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Kyung Park Date

Serum Hemagglutination Inhibition Assay as a Method for Studying the Dynamics of Immune Response to Norovirus Infection

By

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Master in Public Health

Global Health

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B.S., Emory University, 2009

Thesis Committee Chair: Amy E. Kirby, Ph.D., MPH
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Abstract

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By Kyung Park

Norovirus infections are a common cause of epidemic gastroenteritis. It is estimated that over 50% of all gastroenteritis outbreaks worldwide are attributable to norovirus. Norovirus infection is typically acute, mild, and of short duration. However, the disease can present much more severely with longer duration in infants, the elderly, and immune-compromised individuals. Despite norovirus being recognized as a leading cause of major global epidemics, immune response to infection remains poorly understood. In this study, serum hemagglutination inhibition (HAI) assay was used to measure the immune response of 43 volunteers challenged with Norwalk virus (GI.1) and 15 volunteers challenged with Snow Mountain virus (GII.2). Hemagglutination inhibition activity was observed from all 13 of the infected volunteers of the 43 challenged with GI.1, and all 9 of the infected volunteers of the 15 challenged with GII.2. HAI titers increased significantly for individuals infected with either virus. The magnitude of increase was correlated with the magnitude of increase in α-norovirus serum IgG concentration, infection status and presentation of symptoms. Baseline titers were not associated with protection from infection or presentation of symptoms. Although the amount of viral shedding among infected volunteers was found to be associated with HAI titer in the Snow Mountain virus challenge group, there was no significant association found between the amount of viral shedding and HAI titer for the Norwalk virus challenge group. There was no evidence of correlation between duration of viral shedding and HAI titer in either challenge study groups.

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Literature Review

Norovirus background and history

First described by Dr. John Zahorsky in 1929 as the 'winter vomiting disease', norovirus infections are the leading cause of viral gastrointestinal illness among adults and children around the globe[1]. Following the initial report by Zahorsky's team, studies of gastroenteritis outbreaks with similar clinical features were widely suspected to be due to viral infections. However, it was not until 1972 that the virus was first identified from a previous 1968 outbreak at a school in Norwalk, Ohio[2]. Using electron microscopy, noroviruses were identified and confirmed in the stools of infected elementary students in Norwalk, hence the archetype virus was coined as Norwalk virus (NV)[3]. After identifying the causative agent, many other Norwalk-like agents have since been discovered. Noroviruses were formerly recognized as Norwalk-like viruses (NLVs) and small round structured viruses (SRSVs), but are now collectively identified as noroviruses (NoVs)[4].

Over the past several decades, difficulty in the detection of the pathogen has limited the progress of norovirus studies. The lack of available, sensitive, and routine diagnostic methods has impeded the efforts to identify the virus. Recent advances in dissecting the molecular biology of noroviruses, coupled with developments of novel diagnostic techniques, have substantially benefitted our knowledge of the worldwide burden of disease due to noroviruses [4].

Virus Classification: Norovirus Structure and Genetic Diversity

Structural Characteristics

Noroviruses are small, and non-enveloped, and are distinguishable by their structural characteristics. The genus Norovirus is in the family Caliciviridae, which is characterized by a positive-sense, single-stranded, non-segmented RNA genome [5, 6]. Norovirus genomes are at least 7.5kb, and are enclosed within an icosahedral capsid approximately 30 to 38 nm in diameter [6, 7]. They are also distinguished by a poly-adenylated tail at the 3' end, and a 15-kDa genome-linked viral protein (VPg) that is covalently attached to the 5'-end [8, 9]. The norovirus VPg functions as a primer in viral RNA replication[10].

Norovirus genomes are typically organized into three open reading frames (ORFs). ORF1 encodes an RNA polymerase, and non-structural proteins: a helicase and protease [7]. ORF2 encodes the major viral capsid protein, VP1 [7]. ORF3 encodes a highly basic minor capsid protein, VP2 [7, 8]. The 5' region of the norovirus genome encodes all of the nonstructural proteins in ORF1, while the 3' region encodes the major and minor structural proteins in ORF2 and ORF3, respectively [5, 11, 12].

Genetic Diversity and Classification

Until recently, noroviruses have been genetically classified into six genogroups (GI, GII, GIII, GIV, GV, and GVI), three of which (GI, GII, and GIV) contain primarily human viruses [7, 13]. Viruses belonging to GII, particularly GII.4, have emerged as the predominant strain in the human population over the last decade[14]. However, multiple porcine noroviruses have also been placed in GII [15-17]. GIII and GV contain bovine noroviruses and murine noroviruses, respectively [18-22]. GVI primarily infects canines,

but recent findings suggest that canine norovirus may infect humans [23]. The genogroups are further subdivided into at least 31 distinct clusters or genotypes – 8 GI, 19 GII, 2 GIII, 1 GIV, and 1 GV genotypes [5, 7]. This nomenclature system of genogroups was based on the homology of the major VP1 capsid protein sequence in ORF2 [7]. However, the identification of new strains and recombinant viruses has ushered in a proposal for a new system that better addresses the continuing emergence of norovirus lineages. In 2013, Kroneman et al., proposed a dual nomenclature system that utilizes both ORF1 and VP1 sequences for classification [24]. This system suitably acknowledges the rise in prevalence of recombinant strains.

Noroviruses are also commonly referred to by their genogroup, along with their genotype. The Snow Mountain (SMV) strain is a GII.2 strain, as SMV belongs in genogroup II, genotype 2[25, 26]. Noroviruses contain a wide degree of genetic variability in terms of their genogroup and genotype. Members within a genogroup differ by approximately 45.0-61.4% in their capsid genes, members within a genotype differ by 14.3-43.8%, and strains within a genotype differ by 0-14.1% [7]. Strains with differences above 61.4% may represent a new genogroup. The range of differences between genogroup and genotype demonstrates the high genetic and antigenic diversity among noroviruses. Noroviruses have a high variation of intra-genus range compared to other genera of positive-sense, single stranded RNA virus families [7].The expansive genetic diversity of noroviruses is likely a factor that prevents the generation of a broadly-protective norovirus immunity, either by natural infection or by vaccination.

Global Impact of Norovirus

Burden of Norovirus in Developing Countries

Researchers in the 1970s and 1980s demonstrated that noroviruses, notably Norwalk virus, were distributed worldwide, but methods to detect these viruses at that time were limited to a few diagnostic procedures [1, 27, 28]. Electron microscopy and radio-immunoassays of stool and serum samples, respectively, were the primary diagnostic tools for detecting Norwalk virus [27, 28]. Data from more recent surveillance studies suggest there is varied distribution and diversity among circulating noroviruses in populations of developing countries.

Many molecular epidemiologic studies have shown that norovirus infections are especially prevalent among pediatric populations in developing nations. In resource poor settings, the burden of norovirus infections affects both pediatric and adult populations. However, younger children are especially affected by repeated episodes of acute gastroenteritis [29]. Through a passive community and hospital pediatric diarrhea surveillance program in Leon, Nicaragua, a study of 542 stool samples collected between March 2005 and February 2006 suggested that norovirus is an important etiological agent of acute diarrhea among children in Nicaragua. 65 of 542 samples were found to have norovirus, with most strains (88%) belonging to genogroup II [30]. In a hospital-based study of gastroenteritis in Vellore of Southern India, 350 fecal samples were studied from children with gastroenteritis. Norovirus infection accounted for 15.1% of hospitalized children in this surveillance study [31]. In a longitudinal community cohort study between March 2003 and March 2006, 442 children aged 0 to 5 years in a rural town in the Peruvian Amazon were randomly selected for stool samples. Within the cohort, symptomatic norovirus infection was highly associated with age and the odds ratio for infection fell by

2.8% per month [32]. In a 2005 pediatric hospital study of 260 children under 5 years with acute gastroenteritis in Kurdistan, Iraq, 78 (30%) tested positive for norovirus [33].

In some populations, especially in developing countries, antibodies appear to be acquired more rapidly in children. The percentage of children 0 to 5 years of age from developing nations with detectable Norwalk antibody in serum was significantly higher than that of children from the United States. Greater than 70% of children from Bangladesh and Ecuador had serum antibody to Norwalk virus, whereas it was demonstrated in less than 20% of children in the United States [27]. This finding may indicate that norovirus infection occurs earlier in life in developing countries. As evidenced by these studies, noroviruses are recognized as significant health risks in the pediatric populations of developing countries.

While children are especially affected by repeated episodes of acute gastroenteritis, the burden of norovirus infections on the adult population in developing countries is also cumbersome. In a multiple nation serum sample study of adults and children, findings indicated that norovirus infections are relatively ubiquitous in age and geography. Serum samples were obtained from donors aged 0 to greater than 50 years from Bangladesh, Belgium, Ecuador, Nepal, Switzerland, United States and Yugoslavia. The prevalence of serum antibody to Norwalk virus was present in all age groups. There was no striking difference in prevalence among adult age groups between developed and less developed countries, with antibody prevalence ranging from 53.9 to 89.7%[27]. Some studies, like those conducted in Ho Chi Minh City, Vietnam, Cheonan, South Korea, and Belèm, Brazil, found that norovirus infection recurs in the population on a seasonal basis, indicating that the virus remains a persistent global threat [34-36].

A meta-analysis and systematic review of 293 unique articles conducted by Ahmed et al., concluded that norovirus infection is a wintertime phenomenon, at least in the temperate northern hemisphere [37]. In the southern hemisphere, conflicting findings on seasonality have made it difficult to determine conclusive patterns. Surveillance studies in Argentina, Australia, Brazil, Cameroon and Malawi, have found winter months to have marked peaks of infection [36, 38-41]. Other studies in Australia and Madagascar have found that warmer months were associated with higher norovirus infections, while studies in New Zealand and Peru have found little to no seasonal correlation [42-45].

Burden of Norovirus in Developed Countries

The burden of norovirus infections is not limited to populations of developing nations. Norovirus surveillance studies have provided comparisons of the estimates of disease burden in various developed countries. According to the Centers for Disease Control and Prevention (CDC), noroviruses cause 19 to 21 million cases of acute gastroenteritis each year, resulting in 400,000 emergency department visits, 56,000 to 71,000 hospitalizations, and 570 to 800 deaths in the United States [46]. Noroviruses are also the most common cause of foodborne-disease outbreaks in the United States. Data from active and passive surveillance of foodborne gastrointestinal illness in the United States showed that most (58%) illnesses were caused by norovirus[47]. A European surveillance network set up to study foodborne viruses across 10 different countries found that norovirus was responsible for over 85% of all non-bacterial outbreaks reported from 1995 to 2000 [48].A regression analysis conducted from surveillance data in England and Wales between the years 2001 and 2006 projected that 20% of deaths caused by infectious intestinal disease in people ≥65 years were associated with norovirus infection [49]. It is

estimated that approximately 80 deaths each year may be associated with norovirus infection for this age group in just England and Wales. With the emergence of a novel predominant norovirus variant of genogroup II.4, circa 2002, increases in outbreaks have occurred across all collaborating countries of The European Foodborne Viruses Network [48, 50].

Norovirus surveillance studies compare outbreaks and provide approximations of disease, while norovirus outbreak investigations help provide information on the source of disease. An investigation of a large-scale norovirus outbreak in six nursing homes in the Tel-Aviv district of Israel during 3 weeks in 2002 found that person-to-person spread was suspected in all nursing homes. The outbreak affected 246 residents and 33 staff members, resulting in five fatal cases associated with the viral infection [51]. In another investigation, norovirus outbreaks occurred in 236 elderly healthcare facilities in Japan between 2004 and 2005, resulting in 12 fatal cases reported in six prefectures[52]. In an investigative report conducted by the Victorian Infectious Diseases Reference Laboratory (VIDRL), norovirus was identified in 30 of 59 gastroenteritis outbreaks in the state of Victoria, Australia in 2001 by RT-PCR or electron microscopy (EM)[53].

Although it is clear from these surveillance and outbreak studies that acute gastrointestinal illness due to norovirus is a significant burden in both developed and developing countries, there still exists a substantial amount of underreporting which fails to accurately measure the true burden. This is possibly attributed to the lack of a norovirus surveillance network to report to in several geographic locations, the challenges in laboratory detection, and the reluctance to seek medical care due to a mild or brief presentation of illness.

Epidemiology of Norovirus: Epidemiology, transmission and clinical presentation

Epidemiology

Norovirus causes worldwide outbreaks in various epidemiological settings, which includes hospitals, nursing homes, schools, and a variety of social settings like restaurants and cruise ships[53-56]. In many cases, norovirus infections commonly occur in dense populations and demonstrate expansive variability in outbreak setting. In the United States, recent outbreaks have demonstrated the variety of settings affected by norovirus. Outbreaks have occurred on cruise ships [57], college campuses [58], in restaurants [59], geriatric long-term care facilities [60-64], hospital wards [55, 65], schools [66], and military settings [67-69]. The Australian VIDRL investigation revealed that during 2001 norovirus outbreaks occurred in hostels/nursing homes (27%), hospitals (13%), youth refuges (3%), social gatherings associated with food consumption (27%), school outings/camps (13%), and pre-school/child-minding centers (17%) [53].

