

## ABSTRACT

OUTLAW, JANIE ELIZABETH. Review of the Practical Considerations for Human Noroviruses Detection in Foods and Environmental Samples. (Under the direction of Dr. Lee-Ann Jaykus).

The lack of rapid and sensitive assays to detect human norovirus (HuNoV) contamination in food and environmental samples is one factor limiting the ability to control transmission. Nucleic acid aptamers are emerging ligands for use in pathogen capture and detection because of their high target binding affinity, ease of synthesis and functionalization, and stability. The purpose of this study was to evaluate candidate ssDNA aptamers for their binding affinity to HuNoV and to provide proof-of-concept that they could be used in a magnetic capture-detection method. Eight aptamer candidates previously produced using a GI virus-like particle (VLP) cocktail as target, and two aptamer candidates targeting a GII norovirus specimen, were screened for binding specificity and intensity using an Enzyme-Linked Aptamer Sorbent Assay (ELASA) performed on a panel of HuNoV VLPs. Similar assays were done using 20% stool suspensions obtained from infected individuals and fresh produce wash water inoculated with HuNoV. Aptamer AP4-GI bound to both GI and GII genogroup VLPs tested. Binding absorbance ratios for GI VLPs ranged from  $4.8 \pm 0.5$  (GI.8) to  $12.0 \pm 0.6$  (GI.1); and for GII VLPs ranged from  $6.5 \pm 2.7$  (GII.1) to  $19.8 \pm 5.0$  (GII.2 Snow Mountain). Aptamer affinities for virus in diluted human fecal samples and fresh produce wash water were dampened. A previously selected aptamer candidate, M6-2, was used in a magnetic-bead mediated capture assay followed by RT-qPCR for detection of Tulane Virus, a HuNoV surrogate. The presence of the aptamer on the beads improved RT-qPCR assay detection limits by 1-2  $\log_{10}$  PFU, corresponding to  $10^3$ - $10^4$  PFU. This study presents proof-of-concept of the utility of ssDNA aptamers for capture and detection of HuNoV, adding

another tool to the arsenal as we seek to develop better methods of detection for virus contamination in food and environmental samples.

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Review of the Practical Considerations of Human Norovirus Detection in Foods and  
Environmental Samples

by  
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## **DEDICATION**

To my family (Karen, Gordon, Ashlee, Larry, Snoball, and the 206 crew) and those who invested and believed in me when I did not notice my potential.

## BIOGRAPHY

Janie Elizabeth Outlaw was born November 10<sup>th</sup>, 1992 in Baltimore, Maryland to Gordon and Karen Outlaw. The youngest of three, Janie had the opportunity to learn many life lessons early on by observing her older brother Larry (7/12/1974) and older sister, Ashlee (7/26/1985). Although sometimes sheltered by her siblings, they pushed Janie to grow throughout her early years, followed by encouraging her to move to North Carolina for secondary education.

Janie attended North Carolina Central University (NCCU) in Durham, NC where she did not know anyone. Her first time away from home and her family, she had no problem meeting new people due to her friendly personality. Janie always had an interest in sports because of her early involvement in playing Basketball and Volleyball. Since she did not want to play collegiate sports, she decided to help manage the NCCU football team. In addition, Janie wanted to learn more about athletic training. Quickly learning that athletic training was not her passion, she immediately took interest in another sector of sports medicine, Orthopedics. Aspiring to become a team physician, Janie declared her major as Biology, Pre-Medicine but re-declared her major to Pharmaceutical Sciences with a minor in Chemistry at the end of her sophomore year. The decision was a result of the required year of research and summer research internship incorporated into the Pharmaceutical Sciences curriculum. Janie's ability to disregard the extended time added as a consequence of the redeclaration rewarded her because she completed a NoroCORE research internship at NC State University (NCSU) under the supervision of Dr. Lee-Ann Jaykus. The 2014 summer at

NCSU introduced Janie to the world of Food Science. Her interest expanded as she worked in the laboratory and read research articles about foodborne pathogens. Prior to graduating from undergraduate and the split between applying to medical school or pursuing a research graduate degree, Janie followed passion to learn more about food safety at NCSU.

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I also send thanks to my North Carolina Central University support system, Dr. Benjamin Crowe and Dr. Liju Yang. Thank you for pushing me through and sending recommendation letters on my behalf.

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## CHAPTER 1

### Literature Review: Review of the Practical Considerations for Human Norovirus Detection in Foods and Environmental Samples

#### INTRODUCTION

##### Norovirus

Human noroviruses (HuNoV) are the leading cause of acute, non-bacterial gastroenteritis accounting for many outbreaks worldwide (Cheong et al., 2009; Lamhoujeb et al., 2008; Baert et al., 2008). They are responsible for 19-21 million illnesses annually in the United States with roughly 5.5 million of those occurring because of foodborne transmission (CDC). Most commonly associated with restaurants, schools, nursing homes, and other venues where people are in close proximity to one another (e.g., cruise ships), the pathogen is environmentally persistent, resistant to many inactivation methods, and often difficult to capture and detect (Bull et al., 2011). The first recorded case of a HuNoV infection happened in Norwalk, Ohio in 1968. It caused a major gastroenteritis outbreak at an elementary school leaving many persons ill (Estes et al., 1979; Kapikian et al., 1972). This seemingly sporadic outbreak ignited a scientific conundrum leading researchers to try to better characterize the cause of the “winter vomiting bug.” Currently, leading research teams are still facing the unanswered question of how to rapidly detect HuNoV in food and environmental samples, and how to eradicate the virus.

HuNoV enteric viruses are a member of the *Caliciviridae* family. They are able to survive and remain stable outside of the host for long periods of time (Jaykus, 2000), at different temperature ranges (Duizer et al., 2004), and withstand treatment with different

disinfectants and sanitizers (Barclay et al., 2014). For instance, HuNoV remains infectious in foods from two days up to four weeks (Butot et al., 2008; Hewitt and Greening, 2004; Stals et al., 2012), and are extremely resistant to heat, high or low pH, drying, light and UV exposure (Stals et al., 2012).

Human NoV are capable of infecting only humans, with individual susceptibility mediated by tissue characteristics, particularly histo-blood group antigen and blood group type (Huang et al., 2005; Hutson et al., 2002; Tan et al., 2005; Tan et al., 2008). The disease is characterized by diarrhea, vomiting, abdominal pain and fever lasting up to 48 hours (Bull and White, 2011). Most people recover without incident, but the young, old, pregnant, and immunosuppressed (YOPI) subpopulations can experience more serious disease. Fecal-oral transmission, or sometimes vomitus-oral, is the source of HuNoV transmission. Propagation of epidemics occurs as a consequence of a low infectious dose of  $<10$  viral particles (Teunis et al., 2008); high titer ( $>10^6$  infectious units/g feces) and prolonged viral shedding (Patel et al., 2009); and a lack of long-term immunity due to strain differentiation (Bull et al., 2011). The lack of treatment and vaccination further contributes to high disease burden.

Contamination of foods can occur at various points within the farm-to-fork chain, always originating from human bodily waste. This includes, for instance, fecally contaminated irrigation waters, asymptomatic food handlers, and sewage-contaminated source waters. In addition, there have been studies recording transmission through person-to-person contact, consumption of raw or undercooked foods, and contaminated ready-to-eat edibles (Lopman et al, 2012; Rutjes et al., 2006). Clinical studies have shown the virus to be shed in low titers for up to 8 weeks in previously healthy persons and for more than a year in immunocompromised patients (Glass et al., 2009). Aerolization from vomitus particles may

also contaminate hard surfaces posing a threat of cross contamination that is exacerbated by the lack of effective disinfectants against HuNoV.

These hardy, non-enveloped, single-stranded positive-sense, polyadenylated RNA viruses consist of a negatively charged capsid that forms a small round virion sizing between 27-38 nm in diameter, in addition to an internal genome of 7.5-8.4 kb. The genome contains three open reading frames (ORFs) encoding nonstructural and structural proteins (Bull et al., 2011; Mattison et al., 2007). ORF1 encodes six non-structural proteins including an RNA-dependent RNA polymerase (Bull et al., 2011; Mattison et al., 2007) that is believed to self-cleave into six to seven proteins (NS1-NS7) (Moore et al., 2015b). ORF2 and ORF3 encode structural proteins for the major (structural), VP1, and minor (unknown function), VP2, capsid proteins. The viral capsid is an icosahedron with a triangulation number of  $T=3$  (Cuellar et al., 2010) composed of 180 proteins organized into dimers. The major capsid protein, which provides for the structural integrity of the virus, contains three domains: a highly conserved internal shell (S) domain, the P1 domain, and P2 domain, both of which are protruding (Bull et al., 2011) and are of interest as detection targets. The P2 domain includes several motifs that are involved in host cell binding and responsible for the immunological variability of the virus (Siebenga et al., 2077).

The genus norovirus is subclassified into six genogroups (GI-GVI) based on the amino acid sequence of the VP1 protein (Karst et al., 2015). They are further subdivided into multiple genotypes based on nucleotide divergence creating genetic clusters (Bull & White, 2011; Moore et al., 2015b; Patel et al., 2009). The diversity among noroviruses has been caused by point mutations with error prone RNA replication and to recombination between related viruses (Glass, et al., 2009). Genogroups I and II (GI and GII) infect only humans,

with genogroup GII.4 being the epidemic strain (Bull et al., 2011). The GII.4 strains in particular appear to evolve rather rapidly, with new epidemic strains emerging every two to three years (Bull & White, 2011; van Beek et al., 2013). This fact complicates the development of broadly reactive and reliable detection methods. From a detection standpoint, the complexity of HuNoV offers a range of potential directions for investigational studies. Depending upon the contamination source, location, and nature of the outbreak, sample types vary.

## **Epidemiology**

According to the Centers for Disease Control and Prevention (CDC) Foodborne Outbreak Online Database (FOOD Tool), there are six enteric virus types that cause foodborne outbreaks in the U.S. Adenovirus, astrovirus, hepatitis A and E, norovirus, rotavirus, and sapovirus have all caused notable food related outbreaks between 1999-2014. Of the six, viral foodborne pathogen awareness focuses on hepatitis A virus (HAV), norovirus, and rotavirus, with HAV and norovirus in particular documented most frequently (Newell et al., 2010). Scallan et al. (2011) estimated that 59% of all foodborne illnesses were caused by viruses, with HuNoV leading at 98% of all foodborne virus infections.

Clearly, foodborne virus outbreaks are more common than previously recognized. Table 1.1 portrays a list of large recent outbreaks occurring with either HuNoV or HAV. While most are small point source outbreaks (not described here), large outbreaks covering widespread regions occur due to our increasingly widespread food distribution system, including the global food market. Water, fresh produce, and shellfish are modes of transmission that are often involved in epidemics. Widdowson et al. (2005), using the CDC

National Outbreak Reporting System (NORS), reported foodborne outbreaks from 1991 to 2000 ranged from 411(1992) to 1,414 (2000). In regards to HuNoV, a total of 305 laboratory confirmed outbreaks occurred, with 76 outbreaks strongly associated with RTE foods. Salads (26%), sandwiches (13%), fresh produce/fruit (17%), meat dishes (11%), fish dishes (5%), bakery products (7%), oysters (3%), and various other foods (18%) were the main implicated products reported (Widdowson et al., 2005). Similarly, Hall et al. (2013) reported acute gastroenteritis outbreaks using NORS data, also finding HuNoV to be the leading cause of outbreaks and associated with 69,145 illnesses, 1,093 hospitalizations, and 125 deaths. Out of the 1,908 outbreaks they studied, primary transmission modes included, but were not limited to, person-to-person (1,261), foodborne (494), waterborne (4), and environmental (5) transmission routes. Patel et al. (2009) also stated the four-typical transmission paths being person-to-person contact, contaminated food, water, and environmental contamination. The numbers of HuNoV illnesses reported in the epidemiological literature are vastly under-reporting and the disease burden is thought to be magnitudes of what is reported in the CDC literature.

## DETECTION

### **History of Enteric Virus Detection**

The absence of *in vitro* cultivation for HuNoV, despite almost 50 years of attempts, has delayed the advancement of research on this virus. Preventing HuNoV outbreaks is the main goal with discovering sensitive and rapid assays for detection. *In vivo* cultivation, which would require human subjects, has been done (Dolin et al., 1971; Gary et al., 1987) but for obvious reasons, ethical and health concerns arise. Wyatt et al. (1978) inoculated

chimpanzees to observe Norwalk symptoms, and to detect the agent from fecal samples, but similar to human subjects, ethical inquiries would arise. Soon after discovery of the Norwalk virus, Alder et al. (1969) processed stool samples from the original Norwalk outbreak to determine if the “Winter Vomiting Disease” was caused by a bacterial agent. In 1968 and 1971, Wyatt et al. (1974) obtained diarrheal stool samples from families in Norwalk, OH, Honolulu, HA, and Montgomery Co., MD to determine the cause of similar illnesses. Similarly, Dolin et al. (1971) processed stool and rectal swab samples obtained from a U.S. navy ship implicated in an outbreak. In all of these cases, virus was visualized after fecal sample purification using electron microscopy or immune electron microscopy. Later, radioimmunoassays were developed to test the presence of Norwalk virus antibody in patients’ blood serum and to observe antigen and antibody interaction (Gary et al., 1985; Greenberg et al., 1978; Kaplan et al., 1982). More popular, an enzyme-linked immunosorbent assay (ELISA) further enhanced the assay design of using antibodies for capturing virus from stool samples without the need for radioisotopes (Grauballe et al., 1981). Today, RT-qPCR is used for clinical diagnostics (Goodgame, 2007; Phillips et al., 2009) whereas ligand capture methods are emerging tools to facilitate HuNoV detection in food and environmental samples.

### **Detection in Different Sample Types**

Food and environmental samples are frequently the initial source of HuNoV transmission. Therefore finding a reliable assay for detection in these sample types would be of great value in detecting and preventing outbreaks. Despite what some might think, food and environmental samples are more complex than are clinical samples when it comes to

enteric virus detection. Clinical samples are typically stool samples which often have very high concentrations of virus (i.e.,  $10^6$ - $10^9$  infectious particles per g). On the other hand, environmental and food samples introduce the possibility of a low viral load, high sample volumes, complex sample matrices, and sample-associated inhibitors. This is juxtaposed to the very low infectious dose of HuNoV (probably <100-1,000 infectious particles).

Because HuNoV cannot be cultivated outside of humans, complex sample matrices must be processed to concentrate and purify viral agents before the application of detection methods. However, depending upon the sample type, preparation approaches will vary considerably. Contaminated water presents an issue due to the consumption of raw shellfish (Melnick et al., 1978). Similarly, shellfish have been often the focus of development of detection methods for HuNoV (Richards, 1985). More recently, ready-to-eat foods like fresh produce and baked goods have been the subject of concern, and rightly so because of the high risk of bare hand contact and contamination with human feces (Baert et al., 2008). Regardless of the matrix, all food and environmental samples undergo concentration and purification procedures. In fact, prior to any concentration step it is sometimes necessary to dilute the samples to reduce the amount of unwanted natural biota, carbohydrates, proteins, and other similar substances. Historically, studies have shown samples to be diluted in veal infusion broth with bovine serum albumin (BSA) (Dolin et al., 1971; Thornhill et al., 1975), phosphate-buffered saline (PBS) (Kapikian et al., 1972), tryptose phosphate broth (TPB) (Lewis et al., 1988), veronal-buffered saline (VBS), tris(hydroxymethyl)aminomethane (TRIS) buffer, and ethylenediaminetetraacetic acid (EDTA) (Moritsugu et al., 1976). While they all offer different biological or chemical properties for diluting samples, PBS is more

commonly used due to the low introduction of matrix interference, similarity to the human body's isotonic environment, and its non-toxic nature to most cells and viruses.

