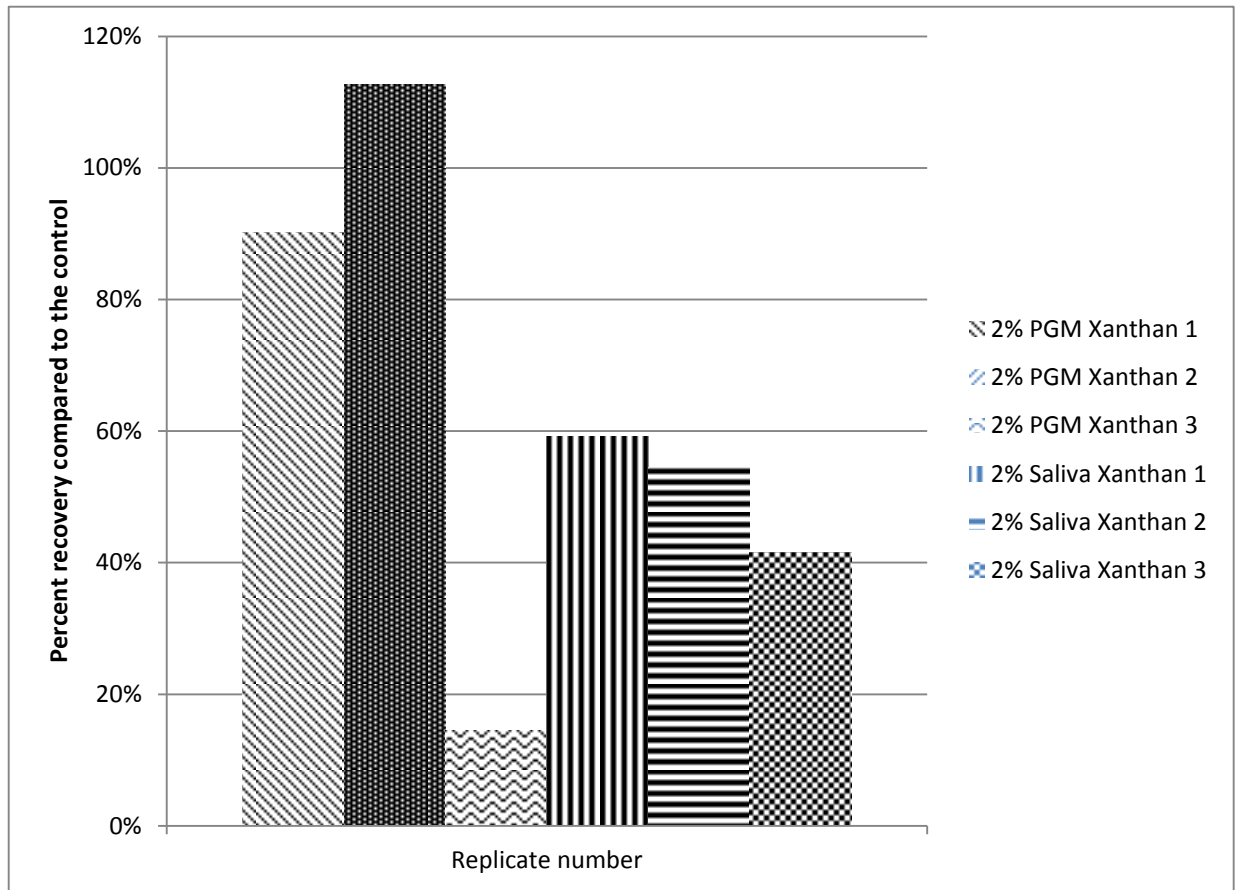


Figure 9: Capture of Norwalk virus (GI.1) in glycine buffer (pH 7.0) using a mucus plug synthesized from xanthan gum containing 2% PGM or saliva. Percent recoveries indicate capture by the ligand compared controls that do not include PGM or saliva. A value over 100% indicates an improvement of the capture and recovery due to the addition of the ligand. Legend indicates the replicate of the assay and the method used.