Documented foodborne norovirus outbreaks derive from a range of sources, which includes contaminated drinking water[70-72], ice consumption [73], raspberries [74, 75], delicatessen meats [76], wedding cakes [77], and shellfish [78-85]. Little research has been done on the detection of norovirus in foods other than shellfish due to the time-consuming, and often unsuccessful, nature of detecting causative agents of foodborne diseases, especially norovirus . In one study, however, feline calicivirus was used to determine virus persistence on lettuce, strawberries, ham and stainless steel surfaces. Infectious virus was recoverable until day 7 from lettuce, ham and stainless steel. Statistically higher titers of calicivirus were recovered in ham over a 7 day period compared to titers recovered from lettuce, strawberries, and stainless steel surfaces [86].

Data from active and passive surveillance of foodborne gastrointestinal illness in Belgium also found that norovirus is the most detected agent in foodborne outbreaks. Data collected in Belgium from 2000 to 2007 of forty foodborne and waterborne norovirus outbreak events revealed that 42.5% of these events were linked back to food handlers[87]. These findings were in accord with internationally recorded data of food handlers commonly being responsible for norovirus transmission events [88-90]. The CDC estimates that nearly 50% of all outbreaks of norovirus infection are related to ill food industry workers who remain on work premises while ill [90].

Transmission

Norovirus infection occurs in all age groups and its transmission occurs mainly through the fecal-oral pathway. There are multiple mediums that can facilitate its transmission; food and water, person-to-person, and environmental contamination [53, 54, 56]. With an estimated median infectious dose of 18 viruses, a minimal amount of fecal matter containing norovirus can pose a risk of transmission via hands, food, objects, aerosols, or water [90, 91]. Outbreaks of norovirus infection have high attack rates and secondary transmission rates, which is likely due to its low infectious dose and its ability to survive for extended periods of time [90, 92]. One study of 99 NoV-infected subjects showed that viral shedding can occur for as long as 3 weeks in certain individuals [93]. This was seen in 26% of infected subjects in the study, however, the proportion was higher in children <1 year of age. In addition, Moe et al. observed that approximately 20% of people infected with norovirus do not display symptoms [90]. Some literature suggests that it is possible for asymptomatic individuals to unknowingly shed virus and infect others without having knowledge of ever being infected [88, 94]. In an investigation of 55 outbreaks and

35 sporadic cases by Ozawa et al., stool samples were taken from norovirus infected symptomatic and asymptomatic food handlers. Similar viral loads were found among both infected groups, indicating the potential hazard of viral transmission from infected asymptomatic individuals [94]. However, in a modeling study of nosocomial infections conducted by Sukhrie et al., the study found that symptomatic individuals were more often involved in transmission events than asymptomatic individuals. Asymptomatic individuals rarely contributed to transmission, despite high levels of virus shedding in stool [95]. It is clear that more work needs to be done to assess the transmission of norovirus infection from asymptomatic individuals, as a conclusive risk is still unknown.

The high attack rate of noroviruses is attributable to several factors, including the small amounts of virus required to cause an infection, its persistence in the environment, and its resistance to common disinfectants [86, 91, 96]. Although the estimated median infectious dose of norovirus is 18 viruses, it has been shown that a single virus particle has nearly 50% likelihood to infect a susceptible individual. Teunis et al. found that the average probability that a single Norwalk virus will cause an infection in an individual is 49%. However, the probability that infected subjects become ill is also dose dependent; infectivity increases with higher dosage[91]. Moreover, noroviruses appear to remain stable following prolonged storage in freezing temperatures and multiple passages through individuals. In multiple challenge experiments, subjects were inoculated with virus isolated from stool originating from the prototypic 1971 Norwalk virus isolate [91, 97]. Virus prepared from the first challenge study was used as inoculum for a subsequent challenge study, accounting for a total of 2 passages originating from the 1971 Norwalk virus isolate

[97]. An infectious dose response analysis from the challenge studies showed that infectivity did not decrease following isolation between passages [91].

Clinical Presentation of Infection

Norovirus infections are typically considered to be short-lived, lasting for only a few days[98]. In healthy adults without underlying systemic illnesses, the course of norovirus infection is rapid, with an incubation period of 24-48 hours [99]. Common symptoms include acute and severe vomiting and diarrhea, with nausea and abdominal cramps which are likely, but not always present. Chills, headache, low-grade fever, malaise and myalgia can also develop[100]. The resolution of symptoms occurs within 12-72 hours of onset [99, 100].

While norovirus infection is typically characterized by an acute bout of gastroenteritis that resolves within hours to a few days from onset of symptoms, norovirus-induced illness has the potential to be much more damaging and severe in specific risk groups. Norovirus infection can be particularly severe in the elderly, and can result in death in some cases [56, 101, 102]. Patients with inflammatory bowel disease may also have severe symptoms, such as bloody diarrhea, upon norovirus infection [103]. Similarly, norovirus infection in infants and young children can develop chronic gastroenteritis, with symptoms lasting up to six weeks [104-107]. Globally, norovirus has been reported to be second only to rotavirus in causing severe acute gastroenteritis in infants and children [4, 106, 107]. It is possible that norovirus may surpass rotavirus as the most common cause of severe gastroenteritis following the adoption of a rotavirus vaccination schedule in children. In a surveillance study published in 2013, Payne et al. established that norovirus has become the leading cause of acute gastroenteritis among

children less than 5 years of age in the United States since the introduction of a rotavirus vaccine [46, 108].

Norovirus Immunity and Susceptibility

Susceptibility to Norwalk Virus (GI.1)

Human susceptibility to norovirus infections is multi-factorial. Several of the norovirus challenge studies have reported that a large proportion, as high as 80%, of volunteers was susceptible to Norwalk virus (NV) infection (GI.1), while a smaller percentage of their volunteers were resistant to infection [109-111]. Some of these studies have suggested that the presence of a genetic factor may be responsible for the infection outcome of individuals. Hutson et al., investigated the relationship between a person's ABO histo-blood group type and the risk of norovirus infection and symptomatic disease via clinical challenge with NV. Results from this initial study found that individuals with an O phenotype have an increased susceptibility to NV infection and symptomatic disease than those individuals expressing the B phenotype [112].

A major finding from further NV challenge studies is that NV infection and symptomatic disease is associated with the ABO histo-blood group family and the expression of the H type-1 oligosaccharide ligand [100, 109, 112]. In a human challenge model, Lindesmith et al. found that 29% of the study population was homozygous recessive for the non-functional allele of the α(1,2)fucosyltransferase gene (FUT2), and did not express the H type-1 ligand. In this subset of the study population, individuals did not become infected after challenge, regardless of the infectious dose [91, 109, 113]. Furthermore, individuals with the functional FUT2 allele developed symptomatic infection following challenge.

FUT2 encodes an enzyme that is required for the production of sugars called histo-blood group antigens (HBGA), that are expressed on the surface of epithelial cells, erythrocytes, and in mucosal secretions [114-116]. Individuals who lack a functional FUT2 gene are unable to generate ABH and Lewis antigens in mucosal secretions, and therefore, are termed non-secretors (Se-)[109]. In these individuals, ABH and Lewis antigens are still produced but are no longer secreted into body fluids. Those individuals who possess functional FUT2 alleles are termed secretors (Se+), and ABH and Lewis antigens are present in saliva and other mucosal secretions and are free to bind to the surface of epithelial cells. Norwalk virus is only capable of infecting Se+ individuals[109, 113]. Individuals who are heterozygous for functional FUT2 alleles are called partial, or weak, secretors (Se^w). In a study of randomly selected individuals, Kelly et al. found that approximately 20% of their study population was homozygous for a mutation of the FUT2 alleles, in correspondence with challenge studies that observed similar prevalence of norovirus-resistant volunteers [114]. Lindesmith et al. found that an estimated 29% of their NV challenge study population was Se-[109]. Hutson et al., also found that 18% of the volunteers in their NV challenge study were Se- [117].

The lack of a norovirus culture system has made it difficult to understand the nature of norovirus infections. However, *in vitro* studies have identified putative norovirus receptors using in vitro cell culture systems. ABH and Lewis antigens are thought to act as cellular receptors for Norwalk virus, and possibly other noroviruses such as GII.4 [118]. By using Norwalk virus-like particles (VLPs), studies have discovered that VLPs attach to HBGAs, resulting in VLP internalization in epithelial cells. VLPs were found to preferentially bind to A, H type 1 and Le^b carbohydrates [113, 119-121]. These experiments have

hypothesized that a successful *in vitro* cell culture system would most likely possess these receptors to support norovirus replication.

Susceptibility to Snow Mountain virus (GII.2) and other noroviruses

Unlike Norwalk virus, not all norovirus infections are associated with ABO blood type, Lewis type, or secretor status. Salivary binding assays have suggested that Snow Mountain virus (SMV) VLP binding is possibly associated with expression of the B blood group antigen (BGA) [120]. In a human challenge study, Lindesmith et al. demonstrated that SMV infection was independent of secretor status and BGA expression [26]. The study also showed that the serum IgG, salivary IgA, and effector T cell responses following SMV infection were cross-reactive between strains within a genogroup, but not between strains in different genogroups [26]. In contrast to Norwalk virus infection and the SMV findings by Harrington et al., this human challenge study concluded that SMV infection is not associated with the presence of a functional FUT2 gene or with expression of any of the H type 1-related molecules. Instead, an increase in Th1 cytokine response was identified in a subset of uninfected volunteers following SMV challenge. Therefore, Lindesmith et al., suggest that activation of a Th1 cellular immune response may be associated with protection from infection after challenge in some SMV-susceptible volunteers[26]. However, findings from this study were limited due to a small sample size.

Other outbreak studies involving different noroviruses have also found that not all norovirus infections are defined by secretor status or ABO blood type. In an investigation of a foodborne outbreak of GI.3 in Sweden, symptomatic disease was as likely to develop in Se- individuals as in Se+ individuals. There was also no indication that an ABO phenotype provided protection to GI.3, or was associated with a higher risk of infection. However,

persons with blood type B exhibited lower frequency of symptomatic infection, as was evidenced in NV challenge studies [122].

In a different outbreak investigation of hybrid GII.12/GII.g in Ohio, Takanashi et al., observed that this emerging norovirus infected humans regardless of ABO blood type. HBGA typing showed that individuals of various ABO blood types were susceptible to these norovirus strains, but susceptibility was still limited to Se+ individuals. These results indicate that some noroviruses may be independent of ABO blood group, yet still dependent on secretor status. Another critical finding from Takanashi et al. was that *in vitro* binding patterns of noroviruses may not accurately reflect *in vivo* HBGA usage. Although the hybrid strain was found to infect Se+ individuals of varying blood groups, GII.12 VLP did not bind substantially to ABO antigens by synthetic HBGA binding, hemagglutination, or saliva binding assays [123].

While much progress has been made in the human norovirus field, it is still limited by the lack of an efficient cell culture model. The immune system response to infection is not well understood, thus it is unclear which noroviruses are dependent on the FUT2 gene for susceptibility.

Immunity

Immune response to norovirus relies upon multiple factors including secretor status, strain type, and time between reinfections. Immune response to norovirus infection is best emphasized in human challenge studies in which volunteers are exposed to homologous virus inoculum [26, 97, 109-112, 120]. In the Norwalk virus challenge study by Lindesmith et al., 50 to 69% of Se+ individuals became infected after being challenged with varying doses of inoculum [109]. Around 14 days post-challenge, this group had a greater

than seven-fold increase in their baseline antibody titer. In contrast, Se+ individuals that remained uninfected did not show a significant increase in Norwalk-virus specific antibody production. There was a two-fold increase within the first 5 days post-challenge, but tapered back down to baseline by 8 days post-challenge. This difference in immune response between the two Se+ groups suggests that there may be some acquired resistance to infection. Se- individuals in this study population did not display changes in the levels of norovirus-specific antibody throughout the challenge period.

In a Snow Mountain virus study by Lindesmith et al., 67% of Se+ individuals and 50% of Se- individuals became infected following challenge – results from this study may be limited due to a small sample size [26]. The infected volunteers produced anti-Snow Mountain virus immune response levels that were significantly higher than their pre-challenge titers, with a median increase of 23.8 fold. Volunteers who were uninfected did not display a significant rise from their pre-challenge titer.

In another Norwalk virus challenge study by Johnson et al., 19 individuals were challenged 3 consecutive times, each spaced 6 months apart. At the time of the first challenge, 73.8% of volunteers became infected. During the second challenge, only 18% of individuals became re-infected. By the third challenge, no individuals showed signs of infection, suggesting that the rate and frequency of reinfection plays a role in generating resistance to infection [111].

Hemagglutination Inhibition as a Method for Studying Immune Response to Norovirus Infection

In vivo, the binding of Norwalk virus to HBGA in Se+ individuals has been found to be essential in establishing infection [109, 113, 117, 124]. Therefore, an assay that can measure the ability of serum antibody to block the norovirus binding to HBGA is helpful in studying the immune response to norovirus infection. Since human erythrocytes are a natural source of HBGA, Czakò et al., have optimized an assay commonly used for measuring the correlate of protection against influenza to quantify protective response to norovirus [125].

Czakò et al., has shown that hemagglutination inhibition is a useful method in measuring the protective antibody response to norovirus exposure and in detecting potential cross-reactive serum antibodies to other noroviruses. To understand the mechanism behind hemagglutination inhibition, the process of hemagglutination must first be understood. A standardized method of hemagglutination is often used to quantify the levels of virus, or bacteria, present in a sample. The standardized tests utilize a constant phosphate-buffered saline diluent, along with constant volumes of serum, antigen, and standardized erythrocyte suspension [126]. Receptors in the capsid protein of noroviruses are thought to bind to HBGAs on the surface of erythrocytes allowing the agglutination of erythrocytes [124].

