2012

Environmental sampling for detection of norovirus using a real-time RT-PCR Assay: A Tool for Foodborne Outbreak Investigations

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ENVIRONMENTAL SAMPLING FOR DETECTION OF NOROVIRUS USING A REAL-TIME RT-PCR ASSAY: A TOOL FOR FOODBORNE OUTBREAK INVESTIGATIONS

by

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A thesis submitted in partial fulfillment
of the requirements for the
Master of Science degree in Epidemiology
in the Graduate College of
The University of Iowa

July 2012

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CERTIFICATE OF APPROVAL

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MASTER'S THESIS

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This is to certify that the Master's thesis of

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This project is dedicated to my supportive and loving family; my husband who always had words of encouragement and motivated me to the end, my children who were understanding when I could not take them to the pool or when "Mom" just had to work, and my parents who lovingly cared for my children when I was in class, conducting research, or studying. Thank-you!

ACKNOWLEDGMENTS

I would like to thank and acknowledge my thesis committee; Dr. Lucy DesJardin, Dr. Michael Pentella, Dr. Tara Smith, and Dr. James Torner. Trish Kreman and Nancy Hall, your mentoring and project assistance were invaluable. Jennine Wolf, your passion and dedication to serving your community was inspiring. Thank-you all.

ABSTRACT

Noroviruses (NoVs) are a leading cause of acute gastroenteritis. They are highly infectious and can be transmitted from infected individuals to susceptible individuals via multiple routes. Epidemiological investigations of outbreaks can aid public health interventions and understanding the mechanisms of transmission can prevent additional infections. This project was designed to develop a method for the collection of environmental samples during prolonged NoV outbreak investigations, and to adapt real-time RT-PCR assays to analyze environmental samples for GI and GII noroviruses.

Real-time RT-PCR assays for the detection of GI and GII NoVs were developed by adapting the State Hygienic Laboratory clinical GI and GII assays to the AB 7500 Fast platform. The performance characteristics of the tests were determined, and showed the tests to be sensitive with high amplification efficiencies. Optimum efficiencies range from 95%-105%, with a 100% efficiency indicating exponential amplification of targeted nucleic acid. Analysis of the GI assay performance yielded a slope = 3.28, $R^2 = 0.999$ and a calculated amplification efficiency of 102%. The GII assay yielded a slope = 3.39, $R^2 = 0.999$ and a calculated amplification efficiency of 97%.

To develop a method for the collection of environmental samples, multiple swab types were tested to determine their ability to recover NoV from laboratory spiked environmental surfaces. It was determined that foam swabs moistened with viral transport media were most effective in recovering NoV from spiked surfaces.

A field test of the environmental sampling method was conducted by sampling environmental surfaces in four restaurants in one Iowa community. NoVs were not detected in these samples, however this method may be of value in future outbreak investigations and requires further study.

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INTRODUCTION

Study Introduction

Noroviruses (NoVs) are a leading cause of acute gastroenteritis. They are highly infectious and can be transmitted from infected individuals to susceptible individuals via multiple routes. This study proposes the development of laboratory methods to detect NoVs from environmental samples and the utilization of environmental sampling data to facilitate NoV outbreak investigations.

Norovirus Disease Burden

Worldwide, noroviruses are the leading cause of outbreak associated gastroenteritis in all age groups (Patel et al., 2008; Blanton et al., 2006; Fankhauser et al., 2002). NoVs are attributable for approximately 50% of all infectious causes of outbreak associated gastroenteritis (Patel et al., 2008). Children less than five years of age experience NoV infection rates at five times the rate of the general population (Lopman et al., 2012). In developing countries, 124 million childhood hospitalizations and 1.8 million deaths are annually attributed to NoVs; as compared to developed countries, where 1.1 million childhood hospitalizations and 218,000 deaths are annually attributed to NoVs (Patel et al., 2008).

In the United States, it is estimated that approximately 23 million NoV illnesses occur annually (Mead et al., 1999). Approximately 9.2 million (40%) of these illnesses were acquired via foodborne NoV transmission (Mead et al., 1999). Outbreak investigations have determined that NoV outbreaks often utilize multiple routes of transmission, which can prolong outbreaks and complicate public health intervention efforts (Boxman et al., 2009a; Cheesbrough et al., 2002; Johansson et al., 2002; Isakbaeva et al., 2005b; Mathews et al., 2012).

Of the 54 foodborne outbreaks in Iowa that were investigated by public health departments between the years 2006-2010, 23 (43%) were attributed to NoV. Also during this time period the Iowa Department of Public Health (IDPH) determined that 71 of 92 (77%) outbreaks of undetermined route of transmission were caused by NoV (IDPH 2006-2011).

There is no vaccine to prevent NoV infection nor is there a specific medical treatment; therefore, the best public health initiatives promote infection prevention. Early and appropriate public health intervention at the beginning of an outbreak can contain the spread of infection and limit secondary outbreaks. Early laboratory confirmation of the cause of the outbreak and mechanism of transmission can aid these public health efforts.

History of Norovirus

Historically viruses have long been suspected of causing outbreaks of gastroenteritis. In 1929, Dr. John Zahorsky proposed the term, "Winter Vomiting Disease" to describe the condition (Zahorsky, 1929). In 1947, a large outbreak of non-bacterial gastroenteritis occurred in a hospital setting. Stools from these patients were pooled and filtrates were used to experimentally infect human volunteers, who subsequently developed non-bacterial gastroenteritis. The filtrate was not infective post heat-treatment and all attempts to culture the filtrate using embryonated eggs failed (Gordon et al., 1947). All subsequent attempts to cultivate NoV have been unsuccessful (Duizer et al., 2004).

In 1968, the Centers for Disease Control and Prevention (CDC) investigated an outbreak of non-bacterial gastroenteritis that occurred in an elementary school in Norwalk, Ohio. The outbreak primary attack rate was 50%, with a secondary attack rate of 33%. As with previous non-bacterial gastroenteritis outbreaks, the causative agent was not cultivable, and viral etiology was suspected (Adler and Zickl, 1969). In 1972, using immune electron microscopy, viral particles were identified in stool filtrates from the

1968 Norwalk, Ohio elementary school gastroenteritis outbreak (Kapikian et al., 1972). The identified viral particles were described as small, round, structured viruses (SRSVs) and the virus referred to as Norwalk virus. Norwalk is the parent strain of viruses later classified as Noroviruses (Atmar and Estes, 2006).

Clinical Norovirus Illness

NoV infections have a short incubation period typically ranging from 12-48 hours. Infections result in acute gastroenteritis in persons of all ages; children are more likely to experience vomiting and adults are more likely to experience diarrhea. Up to 50% of infected individuals also experience headaches, low grade fever, chills and muscle aches (MMWR, 2011; Rockx et al., 2002; Kaplan et al., 1982). Infections in previous healthy individuals are most often self-limiting with illness ranging from 24-72 hours. Illness in the elderly and in hospitalized populations is often more severe and prolonged ranging from 4-6 days (Wu et al., 2005; Lopman et al., 2004).

