MOLECULAR DETECTION OF INFECTIOUS HUMAN NOROVIRUS: VIRUS AND SAMPLE PROPERTIES INFLUENCING METHOD RELIABILITY

by

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(Under the Direction of Jennifer L. Cannon)

ABSTRACT

Human norovirus (HuNoV) is responsible for an estimated 5.5 million illness per year in the U.S., which is about 58% of all foodborne illnesses attributed to known pathogens (Scallan et al., 2011b). The most common means of detection of HuNoV is through the use of RT-PCR, which signifies presence of the viral genomic material in a tested sample, but does not distinguish between infectious and non-infectious HuNoV. The aim of this study was to determine how sample properties such as virus titer, strain, suspension matrix, and inactivation methods influence the reliability of potential methods for detecting infectious HuNoV. Two possible methods of detecting infectious HuNoV; enzymatic pre-treatment with Proteinase K and RNase A, and the use of porcine gastric mucin (PGM) binding, each method prior to RT-PCR, were evaluated against these sample properties. The result of the enzymatic pre-treatment study showed that virus titer and suspension matrix impacted estimation of infectious HuNoV and observations also varied from one viral strain to another. Compared to plaque assay in a cultivable surrogate (MNV-1), there was no correlation in estimation of infectivity; while plaque assay estimated up to 6 log reduction in infectivity after heat treatment, the enzyme pre-

treatment method estimated a maximum of 2.94 log reduction in viral RNA. Evaluation of the PGM-binding method shows variation in estimation of infectivity across inactivation methods and viral strains. By subjecting virus samples to PGM-binding prior to RT-PCR, complete elimination of RT-PCR signals was observed in virus samples treated to a lev/SDS (0.5% levulinic acid + 0.1% sodium dodecyl sulphate) sanitizer, while positive RT-PCR signals were observed in virus samples heat treated at 99^{o}C for 1 min. Non-specific binding of virus samples to testing surfaces was also observed, a variable not considered or evaluated in previous studies. The impact of food matrix and RNA extraction methods on RT-qPCR inhibition and norovirus recovery was also investigated. This study suggests that the efficiency of each RNA extraction method in reducing PCR inhibition varied with the sample matrix being tested in GII.4 samples ($p<0.0001$), but sample matrix was not a significant ($p=0.1675$) contributor in GI.1 signal inhibition.

INDEX WORDS: Human norovirus, detection, infectivity, food, molecular, porcine gastric mucin, enzymatic pre-treatment, PCR inhibition, RNA extraction

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DEDICATION

To my husband, Olumuyiwa, who offered me the love, support and encouragement to achieve this dream, I couldn't have done this without you, thank you. To my parents whose continuous support and love has afforded me this accomplishment. To my sister-in-laws, your love and support cannot be overemphasized.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Human Noroviruses (HuNoV) are major etiological agents of non-bacterial gastroenteritis, often associated with contaminated food, water and environmental surfaces. Despite numerous attempts over the last four decades, HuNoVs remain recalcitrant to laboratory culture (Atmar, 2010; Duizer et al., 2004). The most common means of HuNoV detection is by the use of RT-PCR, which amplifies and confirms the presence of viral genomic material in a sample. However, the ability of a detection method to distinguish between infectious and non-infectious HuNoV is of utmost importance, because the presence of a genomic material does not necessarily mean presence of an infectious virus particle. Alternative methods are thus needed to discriminate between infectious and noninfectious viruses in a food, environmental and clinical samples.

Previous studies have attempted detecting infectious HuNoV by employing methods that assess the integrity of the viral capsid, the viral genome, or a combination of the two (Cannon and Vinje, 2008; Kostela et al., 2008; Rodriguez et al., 2009; Sano et al., 2010). The most promising of these methodologies have been those that employ enzyme pretreatment of samples to avoid false positives in RT-PCR (Nuanualsuwan and Cliver, 2002), binding of HuNoV to epitope-recognizing substances such as Histo blood group antigens (HBGA) and porcine gastric mucins (PGM) (Cannon and Vinje, 2008; Dancho et al., 2012; Morton et al., 2009; Tian et al., 2010), and long template RT-PCR (Kostela

et al., 2008; Wolf et al., 2009). While some studies have yielded promising results, some inconsistency exists across studies. A direct comparison of these studies has been difficult due to differences in the preparation of the virus stocks used by different research groups. A key variable is the matrix in which the virus is suspended during treatment with an inactivation process or during application of the method used to estimate virus infectivity. Components of the matrix may limit the accessibility of the virus to inactivation treatments, or interfere with methods of virus detection and/or infectivity estimates. This could cause significant differences in the results obtained between research groups, potentially masking successful results.

A promising method for detecting infectious HuNoV by binding to porcine gastric mucins (PGM), treatment with RNase A, and followed by RT-PCR was recently developed. Binding to HBGAs is a necessary precursor to human infection for some HuNoV strains. These strains must have an intact (not damaged) HBGA-binding site on their viral capsid in order to bind to HBGAs and they must have an intact viral RNA genome in order to cause infection in a host cell. Treating viruses with RNase A is another means of discriminating between intact and non-intact viruses, as the genomes of intact viruses are protected from the RNA-degrading enzyme. Therefore, HuNoVs that bind to HBGAs found in PGM, resistant to RNase A treatment, and can be subsequently detected by RT-PCR are considered to be potentially infectious.

One major obstacle in detection of HuNoV in samples (food, environmental water and/or clinical samples) is the presence of inhibitors in samples. Inhibitors such as organic matter reduce the efficacy of detection methods that estimate infectivity. Inhibitors sequester viruses in the sample by competing with viruses for capture ligands

used for recovery. Sometimes virus may aggregate. This aggregation protects viruses that are in the core of the aggregate. Organic matter can also assist viruses in aggregation and offer protection to viruses hiding themselves within the organic matter. Also, inhibitors in samples could compete or interfere with enzymes used in researching capsid integrity (i.e. RNase and Proteinase K). Some inhibitors may inhibit RT-PCR, while others may present themselves as RNases, thus degrading RNA. There is therefore a need to find an effective way to remove inhibitors in all samples being used.

The ultimate goal of this study is to assess the impact of sample matrix, virus titer and sample preparation methods on detection of infectious HuNoV with the intention of suggesting a reliable method of detecting infectious HuNoV in samples. We will address the ultimate goal in the following specific aims. (i) Investigate the impact of matrix and virus titer on enzyme pretreatment methods in estimating infectivity. (ii) Evaluate the use of porcine gastric mucin (PGM) binding and RT-qPCR as a method of estimating infectivity after virus inactivation. (iii) Compare extraction methods of viral RNA from food and soil samples to assess the impact of sample matrix and RNA extraction methods on the inhibition of RT-qPCR.

1.0. LITERATURE REVIEW

1.1. Gastroenteritis; a continuous source of concern in society

Foodborne illnesses continue to be a source of concern in the United States and globally. An estimated 178.8 million acute gastroenteritis illnesses occurred each year in the United States with 37 million of these attributed to known pathogens, leaving the remaining 141.8 million illnesses caused by unspecified agents (Scallan et al., 2011a). Of the 37 million illnesses accountable to specific pathogens, 9.4 million episodes are foodborne, and are credited to 31 major pathogens which included bacteria, viruses and parasites, with norovirus responsible for the largest portion (58%) of foodborne illnesses (Scallan et al., 2011b).

Most common symptoms of gastroenteritis include abdominal cramps, watery diarrhea, vomiting, fatigue, and occasionally fever, headache and nausea. Depending on the pathogen culpable for the infection, these symptoms may occur in different combinations with some symptoms being more dominant than others. Norovirus infections are mostly characterized by vomiting and watery diarrhea; vomiting is the more common symptom in children and watery diarrhea is more common in adults (Glass et al., 2009). Infection with HuNoV is generally self-limiting with most people feeling better after only a few days; however, certain groups are at higher risk of having a more severe episode if infected, these includes children, the elderly and immuno-compromised individuals whose immune system may not be strong enough to suppress the virus from being highly pathogenic. To bring this into context, of the estimated 797 deaths associated with norovirus between 1999 - 2007, 718 (90%) were persons 65 years of age

or older (Hall et al., 2012). An underlying feature of the majority of these deaths is dehydration.

1.2. The significance of norovirus in gastroenteritis in the United States

The first virus identified as causing gastroenteritis in humans was the Norwalk agent; however, due to lack of available sensitive and routine diagnostic tools, recognition of its importance as a pathogen was limited (Glass et al., 2009). Now known as noroviruses, taxonified in the genus *Norovirus* and in the family *Caliciviridae*, there has been more interest in HuNoV in the last two decades due to the greater availability of molecular methods that has improved the ability to detect and identify HuNoV.

As previously stated, HuNoV is now the leading cause of foodborne gastroenteritis. Of the 55,961 persons hospitalized as a result of consuming contaminated food, HuNoV is second only to *Salmonella* (35%) as the pathogen responsible for the highest number of hospitalizations (26% or 14,663 persons hospitalized), and an estimated 149 deaths (Scallan et al., 2011b). These estimates were based on US population in 2006. In 2009, the Centers for Disease Control and Prevention (CDC) launched NORS - National Outbreak Reporting System, a web-based platform collecting reports of enteric disease outbreaks caused by bacterial, viral, parasitic, chemical, toxin, and unknown agents, as well as waterborne outbreaks of non-enteric disease (CDC, Nov. 2013). Based on data from NORS, Hall et al. (2013) reported that between 2009 - 2010, there were 88,958 acute gastroenteritis illnesses with a suspected or confirmed single etiology, with norovirus as the leading cause, being responsible 78% (69,145) of these illnesses and 86% (125) of the 146 deaths.

The mode of transmission of HuNoV is a topic of interest to many researchers and food processors. Many argue that transmission is mostly by person-to-person contact rather than foodborne. HuNoV can be transmitted through several means including consumption of food contaminated with feces, hand contact with contaminated surfaces or fomites (Sharps et al., 2012), exposure to aerosol from an infected person or by touching or consuming items from a room where an infected person vomited (Repp and Keene, 2012), and person-to-person contact (Wikswo and Hall, 2012). The importance of hand hygiene, therefore, cannot be over-emphasized in preventing HuNoV spread as well as in combating gastroenteritis. Bidawid et al. (2004), in their studies showed that hand washing either with water or with both topical agent and water to be significantly effective in reducing Feline Calicivirus (FCV), a surrogate of HuNoV, from finger tips. Liu et al. (2010) also showed hand washing with either water alone or antibacterial liquid soap with water to be more significant in reducing quantities of Norwalk Virus on finger pads than the use of alcohol-based hand sanitizer.

1.3. Norovirus: The virus

Norovirus is a genus in the family *Caliciviridae*; a non-enveloped, icosahedral group of viruses with single-stranded, positive sense linear RNA genome. The capsid is about 28-35 nm and the genome therein is about 7.5 to 7.7 kilobases, composed of three open reading frames (ORFs). ORF 1 encodes a large polyprotein from which six non-structural proteins are produced by proteolytic cleavage, ORF 2 encodes the major structural protein, VP1, which forms the capsid, and ORF 3 encodes minor structural protein, VP2 (Atmar, 2010). The coding order in ORF 1 are p48 (at the N Terminus), NTPase, p22,

VPg, $3CL^{pro}$ (PRO) and a RNA-dependent RNA polymerase (POL) on the C terminus (Hardy, 2005). Figure 1.1 shows the ORFs, the proteins encoded and their translational products.

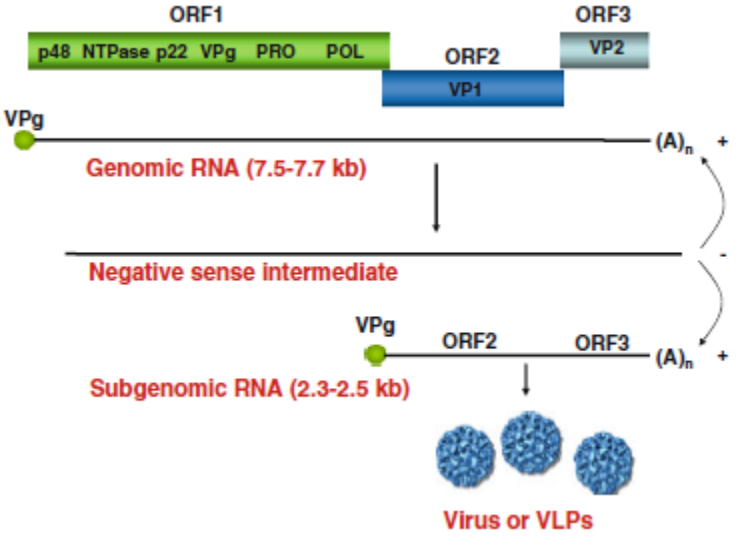

Figure 1.1: Norovirus genomic organization and replication strategy. Figure taken from (Atmar, 2010).

Noroviruses can be classified into five groups (genogroups I to V), the two largest groups, GI and GII, include most of the diverse and common NoVs (Zheng et al., 2006). These are classified into 9 clusters for GI and 22 clusters for GII (Figure 1.2). The prototype HuNoV strain, Norwalk virus, belongs to genogroup I and genotype 1, and is thus designated GI.1 (Atmar, 2010). Consecutive numbers are assigned to each cluster and added onto the tree as new clusters are defined (Zheng et al., 2006). Genotyping has been previously carried out on the basis of partial capsid and RdRp sequences (Kageyama et al., 2004), this criteria was specifically based on variability in the capsid N-terminal/shell domain gene (capsid N/S domain) (Katayama et al., 2002b). Zheng et al.

(2006) proposed a method which requires analysis of the whole VP1 capsid sequence which was generally accepted by attendees at the 3rd International Calicivirus Conference (ICC) held in Cancun, Mexico, in 2007 (Atmar, 2010).

At the 4th ICC held in Chile in 2010, a group of researchers was charged to develop practical standards for NoV nomenclature and typing system (Kroneman et al., 2013) and an update on the progress of these efforts was presented at the 5th ICC in Beijing in 2013 by Dr Marion Koopmans (RIVM, Netherlands). Kroneman et al. (2013) proposed a new classification criteria; "the average distance between all sequences within a newly identified cluster and its nearest established cluster should not overlap within 2 standard deviation (SD) of each other (2×SD criterion)". Using this criteria, one new GI (GI.9) and three new GII clusters (GII.20, GII.21, and GII.22) were identified in addition to previously defined genotypes, a few of previously established genotypes did not comply with the 2xSD criterion. All sequences in GI and GII are of human origin, with the exception of GII.11 and GII.19 that are from porcine viruses, GIII are of bovine origin and GV of murine origin (Kroneman et al., 2013; Zheng et al., 2006).

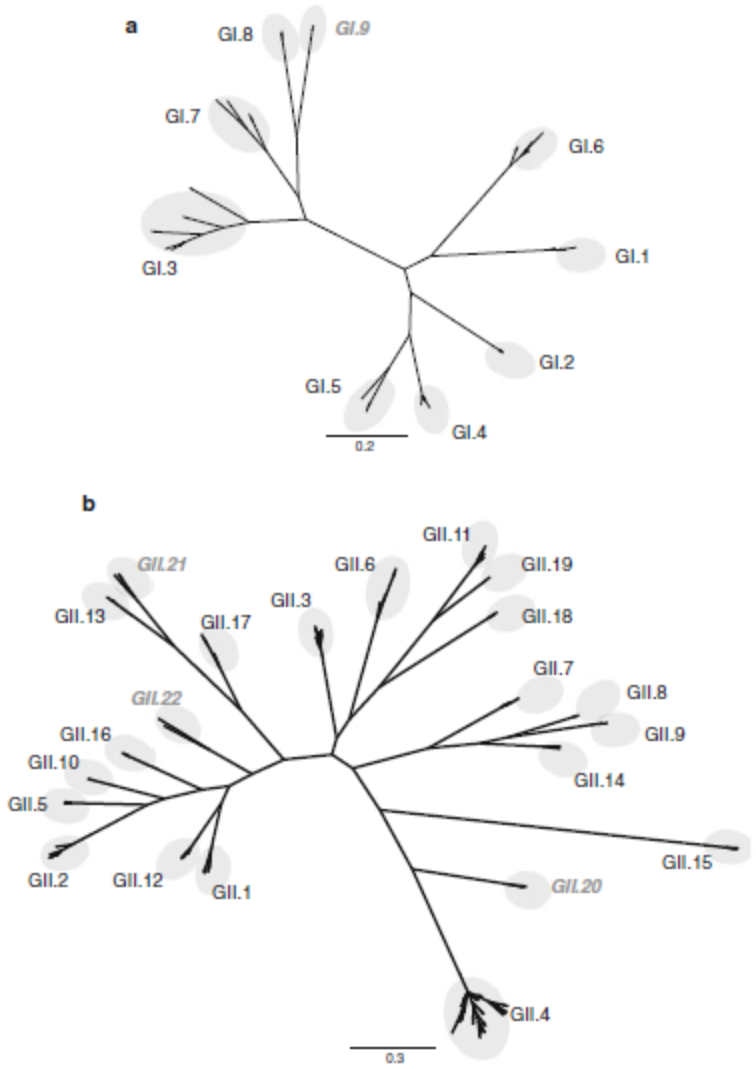

Figure 1.2: Phylogenetic trees of norovirus GI (a) and GII (b) showing updated and newly identified clusters, derived from Kroneman et al., 2013.

1.4. Norovirus infectivity

Infection processes of viruses generally involve binding or adsorption onto the cell surface, penetrating the host cell membrane, entry into the cell and using cellular components to produce and assemble viral components (genomic material and structural and non-structural proteins) to ultimately generate infectious viral particles. Viruses that

complete this process successfully, without error are defined as infectious. A break-down at any point in this process essentially renders the virus non-infectious.

Interaction of norovirus with the host cell surface is through the protein capsid, which possesses epitopes specific to cellular molecules used to gain entry into the cell. The capsid also has the function of protecting the viral genome from degradation by nucleases and abiotic stresses, such as humidity, pH, UV radiation, and temperature (Rodriguez et al., 2009). While the viral capsid is critical for infectivity, intact genomic material is also crucial for replication of genomic RNA, translation of non-structural proteins and production of sub-genomic RNA, which is translated into viral structural proteins. Thus, both components are vital to the successful production of infectious viral particles.

Binding of HuNoV to HBGAs has been suggested to be a necessary precursor to human infection with HuNoVs (Marionneau et al., 2002), the study reported attachment of recombinant norwalk virus-like particles (rNVLPs) to surface epithelial cells of the gastroduodenal junction and saliva of individuals of known ABO, Lewis and secretor phenotypes but does not bind in non-secretors. Binding of HuNoV and rNV-VLPs have been reported in other studies as well (Lindesmith et al., 2003). Hutson et al. (2002) observed that ABO blood type is associated with HuNoV infection; individuals with O phenotype were more likely to be infected, while volunteers with B histo blood group antigen had a lower risk of infection. Cannon and Vinje (2008) recovered low copy numbers of NoVs using a synthetic HBGA binding assay. Due to the difficulties and costs associated with obtaining HBGAs for research, several researchers have used PGM as an alternative (Dancho et al., 2012; Kingsley et al., 2014; Tian et al., 2008). Tian et al.

(2005) studied the inhibition of binding of rNVLP to HBGAs by PGM, and reported that PGM binds effectively to rNVLPs and competitively inhibits rNVLPs binding to human HBGAs.

1.5. Virus detection in food and environmental samples

Detection of microorganisms usually involves culture of the investigative sample on either a nutrient supplying medium, as is in the case of bacterial organisms, or on monolayers of susceptible cell lines as found in the case of many viruses. As previously mentioned, one of the most important food-borne viruses, HuNoV, does not grow in cell cultures, thereby hampering progress in research geared towards HuNoV outbreak control and vaccine development, and limiting our understanding of certain features of HuNoV such as survival in food, water and the environment, and effectiveness of virucidal agents and industrial kill steps in eliminating HuNoV contamination. Also, for confirmation of outbreak sources, detection of infectious HuNoV is important. Currently, confirmation of presence of HuNoV in food or environmental samples follows a general sequence of sample collection, concentration, and detection. Each of these steps involves several other sub steps, and may include differing methods of carrying out each step successfully, depending on the matrix of the contaminated sample; this will be discussed in the following section.

1.5.1. Sample collection

Collection of samples for norovirus detection can be in several formats depending on suspected sources of contamination. Food and soil samples can be simply collected in

sample bags and transferred on ice to the laboratory. Water sample collection depends on the sample of interest; if it is to be collected from a large body of water, collection may require the use of a pump for passing the water through filters which makes the collection step often overlapping with concentration step. Samples are thereafter transferred to the laboratory in an ice chest. Hall et al. (2011) gives recommendations and guidelines about sample collection for HuNoV, an important erratum to this report states that "food samples should be stored frozen at -4°F (-20°C), and water samples should be stored refrigerated or chilled on ice at 39°F (4°C)."

1.5.2. Sample concentration

Due to usually low concentrations of HuNoV in food and environmental samples, such as water samples, there is usually a need for sample concentration into smaller volumes (Rodriguez et al., 2009). While these concentration and purification steps are designed to reduce sample volumes, they are also designed to remove some of the matrix while recovering most of the contaminating viruses (Knight et al., 2013). For food samples, many virus concentration and purification methods employ manipulation of pH and/or ionic conditions to favor virus adsorption to, or elution from, the food matrix (Knight et al., 2013).

Pepper et al. (2009) describes the two available types of filters used in virus concentration; electropositive (positive surface charge) and electronegative (negative surface charge) filters. The authors explain that when electronegative filters are used, aside the reduction of solution pH to about 3.5, cationic salts such as $MgCl_2$ or $AlCl_3$ are required to help reduce the net negative charge usually associated with viruses allowing

adsorption to be maximized. da Silva et al. (2011) observed a decrease in attachment efficiency of GI.1 virus like particles (VLPs) to silica when the pH of the solution was increased above the isoelectric point, which is reported to be between pH 5 and 6 (da Silva et al., 2011; Goodridge et al., 2004), and found the addition of Ca^{2+} and Mg^{2+} to improve the attachment efficiency dramatically. Following adsorption of virus to the membrane, an acidic rinse (H_2SO_4 - pH 3.0) is used to remove cations prior to viral elution with an alkali solution (NaOH - pH 10.5) (Katayama et al., 2002a). The increase in pH due to the alkali solution increases negative charge on the virus which allows for elution of the virus from the negatively charged membrane.

As opposed to negatively charged filters where manipulation of pH is required to facilitate adsorption of virus, when positively charged filters are employed, preconditioning of water is usually not required (Karim et al., 2009). Electropositive filters may be composed of fiberglass or cellulose containing a positively charged organic polymeric resin (1MDS), or nano alumina fibers (NanoCeram) which create a net positive surface charge to enhance adsorption of the negatively charged virus (Pepper et al., 2009). Karim et al. (2009) used a 1.5% beef extract solution (pH 9.0) containing 0.05 M glycine for elution of the virus from the filter. This elution mechanism is facilitated by the more abundant beef extract proteins or glycine which are negatively charged, competing for space on the filter surface thereby displacing the virus and enabling virus particles to elute off the filters. The use of charged filters are mainly in liquid-phase samples.

1.5.3. Secondary concentration and purification of samples

Once the volume of water samples has been reduced (for example from 10 L to 30 ml), further concentration into a much smaller volume is often needed. Polyethylene glycol is often used to as a secondary concentration step for virus from water samples, as well as food matrices (Baert et al., 2008). PEG facilitates phase separation in the sample, and virus in the solution can be concentrated by centrifugation at 9000 x g for 30 min at 4^{o}C; the PEG supernatant is then disposed. The sediment obtained is usually chloroform-extracted which further removes organic materials or food matrix. Ultracentrifugation, and ultrafiltration methods can also be employed as a secondary concentration step to further reduce the volume of water samples. These methods are based on particle size; while ultrafiltration separate particles based on size (diameter), ultracentrifugation separates particles based on their sedimentation rate (Croci et al., 2008; Mattison and Bidawid, 2009).

Immunomagnetic separation (IMS) is a method of concentrating target cells or other particles which involves coating magnetic beads with antibodies capable of binding to target antigens. IMS has been used extensively in identification of different bacteria, viruses and parasites; its use in identification and characterization of microorganisms are reviewed extensively by Olsvik et al. (1994). IMS was first used for isolating microorganisms in food by Skjerve et al. (1990), when *Listeria monocytogenes* was extracted from inoculated cheese and ham samples. The use of IMS in detection of HuNoV in food and fecal samples have been previously studied (Park et al., 2008; Yao et al., 2009). However, the use of antibody capture in this manner has been limited due to the unavailability of commercially accessible broadly reactive antibodies for HuNoVs,

which are antigenically diverse (Knight et al., 2013). Because of their structure, with the P2 region on the outermost portion of the capsid under constant immunoselection, finding broadly reactive antibodies for HuNoV has been very difficult. This challenge motivated research on the use of ligands, such as cell-surface carbohydrates that have been reported to bind to the viral capsids, in magnetic separation of HuNoV. It is possible that just a few HBGAs may be able to bind the majority of HuNoV genotypes with high affinity, and therefore may be a better ligand for magnetic separation.

Cannon and Vinje (2008) described the use of HBGAs bound to magnetic beads, in recovery of low numbers HuNoV particles. HBGAs are complex carbohydrate moieties linked to glycoproteins or glycolipids that are present on red blood cells, mucosal epithelial cells, and also in mucosal secretions, such as intestinal contents, blood and milk (Huang et al., 2005). Other studies have also reviewed the use of HBGAs as a means of purification of HuNoV from clinical specimens (Harrington et al., 2004), and the binding patterns of HuNoV to HBGAs (Huang et al., 2003). The use of porcine gastric mucins (PGM) have also been studied as an alternative to HBGA for concentration or purification of HuNoV from samples (Tian et al., 2005; Tian et al., 2008; Tian et al., 2010).

