Capsid properties contributing to Norovirus persistence in humans

Jennifer Lauren Cannon

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the School of Public Health (Department of Environmental Sciences and Engineering).

Chapel Hill
2008

Approved by:

Jan Vinjé, Ph.D. (research advisor)

Mark Sobsey, Ph.D.

Ralph Baric, Ph.D.

Lee-Ann Jaykus, Ph.D.

Louise Ball, Ph.D.

Otto Simmons III, Ph.D.

ABSTRACT

Jennifer Cannon: Capsid properties contributing to Norovirus persistence in humans
(Under the direction of Jan Vinjé)

Human noroviruses (NoVs) are the leading cause of acute gastroenteritis in adults and contribute to an estimated 30-50% of all foodborne illnesses in the United States. They are listed on the US EPA's drinking water contaminant list and have been detected in environmental waters. No routine cell culture or small animal model is yet available, so there are limited options for testing the efficacy of prevention and control strategies. Recently, several areas of research have provided us with new tools for addressing important questions about the fate of NoV outside the human host. In this study, we examined the role of unique properties of the viral capsid protein involved in histo-blood group antigen (HBGA) receptor binding and the stability of the virus in the environment to better understand NoV persistence in human populations. Using a novel murine norovirus surrogate, we determined that NoVs are likely stable at extremes of pH, after treatment with organic solvents, and after incubation at ambient and refrigerated temperatures, but are inactivated at high temperatures. Exploiting human NoV binding to HBGA receptors, we demonstrated that potentially infectious virus can be concentrated and recovered from environmental waters. Finally, using a surrogate neutralization assay, we compared differences in the ability of human NoV outbreak convalescent patient sera collected over a 21 year period to block NoV virus-like particle (VLP) binding to HBGAs. Our results suggest that outbreak patient sera supports antigenic drift of genogroup II, genocluster 4

NoVs in the face of herd immunity. Taken together, the results of this study will enable researchers with novel tools for evaluating the efficacy of prevention and control strategies for human NoVs.

ACKNOWLEDGEMENTS

Without my family and friends who supported me and believed in me, none of these words could have been written. To my parents, Shirley, Dan and Aleta, my grandparents, Catherine and Richard and Isabel and Archie, my brother Hambone, and my Sanders, Will, I want to express my sincere gratitude. I also thank the members of my committee, especially my advisor, Jan for your scientific and personal guidance throughout my time at UNC. And, to my labmates and friends at UNC and the CDC, thank you for your professional support and most of all, thanks for all the laughs.

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3-D	three dimensional
AGE	acute gastroenteritis
ANOVA	analysis of variance
AP	alkaline phosphatase
ATCC	American Type Culture Collection
BoNoV	Bovine norovirus
BT50	50% antibody blockade titer
CaCV	Canine calicivirus
CDC	Centers for Disease Control and Prevention
CMS	carbohydrate magnetic separation
CPE	cytopathic effect
CRFK	Crandell Reese Feline Kidney
Ct	cycle-threshold
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
DNase	deoxyribonucleic
D-value	decimal reduction time
EPA	Environmental Protection Agency
FBS	fetal bovine serum
FCS	fetal calf serum
FCV	feline calicivirus
FUT	fucosyltransferase

GI	genogroup I
GII	genogroup II
GuSCN	guanidinium thiocyanate
HAV	Hepatitis A virus
HBGA	histo-blood group antigen
HCl	hydrogen chloride
HOCl	hypochlorous acid
HTST	high temperature, short time
HuNoV	human norovirus
IEM	immune electron microscopy
Ig	immunoglobulin
IMS	immuno-magnetic separation
kb	kilobase
kD	kilodalton
Le	Lewis
LTLT	low temperature, long time
mAB	monoclonal antibody
MEM	minimum essential medium
mLT	mutant heat-labile toxin
mM	millimolar
MNV	murine norovirus
MPa	Mega Pascal
NaCl	Sodium chloride

NaOH	Sodium hydroxide
NoV	norovirus
ORF	open reading frame
P1	protruding subdomain 1
P2	protruding subdomain 2
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEC	Porcine enteric calicivirus
PEG	polyethylene glycol
PFU	plaque forming unit
pNPP	para nitrophenyl phosphate
ppm	parts per million
PV	poliovirus
RdRp	RNA dependent RNA polymerase
RNA	ribonucleic acid
RNase	ribonuclease
RTE	ready-to-eat
RT-PCR	reverse transcriptase polymerase chain reaction
rVLP	recombinant virus-like particle
S	shell domain
SaV	Sapovirus
Se	secretor phenotype

SRSV	small round structured viruses
SwNoV	Swine norovirus
T_h	T-helper cell
UV	ultra-violet
VEE	Venezuelan equine encephalitis
VLP	virus-like particle
VP	viral protein
VRP	VEE replicon particles

CHAPTER 1

INTRODUCTION

Human noroviruses (NoVs) are the leading cause of outbreaks as well as sporadic cases of acute gastroenteritis (AGE) worldwide, causing an estimated 30-50% of all foodborne illnesses in the United States (Mead et al, 1999; Widdowson et al, 2005a). The most common transmission routes are person-to-person and foodborne but exposure to contaminated water has also been reported, bringing about their listing on the US EPA's drinking water contaminant list. NoVs exhibit extreme genetic diversity. They are categorized into 5 genotypes (I-V), including 3 (I, II, and IV) that infect humans. They are further divided into genotypes or genoclusters based on amino acid sequence identity of the major capsid protein. NoVs of genogroup II, genotype 4 (GII.4) are the most common cause of AGE globally. During the past 13 years, GII.4 NoVs have been associated with several epidemic seasons of AGE during which new GII.4 variants emerged and displaced the resident GII.4 viruses (Lopman et al, 2004a; Noel et al, 1999; Siebenga et al, 2007). Other NoV genotypes have also persisted in the population but with relatively few structural changes over two decades. Some of these genotypes are thought to be more stable in environmental waters than other genotypes.

Since no routine cell culture or small animal model is yet available, efforts to design effective prevention and control strategies have been hampered. Our understanding of the environmental stability and the fate of infectious NoV after disinfection is based on

laboratory experiments using distantly related surrogate viruses such as the respiratory virus, feline calicivirus. Since molecular methods used for NoV detection cannot distinguish between infectious and noninfectious virus, we cannot be certain whether a positive RT-PCR signal also indicates the presence of virus that is infectious. Furthermore, the current knowledge about basic NoV virology and the correlates of immune protection have primarily come from a handful of human challenge studies, *in vitro* assays using recombinant virus-like particles (VLPs) or laboratory experiments done with surrogate viruses (Daughenbaugh et al, 2006; Dolin et al, 1972; Harrington et al, 2002; Huang et al, 2005; Lindesmith et al, 2003; Lindesmith et al, 2005; Parrino et al, 1977; Souza et al, 2007; Tacket et al, 2003; Wobus et al, 2006). The mechanisms of NoV persistence in human populations are not clearly understood so interventions have been limited.

Recently, several important developments in NoV research have opened new avenues for developing novel prevention and control strategies**.** First**,** a novel murine NoV (MNV) was recently discovered that can be propagated in cell culture (Karst et al, 2003) and quantified by plaque assay (Wobus et al, 2004). The fact that MNV is genetically closely related to human NoVs, an enteric pathogen (Wobus et al, 2006) and is transmitted via the fecal-oral route likely makes this virus a reliable surrogate to measure NoV infectivity (or loss there of). Second, histo-blood group antigens (HBGAs) were recently identified as markers of genetic susceptibility and resistance for Norwalk virus (the prototype GI.1 NoV) infection (Hutson et al, 2002, Lindesmith et al, 2003). These molecules are thought to be receptors for NoVs as individuals who do not display certain HBGAs on their intestinal mucosa or other secretions (known as "non-secretors"), have been shown to be resistant to infection (Hutson et al, 2002, Lindesmith et al, 2003). About 80% of the European and North American

populations are “secretors” (Marionnau et al, 2001). Recent epidemiological studies indicate that there is an association between HBGA expression and genetic susceptibility to NoV strains of genogroup II, genotype 4 (GII.4), although, the association is not as strong as it has been with Norwalk virus (Larsson et al, 2006; Thorven et al, 2005). Furthermore, NoVs or their VLPs can specifically bind to immobilized, synthetic HBGAs (Harrington et al, 2002; Harrington et al, 2004; Huang et al, 2003; Lindesmith et al, 2003), making receptor-binding assays potentially a valuable tool for studying NoV infectivity. Finally, our group recently reported herd immunity in the human population as a driving force for antigenic drift, and a possible mechanism for GII.4 NoV persistence in the population (Lindesmith et al, 2008). With an improved understanding of immune protection in the human population, we will be better able to design VLP based vaccines for preventing NoV disease.

With the aforementioned advances in the field of NoV virology as a back-drop, our studies will address NoV persistence in the human population. We hypothesize that such persistence depends on unique properties of the viral capsid protein involved in HBGA receptor binding and also on stability of the virus in the environment. Because there is no convenient infectivity assay for human NoVs, we validate and employ surrogates to specifically address the following research questions:

1. To what degree does NoV remain infectious after physical treatments that target the integrity of the capsid? To determine this, we used the novel, cultivable MNV as a surrogate for human NoV infectivity. We examined MNV stability at extremes in pH, at high temperatures, upon organic solvent extraction, and after incubation at ambient and refrigerated temperatures under wet and dry conditions. Further, we

compared the stability of MNV to the classically used surrogate infectivity model for human NoVs, the feline calicivirus (FCV).

2. Can successful binding to HBGA receptors followed by detection of viral RNA be used to detect potentially infectious NoV particles found in environmental waters? To further explore NoV stability in the environment, we designed a proof-of-concept model that exploited NoV binding to HBGA in an assay for concentrating virus from environmental waters. Because the viruses we detect bind to their receptors (HBGAs), which is presumably necessary for NoV infection, we propose this assay may be a surrogate for detection of potentially infectious NoV.
3. Do differences in the ability of NoV outbreak sera to block GII.4 VLP binding to HBGA support the hypothesis for the mechanism of GII.4 NoV evolution? Using a large panel of human convalescent sera pairs collected from norovirus outbreaks over 21 years, between 1985 and 2006, we studied differences in HBGA blockade patterns of historic and contemporary GII.4 strains. In addition we examined the role of heterotypic genogroup II NoV strains in herd immunity to GII.4 NoV infection.

Our studies begin to address NoV persistence in humans by validating and applying three different surrogates, better enabling the assessment of NoV stability in the environment and in human populations. Regulatory authorities adopting prevention and control strategies for human NoVs will benefit from our studies, as we address nove alternative methods for estimating NoV inactivation, the infectivity of environmental viruses, and novel approaches needed for predicting which NoV strains to include in a VLP vaccine.

CHAPTER 2

LITERATURE REVIEW

Discovery of Norwalk virus

Acute gastroenteritis (AGE) or winter vomiting disease was first described as early as 1935 (Rischel et al, 1935). During the 1940-1950s, several studies demonstrated the transfer of this gastroenteric disease to healthy volunteers by oral administration of a bacteria-free stool filtrate (Gordon et al, 1955; Jordan et al, 1953; Kojima et al, 1948, Reimann et al, 1945). Because the infectious agent was transmissible after filtration, a viral etiology was suspected. However, when mammalian tissue culture became available to grow viruses in the 1960s, no virus could be detected using standard tissue or organ cell lines (Blacklow et al, 1972). In 1972, Albert Kapikian employed immune electron microscopy (IEM) to examine stool samples from ill adult volunteers who had been administered bacteria-free filtrates derived from a rectal swab of an individual involved in a gastroenteritis outbreak at an elementary school in Norwalk, Ohio (Adler et al, 1969; Kapikian et al, 1972). These volunteers demonstrated gastrointestinal disease (Dolin et al, 1971 and 1972) and small round viruses with a diameter of 27-32 nm were visible by IEM, which in subsequent serological studies were confirmed as the etiologic agent of the original Norwalk outbreak (Kapikian et al, 1972 and 1973).

Over the two decades following the discovery of Norwalk virus, viruses similar to Norwalk virus in morphology and clinical manifestations, but yet antigenically distinct, were discovered (Dolin et al, 1982; Kapikian et al, 1972; Thornhill et al, 1977) and provisionally

classified as “small round structured viruses” (SRSV) (Caul et al, 1982). Analysis of partially purified virus by polyacrylamide gel electrophoresis (PAGE) revealed that the protein composition of Norwalk virus was most similar to viruses belonging to the family *Caliciviridae* (Greenberg et al, 1981), named for their characteristic cup-like depressions on the surface of the virions (from the Latin word “calyx”, meaning cup or goblet).

Molecular Era

In the early 1990s, the Norwalk virus genome, as well as the Southampton virus genome, were cloned and sequenced, establishing these viruses as members of the family *Caliciviridae* (Jiang et al, 1990; Jiang et al, 1993; Lambden et al, 1993). Following these hallmark reports, complete or partial genome sequences of other SRSV reference strains such as Snow Mountain virus (Wang et al, 1994), Hawaii virus (Lew et al, 1994a), Toronto virus (Lew et al, 1994c), Desert Shield virus (Lew et al, 1994b), and Lordsdale virus (Dingle et al, 1995) allowed classification of these viruses into a new genus termed “Norwalk-like viruses” (NLVs) and more recently called *Norovirus* (NoV) (Green (Fields), 2007). A virus with a classical calicivirus morphology was associated with gastroenteritis in an infant home in Sapporo, Japan, in 1977 (Chiba et al, 1979). This virus was found to be antigenically different from Norwalk virus by serologic assays. Cloning and sequencing of this and related viruses from the UK (Liu et al, 1995) confirmed that these viruses cluster into a separate genus called “Sapporo-like viruses” (SLVs), now designated *Sapovirus* (SaV) (Green (Fields), 2007). Although viruses from both NoV and SaV genera are able to cause acute gastroenteritis in humans, NoVs are morphologically as well as genetically distinct from sapoviruses. In addition, SaV infections are more common in children under 5 years of age

and are rarely the cause of outbreaks in adults, whereas NoVs are associated with outbreaks of gastroenteritis in people of all ages (Green (Fields), 2007). Other genera belonging to the *Caliciviridae* family include *Vesivirus* and *Lagovirus*, which do not infect humans, but infect a wide range of animals including cats, dogs, swine, cows, whales, and rabbits (Green (Fields), 2007). Recently, caliciviruses genetically different from all other genera have been reported, and have provisionally been named *Nabovirus* or *Becovirus* (Oliver et al, 2006; Smiley et al, 2002).

Human Noroviruses

Noroviruses are 27-32 nm non-enveloped viruses that contain a single stranded, positive sense RNA genome surrounded by an icosahedral capsid. The genome is 7.5-7.7 kb in length and contains three open-reading frames (ORFs). ORF1 (nucleotides 146-5359) encodes a polyprotein of six nonstructural proteins including p48, NTPase, p22, VPg (binds to 5' end to initiate translation), $3CL^{pro}$ (protease), RdRp (RNA dependent RNA polymerase). ORF 2 encodes the major structural protein (VP1) of approximately 60 kD that folds into an S (shell) and a P (protruding) domain that is further divided into P1 and P2, P2 being the most hypervariable region of the genome and responsible for HBGA receptor binding (Chen et al, 2004). ORF3 (nucleotides 6938-7573) encodes a minor structural protein (VP2) with an unknown function but in vitro studies have shown that this gene regulates the expression and stability of VP1 (Bertolotti-Ciarlet et al, 2003).

Noroviruses are classified into five genogroups (GI-V) (Figure 2.1) based on a >60% amino acid identity in VP1 (Green et al, 2001; Vinjé et al, 2000; Zheng et al, 2006). Human

disease has been associated with GI, GII and GIV. Each genogroup can be further divided into genoclusters or genotypes based on >80% identity in amino acid sequence of

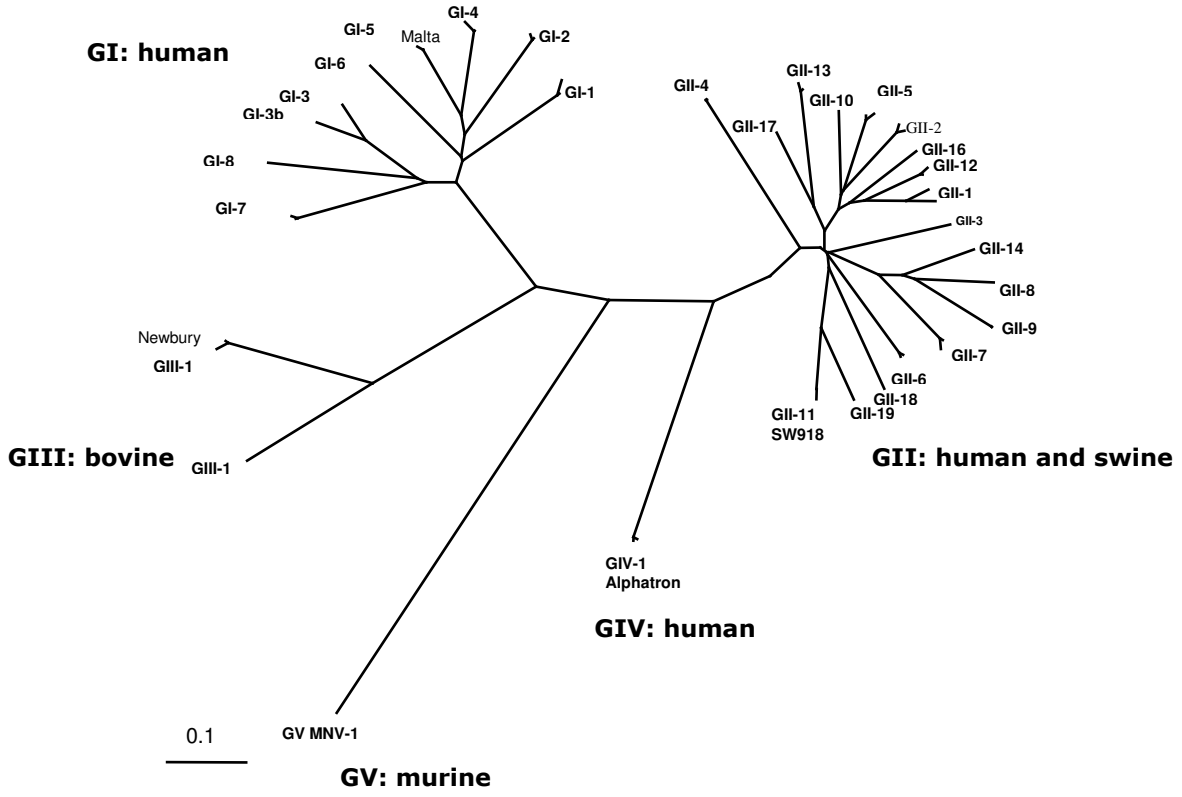


Figure 2.1: Phylogenetic relationships of noroviruses (adapted from Zheng et al, 2006). NoVs are classified into five genogroups (GI-GV) which can be further divided into genotypes (e.g., GII.4 represents genogroup II, genotype 4 viruses). Viruses in genogroups I and IV have been found uniquely in humans; genogroup II viruses cause disease in both humans and swine and genogroup V consist of the newly discovered murine norovirus.

VP1 (Green et al, 2001; Vinjé et al, 2000). Genotypes can be further divided into strains based on specific amino acid changes in the P2 domain of VP1 (Lindesmith et al, 2008). GI NoV currently contains eight genotypes (1-8) and genogroup II (GII) contains 19 genotypes

(1-19) (Wang et al, 2005: Zheng et al, 2006) although more genotypes exist based on partial capsid sequences (Kageyama et al, 2004). NoVs of genetic cluster GII.4 have been predominant in outbreaks throughout the world, whereas GIV viruses are very rare and only a few outbreaks in humans have been reported.