NoV shedding in feces peaks 2-5 days post infection but persists for a median of four weeks after signs and symptoms have abated (Atmar et al., 2008; MMWR, 2011; Akihara et al., 2005; Rockx et al., 2002). Approximately 30% of NoV infections are asymptomatic. Individuals with asymptomatic NoV infection have similar fecal viral loads as do individuals with symptomatic NoV infection (Ozawa et al., 2007). In addition, individuals with asymptomatic NoV infections are also capable of shedding NoVs in their feces for up to four weeks (Ozawa et al., 2007; Rockx et al., 2002). The clinical significance of asymptomatic carriers in NoV transmission is not known.

Norovirus Transmission and Stability

NoVs are highly infectious with a low infectious dose (18-1000 virions) (Lopman et al., 2012; Teunis et al., 2008). NoVs can be transmitted via fomites, food, water, or person-to-person. Direct person-to-person transmission occurs via the fecal-oral route or via ingestion of aerosolized vomitus (Becker et al., 2000; Marks et al., 2003; Marks et al.,

2000; Wikswo et al., 2011). Indirect person-to-person transmission can occur via exposure to fecal or vomitus contaminated fomites such as utensils or cutting boards, or via contaminated environmental surfaces such as door handles and food preparation surfaces (Cheesbrough et al., 2000; MMWR, 2011; Green et al., 1998; Evans et al., 2002; Thornley et al., 2011; Isakbaeva et al., 2005b).

NoVs are environmentally stable and have been recovered from environmental surfaces up to seven days post contamination (D'Souza et al., 2006; Kramer et al., 2006). Numerous NoV outbreak investigations have concluded that contaminated surfaces contributed to sequential NoV outbreaks (Thornley et al., 2011; Isakbaeva et al., 2005b; Evans et al., 2002; Cheesbrough et al., 2000). In 2011, a vomiting incident in an airplane cabin resulted in NoV illness in separate flight crews over a five day period. The common variable linking the five flight crews was having had worked on the same airplane (Thornley et al., 2011). In 2002, a cruise ship reported elevated numbers of passengers with gastroenteritis over six consecutive cruises; NoV was the confirmed etiologic agent (Isakbaeva et al., 2005b). In a concert hall in 1999, a vomiting incident resulted in over 300 NoV illnesses over a five day period (Evans et al., 2002). In 1996, a NoV outbreak extended over a five month time period at a large hotel causing illness in over 1000 individuals (Cheesbrough et al., 2000). In each of these investigations, contaminated surfaces contributed to prolonged outbreaks infecting large numbers of individuals. The ability to determine what surfaces were contaminated and if they had been appropriately disinfected might have prevented additional illnesses in these incidents.

With a low infectious dose, a long environmental stability, a short incubation period, and transmissibility by multiple routes, NoV outbreaks can rapidly infect large numbers of individuals and lead to secondary outbreaks. Outbreaks associated with restaurants or catered events have attack rates ranging from 50-60% (Mathews et al., 2012; Blanton et al., 2006). NoV outbreaks are especially problematic in susceptible and

or semi-contained populations; outbreaks in nursing homes, hospitals, and retirement facilities have attack rates ranging from 36-50%; and outbreaks in daycare centers and schools have attack rates ranging from 27-37% (Mathews et al., 2012; Blanton et al., 2006). In addition to these high NoV attack rates; outbreak amplification occurs due to person-to-person transmission leading to high secondary attack rates which often exceed 30% (Atmar and Estes, 2006).

Norovirus Characteristics

NoVs are nonenveloped, single stranded, positive sense RNA viruses belonging to the *Caliciviridae* family. NoVs are non-cultivable (cannot be grown in culture), and are highly genetically and antigenically variable.

The genomes of NoVs are approximately 7.5 – 7.7 kilobases with three open reading frames (ORFs) (Atmar and Estes, 2006; Bull et al., 2007; Katayama et al., 2002). The first ORF encodes non-structural gene products including RNA-dependent RNA polymerase which is necessary for RNA virus replication. The second and third ORFs encode structural gene products. The major viral capsid gene (VP1) is encoded on ORF2 and the minor viral structural gene (VP2) is encoded on ORF3. Five genogroups, based on molecular analysis of VP1, have been identified. Genogroups GI, GII, and GIV are associated with human gastroenteritis with GI and GII of most clinical significance. Molecular analysis has further classified GI into 8 genotypes and GII into 21 genotypes (Zheng et al., 2006).

Norovirus Detection Methods

Because NoVs are non-cultivable, laboratory detection methods rely on electron microscopic identification, enzyme immunoassays, or molecular analysis.

Immune electron microscopy (IEM) for detection of NoV in stool specimens was first developed in 1972 (Kapikian et al, 1972). IEM requires extensive specimen preparation, expensive laboratory equipment, and highly skilled laboratory technicians.

Stool specimens must be concentrated, clarified, and incubated with reference serum (antibodies). Virus/antibody complexes are collected by centrifugation, suspended in solution, stained and then viewed by electron microscopy (Kapikian et al., 1972; Atmar and Estes, 2001). Sensitivity of IEM NoV detection is low requiring a high viral load (10^6 particles per ml of stool) (Kageyama et al., 2003). Due to low sensitivity, extensive specimen preparation, costly laboratory equipment, and need for highly skilled laboratory technicians, IEM is not applicable for detection of NoV in large epidemiological studies.

Enzyme immunoassays (EIAs) utilize antibody/antigen interaction to detect NoV in stool specimens. EIA testing yields rapid results with minimal sample preparation and laboratory equipment; however, the variability of NoVs lowers the sensitivity and specificity of EIAs. In 2011, RIDASCREEN® Norovirus 3rd Generation EIA (R-Biopharm, Washington, MO) was approved by the FDA for rapid testing of multiple specimens during NoV outbreak investigations. Samples that test negative must be confirmed by molecular testing methods as this EIA is only 63-77% sensitive and 96-98% specific when compared to molecular detection methods (Kirby et al., 2010; Geginat et al., 2012).

Molecular detection of NoVs using real-time reverse transcriptase-polymerase chain reaction (real-time RT-PCR) is considered the gold standard NoV test method (Kageyama et al., 2003; Trujillo et al., 2006; Park et al., 2008; Stals et al., 2009). Molecular tests target the highly conserved RNA polymerase-capsid junction (ORF1-ORF2 junction) of the NoV genome (Katayama et al., 2002; Kageyama et al., 2003; Trujillo et al., 2006). Real-time RT-PCR continuously detects the increase in amplification of targeted nucleic acid sequences making the test sensitive, specific, and semi-quantitative. Real-time RT-PCR has numerous advantages over conventional RT-PCR. Nucleic acid amplification is continuously detected rather than detected at end point, allowing for semi-quantification of the targeted nucleic acid. Samples do not require post PCR processing, which minimizes potential contamination, reduces use of

mutagenic materials such as ethidium bromide, and allows for automation of laboratory processes.