Both physical and chemical methods have been used to concentrate and purify the sample for virus extraction. Centrifugation, a physical method, has been quite popular. For instance, Bawden et al. (1945) used low speed centrifugation to observe separation properties of tobacco mosaic virus, whereas Kapikian et al. (1972) used a higher centrifugation speed to prepare fecal specimens to observe a 27-nm particle associated with the original Norwalk virus gastroenteritis outbreak. Many others have also used various centrifugation methods for sample preparation (Bishop et al., 1974; Haramoto et al., 2009; Schwab et al., 1995).

Since the size (diameter) of HuNoV has been observed to be around 27-38 nm, filtering samples has been a method widely used to capture viral agents prior to detection. This is a physical method that separates based on size, with the viruses much smaller than food particles and bacteria. Studies have shown stool supernatant serially filtered through 1.2 $\mu$ m and 0.5 $\mu$ m pore sizes (Thornhill et al., 1975; 1977) prior to use in immune electron microscopy. Dolin et al. (1971) filtered fecal suspensions through a 1.2 $\mu$ m Millipore filter, similar to Kapikian et al. (1972), to remove bacteria so as to make virus particles safe to administer to volunteers.

Alternatively, elution methods are also sample preparation steps for virus detection. Elution is the process of promoting the dissociation of viruses from matrix-associated materials to which they are bound. Viruses are usually attached to such molecules via electrostatic interactions influenced by charge. Elution buffers vary in ionic composition, salt concentration, pH, elution time and temperature, all of which can elution recovery of viral particles. For example, HAV binding to cellular receptors was enhanced when elution buffers

were low in pH or contained calcium ions that resulted in reduced elution efficiency (Bishop & Anderson, 1997). Tsai et al. (1983) recognized the influence of pH on elution of enteric viruses from estuarine sediments. They investigated four eluent solutions: a glycine buffer, skim milk and isoelectric casein solution, a beef extract preparation, and a nutrient broth. Comparatively, the greatest percent recovery for poliovirus, ranging from 37.1 to 65%, was obtained using 3% beef extract solution.

Precipitation, the creation of a solid from a liquid solution, is another chemical method that allows for concentration of viruses (Hagen et al., 1996). Lewis et al. (1988) found polyethylene glycol (PEG) precipitation to be effective for concentrating viruses from environmental samples with the advantages of variability, gentle effects on viruses, and neutral pH. Bishop et al. (1974) also studied PEG precipitation to extract viruses in clinical samples from children with gastroenteritis. Since viruses act as proteins in solution, PEG precipitation is an ideal method as its hygroscopic nature binds water in the solution to which it is added, allowing proteins (including viruses) to fall out of solution and be easily concentrated by simple low speed centrifugation.

Solvent extraction methods (chemical in nature) have also been used to prepare samples for viral detection (Pike et al., 1977). Enteric viruses are exquisitely resistant to many organic solvents, including ethanol, Freon, and chloroform, all without jeopardizing virus infectivity (Smith et al., 1959). Solvent extraction can therefore be used to remove cell debris and lipid-containing compounds from the sample matrix without affecting contaminating virus (Schlindwein et al., 2009). For instance, Grohmann et al. (1980) used organic solvent extraction to partially purify Norwalk-like particles from fecal specimens to determine antibody levels. Not only valid for stool specimen extraction, Hewitt et al. (2004)

applied chloroform extraction for mussel shellfish to purify HuNoV, HAV, and feline calicivirus in preparation for detection by RT-PCR. There are many other examples of the use of this method in virus extraction from food and environmental samples.

Separation and concentration methods such as the ones described above (i.e., centrifugation, filtration, elution, precipitation, and solvent extraction) are still extensively used in the 21<sup>st</sup> century. In fact, they have set the foundation for current methods to prepare complex sample matrices for downstream enteric virus detection assays. The search for a rapid and sensitive detection assay therefore relies on the classical methodology developed in the late 20<sup>th</sup> and early 21<sup>st</sup> centuries.

### **Current Detection Methods**

At a very high level, one can view virus extraction methods as applied to food and environmental samples as falling into one of three approaches: (1) one or more sequential physical and/or chemical methods to concentrate and purify the viruses prior to detection; (2) direct viral RNA extraction from a matrix without pre-processing steps; and (3) virus extraction using enzymatic digestion treatment (e.g., proteinase K treatment) (Stals et al., 2012). However, there are almost as many methods as there are sample matrices, and indeed, methods are frequently designed in a matrix-specific manner. By way of example, early work by Schwab et al. (2000) used a combination of solvent extraction and PEG precipitation to concentrate Norwalk virus from sandwich components. Interestingly, some eight years later, Baert et al. (2008) processed artificially contaminated foods for virus concentration using a combination of PEG precipitation and solvent extraction as well. In short, more or less the same method was used.

For the sake of simplification, Moore et al. (2015b) developed a flow diagram of different virus extraction approaches based on general sample type. Hard surfaces required a pre-moistened swab for sample collection followed by elution of the virus and/or direct RNA extraction from the swab and/or eluent. Buffer types vary widely from PBS to glycine saline to beef extract, or some combination thereof (e.g., tris-glycine-beef extract or TGBE) (Moore et al., 2015b). Ready-to-eat (RTE) or complex matrices like sandwiches require multiple concentration and purification steps to make the matrix amenable to downstream nucleic acid extraction, for example dilution, filtration, PEG precipitation, and solvent extraction (Moore et al., 2015b). Analogous to complex matrices like sandwiches, Butot et al. (2007) released viruses from fresh or frozen berries and vegetables using an elution buffer (50mM glycine, 100mM Tris, 1% [wt/vol] beef extract [pH 9.5]) following by either PEG precipitation or ultracentrifugation. Using a similar method, Stals et al. (2011) reported virus recoveries between 7.4-61.1% from raspberries and frozen forest fruit mix containing strawberries, raspberries, blackberries, blueberries, and black currants. Different from fresh produce, bivalve molluscan shellfish are more easily processed using a proteinase K treatment followed by RNA extraction (Jothikumar et al., 2005; Moore et al., 2015b). Validated methods for certain products have been recently released and these are discussed later.

After matrix purification, RNA isolation methods are used to prepare samples for nucleic acid amplification. Circa 1990, Boom et al. (1990) introduced a particularly efficient nucleic acid purification method for application to human serum or urine. The method uses chaotropic reagents such as guanidinium isothiocyanate to lyse cells or viruses, followed by phenol-chloroform extraction to remove residual proteins. Due to the toxicity of phenol, silica was applied instead to purify the RNA (Chungue et al., 1993). The so-called Boom

method yields highly purified RNA with efficient yields and forms the basis for most commercial methods, although additional steps may be added to further purification. Baert et al. (2008) compared four manual methods for RNA extraction from RTE food concentrates, finding the best approach to include a direct RNA isolation using the commercial Trizol reagent in conjunction with the RNeasy Mini kit. The RNeasy kit has been used by others as well (Baert et al., 2008; Rutjes et al., 2006). In their comprehensive comparison, Arnal et al. (1999) looked at seven extraction methods for recovering HAV RNA from stool and shellfish samples, finding antibody coated beads and RNAzol (a phenol-chloroform solution) to be the best. Automated nucleic acid procedures were introduced some 15 years ago and improve the efficacy and quality of nucleic acid extractions although they tend to be expensive. Examples include the NucliSENS® easyMAG® system (bioMérieux, Durham, NC) and an automated KingFisher magnetic particle processor (Thermo Fisher Scientific, Pittsburg, PA), among others (Gentry-Shields & Jaykus, 2015; Vega et al., 2011).

Regardless the sample preparation or RNA extraction method, detection of viruses in food and environmental samples is almost always done using RT-PCR. Advances in the last 15 years have allowed the method to be ‘real-time,’ by incorporating a fluorescently labeled probe to emit a signal that is directly proportional to the starting nucleic acid target concentration. This is based on the fact that the larger the amount of target, the sooner a fluorescent signal will cross a threshold, also called the threshold cycle or Ct value. Ideally, low Ct values ranging from 20-35 indicate detectable virus, with the lower number corresponding to the higher initial target concentration. By creating a standard curve that correlates nucleic acid copy number to Ct value, real-time RT-qPCR can be made quantitative. Unfortunately, Ct values >35 are difficult to interpret when obtained in

correspondence with food and environmental samples. Another major issue with RT-qPCR is that it is unable to distinguish between infectious and noninfectious viral particles. This is because viral RNA is persistent even when the virus capsid has been destroyed. This and several other issues (e.g., difficulty interpreting RT-qPCR results, residual matrix-associated inhibition, etc.) make nucleic acid amplification a less than ideal method. Unfortunately and despite vigorous efforts, a simple and reliable procedure for *in vitro* cultivation of HuNoV has not yet been discovered.

### **Facing Detection Limitations**

The purpose of the remainder of this review is to identify the barriers to overcome in order to be able to make better conclusions from RT-qPCR data derived from virus detection in complex sample matrices. The focus is on water, fresh produce, and molluscan shellfish. One major difficulty is the fact that virus concentrations in these samples is low, and the sample matrix is highly complex so large sample volumes must be used, meaning that virus concentration is required. This is particularly important because HuNoV has a very low infectious dose. Because there are so many HuNoV strains, concentration and purification methods must be applicable to all strains, and preferably, results should be consistent and dependable when applied to different sample types. Discrimination of infectious and noninfectious viral particles (the infectivity dilemma) is critically important as only infectious virus causes disease, while even inactivated virus can be detected by RT-qPCR.

## SAMPLE PREPARATION

### **Preparation as a Barrier**

As documented in the narrative above, the quality of sample preparation is critical to our ability to detect viruses in foods. According to Stals et al. (2012), there are three main food categories for which virus detection is commonly applied: foods composed of carbohydrate and water (e.g., fresh produce); foods including fat and proteins (e.g., deli meats, frostings); and molluscan shellfish that are rich in glycogen and accumulate and concentrate pathogens in the gut. The type of sample preparation applied is contingent on the matrix type, e.g., the need for fat degradation (using solvent extraction) or protein cleavage (using enzymes). Even so, sample preparation remains a major barrier to virus detection because it tends to be inefficient (loss of virus during extraction) and does not remove all matrix-associated compounds, which results in residual RT-qPCR inhibition (Gentry-Shields & Jaykus, 2015).

There are many examples of poor recovery and detection limits for viruses in foods. For example, Cheong et al. (2009) noted the difference in detection limits for strawberries and lettuce as a result of the matrix type, drying conditions, viral strain, sample size, and seed or leaf debris. Rutjes et al. (2006) also expressed the difficulty in detecting HuNoV from inoculated whipped cream and leaf lettuce, as well as naturally contaminated dairy, meat, vegetable, and grain food samples. All food samples in that study, both inoculated and naturally contaminated, were found negative for HuNoV. These studies, along with many others, illustrate the problems of both poor virus recovery efficiency and residual inhibitors after virus concentration and RNA extraction, all of which impact the analytical sensitivity of the assay (Cheong et al., 2009; Gentry-Shields & Jaykus, 2015; Rutjes et al., 2006; Stals et

al., 2012). This is further illustrated in the frequent absence of laboratory confirmation of virus in foodborne outbreaks, even if the implicated food product is definitively identified. In fact, it is the exception rather than the rule when HuNoV or HAV are found in a food suspected of being contaminated.

### **Recent Developments in Addressing Barriers**

In summary, detecting viruses in food matrices is difficult with barriers such as low particle counts, high sample volumes, the complexity of matrices, residual inhibitory compounds, and the need to detect only a few viruses due to low infectious dose. It is truly like finding a needle in a haystack when attempting to detect HuNoV in these types of samples. To address the absence of validated detection methods for enteric viruses in foods, the International Organization for Standardization (ISO) embarked on an effort in 2010 to create standard methods. These were released in 2013 for bottled water, leafy greens, berries, and molluscan shellfish and illustrate a range of complex environments from which viruses can be concentrated and purified.

Bottled water is assumed to have little to no inhibitors compared to wastewater or tap water, hence extracting viral particles from bottled water is often used as the baseline or control for concentration and purification studies. Throughout the literature, most studies have started with 1.5L of bottled mineral water. In three separate studies (Huguet et al., 2012; Perelle et al., 2009; Schultz et al., 2011) investigators seeded bottled mineral water with final concentrations of HAV, poliovirus, MS2 bacteriophage, feline calicivirus, and HuNoV fecal suspensions between  $1 \times 10^4$  to  $1 \times 10^5$  particles and used similar filtration methods based on positively charged membranes. Direct RNA extraction from the

membranes was performed with either a viral RNA extraction kit, or by an automated extraction machine (Huguet et al., 2012; Perelle et al., 2009). Schultz et al. (2011) used a different approach by ultracentrifugation of the membrane and re-suspending the pellet for subsequent viral RNA extraction. Collectively the best overall recoveries were observed after directly extracting viral RNA from charged membranes through which water was filtered. This result supported design of ISO 7704:1985, a validated procedure for filtration-based concentration of viruses from bottled water using positively charged porous membranes.

Unlike water, berries and leafy greens are comprised of complex carbohydrates, creating a necessity for multiple concentration and purification steps. In the Butot et al. (2007) study, both assorted berries and vegetables were seeded with either HAV or HuNoV and the virus eluted with a glycine, TRIS, and beef extract elution buffer. This combination elution buffer provided better elution conditions because: (1) glycine reduces non-specific adsorption of protein or virus; (2) TRIS prevents pH reduction caused by acidic plant juices; and (3) beef extract facilitates flocculation of HuNoV on subsequent PEG precipitation (Stals et al. 2012). To address the pectin found in plant cells (a well known RT-PCR inhibitor), pectinase was added to assist in its degradation. This step was followed by either PEG precipitation or ultrafiltration, which were compared side-by-side. Ultrafiltration yielded better virus recoveries in berries compared to PEG precipitation. However, neither was ideal, as PEG precipitation requires pH neutralization of virus eluate and ultrafiltration is undependable if vegetable or berry matter is present (Butot et al. (2007; Stals et al., 2012). Butot et al. (2007) recovered HuNoV or HAV from produce using a series of steps following the ISO method for virus recovery from foods. An elution buffer (50mM Glycine, 100mM Tris, 1% beef extract) was added and agitated with the produce until filtration occurred.

Following filtration, centrifugation helped to remove particulate debris. The remaining supernatant contained virus particles and underwent centrifugation and filtration for virus concentration. Elution buffer removed viral particles from the filter device and RNA extraction and real-time RT-PCR quantified the data (Butot et al., 2007). Stals et al. (2012) reviewed literature on the different extraction procedures for concentrating food-borne viruses from food samples. The review highlights similar methods used in Butot et al., 2007, with minor alterations varying within studies.

Particularly in Europe, the high public health burden associated with viral contamination of molluscan shellfish has created a push for good methods of detection. Bivalve shellfish accumulate viruses in their digestive tracts, and there are significant tissue components that can inhibit RT-qPCR. The first step of the ISO method, is to chop or blend the digestive organs followed by adding a proteinase K solution to degrade the tissue (Jothikumar et al., 2005; Loisy et al., 2005; Stals et al., 2012). After degradation, proteinase inactivation is done by heat and centrifugation used to collect the soluble portion of the shellfish which is further analyzed using RNA extraction and RT-PCR.