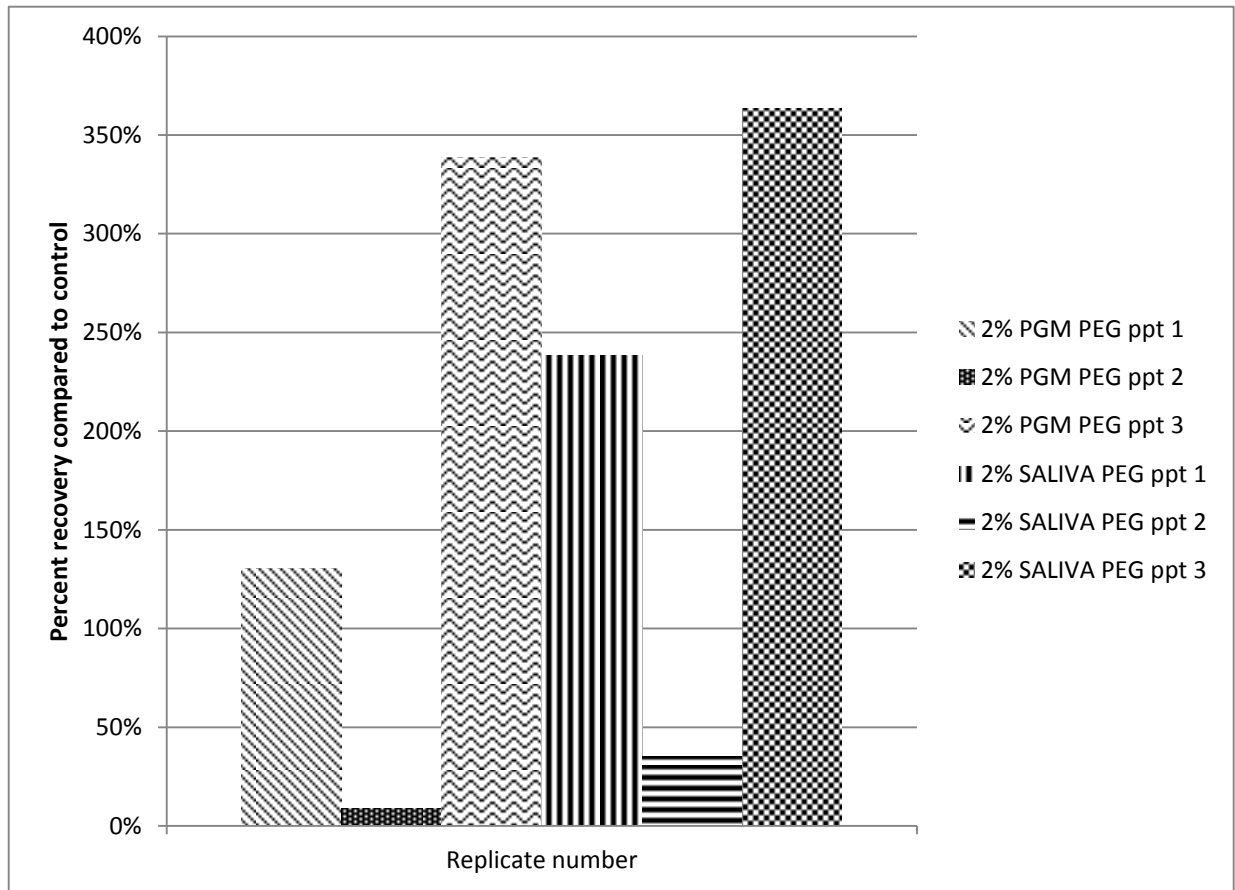


Figure 10: Capture of Norwalk virus (GI.1) using 2% PGM or saliva to enhance polyethylene glycol precipitation. Percent recoveries indicate capture by the ligand compared controls that do not include PGM or saliva. A value over 100% indicates an improvement of the capture and recovery due to the addition of the ligand. Legend indicates the replicate of the assay and the method used.