1.5.4. RNA extraction and detection

Once virus samples have been concentrated into a smaller volume, detection of virus in samples is next in line; this is typically done either by cell culture or through molecular methods. Cultivable viruses employ culture of viruses on susceptible cell monolayers while non-cultivable viruses, such as HuNoV, are limited to molecular

methods such as RT-PCR. While there are other methods employed for detection of viruses in clinical samples such as enzyme immunoassay, these methods are often not employed in the detection of viruses in food because the number of viruses present in food samples is usually too small to be detected by such methods, even though these low level contamination can still cause infection in a susceptible host (Goyal, 2006).

Viral detection by RT-PCR is usually preceded by extraction of the viral genomic material. This can be done by releasing viral RNA from capsids using heat treatment at 99^{o}C for 1 min or more; however, this is rarely performed because residual matrix components of samples can interfere with RT-PCR (Knight et al., 2013). An extraction method needs to be efficient in obtaining viral nucleic acids that are as pure as possible, especially for enteric RNA viruses (Croci et al., 2008), because RNA is generally less stable than DNA and is susceptible to endogenous RNases which are often found in unpurified samples. The use of guanidine thiocyanate-based lysis buffer for capsid destruction as described in Boom et al. (1990), followed by binding extracted RNA to silica, is the most common method of RNA extraction. Kits for this method are available through several bio-diagnostic tool supplying companies. (Berensmeier, 2006) also described the use of magnetic carriers with immobilized affinity ligands or biopolymers exhibiting affinity for the target nucleic acid as another means of isolating viral RNA.

Reverse Transcription-quantitative PCR (RT-qPCR) analysis of the extracted RNA is usually the concluding step in norovirus detection. This involves the use of specific sets of primers and probes (Jothikumar et al., 2005; Kageyama et al., 2003), which have been successfully used by several researchers due to their efficiency and broad reactivity. Even though conventional RT-PCR can also be performed, this option

requires the use of gel electrophoresis to detect the presence of target genomic material. Sequencing is, however required for confirmation and strain identification, regardless of whether RT-qPCR or conventional RT-PCR is employed. Conventional RT-PCR is appropriate for epidemiological purposes; however, it is time consuming compared to RT-qPCR which has a lower detection limit and thus may be more appropriate for research purposes.

One step RT-qPCR uses the reverse PCR primer as a specific primer for reverse transcription yielding the complementary DNA (cDNA), which is then amplified using the forward primer, allowing for the whole process to be done in a single well or tube as applicable. The probe used in RT-qPCR is designed with a fluorophore at the 5' end and a quencher at the 3' end, with a proximity close enough to prevent the fluorophore from fluorescing (Logan, 2009). When the probe binds to the target DNA, the 5' exonuclease activity of the *Taq* polymerase can extend the sequence, causing the fluorophore to fall off. This causes the fluorophore, separated from the quencher, to fluoresce upon excitation from the lamp/laser of the real time thermocycler, and to be detected by the unit's optics (Saunders, 2013). As amplification cycles increase, the concentration of target DNA increases accordingly, this translates into an increased fluorescent signal. A threshold line is set by the computer software to discriminate between the signals and background noise. The amplification cycle at which the concentration of the target DNA (determined by fluorescent signal) increases enough for the signal to cross the threshold line is referred to as the C_T or cycle threshold. Production of a C_T value usually corresponds with a positive sample, if the C_T value is within a reasonable range. The C_T value can also be used to estimate the concentration of the viral genomic copies (GC) in

the sample, this is done by running a dilution series of a standard (genotype specific transcript) alongside the sample of interest. Declaring a sample as positive or negative can be difficult, especially when high C_T values ($C_T > 35$) are produced; therefore, a case by case evaluation of the amplification curves is often required for these samples. Curves that show a consistent increase as the amplification cycle continues, until they flatten out as amplification reaches its plateau (S-shaped curve) are likely to be declared positive, while curves with a jagged up and down random shape should be considered negative.

1.6. Challenges in norovirus detection

The major challenge with HuNoV detection is the unavailability of a cell culture method of detecting the virus, thereby making discrimination of infectious and non-infectious viral particles impossible. Aside from the inability to determine infectivity by molecular methods, PCR of samples containing PCR inhibitors is a major problem. Food samples often consist of a complex matrix containing PCR inhibitors. An example is shellfish, which depending on the season of the year, type of algae and other substances consumed, and the geographical area where they are obtained, may contain varying levels of potential inhibitory substances (Richards, 1999). These inhibitors reduce the efficiency and sensitivity of PCR unless a pre-requisite step is taken to eliminate or reduce inhibitors. Limitation in the amount or volume of sample that can be analyzed in PCR is another drawback in HuNoV detection. The need to concentrate samples into smaller volumes without losing the pathogen of interest significantly increases the time required for detection, while also increasing labor and cost.

The notable variation in the matrices of samples that may require testing for the presence of HuNoV including fecal sample, vomitus, various food types, water, and environmental samples, calls for a set of standardized methods that are capable of detection in most categories of samples of interest. The sequence for sample processing depends on the matrix of the starting sample. Figure 1.3 below shows an example of a decision making tree for choosing necessary steps of sample processing for HuNoV recovery from different samples. It is also important that methods of choice for sample processing be effective in removing or minimizing inhibitors which could otherwise be detrimental to downstream processes such as RT-qPCR for detection.

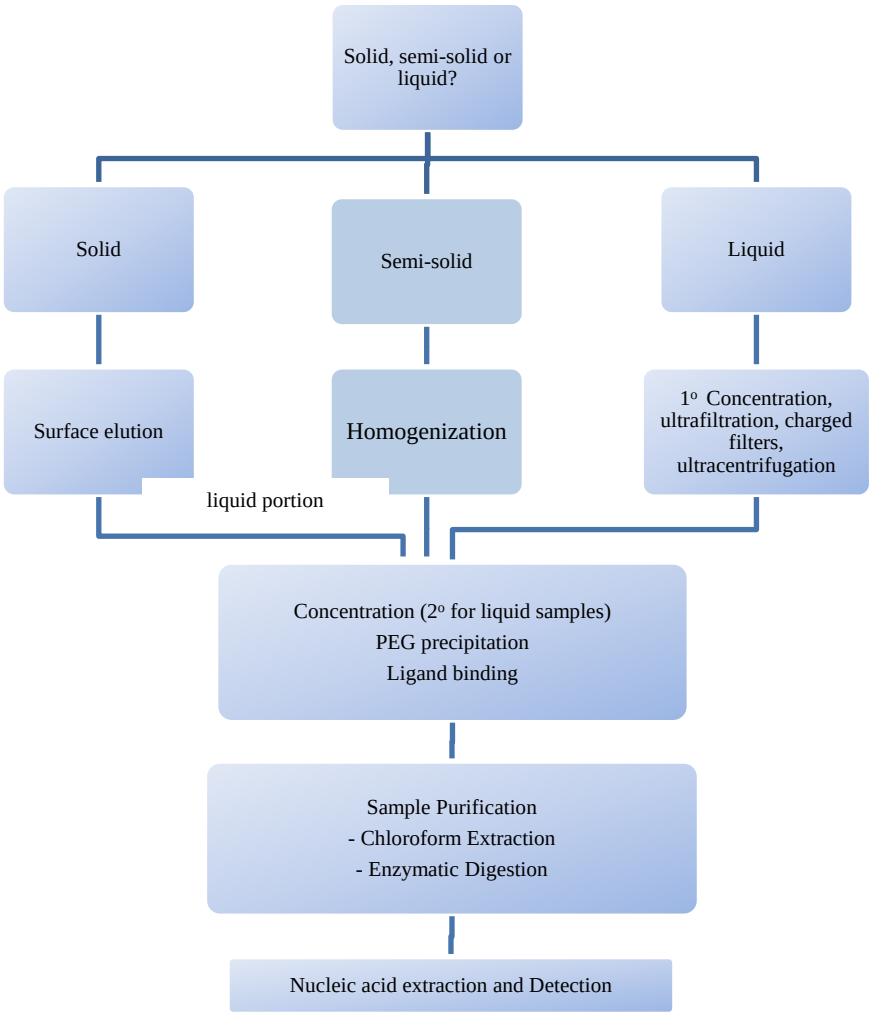

Figure 1.3: An example of a decision making tree for sample processing for norovirus detection.

1.7. Importance of discrimination between infectious and non-infectious HuNoV

Gassilloud et al. (2003) studied the infectious risk posed by the presence of viral genomes in water. This study showed that presence of viral genomes does not equal to presence of infectious virus, employing FCV and Poliovirus 1 (PV-1) as examples of enteric viruses. They demonstrated that while stability of infectious viruses is greatly

affected by the temperature of mineral water (10, 20, and 35°C), temperature has little effect on the corresponding genomes. The study also demonstrated that infectious particles are degraded more rapidly than viral genomes at all temperatures studied. At 35^{o}C, PV-1 was reduced by 4 logs after 19 days, while the predictive model used in the study estimated 75 years for a similar reduction in viral genome. It can be therefore be argued that detection of inactivated virus by RT-PCR represents a false positive test result, since inactivated viruses project no threat to human health (Nuanualsuwan and Cliver, 2002).

When HuNoV detection is carried out in clinical samples of patients presenting symptoms consistent with HuNoV infection, presence of the viral genome suggests either current infection or previous infection, which is sufficient in evaluating exposure of the patient to HuNoV. However, in non-clinical samples, viral genomes detected could be from previously inactivated viruses. Drawbacks in the unavailability of a method for distinctively detecting infectious HuNoV therefore has great significance in testing vehicles and fomites (Cliver, 2009).

An effective method that discriminates between infectious and non-infectious HuNoV stands to help us understand better the behavioral characteristics, properties and mechanisms of pathogenesis of HuNoV (Wolf et al., 2009), and comprehension of disinfection processes (Sano et al., 2010) which significantly impact monitoring of water and food safety. Topping et al. (2009) explained that the lack of a simple culture system for HuNoV hindered the development and verification of control measures used in hazard analysis and critical control point (HACCP) systems and pre-requisite programs to prevent and control HuNoV in food processing and preparation environments.

1.8. Methods for discrimination of infectious and non-infectious norovirus

Several researchers have explored different approaches to developing a method that distinguishes between infectious and non-infectious HuNoV. Based on the requirements for infectivity explained earlier in this review, approaches to determining infectivity of HuNoV particles can be those which evaluate the viral capsid integrity and/or viral genome integrity. A review of previously evaluated methods in these categories is discussed below.

1.8.1. Methods evaluating genome integrity

Due to the importance of an intact genome for replication of viruses, a method of detecting the integrity of a viral genome is an important feature in determining infectivity of the virus. To determine genome integrity in viruses, a method needs to be such that it can confirm that the entire genome is intact and complete. While RT-qPCR is currently the most employed method of detection of HuNoV, this approach targets only a portion of HuNoVs entire sequence. The conserved region spanning across the ORF1 and ORF2 junction of HuNoV is the most common target for RT-qPCR analysis (Kageyama et al., 2003). While this detection method may be useful in detecting the presence of HuNoV in a sample, it is not in any way representative of the entire sequence; rather, this region is targeted because it is highly conserved among norovirus genotypes within the norovirus genogroups (Katayama et al., 2002b). RT-PCR carried out with primers and probes designed to target this region detected NoV in 99% - 80 of 81 stool specimen that were positive by electron microscopy (Kageyama et al., 2003). Even though this region

detected by RT-PCR may be intact, inactivation procedures may damage nucleic acid strands outside the targeted region or in any portion of the entire NoV sequence.

The use of long template RT-PCR, a RT-PCR technique which amplifies a longer range of the viral sequence, has been studied by a few researchers. Kostela et al. (2008) attempted amplification of near full-length genome of HuNoV. Near full-length sequences of 7295 and 7360 nucleotides were isolated from clinical samples of sick children in 2002 and 2005, respectively. This sequence was reported to include most of ORF1, the complete ORF2 and most of ORF3. Near- and entire- genome amplification methodologies are promising - given that changes in viral genome can be detected, but such methods are cumbersome and prone to errors, since amplification efficiency decreases as fragment size increases, making this approach relatively insensitive (Knight et al., 2013).

The use of nucleic acid sequence based amplification (NASBA) in comparison with RT-PCR was evaluated in wastewater, lettuce and blueberries spiked with HAV. NASBA was reported to have a lower detection limit than traditional RT-PCR for the samples tested in this study (Jean et al., 2001). Unlike PCR, NASBA works at isothermic conditions to amplify RNA sequences. The process proceeds at 41^{o}C typically for a 90-min period with the major amplification product being anti-sense single-stranded RNA (Logan, 2009) as opposed to DNA products obtained in PCR and RT-PCR. Results obtained from NASBA techniques can be analyzed using either real time RT-PCR assays or traditional RT-PCR with gel electrophoresis.

1.8.2. Methods evaluating capsid integrity

Similar to most positive-sense, single-stranded RNA viruses, it is hypothesized that HuNoV replication is initiated when the viruses adsorb to cellular membranes, through cell receptor(s). Inactivation treatments that destroy the viral capsid (whether partially or completely) are expected to also disrupt binding sites required for attachment to cells and subsequently infection of the host cell. Methods that are capable of estimating HuNoV capsid integrity may be a determinant for estimating infectivity.

The use of Proteinase K and RNase A as an enzymatic digestion step prior to RT-PCR has been the most explored method of discriminating between infectious and non-infectious HuNoV. Nuanualsuwan and Cliver (2003) reported that Proteinase K assists in further degradation of damaged viral capsids, and after subsequent digestion with RNase A, exposed RNA is degraded. A combination of these treatments and RT-PCR may be useful in assessing capsid integrity and thus infectivity. This method was first published by Nuanualsuwan and Cliver (2002), reporting that when 3 logs PFU/ml of inactivated surrogate viruses, FCV, PV-1 and hepatitis A virus (HAV) were pretreated with the two enzymes prior to RT-PCR, no amplicon was observed after RT-PCR. The study employed UV light, hypochlorite and heating at 72^{o} C as inactivation treatments and measured the intensity of bands produced after gel electrophoresis and ethidium bromide staining of amplified product for detection purposes. Initially, this method seemed to be very promising in elimination of false positives from inactivated viruses in RT-PCR. A drawback in this study is the titer of viruses employed, 3 log PFU/ml is a low titer and therefore does not give an idea of the usefulness of this method when applied to samples containing a higher virus titer.

Lamhoujeb et al. (2008) evaluated the enzyme treatment method described above (with some modifications) in combination with NASBA to distinguish infectious from non-infectious HuNoV. They reported a loss in signal as observed through gel electrophoresis when heat-inactivated FCV and GII.4 HuNoV were subjected to Proteinase K and RNase digestion prior to NASBA. The study reported using a 5-log NASBA particle unit input titer; however, the NASBA particle unit is not equivalent to the units (genome copies or RT-PCR units) used in other studies, making it difficult to compare the results to the other studies.

The use of nucleic acid intercalators such as propidium or ethidium monoazide (PMA or EMA) as candidates for estimating capsid integrity and ultimately distinguishing between infectious and non-infectious HuNoV has also been reported. The theory behind this method is that EMA and PMA cannot penetrate intact capsid, but when they penetrate damaged capsids, the photoinducible azide group on these molecules covalently cross-links the RNA, producing stable monoadducts that cannot be amplified by PCR (Knight et al., 2013). Parshionikar et al. (2010) evaluated the use of PMA prior to RT-PCR on enteroviruses and Norwalk virus survival at different temperatures (72^{o} C, 37^{o} C or 19^{o} C) or after treatment with hypochlorite. They reported a loss in signal, as observed in RT-PCR and gel electrophoresis, for all temperature- or hypochlorite-treated viruses except those incubated at 19^{o} C, which produced results similar to those of the virus stocks treated with PMA prior to RT-PCR. A major shortcoming to this method is that nucleic-acid intercalating agents require double-stranded oligonucleotide regions, while most enteric viruses possess a single-stranded RNA genome. PMA interaction with

viral RNA is likely only in the regions with extensive secondary structure (Knight et al., 2013).

1.8.3. Methods evaluating a combination of genome and capsid integrity

Pecson et al. (2009) employed long template RT-PCR preceded by enzymatic treatment (ET) of the inactivated viral surrogate, bacteriophage MS2, as a means of quantifying infectivity. The group reported a significant loss in RT-qPCR signal with MS2 inactivated by heat or UV, and a less consistent loss in RT-qPCR signal when singlet oxygen was used as inactivation treatment. Also, despite the significant loss in RT-qPCR signal observed with these inactivation treatments, the ET-RT-qPCR still significantly underestimated the loss in infectivity measured by plaque assay. Similarly, Wolf et al. (2009) reported a decrease in long-range RT-qPCR amplification when UV exposure was used as inactivation treatment of Murine Norovirus (MNV) and HuNoV GI and GII, but not when heat at 72^{o} C was used as the inactivation treatment. This variation in results when different inactivation treatments are employed significantly impacts the versatility of the method in discriminating infectious from non-infectious noroviruses. However, a virus can be rendered non-infectious by disruption of capsid alone which halts binding ability required for infection, therefore a successful detection of the entire sequence does not infer that the RNA was from an intact particle.

Combining cellular receptor binding of HuNoV with RT-PCR is another promising method of determining infectivity. This approach explores the previously described ability of HuNoV to bind to HBGAs and PGM, combined with RT-PCR of genomic material extracted from viruses bound to these carbohydrate moieties. Dancho et

al. (2012) described the use of NoV binding to PGM-coupled magnetic beads (PGM-MB) to preferentially exclude non-infectious viral particles from subsequent RT-PCR detection. The group reported a reduction in NoV binding to PGM-MB with increased hydrostatic pressure, UV exposure and thermal treatments. Employing the surrogate MNV, in an ELISA-based method, Hirneisen and Kniel (2012) reported a significant decrease in MNV attachment to PGM in heat-treated samples (80 and 100^{o} C for 5 min), but no significant difference in attachment observed after treatment of MNV with high hydrostatic pressure (200, 300, 400 and 500 MPa at 20^{o} C for 5 min), ozone (6.25 ppm for 0.5, 1.5 and 10 min), or UV (60, 120, 240 and 1000 mW s/cm^{2}). This approach has been studied by several researchers with varying levels of success (Kingsley et al., 2014; Li et al., 2011; Li et al., 2013; Tian et al., 2008; Tian et al., 2010; Ye et al., 2014). For example, PGM-binding of HuNoV was reduced by up to 4 log GC/ml in samples inactivated with 189 ppm chlorine (Kingsley et al., 2014) or with high hydrostatic pressure at 600 MPa (Ye et al., 2014), while only ≤ 1 log GC/ml reduction in binding was observed for HuNoV heat-inactivated at 70^{o}C for 3 min (Li et al., 2011).

1.9. Challenges in evaluating methods for estimating HuNoV infectivity

The merit of methods for estimating HuNoV infectivity remains questionable due to variance in the results reported by different researchers. Some report correlate with surrogate infectivity data, while others do not. In addition, discrepancies in results are often seen when data are compared between studies that are evaluating the same or similar methods for estimating HuNoV infectivity. A closer look at these studies, often reveals methodological variations that make direct comparison of results very

complicated. Thus, methodological variables that differ between studies provide the basis for the current study and include variation in the 1) virus type, 2) viral suspension matrix, 3) virus inoculation titer, and 4) inactivation treatment used.

Given that all viruses possess unique capsid properties, the response of different surrogate viruses to inactivation treatments are expected to vary from one virus type to another. MNV replicates in an animal model (mice) and has been studied extensively for its suitability as a HuNoV surrogate; however, the inability of HuNoV to be cultured or replicate in cell culture or a small animal model is a major hindrance to absolute comparison of surrogates to HuNoV. In 2006, (Wobus et al.) described in detail the similarity of MNV -1 in terms of biochemical and genetic features to HuNoV, including the icosahedral shape, 28-35 nm size and possession of similar proteins. However, several studies comparing the stability and persistence of different cultivable HuNoV surrogates have since shown that different surrogates can vary in their environmental stability and response to different inactivating treatments (Cannon et al., 2006; Hirneisen and Kniel, 2013; Park et al., 2010; Topping et al., 2009; Tung et al., 2013).

For example MNV-1 has been reported to be very resistant to a wide pH range (pH 2-10), while FCV was rapidly inactivated at pH <3 and >9; however, FCV was more stable than MNV-1 at 56^{o} C, while both surrogates were similarly inactivated at 63 and 72^{o} C (Cannon et al., 2006). Tung et al. (2013) reported MNV to be more susceptible to 70% and 90% ethanol than FCV and HuNoV (GII.2 and GII.4). From these and other studies, it can be gathered that surrogates do not behave identically in their response to inactivation treatments. Some studies also suggest higher resistance of HuNoV upon

inactivating treatments than surrogates, based on molecular methods that assess capsid integrity (Escudero-Abarca et al., 2014; Topping et al., 2009). Variations in response to inactivation or disinfection methods have also been reported between HuNoV genotypes; Cromeans et al. (2014) reported a significant difference ($p<0.05$) in GI.1, GI.5 and GII.13 RNA reduction when exposed to 70% and 90% ethanol. The group reported up to 3.5 log reduction in GI.5 compared to a reduction less than 1 log in GII.13.

The suspension matrixes of different virus stocks used as the inoculum have also varied in different studies from partially clarified stool suspensions and cell culture lysates with high concentrations of organic debris and aggregated virus, to highly purified, mono-dispersed viruses derived from purified stool suspensions or cell culture lysates. Researchers have shown norovirus survives for longer periods of time when suspended in fecal material than when the virus is purified or diluted and suspended in PBS. For example, Escudero et al. (2012) observed an increased survival time for NoV suspended in artificial feces and (Topping et al., 2009) observed higher temperature resistance for FCV-9 spiked in a diluted stool specimen as compared to viruses diluted in PBS. Takahashi et al. (2011) reported complete loss of infectivity of MNV-1 (6.2 log MPN/ml inoculation titer) inoculated on food residue-free stainless steel coupons after 30 days; meanwhile, only a 1.4 log MPN/ml reduction was observed for MNV-inoculated coupons contaminated with food residues. Even though the precise mechanism of this protection is unknown, it is possible that solids present in fecal matter or food residues promote enhanced attachment of viruses to surfaces or protection from drying out (Escudero et al., 2012). The matrix in which the virus is suspended may also be

responsible for shielding the virus from environmental hazards or disinfectants, or they may be assisting in the neutralization of disinfectants such as is the case when chlorine is used for disinfecting soiled surfaces. Considering these observed variations in the impact of virus suspension matrix on virus survival and response to disinfectants, there is a possibility that virus suspension matrix can also interfere with methods used to predict HuNoV infectivity. Investigating the effect of virus matrix on methods of infectious norovirus detection is thus necessary.

Viral inoculum titer and inactivation methods employed in different studies geared towards discriminating between infectious and non-infectious HuNoV have also varied from one research group to another. Researchers have used low initial virus titers ($\approx 10^3$ PFU/ml (Nuanualsuwan and Cliver, 2002)), moderate input titers ($\approx 10^5$ PFU/ml (Lamhoujeb et al., 2008)) and high input titers ($\approx 10^8$ PFU/ml (de Abreu Corrêa et al., 2012)). These three groups used a combination of Proteinase K and RNase A or RNase A alone for enzymatic pretreatment (ET) of inactivated virus samples prior to RT-PCR. While the first two groups reported total elimination of PCR signals in samples undergoing ET, the latter reported just 0.5 to 1 log GC/ml reduction in PCR signals. However, these studies are also difficult to compare since they varied in their use of inactivation treatments, including either UV, thermal and/or hypochlorite at varying concentrations, temperatures and treatment times. Studies using low titer can be questioned based on the reasoning that (i.) they do not show effect of the treatments at an higher input titer under the same conditions, (ii.) these input levels are close to detection limits of the assay, (iii.) in practical sense, the 6-log reduction required for sterilization

confirmation would not be predictable from such results. Contrary to our opinions on the use of low titer samples, Topping et al. (2009) explained that even though low log reduction values observed when a high starting copy number sample was used in studies reviewing the use of enzyme pretreatment prior to RT-PCR may appear insignificant, they actually correspond to large differences in copy number due to the exponential nature of the PCR, and this was a justification for using low copy numbers in their studies. However, this argument seems contradictory because the same exponential nature of PCR will apply regardless of initial copy numbers, and logically we would have assumed a higher log reduction in studies with high initial copy number and vice-versa, but this is usually not the case. Therefore, using both low and high titer virus inoculums should be performed when evaluating methods for estimating virus infectivity in order to elucidate the role of virus titer in these apparent discrepancies.

As mentioned above, the use of inactivation methods, such as UV light, different temperature of heat, hydrostatic pressure, ozone, singlet oxygen, and hypochlorite, have made for varying results from the different studies, while an infectivity method may prove to be successful with viruses inactivated with heat for example, the same method may not be successful with other inactivation methods; an example of this was described earlier in studies by Hirneisen and Kniel (2012). A simple explanation for this variation is the differences in mode of inactivation by different inactivation methods, a detection method may or may not be capable of discriminating infectivity depending on the principle of detection. The majority of these inactivating treatments primarily target the viral capsid and only at extreme levels of treatment does RNA get destroyed. Treatment

with germicidal UV light (UVC, short wavelength - 100 to 280 nm, 254 nm commonly used) is an exception, since it primarily targets the RNA for virus inactivation. Capsid-targeting treatments produce characteristic results when milder inactivation conditions are used. The genomic RNA is often unaffected after mild disinfecting treatments; this is determined by the observation that little or no changes in RT-PCR signals are obtained when viruses are inactivated with mild or even moderate treatment levels and are compared to untreated virus. Considering all of these, a method for determining infectivity needs to correlate with the method of inactivation, in other words, if an inactivation method destroys the capsid rather than the RNA, then the method for determining infectivity should be assessing capsid damage.