These standardized methods are those constituting the ISO 15216-1:2017 *Microbiology of the food chain—Horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR—Part 1: Method for quantification*. Regardless of sample matrix complexity and the virus concentration method used, all protocols share a common RNA extraction method for virus capsid disruption before standardized RT-qPCR.

## **Alternatives to the ISO Method**

A major problem with the ISO methods is their inability to discriminate between infectious and non-infectious virus. One way to potentially address this is to link virus concentration to a terminal capture step using a ligand with binding affinity for the virus. In doing so, the specified ligand would target a region only available if the virus is infectious. For example, since HuNoV requires an intact capsid protein in order to be infectious, a ligand designed to attach to multiple epitopes present on the capsid could theoretically be used in a method to discriminate between infectious vs. non-infectious particles. The idea here is that virus infectivity requires an undamaged capsid. If the capsid were damaged, one or more ligands would not be able to bind and could therefore not be detected. On the other hand, the ligand(s) *would* attach to the intact particles and if those bound ligands were detected, it would indicate the presence of infectious viruses. Some ligands offer broad reactivity (i.e., can bind to multiple epitopes and/or types/strains of the target) whereas some are more target-specific. In the case of HuNoV, broad reactivity to the norovirus genus is preferable since we want to detect all HuNoV strains, not just one strain. Hence, the desire is to achieve high sensitivity. Ligands with high target specificity can lead to false negative results because they can detect some, but not all viruses of interest. For HuNoV, ligands to have differing degrees of specificity, with histo-blood group antigens and porcine gastric mucin showing broad reactivity, whereas antibodies, aptamers, and peptides are more target-specific.

Histo-blood group antigens, better known as HBGAs, are carbohydrates linked to glycoproteins or glycolipids that are present on red blood cells and mucosal epithelial cells of the respiratory, genitourinary, and digestive tracts (Tan and Jiang, 2005). They can also be

found as free antigens in biological fluids, such as saliva, intestinal contents, milk, and blood (Huang et al., 2005; Tan and Jiang, 2005). These complex carbohydrates have been recognized as putative receptors for HuNoV via saliva studies (Huang et al., 2005; Patel et al., 2009; Tan and Jiang, 2005). When tethered to a solid surface, HBGA's can be used for virus capture. Different approaches have been applied in this regard. Cannon et al. (2008) and Harrington et al. (2004) used streptavidin-coated magnetic beads with biotinylated HBGAs to capture HuNoV VLPs in non-pristine sample matrices (wastewater or fecal samples). Cannon et al. (2008) created dilutions of 10% stool suspensions (containing 3 to 3 x 10<sup>3</sup> RNA copy number of HuNoV GI.1) and applied HBGA capture, achieving a median detection limit of 300 genome copies after detection by RT-qPCR. Harrington et al. (2004) used synthetic HBGAs to bind to a range of GI and GII HuNoV and VLPs. Their capture method was able to bind most but not all viruses tested. Later work demonstrated differential virus binding to different HBGA types. For instance, Tan et al. (2008) showed that both GII.3 and GII.4 HuNoV strains attached to HBGA having high concentrations of A and B antigens, but variability in H antigen concentration. These studies show the feasibility of HuNoV capture and concentration using synthetic HBGAs

Due to the expensive and variable quality and availability of purified synthetic HBGAs, porcine gastric mucin (PGM) has been used as an alternative. PGM contains a wide variety of naturally occurring HBGAs, and is inexpensive and readily available. PGM purified from porcine stomach mucosa has been coupled to magnetic beads to produce a PGM-magnetic bead suspension (PGM-MB) (Dancho, et al., 2012; Pan et al., 2012; Tian et al., 2011). When HuNoV positive stool samples were added to the PGM-MB solution, followed by washing and magnetic separation, virus could be detected by RT-qPCR (Dancho

et al., 2012; Pan et al., 2012; Tian et al., 2011). Dancho et al. (2012) found an average of 68% of RT-qPCR detectable HuNoV bound to the mucin-coated beads but Pan et al. (2012) showed a much lower percent recovery. Using GII.4 inoculated fresh produce (cherry tomato, blueberries, or mixed salad) and spiked produce wash water with either 20 to 500 real time RT-PCR units (RTUs), showing detection only at the highest inoculum concentration (500 RTU). Similarly, Tian et al. (2011) discovered higher likelihood of detection of HuNoV from spiked romaine lettuce washes having higher viral loads. In some cases, recirculating affinity magnetic separation system (RCAMS) has been done using PGM-MB (Pan et al., 2012; Tian et al., 2011), with this method outperforming PEG precipitation-RT-qPCR. Even though PGM-based magnetic capture has been widely used, results are quite variable. Therefore, alternative target specific ligands have been the subject of recent investigation. Those include antibodies, nucleic acid aptamers, and peptides.

Antibodies are critical to the immune response as they identify and remove foreign antigens. When naturally produced, they tend to be very target specific, which means that most antibodies against HuNoV can bind to some but not all genotypes. Several laboratories have generated polyclonal and monoclonal antibodies using HuNoV capsid protein VP1 as antigens, but the absence of broad reactivity remains problematic (Shiota et al., 2007; Huang et al., 2014). For clinical diagnostics, ELISA assays can be developed to screen for the presence of antibody to a virus (e.g., HAV). When done for HuNoV, Huang et al. (2014) found excellent specificity but limited cross-reactivity, in other words, poor sensitivity. For the antibodies produced by Kitamoto et al. (2002), shared capsid protein epitopes within a genogroup were identified but the antibodies still were unable to detect non-denatured virions or a broad range of strains. This may be different for the GII.4 epidemic strains, as

Lindesmith et al. (2012) reported on three human mAbs that recognized conserved GII.4 epitopes. Hurwitz et al. (2017) characterized phage-displayed single-chain antibodies that detect GI HuNoV through capsid binding. The study found strong binding against GI.1 and GI.7 noroviruses, but requires optimization of the ligands to broaden viral strain reactivity. Nevertheless, the application of the novel ligands still proves the use of receptor-binding mechanisms as promising for virus diagnostic methods. Similarly, Kou et al. (2015) assessed monoclonal and human single-chain antibodies against HuNoV fecal specimens. Both the monoclonal antibody (Mab NV23) and the single-chain antibody (scFv HJT-R3-A9) used in the study identified all HuNoV genotypes present in stool samples. The ligands also reported detecting as little as 10ng of VLP for most HuNoV types. Specificity and sensitivity was demonstrated in Kou et al. (2015) with and without a matrix load present. Overall, the lack of broad reactivity has been a substantial impediment to the development of HuNoV diagnostics but strives towards creating reliable and broad ligands are current as seen in Hurwitz et al. (2017) and Kou et al. (2015).

Nucleic acid aptamers are single-stranded DNA or RNA oligonucleotides that can bind with high affinity to a wide variety of non-nucleic acid target molecules based on their three-dimensional structures, with binding mediated by electrostatic, van der Waals interactions, and hydrogen bonding (Escudero-Abarca et al., 2014; Hamula et al., 2006; Moore et al., 2015b; Tombelli et al., 2005; Stoltenburg et al., 2007). The term aptamer is derived from the Latin word “aptus” which means fitting or to fit (Ni et al., 2011; Stoltenburg et al., 2007). There is something of a ‘lock and key’ relationship between aptamers and their binding targets.

Aptamers are selected from a large library of molecules containing randomly created sequences through an *in vitro* selection process known as systematic evolution of ligands by exponential enrichment (SELEX) (Hamula et al., 2006; Ni et al., 2011; Tombelli et al., 2007). Due to the *in vitro* nature of the selection process, the creation of the oligonucleotides is independent of animal cell lines (Tombelli et al., 2007). Advantageous characteristics of aptamers include their low dissociation constants (Tombelli et al., 2005), ease of synthesis, low cost of production, stability, and ease of regeneration (Escudero-Abarca et al., 2014). Concerns about the stability of RNA aptamers (Ni et al., 2011) have even been allayed as nuclease degradation can be overcome by chemically synthesizing aptamers with modified or inverted nucleotides to prevent terminal degradation.

In regards to HuNoV aptamers, two studies thus far, Escudero-Abarca et al. (2014) and Moore et al. (2015a), have reported on the creation and characterization of aptamers with binding affinity to GII strains. Escudero-Abarca et al. (2014), using a 20% stool suspension as the SELEX target, conducted nine rounds of selection and identified 34 aptamer candidates that were screened for binding affinity to a range of GI and GII VLPs by Enzyme-Linked Aptamer Sorbent Assay (ELASA). Four aptamer candidates with over five sequence repeats per SELEX round were selected for further characterization. Two in particular bound with high affinity to many GI and GII strains (Escudero-Abarca et al., 2014). Moore et al. (2015a) created GII.4 Sydney (SYD) P domain targeted aptamers using a similar SELEX procedure and also used ELASA method to characterize binding of two candidate to GI and GII VLPs. Aptamer M6-2 demonstrated low to strong binding against all VLPs tested. These same investigators showed proof-of-concept that an Aptamer Magnetic Capture (AMC) system could be used to capture virus in stool specimens from outbreaks and in artificially

contaminated produce rinsates (Moore et al. 2015a). These studies lay a foundation for the use of nucleic acid aptamers in virus capture from complex sample matrices.

Peptides are unique, short linear amino acid chains that can be synthesized easily, are more stable and amendable than antibodies, less expensive, and have low immunogenicity (Hwang et al., 2015). Phage display, a powerful technique for screening random peptide sequences, is utilized to screen peptides that are able to bind to a specific target (Rogers et al., 2013). The display relies on genetic material for peptides within the phage chromosome to isolate peptide-displaying phages useful for binding to target molecules. Rogers et al. (2013) isolated HuNoV-specific peptides and evaluated their binding affinity to VLPs and naturally contaminated stool specimens. One phage clone out of three produced was able to bind to the purified HuNoV VLP P domain. The same phage was also used to detect virus from HuNoV positive stool suspensions and interestingly, was not inhibited by any fecal components (Rogers et al., 2013). Analogous to Rogers et al. (2013), Hwang et al. (2015) characterized M13 phage peptides against HuNoV VLPs, finding some binding affinity and proposing them to be potential capture ligands for use in biosensors.

Collectively, antibodies, aptamers, and peptides have promise in addressing the sample preparation issue. However, the reality is that viruses in most samples will need to be pre-concentrated before applying ligand-based capture methods, since sample size is generally greater than that which can be accommodated in magnetic capture. Patharix® (Matrix MicroScience Ltd.) is an automated recirculating extraction system that is able to concentrate pathogens from large and complex sample matrices in preparation for PCR detection (Morton et al., 2009). The system operates via charged or ligand-bound magnetic beads to capture viral particles, essentially reducing sample volume from >100 ml to 1-10 ml.

Morales-Rayas et al. (2010) demonstrated that, with an initial inoculum of  $10^5$  HAV and HuNoV GII viral particles inoculated into a 50 g of food sample (strawberries, raspberries, green onions, and lettuce), at least  $10^2$  particles could be recovered using cationic beads for capture. In another study, Mattison et al. (2009) used a similar Pathatrix® protocol to recover the HuNoV surrogate feline calicivirus (FCV) and HAV from strawberry samples. Virus could be recovered and detected by conventional RT-PCR at inoculum concentrations as low as  $6.0 \times 10^1$  PFU HAV/25 g sample, but detection limits were higher ( $6.0 \times 10^3$  PFU HAV/25 g sample) using real-time RT-PCR. Similar detection limits were observed for feline calicivirus. While Morton et al. (2009) achieved a lower limit of detection ( $10^1$  copies/250 ml) for HuNoV from lettuce, green onion, and strawberry eluates with the Pathatrix® system, they found its performance to be inconsistent. Automated magnetic capture systems have fallen out of vogue for food safety applications, but they may be revived for concentration of human enteric viruses given their unique challenge to detection.

## INFECTIVITY DILEMMA

### **Why is it a barrier and how is it being addressed?**

Whether using traditional or novel methods to concentrate viral pathogens from foods, the infectivity dilemma remains a major barrier. In order to accurately study the infectivity, resistance, and persistence of HuNoV, it is essential to be able to discriminate infectious from non-infectious viruses. HuNoV requires both the capsid and its genome to be considered infectious (Knight et al., 2012). The virus capsid protects the genome from degradation, and loss of capsid integrity releases viral nucleic acids that may be intact but noninfectious due to the damaged capsid (Richards, 1999). Also, considering the persistence

of viral RNA over a period of time, RT-PCR relies on the amplification and detection of nucleic acid sequences. Therefore, if the capsid is damaged, but viral RNA is still present, RT-PCR data shows viral contamination in the analyzed sample. The false positive results overstate the risk to human health and create an unclear conclusion as to whether products are safe or not.

There are two major approaches that are used in conjunction with molecular amplification to aid in discrimination of infectivity status: evaluating either genome integrity or capsid integrity (Moore et al., 2015a). RNase pre-treatment and ligand capture (HBGA, PGM, antibodies, aptamers, peptides) have both been used to help discriminate infectious from non-infectious virus based on capsid integrity. Another method for discriminating infectivity status based on capsid integrity is pre-treatment with nucleic acid intercalating agents such as propidium monoazide (PMA), which bind free nucleic acid (rendering it incapable of serving as a template in PCR) but cannot bind to encapsidated genomes. Escuerdo-Abarca et al. (2014) compared the efficacy of a PMA treatment versus RNase pre-treatment, finding that the PMA method correlated well with RNase-RT-qPCR but was difficult to optimize. Similar results were reported by Kim & Ko (2012). Genome integrity can be assessed using long-range genome amplification with broadly reactive primers. Kostela et al. (2008) provided support for this approach but it is complicated and cumbersome.

## CONCLUSIONS

Concentration, purification, and detection of enteric viruses, including HuNoV, involves complicated methods, some discussed in this review and others beyond the scope.

This hardy, decades old pathogen, that accounts for the largest proportion of foodborne illnesses in the U.S., remains recalcitrant to control. Developing better detection methods, with a focus on infectivity discrimination, can aid in our ability to accurately evaluate the efficacy of candidate control measures. Of greatest interest to us is ligand-based capture as a means by which to discriminate capsid integrity. In order to pursue this, broadly reactive binding ligands must first be identified. In this case, ssDNA aptamers were chosen as those ligands. These previously identified aptamers were characterized for binding affinity to various GI and GII VLPs using an ELISA method in both pristine and complex sample matrices. In addition, a previously identified aptamer having high binding affinity to GII.4 HuNoV strains was shown to efficiently detect HuNoV surrogate after aptamer magnetic capture followed by RT-qPCR detection. This thesis describes the results of these studies.

**Table 1.1**

List of large recent outbreaks related to human norovirus or hepatitis A virus.