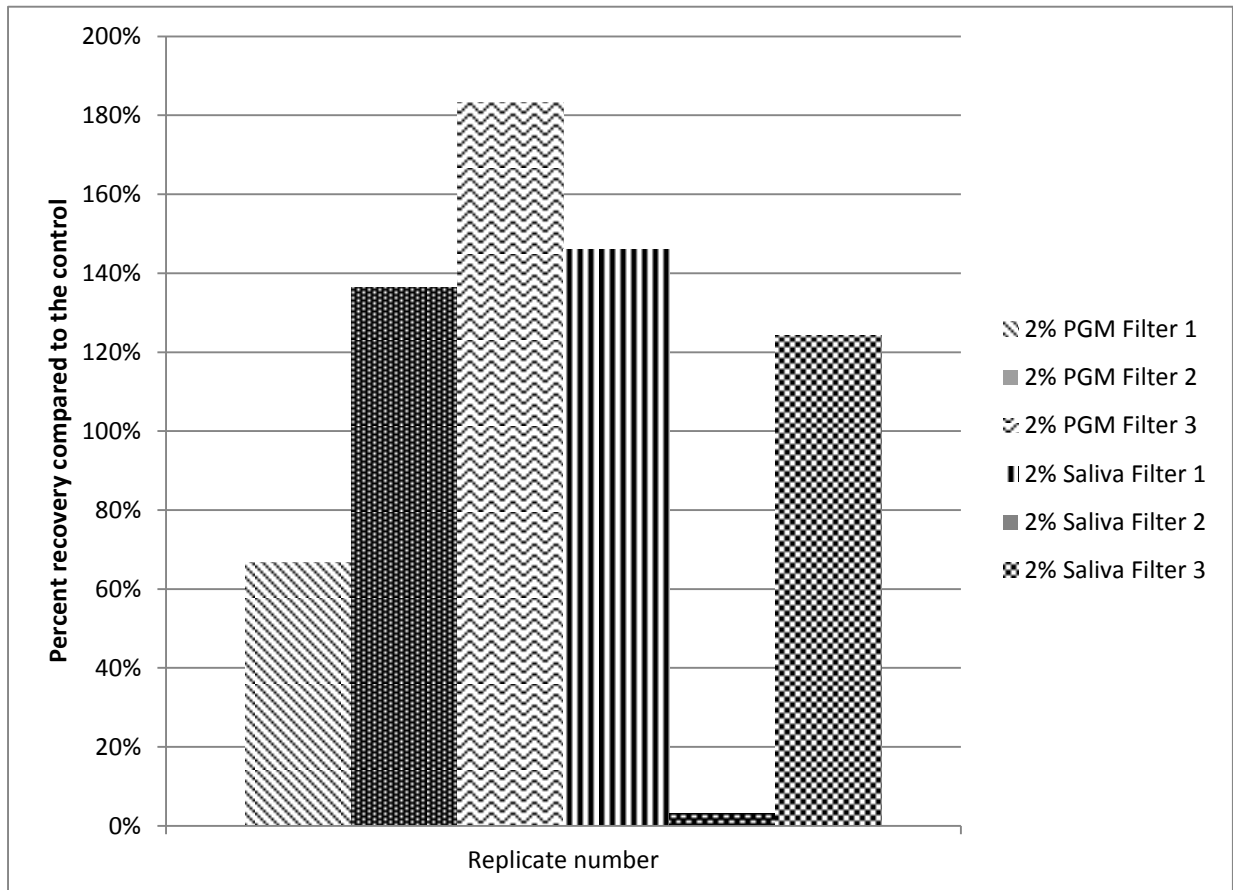


Figure 11: Capture of Norwalk virus (GI.1) using 2% PGM or saliva passed through a 0.2 μm syringe filter. Percent recoveries indicate capture by the ligand compared controls that do not include PGM or saliva. A value over 100% indicates an improvement of the capture and recovery due to the addition of the ligand. Legend indicates the replicate of the assay and the method used.

Table 2: Summary of findings from muco-adhesion, PEG ppt. and filtration studies using PGM or saliva to capture Norwalk virus (GI.1). Results are expressed as method percent recovery, method recovery due to ligand when compared to the control and the ratio of positive results over total number of experiments. Method percent recoveries are the ratio of virus recovered when compared to the inoculum amount. The impact of ligand on percent recovery is calculated by the amount of virus captured by the ligand compared to control samples that did not include PGM or saliva. Therefore, a value over 100% indicates an improvement of the capture and recovery due to the addition of the ligand.