The basis of the inhibition of hemagglutination activity is that the antibodies present in the serum will bind to the HBGA binding site on the virus and prevent the attachment of virus to HBGA. HBGAs are expressed on gastroduodenal cells and are thought to be the cellular receptor for norovirus [113, 127]. Therefore, agglutination of red blood cells is inhibited by the presence of antibodies.

Different noroviruses possess different receptor-binding profiles associated with the ABO, secretor, and Lewis HBGA types [112]. Several of the human challenge studies have shown that human HBGAs serve as receptors for specific norovirus infections, and that the recognition of human HBGAs by some noroviruses is a typical protein-carbohydrate interaction [26, 112, 120, 124, 128, 129]. The protruding domain of the norovirus capsid protein forms an interface with the oligosaccharide side-chains of the HBGA, thereby forming a characteristic latticed layer of virus bound to erythrocytes [130]. Tan et al., suggest that the P domain of norovirus forms a dimer and possesses the essential elements for strain-specific binding to HBGA receptors, although a wide diversity of this mechanism exists among different genotypes and strains [131].

Future of Norovirus Research and Significance of Study

Findings from hemagglutination inhibition (HI) assays can serve as determinants for future improvements in the control and prevention of norovirus infection. HI assays can be used to explore host range and evolution of noroviruses, aiding our understanding of the epidemiology of norovirus gastroenteritis. They can provide new strategies in the development of antiviral and preventative treatment against norovirus, namely through the inhibition of viral attachment to host cells, development of vaccine candidates for host immunity, and the efficacy assessment of novel vaccines.

This study aims to expand the limited knowledge of host immune response to norovirus infection by examining the following objectives:

1) The use of hemagglutination inhibition assays to correlate immune response to host susceptibility of GI.1 and GII.2 virus infection and illness.

2) The use of hemagglutination inhibition assays to define the target histo–blood group antigens and binding specificity for G1.1 and GII.2 virus

Results from this study can aid in the discovery of critical knowledge on norovirus infection and epidemiology in humans. Data from HI assays will be used to evaluate the method's practical application in surveying hemagglutination ability of GI.1 and GII.2 virus and host susceptibility. Achieving these study objectives can benefit the development of future antiviral and vaccine research, thereby decreasing the global burden of norovirus gastroenteritis.

References

1. Cubitt, W.D., D.A. McSwiggan, and W. Moore, *Winter vomiting disease caused by calicivirus.* J Clin Pathol, 1979. **32**(8): p. 786-93.
2. Wobus, C.E., et al., *Replication of Norovirus in Cell Culture Reveals a Tropism for Dendritic Cells and Macrophages.* PLoS Biology, 2004. **2**(12): p. e432.
3. Adler, J.L. and R. Zickl, *Winter vomiting disease.* J Infect Dis, 1969. **119**(6): p. 668-73.
4. Glass, R.I., U.D. Parashar, and M.K. Estes, *Norovirus gastroenteritis.* N Engl J Med, 2009. **361**(18): p. 1776-85.
5. Jiang, X., et al., *Sequence and genomic organization of Norwalk virus.* Virology, 1993. **195**(1): p. 51-61.
6. Hansman, G.S., et al., *Genetic and antigenic diversity among noroviruses.* J Gen Virol, 2006. **87**(Pt 4): p. 909-19.
7. Zheng, D.P., et al., *Norovirus classification and proposed strain nomenclature.* Virology, 2006. **346**(2): p. 312-23.
8. Le Pendu, J., et al., *Mendelian resistance to human norovirus infections.* Semin Immunol, 2006. **18**(6): p. 375-86.
9. Xi, J.N., et al., *Norwalk virus genome cloning and characterization.* Science, 1990. **250**(4987): p. 1580-3.
10. Rohayem, J., et al., *Protein-primed and de novo initiation of RNA synthesis by norovirus 3Dpol.* J Virol, 2006. **80**(14): p. 7060-9.
11. Glass, P.J., et al., *Norwalk virus open reading frame 3 encodes a minor structural protein.* J Virol, 2000. **74**(14): p. 6581-91.
12. Lambden, P.R., et al., *Sequence and genome organization of a human small round-structured (Norwalk-like) virus.* Science, 1993. **259**(5094): p. 516-9.
13. Fankhauser, R.L., et al., *Molecular epidemiology of "Norwalk-like viruses" in outbreaks of gastroenteritis in the United States.* Journal of Infectious Disease, 1998. **178**: p. 1571-1578.
14. Widdowson, M.A., et al., *Outbreaks of acute gastroenteritis on cruise ships and on land: identification of a predominant circulating strain of norovirus--United States, 2002.* J Infect Dis, 2004. **190**(1): p. 27-36.
15. Sugieda, M., et al., *Detection of Norwalk-like virus genes in the caecum contents of pigs.* Arch Virol, 1998. **143**(6): p. 1215-21.
16. Sugieda, M. and S. Nakajima, *Viruses detected in the caecum contents of healthy pigs representing a new genetic cluster in genogroup II of the genus "Norwalk-like viruses".* Virus Res, 2002. **87**(2): p. 165-72.
17. Wang, Q.H., et al., *Porcine noroviruses related to human noroviruses.* Emerg Infect Dis, 2005. **11**(12): p. 1874-81.
18. Deng, Y., et al., *Studies of epidemiology and seroprevalence of bovine noroviruses in Germany.* J Clin Microbiol, 2003. **41**(6): p. 2300-5.
19. Liu, B.L., et al., *Molecular characterization of a bovine enteric calicivirus: relationship to the Norwalk-like viruses.* J Virol, 1999. **73**(1): p. 819-25.
20. Hsu, C.C., L.K. Riley, and R.S. Livingston, *Molecular characterization of three novel murine noroviruses.* Virus Genes, 2007. **34**(2): p. 147-55.

21. Müller, B., et al., *Genetic diversity and recombination of murine noroviruses in immunocompromised mice.* Arch Virol, 2007. **152**(9): p. 1709-19.
22. Kim, M., et al., *Molecular characterization of murine norovirus isolates from South Korea.* Virus Res, 2010. **147**(1): p. 1-6.
23. Kroneman, A., et al., *Proposal for a unified norovirus nomenclature and genotyping.* Arch Virol, 2013. **158**(10): p. 2059-68.
24. Lochridge, V. and M. Hardy, *Snow Mountain Virus Genome Sequence and Virus-like Particle Assembly.* Virus Genes, 2003. **26**(1): p. 71-82.
25. Lindesmith, L., et al., *Cellular and humoral immunity following Snow Mountain virus challenge.* Journal of Virology, 2005. **79**(5): p. 2900-2909.
26. Greenberg, H.B., et al., *Prevalence of antibody to the Norwalk virus in various countries.* Infect Immun, 1979. **26**(1): p. 270-3.
27. Greenberg, H.B., et al., *Role of Norwalk virus in outbreaks of nonbacterial gastroenteritis.* J Infect Dis, 1979. **139**(5): p. 564-8.
28. Parks, C.G., et al., *Genomic diversity of "Norwalk like viruses" (NLVs): pediatric infections in a Brazilian shantytown.* J Med Virol, 1999. **58**(4): p. 426-34.
29. Bucardo, F., et al., *Pediatric norovirus diarrhea in Nicaragua.* J Clin Microbiol, 2008. **46**(8): p. 2573-80.
30. Monica, B., et al., *Human caliciviruses in symptomatic and asymptomatic infections in children in Vellore, South India.* J Med Virol, 2007. **79**(5): p. 544-51.
31. Yori, P.P., et al., *Norovirus highly prevalent cause of endemic acute diarrhea in children in the peruvian Amazon.* Pediatr Infect Dis J, 2009. **28**(9): p. 844-7.
32. Al-Mashhadani MN, et al., *Norovirus gastroenteritis among children in Iraqi Kurdistan.* Journal of Medical Virology, 2008. **80**(3): p. 506-509.
33. Nguyen, T.A., et al., *Diversity of viruses associated with acute gastroenteritis in children hospitalized with diarrhea in Ho Chi Minh City, Vietnam.* J Med Virol, 2007. **79**(5): p. 582-90.
34. Kim, J.K. and J.W. Kim, *Molecular epidemiologic trends of diarrhea-causing virus infection from clinical specimens in cheonan, Korea, in 2010-2012.* J Clin Lab Anal, 2014. **28**(1): p. 47-51.
35. Siqueira, J.A., et al., *Group A rotavirus and norovirus display sharply distinct seasonal profiles in Belém, northern Brazil.* Mem Inst Oswaldo Cruz, 2013. **108**(5): p. 661-4.
36. Ahmed, S.M., B.A. Lopman, and K. Levy, *A systematic review and meta-analysis of the global seasonality of norovirus.* PLoS One, 2013. **8**(10): p. e75922.
37. Tu, E.T., et al., *Norovirus GII.4 strains and outbreaks, Australia.* Emerg Infect Dis, 2007. **13**(7): p. 1128-30.
38. Fernández, M.D., et al., *Environmental surveillance of norovirus in Argentina revealed distinct viral diversity patterns, seasonality and spatio-temporal diffusion processes.* Sci Total Environ, 2012. **437**: p. 262-9.
39. Ayukekbong, J.A., et al., *Monitoring of seasonality of norovirus and other enteric viruses in Cameroon by real-time PCR: an exploratory study.* Epidemiol Infect, 2013: p. 1-10.
40. Trainor, E., et al., *Detection and molecular characterisation of noroviruses in hospitalised children in Malawi, 1997-2007.* J Med Virol, 2013. **85**(7): p. 1299-306.

41. Bruggink, L.D. and J.A. Marshall, *Molecular and epidemiological features of GIIb norovirus outbreaks in Victoria, Australia, 2002-2005.* J Med Virol, 2009. **81**(9): p. 1652-60.
42. Papaventsis, D.C., et al., *Norovirus infection in children with acute gastroenteritis, Madagascar, 2004-2005.* Emerg Infect Dis, 2007. **13**(6): p. 908-11.
43. Greening, G.E., et al., *Molecular epidemiology of norovirus gastroenteritis outbreaks in New Zealand from 2002-2009.* J Med Virol, 2012. **84**(9): p. 1449-58.
44. Rivera, F.P., et al., *Norovirus prevalence in 'pathogen negative' gastroenteritis in children from periurban areas in Lima, Peru.* Trans R Soc Trop Med Hyg, 2011. **105**(12): p. 734-6.
45. Hall, A.J., et al., *Norovirus Disease in the United States.* Perspective, 2013. **19**(8).
46. Scallan, E., et al., *Foodborne illness acquired in the United States--major pathogens.* Emerg Infect Dis, 2011. **17**(1): p. 7-15.
47. Lopman, B.A., et al., *Viral gastroenteritis outbreaks in Europe, 1995-2000.* Emerg Infect Dis, 2003. **9**(1): p. 90-6.
48. Harris, J.P., et al., *Deaths from norovirus among the elderly, England and Wales.* Emerg Infect Dis, 2008. **14**(10): p. 1546-52.
49. Siebenga, J.J., et al., *Norovirus illness is a global problem: emergence and spread of norovirus GII.4 variants, 2001-2007.* J Infect Dis, 2009. **200**(5): p. 802-12.
50. Calderon-Margalit, R., et al., *A large-scale gastroenteritis outbreak associated with Norovirus in nursing homes.* Epidemiol Infect, 2005. **133**(1): p. 35-40.
51. Okada, M., et al., *Genetic analysis of noroviruses associated with fatalities in healthcare facilities.* Arch Virol, 2006. **151**(8): p. 1635-41.
52. Marshall, J.A., A. Dimitriadis, and P.J. Wright, *Molecular and epidemiological features of norovirus-associated gastroenteritis outbreaks in Victoria, Australia in 2001.* J Med Virol, 2005. **75**(2): p. 321-31.
53. Green, K.Y., et al., *A predominant role for Norwalk-like viruses as agents of epidemic gastroenteritis in Maryland nursing homes for the elderly.* J Infect Dis, 2002. **185**(2): p. 133-46.
54. Lopman, B.A., et al., *Linking healthcare associated norovirus outbreaks: a molecular epidemiologic method for investigating transmission.* BMC Infect Dis, 2006. **6**: p. 108.
55. Lopman, B., et al., *Host, weather and virological factors drive norovirus epidemiology: time-series analysis of laboratory surveillance data in England and Wales.* PLoS One, 2009. **4**(8): p. e6671.
56. McEvoy, M., et al., *An outbreak of viral gastroenteritis on a cruise ship.* Commun Dis Rep CDR Rev, 1996. **6**(13): p. R188-92.
57. *Norovirus Outbreaks on Three College Campuses-California, Michigan, and Wisconsin, 2008.*, in *MMWR*. 2009, Centers for Disease Control and Prevention (CDC). p. 1095-1100.
58. Marshall, J.A., et al., *Multiple outbreaks of Norwalk-like virus gastro-enteritis associated with a Mediterranean-style restaurant.* J Med Microbiol, 2001. **50**(2): p. 143-51.
59. Liu, B., et al., *An outbreak of Norwalk-like virus gastroenteritis in an aged-care residential hostel.* N S W Public Health Bull, 2003. **14**(6): p. 105-9.