Product	Virus	Location		Date(s)	# People Ill	Comments	Reference
Unknown	Norovirus	Chipotle Restaurant	Boston, MA	August 2015	234	Investigations have shown the source coming from sick employees. Due to the location difference, assumptions point to two different employees being sick with Norovirus.	Bleach, C. (2016, February 1). UPDATED: CDC declares Chipotle E. coli outbreaks over; cause unknown. <i>Food Safety News: Breaking News for Everyone's Consumption</i> . Retrieved from <a href="http://www.foodsafetynews.com/2016/02/cdc-declares-chipotle-e-coli-outbreaks-over-cause-unknown/#.Vsd9DeZHSJc">http://www.foodsafetynews.com/2016/02/cdc-declares-chipotle-e-coli-outbreaks-over-cause-unknown/#.Vsd9DeZHSJc</a>
			Simi Valley, CA	December 2015	151		
Pomegranate seeds	Hepatitis A	Shipped from Turkey to US states		3/31/2013-7/26/2013	165	9 US states were affected by the Townsend Farms and Harris Teeter Organic Antioxidant Blend that used the contaminated pomegranate seeds. Scenic Fruit Company also used the seeds to make Woodstock Frozen Organic Pomegranate Kernels. It was reported that all ill victims purchased from Coscto markets but no reports from consumers who bought from Harris Teeter. Out of the sick population, 55% were women, 58% were between 40-64 years old, and 44% was hospitalized. There were also 8 secondary cases that confirmed contamination from household contact.	Centers for Disease Control and Prevention (CDC). (2013, October 28). Multistate outbreak of hepatitis A virus infections linked to pomegranate seeds from Turkey (Final Update). <i>Viral Hepatitis</i> . Retrieved from <a href="http://www.cdc.gov/hepatitis/outbreaks/2013/a1b-03-31/">http://www.cdc.gov/hepatitis/outbreaks/2013/a1b-03-31/</a>
Frozen Strawberries	Norovirus	Germany		September-October 2012	10,950 cases	390 institutions in 5 federal states in East Germany were affected during the outbreak period. Schools accounted for 63%, childcare facilities were 36%, and disability care facilities, elder homes, and rehab clinics all accounted for under 1% of facilities affected.	Benard, H., Faber, M., Wilking, H., Haller, S., Höhle, M., Schielke, A., Ducomble, T., Sifczyk, C., Merbecks, S. S., Fricke, G., Hamouda, O., Stark, K., Werber, D., 2014. Large multistate outbreak of norovirus gastroenteritis associated with frozen strawberries, Germany, 2012. <i>Eurosurveillance</i> 1-9.

**Table 1.1 (Continued)**

Frozen Raspberries	Norovirus	Finland	2009	~900	The frozen raspberries were of Polish origin. Investigational research determined the raspberries contamination from an infected ill kitchen worker. The most common detected strain was GI.4 and GI.4 was also found.	Sarvikivi, E., Roivainen, M., Maunula, L., Niskanen, T., Korhonen, T., Lappalainen, M., Kuusi, M., 2012. Multiple norovirus outbreaks linked to imported frozen raspberries. <i>Epidemiology &amp; Infection</i> 140: 260-267.
Bread	Norovirus	Japan	2014	1,000	Norovirus was detected in 3 female employees at the company who supplied bread for school meals. Of the total population ill, 101 elderly patients were admitted to the hospital, 605 children from 14 different schools and 40 staff members. The outbreak also caused 4 deaths in the elderly community.	Xinhua (2014, January 22). 4 die in Japan after norovirus outbreak. <i>China Daily USA</i> . Retrieved from <a href="http://usa.chinadaily.com.cn/world/2014-01/22/content_17250812.htm">http://usa.chinadaily.com.cn/world/2014-01/22/content_17250812.htm</a>
Unknown	Norovirus	Overland Park, KS	2016	~600	The most recent outbreak is still under investigation. The source is unknown but the New Theatre Restaurant has been open since 2004.	News Desk (2016, February 2). New Theatre Restaurant norovirus outbreak infects more than 600. <i>Food Safety News: Breaking News for Everyone's Consumption</i> . Retrieved from <a href="http://www.foodsafetynews.com/2016/02/123136/#.VuUJqgZHSJc">http://www.foodsafetynews.com/2016/02/123136/#.VuUJqgZHSJc</a>
Green Onions	Hepatitis A	Monaca, PA	2003	~555	Of the approximate 555 persons ill, 3 died, 13 restaurant workers were ill, and 75 residents in six other states dined at the same restaurant. The CDC and PA Department of Health investigated the outbreak and of the 207 people with Hep A, 181 persons were determined as the case-patients. 94% of the case-patients reported eating mild salsa. The other 6% reported eating menu items containing green onions.	Centers for Disease Control and Prevention (CDC). (2003, November 28). Hepatitis A outbreak associated with green onions at a restaurant--- Monaca, Pennsylvania, 2003. <i>Morbidity and Mortality Weekly Report</i> . Retrieved from <a href="http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5247a5.htm">http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5247a5.htm</a>

**Table 1.1 (Continued)**

Raw Oysters	Norovirus	United Kingdom	January-March 2010	120	The oyster origin are similar between the 5 countries. Oysters causing the UK outbreak originated from England, Scotland, and Ireland. Oysters eaten in Norway and France originated from Brittany, France. Sweden also had oysters from France, but also some were imported from The Netherlands. Denmark imported oysters from various locations in France. Of all the outbreaks, genogroups I and II were found.	Westrell T, Dusch V, Ethelberg S, Harris J, Hjertqvist M, Jourdan-da Silva N, Koller A, Lenglet A, Lisby M, Vold L., 2010. Norovirus outbreaks linked to oyster consumption in the United Kingdom, Norway, France, Sweden and Denmark, 2010. <i>Eurosurveillance</i> 15(12): 1-4. Retrieved from <a href="http://www.eurosurveillance.org/images/dynamic/EE/V15N12/art19524.pdf">http://www.eurosurveillance.org/images/dynamic/EE/V15N12/art19524.pdf</a>
		Norway		39		
		France		67		
		Sweden		48		
		Denmark		58		

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## CHAPTER 2

### Characterization of Previously Selected Norovirus-Targeted Aptamers Using Two Novel Processes

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#### ABSTRACT

The lack of rapid and sensitive assays to detect human norovirus (HuNoV) contamination in food and environmental samples is one factor limiting the ability to control transmission. Nucleic acid aptamers are emerging ligands for use in pathogen capture and detection because of their high target binding affinity, ease of synthesis and functionalization, and stability. The purpose of this study was to evaluate candidate ssDNA aptamers for their binding affinity to HuNoV and to provide proof-of-concept that they could be used in a magnetic capture-detection method. Eight aptamer candidates previously produced using a GI virus-like particle (VLP) cocktail as target, and two produced using a GII VLP target, were screened for binding specificity and intensity using an Enzyme-Linked Aptamer Sorbent Assay (ELASA) performed on a panel of HuNoV VLPs. Similar assays were done using 20% stool suspensions obtained from infected individuals and fresh produce wash water inoculated with HuNoV. Aptamer AP4-GI bound to both GI and GII genogroup VLPs tested. Binding absorbance ratios for GI VLPs ranged from  $4.8 \pm 0.5$  (GI.8) to  $12.0 \pm 0.6$  (GI.1); and for GII VLPs ranged from  $6.5 \pm 2.7$  (GII.1) to  $19.8 \pm 5.0$  (GII.2 Snow Mountain). Aptamer affinities for virus in diluted human fecal samples and fresh produce wash water were dampened. A previously selected aptamer candidate, M6-2, was used in a magnetic-bead mediated capture assay followed by RT-qPCR for detection of Tulane Virus, a HuNoV

surrogate. The presence of the aptamer on the beads improved RT-qPCR assay detection limits by 1-2  $\log_{10}$  PFU, corresponding to  $10^3$ - $10^4$  PFU. This study presents proof-of-concept of the utility of ssDNA aptamers for capture and detection of HuNoV, adding another tool to the arsenal as we seek to develop better methods of detection for virus contamination in food and environmental samples.

## INTRODUCTION

Per epidemiological evidence, human norovirus (HuNoV) is the leading cause of acute nonbacterial gastroenteritis worldwide (Lamhoujeb et al., 2008). It is also the most common cause of foodborne illness in the United States, resulting in 58% of illnesses, 26% of hospitalizations, and 11% of deaths (Scallan et al., 2011). Due to their high infectivity yet low dose required for infection, these environmentally stable pathogens introduce a major public health concern. Unfortunately, despite their increasingly recognized significance, the availability of streamlined and routine detection methods has been restricted due to the absence of a reliable cell culture model. Although a substantial breakthrough occurred with the recent publication of a reproducible *in vitro* HuNoV cultivation method (Ettayebi et al., 2016), it is not yet ready for widespread use. For the time being, we are still relying on molecular-based amplification and ligand-based capture assays to aid in HuNoV detection.

Molecular amplification, specifically reverse transcriptase quantitative polymerase chain reaction (RT-qPCR), has been the gold standard for HuNoV detection and genome quantification in contaminated food and environmental samples, and to a certain extent, clinical diagnostics (Moore et al., 2015b). Unfortunately, because virus concentrations are often low in food and environmental samples and sample sizes are large, it is necessary to

concentrate and purify the viruses from the matrix before detection. These steps are complicated, time-consuming, and almost always result in virus loss. Even with this so-called pre-analytical sampling (sample prep), residual matrix components frequently remain and result in PCR inhibition (Escudero-Abarca et al., 2014). In addition, the highly sensitive and specific molecular amplification procedures are unable to discriminate between infectious and non-infectious viral agents.

Working towards distinguishing between infectious and non-infectious viral particles, capture ligands (e.g., putative cell receptors, antibodies) can be created against conserved areas of antigens. Antibodies are the most common capture ligand used in food microbiology, and some are available with specificity to HuNoV. However, due to the genetic and antigenic diversity, antibodies lack broad reactivity to bind to all the important human strains of the virus (Shiota et al., 2007). When used in detection assays, the result is poor assay sensitivity. For example, Costantini et al. (2010) evaluated diagnostic accuracy, sensitivity, and reactivity of the IDEIA (enzyme immunoassay) kit for HuNoV detection in clinical samples. The study found the kit to be 57.6% sensitive to the 557 HuNoV samples screened and 91.9% specific. Low sensitivity may occur due to a variety of factors, but the most important is the antigenic diversity of HuNoV strains (Costantini et al., 2010). Besides immunoassay designs, other candidate ligands have been explored such as histo-blood group antigens (HBGAs, the putative HuNoV host cell receptor) and porcine gastric mucin [contains naturally-occurring HBGAs (Huang et al., 2005; Patel et al., 2009; Tan and Jiang, 2005)], but neither has been reported to be completely inclusive of all HuNoV strains. In short, detection of HuNoV could be greatly aided by the availability of broadly reactive ligands but they have yet to be identified.

Nucleic acid aptamers are short single-stranded oligonucleotides (DNA or RNA) that specifically bind to molecular targets such as proteins, viruses, bacteria, and eukaryotic cells. Ranging from 20-100 nucleotides in length (Ni et al., 2011), aptamers are promising ligands for HuNoV capture and detection. They are selected through an *in vitro* biopanning method known as SELEX (Systematic Evolution of Ligands by EXponential enrichment), which entails multiple sequential rounds of target selection and enrichment. As the rounds continue, aptamer pools will show an increased binding affinity to the target that is frequently mediated by an overlap of ligand sequences between aptamers, called motifs. Nucleic acid aptamers offer advantages over previously used ligands because they are easily synthesized, purified, and modified. They are also highly stable and inexpensive. In previous work, aptamers with broad reactivity among different HuNoV strains have been reported (Escudero-Abarca et al., 2014).

The purpose of this study was to evaluate candidate ssDNA aptamers for their binding affinity to HuNoV and to provide proof-of-concept that they could be used in a capture-detection method applied to a model food matrix. Previously selected aptamers (Escudero-Abarca et al., 2014; Moore et al., 2015a) were characterized for strain-specific binding to virus-like particles (VLPs) representative of multiple genotypes. An enzyme-linked aptamer sorbent assay (ELASA) was applied to observe the binding affinity of the selected aptamers to HuNoV VLPs, infectious HuNoV particles, and leafy green produce wash water seeded with VLPs and native virus. In addition, a previously selected HuNoV GII-targeted aptamer (Moore et al., 2015a) was used in aptamer magnetic capture (AMC) of Tulane Virus, a HuNoV surrogate (Tian et al., 2013), followed by detection using RT-qPCR. The collective data show proof-of-concept that ssDNA aptamers with relatively broad

reactivity can have applications for concentrating HuNoV from complex sample matrices such as food and human stool.

## MATERIALS AND METHODS

### **Viruses and Virus-Like-Particles (VLPs)**

Confirmed GII.4 Sydney (Syd), the most prevalent genotype among humans, and GI.6 were obtained as outbreak-derived human fecal specimens courtesy of S.R. Greene (North Carolina Department of Health and Human Services, Raleigh, NC). Norwalk virus (GI.1) stool samples, in addition to HuNoV-negative stools, were received courtesy of C. L. Moe (Emory University, Atlanta, GA) from a human challenge study. All stool samples were suspended 20% in phosphate-buffered saline (PBS) and stored at  $-80^{\circ}\text{C}$  until experimental use. Tulane virus (TV) was propagated and enumerated in LLC-MK2 cells (Farkas et al., 2008) cultured in M199 media (Corning, Manassas, VA) supplemented with 10% fetal bovine serum (FBS; Thermo Scientific, Waltham, MA) and 1% Pen/Strep antibiotic (Thermo Scientific). Virus stock was prepared using 90% confluent monolayers of LLC-MK2 cells that were infected with TV and incubated at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$  for 48 h, or until overt cytopathic effects were observed. Viruses were then harvested by three consecutive rounds of freeze-thaw followed by centrifugation at  $12,000 \times g$  for 20 min, after which virus-laden supernatant was collected and stored at  $-80^{\circ}\text{C}$  until use. Plaque assays for TV quantification were done using LLC-MK2 cells as previously described (Farkas et al., 2008). Tulane virus purification was performed in lab by N. Montazeri-Djouybari (North Carolina State University, Raleigh, NC) and also stored at  $-80^{\circ}\text{C}$  until use. Table 2.3 shows the characteristics of the viruses used in this study. Self-assembled virus-like-particles (VLPs),

which consist of purified virus capsid without the viral genome, were kindly provided by R. Atmar (Baylor College of Medicine, Houston, TX). The VLPs (Table 2.2) used for this study were GI.1 Norwalk, GI.6, GI.7, GI.8, GII.1, GII.2 Snow Mountain (SMV), GII.4 (Grimsby, Syd, and 2012), GII.12, and GII.17. All VLPs were stored at 4°C until use. Collard greens produce wash water was collected from a farm in Faison, NC and stored at -20°C until use.

### **ssDNA Aptamers**

Aptamers (Table 2.1) selected using a graphene oxide-based SELEX (Systematic Evolution of Ligands by EXponential Enrichment) protocol were kindly prepared by B. Escuerdo-Abarca and M. Moore. Aptamers AP1-GI, AP2-GI, AP3-GI, AP4-GI, AP5-GI, AP6-GI, 4S10, and 9S10 (Table 2.1) were all produced using a GI cocktail of VLPs that included GI.1, GI.6, GI.7, and GI.8 as targets. Aptamers AP1-AP6 were selected from the 5<sup>th</sup> SELEX round; 4S10 and 9S10 were obtained from round 10. Aptamers SMV-19 and SMV-21 (Escudero-Abarca et al., 2014) were produced using the whole semi-purified GII.2 Snow Mountain Virus (SMV) as the target. Previously selected aptamers M6-2 Moore et al. (2015a) were produced using the P domain of HuNoV GII.4 2007.