1.10. Importance of current study in estimation of HuNoV infectivity

The factors discussed in section 9 are the influencing factors necessitating the studies reported in this dissertation, the need to compare possible methods of determining infectivity given varying virus titer, suspension matrix and inactivation methods. While finding a cell culture system for HuNoV detection will be a major breakthrough, in the meantime, evaluating promising methods for discriminating between infectious and non-infectious HuNoV is needed. This study minimizes variations observed in previous studies by comparing side by side as many of these variables as practically possible when evaluating promising methods to determine how these factors influence the effectiveness of the methods.

We studied the effect of virus titer and viral suspension matrix on the use of enzyme pretreatment (proteinase K and RNase A) on inactivated samples prior to RT-PCR as a means of determining infectivity of HuNoV. For this study, we employed heat treatment at a high temperature (99^{o} C) for 5 min as the virus inactivation treatment, with a rationale that with complete degradation of the viral capsid at this temperature, other study variables could be more easily investigated.

The second part of this study evaluates the use of PGM-binding followed by RNase A treatment prior to RT-PCR as a means of determining virus infectivity. In the experiments of this study, different virus titers and inactivation methods are used. This section of the study employed the use of heat treatment as used in the first part as well as other commonly employed method of inactivation including ethanol treatments and a sanitizer combination proven to be effective in elimination of bacterial pathogens and HuNoV surrogates (MNV and FCV) - Levulinic acid plus sodium dodecyl sulphate (LEV/SDS) sanitizer (Cannon et al., 2012; Webb et al., 2013; Zhao et al., 2010). This study section puts into perspective how binding of HuNoV to PGM is affected after low and high titer HuNoV have been treated with different commonly employed inactivation treatments.

The third part of this study evaluated different commonly employed RNA extraction methods in an attempt to investigate which of the methods best reduces PCR inhibitors. Four different extraction methods were investigated including a guanidine thiocyanate-based lysis buffer and silica spin column method, a trizol based method, and a commercial kit. This study also included variables such as different food matrices, and

variations in purification steps in the evaluation. Using MNV, HuNoV GI.1 and GII.4 in a virus cocktail inoculated in the different food matrix, this study also seeks to determine if the extraction method and other factors such as the food matrix has any impact on recovery of the different virus type.

Perhaps one of the most important aspects of the studies carried out in this dissertation is the use of a cultivable HuNoV surrogate - MNV-1, alongside both a GI and a GII HuNoV. This enables us to document the different observations related to each part of the research given a commonly used HuNoV surrogate that is cultivable, as well as strains from the two major genogroups of HuNoV. This also gives us the opportunity to correlate results of infectivity determination with plaque assay, as well as evaluating the effectiveness of the different inactivation methods employed in this study on a cultivable surrogate, MNV, by plaque assay. Taken together, the studies carried out in this dissertation provide important insight and progress towards finding a suitable method of discriminating between infectious and non-infectious HuNoV.

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CHAPTER 2

IMPACT OF VIRUS TITER AND SUSPENSION MATRIX ON INFECTIVITY ESTIMATES OF THERMALLY INACTIVATED HUMAN NOROVIRUS USING AN ENZYME PRE-TREATMENT WITH PROTEINASE K AND RNASE A PRIOR TO RT-QPCR[1]

[1]Afolayan, O.T. and J.L. Cannon. To be submitted to *Journal of Applied Microbiology.*

2.1. ABSTRACT

Human norovirus (HuNoV) causes the majority of foodborne illnesses in the U.S., but cannot be grown in cell culture. To predict HuNoV infectivity, enzymatic treatment of HuNoV using Proteinase K and RNase A prior to real time RT-PCR is commonly performed, but false positive results and variability between studies are often reported. This study evaluated the impact of virus suspension matrix and titer when enzymatic pre-treatment (ET) was employed prior to real time RT-PCR to predict HuNoV infectivity after heat inactivation of Murine Norovirus (MNV-1) and HuNoVs GI.1 and GII.4. Plaque assay of MNV-1 was also included for infectivity correlation. Untreated or thermally-inactivated (99^{o}C for 5 min) virus stocks were subjected to ET. The impact of inoculum titer and matrix was evaluated using high and low titer stocks. Virus stocks were purified using three methods: sucrose purification (SP), ultrafiltration (UF), and 0.2 µm syringe-filtration (SF). Virus matrix tested included (SP/UF/SF), SP/UF, SP alone (GI.1 and GII.4) or SP/UF with the addition of 5% fetal bovine serum (FBS) for MNV or stool suspensions for HuNoV. Viral RNA was quantified by real time RT-qPCR. The ET method was more effective in reducing viral RNA from SP/UF purified samples compared to crude samples; however, additional purification (SP/UF/SF) did not improve results. The highest log reduction observed using the ET method with heat-inactivated MNV-1 was 2.94 log genomic copies, however plaque assay revealed approximately a 6 log PFU reduction. Therefore, the ET method as a means of detecting infective HuNoV did not correlate with infectivity as determined by plaque assay. This study suggests that both the virus suspension matrix and the virus titer may impact real-time RT-PCR signals results following ET in thermal inactivation studies.

2.2. INTRODUCTION

Human Noroviruses (HuNoV) are major etiological agents of non-bacterial gastroenteritis, often associated with contaminated food, water and environmental surfaces. Despite numerous attempts over the last four decades, HuNoVs remain recalcitrant to laboratory culture (Atmar, 2010; Duizer et al., 2004). The most common means of HuNoV detection is the use of RT-PCR, which amplifies and confirms the presence of viral genomic material in a sample. However, the ability of a detection method to distinguish between infectious and non-infectious HuNoV is of utmost importance, because presence of a genomic material does not necessarily mean presence of an infectious virus particle. Alternative methods are thus needed to discriminate between infectious and noninfectious viruses in food, environmental and clinical samples.

Noroviruses can be classified into five groups (genogroups I to V), the two largest groups, GI and GII, include most of the diverse and common NoVs (Zheng et al., 2006). These are currently classified into 9 clusters for GI and 22 clusters for GII. The prototype HuNoV strain, Norwalk virus, belongs to genogroup I and genotype 1, and is thus designated GI.1 (Atmar, 2010). Consecutive numbers are assigned to each cluster and added onto NoV phylogenic trees as new clusters are defined (Zheng et al., 2006). One new GI (GI.9) and three new GII clusters (GII.20, GII.21, and GII.22) were recently identified in addition to previously defined genotypes, based on a newly proposed classification criteria, the 2xSD criterion (Kroneman et al., 2013). All sequences in GI and GII are of human origin, with the exception of GII.11 and GII.19 that are from porcine viruses, GIII are of bovine origin and GV is of murine origin (Kroneman et al., 2013; Zheng et al., 2006).

HuNoV studies have largely employed the use of cultivable surrogates to predict the response of HuNoV to disinfection and inactivation methods. Frequently employed surrogates include murine norovirus (MNV-1), feline calicivirus (FCV), and tulane virus (TV), a recently discovered virus, classified into *Recovirus* genus of the *Caliciviridae* family. Given that viruses even of the same genus can possess unique capsid properties, the response of different surrogate viruses to inactivation treatments are expected to vary from one virus type to another. MNV-1 replicates in an animal model (mice) and has been studied extensively for its suitability as a HuNoV surrogate. In a recent study comparing the response of various surrogates to disinfection, inactivation profiles of the surrogates varied for each treatment, but MNV-1 was reported alongside TV to be the most resistant surrogates overall (Cromeans et al., 2014).

Assessing the integrity of the viral capsid, the viral genome, or both the capsid and the genome has been attempted as a means of detecting infectious NoV (Cannon and Vinje, 2008; Kostela et al., 2008; Rodriguez et al., 2009; Sano et al., 2010). Of the most promising methods discussed in literature has been those that employ enzyme pretreatment of samples to avoid false positive signals after RT-PCR. Nuanualsuwan and Cliver (2003) reported that Proteinase K (Prot K) assists in further degradation of damaged viral capsids that have undergone inactivating treatments, and after subsequent digestion with RNase A, exposed RNA is degraded. It has been hypothesized that combination of these treatments with RT-PCR can be used to assess capsid integrity, which may correlate with infectivity. Lamhoujeb et al. (2008) reevaluated the enzyme pretreatment method, adapting it as follows; separating the Prot K treatment step from the RNase A treatment, and also stopping Prot K enzyme action before the addition of

RNase A, as opposed to adding both enzymes at the same time, since Prot K is capable of inactivating RNase A (Yang and Tsou, 1995).

While some studies have yielded promising results, others results have not been convincing. A direct comparison of these studies has been difficult due to differences in the preparation of the virus stocks used by different research groups. Key variables are the matrix in which the virus is suspended, the virus titer, and the genogroup/genotype of norovirus used. Components of the matrix may limit the accessibility of the virus to inactivation treatments or interfere with methods of virus detection and infectivity estimates. Also, it is possible that the effectiveness of the ET pre-treatment method may be different if used with low or high titer samples, since the assay detection limits could differ substantially, moreover, different genotypes of HuNoV may exhibit different responses to ET. These factors may contribute significantly to differences reported between research groups describing the efficacy of ET methods.

The impact of virus suspension matrix, titer and norovirus genogroup on results obtained using the ET method prior to real-time RT-PCR was investigated in this study. Having these variables compared in one study is pivotal to making recommendations on the efficacy of the ET method when used with samples of varying virus titer and the different suspension matrices. This study also seeks to understand the correlation of infectivity as determined by plaque assay compared to infectivity as determined by the ET-RT-PCR, using the cultivable surrogate (MNV). Lastly, the response of two different HuNoV strains to the ET method was compared.

2.3. METHODS

2.3.1. Virus preparation

MNV-1 was a gift from Dr. Herbert Virgin at Washington University. RAW 264.7 cells were obtained from ATCC (Manassas, VA). Virus stocks were prepared by inoculation of MNV-1 on confluent RAW 264.7 cells in T175 flasks, followed by incubation at 37^{o} C with 5% CO_2 for 48 hrs. Infected flasks were taken through three cycles of freezing (-70^{o} C) and thawing (at room temperature) before centrifugation at 2000 x g for 15 min to remove large cell debris and vacuum filtration (0.2 µm PES membrane filter) for further clarification. To make high titer virus stocks, the filtrate was ultracentrifuged in a Beckman Coulter Ultracentrifuge using a Type 35 rotor at 100,000 x g for 1 hour. The pellet was dissolved in sterile PBS overnight at 4°C before sucrose purification (SP) by loading the suspension on top of a 30% sucrose solution in a 5 ml ultracentrifuge tube and centrifugation using a NVT 90 rotor at 100,000 x g for 1 hour at 4°C. The pellet was re-suspended in sterile PBS and the sucrose purification step was repeated. The resulting pellet was suspended in PBS. The virus stocks were further purified by ultrafiltration (UF) using 100K MWCO Amicon Ultrafilter centrifuge tubes. Some virus stocks received additional purification by filtration through a 0.2 µm Syringe Filter (samples denoted: SF). Fetal Bovine Serum (FBS) (5%) was added to select SP/UF virus stocks to mimic a turbid organic matrix such as feces; in these samples, FBS was added after the samples were already SP and UF.

Pea-sized amounts of fecal material from HuNoV GI.1 infected persons (a gift from Dr. Robert Atmar, Baylor College of Medicine), were suspended in 550 µl of PBS (in as many replicates as needed). From a watery stool sample containing GII.4 HuNoV

Sydney strain (a gift from Dr. Jan Vinjé at the CDC, Atlanta, GA) 100 µl-aliquots were suspended in 900 µl-aliquots of PBS, also in as many replicates as needed. The stool suspensions were vortexed thoroughly until fully suspended, centrifuged at 14,000 rpm for 10 min using a Sorvall Legend Microcentrifuge from Thermo Scientific (Waltham, MA). Supernatants were pooled and homogenized and the centrifugation was repeated. Supernatants were then sucrose purified by loading the supernatant on top of 30% sucrose solution in a 5 ml centrifuge tube and centrifuging using a NVT 90 rotor at 100,000 x g for 1 hour at 4°C. The pellet was re-suspended in sterile PBS and the sucrose purification step was repeated. The pellet was re-suspended in sterile PBS and stored at -70^{o}C accordingly. Further purification using ultrafiltration and syringe filtration similar to MNV described above were carried out for GII.4 samples but not for GI.1 samples due to significant loss in virus titer observed when these methods were applied in preliminary work and the relatively low initial titer of the virus in the stool specimen.

2.3.2. Plaque assay

Viral input titer and log reductions in infectious MNV-1 were estimated by plaque assay. Briefly, RAW 264.7 cells reaching ≈90% confluence were infected with 100 µl of thermally treated or untreated samples at appropriate dilutions. After 1 hr of infection with manual gentle rocking at 15-min intervals, infection media was aspirated. An agar overlay containing 50:50 of 1% seakem LE agarose (Lonza, Rockland ME) and 2X minimum essential medium (MEM) was added and plates were incubated at 37^{o}C with 5% CO_2 for 48 hr. The 2X MEM contained MEM (Corning Cellgro, Manassas, VA), 10% Fetal Bovine Serum (Hyclone, Logan, UT), 3% HEPES (Lonza, Rockland,

ME), 2% Penicillin-Streptomycin (Thermo Scientific, Logan, UT), 2% Sodium pyruvate (Corning Cellgro, Manassas, VA), 2% L-glutamine (Lonza, Rockland, ME), 2% Non-essential amino acids (Lonza, Rockland, ME) and 3% Sodium bicarbonate (Corning Cellgro, Manassas, VA). After 48 hours of incubation, a 3.7% formaldehyde solution (Acros Organics, Geel, Belgium) diluted in PBS was added for a minimum of 2 hr to fix the cells on the plates, then the agar layer was removed and plates were stained with 1% Crystal Violet (Alfa Aesar, Ward Hill, MA) in 20% methanol (VWR, West Chester PA). Visible plaques were counted and virus titers in sample were calculated and expressed as particle forming units per ml (pfu/ml), taking into consideration all dilution steps involved.

2.3.3. Thermal inactivation of MNV-1 and HuNoVs

Sample portions (100 µl each) of MNV-1 or HuNoVs were heated in thin-walled PCR tubes in a thermocycler (Mastercycler® ep, Eppendorf Westbury, NY) at 99^{o}C for 5 min, and then held at 4^{o}C until used. Non-heated controls included 100 µl portions of each virus; these were held in a refrigerator at 4^{o}C until used. HuNoV heated samples and non-heated controls were immediately processed by the enzyme pre-treatment (ET) method. For MNV-1, complete heat inactivation was confirmed by plaque assay and also processed by the ET method.

2.3.4. Enzyme pre-treatment method

Proteinase K and RNase A enzymes were used in the enzyme pre-treatment (ET) method of Lamhoujeb et al. (2008) to investigate the method's ability to eliminate

positive RT-PCR signals in thermally inactivated stocks of MNV-1 and GI.1 and GII.4 HuNoV. Briefly, 22 U of Prot K (Amresco, Solon, OH) was added to 100 µl of each sample and incubated at 37^{o}C for 1 hr. Then, 2 µl of 200 µM Phenylmethane sulfonyl fluoride (Sigma-Aldrich, St Louis, MO) was added at room temperature for 30 min to inactivate Prot K. Next, 100 ng of RNase A (Invitrogen, Carlsbad, CA) was incubated with each sample at 37^{o}C for 1 hr, before 80 U of RNase Inhibitor (Ambion) was added at room temperature for 15 min to stop the reaction. For all enzyme treated samples (both heated and non-heated), non-enzyme treated controls were also performed.

Both low and high titer virus stocks prepared by SP/UF/SF, SP/UF only, SP alone (GI.1 or GII.4) or SP/UF + 5% FBS were used to estimate the impact of inoculum matrix and virus titer on pre-treatment efficiency. To further investigate the effect of suspension matrix, in some experiments, sucrose-purified-ultrafiltered (SP/UF) GII.4 virus was suspended in a GI.1-containing stool specimen or a SP/UF + FBS MNV-1 virus stock prior to heat inactivation and ET. For samples containing FBS, 5% FBS was added into the sample only after heat inactivation as heating solidified and changed FBS consistency dramatically.

2.3.5. RNA extraction

RNA was extracted from all samples using a modified form of the guanidine thiocyanate (GuSCN) lysis buffer and silica binding-based method previously described (Boom et al., 1990). The GuSCN lysis buffer was made in-house by dissolving 60g guanidine thiocyanate in 50ml 0.5X TE buffer (this can be heated in a water bath at 56 ^{o}C to facilitate dissolution); after dissolving, 5.5 ml of 5M sodium chloride, 5.5 ml of 3M

sodium acetate, and 1.1 ml polyadenylic acid were added. This was then mixed together and stored at room temperature in a dark container or by covering the bottle completely with foil, since the lysis buffer is light sensitive. Briefly, the GuSCN lysis buffer was added to each sample at a volume equal to the sample volume. Each sample was vortexed briefly and incubated for 15 min at room temperature before 2x the sample volume of 100% ethanol was added to each sample. Samples were then loaded onto a spin column (Omega Bio-Tek, Norcross, GA) and centrifuged at 17,000 x g for 1 min. Each column was washed with 500 µl 75% ethanol and centrifuged again at the same speed. A dry spin was then performed to remove residual ethanol before the RNA on the column was eluted with 50 µl nuclease-free water (EMD Millipore, Billerica, MA).

2.3.6. Real-time RT-PCR

Viral RNA was quantified by real-time reverse transcriptase polymerase chain reaction (RT-qPCR). Real time RT-qPCR was carried out with the following primer/probe pairs; MNV-1, probe G54808P (CTA CCC ACC AGA ACC CCT TTG AGA CTC) and primers G54763F (TGA TCG TGC CAG CAT CGA) and G54863R (GTT GGG AGG GTC TCT GAG CAT) (Park et al., 2010); GI.1, probe Ring1C (AGA TYG CGI TCI CCT GTC CA) (Hill et al., 2010) and primers Cog1F (CGY TGG ATG CGI TTY CAT GA) and Cog1R (CTT AGA CGC CAT CAT CAT TYA C) (Kageyama et al., 2003); GII.4, probe Ring 2 (TGG GAG GGC GAT CGC AAT CT) and primers Cog 2R (TCG ACG CCA TCT TCA TTC ACA) (Kageyama et al., 2003) and JJV2F (CAA GAG TCA ATG TTT AGG TGG ATG AG) (Jothikumar et al., 2005). Amplification was carried out in temperature-time combinations of 50^{o}C for 30 min,

95°C for 15 min and 40-50 cycles of 95°C for 10 s followed by 55°C for 30 s and 72°C for 30 s on the Stratagene Mx3005P qPCR System (Agilent Technologies, Santa Clara, CA). The MxPro software was used for data collection and quantification based on standard curves (10-fold serial dilutions) performed with each MNV-1 and GII.4 assay. For GI.1, C_T values (the number of amplification cycles required for a signal to cross the threshold) were recorded for relative quantification (see results analysis section). All samples and controls were assayed in duplicate wells. RNA transcripts were prepared by *in vitro* transcription of MNV-1 and HuNoV GI and GII cDNA using the MEGAshortscript high yield transcription kit (Ambion, Austin, TX). The range of efficiency values considered acceptable for the standard curves were 85 - 115 %.

2.3.7. Result analysis

To evaluate the ability of the ET method in eliminating RT-PCR-positive signals in heat-treated samples, concentrations of viral RNA were quantified for ET and non-ET samples and for heated and un-heated samples. For all MNV-1 and GII.4 samples, the genomic copy number of MNV-1 and GII.4 HuNoV were determined by comparison with a RNA standard curve obtained from serial dilution of transcripts specific to each virus type. This was used to calculate log reductions in genome copy numbers observed due to ET. This calculation was performed for both heated and unheated samples. In some cases, a complete loss of signal could be observed, for such samples, a cut-off limit for C_T values, which in this study was 40 $C_{T,}$ was determined and genomic copy numbers were extrapolated for this C_T value using the RNA standard curve equation.

In the case of GI.1, Relative Differences (RD) in C_T values generated with each set of experiments were used as a relative measure of the concentration of target RNA present before and after ET using the following equation (Equation 1):

$$\boldsymbol{RD = Ct_E - Ct_{NE}} \quad \text{.....Eqn 1}$$

Where Ct_E = Avg. C_T Value of enzyme pre-treated sample

Ct_{NE} = Avg. C_T Value of untreated sample.

2.3.8. Statistical analysis

Log reductions in genome copy numbers of viral RNA and RD values for each set of experiments were analyzed by ANOVA using JMP Pro 11 (SAS Institute Inc., Cary, NC). Significant differences in the means due to treatment variables were compared using a student's t-test. For all significance tests, $\alpha = 0.05$ was used.

2.4. RESULTS

2.4.1. Thermal inactivation of MNV-1 measured by the ET method and impact of virus titer and suspension matrix

Figure 2.1 depicts the log reduction in genomic copies per ml (GC/ml) of MNV-1 after thermal treatment (99°C for 5 min) as determined by the ET method prior to RT-qPCR; control samples without thermal treatment were also included. Both high (~10 log GC/ml or 6 log PFU/ml) and lower (~7 log GC/ml or 3 log PFU/ml) titer inoculums with differing levels of purification (SP/UF/SF > SP/UF > SP/UF + 5% FBS) were used to examine the impact of virus titer and suspension matrix on the ET method. For the MNV-1 virus stocks prepared with high degrees of purification (SP/UF/SF and SP/UF)

and using the ET method prior to real-time RT-PCR, the highest log reductions were observed. For heated virus stocks, log reductions reached 2.75 and 2.94 GC/ml for high-titer SP/UF/SF and SP/UF stocks, respectively. For unheated SP/UF/SF and SP/UF virus stocks, log reductions were 0.93 and 0.70 GC/ml, respectively, these observation were in the high titer stocks. Differences in log reductions between thermally inactivated and unheated samples were statistically significant for all SP/UF/SF and SP/UF samples, regardless of virus titer ($p < 0.05$). In contrast, log reductions of low and high titer SP/UF + 5% FBS MNV-1 virus stocks were ≤ 1.06 log GC/ml following ET and real-time RT-PCR, regardless of thermal treatment. There was no statistically significant difference in log reduction by ET when comparing heated and non-heated samples in this matrix ($p = 0.3377$ and 0.4900 for low and high titer virus stocks, respectively). Virus suspension matrix significantly impacted the results obtained by ET prior to real-time RT-qPCR for thermal-inactivated MNV-1 ($p= 0.0061$), but the virus titer did not ($p=0.0546$). Using the ET-RT-PCR method, the highest log reduction observed overall in thermally inactivated MNV-1 samples was 2.94 log GC/ml; meanwhile, plaque assay infectivity results revealed complete elimination of infectivity (up to 6 log PFU/ml after thermal inactivation) (data not shown).

2.4.3. Thermal inactivation of human norovirus GI.1 and GII.4 as measured by the ET method and impact of virus titer and suspension matrix

Due to the low titer of GI.1 in the original stool specimen and inefficiencies in the UF and SF methods, further purification after sucrose purification (SP) was not possible. A wider range of GI.1 titers (beyond 3 and 4 log GC/ml) and purification levels,

therefore, could not be compared. Relative differences in C_T values obtained when comparing ET and non-ET samples are reported in Figure 2.2 for heated and unheated samples. A 1 log GC/ml reduction of viral RNA detected is equivalent to approximately a 3.3 value increase in C_T value. Therefore, reductions in GI.1 GC/ml were less than 1 log GC/ml after heat treatment at the two virus titer levels tested (Figure 2.3). ET was not significant in reducing GI.1 RNA after thermal inactivation ($p=0.2191$).

Figure 2.3 depicts log reductions in genomic copies per ml (GC/ml) of GII.4 after thermal treatment (99°C for 5 min) or without heat treatment as determined by the ET method prior to real-time RT-PCR. Stool samples containing high titer GII.4 HuNoV (~7 log GC/ml) were purified at different levels (SP/UF/SF & SP/UF) and diluted 100x or simply diluted 1000x without purification (labeled as Stool Suspension) to reach target inoculum titer for each. Overall, significantly higher log reductions were observed using the ET-RT-PCR method for heated samples when compared to unheated samples (Fig. 2.3) ($p<0.0001$). The highest log reduction observed in unheated GII.4 stocks was 0.80 log GC/ml, while the highest log reduction observed for heated GII.4 stocks was 2.68 log GC/ml; both were in low titer SP/UF/SF samples. The viral suspension matrix was statistically insignificant ($p=0.4002$), while virus titer was significant ($p=0.0006$) (Fig. 2.3), revealing greater log reductions consistently observed for low titer GII.4 stocks when compared to high titer virus stocks.