Norovirus Prevention and Control

Due to a lack of vaccine and treatment options, public health professionals and managers utilize prevention and control methods to combat NoV infections. Methods include hand washing, cleaning and sanitation, and exclusion or isolation of infected persons. Hand washing is the primary method utilized to prevent NoV infections and to control NoV transmission (MMWR, 2011; Sickbert-Bennett et al., 2005). Hand washing physically removes soil and contaminates using water, soap, and vigorous rubbing of the hands for a minimum of 20 seconds (Sickbert-Bennett et al., 2005; Iowa Food Code section 2-301.12). Effective hand washing breaks the route of transmission from infected individuals to fomites, food, water, or directly to susceptible persons.

The second method of preventing and controlling NoV infections and transmission is cleaning and sanitation. Surfaces and fomites must first be cleaned to remove visible soil and contamination. Then surfaces and fomites must be sanitized using a 5000 ppm sodium hypochlorite solution (chlorine bleach) or another disinfectant registered with the EPA as effective against NoVs (MMWR, 2011; Lopman et al., 2012; Doultree et al., 1999; Girard et al., 2010; Barker et al., 2004). As NoVs are extremely infectious, isolating infected individuals is often utilized to prevent viral transmission. Excluding infected individuals from contacting at risk populations or from preparing foods for susceptible persons is also effective in reducing transmission of NoVs to fomites, food, water, or the environment (MMWR, 2011). These prevention and control methods depend on individuals to practice effective hand washing, utilize effective cleaning and sanitation practice, and self report their illness to their employers.

Food Safety Regulations

Federal, state, and local governments have enacted numerous regulations to limit the spread of foodborne pathogens, including NoV.

Food establishments are regulated by code to abide by established food safety guidelines. Iowa Food Code section 2-102.11 requires food establishments to designate a certified food protection manager as the person in charge of food safety during establishment operations. The following food protection manager training programs are accredited by the American National Standards Institute (ANSI): 1) National Restaurants Association ServSafe®, 2) National Environmental Health Association MindLeader, and 3) National Registry of Food Safety Professionals. Successful completion of one of these certified food protection manager training programs fulfills the criteria established in section 2-102.11.

Food establishments are inspected by regulatory authorities to verify compliance to food safety codes. Iowa Food Code section 8-401.10, regulates the minimum frequency of food establishment inspections and section 8-401.20 provides justification for increased frequency of inspections. Inspections are categorized as follows: 1) start-up of a new establishment or after establishment renovations, 2) routine inspection, 3) follow-up to verify implementation of corrective actions, and 4) response to consumer complaint or illness.

Under Iowa Food Code section 4, food establishments are required to utilize cleanable, non-porous, non-absorbent food contact surfaces, equipment, and utensils. Materials frequently utilized include stainless steel, ceramic, plastic, and laminate. All food contact surfaces, equipment, and utensils must be cleaned to remove food residue, rinsed, and then sanitized using an EPA approved disinfectant. Disinfectants frequently utilized are chlorine bleach or quaternary ammonia compounds.

Under Iowa Food Code section 6, food establishments are required to maintain the cleanliness and condition of non-food contact surfaces in their facilities. This

includes surfaces such as floors, walls, ceilings, restrooms, light fixtures, ventilation units, booths, and chairs. Not only is proper facility cleanliness and maintenance important in controlling pests such as rodents and insects but is also important in reducing the spread of infectious pathogens.

When prevention and control methods fail in food establishment settings, NoV infections can spread rapidly. NoV infected food handlers shed virus and are capable of contaminating food, fomites, water, or surfaces (Ozawa et al., 2007; Gotz et al., 2002; Lo et al., 1994; Isakbaeva et al., 2005b; Johansson et al., 2002). In addition to potential NoV infected food handlers, NoV infected patrons are also capable of transmitting NoVs to fomites and surfaces in food establishments.

Norovirus Foodborne Outbreak Investigation Procedures

The Iowa Department of Public Health (IDPH) has published a Foodborne Outbreak Investigation Manual, which clearly describes the procedures to be followed when investigating a suspect foodborne outbreak. The field work for investigating an outbreak as detailed in the manual can be summarized into four sections: 1) initiation of investigation, 2) identification of at risk populations and suspect foods, 3) collection of clinical samples (stool or vomitus) and samples of suspect foods, and 4) identification and verification of corrective actions (IDPH, 2006). The outbreak investigation guidelines specify the collection of food and stool specimens, but do not specify the collection of environmental surface and fomite samples.

During an outbreak investigation, identification of NoVs from food samples is problematic for many reasons. First, suspect foods are often no longer available for sampling. When suspect foods are available for sampling, identification of NoVs from these foods is challenging. Contaminated foods have a low viral load which requires tedious sample concentration making NoV recovery difficult. Finally, inherent food

components often inhibit nucleic acid amplification which reduces the sensitivity of real-time RT-PCR (Stals et al., 2011; Kim et al., 2008).

NoVs are readily transferred to environmental surfaces and fomites (Cheesbrough et al., 2000; Boxman et al., 2009a; Boxman et al., 2009b: Evans et al., 2002; Green et al., 1998; Isakbaeva et al., 2005b), are stable on these sites for up to seven days, and can be transferred by direct or indirect contact from these sites to foods (D'Souza et al., 2006). These viral characteristics give outbreak investigators the opportunity and rationale to collect samples from suspect surfaces; surfaces that are frequently touched by food handlers and or by consumers. However, there is not a standardized laboratory method for collecting environmental samples and testing them for NoVs. A method for collecting and testing environmental surface samples for NoV could help epidemiologists with their investigations by ruling in or out specific routes of NoV transmission.

Specific Aims

Completion of this thesis project will yield an environmental sampling method and real-time RT-PCR assays for the testing of NoV GI and GII in environmental surface samples. In addition, suggestions for environmental surface sampling collected in coordination with NoV outbreak investigations will be developed.

The ability to detect NoVs in environmental samples could aid public health professionals in their outbreak investigations and increase their understanding of NoV transmission pathways. This method could also be used to ensure adequate decontamination after an outbreak has occurred. A better understanding of transmission could provide an opportunity to make additional recommendations to prevent transmission from contaminated surfaces and fomites and decrease illness attributed to NoV.

Specific Aim #1, Develop a real-time RT-PCR assay for the detection of NoV GI and GII from environmental samples.

- Adapt the current SHL clinical NoV assays for analysis of environmental surface samples.
- Develop a method to recover NoV from environmental surfaces.