60. Marx, A., et al., *An outbreak of acute gastroenteritis in a geriatric long-term-care facility: combined application of epidemiological and molecular diagnostic methods.* Infect Control Hosp Epidemiol, 1999. **20**(5): p. 306-11.
61. Milazzo, A., et al., *A large, prolonged outbreak of human calicivirus infection linked to an aged-care facility.* Commun Dis Intell Q Rep, 2002. **26**(2): p. 261-4.
62. Miller, M., et al., *Norwalk-like virus outbreak in Canberra: implications for infection control in aged care facilities.* Commun Dis Intell Q Rep, 2002. **26**(4): p. 555-61.
63. Navarro, G., et al., *An outbreak of norovirus infection in a long-term-care unit in Spain.* Infect Control Hosp Epidemiol, 2005. **26**(3): p. 259-62.
64. Lynn, S., et al., *Norovirus outbreaks in a hospital setting: the role of infection control.* N Z Med J, 2004. **117**(1189): p. U771.
65. Marks, P.J., et al., *A school outbreak of Norwalk-like virus: evidence for airborne transmission.* Epidemiol Infect, 2003. **131**(1): p. 727-36.
66. Chapman, A.S., et al., *Norovirus outbreak associated with person-to-person transmission, U.S. Air Force Academy, July 2011.* MSMR, 2011. **18**(11): p. 2-5.
67. Sharp, T.W., et al., *Epidemiology of Norwalk virus during an outbreak of acute gastroenteritis aboard a US aircraft carrier.* J Med Virol, 1995. **45**(1): p. 61-7.
68. Oyofo, B.A., et al., *Norwalk-like virus and bacterial pathogens associated with cases of gastroenteritis onboard a US Navy ship.* Am J Trop Med Hyg, 1999. **61**(6): p. 904-8.
69. Martinelli, D., et al., *Large outbreak of viral gastroenteritis caused by contaminated drinking water in Apulia, Italy, May-October 2006.* Euro Surveill, 2007. **12**(4): p. E070419.1.
70. Kukkula, M., et al., *Outbreak of Viral Gastroenteritis Due to Drinking Water Contaminated by Norwalk-like Viruses.* The Journal of Infectious Diseases, 1999. **180**(6): p. 1771-1776.
71. Maunula, L., I.T. Miettinen, and C.H. von Bonsdorff, *Norovirus outbreaks from drinking water.* Emerg Infect Dis, 2005. **11**(11): p. 1716-21.
72. Khan, A.S., et al., *Norwalk virus-associated gastroenteritis traced to ice consumption aboard a cruise ship in Hawaii: comparison and application of molecular method-based assays.* J Clin Microbiol, 1994. **32**(2): p. 318-22.
73. Pönkä, A., et al., *An outbreak of calicivirus associated with consumption of frozen raspberries.* Epidemiol Infect, 1999. **123**(3): p. 469-74.
74. Ponka, A., et al., *Outbreak oof calicivirus gastroenteritis associated with eating frozen raspberries.* Euro Surveill, 1999. **4**(6): p. 66-69.
75. Malek, M., et al., *Outbreak of norovirus infection among river rafters associated with packaged delicatessen meat, Grand Canyon, 2005.* Clin Infect Dis, 2009. **48**(1): p. 31-7.
76. Friedman, D.S., et al., *An outbreak of norovirus gastroenteritis associated with wedding cakes.* Epidemiol Infect, 2005. **133**(6): p. 1057-63.
77. Benabbes, L., et al., *Norovirus and other human enteric viruses in moroccan shellfish.* Food Environ Virol, 2013. **5**(1): p. 35-40.
78. Le Guyader, F.S., et al., *Aichi virus, norovirus, astrovirus, enterovirus, and rotavirus involved in clinical cases from a French oyster-related gastroenteritis outbreak.* J Clin Microbiol, 2008. **46**(12): p. 4011-7.
79. Thebault, A., et al., *Infectivity of GI and GII noroviruses established from oyster related outbreaks.* Epidemics, 2013. **5**(2): p. 98-110.

80. Atmar, R.L., et al., *Detection of Norwalk virus and hepatitis A virus in shellfish tissues with the PCR.* Appl Environ Microbiol, 1995. **61**(8): p. 3014-8.
81. Bellou, M., P. Kokkinos, and A. Vantarakis, *Shellfish-borne viral outbreaks: a systematic review.* Food Environ Virol, 2013. **5**(1): p. 13-23.
82. Ma, L.P., et al., *The presence of genogroup II norovirus in retail shellfish from seven coastal cities in China.* Food Environ Virol, 2013. **5**(2): p. 81-6.
83. Rodriguez-Manzano, J., et al., *Adenovirus and Norovirus Contaminants in Commercially Distributed Shellfish.* Food Environ Virol, 2013.
84. Schaeffer, J., et al., *Norovirus contamination on French marketed oysters.* Int J Food Microbiol, 2013. **166**(2): p. 244-8.
85. Mattison, K., et al., *Survival of calicivirus in foods and on surfaces: experiments with feline calicivirus as a surrogate for norovirus.* J Food Prot, 2007. **70**(2): p. 500-3.
86. Baert, L., et al., *Reported foodborne outbreaks due to noroviruses in Belgium: the link between food and patient investigations in an international context.* Epidemiol Infect, 2009. **137**(3): p. 316-25.
87. Barrabeig, I., et al., *Foodborne norovirus outbreak: the role of an asymptomatic food handler.* BMC Infect Dis, 2010. **10**: p. 269.
88. Tuan Zainazor, C., et al., *The scenario of norovirus contamination in food and food handlers.* J Microbiol Biotechnol, 2010. **20**(2): p. 229-37.
89. Moe, C.L., *Preventing norovirus transmission: how should we handle food handlers?* Clin Infect Dis, 2009. **48**(1): p. 38-40.
90. Teunis, P.F., et al., *Norwalk virus: how infectious is it?* J Med Virol, 2008. **80**(8): p. 1468-76.
91. Glass, R.I., et al., *The epidemiology of enteric caliciviruses from humans: a reassessment using new diagnostics.* J Infect Dis, 2000. **181 Suppl 2**: p. S254-61.
92. Rockx, B., et al., *Natural history of human calicivirus infection: a prospective cohort study.* Clin Infect Dis, 2002. **35**(3): p. 246-53.
93. Ozawa, K., et al., *Norovirus infections in symptomatic and asymptomatic food handlers in Japan.* J Clin Microbiol, 2007. **45**(12): p. 3996-4005.
94. Sukhrie, F.H., et al., *Nosocomial transmission of norovirus is mainly caused by symptomatic cases.* Clin Infect Dis, 2012. **54**(7): p. 931-7.
95. Belliot, G., et al., *Use of murine norovirus as a surrogate to evaluate resistance of human norovirus to disinfectants.* Appl Environ Microbiol, 2008. **74**(10): p. 3315-8.
96. Dolin, R., et al., *Transmission of acute infectious nonbacterial gastroenteritis to volunteers by oral administration of stool filtrates.* J Infect Dis, 1971. **123**(3): p. 307-12.
97. Karst, S.M., *Pathogenesis of noroviruses, emerging RNA viruses.* Viruses, 2010. **2**(3): p. 748-81.
98. Estes, M.K., B.V. Prasad, and R.L. Atmar, *Noroviruses everywhere: has something changed?* Curr Opin Infect Dis, 2006. **19**(5): p. 467-74.
99. Hutson, A.M., R.L. Atmar, and M.K. Estes, *Norovirus disease: changing epidemiology and host susceptibility factors.* Trends Microbiol, 2004. **12**(6): p. 279-87.
100. Prevention, C.f.D.C.a., *Norovirus activity--United States, 2006-2007*, in *MMWR*, C.f.D.C.a. Prevention, Editor. 2007. p. 842-846.
101. Lopman, B.A., et al., *Clinical manifestation of norovirus gastroenteritis in health care settings.* Clin Infect Dis, 2004. **39**(3): p. 318-24.

102. Khan, R.R., et al., *Gastrointestinal norovirus infection associated with exacerbation of inflammatory bowel disease.* J Pediatr Gastroenterol Nutr, 2009. **48**(3): p. 328-33.
103. Kirkwood, C.D. and R. Streitberg, *Calicivirus shedding in children after recovery from diarrhoeal disease.* J Clin Virol, 2008. **43**(3): p. 346-8.
104. Murata, T., et al., *Prolonged norovirus shedding in infants <or=6 months of age with gastroenteritis.* Pediatr Infect Dis J, 2007. **26**(1): p. 46-9.
105. Sakai, Y., et al., *Clinical severity of Norwalk virus and Sapporo virus gastroenteritis in children in Hokkaido, Japan.* Pediatr Infect Dis J, 2001. **20**(9): p. 849-53.
106. Zintz, C., et al., *Prevalence and genetic characterization of caliciviruses among children hospitalized for acute gastroenteritis in the United States.* Infect Genet Evol, 2005. **5**(3): p. 281-90.
107. Payne, D.C., et al., *Norovirus and medically attended gastroenteritis in U.S. children.* N Engl J Med, 2013. **368**(12): p. 1121-30.
108. Lindesmith, L., et al., *Human susceptibility and resistance to Norwalk virus infection.* Nat Med, 2003. **9**(5): p. 548-53.
109. Graham, D.Y., et al., *Norwalk virus infection of volunteers: new insights based on improved assays.* J Infect Dis, 1994. **170**(1): p. 34-43.
110. Johnson, P.C., et al., *Multiple-challenge study of host susceptibility to Norwalk gastroenteritis in US adults.* J Infect Dis, 1990. **161**(1): p. 18-21.
111. Hutson, A.M., et al., *Norwalk virus infection and disease is associated with ABO histo-blood group type.* J Infect Dis, 2002. **185**(9): p. 1335-7.
112. Marionneau, S., et al., *Norwalk virus binds to histo-blood group antigens present on gastroduodenal epithelial cells of secretor individuals.* Gastroenterology, 2002. **122**(7): p. 1967-77.
113. Kelly, R.J., et al., *Sequence and expression of a candidate for the human Secretor blood group alpha(1,2)fucosyltransferase gene (FUT2). Homozygosity for an enzyme-inactivating nonsense mutation commonly correlates with the non-secretor phenotype.* J Biol Chem, 1995. **270**(9): p. 4640-9.
114. Kudo, T., et al., *Molecular genetic analysis of the human Lewis histo-blood group system. II. Secretor gene inactivation by a novel single missense mutation A385T in Japanese nonsecretor individuals.* J Biol Chem, 1996. **271**(16): p. 9830-7.
115. Hakomori, S., *Antigen structure and genetic basis of histo-blood groups A, B and O: their changes associated with human cancer.* Biochim Biophys Acta, 1999. **1473**(1): p. 247-66.
116. Hutson, A.M., et al., *Norwalk virus infection associates with secretor status genotyped from sera.* J Med Virol, 2005. **77**(1): p. 116-20.
117. Bucardo, F., et al., *Asymptomatic norovirus infections in Nicaraguan children and its association with viral properties and histo-blood group antigens.* Pediatr Infect Dis J, 2010. **29**(10): p. 934-9.
118. Choi, J.M., et al., *Atomic resolution structural characterization of recognition of histo-blood group antigens by Norwalk virus.* Proc Natl Acad Sci U S A, 2008. **105**(27): p. 9175-80.
119. Harrington, P.R., et al., *Binding of Norwalk virus-like particles to ABH histo-blood group antigens is blocked by antisera from infected human volunteers or experimentally vaccinated mice.* Journal of Virology, 2002. **76**(23): p. 12335–12343.

120. Huang, P., et al., *Norovirus and histo-blood group antigens: demonstration of a wide spectrum of strain specificities and classification of two major binding groups among multiple binding patterns.* J Virol, 2005. **79**(11): p. 6714-22.
121. Nordgren, J., et al., *Norovirus gastroenteritis outbreak with a secretor-independent susceptibility pattern, Sweden.* Emerg Infect Dis, 2010. **16**(1): p. 81-7.
122. Takanashi, S., et al., *Characterization of emerging GII.g/GII.12 noroviruses from a gastroenteritis outbreak in the United States in 2010.* J Clin Microbiol, 2011. **49**(9): p. 3234-44.
123. Hutson, A.M., et al., *Norwalk virus-like particle hemagglutination by binding to h histo-blood group antigens.* J Virol, 2003. **77**(1): p. 405-15.
124. Czakó, R., et al., *Serum hemagglutination inhibition activity correlates with protection from gastroenteritis in persons infected with Norwalk virus.* Clin Vaccine Immunol, 2012. **19**(2): p. 284-7.
125. Hierholzer, J.C. and M.T. Suggs, *Standardized viral hemagglutination and hemagglutination-inhibition tests. I. Standardization of erythrocyte suspensions.* Appl Microbiol, 1969. **18**(5): p. 816-23.
126. Cheetham, S., et al., *Binding patterns of human norovirus-like particles to buccal and intestinal tissues of gnotobiotic pigs in relation to A/H histo-blood group antigen expression.* J Virol, 2007. **81**(7): p. 3535-44.
127. Tan, M., et al., *Outbreak studies of a GII-3 and a GII-4 norovirus revealed an association between HBGA phenotypes and viral infection.* J Med Virol, 2008. **80**(7): p. 1296-301.
128. Tan, M. and X. Jiang, *Norovirus gastroenteritis, carbohydrate receptors, and animal models.* PLoS Pathog, 2010. **6**(8): p. e1000983.
129. Tan, M. and X. Jiang, *Norovirus and its histo-blood group antigen receptors: an answer to a historical puzzle.* Trends Microbiol, 2005. **13**(6): p. 285-93.
130. Tan, M., R.S. Hegde, and X. Jiang, *The P domain of norovirus capsid protein forms dimer and binds to histo-blood group antigen receptors.* J Virol, 2004. **78**(12): p. 6233-42.