2.4.4. Impact of GI.1 stool and MNV-1 suspended in 5% FBS on thermal inactivation of GII.4 as measured by the ET method

Insignificant findings due to suspension matrix were observed for GII.4 samples across the Stool Suspension, SP/UF and SP/UF/SF sample matrices (Fig. 2.3). However, significant findings due to suspension matrix were observed for MNV-1 with 5% FBS and for G1.1 stool suspensions. It was therefore unclear if such results were due to unique properties of the GII.4 virus, or if the GII.4 stool suspension may have been just too diluted to see such an effect due to stool matrix. GII.4 SP/UF virus stocks (6 log GC/ml) were therefore suspended in 10X diluted GI.1 stool samples (3 log GC/ml) or MNV-1 + 5% FBS virus stocks. Both of these virus stocks (without the addition of GII.4 HuNoV) were previously observed to yield no statistically significant differences between heated and unheated samples (Figures 2.1 & 2.2), or between ET and non-ET samples. Fig. 2.4 reveals negligent log GII.4 GC/ml reductions using the ET method for combined GII.4/GI.1 and GII.4/MNV-1 + 5% FBS preparations ($p = 0.2888$), suggesting the ET method is negatively impacted by fecal matrix and high levels of FBS in the virus stock. Also, little difference is observed when comparing RNA recovered from heated vs un-heated virus stocks. In these set of samples, the highest average log reduction of GII.4 was 0.19 log GC/ml using the ET method, regardless of heat inactivation.

2.5. DISCUSSION

As efforts to combat HuNoVs continue to mount, researchers are investigating factors that influence HuNoV transmission during outbreaks and develop intervention strategies to control their spread. However, the inability to discriminate between infectious

and non-infectious HuNoV often leads to inconclusiveness when drafting recommendations and guidelines for HuNoV prevention and control. Without the ability to culture HuNoV, different methods of estimating HuNoV infectivity have been investigated over the last 12 years. ET prior to RT-PCR has been reported by some groups to be effective in reducing or eliminating RT-PCR signals from non-infectious virus in inactivated samples (Lamhoujeb et al., 2008; Nuanualsuwan and Cliver, 2002); however, de Abreu Corrêa et al. (2012) reported only a 0.5 to 1 log reduction in RNA when 10^8 PFU/ml chlorine inactivated MNV-1 samples were treated with RNase prior to nucleic acid extraction. The primary variables that differ between studies that report positive findings and those that do not pertain to preparation of the virus stock (suspension matrix), the virus titer, and the strain of norovirus used. Therefore, in this study those variables were included and evaluated side by side to understand their effect on the ET efficiency.

HuNoV can be associated with a variety of sample matrices, including food, water, environmental and clinical samples. But since they are spread through the fecal-oral route either by direct contact with an infected person or by indirect transmission when a person consumes contaminated food or water, the viruses are naturally associated with either feces or vomit. When working with fecal or vomit samples containing HuNoV in the lab, the fecal matrix is often purified from the virus so that the virus can be studied without interfering substances of the matrix. In some scenarios, a more diluted fecal/vomit matrix may be found naturally, for instance if water is contaminated with HuNoV. Such samples may resemble the purified virus stocks used in the lab, but otherwise, the natural matrix of the virus is more likely to be complex.

In this study, ET prior to real-time RT-PCR reduced the levels of RNA recovered from heated (99 ^{o}C for 5 min) virus samples, when compared to samples that were not heated. There were, however, exceptions to this finding. For the MNV-1 + 5% FBS and the GI.1 SP samples and the combinations of GII.4/MNV-1 +5% FBS and GII.4/GI.1 SP samples, reductions in viral RNA detected in heated samples and unheated samples did not differ significantly from each other and were all estimated to be < 1 log GC/ml as determined by the ET method. Interestingly, log reductions (GC/ml) determined by the ET method were similar for the heated GII.4 Stool Suspension samples (up to 2.55 log GC/ml) and the more purified GII.4 matrixes (SP/UF and SP/UF/SF) that were heated (up to 2.68 log GC/ml). However, when either GI.1 stool or MNV-1 + 5% FBS was added to the GII.4 SP/UF sample, little or no reduction in RNA copies was observed for the heated GII.4 samples, as determined by the ET method. These observations suggest ET efficiency may be reduced in the presence of impurities found in a fecal or vomitus sample or in the presence of high protein levels (5% FBS). We hypothesize that the fecal material present in the GI.1 samples and the 5% FBS mixed into the MNV-1 samples prevented the RNase A enzyme of the ET method from accessing viral RNA exposed after heat treatment. The GII.4 stool suspension used in this study was a very high titer stock (11 log GC/ml) which had to be diluted in PBS to reach the desired virus titer (5 log GC/ml) used in this set of experiments. It is possible that there was a lack of variation in stool suspension, SP/UF/SF and SP/UF samples observed in Fig 2.3 because of significant dilution of fecal sample in PBS, such that the virus was more suspended in PBS than in fecal matrix. The results from these cleaner matrix samples (Fig 2.3) compared to the same GII.4 suspended in GI.1 stool or MNV + 5% FBS matrix (Fig 2.4) suggests that samples obtained from clinical specimens,

food items, environmental waters, environmental surfaces, or even crude cell culture lysates may have to be purified prior to testing by the ET method.

Another factor to consider is the impact of stool suspension or high organic loads on virus survival and response to disinfection. Studies have shown that norovirus survives for longer periods of time when suspended in fecal material than when the virus is purified or diluted and suspended in PBS. For example, Escudero et al. (2012) observed an increased survival time for NoV suspended in artificial feces. Topping et al. (2009) observed higher temperature resistance for FCV-9 spiked in a diluted stool specimen as compared to viruses diluted in PBS. Takahashi et al. (2011) also reported complete loss of infectivity of MNV-1 (6.2 log MPN/ml inoculation titer) inoculated on food residue-free stainless steel coupons after 30 days; meanwhile, only a 1.4 log MPN/ml reduction was observed for MNV-inoculated coupons contaminated with food residues. Similarly, Cromeans et al. (2014) showed significantly higher log reductions of semi-purified HuNoVs after exposure to 70% and 90% ethanol, as determined by the RNase pre-treatment method, when compared to the non-purified stocks. In this study, clarified stool suspensions were buffer exchanged into MEM + 10% FBS using Amicon™ 50 KDa (Ultra-15) ultrafilters.

When only low titer stocks are used in enzyme treatment studies, complete elimination of signal is often reported. This may be because these input levels are close to detection limits of the assays or because the inactivation effects are more sensitively detected at low input titers. Complete elimination of signals using ET prior to conventional RT-PCR of heat inactivated (72^{o}C) FCV, HAV and PV-1 (Nuanualsuwan and Cliver, 2002) and NoV and FCV heated at 72^{o}C for 45 - 60 min (Lamhoujeb et al., 2008) was reported.

Nuanualsuwan and Cliver (2002) treated virus stocks containing ~10^3 PFU/ml with Prot K and RNase A after inactivating treatments to obtain negative results for enzyme treated, heat-inactivated samples. This group as well as Topping et al. (2009) employed the use of low titer viruses alone in their studies justifying the choice because in low titer samples, the sensitivity of the ET method could more accurately be determined. It was hypothesized that virus aggregates form in high titer virus stocks. Viruses on the outside surface of these aggregates are more exposed to the inactivating treatments and are readily inactivated, but internal viruses are protected from the inactivating treatments. Therefore, when high titer virus stocks are inactivated, a mixture of inactivated and non-inactivated viruses often results; this can be prevented by using low titer samples. While this explanation may be valid, not knowing the efficiency of the ET method in higher titer samples creates a gap in the applicability of the method. It is important to have such information so that potential uses of the ET method can be guided with the results. Add a sentence to rap this paragraph up.

Consistently higher log reductions were observed for heated low titer GII.4 samples than heated high titer samples (Figure 2.3) as determined by the ET method. However these findings were not consistent for heated MNV-1 samples of different virus titers, as determined by the ET method. This difference between MNV and GII.4 may be due to inherent differences between the virus types. Topping et al. (2009) reported differences in GII.4 and FCV responses to heat inactivation reporting that GII.4 has a greater thermostability compared to FCV, as FCV RNA was maximally exposed at 63.3 ^{o}C while up to 76.6 ^{o}C heat treatment of GII.4 was required for RNA to be maximally exposed as determined by RNase treatment of the heated virus samples. Cromeans et al.

(2014) also reported a higher RNA reduction in MNV samples that were ethanol treated and RNaseOne treated prior to RNA extraction compared to GII.13 and GI.5 HuNoV samples similarly treated.

However, an alternative explanation for the variations observed would be the difference in virus titer, C_T values in untreated (control) low titer samples are typically close to limit of detection of RT-PCR, and therefore it would make sense that elimination of RT-PCR signals or a seemingly high reduction in signal would be observed in heat treated low titer samples. This rationale would explain the higher log reduction observed in low titer GII.4 samples. However, absence of such observation in the low titer MNV sample used in this study can be explained to be due to the fact that even though the low titer MNV sample had a 3log PFU/ml input titer, the log genomic copies in the same was 7log GC/ml, thus C_T value in this samples were far off the limit of detection, causing a similar observation in RNA reduction to high titer GII.4 samples.

This ET method assay did not correlate directly with infectivity as determined by MNV-1 plaque assay. The highest log reduction observed using the ET method on heated MNV-1 samples was 2.94 log genomic copies (using the SP/UF high titer sample), while plaque assay of heated MNV-1 revealed absence of infectious virus (approximately a 6 log PFU reduction; data not shown). Heat inactivation is expected to damage viral capsids to the point that genomic material is exposed. Degradation of exposed RNA is also expected when in the presence of RNase A. However, there was a ~ 3 log discrepancy observed when comparing plaque assay and ET method plus RT-PCR data, suggesting either that the viral RNA is protected from RNase or the RNase treatment is insufficient. However, the preliminary work carried out prior to commencing this study

tested the efficacy of RNase A on naked MNV RNA (extracted using the GuSCN lysis buffer) and the results showed up to 5log GC/ml reduction in the high titer RNA samples and complete elimination of RT-PCR signals in RNA diluted 100X and beyond. Despite destruction of the outer capsid structure (composed of 180 copies of VP1 structural protein), during heat inactivation, little is known about the other structural viral protein, VP2 after heat treatment. VP2 protein has been reported to be highly basic and hence positively charged (Vongpunsawad et al., 2013). Thus, VP2 may bind to genomic viral RNA, protecting regions of RNA that are amplified by RT-PCR from RNase A degradation. This could be an explanation for positive real-time RT-PCR signals still observed after RNAse treatment of virus stocks inactivated with heat at the extreme temperature employed in this study. Even though the use of Proteinase K prior to RNAse A treatment was presumed to assist in degrading such protein leftovers as suggested by Nuanualsuwan and Cliver (2003), the efficiency of the proteinase K in this regard is questionable based on the results in this study.

2.6. CONCLUSIONS

Generally, molecular methods such as real time RT-PCR are known to overestimate infectious virus titer due to detection of RNA from non-infectious virus; this overestimation was anticipated to be eliminated by pre-treatment with RNase A before real-time RT-PCR. This rationale was the basis for using Prot K and RNase A to treat heated virus samples prior to real-time RT-PCR, since it was anticipated that there would be complete destruction of the viral capsids at the temperature and time used. Therefore, the subsequently exposed RNA would be completely degraded by RNase A. In this study,

the effect of the virus suspension matrix and the virus titer on real-time RT-PCR results following enzyme pre-treatment in thermal inactivation studies varied with the virus type and sample properties. The pretreatment method was more efficient with purified virus stocks. It was also found that the pre-treatment method seems to eliminate RT-PCR signals when using low titer stocks, but the same was not expected in high titer stocks. Also, virus titer was significant with ET-RT-PCR results for GII.4 HuNoV, but it was not for the MNV-1 virus titers tested. It may be that the ET method or the inactivating treatments perform differently when comparing one virus type to another. Therefore, these factors should be considered before applying this method in HuNoV detection studies and more studies may be warranted. Given that the reduction in RNA copies due to the ET method were not consistent across matrices, applicability of this method is limited; however, including a sample purification step(s) when working with samples possessing complex matrices may improve consistency. It is important to note that the (thermal) inactivation methods used in this study were purposefully extreme to guarantee complete inactivation. But even with these extreme temperatures, the ET method was not adequate for eliminating RT-PCR signals in high titer samples. Therefore, using ET methods prior to real-time RT-PCR of virus samples treated with milder inactivation conditions may yield even lower reductions in PCR signals.

2.7. APPENDIX

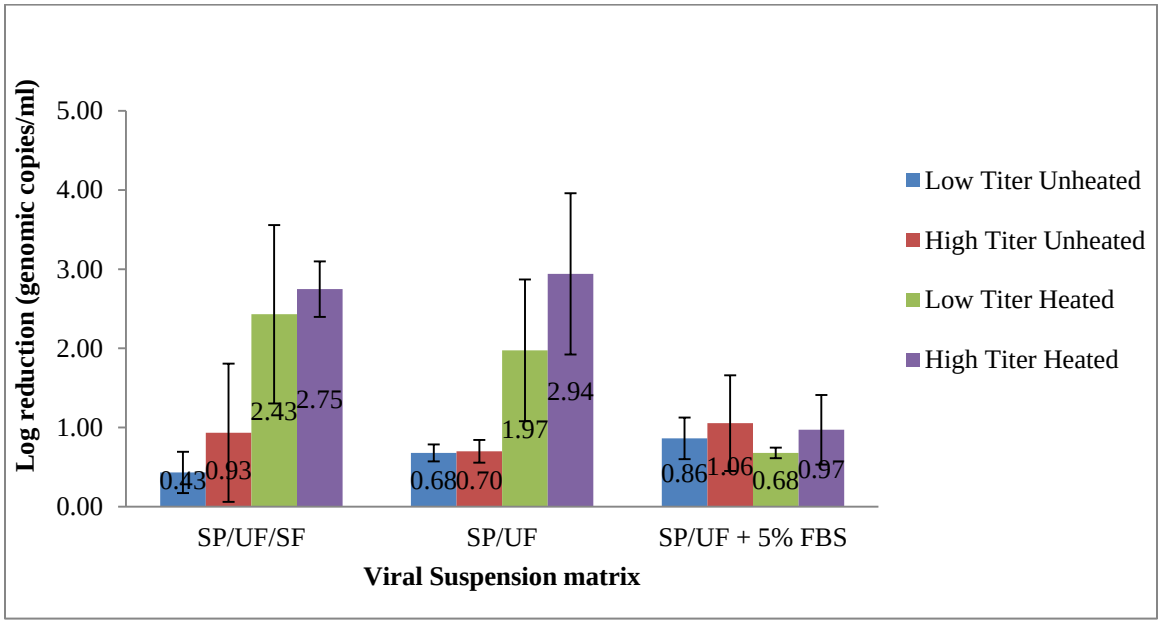

Fig. 2.1: Log reduction of MNV-1 RNA determined by the enzyme treatment (ET) method for virus stocks with different titers and sample matrices. Results reflect the susceptibility of unheated and heated MNV-1 virus stocks to ET, with variables of virus titer and suspension matrix. SP/UF/SF samples are the most purified, while the SP/UF +5% FBS contain the greatest amount of organic material. Low titer samples - 7 log GC/100ul, High Titer samples - 10 log GC/100ul *Error bars represent standard deviation, n=3.

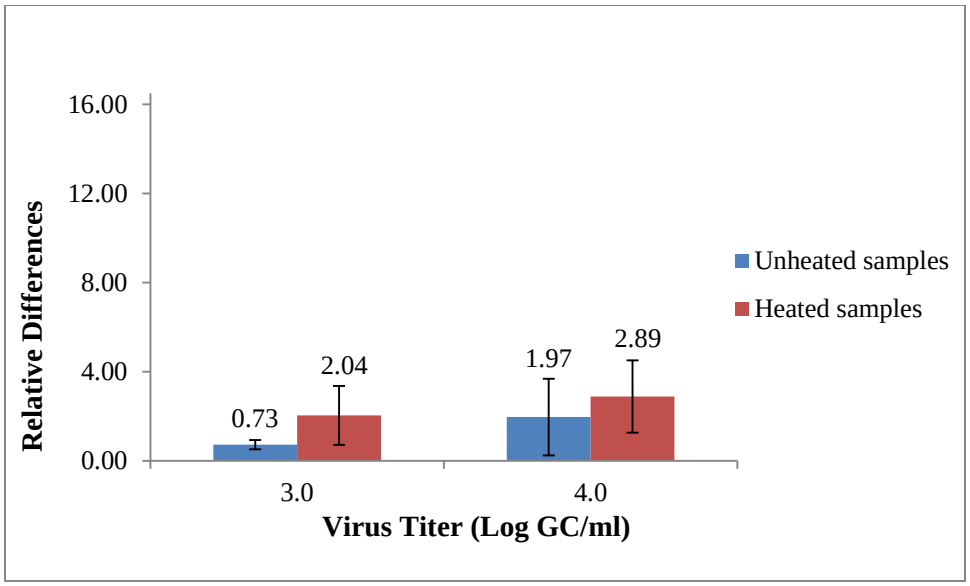

Fig. 2.2: Relative difference (RD) in C_T values with treatment of GI.1 HuNoV with Proteinase K and RNase A. Results reflect the susceptibility of unheated and heated GI.1 virus stocks to ET. C_T values did not justify quantification; RD observed was less than 1 log in all cases. Minimal relative difference in C_T value was observed after ET of GI.1 virus samples. Virus stock was sucrose purified (SP), low titer of virus did not allow for further purification due to loss of titer, therefore only GI.1 SP stock was tested. *Error bars represent standard deviation, n=3.

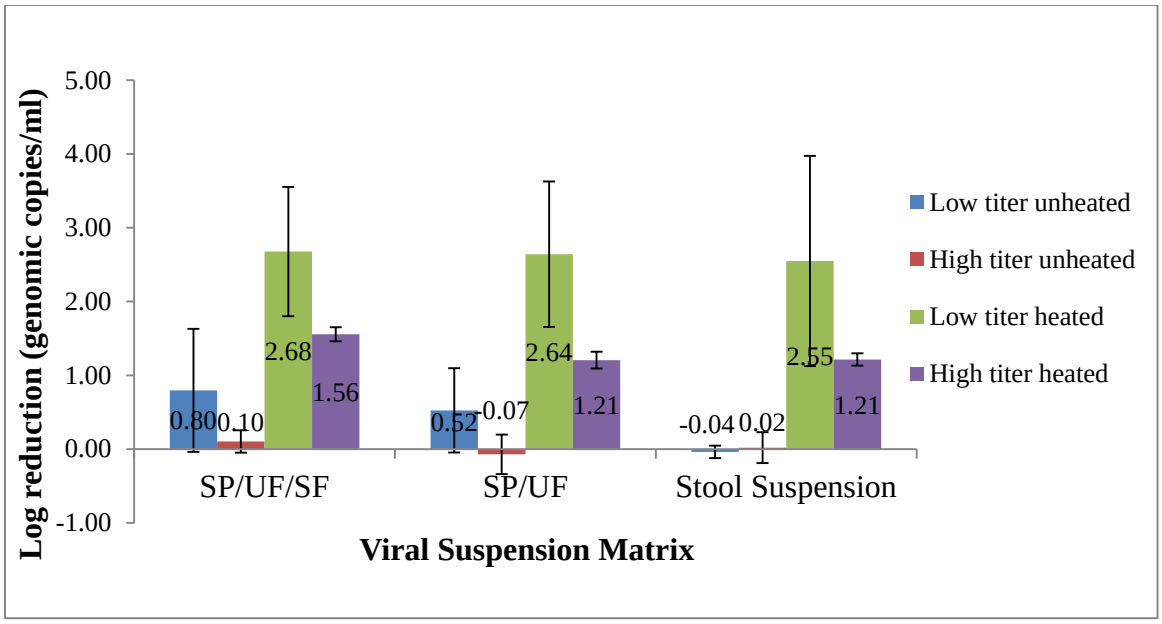

Fig. 2.3: Log reduction in viral RNA recovered after enzyme treatment of GII.4 samples. Results reflect the susceptibility of unheated and heated GII.4 virus stocks to ET, with variables of virus titer and suspension matrix. Stool suspension in order of purification where SP/UF/SF samples are the most purified. Low titer samples - 3 log GC/100ul, High Titer samples - 6 log GC/100ul *Error bars represent standard deviation, n=4.

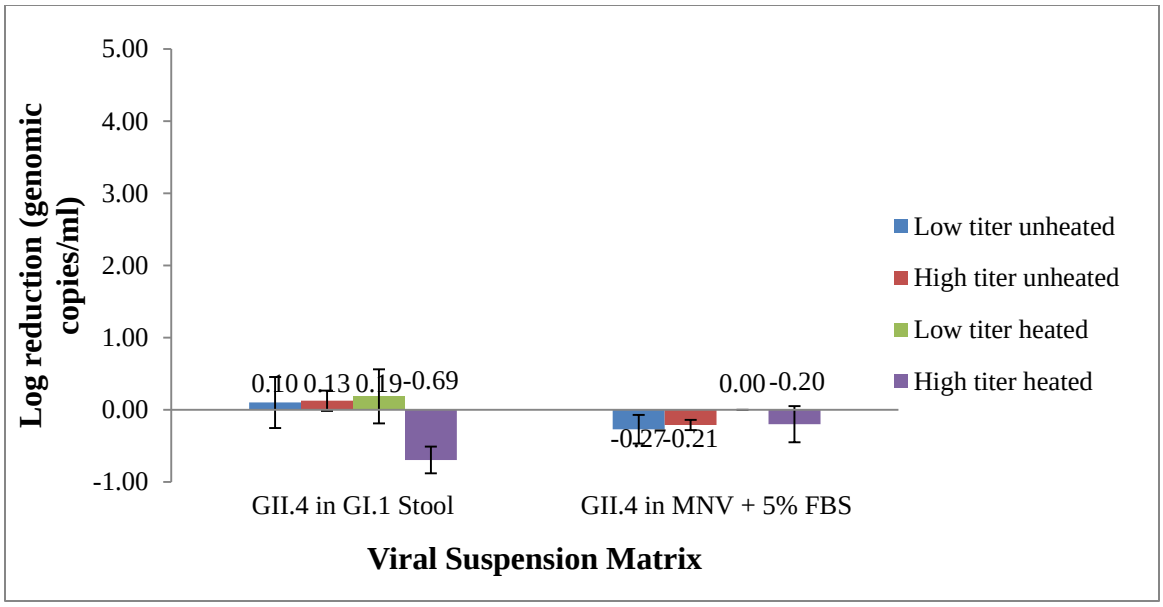

Fig. 2.4: Log reduction in viral RNA recovered after enzyme treatment of GII.4 samples. Results reflect the susceptibility of unheated and heated GII.4 virus stocks to ET, with variables of virus titer and suspension matrix. SP/UF GII.4 virus stock was suspended in GI.1 stool or MNV + FBS. Low titer samples - 3 log GC/100ul, High Titer samples - 6 log GC/100ul *Error bars represent standard deviation, n=4.

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CHAPTER 3

EVALUATION OF A PGM-BINDING METHOD FOR THE DISCRIMINATION OF INFECTIOUS AND NON-INFECTIOUS NOROVIRUS FOLLOWING INACTIVATION BY HEAT, ETHANOL OR A LEVULINIC ACID PLUS SODIUM DODECYL SULFATE SANITIZER[2]

[2]Afolayan, O.T., C.C. Webb and J.L. Cannon. To be submitted to *Food and Environmental Virology*

3.1. ABSTRACT

Human noroviruses (HuNoV) are a major source of foodborne illnesses worldwide. As they cannot be cultured in vitro, alternative methods that discriminate between infectious and non-infectious HuNoV are needed. This study evaluates binding of GI.1 and GII.4 HuNoV to histo-blood group antigens expressed in porcine gastric mucins (PGM) as a surrogate for detecting infectious virus following treatment with heat, ethanol (EtOH) or a levulinic acid plus sodium dodecyl sulfate (LEV/SDS) sanitizer. GI.1 and GII.4 HuNoV (3-5 log genome copies/sample) were inactivated by heat (99^{o}C for 5 min), 70% EtOH, or with liquid sanitizers containing 0.5% Lev/0.01% SDS (low SDS) or 0.5% Lev/0.1% SDS (high SDS) for 1 min. Treated and untreated (control) virus samples were applied to 96-well plates coated with 1 µg/ml PGM. RNase A (100 ng/well) was added to designated wells for each sample to degrade exposed RNA. The number of wells (positive/total) containing bound and potentially intact virus was calculated after real-time RT-PCR. After thermal inactivation, 10% and 16% of PGM coated wells showed positive real time RT-PCR signals for GI.1 and GII.4 with RNAse A treatment, respectively, whereas no positive signals and 66.6% positive signals were observed for non-RNase A treated GI.1 and GII.4 HuNoV, respectively. For both GI.1 and GII.4, the high SDS sanitizer completely eliminated PCR signals, but 100% of wells treated with the low SDS sanitizer were positive. 70% EtOH eliminated positive real time RT-PCR signals for GII.4 samples, while 100% of GI.1 samples similarly treated tested positive. All wells that were not treated with heat or sanitizer were positive for GI.1 and GII.4 RNA. The PGM-binding method is appears to be a promising surrogate for

discriminating between infectious and non-infectious HuNoVs after capsid destruction, but virus strain and inactivation method appear to impact results obtained.

3.2. INTRODUCTION

Human noroviruses (HuNoV) are a major source of foodborne illnesses worldwide. An estimated 5.5 million cases of illnesses for which HuNoV is the causative agent are reported yearly in the US, HuNoV is second only to *Salmonella* (35%) as the pathogen responsible for the highest number of hospitalizations (26% or 14,663 persons hospitalized), and an estimated 149 deaths (Scallan et al., 2011). First identified as Norwalk virus, human noroviruses are taxonified in the genus *Norovirus* and in the *Caliciviridae* family. HuNoV can be transmitted through several means including consumption of food contaminated with feces, person-to-person contact, exposure to aerosolized vomitus from an infected person, or by touching contaminated surfaces or fomites (Repp and Keene, 2012; Sharps et al., 2012; Wikswo and Hall, 2012).