Specific Aim #2, Apply environmental sampling and testing by real-time RT-PCR to environmental samples collected in food establishments. Use results to make possible recommendations for NoV outbreak investigations.

- Complete a ServSafe® food safety course and participate in restaurant inspections conducted by a local public health department inspector to learn best practices of food handling and sanitation of surfaces in food establishments.
- Collect and process environmental samples from selected restaurants to develop test methods and provide data to develop environmental sampling guidelines.

CHAPTER II

APPROACH

Norovirus Prevention and Control in Food Establishments

Completion of a certified food protection manager program and participation in the food establishment regulatory inspection process was utilized to develop a more thorough understanding of food handling and sanitation requirements and regulations implemented to prevent and control transmission of foodborne illnesses such as NoVs in food establishments. In addition, information regarding surfaces and equipment frequently encountered in food establishments, process work flow, and employee practices was utilized to identify surfaces and fomites frequently touched by establishment employees and patrons. These identified surfaces and fomites were targeted for environmental sampling.

ServSafe® curriculum provides detailed information on foodborne pathogens, food handling hygiene, food temperature regulations, cleaning and sanitizing regulations, pest management, employee training, and HACCP (hazard analysis critical control points). The course is presented in a learning environment which encourages active participation and learner engagement. Successful completion of such a course provides food establishment managers the knowledge to combat the transmission of foodborne illnesses such as NoVs in their establishments.

Participation in the regulatory inspections of food establishments was utilized to observe the implementation of food safety regulations and principles. Four food establishment inspections were chosen as they represent the four types of inspections specified by Iowa Food Code section 8-401.20: 1) start-up of a new establishment or after establishment renovations, 2) routine inspection, 3) follow-up to verify implementation of corrective actions, and 4) response to consumer complaint or illness.

During each inspection, the Health Inspector verified implementation of food safety principles. Verification was determined by observing employee practices and facility conditions, by temperature verification of foods, and by questioning managers and staff of food safety principles. Not only did the inspection process fulfill the regulatory inspection requirement, but it was also used as an opportunity to further educate and reinforce food safety principles.

Each inspection, regardless of the reason for the inspection, highlighted common principles of food safety. As stated in Iowa Food Code section 2-102.11, all bare-hand contact of ready-to-eat (RTE) foods is strictly prohibited. During each inspection, strategies to eliminate bare-hand contact of RTE foods were discussed. Successful strategies included wearing of gloves and utilizing aids such as tongs or paper wraps. Proper hand washing was verified at each establishment, which included a facility inspection to verify access to soap, warm water, and a hand drying mechanism and included actual observations of employees washing their hands. Methods to prevent cross contamination were discussed and assessed. Observations of food preparation areas, coolers and freezers were utilized to verify separation of raw and RTE foods. Maintenance of proper food temperatures was verified using a cleaned and sanitized digital thermometer and employees were questioned as to the proper hot hold and cold hold temperatures. Methods to properly chill and heat foods to minimize time in the temperature danger zone (41°F-135°F) were discussed. A common strategy included portioning large quantities of hot food into several shallow pans placed in coolers or ice baths to increase the rate of chilling. It was emphasized that room temperature chilling of hot foods was not acceptable, as the food would be time/temperature abused allowing for a rapid growth of foodborne pathogens.

At each inspection, education of food safety principles was emphasized to all employees of the food establishments. Educational flyers depicting hand washing practices, maintenance of proper food temperatures, and methods to prevent bare-hand

contact of RTE foods were distributed. Flyers were provided in multiple languages (English, Spanish, and Chinese) to improve communication and compliance to food safety principles. Inspections were conducted in an authoritative yet non-threatening manner. The health department's motto, "Education before Regulation", was successfully implemented during each food establishment inspection.

CHAPTER III

METHODS

GI and GII Assay Development

The State Hygienic Laboratory (SHL) clinical NoV GI and GII assays were adapted to test environmental samples on the AB 7500 Fast real-time platform (Life Technologies) using an Invitrogen Superscript® III Platinum® One-Step qRT-PCR enzyme kit (cat# 11732-020). PCR efficiency was determined by performing 10-fold serial dilutions of clinical GI and GII specimens. A dilution series of each assay was tested in triplicate (n=3). The geometric means of the Cycle thresholds (Ct) were plotted to determine the slope and R^2 values for the dilution curves. The amplification efficiency was determined according to the following equation: Efficiency (n) = $\{10(^{-1/slope})\}$-1. The last dilution at which all sample replicates were detected was used as the limit of detection.

Environmental Surface Spiking

GII filtrates were utilized to spike environmental surfaces and were prepared from two clinical GII stool specimens. A pea-sized sample of solid stool or 0.5 ml of liquid stool collected from multiple locations within the stool specimen was suspended in 3ml of phosphate buffered saline (PBS, pH 7.4) in a 15ml conical tube and mixed well. 2ml was transferred to a 2ml O-ring microcentrifuge tube and centrifuged for 20 minutes at 4000 x g. The supernatant was filtered with a 0.2µm Whatman syringe tip filter and collected in a 2ml O-ring microcentrifuge tube. Short-term storage (< 7 days) of stool filtrates was maintained at 4-8°C and long-term storage (≥ 7 days) of stool filtrates was maintained at -70°C (+/- 5°C).

Environmental surfaces consisting of stainless steel, smooth ceramic and plastic surfaces were inoculated with 140µl NoV GII stool filtrate to verify NoV recovery from

the different surfaces and to determine the best sampling swab. Each surface was washed with detergent and hot water, rinsed, soaked in a 10% sodium hypochlorite solution for 15 minutes, triple rinsed, and allowed to air dry. Surfaces were placed in a biological safety cabinet for inoculations of GII filtrate.

Positive controls consisted of 140µl filtrate inoculated in a single spot. Test samples were inoculated with 140µl filtrate deposited in 14 spots randomly placed within a 5cm by 5cm square of the surface being tested. Negative controls were inoculated with 140µl viral transport medium (VTM) (Remel, Lenexa, Kansas) (cat# R12505) in a single spot. All surfaces were allowed to air dry for 2 hours at room temperature in the biological safety cabinet. Three swab types were tested when sampling the spiked environmental surfaces: a dry flocked nasopharyngeal swab (COPAN, Murrieta, California) (cat# 516CS01) (Figure 1), a VTM moistened flocked nasopharyngeal swab (COPAN, Murrieta, CA, USA) (cat# 516CS01), and a VTM moistened foam swab (Puritan, Guilford, ME, USA) (cat# 25-1607 1PF SC) (Figure 2). All swabbing was conducted in a bi-directional pattern. Moistened swabs (flocked nasopharyngeal and foam) were placed in 1ml VTM after sample collection. Single spot positive controls were recovered by repeated pipetting of 140µl VTM twenty times over the inoculation site. Negative controls were sampled utilizing a bi-directional swab pattern. After sampling, all sampled surfaces were soaked in a 10% sodium hypochlorite solution for 15 minutes, exposed to UV light for 15 minutes, then washed with detergent and hot water. Spiked environmental surface samples were held under refrigerated conditions (4-8°C) until processing.