Ligand type	% Mucus	Buffer	Additional treatment	Amount seeded (genome copies)	Mean % Recovery (95% CI) with ligand compared to the control	Number positive /Number exp with ligand
PGM	2%	glycine 7.0	2% Xanthan	200000	72(14-131)	3/3
Saliva	2%	glycine 7.0	2% Xanthan	200000	52(41-62)	3/3
PGM	2%	glycine 7.0	10% PEG 10000	200000	159(-29-348)	3/3
Saliva	2%	glycine 7.0	10% PEG 10000	200000	212(25-400)	3/3
PGM	2%	glycine 7.0	filtration	200000	129(63-195)	3/3
Saliva	2%	glycine 7.0	filtration	200000	91(4-178)	3/3
PGM	20%	none	10% PEG 10000	200000	0	0/3
PGM	20%	none	10% PEG 10000 and filtration	200000	0	0/3

CHAPTER 5

DISCUSSION

Quantification of the viral inoculum was determined using five separate methods and results were compared. Four of five methods yielded similar results, but the use of the GI.3b standard resulted in a titer that was 0.4 to 1 log greater in value than the titers estimated by other methods or calculated by using the GI.1 standard. The value of 2×10^8 genome copies per ml, generated by averaging the virus stock calculations determined with each experiment and after end-point titration of the virus stock, was adopted for the stock concentration, as it was considered to be the most accurate estimate.

In determining the limit of detection (LOD) of viral recovery using ligand-coated functionalized magnetic beads, it was observed that both an increase and decrease of pH decreased binding of the virus to the PGM- or mAB-coated functionalized magnetic beads. The lack of improvement by a decrease in pH is in direct contrast to a previous study that showed an increase in viral binding to PGM-conjugated magnetic beads when using citrate buffer (pH 3.0-6.0) when compared to neutral pH.⁹⁷ The isoelectric point (pI) of GI.1 Norwalk virus is 5.5-6.5¹¹⁵ and the pI of PGM is 2-3¹¹⁶, meaning that in a buffer matrix at a pH of 3.6, the virus would become positively charged and the PGM would remain negatively charged, thus causing an attraction of oppositely charged particles which may increase virus recovery.¹¹⁷ There is also evidence that citrate acts as a fucose molecular mimic by binding to the receptor-binding pocket of the norovirus capsid.¹¹⁸ If this is the case, citrate would serve to effectively block viral binding to the HBGA-containing PGM-coated magnetic beads. Although Tian et al (2010) also

used citrate buffer when conducting the low pH buffer experiments described above, the authors also used carboxyl-derivatized beads instead of the amine-conjugated beads that were used in the experiments described herein. Therefore, it is possible that the blocking aspects of citrate buffer were overwhelmed by an increase in non-specific binding to the carboxyl groups on the beads. It is also worth noting that although ELISA results indicated a clear increase in GI.1 VLP binding to PGM-coated beads in the low pH buffer, the increase in GI.1 virus binding to these beads was minimal (approximately a 1 CT value difference).⁹⁷ Differences in the behavior of GI.1 virus and its VLPs binding to PGM are therefore likely.

PGM-coated amine beads and NV3901-coated tosylactivated beads in the context of glycine 7.0 demonstrated the highest levels of viral recovery and lowest detection limit, but also provided high levels of non-specific binding on both bead types. PGM-coated amine beads in the context of 1 M NaCl PBS, on the other hand, consistently demonstrated a low detection limit with little to no non-specific binding. This indicates that future assays should be performed in a neutral pH buffer and depending on the objectives of the recovery procedure, either glycine buffer (pH 7) or the 1 M NaCl PBS buffer can be used.

Non-specific binding was problematic in many cases. This type of non-specific adsorption of the virus to the functional groups (tosyl, amine or protein G) of the beads, varied depending on the buffer used. Tosyl-activated beads provided more non-specific binding than did amine-linked beads in all buffer types tested. The highest level of non-specific binding to tosyl-activated beads was found when using 1 M NaCl in PBS in both the 0 and 10x dilutions of the inoculum. Because 1 M NaCl typically disrupts the electrostatic virus-surface interaction, this is not what was expected. Amine-conjugated beads provided non-specific virus binding in the undiluted inoculum in the context of glycine buffer at both pH 7.0 and pH 9.5. This is also

not what was expected, as typically, the high protein content of glycine buffer with 3% beef extract increases competitive binding and, therefore, viral elution off of surfaces.