### **Binding Affinity Analysis using Enzyme-Linked Aptamer Sorbent Assay (ELASA)**

Binding affinity assays were completed with 5' biotinylated labeled aptamers (Table 2.1), VLPs (Table 2.2), and in some experiments, using 20% stool suspension (positive for HuNoV or virus-negative) (Table 2.3) using an ELISA-like protocol in which antibodies were replaced with aptamers (Escudero-Abarca et al., 2014). Specifically VLP suspensions (0.9-5.4 mg/ml) were adjusted to a concentration of 3µg/ml in either 1X PBS (pH 7.4),

produce wash water, or 20% stool suspensions (with and without 10-fold serial dilution). One hundred microliters of each suspension were placed in each well of a covered, flat-bottom polystyrene 96-well plate (Costar 3591, Fisher, Pittsburg, PA) and incubated overnight with agitation at 4°C. After coating the wells with the sample, removal of fluid occurred and the wells were blocked with 200 µl of 5% skim milk in PBS containing non-related DNA oligonucleotides [*L. monocytogenes* primers hlyQF/R and L23SQF/R (Rodríguez-Lázaro et al., 2004)]. The plate was incubated overnight at 4°C with gentle agitation and then washed three times with 0.05% PBS-Tween (PBST) before the addition of 100µl of biotinylated aptamer (0.5µM). Aptamer incubation took place for an hour at room temperature (RT) with gentle shaking. After, excess aptamer solution was discarded and the plate was again washed four times with PBST. Subsequently, 100µl of ELISA-grade streptavidin-horseradish peroxidase (1mg/ml, 1:5000, Invitrogen, Carlsbad, CA) was added to each well and incubated at RT for 15 min with mixing. The unbound enzyme was removed and rewashed with PBST thrice followed by addition of 100µl of 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase substrate system [solution A:B (1:1), KPL, Gaithersburg, MD] for color development. After RT incubation for 7 min, 100µl of TMB stop solution (KPL) was added and absorbance was read at 450nm using a microplate reader (Tecan Infiniti M200pro, Tecan Group Ltd., Männedorf, Switzerland). Negative controls consisted of wells without the addition of VLPs or virus. In regards to stool samples, positive stool specimens were naturally contaminated with HuNoV, whereas negative stool samples contained no virus. The produce wash water positive and negative controls were also prepared with and without virus inoculum, respectively. No aptamer controls are ratios of pure 1X PBS, showing a binding

ratio neither aptamer or target present. In the case of the aptamer cocktail experiments, a 1:1 solution with a final concentration of 0.5 $\mu$ M was used.

All ELASA results were replicated on three separate occasions with at least three wells per plate for each treatment. Data were expressed in ratios between the absorbance values for the test samples divided by those of the negative control. Specifically, in the case of VLP binding, ratios were calculated based on absorbance in wells with and without VLP present. For stool samples and produce wash water, ratios were calculated based on a negative control of uninoculated matrix. As per convention (Escudero-Abarca et al., 2014), ratios of absorbance less than 2.0 were considered low to no binding; 2.0-5.0 were considered low binding efficiency; 5.0-10.0 exhibited medium binding efficiency; and >10.0 were considered strong binding. Means and standard deviations for all ratios samples were calculated using Microsoft Excel.

### **Aptamer Magnetic Capture (AMC)**

Following the protocol explained in Moore et al. (2015a), biotinylated aptamer M6-2 was used to concentrate unpurified Tulane virus. Thirty micrograms of Dynabeads® MyOne Streptavidin C1 magnetic beads (Invitrogen-Dynal AS, Oslo, Norway) were suspended in 1 ml PBS and mixed with 0.05% PBST. Following, the beads were captured using the Dynal MPC-M magnetic particle concentrator (Invitrogen-Dynal) and resuspended in 1 ml of 5% skim milk with an overnight blocking step at 4°C with rotation. Thereafter, the beads were washed twice with 500 $\mu$ l PBST and resuspended in 50 $\mu$ l PBST after which they were stored at 4°C until use. These beads were identified as the “blocked beads”. Moving forward, serial dilutions (1:1, 1:10, 1:100, 1:1000, and 1:10000) of crude (cell culture lysate) Tulane virus

were prepared in PBS. One hundred microliters of each dilution was placed into a dedicated tube containing 900µl PBST and 15µl of biotinylated M6-2 aptamer (100µM). Next, samples were rotated for 1 h at 22°C. After rotation, 50µl of blocked beads was added and another rotated incubation occurred for 1 h at 22°C. Beads were magnetically recovered and washed with 500µl PBST once and subsequently washed with 500µl of PBS. Following the washing, the beads were resuspended in 100µl PBS and stored at –80°C until RNA extraction. Negative controls contained 450µl PBST, 450µl Superblock T20 (Thermo Fisher), 100µl of PBS, and 50µl of blocked beads.

### **RNA Extraction and RT-qPCR for Tulane Virus**

For AMC-captured Tulane virus, RNA extraction was performed using the NucliSENS® easyMAG system (bioMerieux SA, Marcy l’Etoile, France) abiding to the manufacturer’s instructions. A final elution volume of 40µl was recovered and the eluted RNA was stored at –80°C until RT-qPCR. Ten-fold serial dilutions of RNA extracts were amplified using RT-qPCR with primers described in Table 2.4. One step RT-qPCR with the Superscript III Platinum One-Step kit (Invitrogen, Grand Island, NY) supplied the ingredients to perform the amplifications. Following the procedure detailed in Gentry-Shields et al. (2015), the RT-qPCR mixture contained 2.5µl of RNA, 400nM of forward and reverse primers, 200nM of fluorescently labeled TaqMan probe, 12.5µl 2X reaction buffer, and 0.5µl RT/Platinum® Taq Mix resulting in a 25µl reaction volume. RT-qPCR amplification was done using a CFX96 Touch™ Real Time PCR Detection System (Bio-Rad, Hercules, CA). Reverse transcription occurred for 30 min at 50°C following initial denaturation for 15 min at 95°C. Directly after denaturation, amplification was done for 45 cycles of 15 s at 95°C and

30 s at 60°C. Amplifiable RT-qPCR units were estimated based on a standard curve of Ct values.

### **Statistical Analyses**

All statistical analyses were performed using R Studio (R Studio, Boston, MA) and data were expressed as mean  $\pm$  standard deviation of three replicates of each experiment, except for naturally contaminated stool samples. One-way analysis of variance (ANOVA) was performed to discover statistical significance for aptamers and VLPs or viruses. If overall statistical significance was found, a pairwise t-test was performed to determine significance between treatments. Values of  $p < 0.05$  were considered statistically significant.

## **RESULTS**

### **Characterization of Binding Affinity of Selected ssDNA Aptamer Candidates to VLPs**

Aptamer candidates from SELEX round 5 (AP1-GI, AP2-GI, AP3-GI, AP4-GI, AP5-GI, and AP6-GI) exhibited positive binding affinities to both GI and GII VLPs [as defined by binding ratios (sample/no sample) over 2.0] by ELASA. For GI VLPs, binding ratios ranged from a low of 2.1 to a high of 12.2. There were statistically significant differences when comparing binding ratios of any one aptamer across all VLPs (Figure 1). However, there were no statistically significant differences in absorbance ratios when comparing a single VLP across aptamer types, except for the GI.8 VLP. These same aptamers displayed binding ratios ranging from 3.0 to 19.8 (Figure 2) when screened using a panel of GII VLPs. Genotype-specific differential binding affinity was observed and significant differences were seen in when comparing binding ratios of any one aptamer across all VLPs. Collectively, all

six aptamers showed variable binding affinity to different HuNoV genotypes, but the highest binding affinity occurred for GII.2 SMV VLP, followed by GI.1 Norwalk VLP (the target for SELEX). Overall, binding affinities were higher for GII VLPs compared to those in genogroup I. Although all of the SELEX round 5 aptamers bound to all of the VLPs screened, aptamer candidate AP4-GI demonstrated medium to strong binding with GI.1 Norwalk, GI.7, GI.8, GII.1, GII.2 SMV, GII.4 GRV, GII.4 HOV, GII.4 SYD, and GII.17 and hence was the most broadly reactive. Consequently, AP4-GI was chosen for use in further studies.

For comparison purposes, SELEX round 10 aptamers 4S10 and 9S10 were also screened against the same VLPs (Figure 3). Both aptamers showed at least some binding affinity to all VLPs tested except for GII.1. Similar to the SELEX round 5 candidates, both 4S10 and 9S10 bound strongly (high absorbance ratios) to the GII.2 SMV VLPs, but comparatively lower binding ratios were observed for the GII.4 VLPs. For this reason, the round 10 aptamers were no longer studied.

### **Binding Affinity to a Cocktail of Two Complementary ssDNA Aptamers**

Aptamers SMV-19 and SMV-21, targeted against partially purified GII.2 SMV stock, were previously reported to show binding affinity for both GI and GII VLPs (Escudero-Abarca et al., 2014). To investigate if combining complementary aptamers having different affinity patterns might improve overall performance of binding, an aptamer cocktail (1:1) of AP4-GI and SMV-19, and a second aptamer cocktail (1:1) of AP4-GI and SMV-21 (final concentration of 0.5 $\mu$ M) were screened against all the VLPs used in the previous experiments. The results are displayed in Figures 4 and 5. Statistical analysis provided

information on the degree of binding efficiency across each VLP type for any one aptamer. The patterns for absorbance ratios were identical when comparing the two cocktails, but the AP4-GI/ SMV-19 combination performed better (i.e., gave higher binding ratios and statistically significant binding ratios across strain types). As was seen in other studies, binding to GII stains was better than that to GI strains, however the use of a cocktail did not enhance the binding efficiencies compared to absorbance ratios of VLPs with AP4-GI.

### **Aptamer Binding Affinity in the Fecal Matrix**

To evaluate the impact of the fecal matrix, ELASA using AP4-GI was performed using serially diluted partially purified outbreak-derived 20% fecal specimens (Figure 6). The quantity of GI.1 positive stool available for these studies was extremely limited, so data were not produced in triplicate. In the ELASA, the fecal matrix produced a large amount of assay interference, and it was necessary to dilute the fecal specimens to as high as  $10^{-3}$  in order to achieve interpretable results. That being said, the dilution of the GI.1 Norwalk stool demonstrated low binding affinity to AP4-GI with an absorbance ratio of 3.8, whereas GI.6 and GII.4 Syd positive stools were not considered positive.

### **The Impact of a Food Matrix on Aptamer Binding Efficiency**

Because of the inhibitory nature of the fecal matrix, we were interested as to whether a food matrix might introduce similar difficulties. A comprehensive study was undertaken to evaluate this using aptamer AP4-GI and fresh produce wash water. In the first phase, serially diluted produce wash water (undiluted to  $10^{-3}$  diluted) was spiked with a standardized concentration (3 $\mu$ g/ml) of GI.1 Norwalk and GII.2 SMV VLPs and subjected to ELASA

(Figure 7). The average absorbance ratios for GII Norwalk VLPs in undiluted wash water fell close the positive cut-off value (ratio of 2.0) but gradually, yet not significantly, increased with further matrix dilution (from 6.4 to 9.3). For the GII.2 SMV VLPs, the same phenomenon occurred and serial dilution of the produce wash water significantly improved absorbance ratios from 11.9 to 20.2. Not unexpectedly, the positive control (VLP spiked in 1X PBS) exhibited the strongest binding of VLPs to AP4-GI. The phenomenon of increasing absorbance ratios corresponding to greater dilutions of the matrix suggests the presence of sample-associated inhibitors.

To further test the hypothesis of reduced aptamer binding affinity due to matrix inhibition, a similar experiment was done but in this case, the 10-fold serial dilutions of produce wash water were seeded with 20% stool, either negative or positive for the presence of HuNoV GII.4 (Syd). Figure 8 displays these results. There were no statistically significant differences when comparing absorbance ratios produced using stool with or without HuNoV. Curiously, both positive and negative stool samples exhibited no statistically significant differences in absorbance ratios as the dilutions increased.

To further understand the inhibition problem, we also inoculated stool (with and without HuNoV) into produce wash water and serially diluted the inoculated wash water (undiluted to  $10^{-4}$  diluted) followed by application of ELASA. There was evidence of aptamer binding affinity for wash water samples inoculated with either HuNoV positive and negative stool, although the ratios for the former were a little bit higher than for the latter (Figure 9). In conclusion, very little trend could be ascertained from all the matrix studies.

## **Aptamer Magnetic Capture (AMC) Followed by RT-qPCR for Concentration and Detection of Tulane Virus**

Previous work in our laboratory has suggested that ELASA performs best when used in pristine samples and with high virus concentrations (i.e., VLPs). For complex samples, magnetic capture methods followed by RNA extraction and nucleic acid amplification are a practical and well-accepted method for detection of a wide variety of foodborne pathogens. As proof-of-concept that ssDNA aptamers could be used for capture of norovirus, aptamer M6-2, previously shown to bind to the HuNoV surrogate Tulane virus (data not shown), was tethered to magnetic beads and used to capture serially diluted virus in suspension. Table 2.4 lists the primers and probe use for TV detection via RT-qPCR. Figure 10 provides a visual display of a RT-qPCR cycle threshold (Ct) values for capture and subsequent detection of serially diluted Tulane virus stocks using magnetic beads alone and those to which aptamer M6-2 was tethered. Although virus did bind to uncoated beads, Ct values for the aptamer-bound beads corresponded to an added value of the aptamer of about 1-2  $\log_{10}$  enrichment in virus concentration.

### **DISCUSSION**

In these studies, previously selected aptamers were tested for their reactivity (quantitative binding affinity) to various human norovirus VLPs and the Tulane virus surrogate using Enzyme-Linked Aptamer Sorbent Assay (ELASA) and Aptamer Magnetic Capture (AMC). Evidence that HuNoV in naturally contaminated human stool and fresh produce wash water can be captured using these aptamers was also sought. So was the use of AMC to concentrate Tulane virus, a HuNoV surrogate, prior to the use of RT-qPCR for

detection. To our knowledge, this is the most comprehensive evaluation of ssDNA aptamer performance for HuNoV, and demonstration that aptamers can be used for virus capture in complex sample matrices and using a magnetic capture method.

Aptamers are oligonucleotides capable of binding with high affinity and specificity to a wide range of target molecules. Selected through SELEX, these stable molecules can fold *in vitro* into structures containing loops or hairpin-loops that are involved with target-receptor binding (Moore et al., 2015a; Tombelli et al., 2005). The three-dimensional structures allow for specific ligand recognition between nucleic acid bases (Hermann and Patel, 2000). Motifs, short recurring patterns in DNA presumed to have biological function, are the hairpin loops that help dictate binding strength through stacking of aromatic rings, electrostatic and van de Waals interactions, and hydrogen bonding (Hermann and Patel, 2000). In addition to structural components, aptamers showing low free Gibbs energy ( $\Delta G$ ) indicate high stability. A compilation of structural motifs and low  $\Delta G$  towards a specific target makes aptamers a promising alternative to antibodies. Since aptamers are created to bind to a specific target, binding sites may be located in different regions of the viral capsid or to more highly conserved areas that occur across genotypes.

In general, the ELASA ratios of absorbance were higher for GII strains, specifically GII.2 SMV, compared to GI strains. This was unexpected for the GI targeted aptamers (produced using a GI cocktail target) because the major capsid protein (VP1) amino acid sequences for GI strains differ substantially from those of GII strains. Despite this, the SELEX round 5 aptamers displayed broadly reactive binding with both GI and GII strains (Figures 1 and 2). Collectively, these aptamers displayed similarly high absorbance ratios when tested with GI.1, GI.6, GI.7, and GI.8 VLPs, less so for the GI.8 VLP. When screened

against GII VLPs (Figure 2), all absorbance ratios were positive per convention (ratio > 2.0). Although statistical significance between binding affinities were noted in some cases, aptamers AP1-GI through AP6-GI were broadly reactive against GI and GII VLPs. Similar to their SELEX round 5 predecessors, the round 10 GI targeted aptamers 4S10 and 9S10 were also reactive to a range of GI and GII VLPs. Like the SELEX round 5 aptamers, both 4S10 and 9S10 performed exceptionally well in binding to the GII.2 SMV VLP. Unlike AP1-GI through AP6-GI, though, 4S10 and 9S10 showed high binding to GI.6 VLP. The major binding trends, which were similar for all the round 5 aptamers and the two round 10 aptamers suggest similarities in structure. All the aptamer candidates displayed similar folding structures and low  $\Delta G$  values (data not shown). This is not surprising as they all were targeted towards the same GI VLP cocktail, although they may well differ in their specific target binding regions.