MANUSCRIPT

Abstract

Norovirus infections are a common cause of epidemic gastroenteritis. It is estimated that over 50% of all gastroenteritis outbreaks worldwide are attributable to norovirus. Norovirus infection is typically acute, mild, and of short duration. However, the disease can present much more severely with longer duration in infants, the elderly, and immune-compromised individuals. Despite norovirus being recognized as a leading cause of major global epidemics, immune response to infection remains poorly understood. In this study, serum hemagglutination inhibition (HAI) assay was used to measure the immune response of 43 volunteers challenged with Norwalk virus (GI.1) and 15 volunteers challenged with Snow Mountain virus (GII.2). Hemagglutination inhibition activity was observed from all 13 of the infected volunteers of the 43 challenged with GI.1, and all 9 of the infected volunteers of the 15 challenged with GII.2. HAI titers increased significantly for individuals infected with either virus. The magnitude of increase was correlated with the magnitude of increase in α-norovirus serum IgG concentration, infection status and presentation of symptoms. Baseline titers were not associated with protection from infection or presentation of symptoms. Although the amount of viral shedding among infected volunteers was found to be associated with HAI titer in the Snow Mountain virus challenge group, there was no significant association found between the amount of viral shedding and HAI titer for the Norwalk virus challenge group. There was no evidence of correlation between duration of viral shedding and HAI titer in either challenge study groups.

Introduction

Noroviruses are a major cause of epidemic and sporadic cases of acute gastroenteritis in adults and children worldwide. Norovirus accounts for greater than 90% of viral gastroenteritis illness and approximately 50% of all viral outbreaks world-wide [132]. Data from past surveillance reports indicate that 58% of food-borne gastrointestinal illness is caused by norovirus in the United States, infecting nearly 21 million American people annually [46, 47]. Despite this widespread incidence, the nature of immune response to norovirus infection remains somewhat unclear. This is largely due to the lack of a tissue culture model, and a lack of a simple and sensitive diagnostic tool for measuring the human response to infection.

Currently, the methods used to detect serum antibody concentrations following norovirus infection are primarily limited to an enzyme-linked immunosorbent assay (ELISA) and a histo-blood group antigen (HBGA) blocking assay. An ELISA is useful in quantitating the amount of serum antibodies present in infected individuals. The humoral immune response of norovirus infection is comparable to that of other infections. High seroconversion, an increase of at least four-fold, of serum α-norovirus IgG is observed following exposure, as confirmed through several serological studies of sera obtained from human challenge studies and outbreaks [26, 133-137]. A subset of antibodies are known to block the binding of norovirus to HBGAs, the proposed cellular receptors for norovirus [112, 120, 138]. HBGA-blocking antibody titers are the first known correlate of protection from illness [113, 119, 120, 124]. Therefore, the HBGA blocking assay has often been used to measure the protective immune response to infection. However, the blocking assay has several technical challenges, as it is sensitive to temperature, pH, and the quality of the

purified HBGAs utilized[125, 134, 139]. The assay is also quite expensive due to the need for synthetic HBGAs.

In an effort to create a reliable, simpler assay that measures the ability of serum antibody to block the virus-HBGA interaction, Czakò et al., have adapted a serum hemagglutination inhibition (HAI) assay that is commonly used for influenza investigations [125]. Norovirus virus-like particles (VLP) have been shown to exhibit hemagglutination activity *in vitro* through HBGA binding. HBGAs are glycans that are expressed on the surface of erythrocytes, as well as epithelial cells and mucosal secretions [114-116]. Analysis of serum samples collected from subjects experimentally challenged with norovirus shows significant increases in HAI activity following infection[124].

In this study, the HAI assay described by Czakò et al., will be used to assess the dynamics of the serum antibody response following Norwalk virus challenge and Snow Mountain virus challenge. Norwalk virus (NV) and Snow Mountain virus (SMV) are the prototype strains of their genogroup clusters, GI.1 and GII.2, respectively [26]. GI.1 and GII.2 are estimated to cause a combined 13% of norovirus outbreaks, with GII.2 responsible for 8% of those outbreaks [26, 140].

Sera used in this study were obtained from previous norovirus human challenge studies. The Norwalk virus challenge study was published in 2011 by Leon et al., and the Snow Mountain virus challenge study was published in 2005 by Lindesmith et al., [26, 141] HAI activity from these sera was examined for correlation with infection status, symptoms, viral shedding, and anti-norovirus IgG levels in serum.

Materials and Methods

Norwalk virus human challenge study

The Norwalk virus serum samples utilized in this study were collected from a human challenge study conducted by Leon et al. in 2009 at Emory University [141]. A total of 51 subjects were challenged in a double-blinded, randomized human challenge study in order to assess the effect of high hydrostatic pressure (HPP) on Norwalk virus infectivity in human volunteers. The participant demographic was healthy men and women, 18-50 years of age, with a genetic susceptibility to Norwalk virus infection (e.g. Secretor positive)[109]. Prior to challenge, saliva, serum and stool samples were collected from each volunteer. Oysters that had been artificially seeded with $1x10^4$ genomic equivalent copies (GEC) of a safety-tested Norwalk virus inoculum were subjected to HPP at varying times and pressures. Each volunteer consumed 6 raw oysters, for a maximum total dose of $6x10^4$ GEC. Study subjects were monitored for gastrointestinal symptoms and vital signs in the Emory University Hospital Clinical Research Network, part of the Atlanta Clinical and Translational Science Institute, three times a day post-challenge. Saliva, serum, and stool samples were collected daily during the first five days post-challenge. Additional serum and saliva specimens were collected during follow-up visits on days 8, 14, 21, 28 and 35 post-challenge.

Snow Mountain virus human challenge study

The Snow Mountain virus serum samples utilized in this study were collected from a human challenge study conducted by Lindesmith et al., at the University of North Carolina [26]. In an aim to examine the infectivity of Snow Mountain virus, 15 healthy adult volunteers were admitted to the University of North Carolina General Clinical Research

Center (GCRC). Prior to challenge, blood, saliva and stool samples were collected. Doses of safety-tested Snow Mountain virus inoculum were prepared from stool filtrates and administered to each study participant. Doses ranged from 10 to 10^5 PCR detectable units, as defined by endpoint titration reverse transcription polymerase chain reaction (RT-PCR), and were randomly distributed amongst study subjects.

Following challenge, study participants remained in the GCRC for the first 5 days for monitoring. Saliva, serum, and stool samples were collected during the first 5 days post-challenge. Participants returned for follow-up visits on days 8, 14, and 21 post-challenge, and sera and saliva were collected at each follow-up visit.

Measurement of anti-norovirus serum IgG levels

The amount of anti-norovirus IgG levels in serum was measured using an enzyme-linked immunosorbent assay (ELISA). Serum IgG measurements were collected prior to this study using methods described by Monroe et al., and *The ELISA Guidebook [142, 143]*. ELISA was conducted using baculovirus-produced Norwalk virus-like particles (VLPs) with alkaline phosphatase-labeled rabbit α-human IgG (Sigma-Aldrich, MO). Antibody concentrations were determined using a standard curve with known IgG concentrations as a measure of comparison. Seroconversion was defined as an increase equal to or greater than four-fold of virus-specific serum IgG units.

Assessment of secretor status using ELISA

An ELISA protocol described by Azevedo et al. (see [144]), was used to determine the secretor status of red blood cell donors in this study. This protocol detects free H-type 1 carbohydrate in whole saliva. Briefly, saliva was diluted 1:1000 in 1X Phosphate Buffer Saline (PBS-1X)(Invitrogen Life Technologies, NY) and 100 µL of diluted saliva was added

to the wells of a polystyrene Medium Binding EIA/RIA plate (Nunc, Corning, MA). The plates were incubated at 4° C overnight.

After washing with 1X PBS-T (PBS-1X + 0.05% Tween 20 Invitrogen), the plates were blocked with 5% w/v defatted milk (Blotto) (Sigma-Aldrich, MO) in PBS-1X for 1 hour in a 37° C humid chamber. Following three washes in 1X PBS-T, plates were incubated with1 mg/ml horseradish peroxidase-conjugated rabbit anti-UEA-I antibody (EY Laboratories, CA) diluted to 1:500 in 5% Blotto/PBS for 1 hour in a 37° C humid chamber. Reactions were developed with 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate (BioFX, MD) and were stopped with 0.18M hydrochloric acid (Fisher Scientific, NH). Plates were read using an ELx800 spectrophotometer (Bio-Tek Instruments, VT) at 450 nm wavelength and analyzed with KC Junior software (Bio-Tek Instruments, VT). Volunteers were considered to be secretor positive if their optical density averages were greater than or equal to four times the average of the negative control. The negative control and positive control was chosen from donor saliva of known secretor status. Furthermore, PBS-1X was included as a negative control, and a secretor positive sample washed without UEA was a control for the TMB substrate. The use of multiple controls ensured accuracy of data and quality control of reagents.

Hemagglutination Inhibition Assay

An HAI assay protocol described by Czakò et al. (see [125]) was performed using serum samples from 43 volunteers challenged with Norwalk virus , and from 15 volunteers challenged with Snow Mountain virus [26, 141]. For the Norwalk virus serum samples, human type O erythrocytes were collected from a donor whose positive secretor status was confirmed using the ELISA protocol. The same measure was completed for Snow Mountain

virus challenged serum samples using human type B erythrocytes from a secretor positive red blood cell donor. Blood was collected in 8.5 mL Buffered Citrate Sodium tubes (Becton, Dickinson and Company, NJ), filtered through 12-ply sterile gauze (Kendall International, MA), and washed with PBS-1X in 4° C for 48 hours until supernatant was visibly clear. A centrifuge was not used to wash blood to reduce hemolysis. The remaining clear supernatant was aspirated to leave a pellet of packed erythrocytes, which were stored at 4° C until ready for use.

To quantitate serum antibody, baculovirus-produced Norwalk virus VLPs produced by Baylor University, made available as part of the NoroCORE Food Virology Collaborative (norocore.ncsu.edu), were used as the hemagglutination (HA) antigen. The HA antigen was titrated prior to the start of the HAI assay. HA antigen was serially diluted by two-fold in PBS-1X across a polystyrene 96-well, V-bottom micro-test plate (Denville Scientific, NJ); the final dilutions were 1:1 through 1:2048. The final row contained only PBS-1X as a quality control of reagents. Fifty microliters of red blood cell (RBC) suspension (0.75% v/v packed erythrocytes in PBS-1X) was added to each well. The plate was gently agitated to mix, and incubated at 4° C for 75 minutes to allow complete settling of cells. The plates were read by eye, and the presence of a latticed well indicated hemagglutination. The highest dilution of VLP that caused complete hemagglutination was considered the HA titration end point. Using the HA titration end point as the starting concentration, the VLP was diluted in PBS-1X pH 5.5 to achieve a final end point of 8 HA units. For example, if the last dilution showing complete agglutination is 1:128, the dilution factor is 1 part VLP and 15 parts PBS-1X pH 5.5.

Serum samples collected pre-challenge were used to determine the baseline hemagglutination inhibition (HAI) titer. Corresponding serum samples collected at time category 5, 6, 8, 9 or 10 (see Table 1) were used to determine post-challenge HAI titers. Sera treatment began by heat inactivation in a 56° C water bath for 30 minutes. Heat-inactivated sera were diluted 1:4 in 25% w/v kaolin (Sigma-Aldrich, MO) suspension in PBS-1X and rotated on a spinner at room temperature for 30 minutes to remove non-specific inhibitors. Supernatant was recovered after spinning at 10k rpm for 10 minutes. To prevent non-specific binding of serum antibodies to the red blood cell surface, packed erythrocytes were added to each serum sample, creating a 1:20 final dilution of erythrocyte in serum. Sera were left to incubate at 4° C for 1 hour, and then spun for 10 minutes at 2000 rpm at 4° C. The supernatant was recovered and the adsorption was repeated one more time, for a total of two times. After a final spin for 10 minutes at 2000 rpm at 4° C, the remaining supernatant was recovered. PBS-1X pH 5.5 was added to each pre-treated serum sample to achieve a final serum dilution of 1:10. Treated serum was stored at -20° C until further testing.

To perform the HAI assay, treated sera was serially diluted by two-fold in PBS-1X down the columns of a 96-well, V-bottom microtiter plate. Final total dilutions of sera, including the addition of PBS-1X during treatment were 1:10 through 1:1280. Column 12 contained only PBS-1X as a quality control for the reagents and the VLP dilution to 8 HA units. Twenty-five microliters of standardized HA antigen was added to each plate well and the plate was gently agitated. Each plate well was then combined with 50 μL of RBC suspension, gently agitated to mix, and left to incubate at 4° C for 75 minutes. The highest dilution of sera that caused complete inhibition of hemagglutination was considered the

HAI titration end point. The HAI titer was documented as the reciprocal of the HAI titration end point.

Statistical Methods

All data analyses were performed using SAS version 9.3 (SAS Institute, NC) to test the significance of association between the following variables: infection status and HAI titer, infection status and seroconversion, infection status and ABO blood type, presentation of symptoms and HAI titer, characteristics of viral shedding and HAI titer, and HAI titer and anti-norovirus serum IgG. Baseline HAI titer measurements were collected from baseline sera, however, final HAI titer measurements were not always from sera collected on the same day for each volunteer. Final HAI titer measurements were collected from the last available serum collection day, ranging from time category 5 to 9 (see Table 1). The characteristics of viral shedding used for analysis include the duration of viral shedding in days, and the amount of viral shedding measured by peak titer.