NoV infection normally causes a self-limiting disease; however, illness in the very young and elderly can be quite severe, leading to hospitalization and sometimes to death (Green, 2002; Lopman et al, 2003a; MMWR, 2007; Sakai et al, 2001; Zintz et al, 2005). Until recently, deaths due to NoVs were thought to be rare, however recently, fatality rates of 1.5 to 2 % have been reported during outbreaks at elderly care facilities in Israel and Japan (Calderon-Margalit et al, 2005; Okada et al, 2006). The symptoms of NoV disease include diarrhea, vomiting, nausea, abdominal cramps, fever, fatigue and general malaise. Onset usually occurs between 12 and 24 hrs after exposure and may last from 12 to 60 hrs during which the virus is shed in vomitus and feces. Recent data shows that NoV illness can last longer than previously recognized (Lopman et al, 2004a; Rockx et al, 2002) and the virus can be detected in feces up to 1-2 weeks after the symptoms have subsided (Okhuysen et al, 1995; Parashar et al, 1998; Patterson et al, 1993; Rockx et al, 2002). In human challenge studies, 18-32% of persons challenged with Norwalk virus or Snow Mountain virus developed an asymptomatic infection (Graham et al, 1994; Hutson et al, 2002; Lindesmith et al, 2003; Lindesmith et al, 2005). Although in one study the incidence of asymptomatic viral shedding in the community is low (5.2%) (de Wit et al, 2001), studies in day-care and hospital settings demonstrated that asymptomatic shedding is a more common occurrence than previously estimated (range 20-33%), especially among children (Akihara et al, 2005; Gallimore et al, 2004; Garcia et al, 2006). Virus shedding by otherwise healthy food

handlers has most likely initiated several NoV outbreaks and presents a significant public health risk (Lo et al, 1994; Patterson et al, 1993).

Early human challenge studies using the NoV prototype strain, Norwalk virus (GI.1), showed that some individuals were resistant to infection, and genetic susceptibility was suggested (Parrino et al, 1977). Recently, it has been shown that human HBGAs are able to bind to NoVs and that successful Norwalk virus infection of humans is dependent on displaying the appropriate HBGA receptor(s) on cells of the gut mucosa (Hutson et al, 2002; Lindesmith et al, 2003). Approximately 80% of the European and North American population express HBGAs on their gastrointestinal and oral mucosa and are known as "secretors", while the remaining ~20% of the population do not display HBGAs on their gastric mucosa ("non-secretors") (Marionneau et al, 2001) and appear to be completely resistant to Norwalk virus infection. Recent research showed that several, but not all, NoVs and their VLPs bind to synthetic HBGAs and binding can be blocked by hyperimmune sera or saliva from secretors by not by saliva from non-secretors (Harrington et al, 2002; Harrington et al, 2004; Huang et al, 2003; Hutson et al, 2003; Lindesmith et al, 2003). Furthermore, it was shown that dimerization of the NoV P2 domains of the VP1 protein forms a specific receptor binding pocket for HBGAs that can be disrupted by changes in amino acid sequences in this region (Tan et al, 2003; Tan et al, 2004). Co-crystallization of the NoV P domain with HBGA was recently described by Cao et al (2007), providing concrete evidence of specific interactions of receptor molecules in the P2 subdomain of the capsid.

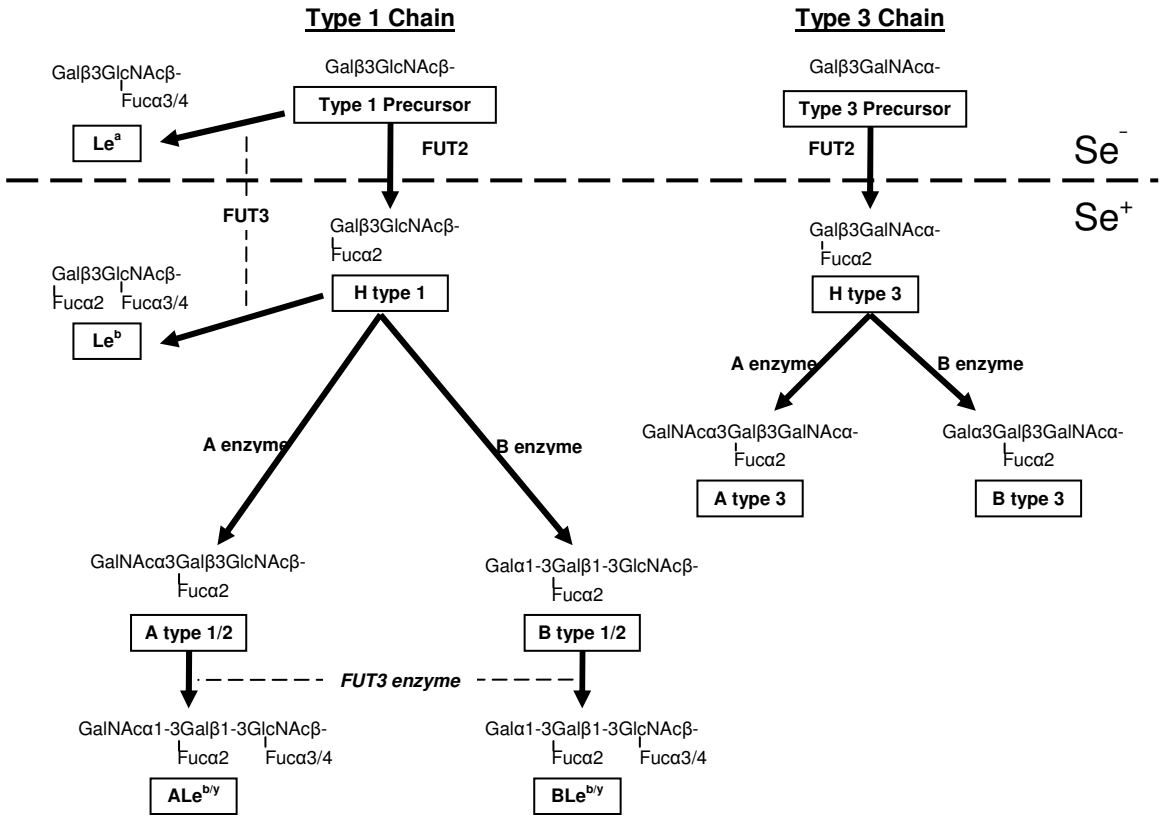


Figure 2.2: Biosynthetic pathway of HBGAs on the H type precursor chains commonly found on gastrointestinal mucosa. The dotted line between Se- and Se+ distinguishes those carbohydrates present on mucosal surfaces of nonsecretors and secretors, respectively. FUT2 represents α1,2 fucosyltransferase and FUT3 represent α1,3 (or α1,4) fucosyltransferase (adapted from Harrington et al, 2002).

Histo-blood group antigens are terminal polysaccharides of glycoproteins or glycolipids and include H type, the ABO blood group, and Lewis carbohydrates. Biosynthesis of HBGAs proceeds by the addition of single sugar molecules to H type precursor molecules by α1,2 fucosyltransferase enzymes (FUT2) to create H type chains, α1,4 (or α1,3) fucosyltransferase enzymes (FUT3) to produce the carbohydrates that determine Lewis type, and A or B enzymes to produce blood group HBGAs (Figure 2.2) (Ravn and Dabelsteen, 2000). The genes encoding these enzymes are polymorphic,

producing either a functional or a nonfunctional enzyme. In the gastric mucosa, where type 1, 2 and 3 HBGA chains predominate (Ravn and Dabelsteen, 2000), a functional FUT2 enzyme (*fut2* gene product) is responsible for the secretor status of an individual, while a functional FUT3 enzyme (*fut3* gene product) is responsible for the Lewis phenotype, and A and B enzymes for A and B phenotypes, respectively. As demonstrated in the cascade diagram in Figure 2.2, an individual designated a "nonsecretor" will only express H type precursor chains and, depending on their Lewis phenotype, Le^a and Le^x carbohydrates, whereas "secretors" have the ability to add more carbohydrates to the cascade.

Transmission

NoVs as well as other enteric viruses are transmitted directly from person to person by the fecal-oral route or indirectly through contaminated food, water or environmental surfaces (Figure 2.3). As many as 1 billion NoV particles can be shed in 1 g of stool (Chan et al, 2006) and direct or indirect contact with emesis or aerosolized droplets presents another important route of NoV transmission. Since the infectious dose of NoV is very low (between 10 to 100 virus particles, Teunis et al, 2008), hands or surfaces that appear clean can still harbor infectious material, contributing to virus spread. Contamination of food can occur anywhere along the food to fork continuum that involves human contact, or indirectly through fecal contamination of waters that come in contact with foods. Since the capsids of enteric viruses have properties that promote survival for long periods of time under harsh conditions such as desiccation, freezing and extremes in pH, they are well adapted to survival in and on foods (Abad et al, 1994; Abad et al, 2001; D'Souza et al, 2006; Green (Fields), 2007; Hollinger and Emerson 2007; Mbithi et al, 1991).

Transmission by food handlers

Despite major improvements in hygiene during the last century, foodborne diseases remain an important public health problem. A recent estimate suggests that foodborne diseases are responsible for about 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths in the U. S. each year (Mead et al, 1999). Of these, an estimated 14 million illnesses, 60,000 hospitalizations and 1,800 deaths are associated with foodborne transmission of known bacteria, viruses, fungi and parasites, whereas the etiologic agent(s) for most cases remains unknown. Among the known pathogens, human noroviruses account for an estimated 2/3 of all disease caused by foodborne pathogens (Mead et al, 1999). Other foodborne viruses including hepatitis A virus (HAV), sapovirus, rotavirus and astrovirus, and these account for only ~0.6% of all cases. Primarily due to more widely available molecular assays, human NoVs have emerged as the most common pathogen of foodborne outbreaks in the developed world (Inouye et al, 2000; Lopman, et al, 2003b; Widdowson, et al, 2005a; Wright et al, 1998).

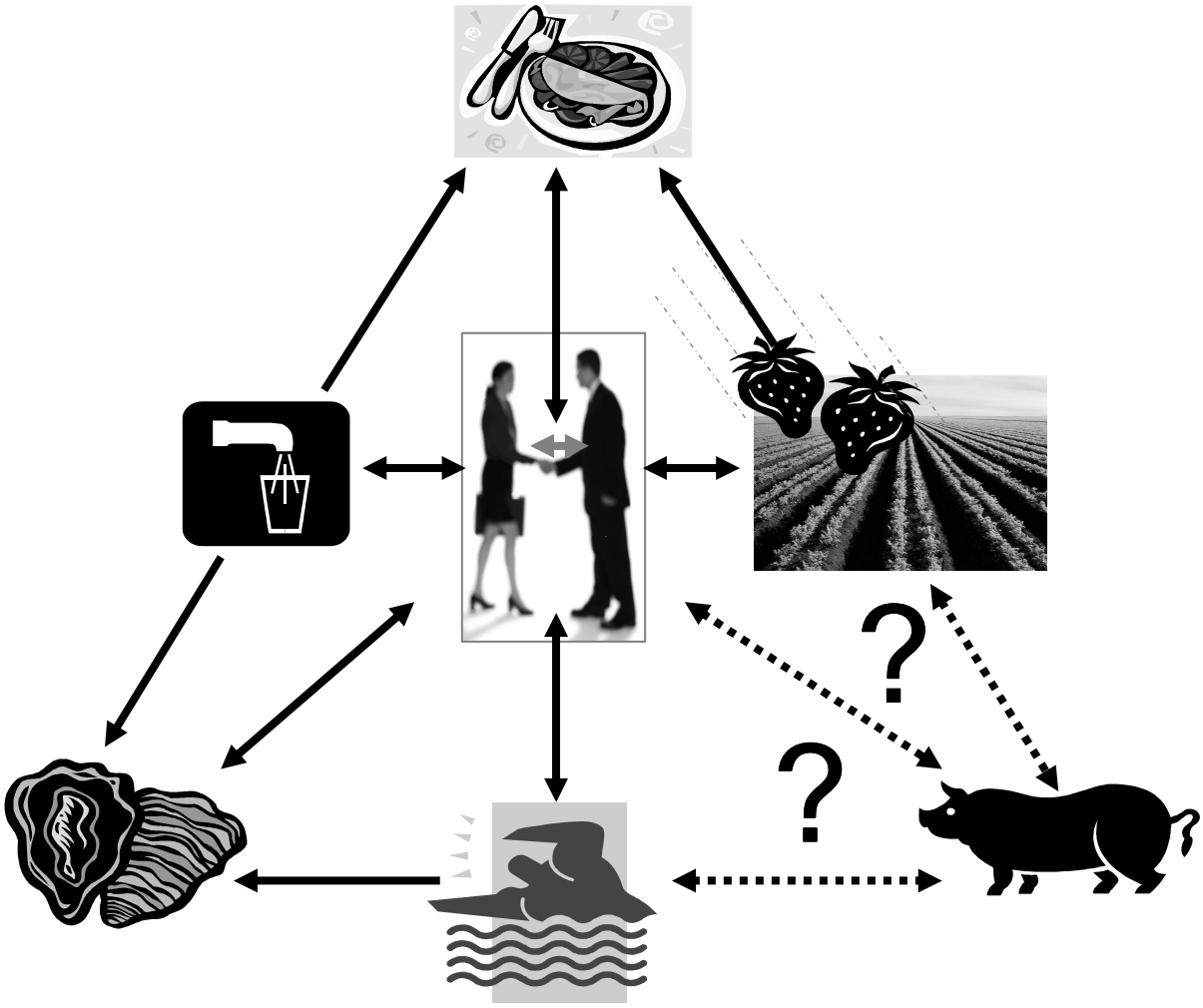


Figure 2.3: Different routes of transmission of norovirus including person-to-person transmission, contamination of foods by fecal matter or vomitus can instigate transmission (pictured at the top of the pyramid and moving clockwise) by prepared or RTE foods, minimally processed foods or irrigation waters, possible zoonotic transmission (not confirmed), recreational waters, shellfish, or drinking water.

The majority of foodborne outbreaks of NoV gastroenteritis are a result of contamination after food handling. The food items that most commonly have been implicated are "ready to eat" (RTE) foods such as salads, bakery items, deli meats and cheeses, fruits and vegetables or other "cold bar" items that do not require cooking before

consumption. Large scale NoV and HAV foodborne outbreaks caused by RTE foods that were contaminated by a food handler during preparation have been documented (Anderson et al, 2001; Daniels et al, 2000; Hirakata et al, 2005; Patterson et al, 1997; MMWR, 2001; MMWR, 2006a). Transmission by this route presents a particular problem because NoV shedding can occur before the onset of clinical symptoms, and can last from less than a day to several weeks, even after all NoV symptoms have subsided (MMWR, 2001; Okhuysen et al, 1995). Reports of NoV outbreaks caused by food contamination from "healthy" foodhandlers have been documented (Lo et al, 1994; Patterson et al, 1993), which has led to recommendations that persons experiencing gastrointestinal illness should not report back to work for at least 24 to 48 hrs after symptoms have subsided (http://www.cdc.gov/ncidod/dvrd/revb/gastro/norovirus-foodhandlers.htm). NoV contamination of foods by persons during picking or packaging of produce items has led to several international foodborne outbreaks (Falkenhorst et al, 2005; Ponka et al, 1999).

Transmission by shellfish/oysters

The consumption of raw or undercooked molluscan shellfish such as oysters, cockles and clams has notoriously been linked to NoV and outbreaks at the national and even international levels (Le Guyader, et al, 2006; Webby et al, 2007). Filter-feeding molluscan shellfish are capable of concentrating viruses from the surrounding waters that have been contaminated with human feces, sewage or vomit and then retain these viruses in their digestive diverticula. Oyster depuration and relaying involves moving harvested oysters sequentially to clean water to allow microbes to be flushed from the digestive tissue of the oysters. Although this process is effective for the removal of some bacteria (such as *E. coli*),

elimination of NoVs by depuration is likely to be less effective because Norwalk virus particles have been shown to bind to carbohydrate structures with a terminal N-acetylgalactosamine on the digestive ducts of oysters (Le Guyader et al, 2006).

Transmission via water

Viral contamination of source water can occur by infiltration of untreated or inadequately treated water or sewage. Water can also be contaminated by contact with stool, vomit (including aerosols), soiled hands, soiled clothing or contaminated surfaces. If this water is then used for drinking, recreation, washing and preparing food, making ice, or irrigating produce, human infection can occur. Several outbreaks have been reported associated with contamination of drinking water, recreational water, and ice (Hoebe et al, 2004; Khan, et al, 1994; Maunula et al, 2004). Contamination of shellfish harvesting waters can also lead to foodborne outbreaks of NoV (Berg et al, 2000; Croci et al, 2007; McDonnell et al, 1997; Shieh et al, 2003), and raspberries contaminated by either irrigation waters or produce wash waters may have been the cause of several international norovirus outbreaks (Ponka et al, 1999).

Zoonotic transmission

Of the NoVs detected in animals, swine NoV is genetically, the closest relative to human NoVs. While recombinant strains have been reported within the bovine, swine, and human NoV genotypes (Oliver et al, 2004; Vinjé et al, 2000; Wang et al, 2005), there is currently little evidence of interspecies recombinants or transmission of NoV from animals to humans. However, clear evidence of replication and diarrhea was recently reported after

challenge with human GII.4 strain in gnotobiotic pigs (Cheetham et al, 2006), and GII.4 VLPs did bind to HBGAs in buccal and duodenal tissues of gnotobiotic pigs expressing A or H antigens (Cheetham et al, 2007; Tian et al, 2007). In another study, antibodies to bovine NoV have been detected in veterinarians and in the general public (Widdowson et al, 2005b), and human NoV specific antibodies have been detected in pigs (Farkas et al, 2005), although more research will be needed to determine if this is true evidence of zoonotic transmission. On the other hand, human and animal NoV strains co-concentrated in oysters were recently reported (Costantini et al, 2006), which may provide another means of proximity of human and animal strains needed for interspecies recombination to occur. Therefore, ingestion and host replication of human and animal strains could potentially be an indirect means for zoonotic transmission by recombination.

Environmental stability

The number of foodborne illnesses caused by enteric bacteria has generally declined over the past decade (MMWR, 2006b). Possible reasons for this trend include better public awareness of safe cooking and hygienic foodhandling practices, improved standards in industrial food processing and better refrigeration and storage (Widdowson et al, 2005c). By contrast, the number of foodborne outbreaks caused by NoVs has remained unchanged or has increased and is now estimated at 30-50% of all foodborne outbreaks (MMWR, 2006b Widdowson et al, 2005a). It is therefore likely that control measures in the food industry and public health awareness campaigns aimed at reducing foodborne bacterial illness have done little to reduce NoV gastroenteritis.

Enteric virus survival on surfaces is largely dependent on the porosity of the surface, the presence of fecal material (or organic matter), temperature and the relative humidity of the environment (Abad et al, 1994; Abad et al, 2001; D'Souza et al, 2006; Mbithi et al, 1991) and no single set of conditions is ideal for promoting the survival of all enteric viruses. For example, human rotavirus, human astrovirus and HAV are much less affected by desiccation and the absence of fecal material than are enteric adenoviruses and poliovirus (PV-1) (Abad et al, 1994; Abad et al, 2001).

Acidification of food, which is done to reduce bacterial growth or spore germination during food processing (Sofos and Busta, 1999), may be inadequate for control of enteric viruses. For example, cell culture adapted HAV can remain infectious after exposure at pH 1 for 5 hr and enteroviruses retain stability at pH 3 (Hollinger and Emerson, 2007). Norwalk virus has been demonstrated to cause infection in human volunteers after incubation for 3 hours at pH 2.7 (Dolin et al, 1972) and resistance of NoV to acidic pH was seen in a large NoV outbreak for which orange juice (pH ~3.5) was implicated as the vehicle of infection (Fleet et al, 2000).