Identification of other novel HuNoV binding ligands has been the subject of recent studies. Rogers et al. (2013) demonstrated the use of phage peptides to capture HuNoV VLPs and compared their sensitivity to that of a HuNoV monoclonal antibody (MAb 3912). The phage were able to bind to the major capsid protein of VLPs, and could be used to detect the virus in positive stool suspensions. They were also found to have a similar limit of detection compared to Mab 3912. Similarly, Huang et al. (2014) compared the use of antibodies against phage peptides for HuNoV capture using an ELISA based method. It was found that both phage peptides and antibodies were able to capture HuNoV VLPs, but ELISA-based diagnostics exhibited poor sensitivity, presumably due to the cross reactivity of the ligands with matrix components. Kou et al. (2015) assessed the performance of monoclonal and polyclonal antibodies against HuNoV VLPs and fecally contaminated stool

specimens. The study found virus capture with the ligands, continuing to strengthen the method of using receptor based binding for virus detection in complex matrices.

An interesting aspect of our study was the use of a combination of aptamers to see if a potential increase in binding affinity or scope of reactivity could be observed. This was done using a 1:1 combination of AP4-GI and SMV-19, and a 1:1 ratio of AP4-GI and SMV-21. Aptamers SMV-19 and SMV-21 were previously reported by Escudero-Abaraca et al. (2014), selected for using GII.2 SMV, and exhibited positive binding ratios for various GI and GII VLPs. Combinations were made based on complementarity of binding affinity. For instance, aptamer SMV-19 did not bind well to GI.8 and GII.12 VLPs whereas AP4-GI did. Similarly, SMV-21 had low binding affinity for GI.1, GI.6, and GI.8 VLPs while AP4-GI showed strong binding affinity for GI.1 and better binding to GI.6 and GI.8 VLPs than the former. Unfortunately, using a combination of the aptamers neither strengthened nor weakened the resulting absorbance ratios. While the reason for this result is unknown, it is possible that the aptamers did not cohesively bind to the targets, or that they competed with each other for target binding, or that they bound to one another instead of to the target. It could also be a function of reagent concentrations or assay design. The idea remains a credible one and deserves further study.

Despite their broadly reactive nature, aptamer binding was inhibited with the introduction of complex sample matrices. For instance, even with dilution, the absorbance ratios of GI.1 GI.6, and GII.4 (Syd) HuNoV-positive stool samples tested with AP4-GI by ELASA were very low ( $\leq 4.0$ ), with reactivity of the aptamer only found for stool positive for GI.1. Extensive studies were undertaken to understand the impact of inhibition in another sample matrix, produce wash water. In early experiments, there was a decrease in aptamer

binding affinity with an increase in matrix complexity. For example, when undiluted VLP-spiked produce wash water (Figure 7) was used in ELASA, low to no binding was observed, yet binding affinities improved as the matrix material was diluted in PBS by up to 1,000-fold. On the other hand, when the same experiment was done using HuNoV inoculum rather than VLPs, a consistent absorbance ratio (i.e., not increasing with subsequent dilution) was observed, suggesting minimal assay inhibition (Figure 8). Finally, when the matrix was seeded with either HuNoV- positive or negative human stool, there was evidence of non-specific binding as demonstrated by positive absorbance ratios for sample seeded even with virus-free human stool (Figure 9). Matrix associated interference with ligand binding has been observed by others (Escudero-Abarca et al., 2014; Kou et al., 2016) but interestingly, Rogers et al. (2013) did not observe it using peptide phage ligands. Our conflicting results could be a function of the ELASA itself, since it tends to have a narrow detection range and higher sensitivity to inhibition. Future work using a different assay design (e.g., aptamer magnetic capture) is warranted.

Ligand-based magnetic capture is frequently used in food microbiology, i.e., as part of the USDA-FSIS detection method for Shiga-toxin producing *E. coli* (United States Department of Agriculture, MLG 5B.05, 2014). Aptamer magnetic capture (AMC) was used in this study as proof-of-concept that these new ligands could be used in a more applied setting. Tulane virus (TV), a surrogate for HuNoV (Kingsley et al., 2014; Predmore et al., 2015), was utilized in the absence of a HuNoV *in vitro* cultivation method. Overall, there was evidence of greater virus capture (by as much as 1-2 log<sub>10</sub> PFU) when the beads were conjugated with aptamer than for unconjugated (control) beads. Detection was possible at a 1:1,000 dilution of Tulane virus, one log<sub>10</sub> better than the control and corresponding to 10<sup>3</sup>-

10<sup>4</sup> PFU. This is similar to the findings reported in Moore et al. (2015a). Harrington et al. (2004) used HBGA coated magnetic beads to capture HuNoV VLPs and virus from naturally contaminated stool samples, finding that high titers were required for efficient capture. Similarly, Morton et al. (2009) used HBGA and porcine gastric mucin (PGM)-coated magnetic beads for HuNoV detection in RTE foods, also finding more reliable results with higher input virus concentrations. Our lower detection limits are at least as good, if not better, than these previous studies.

As previously noted, the inability to discriminate between infectious and noninfectious viral particles is a dilemma faced when developing a reliable, rapid assay for detection of HuNoV. Simply relying on PCR-based assays do not aid in such discrimination because viral RNA can remain stable and amplifiable long after the capsid has been disrupted and infectivity abolished. However, ligand capture followed by RT-qPCR can serve as a proxy for detection of infectious virus is one assumes that only intact, infectious virus can bind to the ligand. Studies to try to prove this phenomenon have been done. For example, Dancho et al. (2012), using PGM attached to magnetic beads to capture particles exposed to three lethal treatments (high temperature, UV irradiation, and high pressure) were able to prove that virus attachment to PGM depends on capsid integrity. Hirneisen & Kniel (2012) used PGM and antibody-coated beads to distinguish between infectious and noninfectious murine norovirus exposed the virus to heat, high pressure, ozone, and ultraviolet light, and included infectivity assay. Their results were mixed. Heat inactivation inhibited binding to both PGM and antibody, but binding was not inhibited after high pressure, ozone, and UV treatment. More studies are needed to definitively establish the value of ligand capture on HuNoV infectivity discrimination, and these are underway in our laboratory.

The ELASA offered a high throughput means by which to evaluate aptamer binding efficiency using VLPs. While the method provided reliable comparative data for VLPs, its utility for screening whole virus capture in food and environmental samples may be limited. Specifically, ELASA tends to have high specificity, low sensitivity, elevated detection limits, and particular sensitivity to matrix effects. Poor or conflicting results when used with more complex samples may occur due to lower titers of virus compared to VLPs; matrix-associated interference with virus binding to plates; or non-specific binding (noise). Another consideration when interpreting these results is the use of VLPs as the target in creation of the GI aptamers. Yes, positive binding affinities were demonstrated for aptamers against VLPs and viruses, but VLPs can have minor structural differences when compared to native virus, a phenomenon that may impact binding affinity. This would be further complicated when evaluating virus binding from complex matrices. Regardless, the data presented here supports the utility of aptamers and suggests that their further development and evaluation are merited.

Overcoming the use of animals for antibody production, aptamers grant non-immunogenicity allowing for wider parameters for target and ligand functionality (Tombelli et al., 2005). Aptamers are also smaller in size relative to antibodies, which makes it easier for aptamers to be synthesized, chemically modified, and enables them to access protein epitopes that may be less accessible (Stoltenburge et al., 2007). Structurally, aptamers are more stable compared to antibodies, and have the ability to be reversibly denatured (Keefe et al., 2010). The broadly reactive and stable nature of aptamers bodes well for their use in HuNoV capture and detection. As seen in the study, the aptamers studied here were broadly reactive to a range of HuNoV VLPs. Data was also presented that ssDNA aptamers could be

used as capture ligands in both ELISA and aptamer-mediated magnetic capture assays. Similar results have been reported by others (Escudero-Abarca et al., 2014; Giamberardino et al., 2013; Moore et al., 2015a). The need for a rapid, reliable assay for HuNoV detection for food and environmental samples remains but aptamers offer a promising tool to help address this issue.

#### ACKNOWLEDGE

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**Table 2.1**

Aptamers screened for binding specificity and affinity using a broad panel of VLPs.

Target	Reference
<b>GI</b>	
AP1	Not published
AP2	
AP3	
AP4	
AP5	
AP6	
4S10	Not yet published
9S10	
<b>GII</b>	
SMV-19	Escudero-Abarca et al., 2014
SMV-21	
M5	Moore et al., 2015
M6-2	

**Table 2.2**

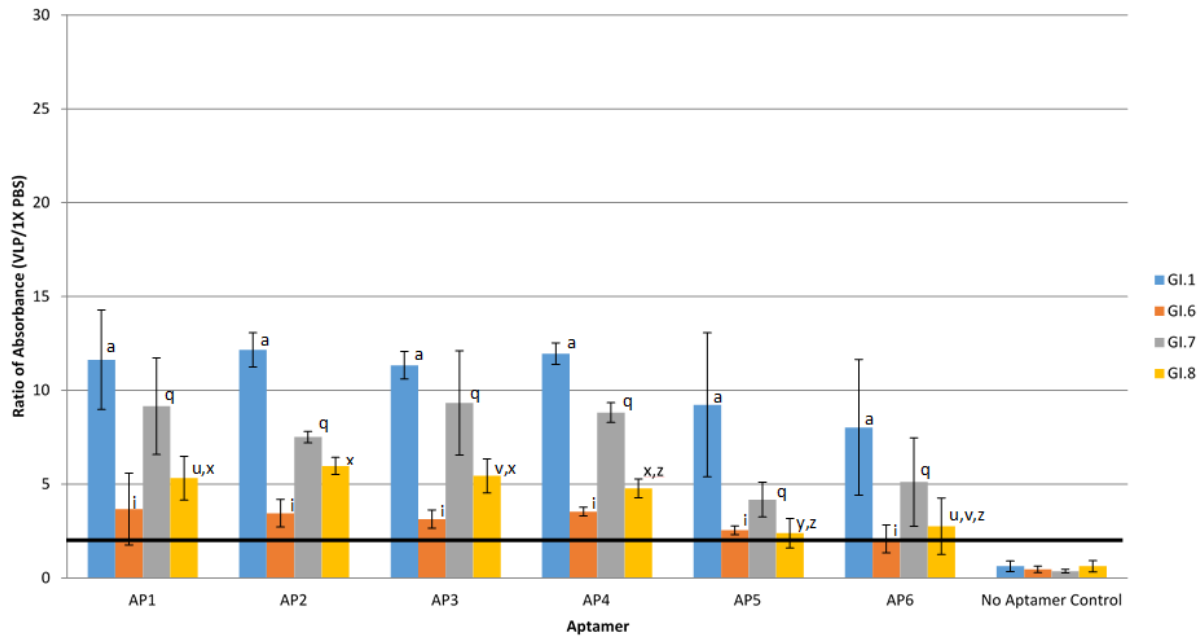
Virus-Like-Particles (VLPs) used in binding affinity studies for select ssDNA aptamers.

GI	GII
GI.1 Norwalk	GII.1
GI.6	GII.2 Snow Mountain
GI.7	GII.4 Grimsby
GI.8	GII.4 Sydney
	GII.4 2012
	GII.12
	GII.17

**Table 2.3**

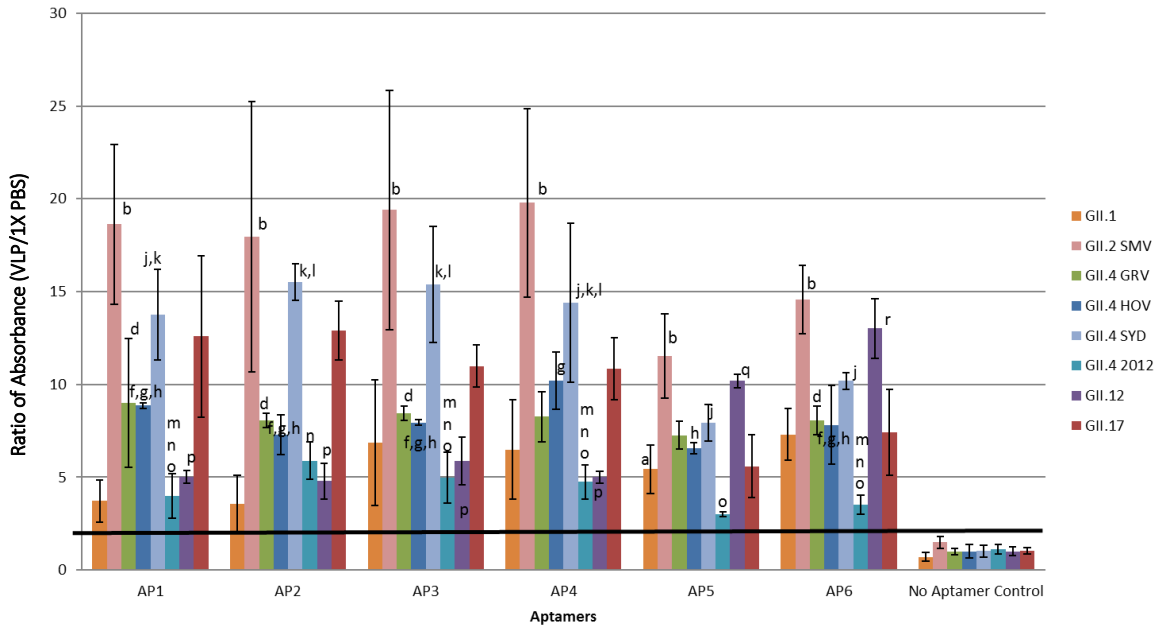
Characteristics of HuNoV and the cultivable surrogate Tulane virus that were used in binding affinity studies for select ssDNA aptamers.