Variables were added to the existing dataset for further analysis. "Fold increase in HAI titer" is a continuous variable calculated by dividing final HAI titer by the baseline HAI titer. "Seroconversion" is a dichotomous variable indicating whether or not there was a four-fold increase between baseline HAI titer and final HAI titer. "Fold increase in IgG" is a continuous variable calculated by dividing the peak serum anti-norovirus IgG concentration by the baseline serum anti-norovirus IgG concentration.

There were 51 volunteers in the Norwalk virus challenge study, but only 43 sera samples were identified for this study. Sera from all 15 volunteers of the Snow Mountain virus challenge study were used for analysis. A p-value less than 0.05 was considered significant.

Results

Volunteer characteristics

To determine the correlation between infection and volunteer characteristics, the following variables were considered: age, sex, race and ABO blood type. Age, sex and race were not found to be correlated with infection for either virus type, with a similar mean age among infected and non-infected groups for both challenge studies (see Table 2 and 8). Among the Norwalk virus challenge volunteers, infection was more common among ABO blood types A and O, but especially among blood type O. This finding is consistent with other Norwalk virus studies [109]. However, there was no significant association found in statistical analysis between ABO blood type and infection status of either study groups (see Table 13). This was likely due to the small sample size of both challenge studies.

All of the volunteers selected for the Norwalk virus challenge study were screened for secretor status, and only secretor positive individuals were chosen for enrollment. Of the 15 Snow Mountain virus challenge volunteers, 12 were secretor positive. Norwalk virus infection has been shown to be dependent on secretor status, however, Snow Mountain virus infection was not found to be associated with secretor phenotype. A total of 67% of infected volunteers were secretor positive, and the remaining infected population was secretor negative [26].

Characteristics of Clinical Illness

The distribution of symptoms among infected volunteers were collected through patient self-reports and clinical vital assessments for both Norwalk virus and Snow Mountain virus challenge studies. The measured symptoms were consistent with other

published Norwalk virus studies including: diarrhea, emesis, nausea, abdominal cramping, headache, chills, myalgia, fatigue, low-grade fever (oral temperature ≥37.6 C), and gastroenteritis [132, 145, 146]. The onset and duration of symptoms appeared rapidly and resolved quickly, ranging from 1 to 2 days, with the exception of one Snow Mountain challenge volunteer (see Table 3 and 9). Infected volunteers were considered to be symptomatic if the presence of one symptom, with the exception of fever or mild cramping, was found to be associated with at least one other symptom [141]. Volunteers who experienced either mild abdominal cramping or low-grade fever were considered asymptomatic. The severity of symptoms were based on a modification of the Vesikari scale, as described by Atmar et al, [147]. The scale ranges from 0 to 17, with the most severe case from both studies found to be at a severity level of 8.

As expected, presentation of symptoms within the Norwalk virus challenge group sub-set (N = 43) was associated with the value of the final titer and with the fold increase in HAI titer from baseline (See Table 4). Surprisingly, there was no significant correlation between HAI titer and clinical illness among the infected volunteers (N = 13). The baseline HAI titer, final HAI titer and fold increase in HAI titer was not found to be associated with presentation of symptoms, which is not consistent with findings from other similar studies [125].

Analysis of clinical illness was not conducted for the Snow Mountain virus challenge group, as all infected volunteers presented with symptoms.

Correlates of Infection

Norovirus infection was defined as a RT-PCR detection of norovirus RNA in any post-challenge stool or vomitus sample. There were 15 infected volunteers in the Norwalk virus challenge study, with data from 13 of the 15 volunteers used for analysis in this study. A total of 9 volunteers were infected in the Snow Mountain virus challenge study, and all infected volunteers were included in the analysis.

There was a significant difference found between the infected and non-infected groups for seroconversion in both virus challenge studies (see Table 5 and 10). Moreover, the final HAI titer and fold increase in HAI titer were both found to have a significant correlation with infection status using bivariate analysis. Analysis was performed using only control subjects for the uninfected group to prevent skewing by subjects that were not exposed to virus inoculum due to HPP treatment.

There was no significant difference found in the mean baseline HAI titer between the infected and uninfected groups of the Norwalk virus and Snow Mountain virus challenge studies (see Table 5 and 10).

Correlates of Viral Shedding

An extended period of viral shedding occurred following infection, as was detected by RT-PCR of stool samples from infected groups of both challenge studies. The duration of shedding documented by both groups ranged from 3 to 35 days (data not shown). The patterns were similar to viral shedding characteristics found in other human challenge experiments [148].

Interestingly, there was found to be no significant relationship between viral shedding and HAI titers for the Norwalk virus challenge study (see Table 6). Although other studies have found that norovirus-specific humoral response is correlated with duration of viral shedding, baseline HAI titer, final HAI titer, and fold increase in HAI titer were not correlated with either the amount or duration of viral shedding for this group [149].

On the other hand, the Snow Mountain virus study group showed a significant association between the amount of viral shedding and HAI titers (See Table 11). The magnitude of increase in HAI titer and the value of post-challenge HAI titer were associated with the amount of virus shed. A greater increase or greater HAI value corresponded with a greater amount of viral shedding as measured by peak titer. The duration of viral shedding was not found to be correlated with HAI titer for the Snow Mountain virus study group.

Correlates of α-norovirus Serum IgG Response

As seen with infection status and seroconversion, there was a strong correlation between the fold increase of HAI titer and the fold increase of α-norovirus serum IgG for the Norwalk virus challenge group (see Table 7). There was found to be a strong, positive linear relationship between those two variables. However, evidence was lacking of a significant relationship between the magnitude of increase in HAI titer and the peak value of α-norovirus serum IgG in infected volunteers.

In the Snow Mountain virus challenge group, HAI seroconversion was a strong indicator of infection status. However, there was no significant correlation found between HAI titers and α-norovirus serum IgG among infected volunteers (see Table 12). This is a direct contrast from what was observed with the Norwalk virus challenge group. Results from the Norwalk virus challenge group suggest that there is a strong, positive linear

relationship between the fold increase in HAI titer and the fold increase of α-norovirus serum IgG. Results from the Snow Mountain virus challenge group did not display this relationship.

Discussion

Noroviruses are major etiologic agents of acute viral gastroenteritis worldwide. However, the immunological response to norovirus infection remains poorly characterized, and immunological methods are limited. In this study, a serum hemagglutination inhibition assay was used to evaluate the human serum antibody response to norovirus infection. The humoral response was measured from volunteers of two separate norovirus challenge studies – Norwalk virus (GI.1) challenge and Snow Mountain virus (GII.2) challenge [26, 141]. Fifty-one volunteers were challenged in the Norwalk virus challenge and 15 volunteers became infected. Of these, 43 challenged volunteers and 13 infected volunteers were included for this study. A total of 15 volunteers were challenged in the Snow Mountain virus study, and 9 volunteers became infected. All challenged and infected volunteers from the Snow Mountain virus challenge were included in this study. The dynamics of the immune response were assessed by measuring the ability of serum antibody to inhibit norovirus binding to human erythrocytes.

Serum Hemagglutination Inhibition Activity

A previous norovirus human challenge study by Czakò et al., indicated that serum hemagglutination inhibition activity can be used to assess serum antibody response to norovirus infection [125]. The results of this study were consistent with the findings by Czakò et al., that HAI activity is highly predictive of infection status. All infected volunteers in this study exhibited seroconversion, suggesting that serum HAI activity is a strong correlate of infection. However, baseline HAI titers and magnitude of seroconversion were not found to be related with protection from gastroenteritis development. Czakò et al.,

were able to find significant differences in baseline HAI titers between symptomatic and infected-but-asymptomatic volunteers, indicating that serum HAI activity correlates with protection from clinical illness among infected individuals. It is reasonable to predict that a higher baseline HAI titer is associated with a stronger immune response to clinical illness. However, this was not consistent with the findings of this study, as this was not displayed in our data. In addition, baseline HAI titer was not correlated with age in this study, which may indicate that any memory response to previous norovirus exposures was not reflected by serum HAI activity.

The results from our study may have differed from that of the Czakò at al., group due to the methods used in the experimental challenge and the hemagglutination inhibition assay. For their study, Czakò at al., used sera that was obtained from a previous challenge study by Atmar et al., [148]. The virus inoculum that was administered to each volunteer was given at one of three different doses prepared from the liquid feces of volunteers who participated in the Atmar et al., study [125, 148]. The sera used in this study was obtained from a human challenge study that used norovirus inoculum prepared from stool filtrates from a subject previously infected with human norovirus. The final virus concentration of the inoculum was standardized for all subjects who were challenged. The method of virus inoculum delivery between this study and that of Czakò et al., was also different. These contrasts in inoculum preparation and source may have elicited differing humoral responses to norovirus infection.

There were also minor differences in the assay methods of this study compared to the Czakò et al., study which may have led to inconsistent results. In this study, the human type O red blood cells came from a single donor whose secretor status was determined

using an ELISA protocol. The same method was used for the human type B red blood cells used in this study. The source of erythrocytes may have played a role in the different patterns of agglutination. It is unclear whether the incongruity in results is attributable to differing study conditions, or if one study is reflecting the true biology of the virus over the other. It is clear, however, that more research is required to understand the dynamics of the immune response to norovirus infection.

This is the first study to examine the relationship between serum HAI activity and the characteristics of viral shedding from norovirus infections. The duration and quantity of viral shedding was considerably variable across all study participants in both study groups. Although the dynamics of viral shedding did not display any significant correlation with serum HAI titers for the Norwalk virus study group, HAI titers were found to be directly correlated with the amount of viral shedding, as measured by peak titer, for the Snow Mountain virus study group. The finding that HAI titers are indicative of greater viral shedding is particularly interesting as the factors that determine viral shedding remain largely unknown [146]. The understanding of norovirus shedding dynamics is critical because of the known infectivity of the virus and its persistence in the environment outside of the human host [86, 90, 91, 93].

Currently, the HBGA blocking assay and ELISA are the primary techniques used to detect humoral response following norovirus infection. Although HBGA blocking and HAI titers have been found to correlate with protection from illness, levels of α- norovirus serum IgG are not predictive of protection from illness [26]. In this study, serum HAI activity was compared to α-norovirus serum IgG levels that were measured using ELISA. There was a strong relationship found between the increase of serum HAI titer and the

increase of α-norovirus serum IgG titer for the Norwalk virus challenge study, which shows that hemagglutination inhibition assays can potentially serve as a viable alternative to detecting the humoral response following certain norovirus infections. However, there was no significant relationship found in this study between the magnitude of HAI titer increase and the highest value of α-norovirus serum IgG measured. It could be hypothesized that a stronger increase in serum HAI titer is correlated with higher serum IgG levels, but this was not supported in our data. Unfortunately, results from the Snow Mountain virus challenge study did not correspond with the HAI titer and α-norovirus serum IgG level findings from the Norwalk virus challenge study. However, these results may have been confounded by study limitations.

Limitations

The main limitation of this study was the limited sample size. Only a sub-set of the full study group was used from the Norwalk virus challenge study because of the inaccessibility of specimen from 8 volunteers, and the absence of data from a few study participants. This further limited the sample size of an already restricted pool of study participants. The Snow Mountain virus challenge study was even smaller than the Norwalk virus challenge study. The small number of participants characterized in this study is not sufficient for identifying trends, especially with variables of considerable fluctuation.

The Norwalk virus and Snow Mountain virus challenge studies utilized self-reporting of some data variables, including age, race and development of symptoms. This introduces potential for information bias, particularly when calculating the severity of symptoms or classifying asymptomatic infection. Another limitation was the recruitment of

participants in both studies. As with most, if not all, human challenge studies, the participants were all healthy adults with no underlying conditions. A final limitation of this study was the artificial inoculation of norovirus in participants of both challenge studies. The calculated inoculum may not be representative of the course of natural norovirus infection, possibly confounding the level of humoral response.

Tables

Table 1. Classification of Days Post-challenge in Time Categories

Time Categories	Days Post-Challenge
1	0
2	2
3	3
4	4
5	5
6	7 to 9
7	13 to 16
8	21 to 26
9	27 to 30
10	35 to 41

Table 2. Volunteer Characteristics of Norwalk Virus Challenge

	Infected N=13	Uninfected N=30	Total N=43
Age Range (years) [a]	19 – 35 (23.2)	20 – 51 (29.7)	19 – 51 (27.2)
Male [b]	7 (53.8)	12 (40.0)	19 (44.2)
Race [c]	**AA** [d] 4 (30.8) **Caucasian** 6 (40.0) **Other** [e] 3 (20.0)	**AA** [d] 11 (36.7) **Caucasian** 14 (46.7) **Other** [e] 5 (16.6)	**AA** [d] 15 (34.9) **Caucasian** 20 (46.5) **Other** [e] 8 (16.6)
ABO blood type [f]	**A** 2 (15.4) **B** 0 (0.00) **AB** 0 (0.00) **O** 11 (84.6)	**A** 8 (26.7) **B** 4 (13.3) **AB** 1 (3.30) **O** 17 (56.7)	**A** 10 (23.3) **B** 4 (9.30) **AB** 1 (2.30) **O** 28 (65.1)
Secretor status [g]	**Se+** 13 (100) **Se-** 0	**Se+** 30 (100) **Se-** 0	**Se+** 43 (100) **Se-** 0

[a] Number in parenthesis indicates mean value.
[b,c,f,g] Number in parenthesis indicates percent within each category.
[d] African American.
[e] Asian, Hispanic, Multi-racial, and other.