Molecular epidemiology

As noted above, human NoVs are a group of genetically diverse viruses that can be classified into multiple genogroups (GI, GII and GIV of importance to humans) and into at least 25 genotypes (Zheng et al, 2006). In most outbreak studies, GII viruses are more common than GI viruses and GIV viruses are rare (Blanton et al, 2006; Hamano et al, 2005). GII viruses are commonly associated with outbreaks in nursing homes, hospitals and cruise ships where person to person transmission is the typical route of transmission. Amidst

extreme diversity in circulating NoV strains, GII.4 viruses (genogroup II; genotype 4) viruses have been responsible for the majority of reported outbreaks over the past 20 years. Increased activity of GII.4 NoV was first observed worldwide in 1995 with the "95/96" GII.4 strain (Noel et al, 1999; Vinjé et al, 1997). Increased GII.4 activity was reported in the US and off-season activity in Europe in 2002, when the 95/96 strain was replaced by two new variant GII.4 strains (Lopman et al, 2004a; Widdowson et al, 2004). In 2004, a new strain emerged causing outbreaks in Australia, Europe and Asia (Bull et al, 2006; Kroneman et al, 2006; Phan et al, 2006) and in 2006, epidemics of two new GII.4 variants were reported worldwide (MMWR, 2007; Phan et al, 2007; Tu et al, 2007; Varhoef et al, 2008). Recent data from our group and others suggests that subtle changes in the HBGA binding region (P2 domain) of the major capsid protein which are driven by antigenic drift in response to herd immunity in the population, maintain GII.4s in the population year after year (Allen et al, 2008; Lindesmith et al, 2008; Siebenga et al 2007). However, in other regions of the world, particularly in underdeveloped countries or tropical, rural communities, GI viruses are more frequently detected than GII NoVs (Chapin et al, 2005; Garcia et al, 2006), raising the question about whether viruses from different genogroups have different survival characteristics. However, as of yet, no particular NoV genotype has been solely associated with foodborne transmission, nor has a particular food item been associated with a single NoV genotype (Blanton et al, 2006). Outbreaks following consumption of contaminated raw shellfish have often revealed the involvement of multiple NoVs genotypes, as contamination is often a direct result of raw sewage contamination of oyster beds (Hamano et al, 2005: Pommepuy et al, 2004).

Detection of viruses in foods and the environment

Foods and environmental waters are complex matrices that may be contaminated with low concentrations of NoVs. Because the minimum infectious dose of NoV, based on Norwalk virus human challenge study data, is very low (10-100 virus particles (Teunis et al, 2008)), sample volumes that approach an average serving size of food (25-100 g) or 1-100 L of water must be tested to accurately measure the exposure risk. Virus and viral RNA concentration methods often result in co-concentration of inhibitors which might lead to false-negative results in the downstream molecular detection methods. Furthermore, all techniques for the detection of NoVs are ultimately based on the detection of viral RNA using molecular methods such as RT-PCR, which cannot determine infectivity.

Successful procedures to concentrate enteric viruses from foods generally include methods such as adsorption-elution, concentration-elution, precipitation or ultrafiltration. Adsorption-elution and concentration-elution methods have demonstrated success in the detection of enteric viruses in foods, especially shellfish (Jaykus, 1996). A secondary concentration step such as polyethylene glycol (PEG) precipitation or immunocapture is often preferred preceding molecular detection, particularly when low levels of contamination are suspected. Alternatively, direct extraction of viral nucleic acids using a guanidinium thiocyanate-based lysis buffer followed by concentration with silica-based beads (Boom et al, 1990) or columns (such as a Qiagen viral RNA mini kit) can be performed. During sample processing, additional purification is often also necessary to remove RT-PCR inhibitors. Organic solvent extraction with chloroform, Freon or an environmentally friendly Freon alternative, Vertrel is often performed when extracting enteric viruses from environmental, food, and water samples (Chung et al, 1996; Hewitt and Greening, 2004; Jaykus et al, 1996;

Legeay et al, 2000; Mullendore et al, 2001). For a comprehensive review of all of these methods for detection of viruses in foods, see Papafragkou et al, 2006.

Prevention and Control

No specific methods exist for the prevention from NoV illness. The CDC currently recommends several measures to prevent virus transmission within healthcare facilities, on cruise ships and for foodhandlers. These measures include avoiding direct contact with feces or vomitus, soiled clothing and bedding or other surfaces contaminated by an ill person. If available, appropriate disposable personal protection equipment such as gloves, lab coats or gowns and surgical masks should be worn for cleaning heavily contaminated areas. Persons on cruise ships should frequently wash their hands (especially after using the restroom, changing a diaper, touching doorknobs or handrails, returning to one's cabin or helping a sick person and before eating, smoking, or brushing one's teeth) with warm water and a generous amount of soap for at least 20 seconds. Food items that may have come in contact with the ill person should be discarded and clothing or linens should be washed with hot water. Frequent handwashing with a mild soap and/or agitation is recommended based on studies using bacteriophage (MS2) as an indicator for the behavior of nonenveloped viruses, as there is currently little evidence that alcohol-based hand sanitizers are effective for non-enveloped viruses (Sickbert-Bennett et al, 2005). In healthcare institutions, containment of NoV outbreaks is dramatically improved by confinement of outbreak patients and restricting admissions to wards where an outbreak is occurring (Lopman et al, 2004b). Similarly, food handlers or preparers are recommended to take 24-48 hr furlough after resolution of symptoms (FDA Foodcode). Since the virus can be transmitted asymptomatically and shed

in feces for up to 2 to 3 weeks after disease onset (Murata et al, 2007), a strict handwashing regimen should be enforced for all employees handling food. Washing raw fruits and vegetables and avoiding the use of water that may have incurred sewage contamination are measures to reduce NoV transmission during food preparation. Since oysters and other shellfish can be contaminated on occasion, consumption of raw or undercooked molluscan shellfish is not recommended. Special care should be taken when preparing shellfish for cooking to avoid indirect contamination of other foods or cooking surfaces.

Surrogates for human NoV infectivity

To date, all attempts to propagate human NoV in routine laboratory cell culture or primary tissue cultures have been unsuccessful (Duizer et al, 2004a). However, a recent report using a 3-D cell culture vessel demonstrated evidence of passage of both GI and GII NoV in vitro (Straub et al, 2007). While this method could prove an exciting new avenue for research, it awaits confirmation by subsequent studies. Human NoVs have been reported to replicate in non-human primates and gnotobiotic pigs (Cheetham et al, 2006; Rockx et al, 2005; Subekti et al, 2002), but no rodent model is currently available. For this reason, most information on persistence of infectious virus after disinfection, stability in the environment, and basic virology questions of replication and immune correlates of protection has come from human challenge studies and studies using model or surrogate viruses. The most frequently used surrogates for NoV infectivity include viruses from other genera within the family *Caliciviridae,* such as feline calicivirus (FCV) and canine calicivirus (CaCV), both belonging to the genus *Vesivirus*, and a cell culture adapted strain of porcine enteric calicivirus (PEC) belonging to the genus *Sapovirus*. Similarities in capsid structure, genomic

material and genomic organization and the ability of these viruses to grow readily in cell culture have made these good surrogates. FCV has been the most frequently used surrogate model for disinfection and survival studies of human NoVs (Doultree et al, 1999; Duizer et al, 2004b; Nuanualsuwan, et al, 2002; Thurston-Enriquez, et al. 2003a; Thurston-Enriquez et al, 2003b; Tree et al, 2005). However, the use of FCV has been criticized because FCV is a respiratory virus and cannot survive at low pH, a necessary characteristic of enteric viruses that must survive passage through the stomach (Duizer et al, 2004b; Kahn and Gillespie 1970). Recently, MNV was discovered in immunodeficient laboratory mice (Karst et al, 2003; Wobus et al, 2004) and this virus can be grown routinely in cell culture (Wobus et al, 2004). Since MNV is an enteric pathogen of mice that is shed at high levels in the feces and can be spread by the fecal-oral route (Karst et al, 2003; Wobus et al, 2006), this virus may be a more appropriate surrogate to measure the effectiveness of sanitizers against human NoV.

Disinfection of surfaces

The CDC recommends the use of 1,000 ppm chlorine bleach (1 part household bleach to 50 parts water) for cleaning of nonporous surfaces that may have come in contact with an ill person or 5,000 ppm chlorine bleach for disinfection of a visibly soiled nonporous surfaces. Other surface disinfectants approved by the US Environmental Protection Agency (EPA) are mainly used as hospital or commercial disinfectants and have manufacturer data to support their efficacy, in most cases based on data using the FCV surrogate. Hypochlorous acid (HOCl) solution (Sterilox® Technologies, Radnor, PA), which can be used as a liquid or fog, is a promising disinfectant which effectively reduced infectivity of MNV by at least 3 $\log_{10}$ (Park et al, 2007). Similarly, ozone gas provided at least a 3 $\log_{10}$ reduction of the

infectivity of FCV, as well as RNA copy number, on contaminated surfaces (Hudson et al, 2007). High pressure inactivation of norovirus can be achieved at <400 MPa based on MNV data even in the context of oyster tissues (Kingsley et al, 2007). But, unlike MNV, human NoVs are capable of binding to internal ligands of oyster tissues (Le Guyader et al, 2006b; Tian et al, 2007) making it possible for oysters to accumulate human NoVs. Therefore, it remains to be seen if high pressure processing of oysters can safely replace cooking.

Infection control

To date, no specific treatment for prevention of human NoV infection and illness exists and in severe cases, dehydration is treated by electrolyte replacement and bed rest, as no specific antivirals are available.

Vaccines

In addition, to being the leading cause of AGE in adults in the developed world, increasing reports of NoV causing severe diarrhea in children of developing countries have recently been described (Long et al, 2007; Monica et al, 2007; Nakagomi et al, 2008), indicating NoVs may be an important cause of diarrhea in developing countries. While the illness is normally self-limiting, reports of severe illnesses and deaths among children and the elderly have been reported more frequently in recent years (MMWR, 2007; Okada et al, 2006; Zintz et al, 2005). Such populations might be ideal targets for a vaccination campaign aimed at reducing or eliminating severe morbidity and mortality caused by human NoVs. Other target populations for vaccination might include persons that routinely are engaging in

interpersonal contact, such as medical personnel in hospitals or nursing homes, food handlers, military personnel and perhaps passengers and crew on cruise ships.

Vaccine development for human NoV has been complicated due to the lack of cell culture or animal model. Nonetheless, several studies have indicated that humoral, mucosal and cellular (Th1/Th2-like) immune responses can be elicited following inoculation of human NoV VLPs in mice (Ball et al, 1998; Guerrero et al, 2001; LoBue et al, 2006; Nicollier-Jamot et al, 2004; Periwal et al, 2003) or upon feeding with transgenic tobacco, tomato or potato plants (Mason et al, 1996; Zhang et al, 2006) or yeast extracts (Xia et al, 2007) that express NoV VLP proteins. Binding of homotypic VLPs to their HBGA ligand can be blocked using convalescent mouse antisera (LoBue et al, 2006; Xia et al, 2007) in a surrogate neutralization assay. Furthermore, a multivalent human NoV VLP inoculated into mice produced antisera capable of heterotypic HBGA blockade of a human NoV VLP genotype that was not included in the inoculum (LoBue et al, 2006). Combined, these results suggest that a human vaccine using multivalent VLPs may be possible. However, oral administration of a VLP vaccine, or alternatively, transgenic potato consumption in humans during phase I clinical trials, elicited humoral, mucosal and cellular responses inferior to native virus challenge, suggesting that an adjuvant and/or boosters may be needed (Ball et al, 1999; Tacket et al, 2000; Tacket et al, 2003). In mice, adjuvant can enhance the immune response to NoV VLPs (Periwal et al, 2003) and trials with a bovine VLP vaccine including a mutant heat-labile toxin (mLT) adjuvant was at least partially protective against bovine NoV challenge in gnotobiotic calves (Han et al, 2006). Currently a Norwalk virus VLP vaccine developed by LigoCyte (Bozeman, MT) is undergoing phase I clinical trials. Because we now have evidence of GII.4 NoV antigenic drift, we are faced with the additional

consideration of changing the vaccine every 1-2 years, much like what is done for the influenza vaccine (Lindesmith et al, 2008).

Conclusions

Noroviruses are now recognized as the most prevalent pathogen of foodborne illness, contributing to more outbreaks than all other known bacterial, viral and protozoan pathogens combined. The number and scope of NoV outbreaks in semi-closed communities, such as cruise ships, schools and other institutional settings and instigated by person to person contact exceed those occurring because of the foodborne route. Since only a few studies have been conducted to ascertain the prevalence of NoV in sporadic AGE, the contribution of this virus to in the overall burden of AGE is currently unknown.

All current recommendations and guidelines for the prevention and control of NoV disease are based on the combined data from molecular epidemiological studies and disinfection and survival studies using surrogate viruses such as FCV which has been criticized for its relevancy as a surrogate for human NoV infectivity. The studies described herein will address this issue by introducing novel surrogates that may better predict the infectivity of human NoVs. Efforts to design antivirals or vaccines against norovirus have been impeded by the lack of a cell culture or animal model for human NoVs. Furthermore, only a handful of human challenge studies have been performed over the last 30 years and can be used to depict the host immune response and viral pathogenesis. Recently, our group has described GII.4 NoV strains are evolving by herd immunity in the population causing a antigenically distinct GII.4 NoV variant strain to emerge about every two years (Lindesmith et al, 2008). Therefore, a vaccine for GII.4 NoVs will have to be updated regularly in order to retain its efficacy. Here, we describe how the use of acute and convalescent sera collected

from NoV outbreak patients can be used to predict NoV strains to be included in a vaccine. Collecting of sera during routine outbreak surveillance will be invaluable for studying NoV epidemiology and immunology that will help us understand NoV persistence in human populations and direct us toward a future with the possibility to reduce the disease burden of human NoVs.

CHAPTER 3

Surrogates for the study of norovirus stability and inactivation in the environment: A comparison of murine norovirus and feline calicivirus

Jennifer L Cannon[1], Efstathia Papafragkou[3], Geunwoo Park[1], Jason Osborne[2] Lee-Ann Jaykus[3], and Jan Vinjé [1*]

[1] Department of Environmental Science and Engineering, University of North Carolina, Chapel Hill, NC
[2] Department of Statistics, NCSU, Raleigh, NC
[3] Department of Food Science, NCSU, Raleigh, NC

This chapter has been previously published: Journal of Food protection 69(11):2761-5.

ABSTRACT

Human noroviruses (NoVs) are the leading cause of food and waterborne outbreaks of acute nonbacterial gastroenteritis worldwide. Due to the lack of a mammalian cell culture model for these viruses, studies on persistence, inactivation and transmission have been limited to cultivable viruses including feline calicivirus (FCV). Recently, reports of the successful cell culture of murine norovirus (MNV) has provided investigators with an alternative surrogate for human NoVs. In this study we compared the inactivation profiles of MNV to FCV in an effort to establish the relevance of MNV as a surrogate virus. Specifically, we evaluated (i) stability upon exposure to pH extremes; (ii) stability upon exposure to organic solvents; (iii) thermal inactivation; and (iv) surface persistence under wet and dry conditions. MNV was stable across the entire pH range tested (2-10) with less than 1 log reduction in infectivity at pH 2, whereas FCV was inactivated rapidly at pH values <3 and >9. FCV was more stable than MNV at 56°C but both viruses exhibited similar inactivation at 63°C and 72°C. Long-term persistence of both viruses suspended in a fecal matrix and inoculated onto stainless steel coupons were similar at 4°C but at room temperature in solution MNV was more stable than FCV. The genetic relatedness of MNV to human NoVs combined with its ability to survive under gastric pH levels makes this virus a promising and perhaps more relevant surrogate than FCV for studying environmental survival of human NoVs.

INTRODUCTION

Human noroviruses (NoVs), members of the family *Caliciviridae*, are now recognized as the leading cause of outbreaks of acute nonbacterial gastroenteritis (Blanton, 2006; Green (Fields), 2007). Epidemiological investigation of outbreaks has shown that the most important modes of transmission are person-to-person contact and consumption of contaminated food (Green (Fields), 2007). The so-called ready-to-eat (RTE) foods are most often associated with NoV outbreaks and include items such as salad bars, deli meats, fresh produce, raw and undercooked molluscan shellfish, and baked desserts (Anderson et al, 2001; Berg et al, 2000; Ponka et al, 1999). NoV outbreaks have also occurred in highly acidic foods such as orange juice (pH = 3.3 – 4.19) and frozen raspberries (pH = 3.18 - 3.26) (Ponka et al, 1999). Recent estimates indicate that as much as 50% of all foodborne disease outbreaks in the U.S. would be shown to be caused by NoVs if specimens from all infected individuals were screened (Widdowson et al, 2005a).

To date, human NoV cannot be grown in cell culture which not only hampers the study of the basic virology of the NoVs, but also hinders studies on the environmental persistence of these viruses and the efficacy of various control measures such as disinfection and cooking. Such studies are important because NoVs have a low infectious dose (10 to 100 virus particles, Teunis et al, 2008), meaning that only a few infectious virus particles can cause infection. Also surfaces, serving dishes or containers, utensils, and food handled by ill persons who are not practicing adequate personal hygiene before preparing food may contribute to illness. Since feline calicivirus (FCV), from the genus vesivirus, can be propagated in cell culture, it has been studied extensively as a surrogate for human NoVs in environmental survival and inactivation studies (D'Souza et al, 1006; Duizer et al, 2004b).

However, FCV is transmitted by the respiratory route and is inactivated at a relatively low pH and hence may not predict human NoV environmental stability or inactivation.

Recently, the first NoV to be propagated in cell culture was reported (Wobus et al, 2004). This virus, designated murine norovirus (MNV), is an enteric pathogen that is shed in mouse feces and is commonly transmitted by the fecal-oral route (Karst et al, 2003). In the present study, we compared inactivation profiles of MNV to FCV in an effort to examine the relevance of MNV as a surrogate for human NoVs. Specifically, we evaluated (i) stability upon exposure to pH extremes; (ii) stability upon exposure to organic solvents; (iii) thermal inactivation; and (iv) surface persistence under wet and dry conditions.

MATERIALS AND METHODS

Viruses, cells, and assays

Macrophage derived, RAW 264.7 cells (ATCC# TIB-71), were cultured in complete minimum essential medium (MEM) containing 10% low endotoxin fetal bovine serum (FBS) (HyClone, Logan, UT). Murine norovirus (MNV), strain P3 (a kind gift of Dr. Skip Virgin, Washington University School of Medicine, St. Louis, MO) was cultured by infecting 80-90% confluent monolayers of RAW 264.7 cells in complete MEM containing 3% low endotoxin FBS. Feline calicivirus (FCV), strain F9 (ATCC# VR-782) was propagated in Crandell Reese Feline Kidney (CRFK) cells (gift of Dr. James Guy, North Carolina State University) using complete Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal calf serum (FCS) (Gibco-Invitrogen). Both viruses were harvested after complete cytopathic effect (CPE) was apparent by repeated cycles of freeze-thaw, followed by centrifugation and storage at -80°C until use. In an effort to mimic the natural state of enteric viruses as they might be found in the environment, we chose not to monodisperse or otherwise further purify the virus stocks.

To determine the infectious titer of MNV and FCV, standard plaque assay techniques were employed as previously reported (Bidawid et al, 2003). Briefly, cells (RAW 264.7 for MNV and CRFK for FCV) were dispensed in 60 mm diameter cell culture plates at a density of $2x10^6$ cells per plate and grown to 80-90% confluence in 5 ml of complete MEM at 37°C. Cell monolayers were infected with ten-fold serial dilutions of the virus for 1 h at 37°C and, after removal of the inoculum, cells were overlaid with 5 ml of overlay medium containing 0.5% agarose and incubated for 48 h. Plaques were subsequently counted 5-8 h after a second agarose overlay (3 ml) including 0.75% neutral red solution (Sigma-Aldrich) was

added. Plates with 5 to 50 plaques were used to determine the virus titer in plaque forming units (PFU).

Virus inactivation studies

pH stability

The pH stability of MNV and FCV was determined by mixing 10 µl of ten-fold serial dilutions of virus solution with 90 µl of each buffer (pH 2, 3, 4, 5 (100 mM citrate buffer), 6, 7, 8, (100 mM phosphate buffer) 9 and 10 (100 mM carbonate buffer) and incubating at 37°C for 30 min and 2 h. The pH was subsequently adjusted to 7 by the addition of 400 µl of complete MEM supplemented with 3% FBS and the drop wise addition of 0.5 M NaOH or 0.5 M HCl.

Organic solvent extraction

Three different organic solvents [chloroform (Fisher Scientific, Atlanta, GA), trichlorofluoromethane (Freon, Fisher Scientific), and Vertrel XF (DuPont, DE)] were tested. A 500 µl volume of organic solvent was mixed with an equal volume of virus stock (1×10^6 PFU to 2.3×10^8 PFU), mixed by vortexing for 1 min, and centrifuged for 5 min at 2000 x g to separate the aqueous from the organic phase. The aqueous phase was recovered, serially diluted in PBS, and immediately assayed.