Noroviruses can be classified into five groups (genogroups I to V), the two largest groups, GI and GII, include most of the diverse and common NoVs (Zheng et al., 2006). These are currently classified into 9 clusters for GI and 22 clusters for GII. One new GI (GI.9) and three new GII clusters (GII.20, GII.21, and GII.22) were recently identified in addition to previously defined genotypes, based on a newly proposed classification criteria, the 2xSD criterion (Kroneman et al., 2013). All sequences in GI and GII are of human origin, with the exception of GII.11 and GII.19 that are from porcine viruses, GIII are of bovine origin and GV of murine origin (Kroneman et al., 2013; Zheng et al., 2006).

Noroviruses are a non-enveloped, icosahedral group of viruses with single-stranded, positive sense linear RNA genome. The capsid is about 28-35 nm and the genome therein is about 7.5 to 7.7 kilobases, composed of three open reading frames (ORFs). ORF 1 encodes a large polyprotein from which six non-structural proteins are produced by proteolytic cleavage, ORF 2 encodes the major structural protein, VP1, which forms the capsid, and ORF 3 encodes minor structural protein, VP2 (Atmar, 2010).

As they cannot be cultured *in vitro*, detection of HuNoV is limited to the use of RT-PCR which gives no information about the infectivity of the virus, and detected RNA could be from infectious or non-infectious HuNoV. HuNoV RNA can be detected in the environment including water bodies for an extended period of time. In a study comparing hepatitis A virus (HAV), feline calicivirus (FCV) and HuNoV survival in mussels over a 4 week period, Hewitt and Greening (2004) reported persistence (no reduction) of RT-PCR-detected HuNoV and HAV RNA after 4 weeks in mussels marinated with acid at pH 3.75, whereas a 1.75 log reduction in infectivity was observed for HAV as measured by a tissue culture infectious dose assay, FCV was inactivated but still detectable by RT-PCR.

Alternative methods that discriminate between infectious and non-infectious HuNoV are thus needed, this can be in a form of manipulations that separate infectious particles in samples prior to RT-PCR. A promising method for detecting infectious HuNoV by binding to porcine gastric mucins (PGM) and treatment with RNase A, followed by real time RT-PCR was recently developed.

HuNoVs specifically recognize histo-blood group antigens (HBGA) expressed on the human gastrointestinal mucosa and also on PGM (Cannon and Vinje, 2008; Dancho

et al., 2012; Morton et al., 2009; Tian et al., 2010). Binding of HuNoV to HBGAs has been positively associated with human infection by some HuNoV strains. For example, both (Lindesmith et al., 2003) and Hutson et al. (2002) observed that ABO blood type is associated with HuNoV infection. Individuals with A or O-type blood were more likely to be infected than volunteers with B-type blood. Also, certain enzymes including Fucosyl transferase-2 (FUT-2 secretor enzyme), FUT-3 (Lewis enzyme) and A and B enzymes are crucial to HBGA synthesis, the FUT-2 enzyme particularly plays an important role in susceptibility to HuNoV infection, thus persons without a functional FUT-2 enzyme (secretor negative) are resistant to certain HuNoV strains (Atmar, 2010). The attachment of recombinant norwalk virus-like particles (VLPs) to the surface epithelial cells of the gastroduodenal junction and saliva was shown for individuals with secretor phenotypes but not for non-secretors (Marionneau et al., 2002). Cannon and Vinje (2008) exploited the ability of HuNoV to bind to HBGA, and developed a HBGA-magnetic bead binding assay using synthetic HBGAs to recover low copy numbers of HuNoVs in environmental samples.

Tian et al. (2005) reported that PGM binds effectively to HuNoV VLPs and demonstrated the competitive inhibition of VLP binding to HBGAs by PGM. Due to the difficulties and costs associated with obtaining synthetic HBGAs for research, several researchers have used PGM as an alternative to synthetic HBGAs (Dancho et al., 2012; Kingsley et al., 2014; Tian et al., 2008). A two log increase in sensitivity for detection of GII HuNoV in a fecal sample using PGM conjugated to magnetic beads (PGM-MB) was reported by Tian et al. (2008). Using standard RNA extraction procedure, the study reported detection in GII HuNoV diluted up to 1 ppm, while concentration of GII HuNoV

by PGM-MB beads followed by heat release of RNA increased detection sensitivity up to a 10 ppb dilution of GII HuNoV.

HuNoVs must have an intact (not damaged) HBGA-binding site on their viral capsid in order to bind to HBGAs. This step appears to be a necessary precursor to infection; however the exact role of HBGAs during HuNoV infection is unclear. In a study investigating binding pattern of RHDV, a calicivirus which binds to HBGAs similarly to HuNoV, the results of the study suggested that HBGAs function as an "attachment factor" rather than the "receptors" in infection (Nyström et al., 2011). For an active infection to proceed, HuNoVs must also have an intact viral RNA genome. Earlier studies into the use of PGM binding of HuNoV did not include and RNase step in their assays (Tian et al., 2005; Tian et al., 2008), while Dancho et al. (2012) rationalized the need for RNAse treatment prior to RT-PCR to ensure that RNA subsequently detected by RT-PCR was not derived from non-encapsidated viral RNA, in other words non-infectious particles.

An intact viral capsid protects the encapsulated viral RNA from degradation, but a compromised capsid exposes the RNA to the possibility of being degraded either by environmental factors or intentional RNAse A treatment. Therefore, treating viruses with RNase A is another means of discriminating between intact and non-intact viruses, as the genomes of intact viruses are protected from the RNA-degrading enzyme. By this rationale, HuNoVs that bind to HBGAs found in PGM are resistant to RNase A treatment, and can be subsequently detected by RT-PCR, are considered to be potentially or putatively infectious.

In this study, the use of PGM-binding and RNase A treatment prior to real time RT-PCR was evaluated as a means of discriminating between infectious and non-infectious HuNoV. Complete inactivation of HuNoVs was attempted by thermal inactivation and treatment with a Levulinic acid plus sodium dodecyl sulphate (LEV/SDS) sanitizer combination proven to be effective in inactivating bacterial pathogens and HuNoV surrogates, murine norovirus (MNV) and feline calicivirus (FCV) (Cannon et al., 2012; Webb et al., 2013; Zhao et al., 2010). The ability of the PGM plus RNase assay to distinguish between inactivated and non-inactivated HuNoV was thus evaluated. HuNoVs stocks belonging to the most common GI and GII genogroups, GI.1 and GII.4 respectively, were employed for this study. Both treated and untreated control stocks were subjected to PGM-binding followed by enzyme treatment with RNAse A and real time RT-PCR. A lower concentration of the LEV/SDS sanitizer that was shown to insignificantly reduce infectious MNV and FCV titers was also used to test the sensitivity of the proposed method. In addition, 70% ethanol which is a disinfectant solution capable of inactivating bacterial pathogens and is often used as a surface disinfectant in both research laboratories and food establishments was also included in this study.

3.3. METHODS

3.3.1. Virus preparation

A fecal sample from a HuNoV GI.1-infected person was gifted by Dr. Robert Atmar at Baylor College of Medicine. GII.4 HuNoV (GII.4 New Orleans) was gifted by Dr. Jan Vinjé at the CDC in Atlanta. Pea-sized amounts of GI.1 and GII.4 samples were suspended in 550 µl portions of PBS. The stool suspensions were vortexed thoroughly

until fully suspended, then centrifuged at 14,000 rpm for 10 min. Each of the supernatants were pooled and redistributed before the centrifugation was repeated. Supernatants were then sucrose-purified by loading the supernatants on top of 30% sucrose solution in a 5 ml centrifuge tube and centrifuging using a NVT 90 rotor at 100,000 x g for an hour at 4°C. The pellet was re-suspended in sterile PBS and stored at -70^{o}C accordingly until used for experiments.

3.3.2. Treatment of viruses with heat or sanitizers

HuNoV GI.1 and GII.4 (3 - 5 log genomic copies (GC) per sample) were inactivated either by heat, ethanol or two different concentrations of the LEV/SDS sanitizer. Briefly, 100 µl aliquots of the virus samples were heated in thin-walled PCR tubes in a thermocycler (Mastercycler® ep, Eppendorf, Westbury, NY) at 99^{o}C for 5 min, and then held at 4^{o}C until used. For the EtOH and LEV/SDS sanitizers, 30 µl of virus stock was added to 270 µl of 70% EtOH, the sub-lethal 0.5% Lev/0.01% SDS (low SDS) sanitizer or the virucidal 0.5% Lev/0.1% SDS (high SDS) sanitizer for 1 min. The virus-treatment solutions (sanitizers) were then added to 2700 µl of a neutralizing solution (100% fetal bovine serum for EtOH-treated samples or minimum essential medium (MEM) containing 10% FBS and 60 µl of 0.1M $NaHCO_3$ for LEV/SDS-treated samples).

3.3.3. The PGM binding plus RNase A method

96-well high-binding plates (Costar, Corning, NY) were coated with 200 µl of 1 µg/ml PGM (M1778 Sigma-Aldrich, St Louis, MO) in 50 mM Sodium carbonate buffer (CBS - carbonate-bicarbonate buffer pH 9.6) overnight at 4^{o}C. Control wells were coated

with just the buffer containing no PGM. Wells were washed with PBS with 0.05% Tween-20 solution (Sigma, St Louis, MO) 3 times and then blocked with 200 µl Blotto (5% dry skim milk in PBS) for 2 hrs at 37^{o}C. Wells were washed again before adding 200 µl of the prepared virus samples (inactivated and untreated stocks) to designated wells and incubation for 1 hr at 37^{o}C. Unbound viruses were removed by aspirating the liquid and 200 µl of RNase A solution (0.5 ng/µl) was added to some of the wells (designated as RNase-treated in order to assess the role of RNase treatment in the overall method). Sterile PBS (200 µl) was added to control wells and all samples were incubated for another 1 hr at 37^{o}C before proceeding directly to RNA extraction.

3.3.4. RNA extraction

RNA was extracted from all samples using a modified form of the Guanidine thiocyanate (GuSCN) lysis buffer and silica binding based method previously described (Boom et al., 1990). The GuSCN lysis buffer used in this study was made in-house by dissolving 60g guanidine thiocyanate (Amresco, Solon, OH) in 50ml 0.5X TE buffer (this can be heated in a water bath at 56 ^{o}C to facilitate dissolution), after dissolving, 5.5 ml 5 M NaCl, 5.5 ml 3M NaOAc (both Fisher Scientific) and 1.1 ml Polyadenylic acid (Sigma, St Louis, MO) were added. These were then mixed together and stored at room temperature in a dark container or by covering the bottle completely with foil since lysis buffer is light sensitive.

For RNA extraction, 200 µl of the GuSCN lysis buffer was added to each well and the plates were shaken abruptly and briefly by hand in the horizontal direction only. After incubation for 15 min at room temperature, the solution was aspirated into 1.5 ml

microcentrifuge tubes. An equal volume of 100% ethanol was added to each sample and vortexed briefly. Samples were then loaded onto a spin column (Omega Bio-Tek, Norcross, GA) and centrifuged at 17,000 x g for 1 min. Each column was then washed with 500 µl 75% ethanol, and centrifuged again at the same speed. A dry spin was performed to remove residual ethanol before the RNA on the column was eluted with 50 µl nuclease-free water (EMD Millipore, Billerica, MA.

3.3.5. Real time RT-PCR

Viral RNA was quantified by real-time reverse transcriptase polymerase chain reaction (RT-PCR). Real time RT-PCR was carried out using the following primer and probe sets; for GI.1 analysis, Ring1C probe (AGA TYG CGI TCI CCT GTC CA) (Hill et al., 2010) and the Cog1F (CGY TGG ATG CGI TTY CAT GA) and Cog1R (CTT AGA CGC CAT CAT CAT TYA C) primers (Kageyama et al., 2003); for GII.4 analysis, Ring 2 probe (TGG GAG GGC GAT CGC AAT CT) and Cog 2R (TCG ACG CCA TCT TCA TTC ACA) (Kageyama et al., 2003), and JJV2F (CAA GAG TCA ATG TTT AGG TGG ATG AG) primers (Jothikumar et al., 2005). Amplification was carried out in a temperature-time combination of 50^{o}C for 30 min, 95^{o}C for 15 min and 40-50 cycles of 95^{o}C for 10 s followed by 55^{o}C for 30 s and 72^{o}C for 30 s. The Stratagene Mx3005P qPCR System (Agilent Technologies, Santa Clara, CA) was used for all genomic quantification. The MxPro software was used to collect results of all tested samples (in duplicate) and was also used to analyze the data accordingly.

3.3.6. Result analysis

With the perspective that RNA signals observed in real time RT-PCR is from viruses bound to PGM (potentially infectious viruses), the results obtained in this study were represented as the ratio of positive signals to total number of samples tested. Positive real time RT-PCR signals represents potentially infectious HuNoV and vice versa. Average C_T values from real-time RT-PCR results of RNA extracted from samples before and after the different inactivation treatments were also reported. Relative Differences (RD) in C_T values generated with each set of experiments were used as a relative measure of the concentration RNA eliminated by the inactivation method. (Equation 1):

$$\boldsymbol{RD = Ct_I - Ct_U}$$ ----------Eqn 1

Where C_{TI} I = Avg. C_T Value of inactivated sample

C_{TU} = Avg. C_T Value of untreated sample.

A 1 log GC/ml reduction of viral RNA detected is equivalent to approximately 3.3 increase in C_T value.

3.4. RESULTS

3.4.1. Thermal inactivation of GI.1 and GII.4 HuNoV as measured by PGM-binding followed by real time RT-PCR.

Average C_T values and the ratio of positive RT-PCR signals to total number of samples tested are reported for heated and unheated viruses subjected to the PGM-binding method, with or without RNase A treatment (Table 3.1). After thermal treatment, complete elimination of real time RT-PCR signals were observed for low-titer

GI.1 HuNoV stocks (3 log GC/ml) which were subjected to PGM-binding with or without RNase A treatment. Since the concentration of virus in unheated stocks was initially low [providing C_T values near the limit of detection (LOD)], less than a 0.5 log GC/ml reduction (relative differences in C_T values were $\leq$ 1.2 units) could be reported due to heat treatment as determined by the PGM-binding assay. The higher-titer GI.1 stocks (4 log GC/ml) provided 20 % positive RT-PCR signals after heat treatment, PGM binding and RNase A treatment, but complete elimination of RT-PCR signal was observed for those samples not treated with RNAse A. Comparing these inactivated stocks to unheated stocks, $\geq$ 83.3 % positive RT-PCR signals were observed for all GI.1 unheated stocks subjected to PGM-binding with or without RNAse A treatment. For the higher-titer GI.1 virus stocks, C_T values ranged from 30.6 to 32.3 for unheated virus stocks, to near or beyond the LOD for the heated virus stocks. Relative differences therefore plateaued at 4.1-4.5 units, corresponding with an estimated 1.2-1.4 log GC/ml reduction as measured by the PGM-binding assay. Slightly greater reductions in RD and log GC/ml were obtained when comparing heated and unheated viruses, when GI.1 samples were not treated with RNase A.

For heat treated GII.4 stocks, a different response was observed compared to GI.1 virus, as measured by the PGM-binding assay. In this set of experiments, 100 % of unheated GII.4 stocks (5 log GC/ml) provided positive RT-PCR signals after subjection to PGM-binding with and without RNAse A treatment. For thermally treated GII.4 stocks subjected to PGM-binding, 16.7 % and 66.7 % of samples provided positive RT-PCR signals when proceeded by RNAse A treatment or without RNase treatment, respectively. C_T values ranged from 28.8 to 30.6 for unheated virus stocks, to near or

beyond the LOD for most of the heated virus stocks. Relative differences therefore plateaued at 7.3 units for the samples that were not treated with RNase A and 9.7 units for sample treated with RNase A. These RD values correspond respectively with an estimated 2.2 and 2.9 log GC/ml reduction as measured by the PGM-binding assay.

Also, assessed was the binding of unheated and heated GI.1 and GII.4 viruses on wells (of the 96-well plate) to which neither PGM nor RNase A was added (control wells). In these experiments, no positive and 10 % RT-PCR positive were observed for the 3 log GC/ml GI.1 stock without and with thermal treatment, respectively. However, at the 4 log GC/ml input titer, 83.3 % and 30 % positive RT-PCR signals were observed without and with thermal inactivation, respectively. However, these positive signals possessed high C_T values in general with average RD value across the two titers with and without heat treatment $\geq$ 35.49 and ranging from 33.9 to C_T values close to or beyond LOD. For similar control stocks (no PGM, No RNase A) in GII.4 samples, 100 % and 66.7 % positive real time RT-PCR signals were observed without and with thermal inactivation, respectively. C_T values in these GII.4 sample ranged from 31.2 to 35.9 and 33.54 to beyond LOD in untreated and heat-treated stocks respectively.

3.4.2. Levulinic acid plus sodium dodecyl sulfate sanitizer inactivation of GI.1 and GII.4 HuNoV as measured by PGM-binding followed real time RT-PCR.

Inactivation of human noroviruses, as determined by the PGM-binding assay, was investigated using a LEV/SDS sanitizer [0.5% Levulinic acid plus two concentrations of the SDS component, 0.01% (low) and 0.1% (high)] (Table 3.2). For both GI.1 and GII.4 HuNoV stocks, 100 % of samples tested resulted in positive RT-PCR signals regardless

of presence of PGM or RNAse A treatment. C_T values in PGM containing wells were however lower than observed in no-PGM control wells; C_T values ranged from 29.0 to 34.0 in PGM wells, while the observed C_T range was 35.3 to 40.1 for GI.1 samples in no-PGM wells. For GII.4 samples, C_T value ranges were 29.6 to 33.2 and 32.9 to 38.9 for PGM wells and no-PGM wells, respectively. Complete elimination of RT-PCR signal was observed when a corresponding set of samples were exposed to the high SDS LEV/SDS sanitizer for 1 min. No difference in C_T value could be observed between samples as all results were beyond the assay LOD. In contrast, all GI.1 and GII.4 stocks exposed to the low SDS treatment retained positive real time RT-PCR signals except for the GI.1 control wells that did not contain PGM and were not treated with RNAse A prior to real time RT-PCR. C_T values were very similar for the low SDS treated samples bound to PGM regardless of RNase A treatment or virus type. C_T values ranged from 30.4 to 33.7 for GI.1 and GII.4 samples, while the wells without PGM provided higher C_T values, ranging from 35.2 to C_T values close to the LOD and 32.6 to 36.45 for GI.1 and GII.4, respectively. For these low SDS treated samples, the average RDs in C_T values were 1.34 and 0.22 for PGM-bound, RNase A treated GI.1 and GII.4 respectively, corresponding to a 0.40 log GC/ml reduction of GI.1 and 0.06 log GC/ml of GII.4 samples.

Elimination of signals obtained with the high SDS treatment were queried if they could be due to the SDS in the sanitizer alone, disrupting virus binding to PGM or if the results were due to a synergistic effect between both Levulinic acid and SDS. Similar experiments were carried out using the two SDS levels used in this study, each without the Levulinic acid. Regardless of the concentration of SDS, positive RT-PCR signals

were detected after applying the PGM and RNase assays (Table 3.3). This observation suggests that inactivation/destruction of HuNoV by the LEV/SDS sanitizer is due to a synergistic effect of both components of the sanitizer. C_T values for these set of samples were similar regardless of the SDS treatment, with values ranging from 26.9 - 27.9 for PGM wells with RNAse treatments, and even though positive signals were also in the samples without PGM in the wells were observed, C_T values were very similar and ranged from 30.85 to 32.57 in untreated and low SDS treated samples, in high SDS only treated samples, C_T value ranged from 34.4 to 36.7. These values suggest a reduced amount of binding compared to the untreated and low SDS only treated samples, but still lower than ≥ 38 C_T value observed in Lev/high SDS sanitizer treated samples.

3.4.3. Ethanol inactivation of GI.1 and GII.4 HuNoV as measured by PGM-binding followed by real time RT-PCR

Both untreated GI.1 and GII.4 stocks provided 100 % RT-PCR positive signals after subjection to PGM-binding and RNAse A treatment prior to RT-PCR (Table 3.4). Correspondingly, treatment of GI.1 and GII.4 stocks with 70 % ethanol resulted in 100% and 0 % positive real time RT-PCR signals, respectively for PGM and RNase A treated wells. For control samples (no PGM) after 70% ethanol treatment, complete elimination of RT-PCR signal was observed for both GI.1 and GII.4 stocks treated with RNase A prior to real time RT-PCR. As estimated by RD, ethanol treated GI.1 samples attained up to a 0.87 log GC/ml reduction, while ethanol treated GII.4 samples attained up to a 3.98 log GC/ml reduction as measured by the PGM-RNase-RT-PCR assay. However, 75 %

and 100 % of control wells (no PGM) containing untreated GI.1 and GII.4 virus were RT-PCR positive.

Comparing C_T values between the PGM coated wells and control (no PGM) wells, C_T values for untreated GI.1 samples ranged from 28.6 to 32.0 and from 30.76 to 40.40, respectively. For corresponding GII.4 samples, C_T values ranged from 26.43 to 27.28 and from 30.11 to 32.9 for samples subjected to PGM and without PGM, respectively. After the 70% ethanol treatment, C_T values generated from PGM bound wells ranged from 29.3 to 33.2, while without PGM, C_T values ranged 35.40 to beyond the LOD. For these ethanol treatment data, even though positive signals were detected in the control (no PGM) samples, the higher C_T values observed when compared to the corresponding wells containing PGM suggests a lower amount of virus binding uncoated wells.

3.5. DISCUSSION

Many researchers are embracing the prospects of PGM-binding in combination with RT-PCR as a promising method of determining HuNoV infectivity, and thus are investigating the applicability of this method. Since Tian et al. (2005) reported that rNV VLPs bind effectively to PGM and PGM competitively inhibits rNV VLPs binding to HBGA and Caco-2 cells, the use of PGM for virus binding studies has increased. Low cost and availability of PGM have also made it an attractive alternative to the use of HBGAs for research purposes.

Observation of 80 to 83 % reductions in the ratio of positive real time RT-PCR signals for thermally inactivated GI.1 and GII.4 HuNoVs that were subjected to PGM-

binding and RNAse A treatment prior to real time RT-PCR suggests that the heat treatment seemed to damage the virus capsids to the point that less capsids were captured by PGM. After subjection of heat treated GI.1 and GII.4 sampled to PGM-binding, subsequent RNAse A treatment reduced number of positive signals in GII.4 from 66.7% to 16.7% while there was no reduction due to RNAse A treatment in GI.1 samples.

Dancho et al. (2012) reported that NoV binding to PGM conjugated to magnetic beads (PGM-MB) was altered above 60^{o}C, with only 6% binding to PGM-MBs after 60 sec at 64^{o}C, and negligible NoV binding to PGM-MBs detectable after 120 sec at 73^{o}C. Li et al. (2012) reported a variation in heat resistance between GI and GII strains when P particles (sub viral particles formed from VP1 proteins) of GI.1, GI.4 GII.9 and GII.4 were studied. The study reported a higher resistance to heat in GII.4 and GII.9 when compared to the GI.1 strain tested, P particles GI.1 was more sensitive to heat treatments than other NoV strains tested. This observation is somewhat similar to a higher ratio of positives observed in our studies in heat treated GII.4 samples compared to GI.1 samples as determined by the PGM-binding-RT-PCR method. Li et al. (2011) reported no significant reduction in PGM binding RT-PCR of GI.8 after heat treatment at 70^{o}C for 3 min, while the current study reports GI.1 log reductions up to 1.37 log GC/ml as estimated by RD. Other than the fact that our study used a different genotype of Genogroup 1 (GI.1) while the Li group used GI.8, heat treatment in our studies was at a higher temperature and longer time, which could have accounted for the reported variation.

Another factor that may be responsible for these differences is the viral suspension matrix; while the Li group's study used fecal suspension of the virus, the

Dancho group used fecal material suspended in DMEM (containing 10% FBS) that was purified to an extent [centrifugation (12, 000 x *g* for 20 min at 4 ^{o}C) and filtration (0.22µM filter)], while the current study used fecal suspensions that were sucrose purified by ultracentrifugation. This variation in suspension matrix can be a factor influencing the differences observed from one study to another. Components of a matrix may compete with HuNoV for PGM-binding, or may inhibit or disrupt binding.

Virus titer is another factor that must be considered when comparing studies. Variation in estimated log reductions by input titer versus relative differences is a challenge in studies employing low titer sample. While results in the current study reported up to 1.37 log GC/ml as estimated by RD, an ~4 log GC/ml reduction in the same sample can be reported as there was either total elimination of signals or signals below detection limit in heat inactivated samples with an initial input titer of 4 log GC/ml. When C_T values are beyond LOD, it makes it difficult to quantify log reduction relatively, causing reports of elimination of positive signals as log reduction up to the input titer in starting samples.

The levulinic acid plus sodium dodecyl sulphate (LEV/SDS) sanitizer combination has been reported to be effective in eliminating bacterial pathogens and HuNoV surrogates (MNV and FCV) (Cannon et al., 2012; Webb et al., 2013; Zhao et al., 2010). The mechanism of inactivation of HuNoV by the high SDS sanitizer is yet to be understood. Using the PGM-binding prior to RT-PCR, complete elimination of RT-PCR signals were observed for the two virus strains treated with high LEV/SDS sanitizer, while RT-PCR signals were not eliminated in virus samples treated at a very high temperature of 99^{o}C. It is hypothesized that this is due to variation in mechanisms of

inactivation between the two treatments. A possibility is that the heat inactivation method, while it may break viral capsids apart, does not disrupt the binding sites required for PGM-binding; thus bits of the broken capsids still bind to PGM. On the other hand, the high LEV/SDS treatment may interact chemically with the virus capsids to disrupt binding sites, thereby completely eliminating PCR signals.