Figure 1: Flocked nasopharyngeal swab

Figure 2: Foam swab

RNA Extraction

Nucleic acid extractions were manually performed using a QIAamp® Viral RNA Minikit (Qiagen, Valencia, CA, USA) (cat# 52906), following the manufacturer's vacuum protocol instructions. First, samples were pulse-vortexed for 1 minute followed by brief centrifugation to remove liquid from the lid of sample containers. Sample lysis was initiated by pipetting 560µl of Buffer AVL into a 1.5ml microcentrifuge tube followed by 140µl of sample. Contents of the microcentrifuge tube were mixed by pulse-vortexing for 15 seconds followed by 10 minute room temperature incubation. A brief centrifugation step was utilized to remove droplets from the lid of the microcentrifuge tube. Next, 560µl ethanol (96-100%) was added to the microcentrifuge tube and mixed by pulse-vortexing for 15 seconds (lysate). Another brief centrifugation step was utilized to remove droplets from the lid of the microcentrifuge tube. A QIAamp Mini column was connected to the VacConnector on a vacuum manifold and 630µl of lysate was

pipetted into the mini column without wetting the rim or touching the column membrane. Vacuum was applied until all lysate was drawn through the membrane. Again following the same procedure, 630 µl of lysate was pipetted into the mini column and vacuum was applied. Then, 750µl of Buffer AW1 was added to the mini column without wetting the rim or touching the membrane followed by vacuum. Following the same procedure, 750µl of Buffer AW2 was added to the mini column followed by vacuum. Next the mini column was placed into a 2ml collection tube and centrifuged at 8000 rpm for 1 minute to dry the membrane. The mini column was then placed into a 1.5ml microcentrifuge tube and 60µl Buffer AVE was added, followed by a 1 minute room temperature incubation. The microcentrifuge tube was then placed in a centrifuge at 8000 rpm for 1 minute.

Short-term storage (< 7 days) of RNA extracts was maintained at 4-8°C and long-term storage (≥ 7 days) of RNA extracts was maintained at -70°C (+/- 5°C).

Real-time RT-PCR

Real-time RT-PCR is a molecular laboratory test that utilizes reverse transcription followed by amplification and detection of fluorescence to detect specific sequences of nucleic acid.

Reverse transcription (RT) is the first phase of real-time RT-PCR and is the process by which the enzyme reverse transcriptase synthesizes complementary DNA (cDNA) using viral RNA as the template. Once reverse transcription is complete, the amplification and detection phase of real-time RT-PCR begins.

Amplification utilizes many cycles of denaturation, targeted annealing, and DNA synthesis (Polymerase Chain Reaction). Denaturation separates double stranded nucleic acid into single strands. Annealing attaches the primers and the probe to targeted single stranded DNA sequences. The annealed primers initiate DNA synthesis which is catalyzed by the enzyme Taq polymerase. The probe is designed with a fluorescent reporter, 6-carboxyfluorescein (FAM) attached at the 5' terminus, and a quencher

molecule, Blackhole Quencher1 (BHQ1), attached at the 3' terminus. In an intact probe, the quencher molecule absorbs the fluorescence emission from the fluorescent reporter. During DNA synthesis, Taq polymerase cleaves the annealed probe releasing the fluorescent reporter from the proximity of the quencher resulting in an increase of fluorescence detection. Real-time RT-PCR continuously detects the increase in fluorescence during each PCR cycle (detected in real time).

The amplification cycle that produces fluorescence greater than the background noise is the Cycle threshold (Ct) of the reaction. Ct values are inversely proportional to the amount of targeted nucleic acid in the original sample; a lower Ct indicates a greater initial quantity of targeted nucleic acid in the sample, and a higher Ct indicates a lower initial quantity of targeted nucleic acid.

Real-time RT-PCR assays in this study were conducted on an AB7500 Fast real-time platform (Life Technologies, Carlsbad, CA) using an Invitrogen Superscript® III Platinum® One-Step qRT-PCR enzyme kit (cat# 11732-020) in a total reaction volume of 25µl. PCR set-up included 5µl of extracted sample, and 400nM final concentration each of primers and probes.

The primers and probes target the highly conserved RNA polymerase-capsid junction (ORF1-ORF2 junction) of the NoV genome (Katayama et al., 2002; Kageyama et al., 2003; Trujillo et al., 2006). GI PCR utilized NoVG1-F and NoVG1-R primers and a NoVG1-P (FAM-BHQ1) probe (Table 1) (Van Stelten et al., 2011). GII PCR utilized Cog 2F and Cog 2R primers and a Ring2 (FAM-BHQ1) probe (Table 1) (Kageyama et al., 2003). The cycling parameters were: 48°C for 45 min for reverse transcription, an initial denaturation at 95°C for 10 min, and then 40 cycles of amplification with denaturation at 95°C for 15 sec and anneal at 60°C for 60 sec.

Table 1: Primer and probe oligonucleotides used for real-time RT-PCR

Primer or Probe	**Targeted Annealing Strand**	**Sequence (5' - 3')**	**Position**
NoVGI-F	Antisense	GCY ATG TTC CGY TGG ATG C	5319[a]
NoVG1-R	Sense	GTC CTT AGA CGC CAT CAT CAT T	5415[a]
NoVG1-probe	Sense	*FAM*-TCG GGC ARG AGA TYG CGR TC-*BHQ1*	5386[a]
Cog2-F (GII)	Sense	CAR GAR BCN ATG TTY AGR TGG ATG AG	5003[b]
Cog2-R (GII)	Antisense	TCG ACG CCA TCT TCA TTC ACA	5100[b]
Ring 2 (GII probe)	Sense	*FAM*-TGG GAG GGC GAT CGC AAT CT-*BHQ1*	5048[b]

[a] Nucleotide positions based on full genome sequence (AB 187514) accessed from GenBank (Source:Van Stelten, A., Kreman, T. M., Hall, N., & DesJardin, L. E. (2011). Optimization of a real-time RT-PCR assay reveals an increase of genogroup I norovirus in the clinical setting. *Journal of Virological Methods*, 175, 80-84.)

[b] Nucleotide positions based on full genome sequence (AF 145896) accessed from GenBank (Source: Kageyama, T., Kojima, S., Shinohara, M., Uchida, K., Fukushi, S., Hoshino, F., et al. (2003). Broadly reactive and highly sensitive assay for Norwalk-like-viruses based on real-time quantitative revere transcription-PCR. *Journal of Clinical Microbiology*, *41* (4), 1548-1557.)