Thermal inactivation

The capillary tube method of Fairchild and Foegeding (1993) was used for the thermal inactivation experiments. Briefly, capillary tubes (Kimble Products, Vineland, New

Jersey) were filled with 50 µl of each virus stock (approximately 5 x 10^5 to 1 x 10^6 PFU), heat sealed, submerged in a waterbath, and held at various temperatures (56°C, 63°C, and 72°C) for times ranging from 5 sec (at 72°C) to 20 sec (at 56°C). At each time point, three capillary tubes were removed and cooled on ice. Each tube surface was then sanitized by immersion in 10% sodium hypochlorite followed by 70% ethanol, crushed, serially diluted in PBS, and assayed by plaque assay. Consistent with published literature (Russell, 1999), virus inactivation was calculated as the decimal reduction time (D-value) which is defined as the time needed to achieve a one log reduction in infectious virus titer at a given temperature.

Stability on environmental surfaces

Virus stocks were mixed with reconstituted artificial feces (Feclone, SiliClone Laboratories, Valley Forge, PA) and aliquots of 50 µl (representing a total of about 5x10^5 PFU of MNV or FCV) were placed on the center of individual sterilized stainless steel coupons (5 x 5 cm). At various time-points (1, 2, 3, 4, 5, 6, and 7 d), viruses were eluted from the coupon surfaces by repeated pipetting (25 times) of the contaminated area with 450 µl of 10 mM glycine-150 mM NaCl buffer, pH 7.0. To assess the effect of desiccation on virus survival, spiked clarified fecal suspensions (20%) were also evaluated. All experiments were done at both 4^{o}C (54% relative humidity) as well as room temperature (22 ± 2^{o}C) (75-85% relative humidity).

Statistical Considerations

All virus inactivation experiments were carried out in triplicate. For thermal inactivation, linear regressions were analyzed in Microsoft Excel (Redmond, WA) and D-

values were plotted as the negative reciprocal of the slope of the regression line. Analysis of variance (ANOVA) (SAS 9.1, SAS Institute, Cary, NC) was used to identify significant differences in virus recovery between treatments and/or viruses.

RESULTS

pH stability

At low pH values of 2, 3 and 4, MNV infectivity was reduced by less than one log (0.6, 0.6 and 0.5, respectively), while the FCV titer was reduced by 2 to 4 log (4.4, 3.7, and 2.3, respectively) (Figure 3.1A). At higher pH values (5-9) the MNV titer dropped less than one log whereas the FCV titer dropped by approximately 2 log. At pH 10, the MNV titer fell by 1.8 log, whereas the infectivity of FCV was reduced by 5.1 log. At all pH values tested, the differences in log reductions between MNV and FCV were statistically significant ($p < 0.05$). Increasing the incubation time from 30 min to 2 h did not result in statistically significant changes in virus stability.

Organic solvent stability

To compare the sensitivity of MNV and FCV to inactivation using organic solvents, aliquots of virus were extracted with chloroform, Freon and Vertrel and assayed for infectivity. Both viruses were relatively resistant to all three organic solvents with recoveries ranging from 95-104% for MNV and 85-91% for FCV (Figure 3.1B). Regardless which organic solvent was used, the recovery of MNV was better than that for FCV ($p < 0.05$).

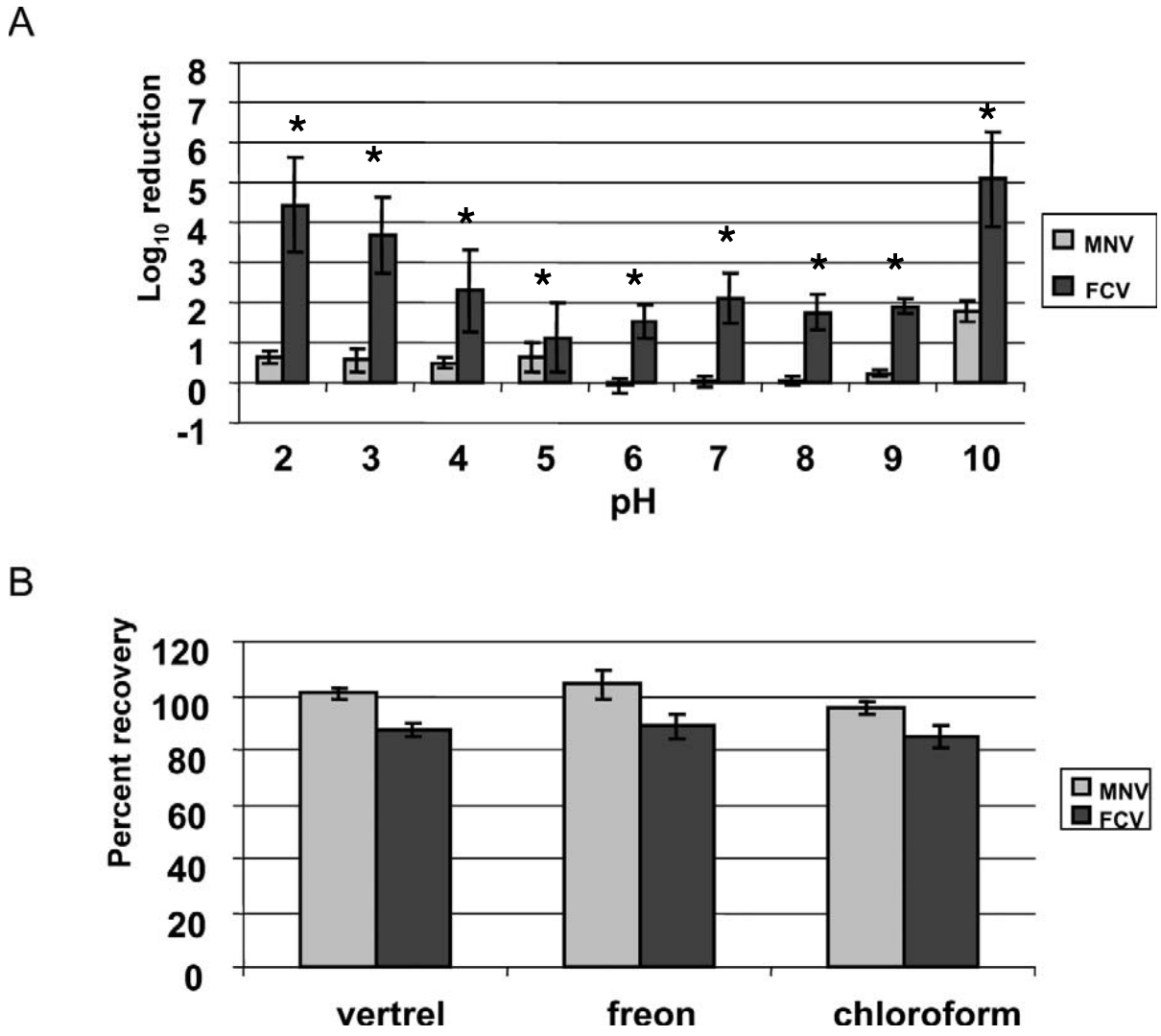


Figure 3.1: Survival of MNV and FCV (A) across a range of pH values (pH 2 to 10) after 30 min at 37° C and (B) after extraction with Vertrel, Freon, and chloroform. Light shading, MNV; dark shading, FCV. Error bars indicate standard deviation (n = 3). * indicate significant differences in $\log_{10}$ reductions between MNV and FCV.

Thermal inactivation

Virus inactivation was measured at 3 different temperatures (56° C, 63° C, and 72° C). At 56°C (which corresponds to the lower temperature limit for "hot bars"), a 1 log inactivation was achieved at 3.5 min and 6.7 min for MNV and FCV, respectively (Table

3.1). At 63°C (consistent with low-temperature, long time, (LTLT) pasteurization) both viruses were inactivated by 1 log in about 25 sec and at 72°C (consistent with high-temperature, short time, HTST pasteurization), both MNV and FCV were inactivated by 1 log in less than 10 sec (MNV=9.9 sec and FCV=7 sec) (Table 1). Survival of MNV and FCV at 56°C differed significantly ($p < 0.05$), but at 63°C and 72°C virus infectivity after temperature treatment was not significantly different ($p => 0.05$).

Table 3.1:. D-values for thermal inactivation of MNV and FCV at different temperatures.

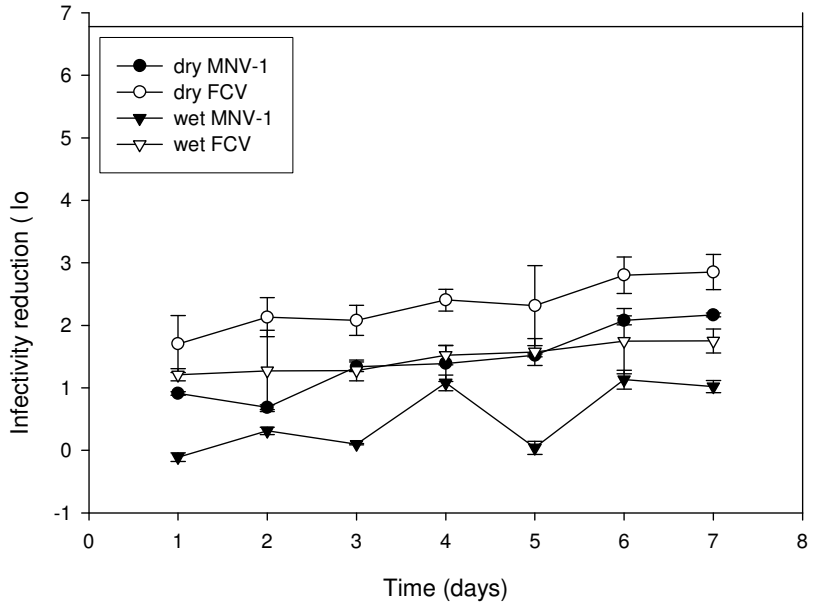
Temperature (°C) [a]	MNV[a]		FCV [a]		F test
	D	σ [b]	D	σ [b]	P value
56	3.473	0.092	6.715	0.010	<0.0001
63	0.435	0.005	0.406	0.012	0.5327
72	0.166	0.016	0.118	0.013	0.3169

[a] Expressed as D-values in min. [b] Standard deviation. Values are the mean of three experiments

Environmental stability

To study the environmental persistence of MNV and FCV, we tested the infectivity of both viruses in suspension as well as dried onto stainless steel surfaces for up to 7 days. At 4°C, both viruses were inactivated at a similar rate (Figure 3.2A). At room temperature, the

A.



B.

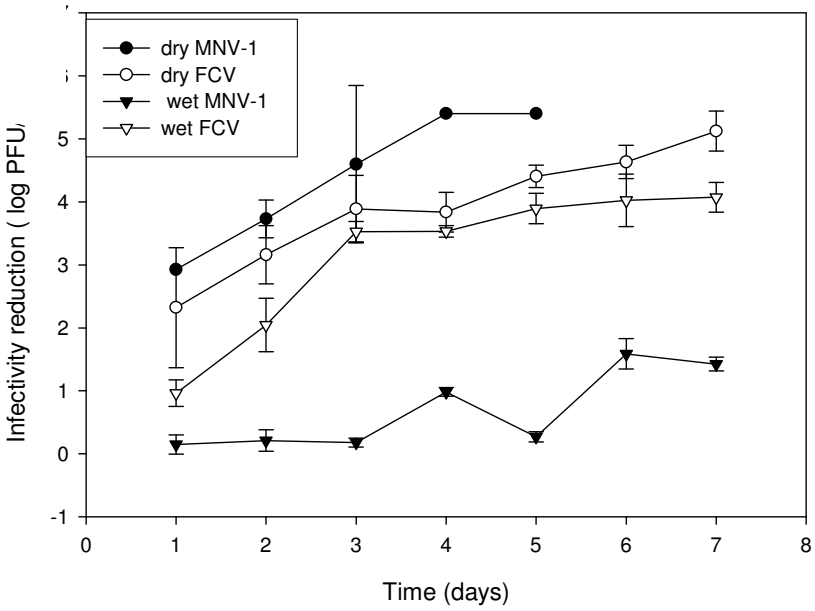


Figure 3.2: Survival of MNV and FCV over a 7-day period at (A) 4°C and (B) room temperature in suspension (wet) and on a stainless steel coupon (dry). MNV is indicated with closed circles and triangles; FCV is indicated with open circles and triangles. Error bars indicate standard deviation (n =3). (MNV was inactivated beyond the detection limit of the plaque assay after day 5 under dry conditions at room temperature; therefore, data for day 6 and 7 are not included.)

difference in inactivation pattern was more profound than at 4°C (Figure 2B). The rates of virus reduction over time for FCV and MNV were similar from day 1 to day 7 for all conditions ($p > 0.05$) except when wet at room temperature, in which case differences were significant ($p < 0.05$).

DISCUSSION

Murine norovirus (MNV), the first norovirus to be routinely propagated in cell culture, is genetically related to the non-cultivable human NoVs and is spread by the fecal-oral route (Karst et al, 2003). To date, several cultivable enteric caliciviruses belonging to other genera within the family *Caliciviridae* have been used as surrogates for the human NoVs, including porcine enteric calicivirus (PEC) (a sapovirus) (Guo et al, 1999), canine calicivirus (a vesivirus) (Mochizuki et al, 1993) and feline calicivirus (FCV) (also a vesivirus (Kahn and Gillespie, 1970)). Of these, FCV has been the most commonly used surrogate to model survival, persistence and inactivation of human NoVs (Bidawid et al, 2003; Duizer et al, 2004b). In the present study, we compared the survival of infectious FCV and MNV under a variety of environmental conditions, including extremes of pH, elevated temperature, and desiccation. Although virus inactivation kinetics associated with the use of disinfectants in water has historically been evaluated using monodispersed viruses, we and others (D'Souza et al, 2006; Duizer et al, 2004b) have employed a more environmentally relevant experimental design by using cell culture lysates as virus inoculum. This approach yields a comparatively more conservative prediction of the behavior of viruses in the environment.

We demonstrated that MNV is considerably more resistant to both high and low pH values with only a minimal loss of infectivity at pH 2. By contrast and consistent with other investigators (Duizer et al, 2004b; Kahn and Gillespie, 1970), FCV is quite unstable at lower pH values. Such acid resistance is probably crucial for successful infection, as enteric viruses must survive the stomach to reach their target cells in the small intestine. Norwalk virus, the prototype human NoV, was reported to cause infection in human volunteers after incubation for 3 h at pH 2.7 (Dolin et al, 1972). Most enteric viruses demonstrate resistance

to low pH; for example, cell culture adapted HAV can remain infectious after being held at pH 1 for 5 h and human enteroviruses retain stability at pH 3 (Pallansch and Roos (Fields) 2001). An ideal surrogate for the human NoVs should have the capability of surviving at low pH and our results demonstrate that MNV is superior in acid tolerance when compared to FCV.

Because organic solvent extraction is often an essential step in concentrating enteric viruses from environmental, food, and water samples (Chung et al, 1996), we evaluated the effect of three commonly used organic solvents on MNV and FCV infectivity. None of the organic solvents, including Vertrel, a Freon substitute not implicated in ozone depletion (Mendez et al, 2000), substantially impacted or otherwise reduced virus infectivity.

Overall, our findings on the thermal resistance of FCV are consistent with or less conservative than the results of others, which might be explained by the use of the capillary tube method which offers more efficient heat transfer than test tube methods (Duizer et al, 2004b; Fairchild and Foegeding, 1993). While the somewhat minimal differences in between MNV and FCV survival upon exposure to high temperatures probably reflects differences in capsid structure, pasteurization by the batch method (63°C for 30 min), classical method (72°C for 2 min) or continuous method (72°C for 15 sec) would most likely result in complete inactivation, unless the initial virus contamination level was high (> 4 log). However it is conceivable that the D values could be higher in the presence of certain food components which might have a protective effect on the thermal stability of viruses (Bidawid et al, 2000).

Our environmental persistence studies were unique in that we suspended virus stocks in artificial feces to mimic fecally contaminated surfaces, as it has been suggested that the

fecal matrix can have protective effects on virus persistence on surfaces (Kampf et al, 2005). We found that both viruses were inactivated at similar rates under all conditions except when wet at room temperature, a condition under which MNV was more stable than FCV. Overall our data for environmental survival of FCV are in agreement with results obtained in previous studies (D'Souza et al, 2006).

The results of this study clearly demonstrate that important differences exist in the stability of MNV and FCV under environmental conditions such as exposure to low pH, high temperature, and desiccation. The most dramatic finding was that MNV is more stable to extremes of pH than FCV and in this regard is more characteristic of the human NoVs. Until an *in vitro* model for the evaluation of human NoV infectivity becomes available, the genetically related MNV, which is easy to grow in the laboratory and is stable at low pH, may be a more relevant surrogate for studying the environmental persistence of human NoVs.

ACKNOWLEDGEMENTS

This research was supported in part by a grant to JV from the National Institute of Environmental Health Sciences (P30ES10126) and to LAJ through the International Life Sciences Institute-North America (ILSI-NA). The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Research Service nor criticism of similar ones not mentioned. We would also like to thank Dorothy Thompson and Douglas Wait at UNC-CH for expertise and technical assistance.

CHAPTER 4

A histo-blood group antigen-binding assay for recovery and purification of norovirus from environmental waters

Jennifer L Cannon[1,2*] and Jan Vinjé [2]

[1] Department of Environmental Science and Engineering, University of North Carolina, Chapel Hill, NC
[2] Gastroenteritis and Respiratory Viruses Laboratory Branch, Division of Viral Diseases, Centers for Disease Control and Prevention, Atlanta, GA

This article was submitted to Applied Environmental Microbiology: June 10, 2008

(AEM01302-08 Version 1).

ABSTRACT

Noroviruses (NoV) are the leading cause of outbreaks of gastrointestinal illness worldwide, and recognized contaminant of recreational and drinking waters. Currently, assays to measure NoVs infectivity are not available, so NoVs detection is limited to molecular methods. Specific histo-blood group antigen (HBGA) carbohydrates have been identified as the putative receptors for NoVs. In the present study, we have evaluated a HBGA binding assay as a proof of concept for the recovery and purification of low numbers of NoVs from environmental waters. Synthetic HBGAs (H type 1) bound to magnetic beads were used to capture Norwalk virus (strain 8FIIb) in the context of environmental waters, and the assay detection limit was determined by conventional and real-time RT-PCR. To examine if the assay was specific for intact viral particles, RNase A was mixed with virus-bound HBGAs in CMS. Inputs as low as 30-300 genomic copies of Norwalk virus could be recovered in up to 1 ml of surface water or wastewater, and bound particles were RNase resistant. In conclusion, we report CMS applicability in the recovery and purification of low copy numbers of NoV in water samples and hypothesize that binding to HBGA followed by RT-PCR may be a surrogate for the detection of infectious virus.

INTRODUCTION

In recent years, human noroviruses (NoVs) have been recognized as the leading cause of outbreaks of acute gastroenteritis worldwide. Large NoV outbreaks have been reported in association with contaminated drinking water from private wells, small water systems, and community water systems and from recreational waters (Borchardt et al, 2003; Carrique-Mas et al, 2003; Hoebe et al, 2004; Kukkula et al, 1999). In addition, consumption of shellfish from fecally contaminated harvesting waters or produce from contaminated irrigation or wash waters has been implicated in several national and international NoV outbreaks (Berg et al, 2000; Croci et al, 2007; Falkenhorst et al, 2005; McDonnell et al, 1997; Shieh et al, 2003). Such reports indicate issues in water sanitation are of continued public health importance even in the developed world. Rapid methods for the recovery of human NoVs from such waters can help in outbreak source identification.

Currently, detection of human NoVs in environmental waters is based on sensitive molecular methods such as RT-PCR and real time RT-PCR to detect viral RNA since NoVs cannot be grown in routine cell culture. Although environmental waters may contain low concentrations of NoVs, exposure to even a small number of NoVs is a public health risk as the infectious dose is very low (10-100 virus particles) (Teunis et al, 2008). Therefore, large sample volumes (1 L to 100 L) must be concentrated into microliter volumes to assess the potential human health risk. Environmental water samples are complex matrices that often contain compounds that inhibit enzymes used in RT-PCR. Hence, virus concentration methods not only need to be able to efficiently concentrate low numbers of virus particles but also include steps to remove RT-PCR inhibitors to avoid false negative results.