Overall, PGM- coated beads out-performed NV3901- coated beads for recovering GI.1 norovirus in all buffers tested. PGM-coated beads demonstrated consistent recovery of both 0 and 10x diluted inoculum in all buffer matrices and demonstrated recovery of 100x dilution in glycine (7.0). Although NV3901-coated beads had recovery of 0 and 10x diluted inoculum in 1 M NaCl, the recovery was limited to the 0 dilution of the inoculum in all other matrices. This is likely due to the broader specificity of binding sites found in the HBGA-containing PGM compared to the mAB and is important to consider in future work with various genotypes. PGM was added to the beads at a concentration of 60 µg/ mg of beads and NV3901 was added at 20 µg/ mg of beads. This is important to note as having a higher concentration of binding sites available may have increased the recovery by PGM versus that of NV3901. It is also possible that there were inhibitors such as interfering proteins or carbohydrates still present from the stool-derived GI.1 sample that competitively bound to the antibody, but not to the PGM. Another consideration is that mutations could have accumulated in the capsid protein of the GI.1 virus using as the inoculum, thereby limiting its interaction with the hyper-specific mAB, but not the PGM. Because the monoclonal antibody (NV-3901) was selected by using a Norwalk virus VLP, it would be possible to compare the sequence of the Norwalk virus derived from the stool samples to the sequence of nucleic acids encoding the VLP in the recombinant baculovirus vector. The mAB NV3901 binding site has been mapped¹¹⁹ therefore, mutations present in that region can easily be determined.

Since it was possible that the mAB NV3901 was not displayed on the tosyl-activated beads in an orientation that was conducive to viral binding, protein G-coated beads were

investigated as an alternative. According to the manufacturer of the tosyl-activated beads (Dynal), the sulphonyl esters found on the bead bind with the more hydrophobic Fab region of the antibody. This differs from the binding that occurs in the Fc region of the protein G beads. Binding at the Fab region shortens the length of the antibody that is presented and may not allow as much flexibility in its presentation. Binding at the Fc region may allow for greater flexibility in presentation, and therefore less steric hindrance created by binding viruses.

The lower LOD was 2×10^4 genome copies (average of three replications) for each NV3901-coated protein G beads and NV3901-coated tosyl-activated beads. Non-specific binding, however, was problematic with both bead types recovering approximately 1.7 log genomic copies of the 100x dilution. Antibody orientation presentation is an important aspect to consider in future investigations.

Exploration of other bead and/or buffer-types could prove useful in further method optimization. Adjustment of pH and/or protein and ionic concentration could aid in driving virus-ligand interactions and increase viral recovery. It is possible that through the commercial processing of PGMs that there is degradation in some of the HBGA binding sites¹²⁰. PGMs that are extracted under non-denaturing conditions have more responsive gel-forming properties than commercially derived PGMs which are extracted under denaturing conditions¹²¹. Therefore, future experiments could be conducted with PGMs harvested from pig stomachs treated with protease inhibitors in lieu of those that are commercially processed.

Based on findings in literature¹²², it is possible that the phosphates present in the PBS used to suspend the virus-bound beads prior to RNA heat-release, may sequester the divalent cations (Mg^{2+}) that are necessary for the RT-qPCR reaction, making them unavailable in the

reaction. An assay was designed to investigate RT-PCR inhibition caused by PBS, when compared to Tris buffer and nuclease-free water. It was determined that PBS demonstrated less than a 1 log or 3.3 CT value difference when compared to water and was not considered inhibitory; however, Tris buffer was slightly less inhibitory (1/2 log) when compared to PBS.

HBGA-containing PGM and saliva were added to other methodologies that are often used during virus concentration or purification procedures to see if their addition could improve the recovery of viruses using these methods. For example, a polyethylene Glycol (PEG) precipitation method using 10% w/v PEG 10,000 molecular weight (MW) was used because it was previously optimized using glycine and 3% beef extract.¹¹⁴ Either PGM or saliva was added during the PEG dissolution step to see if it could improve virus precipitation once centrifuged. Saliva with PEG precipitation yielded the highest percentage of recovery of all the novel assays (PGM yielded second highest), but also displayed the most variability.