Table 3. Distribution of Symptoms in Norwalk Virus Challenge [a]

Volunteer	Number of symptoms presented [b]	Duration of symptoms (days)	Severity of symptoms [c]
3	0	0	1
4	7	1	5
9	0	0	1
12	1	2	1
15	7	1	4
16	1	1	1
17	5	1	3
29	0	0	1
34	6	1	7
37	3	1	4
40	4	1	6
46	6	1	3
54	7	1	8

[a] Among infected volunteers (N=13).
[b] Symptoms include: Diarrhea, emesis, nausea, abdominal cramping, headache, chills, myalgia, fatigue, fever, gastroenteritis.
[c] Severity of symptoms were based on Vesikari scale

Table 4. Bivariate Analysis for Correlates of Symptomatic Infection in Norwalk Virus Challenge

Variable 1	Variable 2	Test Statistic [a] (T-value)	P-value
Symptoms [b]	Fold Increase in HAI Titer [b]	3.47 (41)	0.0013
Variable 1	**Variable 2**	**Test Statistic (R^2)**	**P-value**
Symptoms [b]	Baseline HAI Titer [c]	0.0095	0.7511
	Baseline HAI Titer [b]	0.0143	0.4338
	Final HAI Titer [c]	0.0201	0.6440
	Final HAI Titer [b]	0.3352	<0.0001
	Fold Increase in HAI Titer [c]	0.0046	0.8248
	Fold Increase in HAI Titer [b]	0.2267	0.0013

[a] Number in parenthesis indicates degrees of freedom.
[b] Full study group (N=43); Symptomatic infection was defined as having the presence of one symptom, with the exception of fever or mild cramping, associated with the presence of at least one other symptom.
[c] Among infected volunteers (N=13).

Table 5. Bivariate Analysis for Correlates of Infection in Norwalk Virus Challenge

Variable 1	Variable 2	Test Statistic (T-value) [a]	P-Value
Infection Status [b]	Baseline HAI Titer [c]	1.39 (20)	0.1812
	Final HAI Titer [c]	-3.56 (20)	0.0020
	Fold Increase in HAI Titer [c]	-3.39 (20)	0.0029
Variable 1	**Variable 2**	**Test Statistic (R^2)**	**P-Value**
Infection Status [b]	Baseline HAI Titer [d]	0.0458	0.1580
	Final HAI Titer [d]	0.2317	0.0271
	Fold Increase in HAI Titer [d]	0.2908	0.0271
Variable 1	**Variable 2**	**Test Statistic [a] (T-value)**	**P-value**
Infection Status [b]	Baseline HAI Titer [e]	1.57 (41)	0.1239
Variable 1	**Variable 2**	**Test Statistic (Chi-square)**	**P-value**
Infection Status [b]	HAI Seroconversion [c, f]	12.75	<0.0001

[a] Number in parenthesis indicates degrees of freedom.
[b] Infection status determined by the detection of Norwalk virus RNA in one or more stool or vomitus samples via RT-PCR.
[c] Comparison between infected (N=13) and control group (N=9).
[d] Control group (N=9).
[e] Among infected volunteers (N=13).
[f] Seroconversion defined by at least a 4-fold increase in HAI titer.

Table 6. Bivariate Analysis for Correlates of Viral Shedding in Norwalk Virus Challenge [a]

Variable 1	Variable 2	Test Statistic (Pearson's r)	P-value
Duration of Viral Shedding	Baseline HAI Titer	0.41126	0.1627
	Final HAI Titer	-0.00386	0.9900
	Fold Increase in HAI Titer	-0.2925	0.3321
Peak Viral Shedding	Baseline HAI Titer	0.06465	0.8338
	Final HAI Titer	0.19305	0.5275
	Fold Increase in HAI Titer	0.04609	0.8812

[a] Among infected volunteers (N=13).

Table 7. Bivariate Analysis for Correlates of α-norovirus Serum IgG Response in Norwalk Virus Challenge [a]

Variable 1	Variable 2	Test Statistic (Pearson's r)	P-value
Fold Increase in HAI Titer	Fold Increase in IgG Antibody	0.84648	<0.0001
	Final Total IgG	0.19842	0.5158

[a] Among infected volunteers (N=13).

Table 8. Volunteer Characteristics of Snow Mountain Virus Challenge

	Infected N=9	Uninfected N=6	Total N=15
Age Range (years) [a]	23 – 54 (33.8)	21 – 32 (26.0)	21 – 54 (30.7)
Male [b]	5 (55.6)	2 (33.3)	7 (46.7)
Race [c]	**AA** [d] 3 (33.3) **Caucasian** 6 (66.7) **Other** [e] 0	**AA** [d] 1 (16.7) **Caucasian** 5 (83.3) **Other** [e] 0	**AA** [d] 4 (26.7) **Caucasian** 11 (73.3) **Other** [e] 0
ABO blood type [f]	**A** 2 (22.2) **B** 1 (11.1) **AB** 2 (22.2) **O** 4 (44.4)	**A** 2 (33.3) **B** 0 **AB** 0 **O** 4 (66.7)	**A** 4 (26.7) **B** 1 (6.7) **AB** 2 (13.3) **O** 8 (53.3)
Secretor status [g]	**Se+** 8 (88.9) **Se-** 1 (11.1)	**Se+** 4 (66.7) **Se-** 2 (33.3)	**Se+** 12 (80.0) **Se-** 3 (20.0)

[a] Number in parenthesis indicates mean value.
[b,c,f,g] Number in parenthesis indicates percent within each category.
[d] African American.
[e] Asian, Hispanic, Multi-racial, and other.

Table 9. Distribution of Symptoms in Snow Mountain Virus Challenge [a]

Volunteer	Number of symptoms presented [b]	Duration of symptoms (days)	Severity of symptoms [c]
1	9	2	2
2	9	1	3
3	9	4	5
4	2	1	0
10	7	2	6
11	4	1	2
12	9	1	5
13	9	2	3
15	9	1	3

[a] Among infected volunteers (N=9).
[b] Symptoms include: Diarrhea, emesis, nausea, abdominal cramping, headache, chills, myalgia, fatigue, fever, gastroenteritis.
[c] Severity of symptoms were based on Vesikari scale.

Table 10. Bivariate Analysis for Correlates of Infection in Snow Mountain Virus Challenge

Variable 1	Variable 2	Test Statistic [a] (T-value)	P-value
Infection Status [b]	Baseline HAI Titer [c]	0.45 (9)	0.6621
	Baseline HAI Titer [d]	0.52 (13)	0.7210
	Final HAI Titer [c]	2.49 (13)	0.0271
	Fold Increase in HAI Titer [c]	2.40 (13)	0.0318
Variable 1	**Variable 2**	**Test Statistic (R^2)**	**P-Value**
Infection Status	Baseline HAI Titer [c]	0.0152	0.6621
	Final HAI Titer [c]	0.3229	0.0271
	Fold Increase in HAI Titer [c]	0.3079	0.0318
Variable 1	**Variable 2**	**Test Statistic (Chi-square)**	**P-value**
Infection Status	HAI Seroconversion [c, e]	15.00	0.0001

[a] Number in parenthesis indicates degrees of freedom.
[b] Infection status determined by the detection of Norwalk virus RNA in one or more stool or vomitus samples via RT-PCR.
[c] Full study group (N=15).
[d] Comparison between infected (N=9) and uninfected volunteers (N=6).
[e] Seroconversion defined by at least a 4-fold increase in HAI titer.

Table 11. Bivariate Analysis for Correlates of Viral Shedding in Snow Mountain Virus Challenge [a]

Variable 1	Variable 2	Test Statistic (Pearson's r)	P-value
Duration of Viral Shedding	Baseline HAI Titer	-0.32985	0.3860
	Final HAI Titer	-0.61950	0.0752
	Fold Increase in HAI Titer	0.62462	0.0721
Peak Viral Shedding	Baseline HAI Titer	-0.05194	0.8944
	Final HAI Titer	0.78998	0.0113
	Fold Increase in HAI Titer	0.76323	0.0167

[a] Among infected volunteers (N=9).

Table 12. Bivariate Analysis for Correlates of α-norovirus Serum IgG Response in Snow Mountain Virus Challenge [a]

Variable 1	Variable 2	Test Statistic (Pearson's r)	P-value
Fold Increase in HAI Titer	Fold Increase in IgG Antibody	-0.24873	0.5187
	Final Total IgG	0.00269	0.9945

[a] Among infected volunteers (N=9).

Table 13. Bivariate Analysis for Correlates of Infection

Variable 1	Variable 2	Test Statistic (Chi-square)	P-value
Infection Status	ABO Blood Type[a]	3.7107	0.2944
Infection Status	ABO Blood Type[b]	2.5000	0.4753

[a] Norwalk virus study group (N=43)
[b] Snow Mountain virus study group (N=15)

References

1. Cubitt, W.D., D.A. McSwiggan, and W. Moore, *Winter vomiting disease caused by calicivirus.* J Clin Pathol, 1979. **32**(8): p. 786-93.
2. Wobus, C.E., et al., *Replication of Norovirus in Cell Culture Reveals a Tropism for Dendritic Cells and Macrophages.* PLoS Biology, 2004. **2**(12): p. e432.
3. Adler, J.L. and R. Zickl, *Winter vomiting disease.* J Infect Dis, 1969. **119**(6): p. 668-73.
4. Glass, R.I., U.D. Parashar, and M.K. Estes, *Norovirus gastroenteritis.* N Engl J Med, 2009. **361**(18): p. 1776-85.
5. Jiang, X., et al., *Sequence and genomic organization of Norwalk virus.* Virology, 1993. **195**(1): p. 51-61.
6. Hansman, G.S., et al., *Genetic and antigenic diversity among noroviruses.* J Gen Virol, 2006. **87**(Pt 4): p. 909-19.
7. Zheng, D.P., et al., *Norovirus classification and proposed strain nomenclature.* Virology, 2006. **346**(2): p. 312-23.
8. Le Pendu, J., et al., *Mendelian resistance to human norovirus infections.* Semin Immunol, 2006. **18**(6): p. 375-86.
9. Xi, J.N., et al., *Norwalk virus genome cloning and characterization.* Science, 1990. **250**(4987): p. 1580-3.
10. Rohayem, J., et al., *Protein-primed and de novo initiation of RNA synthesis by norovirus 3Dpol.* J Virol, 2006. **80**(14): p. 7060-9.
11. Glass, P.J., et al., *Norwalk virus open reading frame 3 encodes a minor structural protein.* J Virol, 2000. **74**(14): p. 6581-91.
12. Lambden, P.R., et al., *Sequence and genome organization of a human small round-structured (Norwalk-like) virus.* Science, 1993. **259**(5094): p. 516-9.
13. Fankhauser, R.L., et al., *Molecular epidemiology of "Norwalk-like viruses" in outbreaks of gastroenteritis in the United States.* Journal of Infectious Disease, 1998. **178**: p. 1571-1578.
14. Widdowson, M.A., et al., *Outbreaks of acute gastroenteritis on cruise ships and on land: identification of a predominant circulating strain of norovirus--United States, 2002.* J Infect Dis, 2004. **190**(1): p. 27-36.
15. Sugieda, M., et al., *Detection of Norwalk-like virus genes in the caecum contents of pigs.* Arch Virol, 1998. **143**(6): p. 1215-21.
16. Sugieda, M. and S. Nakajima, *Viruses detected in the caecum contents of healthy pigs representing a new genetic cluster in genogroup II of the genus "Norwalk-like viruses".* Virus Res, 2002. **87**(2): p. 165-72.
17. Wang, Q.H., et al., *Porcine noroviruses related to human noroviruses.* Emerg Infect Dis, 2005. **11**(12): p. 1874-81.
18. Deng, Y., et al., *Studies of epidemiology and seroprevalence of bovine noroviruses in Germany.* J Clin Microbiol, 2003. **41**(6): p. 2300-5.
19. Liu, B.L., et al., *Molecular characterization of a bovine enteric calicivirus: relationship to the Norwalk-like viruses.* J Virol, 1999. **73**(1): p. 819-25.
20. Hsu, C.C., L.K. Riley, and R.S. Livingston, *Molecular characterization of three novel murine noroviruses.* Virus Genes, 2007. **34**(2): p. 147-55.