Recently, histo-blood group antigens (HBGAs) have been identified as candidate receptors for NoVs as symptomatic Norwalk virus infection of human volunteers is dependent on cells displaying the appropriate HBGA receptor(s) (Hutson et al, 2002; Lindesmith et al, 2003). Several, but not all, NoVs and their respective virus-like particles (VLPs) bind to synthetic HBGAs and 8 different binding patterns have been identified (Harrington et al, 2002; Harrington et al, 2004; Huang et al, 2003; Huang et al, 2005; Hutson et al, 2003; Lindesmith et al, 2003). Co-crystallization of the HBGA bound to the receptor-binding pocket of a NoV capsid was recently solved (Cao et al, 2007), and protein mutagenesis in this region obliterated HBGA binding (Tan et al, 2003), providing additional support for the importance of HBGAs in NoV infection.

In this study, we report a proof of concept method for the rapid purification and recovery of low numbers of potentially infectious NoVs employing HBGA carbohydrates in a magnetic separation (CMS) assay. We examined the sensitivity and specificity of CMS for human NoV in the context of other enteric viruses and its applicability in concentrating NoVs from environmental waters.

MATERIALS AND METHODS

Viruses and viral RNA extraction

We used a panel (n = 5) of GI.1 NoV positive stool samples obtained from human volunteers which were kindly provided by Drs. Christine Moe (Emory University, Atlanta, GA) and Robert Atmar (Baylor College of Medicine, Houston, TX). RNA from a GII.4 stool sample was used as a positive control in the RNase A treatment experiments. Viral RNA was extracted from 10% stool suspensions using a Qiagen viral RNA stool kit. Group A rotavirus strain WA (serotype 1) and human astrovirus serotype 1 (Oxford strain) were included to evaluate the specificity of the assay.

Conventional and TaqMan® Realtime Reverse Transcription-Polymerase chain reaction

Conventional RT-PCR was performed using a Qiagen One-Step RT-PCR kit (Qiagen, Valencia, CA) including the Q solution and using Norwalk virus specific primers (pol5' and pol 3') (DeLeon et al, 1992) targeting a partial region of the RNA polymerase gene. Cycling conditions included a 42°C reverse transcription (RT) step for 1 hr, activation of Taq polymerase at 95°C for 15 min., followed by 35 cycles of 94°C, 50°C, and 72°C for 30 sec each and a final extension step of 7 min at 72°C.

TaqMan® realtime RT-PCR was performed using the QuantiTect Probe real-time RT-PCR kit (Qiagen) on an ABI 7500 (Applied Biosystems, Foster City, CA) using GI primers (CogI F/R) and a FAM labeled probe (Ring 1a) targeting the ORF1 - ORF2 junction region of the genome (Kageyama et al, 2003; Tian et al, 2008). Cycling conditions were as follows: reverse transcription at 50°C for 30 min, activation of Taq polymerase at 95°C for 15 min, followed by 45 cycles of 95°C for 10 sec, 55°C for 32 sec and 72°C for 30 sec. Data were collected during the 55°C extension period of each cycle.

RNA transcripts and RNA quantification

GI.1 NoV RNA transcripts were generated by cloning a 3 kb RT-PCR product of Norwalk strain 23-3 in a TOPO-TA vector (Invitrogen) followed by run-off RNA transcription using a MEGAScript kit (Ambion, Austin, TX) and three rounds of DNA digestion (twice with a Megaclear kit (Ambion) and once using an on-column RNase-free DNase kit (Qiagen)). RNA transcripts were quantified using a NanoDrop® (Thermo Fisher Scientific, Waltham, MA) spectrophotometer. Duplicates of 10-fold serial dilutions of RNA transcripts were used to generate a standard curve (slope = -3.63; y-intercept = 42.1; $R^2 = 0.997$) using the ABI 7500 software. Using this program, total Norwalk virus RNA copy numbers (3×10^7) per ml of 10% stool suspensions were estimated and used to determine input virus levels for CMS.

Carbohydrate Magnetic Separation (CMS) Assay

The HBGA-binding assay with magnetic beads previously described by Harrington et al (2004) was used with some modifications. Briefly, streptavidin-coated magnetic beads (25 µl) (Dynal Biotech, Olso, Norway) were washed 3 times in PBS and pre-blocked with blocking buffer [5% fetal bovine serum (FBS) or SuperBlock (Pierce Biotech., Rockford, IL)] for 1 hr before adding 50 µl of synthetic biotinylated H type 1 (or H type 2, H type 3 or Le^b only during optimization of CMS) histo-blood group carbohydrates (1 mg/ml) (Glycotech, Rockville, MD) in 950 µl of blocking buffer [5% fetal bovine serum (FBS) or SuperBlock (Pierce Biotech., Rockford, IL)] for 1.5 hr at room temperature (Figure 1). Beads were subsequently washed 3 times with 0.05% PBS-Tween 20. A magnet (Dynal Biotech, Oslo, Norway) accommodating 1.7 ml microcentrifuge tubes was used during washing steps. Beads were then blocked in 1 ml blocking buffer [5% fetal bovine serum (FBS) or SuperBlock (Pierce Biotech., Rockford, IL)]

supplemented with 0.25% Tween-20 for 1 hr at room temperature followed by 18 h at 4°C. After 3 washes with 0.05% PBS Tween-20, the beads were suspended in 1 ml of blocking buffer, environmental water concentrate, or PBS. Dilutions of stool containing 3,000, 300, 30 or 3 Norwalk virus copy numbers were added to the CMS suspensions and were incubated for 4 hr at room temperature on an end-over-end rotator (Dynal Biotech). The beads were washed 5 times with 0.05% PBS Tween-20 and by 3 times with PBS before they were suspended in 50 µl PBS. The bead suspension was then 10-fold serially diluted in water and viral RNA was released by heat (5 min. at 99°C). Beads were pelleted by a 25 sec spin at 14,000 x g and 2.5 µl or 1 µl of supernatant was analyzed by RT-PCR.

Sensitivity of CMS

To determine the sensitivity of CMS, the detection limit was examined using dilutions of stool containing 3,000, 300, 30 or 3 RNA copy numbers added to carbohydrate-bound beads in SuperBlock blocking buffer and analyzed as described above.

Specificity of CMS

Two sets of CMS reaction tubes were prepared containing 3,000, 300, and 30 RNA copy numbers of Norwalk virus stool suspension in 1 ml of SuperBlock. To one set of tubes, rotavirus group A serotype 1 (strain WA) was added and serotype 1 human astrovirus (Oxford strain) was added to the other set. Inhibition of Norwalk virus binding to the carbohydrate beads was measured by comparison with controls with Norwalk virus only.

Concentration of environmental waters

Surface water (influent drinking water) (n=4) and influent (n=2) and effluent (n=4) wastewater samples were collected from drinking and wastewater facilities in Columbus, GA and Atlanta, GA, respectively and stored at 4°C for no more than 24 hrs before processing. Surface waters were processed as described previously (Vinjé et al, 2004). Briefly, four liters of surface water (influent drinking water) was adjusted to pH 7.0-7.2 before polyethylene glycol (PEG) precipitation (8% PEG 8000, 0.3 M NaCl) at 4°C overnight, with constant stirring. After centrifugation at 7,280 x g for 1 hr at 4°C, PEG-precipitated virus was suspended in PBS (pH 7.2) and extracted with 50% (v/v) chloroform. The supernatants (5-20 ml) were stored at -70°C.

For wastewater, 850 ml of sample was centrifuged at 7,280 x g for 15 min at 4°C to pellet solids. After retaining the supernatant, the pellet was chloroform extracted (50% (v/v)) and centrifuged at 2,000 x g for 10 min at 4°C. The aqueous phase was combined with the supernatant from the first centrifugation and each sample was processed by PEG precipitation as described above for surface waters.

RNase A treatment of HBGA-bound virus

To determine if the Norwalk virus particles bound to H-type 1 coated beads were intact and potentially infectious, 30,000 or 6,000 RNA copy numbers from a 10% NoV stool suspension was treated with 10 ng of ribonuclease A (Invitrogen, Carlsbad, CA) in 0.1 M TE buffer and incubated for 30 min. at 37°C. RNase inhibitor (160 U/ 50 µl of sample) (Invitrogen) was then added to neutralize any remaining RNase activity by incubating for 15 min at 37°C. GII.4 RNA (1 x 10^5 copy numbers) was added to the reaction tube either before

or after RNase treatment to check the efficacy of the RNase A or the RNase inhibitor, respectively. Presence of RNA was detected by TaqMan® real-time RT-PCR and results were reported in Ct (cycle-threshold) values. Statistical significance was measured by a student's *t* test.

RESULTS

Optimization of carbohydrate magnetic separation method

Our first step in optimization of the carbohydrate magnetic separation (CMS) method for the detection of human NoV was to examine three blocking buffers (5% Blotto, 5% FBS, SuperBlock) for their ability to block nonspecific binding to uncoated magnetic beads without interfering with specific binding to H type 1 carbohydrate or the reproducibility of the results. The use of 5% FBS as a blocking buffer did not interfere with specific binding and blocked nonspecific binding in 4 of the 7 experiments with a virus input level of 30 to 30,000 copy numbers, yet inhibition of RT-PCR was often observed for the undiluted sample, but not the 10 -fold diluted sample of virus-bound beads (6 of the 7 experiments). Although not effective at blocking nonspecific binding to uncoated beads at virus concentrations greater than 3,000 copy numbers, SuperBlock demonstrated the most reproducible results allowing detection of low input levels (30 to 3,000 copy numbers), and successfully blocked nonspecific binding when 3,000 copy numbers or fewer viruses were analyzed (data not shown). Furthermore, SuperBlock did not inhibit RT-PCR for the undiluted bead samples in any experiment (n = 8). Therefore, SuperBlock was used in the final format of the CMS.

Next, we compared a panel of synthetic HBGAs in CMS for their ability to capture low (3, 30, 300 or 3,000) input levels of NoV. We found that Norwalk virus (strain 8FIIb 23-3b) bound to H type 1, H type 3, and Lewis while no binding was detectable to H type 2. H type 1 carbohydrate was chosen for subsequent CMS experiments because it bound to Norwalk virus at the lowest input level (3 copy numbers), whereas the lowest detectable input level for the H type 3 and Lewis b carbohydrates was 300 and 3,000 copy numbers, respectively (n = 3).

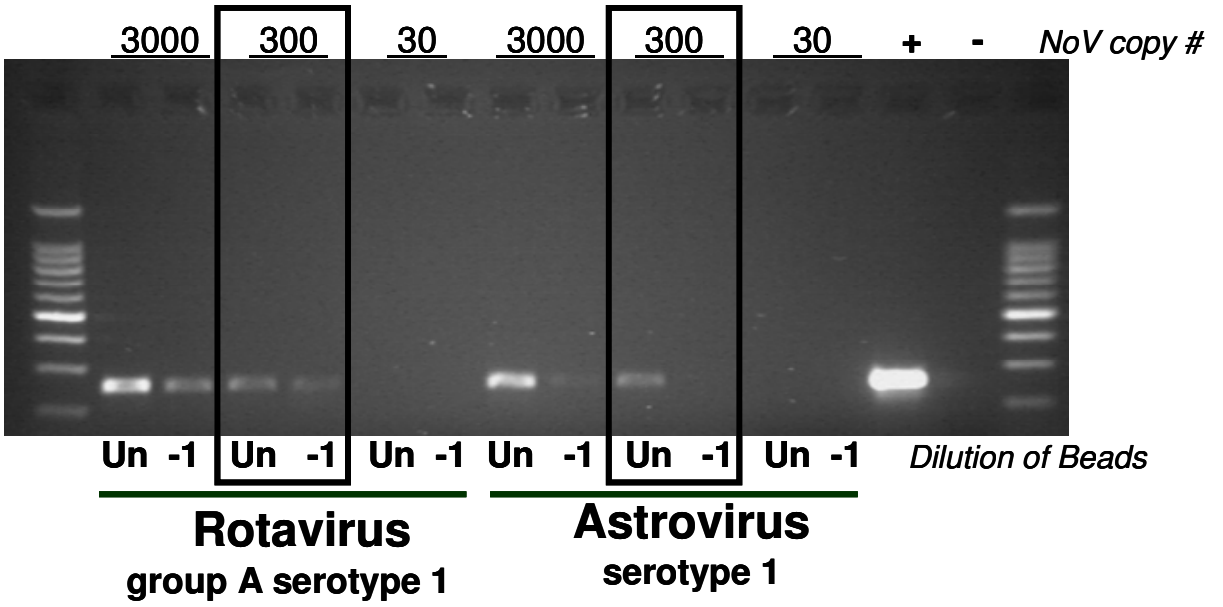


Figure 4.1: Norwalk virus binding to H type 1 in the presence of rotavirus and astrovirus as indicated by the ethidium bromide stained gel. DNA ladders flank wells containing RT-PCR amplicons from CMS experiments performed using 3,000, 300, or 30 NoV copy number input levels in the presence of rotavirus or astrovirus. "Un" and "-1" refer to undiluted and 10-fold dilutions of CMS beads just prior to heat release and RT-PCR.

Sensitivity and Specificity of CMS

A GI.1 stool sample containing 3 x 10^7 genomic copy numbers of Norwalk virus per ml of 10% stool suspension was used to determine the detection limit of CMS. Input levels ranging from 3 to 3,000 viral copy numbers (median = 300 copy numbers; n = 10) per ml of SuperBlock could be detected by CMS. Neither rotavirus nor astrovirus inhibited the binding of Norwalk virus to the H-type 1 carbohydrates as a 300 NoV copy number input level could be detected by CMS in the presence of these enteric viruses (Figure 4.1).

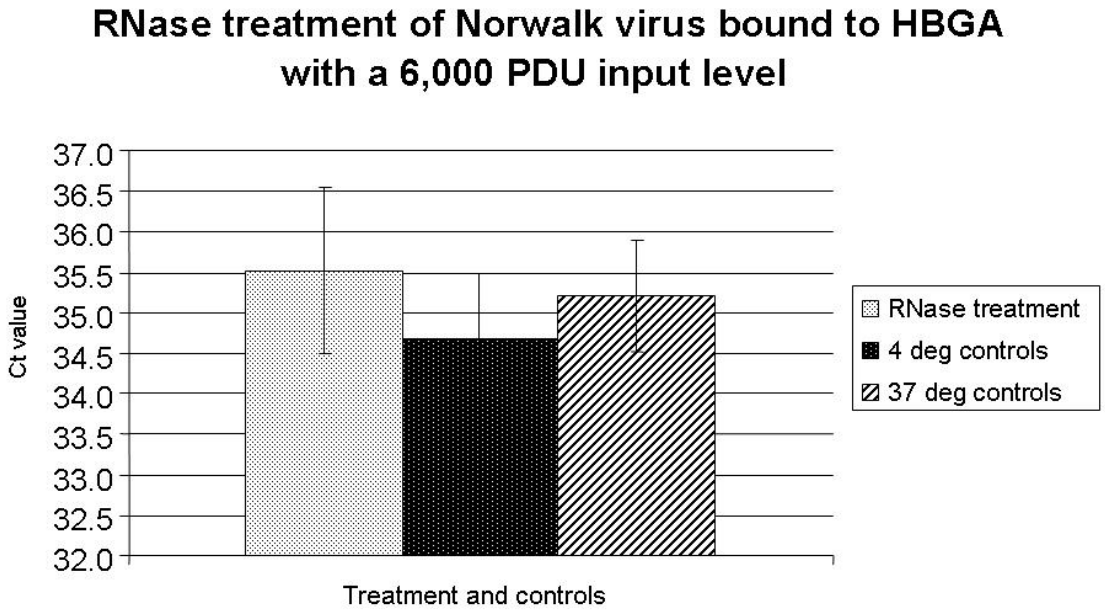


Figure 4.2: Effect of RNase A on an input level of 6,000 copy numbers of Norwalk virus bound to HBGA and processed using CMS. The TaqMan realtime RT-PCR cycle-threshold (Ct) values for the RNase treatment and 4°C and 37° control samples are indicated on the y axis. Error bars indicate standard deviations in 3 replicate experiments.

RNase treatment of HBGA-bound virus

To determine if CMS was specific for concentrating intact, potentially infectious, NoV particles, HBGA-bound NoVs were treated with RNase A before real-time RT-PCR detection of viral RNA. No significant reduction in Ct value between the RNase treated and untreated 37°C controls for the 30,000 (data not shown) or 6,000 (Figure 1) copy number input levels ($p \geq 0.2$) was observed (Figure 4.2). In addition, the difference in Ct value between the 4°C controls and the 37°C controls was not significant at either input level ($p \geq 0.3$). RNase treatment of GII.4 control RNA resulted in complete loss of a realtime RT-PCR signal.

Detection limit of CMS in the context of environmental waters

To address the applicability of CMS in concentrating and purifying NoVs from waters, PEG concentrated environmental water samples (surface, effluent wastewater, influent wastewater) were spiked with 3, 30, 300 or 3,000 RNA copy numbers of Norwalk virus and analyzed. The detection limit of CMS was determined between 30 to 3,000 (median = 300) for all of the water types tested (Table 4.1). Norwalk virus spiked into surface water concentrate could be detected consistently at the 30 copy number input level (n=4) and concentrate from effluent wastewater (treated sewage effluent) was consistently detected at the 300 copy number input level (n=4). The influent wastewater (raw sewage) concentrate was more inconsistent having a detection limit of 300 or 3,000 copy numbers (n=2). While the detection limit of the CMS assay related inversely with the quality of the environmental water sample tested, the assay proved robust for detecting low levels of Norwalk virus in environmental waters.

Table 4.1: At each NoV input level (copy numbers of RNA), the proportion of experiments (n = 4 or 2) resulting in RNA detection following CMS are indicated for each environmental water sample type.

	NoV input (RNA copy numbers)			
Type of environmental water	**3,000**	**300**	**30**	**3**
Surface water	4/4	4/4	4/4	0/4
Effluent wastewater	4/4	4/4	0/4	0/4
Influent wastewater	2/2	1/2	0/2	0/4

DISCUSSION

This study provides a proof-of-concept for using a carbohydrate magnetic separation (CMS) method for concentrating and purifying low numbers of NoVs. The presence of other enteric viruses did not inhibit the method, and we demonstrate the applicability of CMS when analyzing environmental waters suspected of NoV contamination. We confirmed that the HBGA-bound virus particles are intact and hence we predict that CMS, in the absence of a cell culture system, is able to recover infectious NoVs from environmental waters.

Several groups have independently developed successful methods for concentrating human NoVs using immunomagnetic separation (IMS) (Gilpatrick et al, 2000; Myrmel et al, 2000; Park et al, 2008; Schwab et al, 1996). Similarly, a magnetic bead-based technique using pig gastric mucins that contain a mixture of HBGAs was recently described (Tian et al, 2008). Like CMS, several of these methods can detect low numbers of NoVs, even from complex matrices (Gilpatrick et al, 2000, Myrmel et al, 2000; Park et al, 2008; Schwab et al, 1996, Tian et al, 2008). However, CMS uses specific HBGAs that bind human NoVs (Cao et al, 2007), thereby detecting viruses with an intact binding pocket. Although efficient at removal of inhibitory elements from most complex sample matrices, magnetic separation techniques combined with RT-PCR may succumb to inhibition when used with extremely heterogeneous samples such as sewage influent. In our experiments, CMS-RT-PCR detected NoV from sewage influent with a one log decrease in sensitivity. The sewage concentrate may contain inhibitory elements that may lyse the virus before it can bind to receptor, or elements that affect virus binding to receptor such as human IgG binding specifically to the HBGA binding site of the capsid. Alternatively, the overload of inhibitory elements may aspecific bind to the magnetic beads, thereby inhibiting downstream RT-PCR.