Virus	Particle Size (nm. diam.)	Genome length (kb)	Isoelectric Point (pI)	Genus
<b>Human norovirus</b>	27-37	7.5-7.7	5.5-6.0	<i>Norovirus</i>
GI.1 Norwalk				
GI.6 NV12-094				
GII.4 Sydney NV14-016				
<b>Tulane Virus (TV)</b>	40	6.7	10	<i>Recovirus</i>



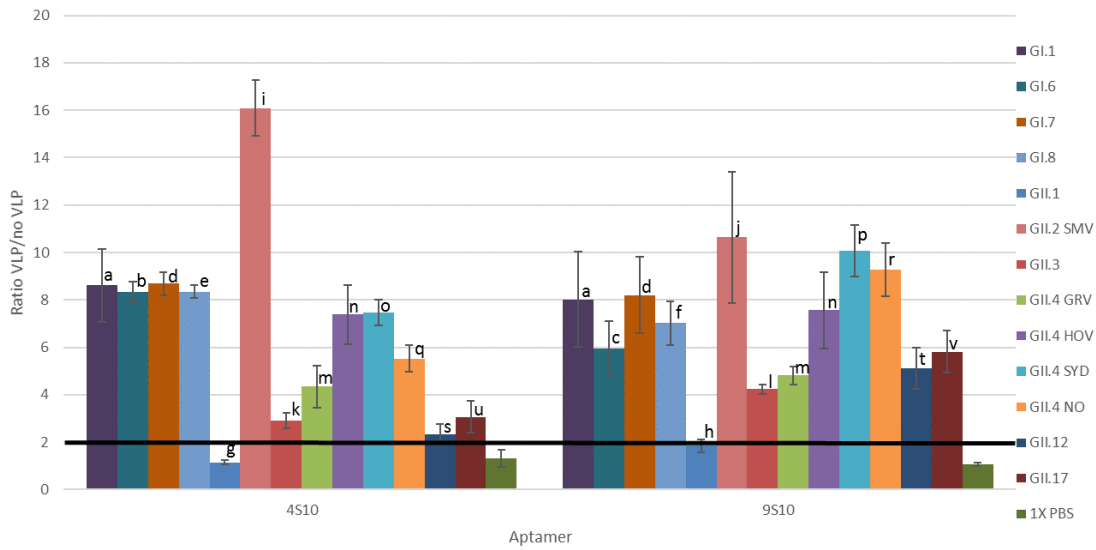
**Figure 2.1**

Binding ratios of GI aptamers against GI and GII VLPs. Data are presented as ratio of absorbance read at 450nm for VLP versus PBS negative control wells. Results less than 2.0 are considered negative per convention; 2.0-5.0 low binding; 5.0-10.0 medium binding; and >10.0 strong binding. There were statistically significant differences when comparing binding affinity of any one aptamer across all VLPs. Different letters indicate statistically significant differences in absorbance ratios when comparing a single VLP across multiple aptamers.

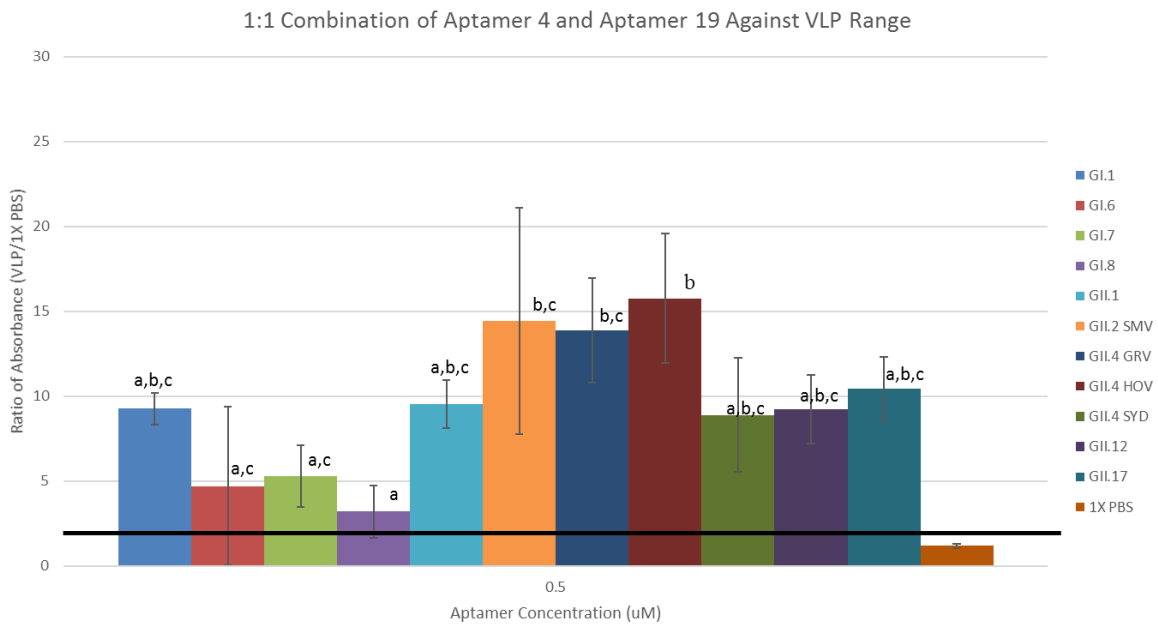


**Figure 2.2**

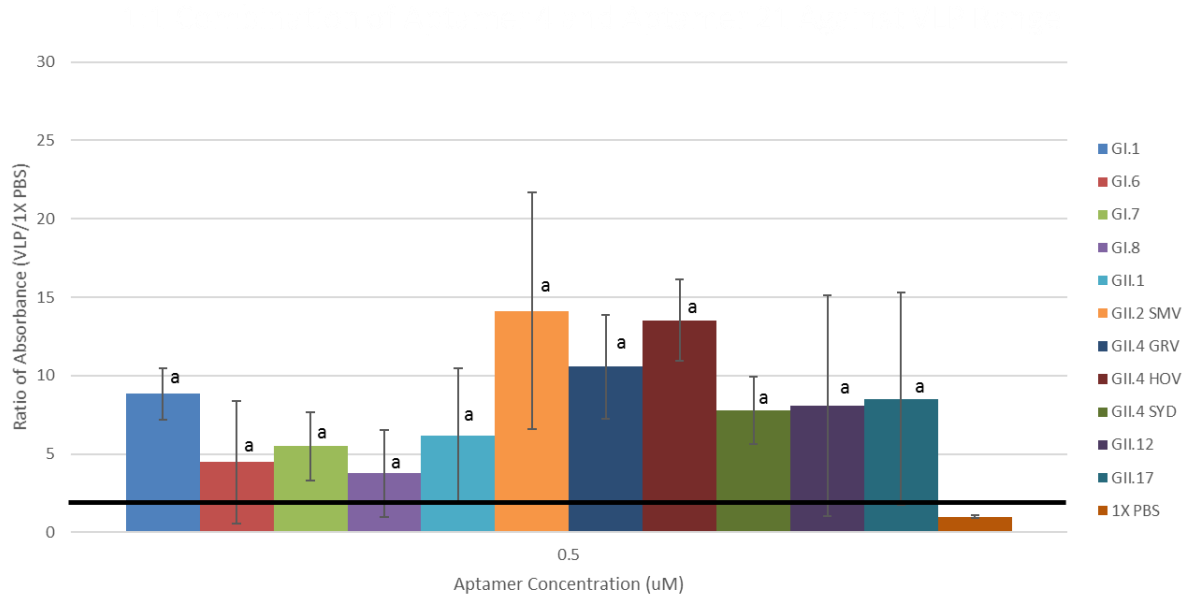
Binding ratios of GII GLPs to selected aptamers by ELASA. Data are presented as ratio of absorbance read at 450nm for VLP versus PBS negative control wells. Results less than 2.0 are considered negative per convention; 2.0-5.0 low binding; 5.0-10.0 medium binding; and >10.0 strong binding. There were statistically significant differences when comparing binding affinity of any one aptamer across all VLPs. Different letters indicate statistically significant differences in absorbance ratios when comparing a single VLP across multiple aptamers.



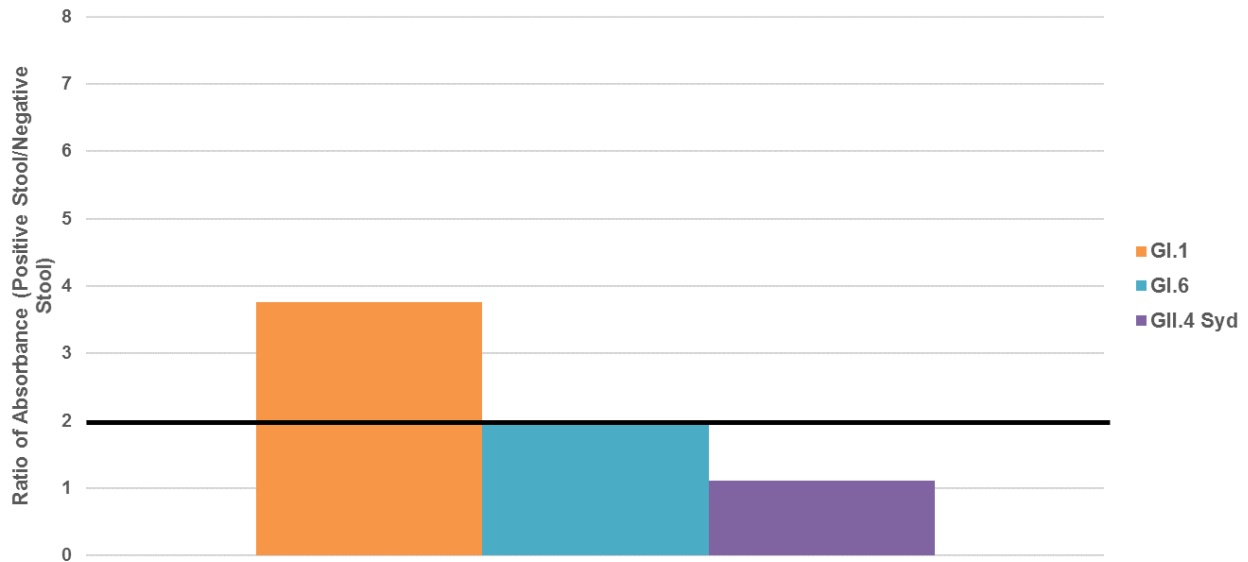
**Figure 2.3**  
Binding ratios of GI and GII VLPs against GI.1 targeted SELEX round 10 aptamers 4S10 and 9S10. Different letters indicate statistically significant differences in absorbance ratios when comparing VLPs to one another for a single aptamer.



**Figure 2.4**  
Binding ratios of GI and GII VLPs against a 1:1 cocktail of GI.1 target aptamer AP4-GI and GII.2 SMV target aptamer SMV-19. Letters indicate a statistically significant difference ( $p < 0.05$ ) between each VLP.

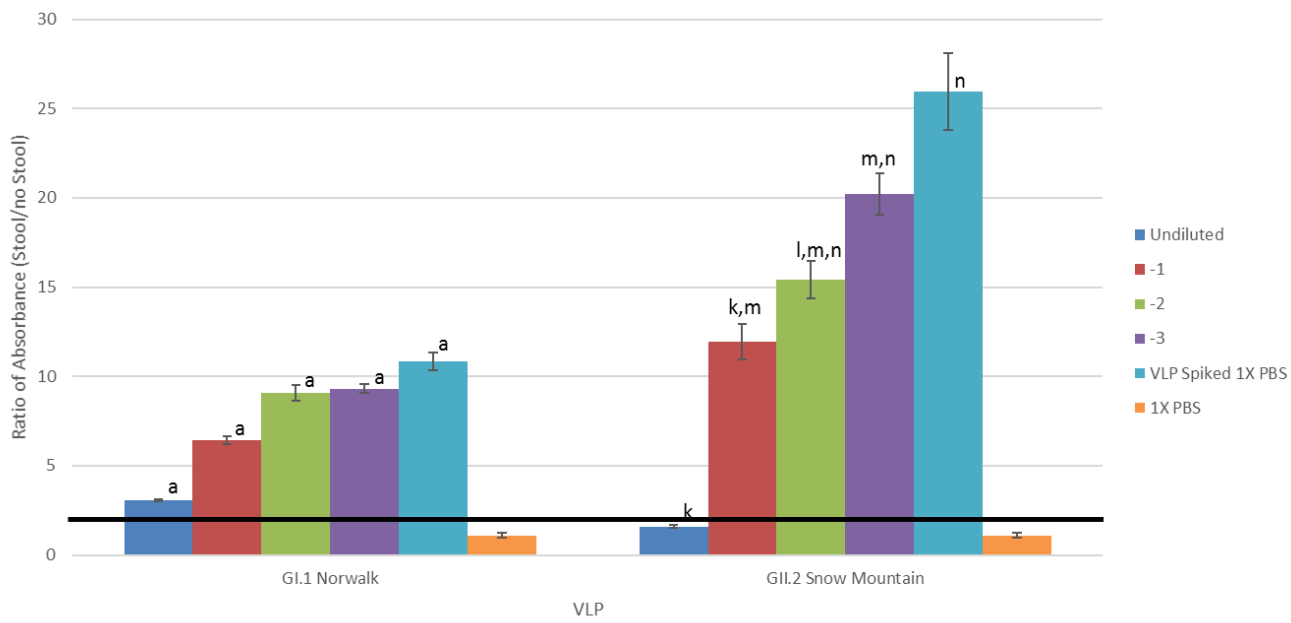


**Figure 2.5**  
 Binding ratios of GI and GII VLPs against 1:1 combination of GI.1 targeted AP4-GI and GII.2 SMV targeted aptamer SMV-21. Letters indicate a statistically significant difference ( $p < 0.05$ ) between each VLP. There is no significant difference for ratios of absorbance between VLPs.



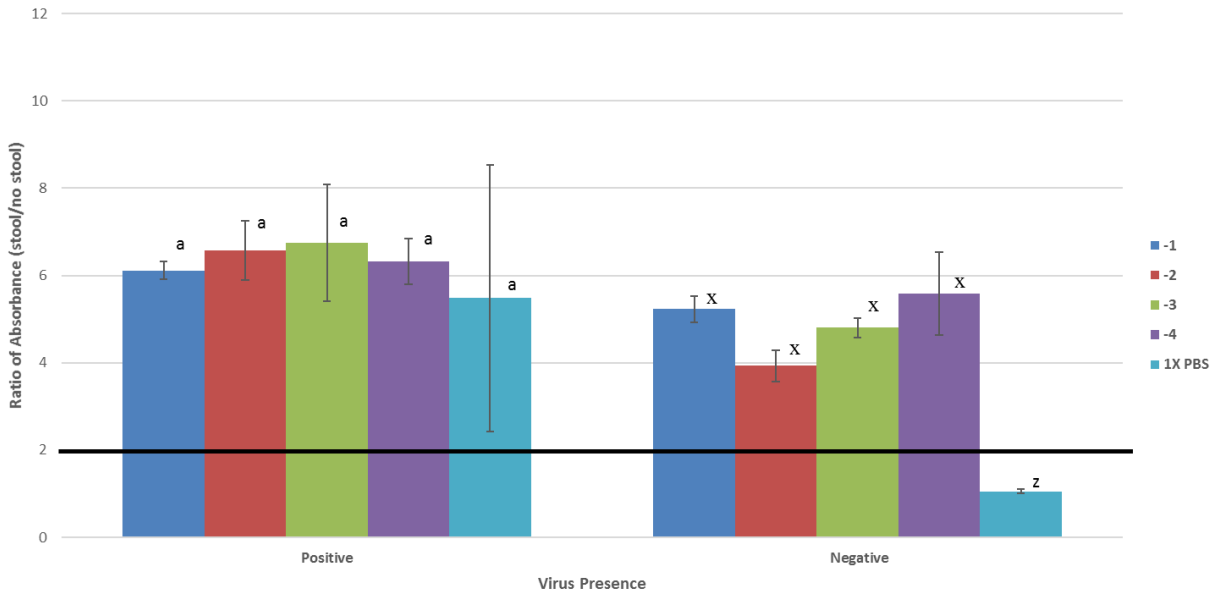
**Figure 2.6**

Naturally contaminated stool dilutions against AP4-GI. Note these are not in triplicate due to the low availability of virally contaminated stool suspensions.