Other novel methods were also explored. Xanthan gum, which is used as a thickening agent in food processing, was mixed with PGM or saliva for the muco-adhesion assay experiments. The purpose of this was to see if a thick mucus-like substance containing HBGAs could be created that would bind norovirus and could be easily precipitated with slow-speed centrifugation. Xanthan gum, unlike other gums (e.g. Guar gum), is stable at a neutral pH and therefore does not need to be dissolved into solution in an acidic pH environment in order to achieve the desired viscosity. Because glycine pH 7.0 yielded the most consistent results with the bead assays, all novel PGM and saliva assays were performed in this buffer. PGM or saliva with xanthan gum contributed the least amount of improvement due to the addition of the ligand when compared to the other novel methods tested.

Typically, ultra-filters are used rather than the 0.2 μm membrane filters used in this study to concentrate norovirus from a liquid sample. This is because the viral particles are $\sim 27\text{-}35\text{ nm}$, so typically the filter would allow the viral particle through. However, it was hypothesized that PGM or saliva added to the samples prior to membrane filtration would surround the viral particle via mucin interaction and would cause the particle to get trapped in the filter. Although PGM and saliva both resulted in three of three positive replicates, PGM out-performed saliva by having a 48% positive impact due to the addition of the ligand versus a 16% impact by saliva. This may be attributed to the viscosity of PGM versus that of saliva in limiting the passage of virus through the filter.

Overall, an addition of PGM and saliva to PEG precipitation yielded the most promising results. There was an expectation that an increase of PGM concentration would increase viral recovery and this was not the case as the assay yielded negative results in all three replicates. Based on this data, testing was performed to determine if PGM was inhibitory and it was determined that it was not.

There was an improvement due to the addition of PGM in all novel assays; although, this improvement was not consistently demonstrated between replicates. Increasing PGM concentration appears to have a negative effect on viral recovery, however; further testing may need to be performed to determine the ideal concentration of mucins since inhibition has been ruled out. In addition, PGM-coated beads out-performed NV3901-coated beads in all buffer matrices tested. Based on this data, PGM appears to be a promising medium for viral recovery.

CHAPTER 6

CONCLUSIONS

This study is an important step in the development of low-cost, sensitive norovirus detection methods. Since ultimately the goal of this line of investigation was to develop methods for norovirus detection in food, it was important that all experiments were conducted with elution buffers that are commonly used in Food Virology. PGM-coated beads proved to be the most versatile and most compatible, out-performing the mAB-coated beads in nearly all buffer matrices tested. However, the PGM-coated beads proved to be most compatible with the neutral pH buffers, glycine buffer (pH 7.0) and the 1 M NaCl PBS buffer. Increasing and decreasing pH had a negative effect on recovery (approximately a 2-log reduction in the undiluted inoculum), but PGM-coated beads continued to out-perform mAB-coated beads in these matrices. Non-specific binding was problematic, especially for uncoated tosyl-activated beads in the context of 1 M NaCl. Due to the possibility that antibody presentation by mAB-coated tosylated beads could have created steric hindrance during virus attachment, protein-G beads coated with NV-3901 were also investigated. Protein G beads performed slightly better than the tosyl-activated beads (5.4 log recovery for protein G and 3.89 recovery in tosyl activated beads); although, LOD was not improved using the protein G beads.

Unbound PGM and saliva appeared to enhance capture and recovery of human norovirus when used in conjunction with PEG precipitation or membrane filtration; however, further optimization is needed as reproducibility was problematic. Changes in mucus concentration, molecular weight and percentage of PEG, or changes in filter size may all have a positive impact on viral recovery using novel methods and will be considerations in future work. It is also

recommended that future work should be performed with native PGMs (extracted under non-denaturing conditions) as these carbohydrates retain their gel-forming properties.

While Norwalk virus binding to PGM-coated beads proved to be more reliable than when using the mAB-coated beads, the limit of detection (2×10^3 genomic copies) was still higher than desired since the number of virus particles required to cause infection is as low as 10 particles. The novel assays explored in this experiment were even less effective and less repeatable for Norwalk virus capture. It is therefore necessary to continue method optimization and development and decrease the assay limit of detection in order to make it an effective means of detection of norovirus during an outbreak investigation. In conclusion, the ligand-based assays investigated in this study demonstrate potential as rapid and cost-effective means of recovering and concentrating norovirus from food and water samples, as they are compatible with buffers commonly used in food virology. Future work need be directed toward lowering the assay limit of detection and expansion to include other norovirus genotypes.

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