Collection of Food Establishment Environmental Samples

Four restaurants in one Iowa community agreed to participate in this study. The restaurants were not targeted for food safety concerns, but were chosen as they represent the wide variety of restaurants available to consumers.

Observations of each food establishment's process flow were utilized to determine suspect NoV sites; which included surfaces that were frequently touched by food establishment employees, by establishment patrons, or surfaces that are difficult to

clean. Twenty-seven candidate sites were identified and categorized as follows: equipment used by food establishment employees, handles touched by food establishment employees, and customer self-serve areas. Each site was sampled using three swab types: 1) dry flocked nasopharyngeal swab, 2) VTM moistened flocked nasopharyngeal swab, and 3) VTM moistened foam swab. Sampling was conducted in a bi-directional pattern over a 5 cm by 5 cm surface of each suspect site. All samples were stored in coolers with ice packs for up to 6 hours then held under refrigeration conditions (4-8°C) until sample processing.

Analysis of Environmental Samples

Environmental samples were manually extracted using a QIAamp® Viral RNA Minikit following the manufacturer's instructions as previously described. Real-time RT-PCR analysis was conducted using the adapted AB7500 Fast GI and GII assays. To test for potential PCR amplification inhibition, each extracted sample was also analyzed in replicate wells with 1µl positive GI and GII extract spiked into the replicate wells.

CHAPTER IV

RESULTS

GI and GII Assay Performance

GI and GII NoV assay performance was determined by analyzing serial dilutions and dilution curves for each assay. Both assays performed within the optimal efficiency range of 95%-105% (Table 2 and Figures 3-4). An amplification efficiency of 100% indicates true exponential amplification of targeted nucleic acid and yields a sensitive assay with a low limit of detection.

Table 2 – GI and GII NoV real-time RT-PCR assay performance

Real-time RT-PCR Assay	**Slope**	**R^2**	**Efficiency (%)**
GI NoV AB7500 Fast Invitrogen enzyme kit	3.28	0.999	102
GII NoV AB7500 Fast Invitrogen enzyme kit	3.39	0.999	97

Figure 3 – GI NoV assay performance

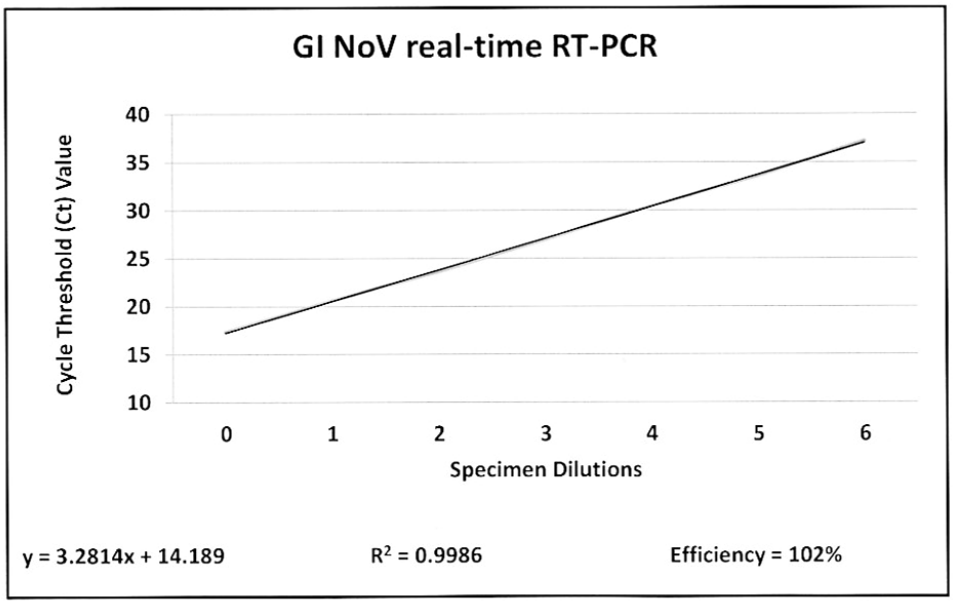
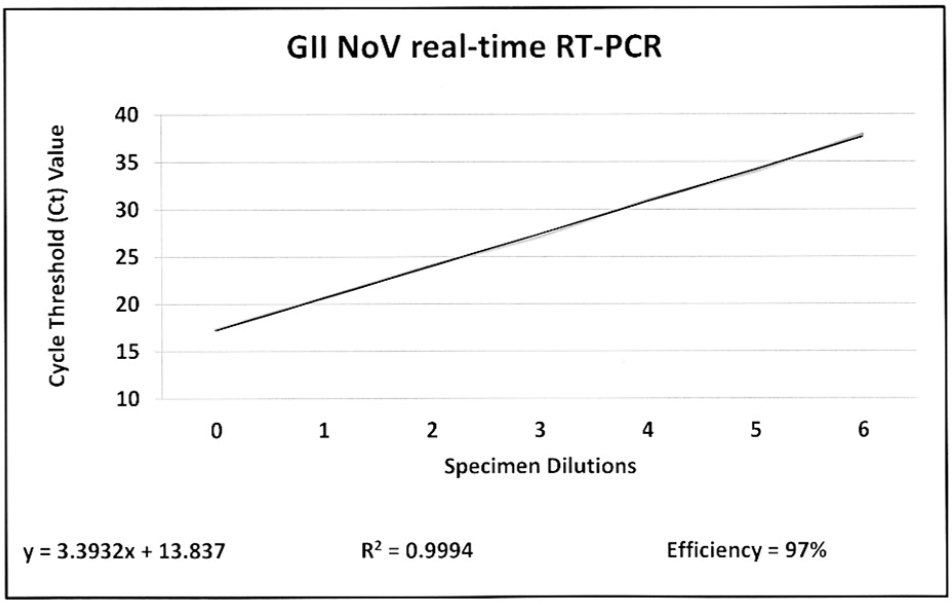


Figure 4 – GII NoV assay performance



Environmental Surface Spiking

NoV was recovered from each of the three laboratory spiked environmental surfaces using each of the three swab types tested (Tables 3-5). The VTM moistened foam swab was most effective in recovering NoV from spiked surfaces, as it had the lowest Ct differences and the lowest fold differences and the swab design was most appropriate for ease of use when sampling in the field.

A comparison of Ct values was utilized to determine NoV recovery. A Ct value is the fractional cycle number of PCR amplification in which detected fluorescence is greater than the background noise. The Ct values of positive controls inoculated with 140µl filtrate in a single aliquot and sampled by repeated pipetting were compared to the Ct values of samples inoculated with 140µl filtrate in 14 random aliquots and sampled using a bi-directional swabbing pattern. The smaller the difference between the positive control Ct and the surface sample Ct, the more effective the NoV recovery.