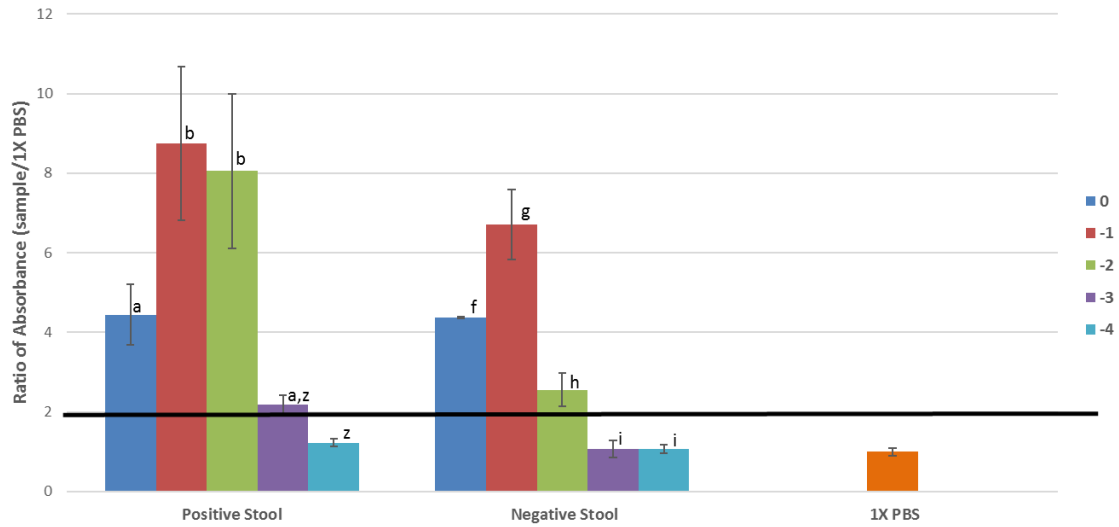
**Figure 2.7**

Produce wash water spiked with GI.1 and GI.2 VLP, respectively. Water dilutions are made prior to spiking VLP and 'VLP Spiked 1X PBS' is the positive control. AP4-GI is used for binding efficiencies against wash water. Letters indicate a statistically significant difference ( $p < 0.05$ ) for each dilution within the VLP.



**Figure 2.8**

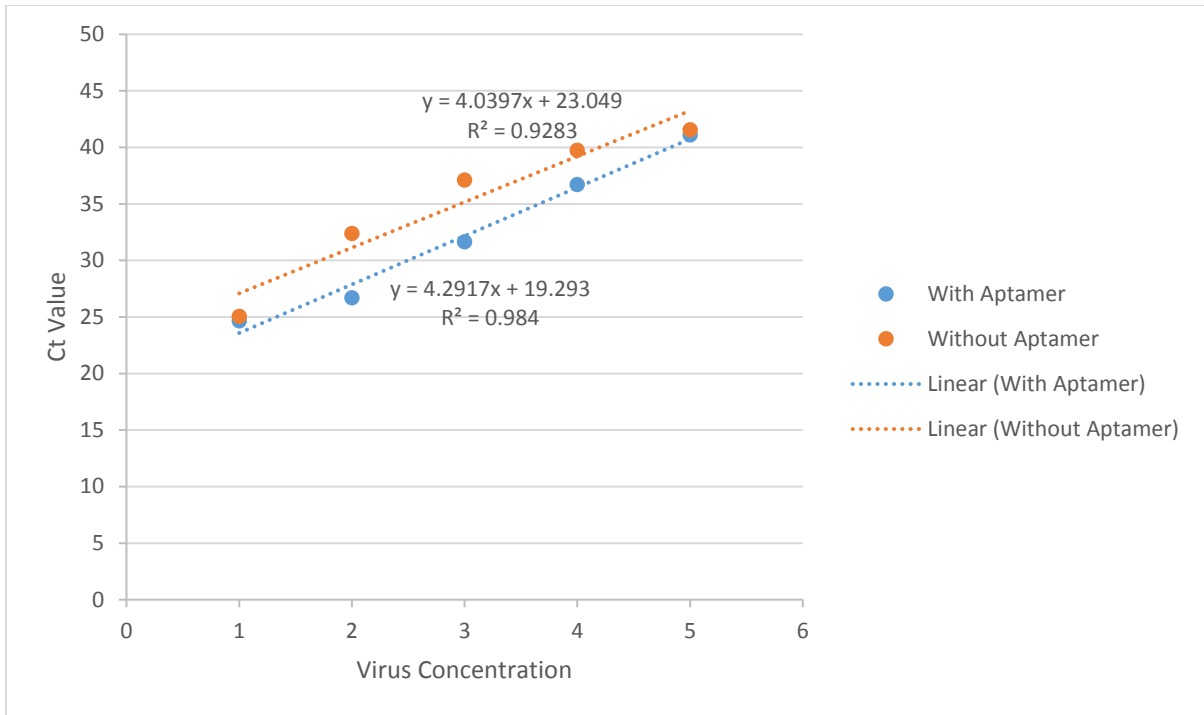
Produce wash water diluted with 1X PBS and spiked with 1:10 dilution of stool. GIL4 NV14-016 naturally contaminated stool indicates positive virus presence whereas negative stool implies no virus present. Letters indicate a statistically significant difference ( $p < 0.05$ ) for each dilution within virus presence.



**Figure 2.9**  
 Produce wash water initially spiked with either naturally contaminated stool or uncontaminated stool and diluted sequentially. Letters indicate a statistically significant difference ( $p < 0.05$ ) between each stool specimen and dilution.

**Table 2.4**  
 Primer and probe names, sequences, genome location, and source for RT-qPCR assays used.

Virus	Name	Sequence (5'-3')	Location	Reference
Tulane Virus	TV-FW	GAGATTGGTGTCAAACACTCTTTG	3645	Sestak et al. 2012
	TV-RV	ATCCAGTGGCACACACAATTT	3800	
	TV-Probe	6-FAM-AGTTGATTGACCTGCTGTGTCA-BHQ1	3697	



**Figure 2.10**  
Standard curve of Ct values obtained from aptamer magnetic capture with Tulane virus against aptamer M6-2

**Table 2.5**  
Average aptamer magnetic capture RT-qPCR results with Tulane virus against aptamer M6-2

Virus Concentration (PFU/ml)	Virus Dilution	Positive (Ct)	Negative (Ct)
$5 \times 10^6$	1:1	24.66	25.04667
$1 \times 10^6$	1:10	26.7	32.38333
$1 \times 10^5$	1:100	31.65667	37.10333
$1 \times 10^4$	1:1000	36.70667	39.74333
$1 \times 10^3$	1:10000	41.115	41.565

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## CONCLUSIONS

Human norovirus is the cause of most worldwide outbreaks of acute, nonbacterial gastroenteritis, and is a hardy pathogen difficult to disinfect and detect. Accounting for roughly 5.5 million foodborne illnesses in the United States, the environmentally stable virus is easily transmitted, has high resistance, and obstructs the progress of creating a reliable, sensitive and specific detection method assay. The lack of dependable, yet rapid detection tools enable the demand to decrease public health concerns as a result of human norovirus outbreaks. Striving to confront the demand, we worked to develop a reliable detection procedure using aptamers in two novel processes. Data presented throughout our studies promote the use of the ligands to capture human norovirus particles in food and environmental samples.

Aptamers, the short ssDNA ligands used, were the focus during both novel processes in our studies. The first process, ELASA, characterized a range of aptamers (genogroups GI and GII targeted) against both GI and GII genogroups VLPs and viruses. Using the ELISA-based method, we provided evidence on the effectivity of the aptamers. Overall, AP4-GI demonstrated confident binding against all VLPs and was the most broadly reactive with binding ratios ranging from 3.5 against GI.6 VLP to 19.8 against GII.2 SMV VLP. Due to the reactive nature, AP4-GI was chosen to complete further ELASA studies.

Previously selected GII-targeted aptamers, SMV-19 and SMV-21, were used to create an aptamer cocktail with GI-targeted AP4-GI, in hopes to expand reactivity against GI and GII VLPs. The SMV-19/AP4-GI and SMV-21/AP4-GI complementary cocktails did not

enhance binding efficiencies compared to AP4-GI alone, therefore further studies continued without the GII-targeted aptamers.

Although aptamers performed well against targets in a pure sample matrix, the addition of soil or fecal load somewhat limited the aptamer's ability to bind to the norovirus target. Partially purified outbreak-derived 20% fecal specimens were subject to the ELASA method following serial dilutions. Specimens were diluted as high as  $10^{-3}$  to observe results, but low affinity against GI.1 Norwalk stool (3.8 ratio) was presented, along with negative binding ratios with GI.6 and GII.4 Sydney stool samples. Moving forward, AP4-GI was used to assess the introduction of a food matrix and if it would exhibit similar inhibition as did the fecal matrix. Serially diluted leafy green produce wash water was spiked with a standardized concentration of a GI and GII VLP, separately. Ratio absorbance improved as the matrix dilution increased which supported the presence of sample-associated inhibitors.

To combine both fecal and food matrices, we inoculated wash water with virally contaminated fecal matter and observed the binding affinities in two fashions. First, we serially diluted the wash water and seeded the dilutions with a standard 20% stool and saw an increase in absorbance ratios as the dilutions increased. There was no seen significant difference ( $p < 0.05$ ) between the samples. Binding ratios ranged from 6.3 to 6.7 for serially diluted wash water, and the positive control (1X PBS spiked with stool) resulted in a binding ratio of roughly 5.5. AP4-GI exhibited similar binding with the standard amount of fecal matter independent of produce soil load presence. Similarly, negative stool seeded into serially diluted wash water displayed no significant difference throughout binding affinities of serial dilutions ( $p < 0.05$ ). Secondly, we introduced stool into undiluted produce wash water, and serially diluted the contaminated solution. This method supports the decrease in

binding ratios with both fecal and food inhibition introduced. The undiluted samples depicted roughly 4.44 and 4.37 binding ratios for positive stool and negative stool, respectively. Both ratios significantly increased ( $p < 0.05$ ) as the dilution increased to -1. On the other hand, as the dilution increased to -2, AP4-GI against positive stool still suggested medium binding (5.0-10.0) whereas negative stool showed a low binding ratio of 2.6. Binding affinity with all negative stool samples show the unspecific binding of the aptamer to fecal matter, or the presence of background noise when reading the absorbance. Ratios were slightly higher than the previous method of diluting the water first, but not much data is available to make any solid conclusions on matrix inhibiting complexes.

Aptamer Magnetic Capture (AMC) was the second novel process we used to capture Tulane virus. A previously selected aptamer, M6-2, gave promising results which suggest the use of aptamers in multiple methods for virus capture. RT-qPCR data showed roughly a 1-2  $\log_{10}$  viral concentration in the presence of aptamer coated magnetic beads compared to without aptamers. Although more data is required, AMC may be productive in the presence of fecal and food matrices.

Aptamers promote promising use for detecting norovirus in food and environmental samples. The overall interpretation of the use of aptamers in both ELISA and AMC is the advancing effectivity against the hardy, forty-nine-year-old highly infectious virus. The ability of the virus to infect populations with ease using a low infectious dose of  $< 10$  viral particles creates a dilemma in performing sensitive, yet specific detection procedures. Not to mention, aerolization from vomitus particles may also contaminate hard surfaces in conjunction with shedding. Although our studies were focused on an application for food and

environmental samples, hard surfaces and food contact areas are also a concern when discussing foodborne illnesses.

Our research provides evidence that although short ssDNA ligands are reliable for norovirus and Tulane virus capture, the introduction of complex matrices (fecal and produce wash water) decrease binding efficiencies against the target. On the other hand, with more work required, the performance of aptamers against norovirus in detection methods are promising. Future studies in our laboratory may include additional selection of aptamers using SELEX for refining candidates. In addition to reducing matrix-associated effects on binding and further investigating magnetic bead-based binding similar to AMC.

## APPENDICES

### Appendix A

The purpose of this work was to determine if it was possible to detect human norovirus from a cooked pork product that was served at a wedding reception in Tennessee and implicated in an outbreak break of Norovirus GII.4. The samples were received on June 9, 2015 and stored in -80°C until processing. Three sample bags of thick cut, cooked seasoned pork were submitted. Two bags contained one slice of pork each and one bag contained eight slices. Slices weighed around 75g each.

As no “standard” extraction methods are available for this product, preliminary studies were undertaken to design a method. The overall virus extraction method consisted of the sequential steps of elution, solvent extraction, proteinase K digestion, and polyethylene glycol (PEG) precipitation. In more detail, a 25g sample was stomached for one minute in 50ml of 0.05M glycine-0.14M saline buffer, pH 9. After recovering the first elution, another 25ml was added to the same sample and stomached again for one minute to produce a second elution. The two elutions were combined and an equal volume of Vertrel™ XF MS-782 (DuPont) was added to each, followed by thorough shaking and centrifugation at 5,000 x g for ten minutes at 12°C. The supernatant was recovered and 100ug/ml Proteinase K (Denville Scientific) was added with incubation by shaking at 320 rpm for one hour in 37°C. This was followed by addition of 0.1mM phenylmethylsulfonyl fluoride (PMSF), an inhibitor for proteinase K, with incubation at 30 minutes. Precipitation of protein (including viruses) was done using 8% PEG/0.9M NaCl with overnight, agitated incubation 4°C. Samples were then

centrifuged for 20 minutes 10,000 x g at 12°C and the pellet recovered. This was suspended in 1ml 1X PBS and stored at -80°C until RNA extraction.

RNA was extracted using the EasyMAG protocol (bioMerieux) with final elution volumes of 40ul. For detection, RT-qPCR with JJV2F/COGR primers (Baert et al., 2008; Escudero-Abarca et al., 2014; Jothikumar et al., 2005; Lamhoujeb et al., 2008) was conducted with and without an internal amplification control (IAC). The IAC was prepared in the lab by amplification of the viral RNA using modified primers complementary to the pUC19 plasmid according to Abdulmawjood A. et al, 2002 protocol. This primer set targets the ORF1/ORF2 junction and is specific for Norovirus genogroup GII. Extraction dilutions of  $10^{-1}$  to  $10^{-3}$  were tested to account for potential matrix-associated inhibition. If samples were presumptively positive (Ct values <42), conventional PCR was done using the MON431F/MON433R primers (Escudero-Abarca et al., 2014; Williams-Woods et al., 2011) that target the more highly variable RNA-dependent RNA polymerase (SuperScript III Platinum One-Step qRT-PCR System, Invitrogen, Life Technologies). When suspicious bands were obtained by gel electrophoresis (~213bp size), they were cut from the gel, purified, and sent for DNA sequencing. The protocol was repeated four times, one sample for bag one, one for bag two, and two samples of bag three, for assurance.

Clear negative results were observed by RT-qPCR for 10/16 dilution samples. Six of 16 dilution samples showed FAM Ct ranging from 39.8 to 41.3, in the high positive, although many samples showing amplification at this range are actually negative. These were confirmed by traditional RT-PCR, and three of the dilution samples displayed bands of the appropriate size with DNA concentrations ranged from 2.5ng/ul to 4.0ng/ul. All controls

tested negative. Unfortunately, none of the cloned and sequenced amplicon sequences corresponded to norovirus.

Overall, there were no positive Norovirus GII.4 New Orleans pork samples identified in these analyses. However, the absence of positive detection should not be construed as absence of contamination in these samples, or negation of accompanying epidemiological evidence. This is because testing is impacted by many factors, for instance (i) low levels of contamination; (ii) non-uniform distribution of the virus in foods (i.e., food was positive but sample was not); and/or (iii) poor assay sensitivity (influenced by extraction and amplification efficiencies, matrix-associated inhibitors). Suffice it to say that due diligence was taken to try to detect virus in the submitted samples.

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