Furthermore, while the PGM-binding method gave elimination of RT-PCR signals in both GI and GII genogroups treated with the high SDS treatment, when the two virus strains were subjected to 70% ethanol, complete elimination of signals was observed for GII.4 HuNoV, but not for GI.1 HuNoV. Complete elimination of signals or C_T values above the cutoff value were observed for ethanol-treated virus stocks subjected to wells with no PGM, but including RNAse A treatment prior to real time RT-PCR. Despite non-specific binding observed in control wells (no PGM), the C_T values obtained from samples in those wells suggests that the non-specific binding was either not as strong or as quantitatively high as was specific binding to PGM. For untreated stocks, C_T values ranged from 28.6 to 32 and from 26.4 to 27.3 for GI.1 and GII.4 subjected to PGM-binding, respectively, while sample that were subjected to control wells (no PGM) ranged from 30.8 to 40.4 (the 30.8 value was abnormal as the next C_T in the range was 33.6) and from 30.11 to 32.86 for GI.1 and GII.4, respectively.

For 70% ethanol treated GI.1 samples subjected to PGM binding and RNAse A treatment, the RD value average was 0.88, which is estimated to be about a 0.27 log GC/ml reduction. However, corresponding GII.4 samples yielded an RD value average of $\geq$ 13.15, which is approximately equivalent to a $\geq$3.98 log GC/ml reduction in virus. Given that both GI.1 and GII.4 stocks were tested at the same input titer level, these

results suggests a either a higher resistance to ethanol treatment for GI.1 samples compared to GII.4 samples or differences in PGM-binding properties across the viral strains.

To our knowledge, there are currently no studies reported that have evaluated PGM binding prior to RT-PCR as a means of estimating infectivity in ethanol treated HuNoV. However, in studies that use the RNAse pretreatment method after virus treatment with ethanol, there does appear to be a difference in response to treatment due to virus strain. For instance, Park et al. (2010) observed negligible reduction (0.1 and 0.6 log GC/ml for 1 and 5 min contact times, respectively) of GII.4 samples treated with 70 % ethanol. However, studies by Cromeans et al. (2014) evaluated MNV and GI.5 and GII.13 HuNoV strains treated with 70% ethanol for 1 min and reported up to 3.5 log reduction in semi-purified GI.5 virus, whereas both semi-purified and non-purified GII.13 stool samples were reduced by less than 1 log. The study also reported a higher resistance to ethanol treatment for GII.13 strains than for GI.5 strains.

Also, significant variations in structural differences between different GI strains has been reported by Shanker et al. (2014), with the knowledge that HuNoVs bind HBGAs through the protruding (P) domain of the major capsid protein VP1, the group studied and compared the structure of the P domain of GI.7, GI.1 and GI.2 strains. Thus, HBGA and/or PGM binding across HuNoV GI strains may also vary.

Positive real time RT-PCR signals for virus stocks bound to wells containing no PGM observed in our studies is supported by previous studies using PGM or cell binding assays. Li et al., 2011 reported no significant difference between the virus binding to PGM and Caco-2 cells (as specific binding receptors) or Bovine Serum Albumin (BSA)

which was used as a non-specific binding control (used in the blocking buffer). Given that there are other factors that could be responsible for nonspecific binding, such as electrostatic interactions on the plate surfaces themselves or non-specific adsorption to the blocking buffer, it is difficult to ascertain that determination of HuNoV infectivity by binding of norovirus to PGM is specific.

3.6. CONCLUSIONS

This study suggests the PGM-binding, RNAse A treatment prior to real time RT-PCR as a means of determining HuNoV infectivity requires more studies to improve specificity of the PGM-binding step. There is also a need to understand why the method appears suitable for determining virus infectivity when certain inactivation treatments are employed and not with others. Heat inactivation reduced positive real time RT-PCR signal of HuNoV subjected to PGM-binding and RNAse A treatment prior to real time RT-PCR. Higher reductions in RT-PCR signals were observed for thermally inactivated GI.1 HuNoV than for GII.4 strains, virus titer variations and differences in virus type such as variations in capsid properties may contribute to the observation. Since intact HBGA binding sites are required for binding of HuNoV to HBGA, positive signal in some heat inactivated samples suggest incomplete destruction of binding sites in the virus. Complete elimination of real time RT-PCR signals for both GI.1 and GII.4 samples by the 0.5% Lev/0.1% SDS sanitizer suggests that this sanitizer is effective in destroying, disrupting, or masking the HBGA binding sites in HuNoV. However, positive signals were observed in all virus samples treated with the 0.5%Lev/0.01% SDS sanitizer which implies the level of SDS in the sanitizer is important for disruption of PGM-binding

ability of the viral capsids. However, HuNoV binding was also observed in wells not coated with PGM which raises questions about the specificity of the PGM-binding method. Blocking buffers need to be evaluated to find a more efficient blocking buffer that improves specificity. More studies are also needed on the suitability of the PGM-binding method for HuNoV inactivated using other commonly employed methods of inactivation.

3.7. APPENDIX

Table 3.1: Positive real time RT-PCR signals observed for GI.1 and GII.4 HuNoV without and with heat treatment (99°C) for 5 min. Ratio of positives are the number of samples testing positive for real time RT-PCR out of the total number of samples tested. The range in C_T values show the lowest and highest C_T values obtained for samples in each category. RD values are calculated according to equation 1.

Virus strain and titer	PGM	ET	Unheated		Heated		Relative Difference in Avg C_T (B - A)	Estimated log reduction**
			A Avg. C_T (range) # (min- max)	Ratio of positives	B Avg. C_T (range) # (min- max)	Ratio of positives		
GI.1 3 log GC/ml	+	+	35.14 (33.82-36.12)	10/12	36* (> 36 - No C_T)	0/10	0.86	0.26
	+	-	34.80 (33.25-35.74)	11/12	36* (> 36 - No C_T)	0/12	1.20	0.36
	-	-	36* (>36)	0/12	35.77(34.07 - No C_T)	1/10	- 0.23	-0.06
GI.1 4 log GC/ml	+	+	31.49 (30.59 - 32.34)	12/12	36* (≥ 36)	2/10	4.51	1.37
	+	-	31.78 (31.15 - 33.17)	12/12	35.83 (34.74 - ≥ 36)	0/12	4.05	1.23
	-	-	35.50 (34.05 - 36.38)	10/12	35.49 (33.87 - ≥ 36)	3/10	-0.01	-
GII.4 5 log GC/ml	+	+	29.69 (29.29 - 30.29)	12/12	39.40 (38.00 - No C_T)	2/12	9.71	2.94
	+	-	29.70 (28.77 - 30.64)	12/12	37 (33.45 - No C_T)	8/12	7.30	2.21
	-	-	32.99 (31.19 - 35.92)	12/12	36.93 (33.54 - No C_T)	8/12	3.94	1.19

***** C_T values of 36 or 40 were the cut off values based on quality of RT-PCR signal curves obtained beyond 36 and 40 in comparison to all other signals for GI and GII assays, respectively. ******Estimated log reduction is determined by dividing RD by 3.3, given that 3.3 C_T value units ≈ 1 log GC/ml.

Table 3.2: Positive real time RT-PCR signals observed for GI.1 and GII.4 HuNoV without and with LEV/SDS sanitizer treatments. Ratio of positives are the number of samples testing positive for real time RT-PCR out of the total number of samples tested. The range in C_T values show the lowest and highest C_T values obtained for samples in each category. RD values are calculated according to equation 1.

Virus strain and titer	PGM	ET	Untreated		Low SDS Treated				High SDS Treated			
			A = Avg. C_T (range) # (min- max)	Ratio of positives	B = Avg. C_T (range) # (min- max)	Ratio of positives	RD B-A	Log red (GC/ml)*	C = Avg. C_T (range) # (min- max)	Ratio of positives	RD C-A	Log red (GC/ml)*
GI.1 4 log GC/ml	+	+	30.47 (29.03 - 32.29)	12/12	31.81 (30.37 - 32.87)	12/12	1.34	0.40	>38 (>38 - No C_T)	0/12	≥7.53	≥2.28
	+	-	32.39 (30.94 - 33.99)	12/12	31.59 (30.46 - 33.66)	12/12	-0.80	-0.24	>38 (>38 - No C_T)	0/12	≥5.61	≥1.7
	-	-	37.45 (35.25 - 40.08)	12/12	>38 (35.15 - >38)	4/12	0.55	0.17	>38 (>38 - No C_T)	0/12	≥0.55	≥0.18
GII.4 5 log GC/ml	+	+	31.27 (30.27 - 32.63)	12/12	31.49 (30.78 - 32.99)	12/12	0.22	0.06	>38 (36.27 - No C_T)	0/12	≥6.73	≥2.03
	+	-	31.53 (29.59 - 33.24)	12/12	31.59 (30.41 - 33.36)	12/12	0.06	-	>38 (>38 - No C_T)	0/12	≥6.47	≥1.96
	-	-	34.52 (32.94 - 38.89)	12/12	34.06 (32.59 - 36.45)	12/12	-0.46	0.14	>38 (>38 - No C_T)	0/12	≥3.48	≥1.06

A C_T value of 38 was the cut off based on quality of RT-PCR signal curves obtained beyond 38 in comparison to all other signals. *****Estimated log reduction is done by dividing RD by 3.3, given that 3.3 C_T value units ≈ 1 log GC/ml. Low SDS = 0.5% LEV, 0.01% SDS, High SDS = 0.5% LEV, 0.1% SDS.

Table 3.3: Positive real time RT-PCR signals observed for GII.4 HuNoV without and with SDS treatments only. Ratio of positives are the number of samples testing positive for real time RT-PCR out of the total number of samples tested. The range in C_T values show the lowest and highest C_T values obtained for samples in each category. Low SDS = 0.01% SDS, High SDS = 1% SDS.

Virus strain and titer	PGM	ET	Untreated		Low SDS Treated		High SDS Treated	
			Avg. C_T (range)	**Ratio of positives**	**Avg. C_T (range)**	**Ratio of positives**	**Avg. C_T (range)**	**Ratio of positives**
GII.4 5 log GC/ml	+	+	27.62 (27.41 - 27.89)	8/8	27.40 (26.90 - 27.76)	8/8	27.32 (27.22 - 27.52)	8/8
	-	+	31.96 (31.24 - 32.57)	8/8	31.44 (30.85 - 32.07)	8/8	35.70 (34.35 - 36.73)	8/8

Table 3.4: Positive real time RT-PCR signals observed for GI.1 and GII.4 HuNoV without and with 70% EtOH treatments. Ratio of positives are the number of samples testing positive for real time RT-PCR out of the total number of samples tested. The range in C_T values show the lowest and highest C_T values obtained for samples in each category. RD values are calculated according to equation 1.

Virus strain and titer	PGM	ET	Untreated		Ethanol Treated		Relative Difference in Avg. C_T (B - A)	Estimated log reduction*
			A Avg. C_T (range) # (min- max)	Ratio of positives	B Avg. C_T (range) # (min- max)	Ratio of positives		
GI.1 5 log GC/ml	+	+	30.32 (28.58 - 31.99)	8/8	31.20 (29.29 - 33.24)	12/12	0.88	0.27
	-	+	36.06 (30.76[1] - >40)	6/8	38.92 (35.40 - >40)	0/12	≥ 2.86	≥ 0.87
GII.4 5 log GC/ml	+	+	26.85 (26.43 - 27.28)	8/8	No C_T	0/12	≥ 13.15	≥ 3.98
	-	+	31.74 (30.11 - 32.86)	8/8	No C_T	0/12	≥ 8.26	≥ 2.50

A C_T value of 40 was the cut off based on quality of RT-PCR signal curves obtained beyond 40 in comparison to all other signals. *****Estimated log reduction is done by dividing RD by 3.3, given that 3.3 C_T value units ≈ 1 log GC/ml. [1]The 30.76 data point was much lower than the other C_T values in the range presented, with the next data point being a C_T value of 33.59.

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CHAPTER 4

IMPACT OF FOOD MATRIX AND RNA EXTRACTION METHOD ON RT-QPCR INHIBITION AND NOROVIRUS RECOVERY[3]

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4.1. ABSTRACT

Detection of Human Norovirus (HuNoV) in food samples is mainly through molecular methods; specifically RT-PCR which could be either real-time or conventional. Due to very small sample volume required for PCR, samples to be tested for presence of HuNoV often go through a viral elution-concentration step, a procedure that inadvertently concentrates PCR inhibitors in the sample as well. This study evaluated four different RNA extraction methods employing Lysis, Unex, and Trizol RNA extraction buffer, and a QiAmp viral RNA extraction kit. Four food sample matrices; mixed berries, iceberg lettuce, lunch meat (ham), and pasta salad, as well as one environmental sample matrix (top soil), were employed in this study. The effects of the various sample matrices and viral RNA extraction methods on PCR inhibition and viral RNA recovery were investigated using three different noroviruses (HuNoVs GI.1 and GII.4 and murine norovirus). PCR Inhibition was estimated through the use of internal amplification controls (IAC), and viral RNA recovery was evaluated by RT-qPCR. Both the sample matrix and method of RNA extraction were significant factors ($p<0.0001$) contributing to RNA recovery and PCR inhibition. Effect of each RNA extraction method in reducing PCR inhibition varied with the sample matrix being tested. Sample matrix was found to be a significant factor to inhibition of RT-qPCR signals of GII.4 RNA signals ($p<0.0001$), but not a significant ($p=0.1675$) contributor in GI.1 signal inhibition. A chloroform extraction step employed during the sample preparation (elution-concentration) was also found to be effective in reducing PCR inhibition and improving viral RNA recovery.

4.2. INTRODUCTION

An estimated 178.8 million acute gastroenteritis (AGE) illnesses occurred each year in the United States, 37 million of these are attributed to known pathogens, leaving the remaining 141.8 million illnesses caused by unspecified agents (Scallan et al., 2011a). Of AGE illnesses, human norovirus (HuNoV) is responsible for 19-21 million cases (CDC, 2013). Foodborne illnesses account for 9.4 million episodes of AGE and human norovirus (HuNoV) is responsible for the largest portion (58%) of foodborne illnesses (approximately 5.5 million episodes each year) (Scallan et al., 2011b). HuNoV can be transmitted through consumption of foods or water indirectly contaminated with human feces or by direct hand contact with ill persons or contaminated surfaces (fomites) ill persons have touched. Raw or undercooked food items especially shellfish, minimally processed items such as mixed salads and greens, and food items involving some level of handling post kill-step are sources of concern for HuNoV contamination.

Due to the unavailability of a cell culture model, detection of HuNoV in food samples have been limited to molecular methods such as RT-PCR. While there are other methods for the detection of viruses in clinical samples such as enzyme immunoassay, these methods are often not employed in the detection of viruses in food because the number of viruses present in food samples is usually too low to be detected by such methods, even though these low levels of contamination can still cause infection in a susceptible host (Goyal, 2006). When testing food and environmental samples for presence of HuNoV, virus elution and concentration are often the first steps. A widely used elution-concentration method involving washing of fruit or vegetable surfaces with a 100 mM Tris-HCl, 50 mM glycine, and 3% beef extract, pH 9.5 buffer was employed.

The elution buffer, a combination of salt, amino acids and protein, is theorized to disrupt electrostatic and hydrophobic interactions between fruit or vegetable surfaces and viruses, forcing viruses into solution (Croci et al., 2008). This buffer or a modified form of it has been employed successfully in several studies (Baert et al., 2008; de Abreu Corrêa et al., 2012; Scherer et al., 2010).

After virus has been eluted into solution, concentration is needed to reduce sample volume into a manageable size and improve detection sensitivity. Given the usually low concentration of viruses in food and water samples, the concentration step is required, moreover a very small sample volume is required for PCR analysis. Polyethylene glycol (PEG) is often used for virus concentration, PEG facilitates phase separation in the sample. The virus in the solution can be concentrated by relatively low speed centrifugation (for example at 9000 x g for 30 min at 4^{o}C) and the supernatant can then be discarded. The sediment obtained is usually chloroform-extracted which further removes organic materials, any bacteria cells present and residual food matrix. Ultracentrifugation and ultrafiltration methods can also be employed as a secondary concentration step to further reduce the volume of larger samples.

Viral detection by RT-PCR is usually preceded by extraction of the viral genomic material. This can be done by releasing viral RNA from capsids using heat treatment at 99 ^{o}C for at least 1 min; however, this is rarely performed because residual matrix components of samples which can interfere with RT-PCR are not separated from the viral RNA in this approach (Knight et al., 2013). An extraction method needs to be efficient in obtaining viral nucleic acids that are as pure as possible, especially for enteric RNA

viruses (Croci et al., 2008), because RNA is generally less stable than DNA and is susceptible to endogenous RNases which are often found in unpurified samples.

The use of guanidine thiocyanate-based lysis buffers for capsid destruction followed by binding extracted RNA to silica, as described in Boom et al. (1990), is the most common method of RNA extraction. Kits for this method are commercially available through several bio-diagnostic companies. Even though, RT-PCR has some advantages including sensitivity and specificity, some of its challenges include its inability to determine infectivity of amplified targets, the very small sample volume required, and its susceptibility to PCR inhibitors. The presence of inhibitors, a major obstacle in RT-PCR, is often as a result of virus concentration. Although this step is often required to reduce sample volumes for RT-PCR, inhibitors can also be concentrated, thereby reducing the efficacy of detection methods. Some inhibitors may inhibit the polymerases of RT-PCR, while others may themselves be RNases, thus degrading RNA. Some common PCR inhibitors include food constituents such as organic and phenolic compounds, glycogen, fats, and environmental compounds (phenolic compounds, humic acids, and heavy metals) (Wilson, 1997).

An RNA extraction method that reduces inhibitors of RT-PCR in a sample and yields good viral RNA recovery rates is desirable. The objective of the current study is to compare commonly employed methods of viral RNA extraction from varying food matrices, to assess the impact of sample matrix and RNA extraction methods on the inhibition of RT-qPCR. Four types of RNA extraction methods were applied to extracted food matrices including, iceberg lettuce, lunch meat (black forest ham), mixed berries and pasta salad, or to soil, all of which were spiked with a virus cocktail of murine

norovirus (MNV) and HuNoVs GI.1 and GII.4. RT-qPCR. Analysis of the extracted viral RNA was carried out in the presence of corresponding HuNoV GI and GII internal amplification controls (IAC) to estimate inhibition in the PCR. Recovery of each virus type from the different food matrices was also evaluated.

4.3. METHODS

4.3.1. Sample matrix preparation

Food matrices were prepared by a typical virus elution-concentration procedure used in the field of Food Virology for extracting viruses from food samples. This elution-concentration procedure is atypical for soil samples; however, we included soil in our study because it is known to contain inhibitors of PCR. Food samples used included (i) head of iceberg lettuce (ii) store brand mixed berries - Triple berry medley containing blackberries, blueberries and red raspberries (iii) store brand packaged deli thin sliced black forest ham (iv) store brand macaroni salad (v) top soil (Sims Bark Co. Tuscumbia AL). Portions (25 g) of each food sample or soil sample (top soil) were weighed in filtered stomacher bags (Fisher Scientific). To each, 75 ml of 50 mM buffer (Fisher Scientific, Fair Lawn, NJ) with 0.1 M Tris-HCl (pH 9.5) (Promega, Madison, WI) and 3% beef extract (212303 BD, Sparks, MD) was added. Bags were closed with a clip, placed in metal racks and placed in a Series 25 Incubator Shaker (New Brunswick Scientific Co., Edison, NJ) at room temperature, for 20 min at 200 rpm. The filtrate was then collected and the pH was adjusted to 7.4 using 0.1N NaOH or 6N HCl (Fisher Scientific). Filtrates were portioned (≈40 ml per tube) into 50 ml tubes and 8% polyethylene glycol (PEG; MW 10,000; Alfa Aesar, Ward Hill, MA) and 0.3 M NaCl

(Fisher Scientific) were then added to each. After briefly shaking by hand, the tubes were placed on a G-33 shaker (New Brunswick Scientific Co., Edison, NJ) at 200 rpm overnight at 4 ^{o}C to allow the PEG to dissolve into solution. The next morning, all tubes were centrifuged at 9000 x *g* for 30 min at 4 ^{o}C using a Thermo Scientific Sorvall Legend X1R centrifuge (Thermo Scientific, Germany). After discarding the supernatant, the pellet was suspended in 1 ml PBS (Corning Cellgro, Manassas, VA). Pellet suspensions from all tubes were pooled together and mixed thoroughly. Half of each suspension was portioned (500 µl) into microcentrifuge tubes and stored at -70 ^{o}C until later use. The other half was chloroform-extracted. Briefly, equal volumes of chloroform were added to the suspension, shaken thoroughly by hand, and then centrifuged at 9000 x *g* for 15 min at 4 ^{o}C. Aqueous phases of each were collected carefully, avoiding disturbance of the interphase. They were then vortexed and centrifuged again at 3,000 x *g* for 20 min at 4 ^{o}C. The upper layer (aqueous phase) was again collected, portioned (500 µl) into microcentrifuge tubes, and stored at -70 ^{o}C until further use.

4.3.2 Virus preparation

A virus cocktail was prepared containing MNV-1, HuNoV GI.1 and GII.4 with a target of ~10^{7} log genomic copies (GC)/ml of each virus type. MNV-1 was a gift from Dr. Herbert Virgin at Washington University (St. Louis, MO). Fecal material from a HuNoV GI.1-infected person was a gift from Dr. Robert Atmar (Baylor College of Medicine) and a watery stool sample containing GII.4 HuNoV Sydney strain was a gift from Dr. Jan Vinjé (CDC, Atlanta, GA). RAW 264.7 cells were obtained from ATCC (Manassas, VA). MNV-1 stocks were prepared by inoculation of MNV-1 on confluent

RAW 264.7 cells in T175 flasks, followed by incubation at 37^{o} C with 5% CO_2 for 48 hrs and confirming CPE. Infected flasks were taken through three cycles of freezing (-70^{o} C) and thawing (at room temperature) before centrifugation at 2000 x *g* for 15 min to remove large cell debris and vacuum filtration (0.2 µm PES membrane filter) for further clarification. Pea-sized amounts of the GI.1 fecal material were suspended in 550 µl of PBS and 100 µl portions of the GII.4 stool sample were suspended in 900 µl of PBS. These stool suspensions were vortexed thoroughly to fully suspend the fecal material and centrifuged at 17,000 x *g* for 10 min using a Sorvall Legend Microcentrifuge (Thermo Scientific; Germany). For each HuNoV, supernatants were pooled, homogenized, and centrifuged again for further clarification of the supernatants. Applicable volumes of MNV-1 cell culture lysate and the HuNoV GI.1 and GII.4 stool suspensions were combined and PBS was added to make a cocktail of all viruses at the target concentration of ~10^7 genome copies per ml per virus.

4.3.3. Virus inoculation

On the day of each experiment, 10 µl of the virus cocktail was added to 90 µl of the previously prepared food matrixes (yielding ~10^5 GC input titer). All RNA extraction methods (see below) were tested side by side and in duplicate. After adding the virus to the matrix, the mixture was vortexed and incubated for 30 min at room temperature to allow adsorption of the virus to the food or soil matrix components. Since the primary interest of this study was on the ability of the RNA extraction methods to reduce RT-qPCR inhibitors that may be present in samples following viral elution-concentration, rather than determining the efficiency of the complete virus recovery procedure,

experiments were carried out in this manner. In this approach, the RNA extraction procedures could be evaluated alone, without having to consider virus loss during the elution-concentration procedure.

4.3.4. RNA extraction

RNA extraction was carried out on virus inoculated matrices using four different methods of RNA extraction. Three of the methods were a modified form of the Guanidine thiocyanate (GuSCN) lysis buffer and silica binding-based method previously described (Boom et al., 1990). The GuSCN lysis buffer of the first method was made in-house by dissolving 60 g of guanidine thiocyanate (Amresco, Solon, OH) in 50ml 0.5X TE buffer (this can be heated in a water bath at 56 ^{o}C to facilitate dissolution). After dissolving, 5.5 ml of 5 M NaCl, 5.5 ml of 3M NaOAc (both Fisher Scientific) and 1.1 ml of Polyadenylic acid (Sigma, St Louis, MO) was added. These were then mixed together and stored at room temperature in a dark container or by covering the bottle completely with foil since the lysis buffer is light sensitive.

For RNA extraction method 1, the GuSCN lysis buffer was added to each sample at a volume equal to the sample volume (100 µl). Each sample was vortexed briefly and incubated for 15 min at room temperature before 2x the sample volume of 100% molecular grade ethanol was added to each sample. Samples were then loaded onto a spin column (Omega Bio-Tek, Norcross, GA) and centrifuged at 17,000 x g for 1 min. Each column was washed with 500 µl of 75% ethanol and centrifuged again at the same speed. A dry spin was performed to remove residual ethanol before the RNA on the

column was eluted with 50 µl nuclease-free water (EMD Millipore, Billerica, MA). Method 1 is abbreviated as the “Lysis” extraction method throughout the text.

The second method is very similar to the first in the steps for RNA extraction; the major difference between the two is the lysis buffer. RNA extraction method 2 used the E-Sphere UNEX buffer (Phthisis Diagnostics, Charlottesville, VA) instead of the GuSCN lysis buffer used in method 1. The UNEX buffer is also a guanidine thiocyanate-based buffer that comes prepared and ready to use by the manufacturer. All subsequent steps after addition of lysis buffer are identical to Method 1. Method 2 is abbreviated as the “Unex” extraction method throughout the text.