When comparing Ct values it is imperative that compared samples be of the same dilution factor. Moistened flocked nasopharyngeal swabs and moistened foam swabs were diluted 10-fold when placed in 1ml VTM. In order to compare swab performance, the dry flocked nasopharyngeal samples and the positive control samples must also be diluted 10-fold. All samples inoculated on smooth ceramic were experimentally controlled for the dilution factor. The dry flocked nasopharyngeal swab samples and the positive control samples inoculated on stainless steel and plastic were not experimentally diluted 10-fold. Therefore, the effect of a 10-fold dilution was estimated using the GII serial dilution curve where a 10-fold dilution resulted in an increase of 3 Ct. Experimental and estimated Ct values are labeled in Tables 3-5.

PCR amplification is exponential; therefore, a Ct difference of 1 is equal to a 2-fold difference in amount of targeted nucleic acid if the real-time RT-PCR assay is 100% efficient. The assays adapted in this study performed within the optimal efficiency range; therefore, the fold difference between compared samples was calculated assuming 100%

efficiency. The fold difference between compared samples was determined according to the following equation: fold difference = 2^n; where n = difference between Ct values. Fold difference results are listed in Tables 3-5.

Three surfaces (stainless steel, smooth ceramic, and plastic) were spiked with GII NoV clinical filtrate. These surfaces were chosen as they represent materials commonly encountered and utilized in food establishments. NoV was recovered from each spiked surface. The range of fold differences for each surface was compared; stainless steel had the smallest range (10-62), followed by smooth ceramic (2-6700) and then plastic (27-10514) (Tables 3-5).

Three types of swabs were tested to determine their ability to recover NoVs from environmental surfaces and for their suitability for use when swabbing environmental surfaces (Tables 3-5). Dry flocked nasopharyngeal swabs had Ct differences of 12.71 on smooth ceramic, 5.96 on stainless steel, and 13.36 on plastic with a fold difference range of 62–10514. VTM moistened nasopharyngeal swabs had Ct differences of 4.02 on smooth ceramic, 5.21 on stainless steel, and 4.87 on plastic with a fold difference range of 16-37. VTM moistened foam swabs had Ct differences of 1.19 on smooth ceramic, 3.28 on stainless steel, and 4.78 on plastic with a fold difference range of 2-27.

To evaluate swab recovery performance, Ct differences and fold differences were compared. The VTM moistened foam swab had the lowest Ct differences and the lowest fold difference range indicating a higher degree of NoV recovery. In addition, the firm plastic shaft of the foam swab allowed for precise swab placement and ease of sample manipulation.

Table 3 – Smooth ceramic environmental surface spiking results and swab performance

Swab Type	Test	Ct[a]	Ct Difference	Fold Difference
Flocked Nasopharyngeal	Control	22.03	12.71	6700
	Sample	34.74		
VTM Flocked Nasopharyngeal	Control	22.03	4.02	16
	Sample	26.05		
VTM Foam Swab	Control	22.03	1.19	2
	Sample	23.22		

[a] Ct values experimentally controlled for dilution effect.

Table 4 – Stainless steel environmental surface spiking results and swab performance

Swab Type	Test	Ct	Dilution Controlled Ct	Ct Difference	Fold Difference
Flocked Nasopharyngeal	Control	16.21[a]	19.21[b]	5.96	62
	Sample	22.17[a]	25.17[b]		
VTM Flocked Nasopharyngeal	Control	16.21[a]	19.21[b]	5.21	37
	Sample	24.42[a]	24.42[a]		
VTM Foam Swab	Control	19.74[a]	22.74[b]	3.28	10
	Sample	26.02[a]	26.02[a]		

[a] Experimental Ct value.

[b] Estimated Ct value corrected for 10-fold dilution effect.

Table 5 – Plastic environmental surface spiking results and swab performance

Swab Type	Test	Ct	Dilution Controlled Ct	Ct Difference	Fold Difference
Flocked Nasopharyngeal	Control	15.74 [a]	18.74 [b]	13.36	10514
	Sample	29.10 [a]	32.10 [b]		
VTM Flocked Nasopharyngeal	Control	15.74 [a]	18.74 [b]	4.87	29
	Sample	23.61 [a]	23.61		
VTM Foam Swab	Control	15.74 [a]	18.74 [b]	4.78	27
	Sample	23.52 [a]	23.52		

[a] Experimental Ct value.

[b] Estimated Ct value corrected for 10-fold dilution effect.

Food Establishment Environmental Samples

Environmental samples were collected from four food establishments. A total of 77 samples were collected and categorized by collection site; equipment (n=22), handles contacted by food establishment employees (n=27), and customer self-service (n=28) (Table 6). The specific locations of the sample sites are listed in table 6.

NoV was not detected in the samples collected in the food establishments (Table 7). To rule out PCR inhibition due to potential sample contaminates such as food particles, dust, and debris, each sample was also analyzed in replicate wells with the addition of 1µl positive NoV GI and GII nucleic acid extracts. NoV was successfully amplified and detected in each of the spiked environmental samples (Table 8).

Table 6: Environmental samples collected in food establishments (n = 77)

Customer Self-Serve **n = 28 (36%)**	**Handle (employee only)** **n = 27 (35%)**	**Equipment (employee only)** **n = 22 (29%)**
Debit card reader	Dishwasher	Stainless steel prep table
Lottery machine	Trash can	Ice machine
Display case handle	Walk-in cooler	Hand wash sink
Ice cream machine	Telephone	Stainless steel mixer
Restrooms	Roaster	Cash register
Cooler	Ice cooler	Spigot of drink dispenser
Condiment containers	Raw meats cooler	
Service counter		
Hand wash station		

Table 7: NoV results of food establishment environmental samples

Site Description	**Number of Samples (n = 77)**	**GI NoV**	**GII NoV**
Customer Self-Serve	28 (36%)	Not Detected	Not Detected
Handle (employee only)	27 (35%)	Not Detected	Not Detected
Equipment (employee only)	22 (29%)	Not Detected	Not Detected

Table 8: NoV results of food establishment environmental samples with NoV spikes

Site Description	**Number of Samples (n = 77)**	**GI NoV**	**GII NoV**
Customer Self-Serve	28 (36%)	Detected	Detected
Handle (employee only)	27 (35%)	Detected	Detected
Equipment (employee only)	22 (29%)	Detected	Detected

CHAPTER V

DISCUSSION

Norovirus Outbreak Investigation

The Iowa Department of Public Health Foodborne Outbreak Investigation Manual specifies the collection of food and stool specimen, but does not specify the collection of environmental surface and fomite samples. The collection and testing of environmental samples could provide data to aid public health professionals in identifying the source of prolonged NoV outbreaks and their routes of transmission. However, there is not a standardized laboratory method for collecting environmental samples and testing them for NoVs.