Human volunteer challenge studies with Norwalk virus (GI.1) have unequivocally demonstrated that FUT2 –dependent HBGAs are candidate receptors for human NoVs (Hutson et al, 2003; Lindesmith et al, 2003). Several other NoV genotypes also recognize HBGA structures on gut epithelial cells of secretor positive individuals (Marionneau et al, 2002), although exceptions have been reported (Lindesmith et al, 2005). While an intact HBGA binding region of the capsid is likely to be required for infection, it is not known if other regions of the capsid such as those involved in secondary receptor binding or internalization may also be required for infection. Since the HBGA binding region is located in a cavity formed by dimerization of P domains (Tan et al, 2004), it is possible that areas of the capsid more surface exposed may be damaged more readily leading to an over estimation of infectivity predicted by CMS. In addition, CMS may not be adequate for predicting infectivity after treatments targeting the genome such as germicidal UV since the RT-PCR region amplifies is small and damage of viral RNA in other regions of the genome may occur undetected thus overestimating infectivity of the HBGA-captured virus by CMS.

Whether or not our CMS binding assay can be generalized for other genogroups, particularly GII.4 NoVs most frequently associated with outbreaks of NoVs which results will likely determine the applicability of CMS as a broadly reactive recovery method. In addition to the HBGA binding of Norwalk virus to H type 1, we have shown GII.2 binding to H type 3 and three GII.4 NoV strains (a Camberwell-like, Grimsby-like and a Farmington Hills-like) binding to H type 3 and/or Le^y carbohydrates adding to and confirming the results of others (Harrington et al, 2004). Thus, future experiments should be directed toward uncovering a cocktail of HBGAs that will be able to bind the majority of circulating strains of NoVs. If successful, CMS may serve as part of a rapid detection method for NoVs in complex matrices

such as environmental waters or food. Furthermore, with validation of HBGA association with infectivity of NoV genogroups other than Norwalk by human volunteer challenge or in vitro cell culture, CMS may serve as a surrogate model for human NoV infectivity.

ACKNOWLEDGEMENTS

We would like to thank Drs. Moe (Emory University) and Atmar (Baylor College of Medicine) for kindly providing Norwalk virus stocks and special thanks to Linda Vermeulen for her assistance on the RNse A experiments. We also extend our appreciation to technical assistance by Dr. Patrick Harrington (UNC-CH) and Douglas Wait (UNC-CH). This project was supported by the American Water Works and Research Foundation (AWWARF) project # 2860.

CHAPTER 5

Herd immunity to GII.4 noroviruses is supported by outbreak patient sera, 1985-2006

Jennifer L Cannon, [1,2,3], Lisa C. Lindesmith[4], Eric F. Donaldson[4], Lauryn Saxe[1], Ralph S. Baric[4], and Jan Vinjé[1]

[1]Centers for Disease Control and Prevention, Atlanta GA 30333,
[2]Univ. of North Carolina-Chapel Hill, Department of Environmental Science and Engineering, NC 27599,
[3]Atlanta Research and Education Foundation, Decatur, GA 30033
[4]Univ. of North Carolina-Chapel Hill, Department of Epidemiology, NC 27599.

This chapter is planned for submission in the Journal of Virology with additional data from our collaborators.

ABSTRACT

Noroviruses (NoVs) of genogroup II, cluster 4 (GII.4) are the most common circulating NoVs causing outbreaks of acute gastroenteritis worldwide. During the past 13 years, GII.4 NoVs caused four epidemic seasons of viral gastroenteritis for which four variants emerged and displaced the resident viruses. Recently, our group has provided evidence for evolution by antigenic drift in the face of herd immunity as a mechanism for GII.4 NoV persistence in the human population. Using a panel of human outbreak sera collected over a 21 year period, we investigate here if there is support for herd immunity to GII.4 NoVs in the US population (1985-2006), and what role other GII genotypes could have played in GII.4 evolution. We investigated the seroprevalence and specificity of GII.4 antibody in the years prior to, during and between three epidemic peaks (1996, 2002 and 2006) using a large collection of acute and convalescent serum pairs (n= 210) collected from 27 outbreaks. In a surrogate neutralization assay, we measured blockade of histo-blood group antigen (HBGA) receptor binding using a panel of GII.4 VLPs representing 1987, 1997, 2002 and 2006 outbreak strains. Convalescent sera dilutions required for 50% HBGA blockade were compared between populations stratified by date and genotype. In general, blockade of GII.4 VLP-HBGA binding was greater with convalescent outbreak sera collected within or near the time the VLP strain was in circulation. Blockade of more distantly related GII.4 VLPs required higher sera concentrations or was not achieved at all. With the exception of GII.3 NoVs, heterotypic genotypes did not contribute to herd immunity against GII.4 NoVs based on their inability to block GII.4 VLP binding to HBGA. These results support herd immunity as a driving force for GII.4 evolution in the US population and suggest that cross-protection across genotypes occurs in humans but is genotype and strain

specific. The information provided by this study emphasizes the need for a revitalization of serologic surveillance for human NoVs. Early evidence of the antigenic differences of emerging GII.4 strains towards the end of a norovirus outbreak season will be important for designing VLP vaccines.

INTRODUCTION

Human noroviruses (NoVs) of the family *Caliciviridae* are the most common cause of acute gastroenteritis worldwide and the leading cause of foodborne illnesses in the United States (Green (Fields) 2007; Widdowson et al, 2005a). Although generally causing a short-lived but acute illness involving diarrhea and/or vomiting, more severe symptoms and fatalities occasionally occur among the elderly, infants and immunocompromised persons (Green, K.Y., 2002; Sakai et al, 2001; Zintz et al, 2005; MMWR 2007). Outbreaks in communal and institutional settings such as hospitals, nursing homes, cruise ships, university dorms, and military barracks are frequently reported; furthermore, these institutions suffer the most significant economic damages during NoV outbreaks due to direct healthcare costs, decontamination efforts and indirect losses (Lopman et al, 2004b).

Currently there is no vaccine or antiviral therapy to treat NoV infections and such efforts have been significantly hampered by the lack of a simple cell culture or small animal model for human NoVs. All information on host-pathogen interactions has come from a handful of human challenge studies and epidemiologic investigations (Lindesmith et al, 2003; Lindesmith et al, 2005; Parrino et al, 1977; Larsson et al, 2006; Rockx et al, 2005). As a result, NoV basic virology and correlates of immune protection are poorly understood. Recently, the use of recombinant virus-like particles (VLPs) and replicon constructs have proved promising lines of research toward vaccine and antiviral development (Chang and George, 2007; Feng and Jiang 2007; LoBue et al, 2006; Tacket et al, 2003) Additionally, computer-generated structural homology modeling of NoV capsid proteins combined with epidemiologic and immunologic investigations are proving to be valuable new tools for more specific targeting of such efforts (Lindesmith et al, 2008; Siebenga et al, 2007).

NoVs have a 7.5-7.7 kb, single stranded, positive-sense RNA genome consisting of 3 open-reading frames (ORFs) packaged in a non-enveloped iscosohedral capsid. Recombinant expression of the ORF2 major capsid protein (VP1) in a baculovirus or Venezuelan Equine Encephalitis (VEE) expression vector has been successful for the production of virus-like-particles (VLPs) that are physically and antigenically similar to native virus particles (Baric et al, 2002; Green et al, 1993). The surface exposed P2 subdomain of VP1 is the most hypervariable region of the genome and responsible for receptor binding (Cao et al, 2007; Chen et al, 2004).

Histo-blood group antigen (HBGA) expression on the gut mucosa and its secretions have shown to be correlated with susceptibility to infection with the prototype Norwalk virus (Hutson et al, 2002; Lindesmith et al, 2003). Several, but not all NoVs specifically bind to HBGAs which are likely used as a receptor for docking and entry into the cell during infection (Harrington et al, 2002; Harrington et al, 2004; Huang et al, 2003; Hutson et al, 2003; Lindesmith et al, 2003). Recent epidemiological studies indicate there is an association between HBGA expression and genetic susceptibility to NoV strains of genogroup II, cluster 4 (GII.4) although, the association is not as clear as it has been with Norwalk virus (Thorven et al, 2005; Larsson et al, 2006).

More than 25 genotypes of human NoVs have been described (Zheng et al, 2006) but GII.4 strains are the most prevalent globally. Over the last two decades, NoV epidemic peaks associated with GII.4 have been reported worldwide in 1996, 2002, and 2006 and at least one novel GII.4 variant strain could be identified during each of these years (Noel et al, 1999; Fankenhauser et al, 2002; Lopman et al, 2004a; Bull et al, 2006; Siebenga et al, 2007). Epidemic seasons were followed by periods of moderate to low NoV activity where GII.4

strains cocirculated indiscriminately along with NoVs of several other genotypes. During the GII.4 epidemic peaks of 1996 and 2002, the proportion of GII.4 strains causing outbreaks in the United States was greater than 50%, but between these years, the GII.4s were responsible for just 10-29% of all NoV outbreaks (CDC, unpublished data). One year after the epidemic peak of GII.4s in 1996, GII.3 NoV circulation increased from about 4% in 1996 to about 25% in 1997 (Noel et al, 1999). After 2002, variant GII.4 strains emerged much more quickly causing epidemics every 1-2 years. This type of epochal evolution has been described by our group and others (Lindesmith et al, 2008; Siebenga et al, 2007).

Recently, our group has proposed a mechanism for persistence of GII.4 in the human population based on the epidemic patterns observed for GII.4 strains during the past 20 years (Lindesmith et al, 2008). Clear evidence of structural changes in the P2 region of GII.4 capsid protein leading to alternative HBGA receptor usage and immune evasion could be observed for GII.4 strains from 1987, 1997, 2002, 2004, and 2005. HBGA binding with representative GII.4 VLPs supported the structural models and receptor blockade data provided by hyperimmune mouse antisera unambiguously demonstrated antigenic variation between GII.4 strains and phenotypic support for immune evasion. Furthermore, blockade patterns of human sera from a GII.4 outbreak supported herd immunity as the driving force for antigenic change (Lindesmith et al, 2008).

Here, we expand upon our previous work by examining receptor blockade patterns of human sera collected during NoV outbreaks over a 21 year period from 1985 to 2006. Our results support a general pattern of receptor blockade expected when herd immunity is the driving force of antigenic change. Furthermore, we demonstrate that the receptor blockade assay has the specificity to discriminate minute differences between heterotypic genogroups

important for understanding cross-protective immunity. Based on these findings, we conclude that in addition to norovirus strain surveillance, analyzing human sera collected during routine outbreak surveillance will be fundamental for choosing strains to include in VLP based vaccine candidates in an approach similar to that for influenza virus.

MATERIALS AND METHODS

Serum specimens

A total of 27 outbreaks for which 210 acute and convalescent serum pairs had been collected between 1985-2006 and archived at the National Calicivirus Laboratory at the CDC (Atlanta, GA) were selected for this study. Serum pairs were chosen from specific populations either prior to or during epidemic peak years for GII.4 NoVs (Figure 5.1). Stratified by date, we chose 91 serum pairs from 10 outbreaks in 1985-1987, 35 serum pairs from 1 outbreak in 1988, 68 serum pairs from 15 outbreaks in 1994-2000 and 6 serum pairs from 1 outbreak in 2006. All outbreaks had an acute serum sample collected between 0-7 days after onset of illness and had a convalescent sera sample taken 13-47 days after the acute sample. Corresponding stool specimens either tested previously for NoV (Fankhauser et al, 2002; Noel et al, 1999) or tested positive for GII NoV by RT-PCR followed by sequence analysis for outbreaks occurring from 1994-2006. No stool samples were available from 10 of the 1985-1988 outbreaks but they were classified as NoV-suspected based on, (1) fulfillment of Kaplan criteria (Kaplan et al, 1982); or 2) positive test for NoV by immune electron microscopy or enzyme immuno assay using convalescent sera form patients infected with Snow Mountain virus or Toronto virus. In addition, serum samples that had demonstrated seroconversion to GI NoV were excluded.

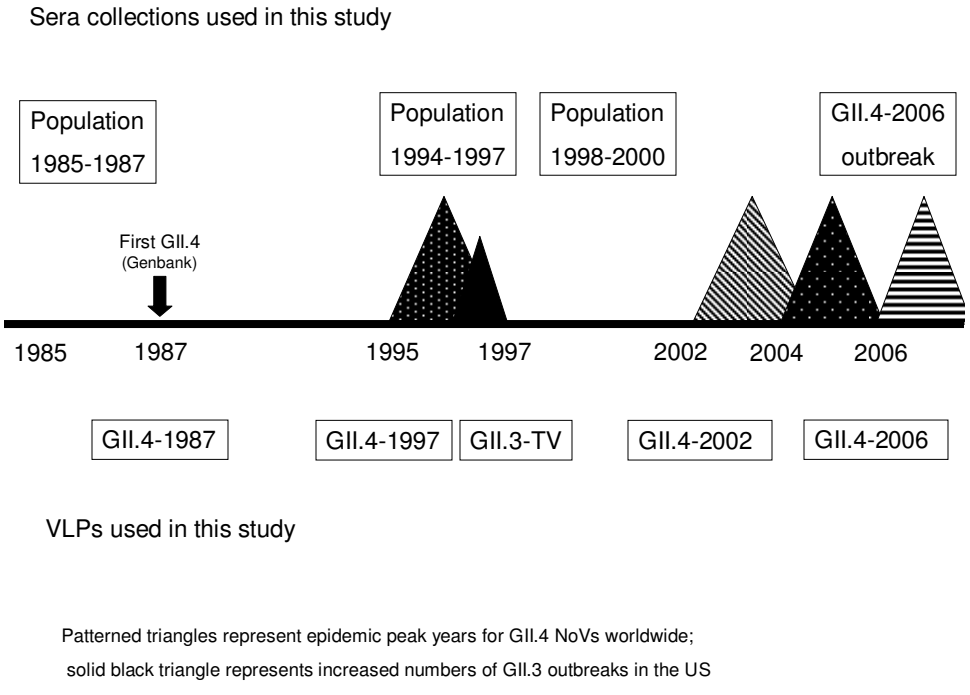


Figure 5.1: Timeline from 1985 to 2006 indicating the populations from which outbreak sera specimens were collected and the VLPs used in this study.

Stool sample processing

Viral RNA was extracted from 10% stool suspensions (PBS) with the Ambion MagMax (Applied biosystems) kit and the KingFisher automated extractor (Thermofisher) and tested for NoV GII by real-time RT-PCR (Trujillo et al, 2006). Positive samples were amplified (Qiagen One-Step RT-PCR, Qiagen, Valencia, CA) in region C (Kojima et al, 2002) and cycling conditions included a 42°C reverse transcription (RT) step for 1 hr, activation of Taq polymerase at 95°C for 15 min., followed by 35 cycles of 94°C, 50°C, and 72°C for 30 sec each and a final extension step of 7 min at 72°C. Amplicons were sequenced using a Big Dye® Terminator Cycle sequencing kit, BigDye® X-terminator Purification kit and the ABI 3130 Genetic Analyzer (ABI, Foster City, CA).

Virus like particles (VLPs)

Complete ORF2 genes of strains representing clusters GII.4-1997, GII.4-2002, GII.4-2006 and GII.3-TV (Figure 5.1) were amplified and cloned into VEE replicons as previously reported (Baric et al, 2002; LoBue et al, 2006). GII.4-1987 ORF2 gene was commercially synthesized by BioBasic (biobasic.com) and inserted into the VEE replicon (Lindesmith et al, 2008). VEE replicon particles (VRPs) were generated and VLPs were expressed in Baby Hamster Kidney (BHK) cells, purified and visualized by negative staining EM as described previously (Baric et al, 2002).

Serology

Between 3 and 19 serum pairs from each outbreak were tested for a four-fold increase in anti-GII.4 VLP IgG titer between acute and convalescent serum by ELISA as previously described (Lindesmith et al, 2003). Briefly, 1 µg/ml of VLPs diluted in PBS were bound to 96-well plates for 4 hrs before plates were blocked over-night in 5% Blotto. Two-fold serial dilutions of acute and convalescent outbreak patient sera were added to the plates, and human IgG specifically reacting with the VLPs was detected using mouse anti-human IgG-AP (Sigma, St. Louis, MO) and pNPP (para-nitrophenol phosphate) (Sigma). Either GII.4-1987 or GII.4-1997 VLPs were used as previous results indicated that they are antigenically very similar (Lindesmith et al, 2008). Geometric mean titers of convalescent serum were calculated by comparison to a standard curve of purified IgG. Comparisons between groups of convalescent serum specimens were done using Mann-Whitney analysis (http://faculty.vassar.edu/lowry/VassarStats.html).

HBGA binding and blockade assays

HBGA binding studies were performed under identical conditions as the HBGA blockade studies only without the addition of human sera (see below).

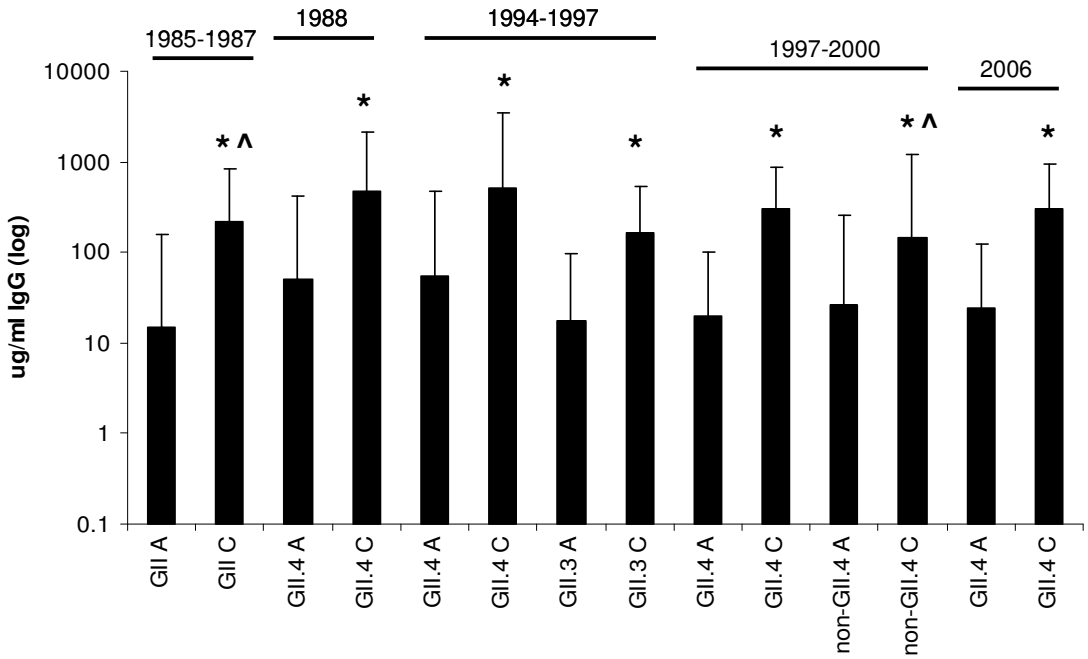
Convalescent sera from outbreak patients (110 specimens from 26 outbreaks) demonstrating seroconversion to GII.4 VLPs were subjected to a surrogate neutralization assay to measure blockade antibody titers as described previously (LoBue et al, 2006; Lindesmith et al, 2008). Briefly, GII.4-1987, GII.4-1997, GII.4-2002, GII.4-2006 and GII.3-TV VLPs (1 µg/ml) were incubated with 2-fold serial dilutions of sera at 37°C for 1 h. After washing plates with PBS-0.05% Tween 20 (PBS-T), sera/VLP sample mixture was added to Neutravidin plates (Pierce) that had been coated with 10-20 µg biotinylated HBGA (Glycotech) and incubated for 1.5 hr at 37°C. Binding or VLP blockade was detected using mouse anti GII.4-VLP sera followed by goat anti-mouse IgG-AP (Sigma) and detection by pNPP (Sigma).

By comparison to a control (VLP binding to HBGA in the absence of human sera), 50% antibody blockade titers (BT50) were determined with two-fold dilutions from 1:200 to 1:6400. Sera with a BT50 less than 0.5% (1:200) or greater than 0.016% (1:6400) were given the values of 1% and 0.008%, respectively. BT50 values were compared between groups of serum samples using Mann-Whitney analysis (http://faculty.vassar.edu/lowry/VassarStats.html).