The third method tested was a Trizol-based RNA extraction method. Briefly 800 µl of Trizol LS reagent (Invitrogen, Carlsbad, CA) was added to 100 µl of sample, followed by addition of 200 µl of chloroform (EMD, Gibbstown, NJ). The mixture was vortexed thoroughly for 2 min, incubated at room temperature for 5 min, and then centrifuged at 6,200 x *g* for 20 min at 4 ^{o}C. The aqueous phase was aspirated into a sterile micrcentrifuge tube avoiding the interphase completely. Viral nucleic acid was precipitated from the aqueous phase by adding 600 µl isopropanol, shaking it thoroughly by hand and incubating at room temperature for 10 min. Samples were then centrifuged at 16,300 x *g* for 10 min at 4 ^{o}C. The supernatant was thereafter discarded and the pellet was washed by adding 500 µl of 70% ethanol to each sample, vortexing briefly and centrifuging for 5 min at 16,300 x *g* at 4 ^{o}C. The supernatant was then carefully removed and washing of the pellet was repeated. The resulting purified RNA was then air-dried in a laminar flow hood for ≈5 - 10 min and re-suspended in 50 µl DEPC water. Method 3 is abbreviated as the “Trizol” extraction method throughout the text.

The fourth method employed the QiAmp Viral RNA MiniKit (Qiagen, Hilden, Germany) following manufacturer's 'Spin Protocol' instructions with some minor modifications. Briefly, 5.6 µl of carrier RNA-AVE was added to 400 µl of the lysis buffer (Buffer AVL). This solution was added to each 100 µl sample (rather than 140 µl) to match the sample and lysis buffer volumes used in methods 1-3. Subsequent steps of the RNA extraction procedure were performed as described in manufacturer's handbook, except 400 µl of 100% ethanol was used rather than the recommended 560 µl. Method 4 is abbreviated as the "Qiagen" extraction method throughout the text.

4.3.5. Real time RT-PCR

Viral RNA was quantified by real-time reverse transcriptase polymerase chain reaction (RT-PCR). Each virus type in the cocktail mix; MNV-1, GI.1 and GII.4 HuNoV, were analyzed in separate RT-PCR mastermixes, but processed at the same time (on the same PCR plate) for each experimental replicate. Following preliminary experiments showing that optimal amplification of the different viral RNA used in this study was attainable with different mastermixes, such were employed in this study. For MNV and GI analysis, qScript™ XLT One-Step RT-qPCR ToughMix®, Low Rox™ (95134, Quanta Biosciences, Gaithersburg, MD) was used, and for GII analysis, Quantitect Probe RT-PCR Kit (204443, Qiagen, Hilden, Germany) were used. Real time RT-PCR was carried out with the following primer/probe pairs; MNV-1, probe G54808P (CTA CCC ACC AGA ACC CCT TTG AGA CTC) and primers G54763F (TGA TCG TGC CAG CAT CGA) and G54863R (GTT GGG AGG GTC TCT GAG CAT) (Park et al., 2010); GI.1, probe Ring1C (AGA TYG CGI TCI CCT GTC CA) (Hill et al., 2010) and primers

Cog1F (CGY TGG ATG CGI TTY CAT GA) and Cog1R (CTT AGA CGC CAT CAT CAT TYA C) (Kageyama et al., 2003); GII.4, probe Ring 2 (TGG GAG GGC GAT CGC AAT CT) and primers Cog 2R (TCG ACG CCA TCT TCA TTC ACA) (Kageyama et al., 2003) and JJV2F (CAA GAG TCA ATG TTT AGG TGG ATG AG) (Jothikumar et al., 2005). Internal amplification controls (IAC) were also used in this study for detecting PCR inhibition. HuNoV GI and GII IAC cDNA (a gift from Dr. Lee-Ann Jaykus, North Carolina State University) were subjected to *in vitro* transcription using MEGAshortscript high yield transcription kit (Ambion, Austin TX); the derived IAC transcripts were used in each HuNoV sample tested by RT-qPCR as follows. Briefly, 1 µl of corresponding IAC template was added to 18 µl of PCR Mastermix corresponding to each virus type. Duplicate samples (1 µl each) were tested for each virus/food matrix/extraction method combination of this study. Amplification was carried out in temperature-time combinations of 50^{o}C for 30 min, 95^{o}C for 15 min and 40-50 cycles of 95^{o}C for 10 s followed by 55^{o}C for 30 s and 72^{o}C for 30 s on the Stratagene Mx3005P qPCR System (Agilent Technologies, Santa Clara, CA). The MxPro software was used for data collection and quantification based on standard curves (10-fold serial dilutions of RNA transcripts) included with each MNV-1, GI.1 and GII.4 assay. RNA transcripts were prepared by *in vitro* transcription of HuNoV GI, GII or MNV-1 cDNA using the MEGAshortscript high yield transcription kit (Ambion). The range of efficiency values considered acceptable for the standard curves were 85 - 115 %.

4.3.6. Results analysis

The impacts of sample matrix and method of RNA extraction on both PCR inhibition and virus recovery were evaluated. PCR inhibition in GI.1 and GII.4 samples was determined by evaluating the IAC C_T values generated for each sample. When the IAC C_T value of a sample exceeded that of a negative control (containing only IAC; no sample) by more than 3.3 C_T values (≈1 log genomic copies), or if no C_T value could be generated, samples were considered to be positive for PCR inhibitors. For estimation purposes a C_T value of 40 (limit of detection - LOD) was assigned to samples which generated no C_T values. Genomic RNA extracted was also quantified using the MxPro software and reported as log GC/ml of MNV-1, GI.1 or GII.4 recovered from each sample matrix for the four different RNA extraction methods.

4.3.7. Statistical analysis

Virus recovery as measured by log genomic copy numbers of viral RNA and PCR inhibition as measured by IAC C_T values were analyzed for each set of experiments by ANOVA using JMP Pro 11 (SAS Institute Inc., Cary, NC). Significant differences in the means due to experimental variables were compared using a student's t-test. For all significance tests, $\alpha = 0.05$ was used.

4.4. RESULTS

4.4.1. Effect of sample matrix and RNA extraction method on inhibition of RT-qPCR signals

Inhibition of RT-qPCR signals as evaluated in this study by the use of IACs is depicted in Figure 4.1. In the figure, average IAC C_T values for the negative controls (containing no sample; only IAC) used with each experiment and the distribution of IAC C_T values generated in evaluating the impact of the various food matrices and the soil matrix (added since they are known to contain PCR inhibitors) and the four different extraction methods. Since the IAC was added to each RT-qPCR mastermix just before sealing plates for thermo cycling, the only contributing variable to a change in C_T value for a sample when compared to the C_T values of the negative controls (sample negative; IAC positive) was the sample itself. A C_T value higher for a sample than the C_T value of the negative control, indicates some level of PCR inhibition by the sample matrix. For every change in C_T value of 3.3 units for the sample compared to the control indicates inhibition of target amplification by approximately 1 log. Using a linear regression model of the complete data by virus type, sample matrix was found to be a significant factor to inhibition of RT-qPCR signals of GII.4 RNA signals ($p<0.0001$), but not a significant ($p=0.1675$) contributor in GI.1 signal inhibition

For GI assays, average IAC C_T values observed for each sample matrix were comparable to the corresponding average IAC C_T values of the negative controls (Fig 4.1). Looking at the distribution of IAC C_T values across the different sample matrices for GI assays, for nearly all sample matrices and extraction methods, maximum differences in average sample C_T values observed were 1.00 or less than corresponding

controls. This maximum difference was observed for a non-chloroform extracted soil matrix from which viral RNA was extracted using the "Unex buffer" method. One exception to this observation was one non-chloroform extracted pasta salad matrix from which RNA was extracted using the “Lysis buffer” extraction method. Both duplicate RT-qPCR wells for this sample yielded no positive IAC signal, suggesting presence of PCR inhibitors in this sample (Figure 4.1).

For GII assays, average IAC C_T values observed across all sample matrices were higher than the average IAC C_T values observed in corresponding (no sample; IAC positive) negative controls (Fig 4.1). The lowest average C_T value (31.72) was observed for the lettuce matrix, which was significantly lower than the next lowest observed average C_T value (34.46) that was observed for the lunch meat. Significantly greater average C_T values were reported for mixed berries (36.60) and pasta salad (36.47), although these values were not significantly different from each other. The highest average C_T value reported was for top soil (38.51). Comparing average C_T values of the negative controls (no sample; IAC positive) with average IAC C_T value in each experimental sample, a minimum difference of -0.5 C_T value was observed in chloroform extracted lettuce matrix from which RNA was extracted using the "Qiagen" method, while a maximum difference of $\geq$8.9 C_T values was observed in the non-chloroform extracted soil matrix from which RNA was extracted by either "Lysis", "Qiagen" or "Unex" method. In these soil samples, no C_T value were detected and the estimated relative difference was calculated to be 40, which was the C_T value determined to be the assay limit of detection (LOD). Based on these differences, PCR inhibition of GII samples were observed, with a maximum of 2.70 log genomic copy reductions of GII

IAC amplification. It is important to note here that the differences observed between GI and GII IACs may not be due to the viruses, since different master mixes (listed in methods) were used for the different assays.

There was a significant effect due to the type of RNA extraction method used on PCR inhibition for nearly all GI and GII assays, as observed by IAC amplification (Table 4.1). Whether or not a chloroform extraction step was included prior to RNA extraction was also a significant factor effecting PCR inhibition ($p=0.0113$). In nearly all cases, inclusion of the chloroform extraction step significantly reduced PCR inhibition for GI and GII assays (Table 4.1). Exceptions included iceberg lettuce for GI assays, where regardless of the RNA extraction method employed, there were no significant differences with and without chloroform extraction in IAC C_T values and the same observation was made for soil matrix samples for GII assays (Table 4.2). The difference between these two is that for lettuce there was no PCR inhibition, while in top soil, the chloroform extraction step did not reduce PCR inhibition.

Generally, the GI IAC assay demonstrated that there was no significant GI inhibition regardless of sample matrix or RNA extraction method, as nearly all average sample GI IAC C_T values were not more than 3.3 C_T values higher than their corresponding IAC negative controls (Table 4.2). An exception was the IAC C_T value generated for the pasta salad matrix that was not chloroform extracted from which RNA was extracted using the "Lysis" buffer method; the relative difference in IAC C_T value of this sample compared to the negative control was 6.5 (or ~2.0 log genomic copies). Aside this pasta salad sample, for all GI assay samples, the highest difference in C_T value observed was 1.0 (or ~0.30 log genomic copies). For the GII assay, there was more PCR

inhibition observed and this varied from one food matrix and RNA extraction method to another. For the GII assay, chloroform extracted lettuce samples did not contribute to PCR inhibition of the GII IAC, while a ≥ 8.9 C_T value difference (approximately ≥ 2.7 log genomic copies) was observed for non-chloroform extracted soil samples regardless of the RNA extraction method, except for "Trizol" extracted samples which showed a 6.81 C_T value difference (~2.1 log genomic copies) between samples and negative controls. Aside from the soil sample matrix, the mixed berries and pasta salad matrices provided generally higher C_T values, especially for non-chloroform extracted samples, while the lettuce and lunch meat sample matrices yielded lower relative differences in IAC C_T values compared to the corresponding negative controls.

4.4.2. Effect of sample matrix and RNA extraction method on viral RNA recovery.

Using a linear regression model of the complete data by virus type, sample matrix was found to be a significant ($p<0.0001$) contributor to viral RNA recovery of MNV-1, GI.1 and GII.4. The lettuce sample matrix consistently yielded the highest log genomic copy number across the three virus types, while the soil sample yielded the lowest GI and GII genomic copy numbers, and pasta salad matrix provided the lowest genomic copy yield for MNV-1. The current study also shows that during sample concentration/clean-up, the chloroform extraction step applied towards the end of the concentration is a significant factor affecting viral RNA recovery ($p<0.0001$). In most cases, the chloroform extraction step increased viral RNA recovery when compared to corresponding non-chloroform extracted matrices (Table 4.5). For example, for lunch meat and pasta samples, including the chloroform extraction step yielded significantly ($p<0.0001$)

greater GII.4 RNA recovery. Non-chloroform extracted lunch meat and pasta matrix samples yielded a lower average GII.4 virus recoveries, at 4.53 and 2.44 log GC/ml, respectively, while the corresponding chloroform extracted samples yielded 5.12 and 4.54 log GC/ml. These differences were statistically significant ($p<0.0001$). Out of the 15 variables in the data sets (five sample matrix, three virus types), only in one instance, GII.4 recovered from lettuce, was the chloroform extraction step not a significant factor ($p= 0.0802$, $\alpha=0.05$). Including the chloroform extraction step during sample preparation therefore significantly ($p<0.0001$) improves recoveries of GII.4 HuNoVs for all food and soil types used in this study except Lettuce ($p = 0.0802$) .

Extraction method was found to be a significant factor influencing viral RNA recovery in the majority of the samples tested. For example, for mixed berries, the extraction method was significant ($p<0.0001$) for MNV-1, GI.1 and GII.4 genome copies recovered (Table 4.3). A student's t-test of the means showed that for GII.4 RNA recovery, the Trizol-based extraction method was significantly different from the other three methods tested, while the Lysis, Unex and Qiagen methods yielded no significant differences from each other (Table 4.2). The Trizol method yielded an average of 2.00 log GC of GII.4 while the Lysis method recovered 5.06 log GC/ml. This is a 3 log difference between the two methods. The corresponding recovery of GI.1 RNA was 3.76 log GC/ml using the Trizol method and 5.89 log GC/ml using the Lysis method (Table 4.2). Similarly, for MNV-1 recovery from the mixed berries matrix, the Trizol extraction method was the least efficient with 3.73 log GC/ml recovery compared to a 6.14 log GC/ml recovery obtained by the Lysis extraction method. Fig 4.2 also shows a representation of viral RNA recoveries for the different food matrices; statistical

inferences made with berry samples are also reported. From the figure, it is evident that only with the use of the Trizol extraction method is viral RNA recovery low for the mixed berries matrix.

4.5. DISCUSSION

With HuNoV as the leading causative agent of foodborne gastroenteritis in the U.S., effective and reliable methods for detecting HuNoV in food samples is crucial for understanding transmission routes and developing effective strategies to reduce foodborne outbreaks of HuNoV. The use of RT-PCR, whether conventional or real-time, for HuNoV detection is faced with a significant hurdle due to PCR inhibition. Given the very small sample volume required for PCR and the usually low number of HuNoV particles found in food and environmental samples, a virus elution-concentration procedure is usually the first step in sample preparation prior to virus detection. The challenge with this process is that concentration of PCR inhibitors can also occur during virus concentration; these inhibitors reduce PCR signals or mask presence of HuNoV particles, resulting in underestimation of viral contents. While a virus elution-concentration method with a high efficiency of virus recovery from samples is crucial, an RNA extraction method capable of reducing or removing PCR inhibitors is also very important.

In this study, the impact of four different RNA extraction methods used on four food sample matrices and a top soil matrix was evaluated for their efficacy in eliminating or reducing PCR inhibition, while maintaining viral RNA recovery. Food matrices were chosen from different categories of food products; fruits (mixed berries), vegetables

(iceberg lettuce), RTE meats (lunch meat) and a complex RTE salad (pasta salad). A soil sample was also tested along with the food sample as a representation of an environmental sample known to contain a high levels of PCR inhibitors, such as humic acids (Wilson, 1997). Presence of inhibitors in soil samples was confirmed in this study with the highest average IAC C_T values reported for both GI and GII assays. Also, average RNA recoveries were generally lower for the soil samples when compared to other food matrices. Given that the virus cocktail used in this study contained MNV-1, GI.1 and GII.4 HuNoV, a limitation of this study was the absence of MNV IAC assay due to unavailability of MNV IAC. However, since we had IAC assays for GI and GII, the most common genogroups of human norovirus, estimates and results obtained from these two assays are likely to be a good representative of PCR inhibition in human norovirus. Also the two different master mixes used in this study as described in the methods section may be responsible for some difference observed. The MNV and GI assays employed the "Quanta" manufactured master mix while the GII assay employed a "Qiagen" manufactured master mix. A generally higher viral RNA recovery is consistent with GI.1 and MNV-1 recovery when compared to GII.4 RNA recovery, while lower PCR inhibition is observed in the GI assays than in GII assays. Even though we employed the different mastermixes based on their amplification efficiency for each viral RNA as determined in preliminary studies, these observations suggests that the efficiency of the "Quanta" mastermix may be unaffected by the presence of inhibitors in samples, as compared to the "Qiagen" mastermix. The Qiagen mastermix was however more efficient in GII.4 amplification especially when close to the LOD (data not shown).

The efficiency of the RNA extraction methods tested in this study varied with the sample matrices tested. While the Trizol method was the least efficient method for MNV-1, GI.1 and GII.4 RNA recovery from mixed berries, this method was the most efficient for recovery of each of the viruses from lunch meat. After elution-concentration of the food sample, the resulting matrix is still representative of the food and will contain different chemical component from one food type to another. For example, the mixed berries matrix (especially samples that did not include the chloroform extraction step) were visually dark in color with a highly viscous consistency, while the lunch meat matrix, was a less viscous, colorless matrix. Also, considering that the different food matrix vary in fat and carbohydrate content; for example comparing the pasta salad and the lunch meat samples, fat content were 16% and 4%, and carbohydrate contents were 25% and 3% respectively, some variations in viral RNA recovery and PCR inhibition is expected from one sample to the other. Baert et al. (2008) in their studies compared different virus concentration-extraction methods for GII.4 seeded into RTE food samples. Based on their results, two categories of RTE food products, fruits and vegetables grouped together in one category, and RTE dishes (penne and tagliatelle salads) which are rich in proteins and fat formed another category. The group explained that despite the elution-concentration procedure, the variability observed in the results indicated that interfering components from the food samples were still present. The results of the current study strongly support this observation. However, considering that virus cocktail was added after the elution-concentration steps, variation in our results cannot be attributed to the elution-concentration step, but rather to the resulting sample matrix and the ability of RNA extraction methods to remove PCR inhibitors.

Higher RNA recoveries were observed for samples that were chloroform-extracted after sample concentration and before RNA extraction. Of the 120 averages reported (Table 4.5), in only 2 of these (mixed berry matrix, Lysis extracted MNV, and Unex extracted GI.1) was the RNA recovery the same with and without the chloroform extraction step. This suggests that chloroform extraction is a necessary step for either reducing the amount of PCR inhibitors in the sample matrix, reducing the complexity of the sample matrix which may in turn increase extraction buffer efficiency, or a combination of both. This observation was also similar regardless of virus genogroup and/or virus genotype. Contrary to our observations, Baert et al. (2008) observed that extra purification steps could negatively influence virus recovery; such was observed when a proteinase K/CTAB treatment followed by a phenol/chloroform/isoamylalcohol purification was employed. A major variation between the current study and the former is that in the current study, the sample matrix was prepared sans virus, first preparing a food matrix using the elution-concentration steps and afterwards adding the virus cocktail. The previous study seeded virus directly on the food samples prior to elution-concentration. In such an experimental design, it cannot be determined how much of the virus loss encountered was a result of the elution-concentration procedure, the chloroform extraction step or the RNA exaction method. The current study added virus cocktail directly to the extracted and concentrated food matrix in order to focus on impact of the RNA extraction methods and chloroform extraction steps on reducing PCR inhibition.

Rutjes et al. (2006) compared three virus concentration methods (PEG-NaCl, ultracentrifugation and ultrafiltration) and two RNA extraction methods (Trizol and Qiagen Kit) for detection of norovirus in whipped cream and lettuce samples and

reported that the PEG-NaCl with Trizol method was the most efficient for detection of viruses in whipped cream, while the ultracentrifugation with Qiagen kit method was best for detection on lettuce. The results in the current study support the Baert et al. (2008) and Rutjes et al. (2006) recommendations that different concentration and RNA extraction methods be employed for different food matrices rather than a one-size-fits-all approach.

4.6. CONCLUSIONS

The results of this study show that the efficiency of RNA extraction methods in reducing RT-qPCR inhibition depends on the sample matrix and other factors such as the chloroform extraction step in the elution-concentration procedure. The chloroform extraction step reduced PCR inhibitors in the sample and improved viral RNA recovery in nearly all cases. The soil sample matrix showed the highest level of PCR inhibition followed by pasta the salad matrix; the least amount of PCR inhibition observed was from the lettuce sample matrices. The GuSCN Lysis buffer and the Esphere Unex buffer were generally comparable in both viral RNA recovery and PCR inhibition, while the Qiagen QiAmp kit and Trizol buffer method efficiency varied with sample matrix. PCR inhibition observed in this study was mainly in the GII assay rather than GI assay, considering that for each assay, the sample matrix tested were the same, a variation in the RT-PCR mastermix used may be responsible for the observed differences.

4.7. APPENDIX

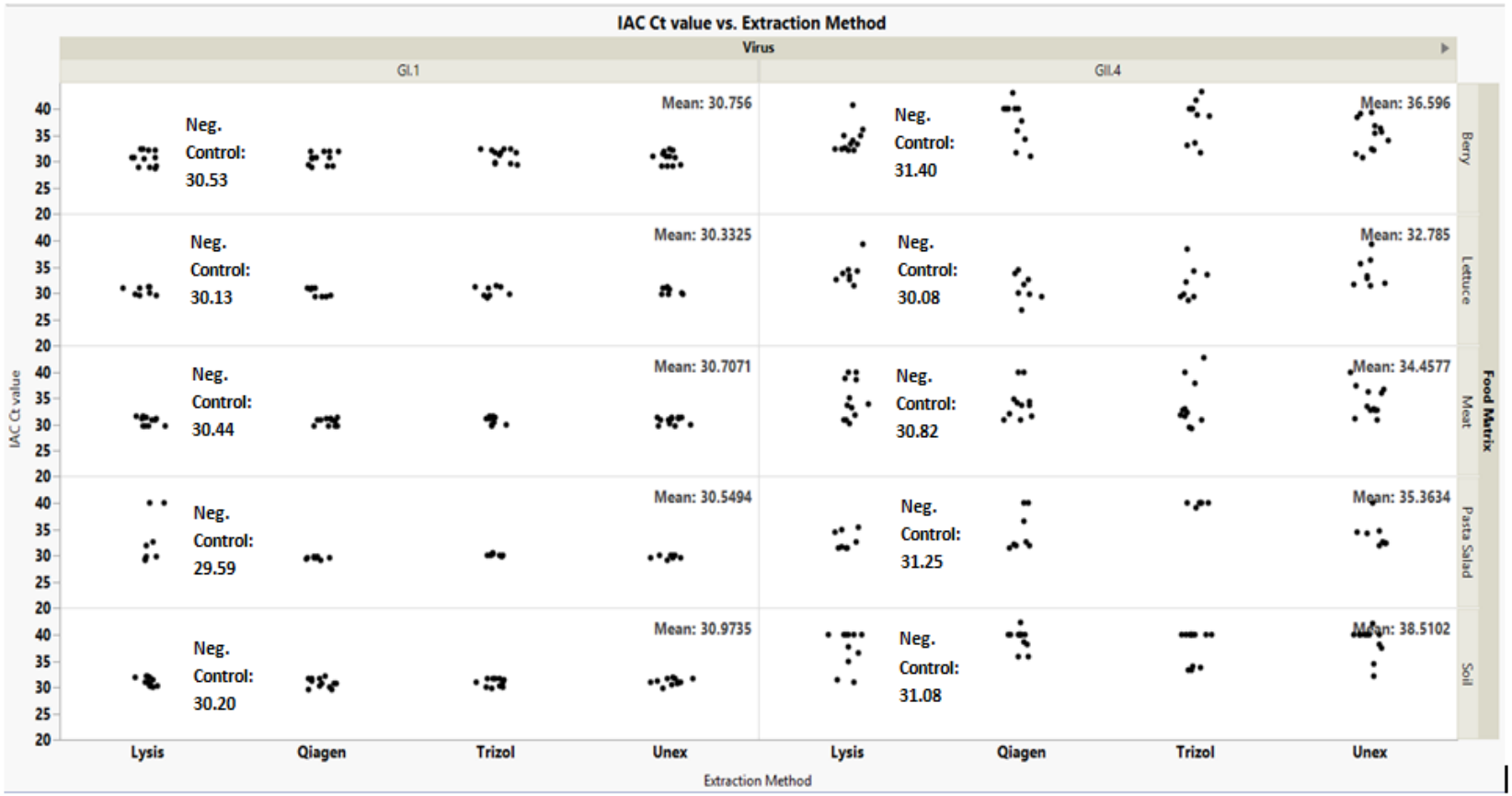


Figure 4.1: Internal Amplification Control (IAC) C_T value distribution across the different sample matrices when four RNA extraction methods are employed. Negative control IAC C_T values (no sample but IAC positive) are reported for each

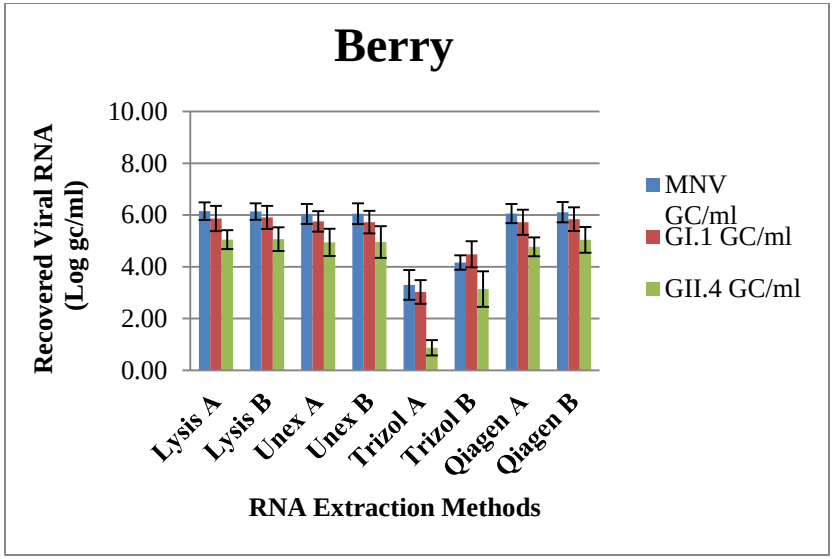
data set and indicated on the figure. Black dots represent the distribution of IAC C_T values generated after amplification of IACs in the presence of different sample matrices extracted by different methods of RNA extraction. C_T values greater than the average control C_T value indicate some level of PCR inhibition due to the sample matrix; the greater the difference, the greater the sample inhibition.