This research provides a method for collecting environmental samples and using real-time RT-PCR to test the samples for NoVs. The use of VTM moistened foam swabs was shown to recover NoV from three types of surfaces contaminated in the laboratory. The collection and processing of environmental samples may provide epidemiological data to guide prolonged NoV outbreak investigations.

Environmental sampling for NoVs is applicable in public health interventions. Environmental sampling could be utilized to verify decontamination efforts after known NoV contamination. Such verification could reduce the continued transmission of NoVs in high risk and or semi-confined populations. Public and private institutions such as schools, hospitals, nursing homes, restaurants, recreational water parks, cruise-ship companies and airlines could re-open with increased confidence.

Data from NoV environmental samples could be submitted to CaliciNet, the CDC's NoV outbreak surveillance network. CaliciNet was established in 2009 as a tool to link NoV outbreaks caused by common sources, to recognize common routes of NoV transmission, and to identify emerging strains of NoVs (MMWR, 2011; Vega et al., 2011). The inclusion of environmental sample data could provide additional information

on the epidemiology of NoVs. This would assist epidemiologists in linking the strain of NoV found in the patient to that found in the environment; therefore, establishing a likely exposure event.

GI and GII Assay

This research provides real-time RT-PCR assays for the detection of GI and GII NoVs in environmental samples. The assays are highly efficient; GI assay was 102% efficient and GII assay was 97% efficient.

Environmental Sampling

This study developed environmental sampling procedures that demonstrated the ability to recover NoV from stainless steel, smooth ceramic, and plastic surfaces spiked in a controlled laboratory setting. When comparing Ct differences and fold differences, it was found that more NoV was recovered from stainless steel surfaces and less NoV was recovered from plastic surfaces. The smoother surfaces of stainless steel and ceramic may have allowed for more virus recovery than the more textured plastic surface. The grooves of the textured surface may have reduced the thoroughness of swab contact with the surface which in turn may have reduced virus recovery.

On each surface, three swab types were tested. It was determined that VTM moistened swabs recovered more NoV than dry swabs. When comparing VTM moistened swabs, the foam swab was more effective in NoV recovery as it had the lowest Ct differences and the lowest fold difference range. The foam swab is designed with a larger sampling surface area than the nasopharyngeal swab. The larger sampling surface area combined with a firm plastic shaft allows for precise swab manipulation which may have resulted in the recovery of more NoV.

Environmental sampling was conducted on surfaces in four food establishments. Although NoV was not detected in the environmental samples, the sampling process confirmed the ease of use of the VTM moistened foam swab in an uncontrolled setting.

To test for potential PCR inhibition, laboratory spiked samples and environmental samples were tested in replicate wells with clinical GI and GII spikes. PCR inhibition was not detected in the laboratory spiked samples or in the environmental samples collected from surfaces in the food establishments. This provides support for the method; that if NoV had been present on the sampled environmental surfaces above the detection limits of the assays, the target RNA should have been detected.

Study Limitations

NoVs are non-cultivable and highly genetic and antigenically variable; therefore, detection of NoV RNA by real-time RT-PCR is the gold standard NoV detection method (Kageyama et al., 2003; Trujillo et al., 2006; Park et al., 2008). However, it is important to note that laboratory detection of NoV RNA by real-time RT-PCR does not equal detection of infectious virions, as NoVs must have an intact viral capsid to maintain infectivity. Non-encapsidated viral RNA is rapidly degraded by environmental RNase enzymes, and once degraded is no longer detectable using real-time RT-PCR (D'Souza et al., 2006; Duizer et al., 2004; Girard et al., 2010; Gassilloud et al., 2003), however it is still possible to detect a non-encapsidated viral RNA prior to it being degraded by environmental RNase enzymes (D'Souza et al, 2006). This means that real-time RT-PCR could potentially detect non-infectious NoV.

The collection of environmental samples in four food establishments was not coordinated with a NoV outbreak investigation. It is possible that NoV was not present at detectable levels in the community at the time of sampling and may have impacted the lack of NoV detection in the environmental samples. It is also possible that the test method is not sensitive enough to detect NoV in an uncontrolled field setting. Additional environmental testing conducted in coordination with an active NoV outbreak investigation could provide data to evaluate the epidemiological benefits of environmental sampling.

To determine which swab type was most effective in recovery of NoV, laboratory spiked environmental surfaces were allowed to air dry for two hours before sampling. The two hour air dry period was utilized to determine swab performance, not to establish the longevity of NoV stability. To further evaluate swab performance, additional environmental samples and laboratory spiked samples could also be analyzed for total bacterial plate counts. The addition of total bacterial plate count data could further determine the ability of swabs to collect organisms from environmental surfaces and to release organisms during sample preparation.

GII stool filtrates from two clinical specimens were utilized to spike environmental surfaces. The swab performance and virus recovery detected in this study could be attributed to the specific GII viruses tested. Further spiking experiments with multiple GI and GII clinical specimens would provide additional data on the environmental recovery of clinically significant NoVs.

Swab recovery of GII from spiked environmental surfaces was not 100% as determined by the Ct differences and the fold differences listed in Tables 3-5. Low virus recovery could be attributed to the stability of NoV on clean non-porous surfaces. It is possible that NoV has a shorter period of stability on a clean non-porous surface as compared to unclean or porous surfaces. Additional environmental surface spiking experiments should be conducted on various surfaces, such as porous and nonporous, and in various states of cleanliness. In addition, the samples should be collected in a time series to better depict the environmental stability of NoVs.

The sampling of the GII spiked stainless steel and plastic surfaces was not experimentally controlled for sample dilution; therefore, the Ct effect of sample dilution was estimated using the GII serial dilution curve. Although estimating the Ct effect of sample dilution is not ideal, the GII assay was 97% efficient, which increases the confidence of estimated Ct values. This confidence is demonstrated by the results obtained from the GII positive stool filtrate used to spike the smooth ceramic samples. A

sample of this filtrate was experimentally controlled for sample dilution and was compared to a sample that was not controlled for sample dilution. The experimental Ct value of 20.65 was comparable to the estimated Ct value of 20.66. To eliminate estimated effects of sample dilution, all future NoV real-time RT-PCR tests should be experimentally controlled for sample dilution.

Conclusions and Recommendations

This study shows that VTM moistened foam swabs can be used to recover NoV from environmental surfaces contaminated in controlled laboratory conditions. It may also be possible that VTM moistened foam swabs could recover NoV from environmental surfaces or fomites contaminated in an uncontrolled setting such as a food establishment. Testing environmental samples using real-time RT-PCR for detection of GI and GII NoVs could aid in outbreak and decontamination investigations.

The analysis of environmental samples could provide additional data to aid public health professionals in identifying sources of prolonged NoV outbreaks and their routes of transmission. The increase in NoV epidemiological data could better guide public health interventions and ultimately decrease illness attributed to NoV.

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