RESULTS

Serologic relatedness among outbreak sera, 1985-2006

In all, 110 (52%) of the 210 serum pairs seroconverted to GII.4, including at least one serum sample from 26 of 27 outbreaks. Stratified by date, 37 (41%), 17 (49%), 50 (64%) and 6 (100%) of serum pairs seroconverted to GII.4 NoV VLPs in 1985-1987, 1988, 1994-2000 and 2006, respectively. Figure 5.2 indicates the percentage of persons seroconverting to GII.4 and the log concentration (µg/ml) of IgG reactive with GII.4 VLPs in acute and convalescent sera stratified by date and by genotype of the outbreak (non-GII.4 represents serum from outbreaks caused by GII.1, GII.2, GII.10 and GII.14 NoVs). For all seroconvertants, the mean convalescent IgG titer was greatest for the 1995-1997 GII.4 outbreak group (480 µg/ml). When compared to this group mean convalescent IgG titers did not significantly differ from those of any of the GII.4 groups of seroconvertants ($p \geq 0.5222$) in figure 5.2 (convalescent IgG titers 325, 447 and 297 µg/ml the 1995-1997, the 1997-2000 and the 2006 groups, respectively) or for the GII.3 seroconvertants in the 1995-1997 group (163 µg/ml IgG; $p = 0.1585$). On the other hand, the GII 1985-1987 group and the non-GII.4 group in 1997-2000 had a mean convalescent IgG titers of 215 µg/ml and 149 µg/ml which were significantly lower than that of the 1995-1997 GII.4 group ($p = 0.0193$) (Note that the IgG titer for the non-GII.4 group is higher than the titer of the GII.3 group, but the p values do not reflect this observation. This is due to one sample in the non-GII.4 group that had a convalescent IgG titer of 1081 µg/ml. If this data point was excluded from the statistical analysis, the mean IgG titer would be 76 µg/ml.)



% seroconvert	41 %	49 %	70 %	80 %	91 %	44 %	100 %
# sc / # total	37/91	17/35	14/20	12/15	10/11	14/32	6/6

* Significant increase in titer between acute and convalescent sera ($p < 0.05$)

^ Significantly lower convalescent titer compared to the 1995-1997 GII.4 convalescent titer

Figure 5.2: Log concentrations of mean IgG titers of acute and convalescent sera from outbreak patients seroconverting to GII.4 VLPs. The percentage of persons seroconverting to GII.4 (% seroconvert) and the number of seroconvertants over the total number of sera pairs tested are given (#sc / # total). Data are stratified by date and by genotype of the outbreak (non-GII.4 represents sera from outbreaks caused by GII.1, GII.2, GII.10 and GII.14 NoVs). Bars reflect mean values and error bars indicate the maximum concentration of IgG for each population.

HBGA blockade of GII.4 VLP binding to HBGA by outbreak sera

Many of the sera pairs tested by ELISA demonstrated antigenic relatedness to GII.4 NoV by cross-reacting with GII.4 VLPs. To further determine the specificity of serum antibodies for the region around the HBGA binding pocket. Convalescent sera from the 91 serum pairs seroconverting to GII.4 VLPs were further tested for their ability to block GII.4 VLP binding to HBGAs. GII.4-2006 VLP binding to HBGA could be efficiently blocked by the 2006 convalescent sera from all 6 patients involved in the 2006 outbreak. The GII.4-2006 VLP was used a positive control for HBGA blockade since it was constructed based on the NoV capsid sequence obtained from stool specimens from the 2006-Minerva outbreak from which our sera samples were derived. The 6 convalescent serum samples from the 2006 GII.4-Minerva outbreak had mean BT50 values that did not significantly differ ($p \geq 0.1499$) between the GII.4-1987 (0.18%), GII.4-1997 (0.09%) GII.4-2002 (0.07%) or the GII.4-2006 (0.06%) VLPs (Figure 5.3a).

Alternatively, we examined GII.4 VLP blockade by GII outbreak sera from a time prior to the discovery of the first GII.4 sequence (GII.4-1988; or Camberwell) to be sequenced. While the 37 sera pairs collected from patients from 1985-1987 demonstrated seroconvertion to GII.4 VLPs, the mean concentration of sera required to block VLP-HBGA binding by 50% (mean BT50) was high for all VLPs tested (0.46% for GII.4-1987, 0.31% for GII.4-1997 and 0.49% for GII.4-2002) (Figure 5.3b). The mean BT50s did not differ between GII.4-1987 and GII.4-1997 VLPs ($p = 0.0801$) or for GII.4-1987 and GII.4-2002 ($p=0.3843$), but differed when comparing GII.4-1997 and GII.4-2002 VLPs ($p=0.0053$).

As noted above, no significant differences between mean BT50 values could be observed for GII.4 VLP blockade by the 2006 outbreak sera, even though there appeared to

be a trend toward higher mean BT50s for GII.4-1987 and GII.4-1997, when compared to the contemporary GII.4 VLPs. However, if we compare the panel of mean BT50 values for the 2006 sera with the panel of mean BT50 values for the 1985-1988 sera, significant differences can be noted. The mean BT50s for the GII.4-2004 and GII.4-2006 VLPs using 2006 sera are significantly lower than all of the mean BT50s of the GII.4 VLPs using 1985-1988 sera. On the other hand, the mean BT50 values for GII.4-1987 and GII.4-1997 using 2006 sera do not differ from the BT50 values for GII.4-1987 and GII.4-1997 using the 1985-1988 sera ($p \geq 0.1416$), indicating that a higher concentration of 2006 sera is required to block GII.4-1987 and GII.4-1997 VLPs than is required for the contemporary GII.4 VLPs.

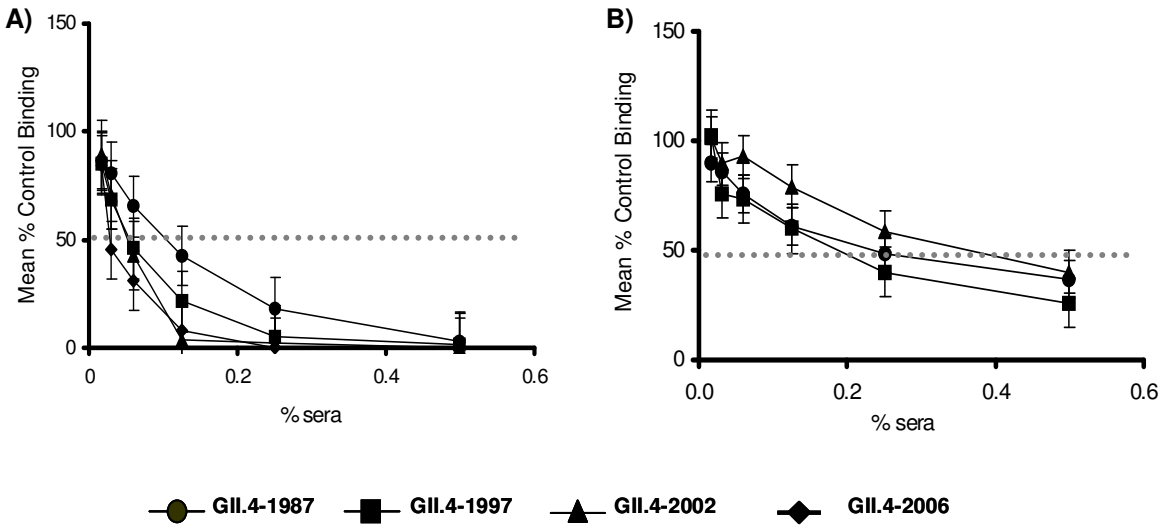


Figure 5.3: Blockade of GII.4 VLPs with A) 2006 outbreak sera 2006 (6 persons from 1 outbreak and B) 1985-1988 outbreak sera (n=37 persons from 10 outbreaks). The mean % control binding indicates the degree to which outbreak sera is capable of blocking GII.4 VLP binding to HBGA as compared to a no sera control.

Herd immunity to GII.4 NoVs

To expand upon the HBGA blockade data reported above and to find out if blockade patterns indicative of herd immunity could be revealed using outbreak sera from patients infected with GII NoVs, 65 convalescent sera specimens collected from 15 outbreaks ranging from 1988 to 2000 were tested in the surrogate neutralization assay. In addition to the 1985 (1985-1987) and 2006 outbreaks already noted, three additional outbreak years (1988, 1995, and 1999), were included, representing outbreaks occurring in July 1987-June 1988, July 1994-June 1997, and July 1997-June 2000. First, sera from outbreaks that were confirmed to be caused by GII.4 NoV (45 specimens from 8 outbreaks) were examined for their ability to block GII.4 VLP binding to HBGA (figure 5.4A). In 1988, mean BT50 values were similar for GII.4-1987 (0.27%) and GII.4-1997 (0.24%) VLPs, but differed significantly from the mean BT50 value for GII.4-2002 (0.52%) ($p \leq 0.0285$). Similarly, in 1995, the mean BT50 value for GII.4-2002 (0.24%) was greater than the mean BT50 values for GII.4-1987 (0.09%) and GII.4-1997 (0.08%) ($p \leq 0.0324$). However, in 1999, mean BT50 values did not differ between the three VLPs (0.12%, 0.09% and 0.14% for GII.4-1987, GII.4-1997 and GII.4-2002, respectively ($p \geq 0.2005$)). A clear trend toward lower BT50 values over time was demonstrated with the GII.4-2002 VLP using GII.4 outbreak sera. However, such analysis for GII.4 outbreak sera alone cannot reflect herd immunity at the population level because NoV strains other than GII.4 were in circulation during this time frame.

Outbreak sera from other GII NoV strains in circulation from July 1994-June 2000, including GII.1, GII.2, GII.3, GII.10, and GII.14, were subjected to the surrogate neutralization assay. Using the same classification scheme as above, but also including GII outbreak sera from 1985-1987 (classified as 1985), mean BT50 values were combined with

the GII.4 data and plotted over time (Figure 5.4B). In 1995, there was little change in the mean BT50 values for GII.4-1987 (0.15%), GII.4-1997 (0.1%), and GII.4-2002 (0.24%). Mean BT50 values for GII.4-2002 were significantly higher than those of GII.4-1997 ($p = 0.0036$), but not GII.4-1987 ($p = 0.1738$). For the mixed genotype data set from1999, mean BT50 values were greater than those of the 1999 GII.4 data set, for the GII.4-1987 (0.36%), GII.4-1997 (0.29%), and GII.4-2002 (0.39%), but did not significantly differ from each other ($p \geq 0.2005$).

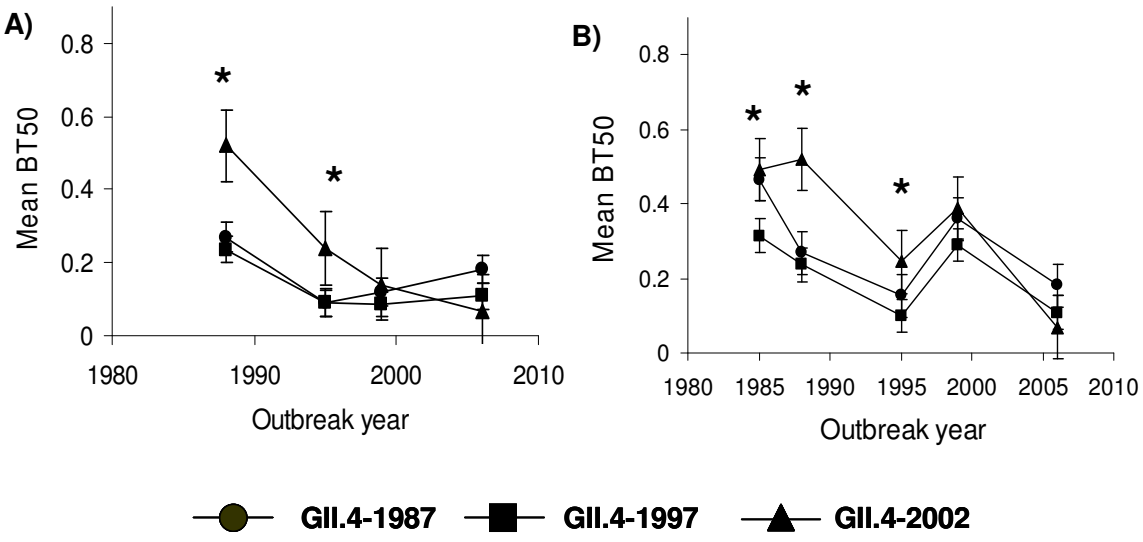


Figure 5.4: Blockade of GII.4 VLP binding to HBGA by A) GII.4 outbreak sera collected 1988-2006 and B) by heterotypic outbreak sera 1985-2006 which includes GII(unknown) samples in 1985-1987 GII.4 samples in 1988-2006, GII.3 samples in 1995, and GII.1, GII.2, GII.10, and GII.14 samples in 1999. * indicates significant differences between GII.4-1987, GII.4-1997 or GII.4-2002 VLPs within each chronological group.

Looking specifically at the ability of outbreak sera from heterotypic NoV genotypes to block HBGA binding of GII.4 VLPs, two patterns emerged. Convalescent sera from GII.1, GII.2, GII.10 and GII.14 outbreaks in 1999 did not block GII.4 VLP binding as mean

BT50 values were 0.48%, 0.38%, 0.56% for GII.4-1987, GII.4-1997, and GII.4-2002, respectively (Figure 5.5a). On the other hand, convalescent sera from GII.3 outbreaks in 1995 blocked binding of GII.4 VLPs with mean BT50 values of 0.22% for GII.4-1987, 0.11% for GII.4-1997 and 0.25% for GII.4-2002 (Figure 5.5b). None of these values differed from the mean BT50 values for GII.3 sera blockade of a control, GII.3-TV VLP (mean BT50 = 0.22%; $p \geq 0.5619$).

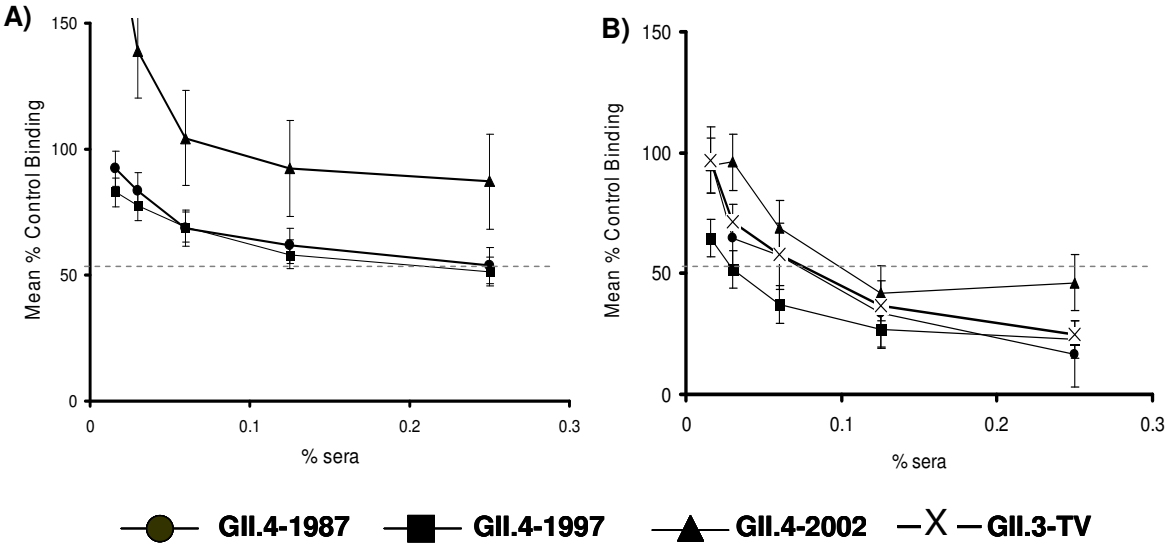


Figure 5.5: Blockade of GII.4 or GII.3 VLP binding to HBGA by A) non-GII.4 sera from 1999 or B) GII.3 sera from 1995.

Although GII.3 outbreak sera effectively blocked GII.4-1997 with a mean BT50 value (0.11%) that did not differ significantly from GII.4-1997 VLP blockade by GII.4 outbreak sera from 1995 (BT50 = 0.09%) ($p = 0.749$), the opposite was not observed. GII.4

outbreak sera from 1995 did not block GII.3-TV binding to HBGA as the mean BT50 was (0.56%) (Figure 5.6).

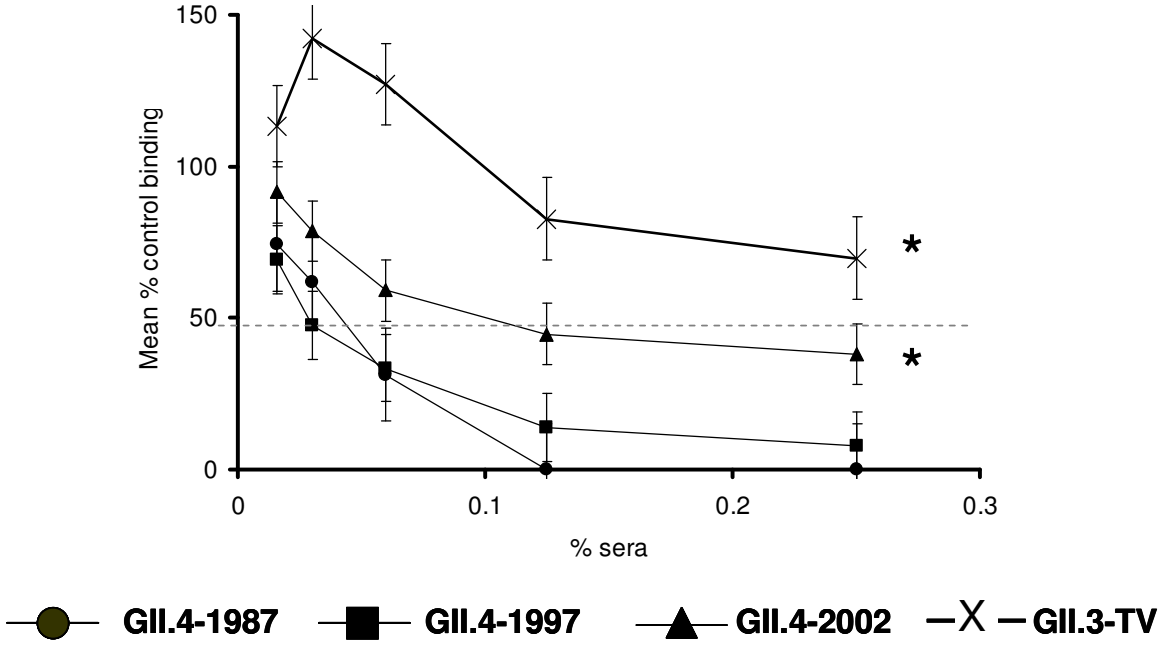


Figure 5.6: Blockade of GII.4 or GII.3 VLP binding to HBGA by GII.4 outbreak sera in 1995. * indicate statistically significant differences in BT50 values between when compared to the BT50 values of GII.4-1987 or GII.4-1997.

DISCUSSION

In this paper, we support herd immunity as the driving force for antigenic evolution of NoV GII.4 strains using sera collected during 1985-2006 outbreaks. Furthermore, we describe specific cross-reactivity between GII.3 and GII.4 strains, with sera from GII.3 outbreaks blocking GII.4 VLP binding to HBGA. We also show how convalescent sera from patients infected by other heterotypic strains (GII.1, GII.2, GII.10, or GII.14) do not block GII.4 binding to HBGA. Our results demonstrate the need for continued serologic surveillance of human NoVs as outbreak sera can be used to discriminate minute differences between strains, better define cross-reactivity between genotypes, and estimate the susceptibility of the population to an emerging NoV.

The GII.4-Camberwell strain detected in a 1987 norovirus outbreak was the first characterized GII.4 strain (Cauchi et al, 1996) and to date, no prior sequences have been obtained from GII.4 outbreaks. The GII outbreak sera in the 1985-1988 collection required high concentrations of convalescent sera to effectively block HBGA binding of GII.4-1987, GII.4-1997 and GII.4-2002 VLPs. This finding as well as the low level of seroconvertants that could be detected during this time period is evidence for low levels of GII.4 circulation in the population between 1985 and 1987. However, we cannot rule out the possibility that ancestral GII.4 strains antigenically dissimilar to the GII.4 VLPs used in this study circulated during this time.