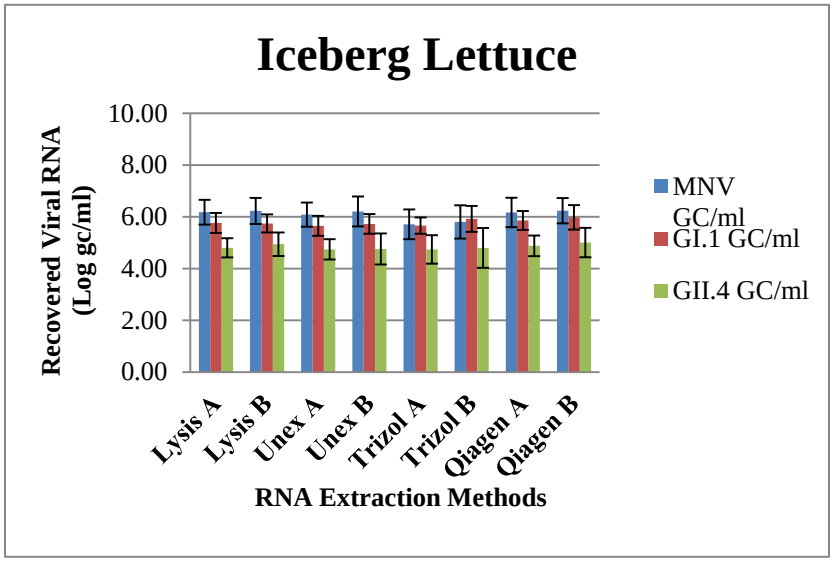
Table 4.1: Impact of RNA extraction method and inclusion of a chloroform extraction step prior to RNA extraction on GI or GII IAC amplification in the context of different sample matrices. For each sample matrix, p-value are reported indicating whether or not the RNA extraction method used or incorporation of a chloroform extraction step prior to RNA extraction impacted the amplification of IACs in the GI or GII assays.

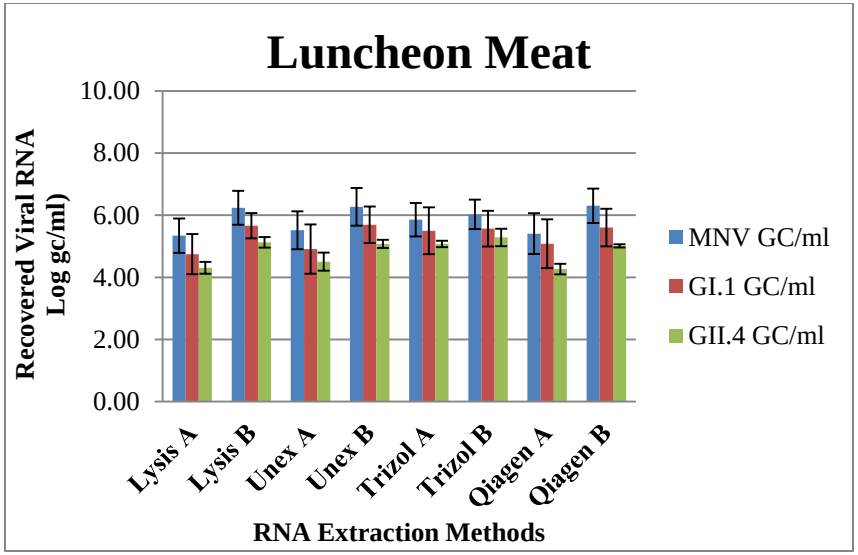
IAC	Effect test	Mixed Berries	Iceberg lettuce	Lunch Meat	Pasta Salad	Top Soil
GI assay	Extraction Method	<0.0001	0.0309	0.0018	0.0006	0.0184
	Chloroform Extraction Step	0.0165	0.5435	0.0824	0.0034	0.0002
GII assay	Extraction Method	0.0003	0.0033	0.7922	<0.0001	0.6818
	Chloroform Extraction Step	0.0054	0.0004	0.0045	0.2316	0.0332

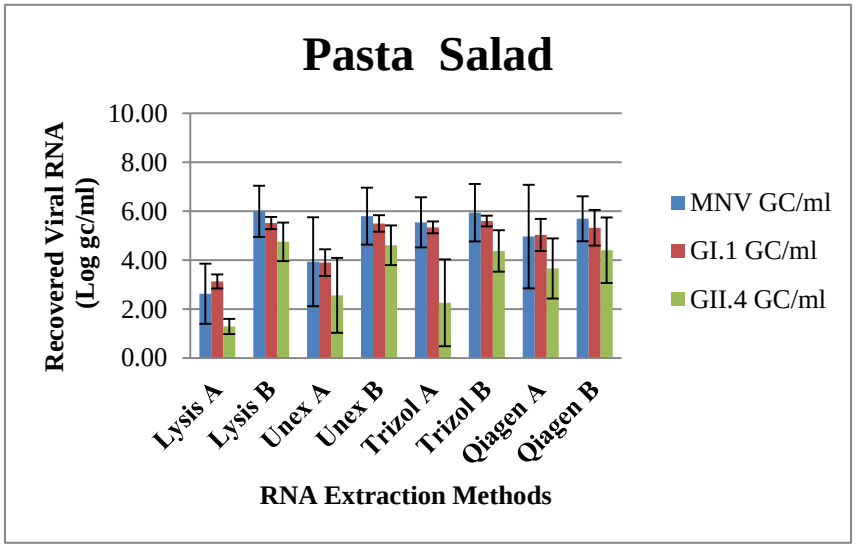
Table 4.2: Average IAC C_T values show the impact of the different RNA extraction methods and the chloroform extraction step in reducing inhibition of RT-PCR in the context of the different sample matrices. For each sample matrix, the average IAC C_T value of the negative control (no positive sample, only IAC in master mix) are also displayed as a reference value. IAC values with the same lowercase superscript along each row were not significantly different and for values with the same uppercase letter superscript there was no difference between C_T values generated with and without the chloroform extraction step within each matrix (α=0.05).

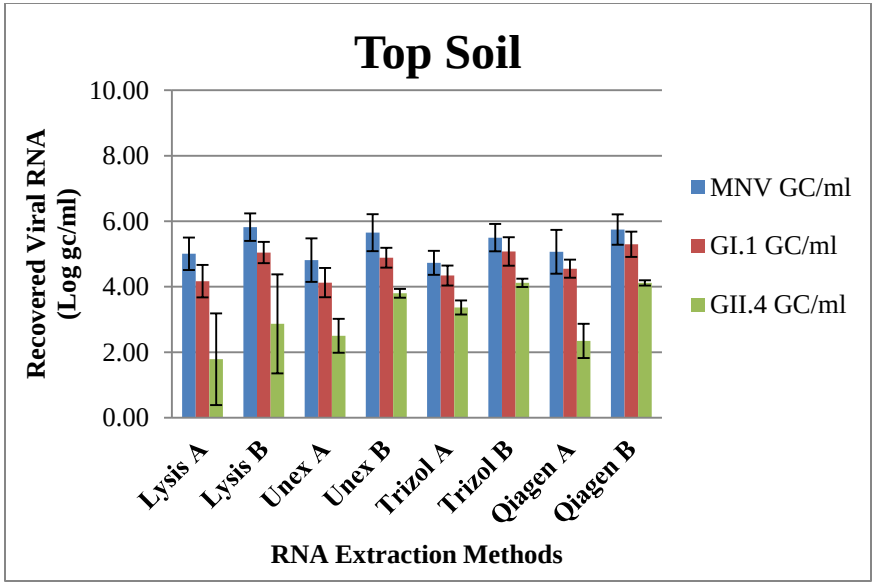
IAC	Sample Matrix	Negative Control Average	Chloroform extraction step	RNA Extraction Method			
				Lysis	Qiagen	Trizol	Unex
GI assay	Mixed berries	30.46	-	30.64^{bA}	30.62^{bA}	31.24^{aA}	30.83^{bA}
			+	30.62^{bA}	30.51^{bA}	31.05^{aA}	30.53^{bB}
	Iceberg Lettuce	30.13	-	30.41^{aA}	30.16^{aA}	30.19^{aA}	30.49^{aA}
			+	30.40^{abA}	30.15^{bA}	30.46^{aA}	30.41^{abA}
	Lunch Meat	30.44	-	30.57^{bcB}	30.42^{cB}	30.91^{aA}	30.75^{abA}
			+	30.83^{aA}	30.64^{aA}	30.81^{aA}	30.75^{aA}
	Pasta Salad	29.59	-	36.13^{aA}	29.42^{bA}	30.24^{bA}	29.84^{bA}
			+	29.49^{bB}	29.66^{abA}	29.97^{aA}	29.65^{bA}
	Top Soil	30.19	-	31.14^{aA}	30.89^{bA}	31.16^{aA}	31.19^{aA}
			+	31.01^{aA}	30.74^{bA}	30.79^{bB}	30.88^{abB}
GII assay	Mixed berries	31.40	-	35.43^{cA}	39.84^{abA}	40.11^{aA}	35.86^{bcA}
			+	32.75^{cA}	36.76^{abB}	37.58^{aA}	34.44^{bcA}
	Iceberg Lettuce	30.08	-	34.91^{aA}	32.65^{aA}	32.76^{aA}	35.87^{aA}
			+	33.13^{aA}	29.54^{cB}	31.07^{bcA}	32.36^{abA}
	Lunch Meat	30.82	-	32.81^{bA}	32.67^{bA}	35.37^{aA}	32.89^{bB}
			+	36.71^{aB}	35.13^{aA}	34.10^{aA}	35.98^{aA}
	Pasta Salad	31.25	-	31.52^{cB}	36.03^{abA}	40.00^{aA}	32.29^{bcB}
			+	34.33^{aA}	33.13^{cA}	39.77^{aA}	35.84^{bA}
	Top Soil	31.08	-	40.00^{aA}	40.00^{aA}	37.89^{bA}	40.00^{aA}
			+	36.32^{aA}	37.47^{aA}	37.87^{aA}	37.47^{aA}


Berry
Recovered Viral RNA (Log gc/ml)
10.00
8.00
6.00
4.00
2.00
0.00
Lysis A
Lysis B
Unex A
Unex B
Trizol A
Trizol B
Qiagen A
Qiagen B
RNA Extraction Methods
MNV GC/ml
GI.1 GC/ml
GII.4 GC/ml


Iceberg Lettuce
Recovered Viral RNA (Log gc/ml)
10.00
8.00
6.00
4.00
2.00
0.00
Lysis A
Lysis B
Unex A
Unex B
Trizol A
Trizol B
Qiagen A
Qiagen B
RNA Extraction Methods
MNV GC/ml
GI.1 GC/ml
GII.4 GC/ml


Luncheon Meat
Recovered Viral RNA
Log gc/ml)
10.00
8.00
6.00
4.00
2.00
0.00
Lysis A
Lysis B
Unex A
Unex B
Trizol A
Trizol B
Qiagen A
Qiagen B
RNA Extraction Methods
MNV GC/ml
GI.1 GC/ml
GII.4 GC/ml


Pasta Salad
Recovered Viral RNA
(Log gc/ml)
10.00
8.00
6.00
4.00
2.00
0.00
Lysis A
Lysis B
Unex A
Unex B
Trizol A
Trizol B
Qiagen A
Qiagen B
RNA Extraction Methods
MNV GC/ml
GI.1 GC/ml
GII.4 GC/ml


Top Soil
Recovered Viral RNA (Log gc/ml)
10.00
8.00
6.00
4.00
2.00
0.00
Lysis A
Lysis B
Unex A
Unex B
Trizol A
Trizol B
Qiagen A
Qiagen B
RNA Extraction Methods
MNV GC/ml
GI.1 GC/ml
GII.4 GC/ml

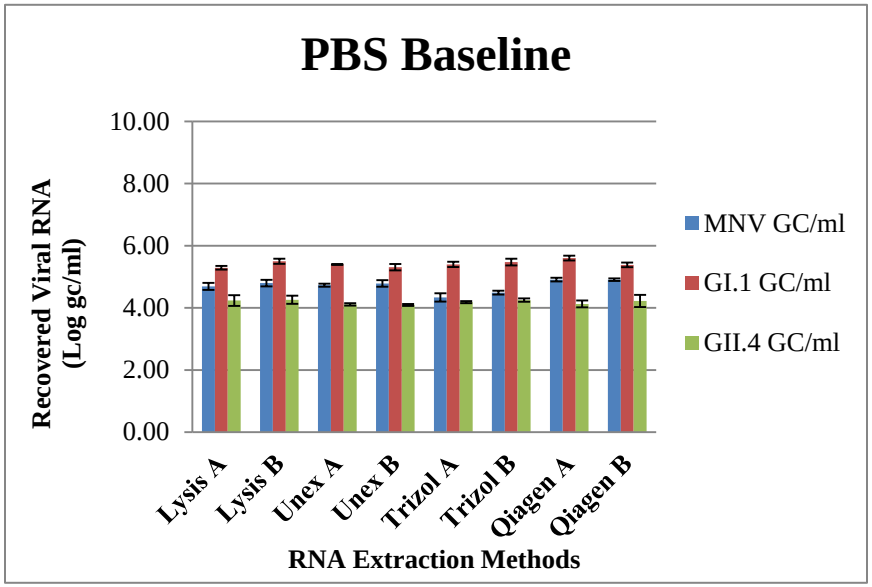

PBS Baseline
Recovered Viral RNA (Log gc/ml)
10.00
8.00
6.00
4.00
2.00
0.00
Lysis A
Lysis B
Unex A
Unex B
Trizol A
Trizol B
Qiagen A
Qiagen B
RNA Extraction Methods
MNV GC/ml
GI.1 GC/ml
GII.4 GC/ml

Figure 4.2: Recovery of MNV-1, GI.1 and GII.4 viral RNA from different sample matrices using four RNA extraction methods with or without a preceding chloroform extraction step. In each chart, average log GC/ml of MNV-1, GI.1 and GII.4 viral RNA recovered from each of the six sample matrices (including the PBS control) using each of the four RNA extraction methods either with or without chloroform extraction are reported. With each extraction method, the letter "A" designates absence of the chloroform extraction step and "B" represents presence of the chloroform extraction. Error bars indicate standard deviations from the mean.

Table 4.3: Average recovery of MNV-1 and GI.1 and GII.4 HuNoV RNA from different sample matrices. Log_{10} genomic copies of viral RNA per ml (Log GC/ml) recovered from each sample matrix inoculated with ~10^5 Log GC/ml is reported. The p-value shows effect of the sample matrix on viral RNA recovery. Values with the same superscript along each row were not significantly different (α=0.05).

Virus recovered	Matrix Effect p-value	Mixed Berries	Iceberg Lettuce	Lunch Meat	Pasta Salad	Top Soil
MNV-1 (Log GC/ml)	<0.0001	5.50^{b}	6.08^{a}	5.87^{a}	5.06^{c}	5.29^{bc}
GI.1 (Log GC/ml)	<0.0001	5.29^{b}	5.79^{a}	5.35^{b}	4.92^{c}	4.67^{d}
GII.4 (Log GC/ml)	<0.0001	4.23^{b}	4.83^{a}	4.83^{a}	3.49^{c}	3.11^{d}

Table 4.4: Impact of RNA extraction method and inclusion of a chloroform extraction step prior to RNA extraction on MNV, GI or GII RNA recovery in the context of different sample matrices. For each sample matrix, p-values are reported indicating whether or not the RNA extraction method used or incorporation of a chloroform extraction step prior to RNA extraction impacted the recovery of viral RNA from the matrix.

Virus and Response	Test variable	Mixed Berries	Iceberg lettuce	Lunch Meat	Pasta Salad	Top Soil
MNV - Genomic Copies	Extraction Method	<0.0001	<0.0001	0.0791	<0.0001	0.0002
	Chloroform Extraction Step	0.0003	0.0046	<0.0001	<0.0001	<0.0001
GI.1 Genomic Copies	Extraction Method	<0.0001	0.0043	0.0065	<0.0001	<0.0001
	Chloroform Extraction Step	<0.0001	0.0146	<0.0001	<0.0001	<0.0001
GII.4 Genomic Copies	Extraction Method	<0.0001	0.0183	<0.0001	0.0060	0.0002
	Chloroform Extraction Step	<0.0001	0.0802	<0.0001	<0.0001	<0.0001

Table 4.5. Average log_{10} genomic copy numbers reported reveal the impact of the different RNA Extraction methods and inclusion of the chloroform extraction step for MNV-1, GI.1 and GII.4 HuNoV RNA recovery in the context of the different sample matrices. Average log GC numbers with the same lowercase superscript along each row were not significantly different and for values with the same uppercase letter superscript, there was no difference between log GC recoveries achieved with and without the chloroform extraction step within each sample matrix (α=0.05).

Effect Test	Sample Matrix	Chloroform Extraction	RNA Extraction method			
			Lysis	Qiagen	Trizol	Unex
MNV-1 (Log GC/ml)	Mixed berries	-	6.15^{aA}	6.06^{aA}	3.3^{bA}	6.04^{aA}
		+	6.14^{aA}	6.11^{aA}	4.17^{bB}	6.05^{aA}
	Iceberg Lettuce	-	6.18^{aA}	6.17^{aA}	5.71^{bA}	6.09^{aA}
		+	6.23^{aB}	6.24^{aA}	5.80^{bB}	6.21^{aB}
	Lunch Meat	-	5.34^{bA}	5.41^{bA}	5.86^{aA}	5.52^{bA}
		+	6.24^{aB}	6.31^{aB}	6.03^{bB}	6.27^{aB}
	Pasta Salad	-	2.63^{cA}	4.97^{aA}	5.55^{aA}	3.94^{b}
		+	6.00^{aB}	5.69^{aA}	5.94^{aB}	5.80^{a}
	Top soil	-	5.01^{abA}	5.07^{aA}	4.73^{cA}	4.81^{bcA}
		+	5.82^{aB}	5.75^{abB}	5.50^{cB}	5.65^{bB}
GI.1 (Log GC/ml)	Mixed berries	-	5.87^{aA}	5.72^{aA}	3.03^{bA}	5.76^{aA}
		+	5.91^{aA}	5.84^{aA}	4.48^{cB}	5.73^{bA}
	Iceberg Lettuce	-	5.76^{bA}	5.86^{aA}	5.66^{cA}	5.65^{cA}
		+	5.75^{bA}	5.98^{aA}	5.92^{abA}	5.73^{bA}
	Lunch Meat	-	4.75^{cA}	5.08^{bA}	5.50^{aA}	4.91^{bcA}
		+	5.66^{aB}	5.61^{aB}	5.57^{aA}	5.69^{aB}
	Pasta Salad	-	3.13^{cA}	5.03^{aA}	5.34^{aA}	3.91^{bA}
		+	5.52^{aB}	5.33^{aA}	5.60^{aB}	5.50^{aB}
	Top soil	-	4.17^{cA}	4.55^{aA}	4.34^{bA}	4.13^{cA}
		+	5.05^{bB}	5.30^{aB}	5.08^{bB}	4.88^{cB}
GII.4 (Log GC/ml)	Mixed berries	-	5.05^{aA}	4.77^{bA}	0.87^{cA}	4.95^{abA}
		+	5.07^{aA}	5.04^{aB}	3.14^{bB}	4.96^{aA}
	Iceberg Lettuce	-	4.81^{abA}	4.88^{aA}	4.74^{bA}	4.74^{bA}
		+	4.94^{abB}	5.01^{aA}	4.80^{bA}	4.76^{bA}
	Lunch Meat	-	4.31^{cA}	4.27^{cA}	5.08^{aA}	4.50^{bA}
		+	5.13^{abB}	5.01^{bB}	5.28^{aA}	5.08^{abB}
	Pasta Salad	-	1.29^{cA}	3.66^{aA}	2.26^{bA}	2.56^{bA}
		+	4.75^{aB}	4.41^{aA}	4.38^{aB}	4.61^{aB}
	Top soil	-	1.79^{bA}	2.35^{bA}	3.37^{aA}	2.5^{abA}
		+	2.87^{bB}	4.12^{aB}	4.12^{aB}	3.80^{aB}

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CHAPTER 5

SUMMARY AND CONCLUSIONS

Human noroviruses are major etiological agents of non-bacterial gastroenteritis, often associated with contaminated food, water and environmental surfaces. As the leading cause of foodborne illness in the US, detection of HuNoV in food samples is important for epidemiological purposes and intervention strategies. The most common means of HuNoV detection is by the use of RT-PCR, which amplifies and confirms the presence of viral genomic material in a sample, however, the presence of a genomic material does not necessarily mean presence of an infectious virus particle. Alternative methods are thus needed to discriminate between infectious and noninfectious viruses in a food, environmental and clinical samples. The current study evaluates two possible methods of detecting infectivity.

With RT-PCR as the only method of HuNoV detection currently available, a major obstacle with detection in samples (food, environmental water and/or clinical samples) is the presence of inhibitors in the samples. Inhibitors such as organic matter and humic acid reduce the efficacy of detection methods that estimate infectivity. Extraction of RNA from virus in samples is a necessary precursor to RT-PCR analysis. This study therefore also seeks to evaluate different RNA extraction methods for their ability to reduce or eliminate PCR-inhibitors and also compare the efficiency of the methods for RNA recovery in different sample matrixes.

The results from the three studies of this dissertation suggest that molecular detection of infectious HuNoV may not be impossibility. However several factors were identified that influence the reliability of such methods, including; the virus type, the virus suspension matrix, virus titer, and type of virus inactivation n method employed in the study.

In the enzyme pretreatment study using Proteinase K and RNAse A treatment prior to RT-qPCR of thermally inactivated viruses, virus suspension matrix and virus titer were significant factors impacting the observed results. Suspension of a virus sample in stool, as compared to a purified matrix such as PBS reduces efficiency and reliability of the method. Dilution of samples may reduce or dilute out the effect of the suspension matrix, however this also reduces the concentration of viruses in the sample which works against the need for a concentrated virus sample required for RT-PCR. While the method seemed to be efficient in eliminating false positive signals in inactivated samples when a lower input titer of virus was used, the same results were not be observed when a higher titer virus sample was used. The maximum log reduction observed using this method was 2.94 log genomic copies heat inactivated MNV-1, however the reduction does not correlate with a 6 log PFU/ml reduction observed as determined by plaque assay. The results obtained in this study suggest a need for purification of samples to ensure efficacy of the ET method.

In the study evaluating the PGM-binding plus RNAse A treatment method prior to RT-qPCR, virus input titer and genotype appeared to also be influential in the results obtained. The efficiency of the PGM-binding-RNAse A method in estimating infectious HuNoV also varied across the inactivation methods tested. However, non-specific binding observed in the study indicates the need for improving the specificity of this

binding method which could be a potential method of detecting infectious HuNoV. Reduction and elimination of positive RT-PCR signals from non-infectious viruses also varied depending on the inactivation method used. Elimination of RT-PCR signals by the PGM-binding-RNAse A method was observed more when Lev/high SDS sanitizer was the inactivation method compared to other methods tested; thermal inactivation and 70% ethanol treatment. Results also varied from one virus type to another, even though this variation may be attributed to differences in virus titer.

The third study shows the influence of sample matrices and RNA extraction methods on PCR inhibition and recovery of viral RNA; this study suggests that different food samples have different components which may influence viral RNA recovery and PCR inhibition. PCR inhibition and viral RNA recovery also varied from one extraction method to another and between the different norovirus strains studied, more PCR inhibition were observed in GII assays than in GI assays, and RNA recovery was higher for MNV and GI.1 than it was for GII.4 HuNoV. Mastermix used in the RT-PCRs assays were suspected to have an effect on PCR inhibition and viral RNA recovery. Chloroform extraction step in the virus elution-concentration step was observed to be a significant factor in reducing PCR inhibition in GII assays. The results of this study provide information that can be used to enhance/improve HuNoV detection in food samples.

Further research should focus on improving method reliability, for example improving specificity in PGM-binding-RNAse A method, testing other commonly employed inactivation methods and other norovirus strains. Also, any study into molecular detection of infectious HuNoV should consider variables such as virus titer, suspension matrix and virus inactivation methods.

Finally, the importance of study/process controls cannot be overemphasized, the use of negative and positive controls as may be applicable is important in making reasonable conclusions from research results. In a portion of this dissertation evaluating PGM-binding prior to RT-PCR as a means of detecting infectivity, the study incorporated controls that has not been considered in previous studies on PGM-binding. Since the principle behind this method is that intact virus capsids will bind to PGM, which can subsequently be detected in RT-PCR, it was therefore important to test that in the absence of PGM, there will be no positive RT-PCR signals in tested samples. The results in this study suggests non-specific binding of virus particles to ELISA plate wells, this is the first research work reporting this observation. Eliminating non-specific binding in this method will be a giant stride towards confirming a means of detecting norovirus infectivity.