During the 1995-1996 winter season, epidemic proportions of GII.4 NoV outbreaks were reported worldwide (Noel et al, 1999; Vinjé et al, 1997). The following year, GII.4 NoVs continued to circulate, although in diminishing proportions, in the US and in Europe (Noel et al, 1999; Koopmans et al, 2000; Fankenhauser et al, 2002). This was followed by a

five year period of relative quiescence where different NoV genotypes co-circulated without any apparent epidemic pattern (CDC, unpublished). In 2002, a new GII.4 strain (Farmington Hills) emerged, causing the second reported NoV pandemic (Widdowson et al, 2004; Lopman et al, 2004a). We compared antibody blockade patterns from sera collected from outbreaks spanning the 1995-1996 pandemic (July 1994-June 1997) with sera collected from outbreaks during a period of relatively low GII.4 circulation (July 1997- June 2000). The 1994-1997 sera showed a blockade pattern expected for a population recently exposed to GII.4-1997 but unexposed to the GII.4-2002 virus. All sera blocked binding of GII.4-1997 VLPs (and the antigenically similar GII.4-1987 VLPs) to HBGA, but were less capable of blocking binding of GII.4-2002 VLPs to HBGA. However, during the period between 1997 and 2000, outbreak sera from this study blocked all GII.4 VLPs to the same degree, even though the GII.4-2002 strain had not emerged.

During the peak of the GII.4 NoV epidemic in 1995-1996, 69% of the NoV outbreaks were caused by GII.4s (Noel et al, 1999). The following year, the number of GII.4 outbreaks dropped to 31% but the number of GII.3 outbreaks increased from about 4% in 1995-1996 to about 25% in 1996-1997 (Noel et al, 1999). We showed that convalescent GII.3 outbreak sera effectively blocked GII.4 VLP binding to HBGAs to the same degree as it blocked GII.3 VLP binding to HBGA. However, structural relationships between GII.4 and GII.3 are complex, as GII.4 sera from the 1994-1997 outbreaks did not block HBGA binding of the GII.3-TV VLP, suggesting the sharp rise in the number of GII.4 outbreaks in 1996 could not prevent the rise in GII.3 outbreaks the following year. But since GII.3 outbreak sera blocked the binding of GII.4-1997 VLPs to HBGA, the concurrent rise in the number of GII.3

outbreaks with continuing GII.4-1997 circulation in 1996-1997 could have helped generate sufficient herd immunity, leading to the quelling of the 1996 GII.4 epidemic.

In the years leading up to the next GII.4 epidemic in 2002, GII.4 strains circulated at relatively low levels, causing only about half of the total number of NoV outbreaks annually (Lopman et al, 2003b). By 2000-2001, GII.4 NoVs were detected in only 16% of outbreaks, but increased to 61% the following winter season (Blanton et al, 2006). About 50% of our 1998-2000 sera specimens were from GII.4 outbreaks and the others were from other GII genoytpes. Serum from non-GII.4 outbreaks was unable to block the binding of GII.4 VLPs to HBGA. A possible explanation is that these viruses share some common capsid epitopes with GII.4 VLPs, but likely lacked specific neutralizing epitopes as the heterotypic sera blocked GII.4 VLPs to a lesser extend compared to the homotypic sera. Therefore, the serologic response against the heterotypic GII strains examined in this study (GII.1, GII.2, GII.10 and GII.14) does not likely contribute to herd immunity against GII.4 NoVs. However, the GII.4 outbreak sera from our 1998-2000 sample set blocked binding of GII.4-1987, GII.4-1997 and GII.4-2002 VLPs. Previous exposure to strains in circulation during that time period that were antigenically similar to GII.4-2002 (CDC, unpublished data) is a possible explanation of this observation. However, even if the 1998-2000 population was exposed to such a strain, the proportion was still too low to generate sufficient herd immunity to prevent the 2002 epidemic.

The 2006 GII.4 outbreak sera blocked HBGA binding for all GII.4 VLPs tested. Therefore, persons recently exposed to GII.4-2006 would likely be protected from infection by GII.4-1987, GII.4-1997, GII.4-2002 and GII.4-2006. While it is possible that all persons in the 2006 GII.4 outbreak had been previously exposed to all of the GII.4 strains used in this

study, the ability to block the chronologically older strains after a recent infection with a contemporary strain does not necessitate infection by the older strain. Mouse antisera raised against GII.4-2004 and GII.4-2005 strains block both GII.4-2002 and GII.4-1997 VLPs (Lindesmith et al, 2008). Some cross-reactive epitopes of chronologically older GII.4 strains may be conserved in contemporary GII.4 strains. Infection by an emergent strain will likely confer protection from strains of recent years and may provide some level of protection to strains of previous decades.

This study has many limitations because it was designed retrospectively and the specimens used in our analysis were drawn from a subset of the population that may not be representative of the US population. Because norovirus outbreak surveillance is passive, the samples received by CDC may be biased towards large scale outbreaks or outbreaks with more severe clinical symptoms. In addition, we do not have exposure history information for the outbreak patients in our study and thus we do not know how much of the measured antibody reactivity is a reflection of a memory response from previous NoV infections. The seroconvertants included in this study had GII.4 NoV cross-reactive antibodies with titers ranging from 1 µg/ml to 419 µg/ml in their acute sera, but none significantly blocked GII.4 VLPs when a subset was tested. This provides evidence for the apparent lack of a recent GII.4 NoV infection, but does not exclude any possible contributions from a memory response.

In addition, only convalescent serum samples were available for our analyses, as no salivary or mucosal specimens were available, and fecal immunoglobulins are not appropriate due to the relatively poor detection limit of the surrogate neutralization assay (LoBue et al, 2006). While an early salivary IgA response is correlated with protection from

Norwalk virus but not Snow Mountain Virus (SMV) (Lindesmith et al, 2003; Lindesmith et al, 2005), the contribution of serum IgG in the immune response against NoV infection remains unclear. However, at lower levels, mucosal antibodies are produced after vaccination and natural infection. In addition, IgG in serum can transfer to the gut via transudation and IgG from the gut and spleen are capable of blocking 100% of VLP binding to HBGA (LoBue et al, 2006).

Currently there is no vaccine or antiviral therapy for controlling NoVs. Efforts have been significantly hampered by the lack of an in vitro culture system or small animal infectivity model. The most promising avenues of research have been using NoV replicons in mammalian cell culture for testing antiviral efficacy (Chang and George, 2007) and the development of recombinant VLP vaccines (LoBue et al, 2006). Several studies have indicated that humoral, mucosal and cellular (Th1/Th2-like) immune responses can be elicited following inoculation of rVLPs in mice (Ball et al, 1998; Guerrero et al, 2001; LoBue et al, 2006; Periwal et al, 2003; Nicollier-Jamot et al, 2004) or upon feeding with transgenic tobacco, tomato or potato plants (Mason et al, 1996; Zhang et al, 2006) or yeast extracts (Xia et al, 2007) that express NoV rVLP proteins. Development of a human vaccine administered with adjuvant using multivalent rVLPs may be possible for preventing the majority of circulating NoV infections. However, with our current knowledge of NoV evolution, such a vaccine will likely have to be updated every 1-2 years, in a fashion similar to that of the influenza vaccine, in order to protect against emergent strains (Lindesmith et al, 2008).

This study has provided us with serologic data to support herd immunity against GII.4 NoVs. Using a surrogate neutralization assay, we were able to uncover minute antigenic

differences of NoVs that cannot easily be distinguished by phylogenetic analysis or basic serologic assays (ELISA). This study suggests a need for continued serologic surveillance for human NoVs. Surrogate neutralization data along with outbreak sera can help us identify antigenically distinct, emerging GII.4 strains that may begin circulation toward the end of an epidemic, as neutralization assays help identify influenza virus vaccine candidates. Well designed prospective studies including serologic and HBGA blockade studies, will become increasingly important as VLP vaccines for human NoVs become a reality.

ACKNOWLEDGEMENTS

We would like to thank the Gastroenteritis and Respiratory Viruses Laboratory Branch at the Centers for Disease Control, Atlanta, GA, and Atlanta Research and Education Foundation, Decatur, GA, for funding this research (Grant G01).

We would also like to thank Boyd Yount, UNC-Chapel Hill, for technical assistance and work in production of VEE replicon particles and scientific discussions with Dr. Charles LeBaron, CDC, Atlanta. We would also like to extend our appreciation for members of the GRVLB at the CDC, Atlanta, including Nicole Gregoricus, DuPing Zheng, Susan Owen-Edwards and Jon Gentsch.

CHAPTER 6

FINAL DISCUSSION

Human NoV survival on environmental surfaces is likely to have contributed to transmission of the viruses during outbreaks on cruise ships (Widdowson et al, 2004; Isakbaeva et al, 2005), hotels (Cheesbrough et al, 2000), hospitals (Green et al, 1998), and long-term care facilities (Wu et al, 2005). Waterborne outbreaks reported from contamination of drinking water, recreational waters or ice (Hoebe et al, 2004; Khan, et al, 1994; Maunula et al, 2004), and foodborne outbreaks resulting after contamination of irrigation waters and shellfish harvesting waters (Berg et al, 2000; Shieh et al, 2003) further exemplify NoV environmental stability. Like other enteric viruses that lack a lipid envelope (such as poliovirus (PV-1) and hepatitis A virus (HAV)), NoVs have a durable capsid structure allowing them to withstand temperature ranges from -80°C up to ~60°C and survive at low pH (2.7 for 3 hr) (Green, 2007). Unlike poliovirus and HAV, NoVs are not yet preventable by vaccine and continue to be the leading cause of adult AGE.

In the absence of a true infectivity model for human NoVs, several surrogate models have provided information about the fate of infectious virus after exposure to physical or chemical inactivating treatments or disinfectants (Doultree et al, 1999; Duizer et al, 2004; Tree et al, 2005). Distantly related caliciviruses, such as FCV, have classically been used as surrogates for NoV in such studies. The results of our studies suggest MNV, the first norovirus to be cultured, is a more relevant surrogate for human NoV than FCV. MNV is not

only more closely related to human NoV (Wobus et al, 2004), but like other enteric viruses, is stable at a low pH. Our studies also indicate that MNV survives longer than FCV at ambient temperatures.

While MNV is structurally more similar to human NoV than FCV, a recent publication by Katpally et al. (2008) revealed significant structural differences between the native MNV crystal structure and that of the Norwalk virus (GI.1) VLP. The P domains of the MNV particle are rotated about 40° clockwise and form what appears to be a second layer about 16Å above the shell domain of the capsid structure. Extensive interactions between the P1 and P2 domains lift the P1 domain toward the surface, forming a pocket between the S and P domains which in turn causes a "swelling" of the capsid structure, increasing its radius by about 10%. In contrast, the P domains of the Norwalk VLP do not form such extensive interactions such that the P1 domain occupies the space between the S and P2 domains. It remains to be seen if native Norwalk virus particles would show a similar conformation, if this is a property of norovirus maturation, or perhaps a structure unique to MNV.

Human NoVs bind to HBGAs, and monoclonal antibodies (mAb) mapped to the P2 subdomain near the receptor binding domain of the capsid are capable of blocking receptor binding (Cao et al, 2007; Lochridge et al, 2005). Similarly, a neutralizing mAb maps to the P2 subdomain of MNV (Lochridge and Hardy, 2007). This suggests that like human NoVs, MNV may have a receptor binding site in the P2 subdomain of the capsid. Although MNV exhibits tropism for cells of the hematopoietic lineage (such as macrophages and dendritic cells), MNV VLPs do not bind to any synthetic human HBGAs tested so far (personal communication, Lisa Lindesmith). Monocytic cells are capable of phagocytosis of microbes without specific receptor interaction so it is possible that MNV does not enter the cell by the

same mechanism as human NoVs. Once the mechanisms of cell entry are delineated, it will be important to compare and contrast such mechanisms of MNV and human NoV attachment and entry into cells in order to validate the use of MNV as a model system for studying the behavior of human NoV.

In the absence of a cell culture system for human NoVs, we demonstrate that a novel carbohydrate magnetic separation (CMS) method using HBGAs that bind to complete Norwalk virus particles may be an excellent method for concentrating infectious virus from complex sample matrices, followed by detection of viral RNA. This method proved applicable to environmental water. Furthermore, HBGA bound viruses were resistant to treatment with RNase A, suggesting the viruses detected were intact and potentially infectious.

We provided evidence that CMS can by applied for specific binding of Norwalk (GI.1) virus to H type 1 HBGAs. The P2 region of the capsid protein is encoded by the most hypervariable region of the genome which corresponds to the receptor binding region. Changes in this region result in changes in receptor binding patterns (Lindesmith et al, 2008), creating diversity in binding among different norovirus strains. Whether or not CMS will be applicable to the concentration and purification of other NoV genogroups will likely determine its usefulness.

While an overwhelming majority of NoV outbreaks continue to be reported as GII.4s, NoVs of genogroup I (GI) are more often detected in environmental waters, in association with shellfish or wastewater effluents (Bon et al, 2005). Detection of GI viruses in such harsh environments raises the question whether GI NoVs are actually more robust survivors than are GII.4 viruses.

Once validated for detection of other NoV genotypes, the CMS surrogate may be used to characterize differences in stability between NoV genotypes. Disinfectants or other inactivating treatments can be applied prior to detection with CMS to estimate NoV infectivity. Furthermore, we can compare the MNV and CMS-binding models to determine if loss of infectivity in the MNV model correlates to loss of detection by CMS. We expect all treatments that result in capsid protein damage will likely correlate with the infectivity model. However, since MNV does not bind to any of the synthetic HBGAs tested to date, and human NoV cannot yet be cultured, the two methods cannot be directly compared. As an intermediate between the two surrogate assays, an immunomagnetic separation (IMS) technique using monoclonal antibodies against human NoV and MNV could be applied. Using all four methods (CMS, both IMS assays and the MNV plaque assay), we can potentially make a more robust assessment of how CMS relates to NoV infectivity. Further, we can clarify genotypic differences of human NoV survival and inactivation.

Another interesting and relevant question focuses on the origins and evolution of the epidemic GII.4 NoVs. Our group has recently described GII.4 NoV evolution by antigenic drift in response to herd immunity in the population (Lindesmith et al, 2008). In studies described here, we provide evidence that sera collected from patients involved in NoV outbreaks over a 21 year period (1985-2006) support this theory. On the other hand, GI.1 viruses have changed very little over time as very few amino acid changes occurred in the P2 region of the genome of the NoV GI 8FIIa strain (first detected in 1968) compared to a 2001 GI.1 outbreak strain. Further, convalescent sera from a human challenge with the 8FIIa strain blocked HBGA binding of GI.1-1968 and GI.1-2001 VLPs (designed to reflect the 1968 and 2001 GI.1 strains, respectively) equally well (personal communication with Lisa

Lindesmith). Amino acid changes in the receptor binding region of GII.4 capsid structures can create changes in the repertoire of HBGAs they can bind. Since the genes encoding human HBGA catalization are polymorphic, such changes in receptor binding repertoire could allow for the virus to reach previously inaccessible populations (Lindesmith et al, 2008). The GI.1-1968 and GI.1-2001 strains do not appear to differ in their receptor binding patterns (personal communication with Lisa Lindesmith), indicating the populations they are capable of infecting, have either not changed in over 30 years or there has been a reversion of the contemporary strain to become antigenically similar to the 1968 GI.1 NoV. On the other hand, GI.1 NoVs may not evolve in the same manner as GII.4 NoVs because they are limited by the types of amino acids changes possible with maintenance of viral infectivity. If subtle amino acids changes lead to capsids that do not fold properly and/or are nonfunctional, the virus is unlikely to be able to initiate infection.

One hypothesis for why such disparities in evolutionary change are occurring between GI.1 and GII.4 NoVs has to do with the types of HBGA receptors each virus uses. In addition, GI.1 VLPs and native viruses bind to HBGAs strictly of "secretor" lineages, which is dependent on the polymorphic *fut2* gene product. Specifically, GI.1 VLPs do not bind to HBGAs of the H type 2 lineage that are not typically under control of the FUT1 enzyme. Several GII.4 strains bind to HBGAs of the H type 2 lineage (Le^y), and at least one strain binds to HBGAs expressed only by "non-secretors". If we hypothesize that strains binding to non-polymorphic ligands have a higher virulence than do strains binding to polymorphic HBGAs because they are not restricted by a genetically susceptible population, GII.4 strains might be considered more "virulent". Under this hypothesis, GI.1 strains might

be shed longer and contribute to more asymptomatic shedding. Such differences in virulence may contribute to evolutionary disparities.

Alternatively, a general biological concept is that microbes (viruses) that bind too well to their receptor(s) are less successful at the population level and less virulent than those that have a weaker binding affinity. We know that native GI.1 viruses from stool readily bind HBGAs, as an input as low as 3 NoV RNA copy numbers can be detected by RT-PCR. However, input levels approaching 5,000-10,000 NoV RNA copy numbers of GII.4 NoV from stool must be used in order to be detected by the HBGA binding assay (unpublished data from the Vinjé lab). Strains of polyoma virus that bind with less affinity to their carbohydrate receptor are readily released from cell debris after lytic infection and are more virulent than are their high affinity binding counterparts (Stehle and Harrison, 1996). Like polyoma viruses, human NoVs may also interact with cell debris. But since saliva and other mucosal secretions of the gastrointestinal tract are known to contain HBGAs, NoVs may be coated with HBGAs before reaching the cells of the intestines. Viruses that bind with low affinity to salivary or mucosal HBGAs may be easily dissociated from the carbohydrates after passing through the stomach or in the intestines, allowing them to attach to cell surfaces. Alternatively, those that bind with high affinity would still be bound in the small intestine, leaving few epitopes for HBGA binding in the gastrointestinal epithelial cells. If GI.1 viruses truly bind with higher affinity to HBGAs, they might be considered less virulent and perhaps follow a different evolutionary pattern.

In our serologic studies, we found antigenic similarities between GII.3 and GII.4 NoVs, but few antigenic similarities between GII.4 and GII strains other than GII.3. Epochal evolution has not yet been described for any GII strain other than GII.4. However,

preliminary analysis of amino acid sequences published on GenBank spanning the years 1983 to 2002 indicate that, like GI.1 NoVs, significant changes in the capsid of GII.3s do not seem to occur over time (unpublished data from the Vinjé lab). Outbreaks of GII.3 NoVs are not as commonly reported as GII.4 NoVs, but a high frequency of sporadic illnesses caused by GII.3 NoVs have recently been reported in Hong Kong, Australia, and Canada (Kirkwood and Bishop 2001; Lau 2004; Lee et al, 2008). A more in-depth analysis of the similarities and differences between these two genotypes may be helpful to identify structural, antigenic and evolutionary differences between GII.4 and GII.3 NoVs that may provide an indication to why GII.3 NoVs appear to cause more sporadic illnesses than outbreaks.

In this study, we addressed unique capsid properties of human NoV that seem to be associated with environmental stability and receptor binding and hence contribute to their persistence in the environment and in the human population. To do this, we used two surrogate assays to measure human NoV infectivity. Using the MNV model, we documented the ability of a culturable NoV to survive in extremes of pH for at least 2 hrs and in suspension and on surfaces for at least a week. In addition, we demonstrated that the MNV infectivity assay is a more relevant model than the classically used FCV model when evaluating virus resistance to extremes of pH. This may mean that it is a better surrogate for human NoV behavior under the same conditions. Using the CMS method, we hypothesize that binding of human NoVs to their HBGA receptors may be a surrogate for detecting potentially infectious virus. Furthermore, we showed that this method could be used to prepare environmental waters for virus detection using RT-PCR. Lastly, we described how human outbreak sera pairs can be used to investigate the mechanism of GII.4 evolution by antigenic drift in response to herd immunity in the population based on the use of a surrogate

neutralization assay. We examined antigenic similarities and differences among heterotypic NoV genotypes, with emphasis on GII.3 NoVs, to make predictions about their contribution to GII.4 evolution.

The results of this study provide important information for designing strategies for prevention and control of human NoVs, and for making recommendations for outbreak management. Using the surrogate infectivity models described here, we should be able to evaluate the efficacy of disinfectants or other antiviral treatments commonly used for hands, food products, environmental surfaces or water. From our serologic studies using the surrogate neutralization assay, we predict that prospective studies using human outbreak serum pairs can help us identify antigenically-distinct human NoV strains appearing toward the end of an epidemic season. Once a framework has been laid, as has been done for influenza virus vaccine production, such information will be important for predicting potential epidemic strains prior to their emergence, which may facilitate the design of effective NoV vaccines in the future.

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