21. Müller, B., et al., *Genetic diversity and recombination of murine noroviruses in immunocompromised mice.* Arch Virol, 2007. **152**(9): p. 1709-19.
22. Kim, M., et al., *Molecular characterization of murine norovirus isolates from South Korea.* Virus Res, 2010. **147**(1): p. 1-6.
23. Mesquita, J.R., et al., *Presence of antibodies against genogroup VI norovirus in humans.* Virol J, 2013. **10**: p. 176.
24. Kroneman, A., et al., *Proposal for a unified norovirus nomenclature and genotyping.* Arch Virol, 2013. **158**(10): p. 2059-68.
25. Lochridge, V. and M. Hardy, *Snow Mountain Virus Genome Sequence and Virus-like Particle Assembly.* Virus Genes, 2003. **26**(1): p. 71-82.
26. Lindesmith, L., et al., *Cellular and humoral immunity following Snow Mountain virus challenge.* Journal of Virology, 2005. **79**(5): p. 2900-2909.
27. Greenberg, H.B., et al., *Prevalence of antibody to the Norwalk virus in various countries.* Infect Immun, 1979. **26**(1): p. 270-3.
28. Greenberg, H.B., et al., *Role of Norwalk virus in outbreaks of nonbacterial gastroenteritis.* J Infect Dis, 1979. **139**(5): p. 564-8.
29. Parks, C.G., et al., *Genomic diversity of "Norwalk like viruses" (NLVs): pediatric infections in a Brazilian shantytown.* J Med Virol, 1999. **58**(4): p. 426-34.
30. Bucardo, F., et al., *Pediatric norovirus diarrhea in Nicaragua.* J Clin Microbiol, 2008. **46**(8): p. 2573-80.
31. Monica, B., et al., *Human caliciviruses in symptomatic and asymptomatic infections in children in Vellore, South India.* J Med Virol, 2007. **79**(5): p. 544-51.
32. Yori, P.P., et al., *Norovirus highly prevalent cause of endemic acute diarrhea in children in the peruvian Amazon.* Pediatr Infect Dis J, 2009. **28**(9): p. 844-7.
33. Al-Mashhadani MN, et al., *Norovirus gastroenteritis among children in Iraqi Kurdistan.* Journal of Medical Virology, 2008. **80**(3): p. 506-509.
34. Nguyen, T.A., et al., *Diversity of viruses associated with acute gastroenteritis in children hospitalized with diarrhea in Ho Chi Minh City, Vietnam.* J Med Virol, 2007. **79**(5): p. 582-90.
35. Kim, J.K. and J.W. Kim, *Molecular epidemiologic trends of diarrhea-causing virus infection from clinical specimens in cheonan, Korea, in 2010-2012.* J Clin Lab Anal, 2014. **28**(1): p. 47-51.
36. Siqueira, J.A., et al., *Group A rotavirus and norovirus display sharply distinct seasonal profiles in Belém, northern Brazil.* Mem Inst Oswaldo Cruz, 2013. **108**(5): p. 661-4.
37. Ahmed, S.M., B.A. Lopman, and K. Levy, *A systematic review and meta-analysis of the global seasonality of norovirus.* PLoS One, 2013. **8**(10): p. e75922.
38. Tu, E.T., et al., *Norovirus GII.4 strains and outbreaks, Australia.* Emerg Infect Dis, 2007. **13**(7): p. 1128-30.
39. Fernández, M.D., et al., *Environmental surveillance of norovirus in Argentina revealed distinct viral diversity patterns, seasonality and spatio-temporal diffusion processes.* Sci Total Environ, 2012. **437**: p. 262-9.
40. Ayukekbong, J.A., et al., *Monitoring of seasonality of norovirus and other enteric viruses in Cameroon by real-time PCR: an exploratory study.* Epidemiol Infect, 2013: p. 1-10.
41. Trainor, E., et al., *Detection and molecular characterisation of noroviruses in hospitalised children in Malawi, 1997-2007.* J Med Virol, 2013. **85**(7): p. 1299-306.

42. Bruggink, L.D. and J.A. Marshall, *Molecular and epidemiological features of GIIb norovirus outbreaks in Victoria, Australia, 2002-2005.* J Med Virol, 2009. **81**(9): p. 1652-60.
43. Papaventsis, D.C., et al., *Norovirus infection in children with acute gastroenteritis, Madagascar, 2004-2005.* Emerg Infect Dis, 2007. **13**(6): p. 908-11.
44. Greening, G.E., et al., *Molecular epidemiology of norovirus gastroenteritis outbreaks in New Zealand from 2002-2009.* J Med Virol, 2012. **84**(9): p. 1449-58.
45. Rivera, F.P., et al., *Norovirus prevalence in 'pathogen negative' gastroenteritis in children from periurban areas in Lima, Peru.* Trans R Soc Trop Med Hyg, 2011. **105**(12): p. 734-6.
46. Hall, A.J., et al., *Norovirus Disease in the United States.* Perspective, 2013. **19**(8).
47. Scallan, E., et al., *Foodborne illness acquired in the United States--major pathogens.* Emerg Infect Dis, 2011. **17**(1): p. 7-15.
48. Lopman, B.A., et al., *Viral gastroenteritis outbreaks in Europe, 1995-2000.* Emerg Infect Dis, 2003. **9**(1): p. 90-6.
49. Harris, J.P., et al., *Deaths from norovirus among the elderly, England and Wales.* Emerg Infect Dis, 2008. **14**(10): p. 1546-52.
50. Siebenga, J.J., et al., *Norovirus illness is a global problem: emergence and spread of norovirus GII.4 variants, 2001-2007.* J Infect Dis, 2009. **200**(5): p. 802-12.
51. Calderon-Margalit, R., et al., *A large-scale gastroenteritis outbreak associated with Norovirus in nursing homes.* Epidemiol Infect, 2005. **133**(1): p. 35-40.
52. Okada, M., et al., *Genetic analysis of noroviruses associated with fatalities in healthcare facilities.* Arch Virol, 2006. **151**(8): p. 1635-41.
53. Marshall, J.A., A. Dimitriadis, and P.J. Wright, *Molecular and epidemiological features of norovirus-associated gastroenteritis outbreaks in Victoria, Australia in 2001.* J Med Virol, 2005. **75**(2): p. 321-31.
54. Green, K.Y., et al., *A predominant role for Norwalk-like viruses as agents of epidemic gastroenteritis in Maryland nursing homes for the elderly.* J Infect Dis, 2002. **185**(2): p. 133-46.
55. Lopman, B.A., et al., *Linking healthcare associated norovirus outbreaks: a molecular epidemiologic method for investigating transmission.* BMC Infect Dis, 2006. **6**: p. 108.
56. Lopman, B., et al., *Host, weather and virological factors drive norovirus epidemiology: time-series analysis of laboratory surveillance data in England and Wales.* PLoS One, 2009. **4**(8): p. e6671.
57. McEvoy, M., et al., *An outbreak of viral gastroenteritis on a cruise ship.* Commun Dis Rep CDR Rev, 1996. **6**(13): p. R188-92.
58. *Norovirus Outbreaks on Three College Campuses-California, Michigan, and Wisconsin, 2008.*, in *MMWR*. 2009, Centers for Disease Control and Prevention (CDC). p. 1095-1100.
59. Marshall, J.A., et al., *Multiple outbreaks of Norwalk-like virus gastro-enteritis associated with a Mediterranean-style restaurant.* J Med Microbiol, 2001. **50**(2): p. 143-51.
60. Liu, B., et al., *An outbreak of Norwalk-like virus gastroenteritis in an aged-care residential hostel.* N S W Public Health Bull, 2003. **14**(6): p. 105-9.

61. Marx, A., et al., *An outbreak of acute gastroenteritis in a geriatric long-term-care facility: combined application of epidemiological and molecular diagnostic methods.* Infect Control Hosp Epidemiol, 1999. **20**(5): p. 306-11.
62. Milazzo, A., et al., *A large, prolonged outbreak of human calicivirus infection linked to an aged-care facility.* Commun Dis Intell Q Rep, 2002. **26**(2): p. 261-4.
63. Miller, M., et al., *Norwalk-like virus outbreak in Canberra: implications for infection control in aged care facilities.* Commun Dis Intell Q Rep, 2002. **26**(4): p. 555-61.
64. Navarro, G., et al., *An outbreak of norovirus infection in a long-term-care unit in Spain.* Infect Control Hosp Epidemiol, 2005. **26**(3): p. 259-62.
65. Lynn, S., et al., *Norovirus outbreaks in a hospital setting: the role of infection control.* N Z Med J, 2004. **117**(1189): p. U771.
66. Marks, P.J., et al., *A school outbreak of Norwalk-like virus: evidence for airborne transmission.* Epidemiol Infect, 2003. **131**(1): p. 727-36.
67. Chapman, A.S., et al., *Norovirus outbreak associated with person-to-person transmission, U.S. Air Force Academy, July 2011.* MSMR, 2011. **18**(11): p. 2-5.
68. Sharp, T.W., et al., *Epidemiology of Norwalk virus during an outbreak of acute gastroenteritis aboard a US aircraft carrier.* J Med Virol, 1995. **45**(1): p. 61-7.
69. Oyofo, B.A., et al., *Norwalk-like virus and bacterial pathogens associated with cases of gastroenteritis onboard a US Navy ship.* Am J Trop Med Hyg, 1999. **61**(6): p. 904-8.
70. Martinelli, D., et al., *Large outbreak of viral gastroenteritis caused by contaminated drinking water in Apulia, Italy, May-October 2006.* Euro Surveill, 2007. **12**(4): p. E070419.1.
71. Kukkula, M., et al., *Outbreak of Viral Gastroenteritis Due to Drinking Water Contaminated by Norwalk-like Viruses.* The Journal of Infectious Diseases, 1999. **180**(6): p. 1771-1776.
72. Maunula, L., I.T. Miettinen, and C.H. von Bonsdorff, *Norovirus outbreaks from drinking water.* Emerg Infect Dis, 2005. **11**(11): p. 1716-21.
73. Khan, A.S., et al., *Norwalk virus-associated gastroenteritis traced to ice consumption aboard a cruise ship in Hawaii: comparison and application of molecular method-based assays.* J Clin Microbiol, 1994. **32**(2): p. 318-22.
74. Pönkä, A., et al., *An outbreak of calicivirus associated with consumption of frozen raspberries.* Epidemiol Infect, 1999. **123**(3): p. 469-74.
75. Ponka, A., et al., *Outbreak oof calicivirus gastroenteritis associated with eating frozen raspberries.* Euro Surveill, 1999. **4**(6): p. 66-69.
76. Malek, M., et al., *Outbreak of norovirus infection among river rafters associated with packaged delicatessen meat, Grand Canyon, 2005.* Clin Infect Dis, 2009. **48**(1): p. 31-7.
77. Friedman, D.S., et al., *An outbreak of norovirus gastroenteritis associated with wedding cakes.* Epidemiol Infect, 2005. **133**(6): p. 1057-63.
78. Benabbes, L., et al., *Norovirus and other human enteric viruses in moroccan shellfish.* Food Environ Virol, 2013. **5**(1): p. 35-40.
79. Le Guyader, F.S., et al., *Aichi virus, norovirus, astrovirus, enterovirus, and rotavirus involved in clinical cases from a French oyster-related gastroenteritis outbreak.* J Clin Microbiol, 2008. **46**(12): p. 4011-7.
80. Thebault, A., et al., *Infectivity of GI and GII noroviruses established from oyster related outbreaks.* Epidemics, 2013. **5**(2): p. 98-110.

81. Atmar, R.L., et al., *Detection of Norwalk virus and hepatitis A virus in shellfish tissues with the PCR.* Appl Environ Microbiol, 1995. **61**(8): p. 3014-8.
82. Bellou, M., P. Kokkinos, and A. Vantarakis, *Shellfish-borne viral outbreaks: a systematic review.* Food Environ Virol, 2013. **5**(1): p. 13-23.
83. Ma, L.P., et al., *The presence of genogroup II norovirus in retail shellfish from seven coastal cities in China.* Food Environ Virol, 2013. **5**(2): p. 81-6.
84. Rodriguez-Manzano, J., et al., *Adenovirus and Norovirus Contaminants in Commercially Distributed Shellfish.* Food Environ Virol, 2013.
85. Schaeffer, J., et al., *Norovirus contamination on French marketed oysters.* Int J Food Microbiol, 2013. **166**(2): p. 244-8.
86. Mattison, K., et al., *Survival of calicivirus in foods and on surfaces: experiments with feline calicivirus as a surrogate for norovirus.* J Food Prot, 2007. **70**(2): p. 500-3.
87. Baert, L., et al., *Reported foodborne outbreaks due to noroviruses in Belgium: the link between food and patient investigations in an international context.* Epidemiol Infect, 2009. **137**(3): p. 316-25.
88. Barrabeig, I., et al., *Foodborne norovirus outbreak: the role of an asymptomatic food handler.* BMC Infect Dis, 2010. **10**: p. 269.
89. Tuan Zainazor, C., et al., *The scenario of norovirus contamination in food and food handlers.* J Microbiol Biotechnol, 2010. **20**(2): p. 229-37.
90. Moe, C.L., *Preventing norovirus transmission: how should we handle food handlers?* Clin Infect Dis, 2009. **48**(1): p. 38-40.
91. Teunis, P.F., et al., *Norwalk virus: how infectious is it?* J Med Virol, 2008. **80**(8): p. 1468-76.
92. Glass, R.I., et al., *The epidemiology of enteric caliciviruses from humans: a reassessment using new diagnostics.* J Infect Dis, 2000. **181 Suppl 2**: p. S254-61.
93. Rockx, B., et al., *Natural history of human calicivirus infection: a prospective cohort study.* Clin Infect Dis, 2002. **35**(3): p. 246-53.
94. Ozawa, K., et al., *Norovirus infections in symptomatic and asymptomatic food handlers in Japan.* J Clin Microbiol, 2007. **45**(12): p. 3996-4005.
95. Sukhrie, F.H., et al., *Nosocomial transmission of norovirus is mainly caused by symptomatic cases.* Clin Infect Dis, 2012. **54**(7): p. 931-7.
96. Belliot, G., et al., *Use of murine norovirus as a surrogate to evaluate resistance of human norovirus to disinfectants.* Appl Environ Microbiol, 2008. **74**(10): p. 3315-8.
97. Dolin, R., et al., *Transmission of acute infectious nonbacterial gastroenteritis to volunteers by oral administration of stool filtrates.* J Infect Dis, 1971. **123**(3): p. 307-12.
98. Karst, S.M., *Pathogenesis of noroviruses, emerging RNA viruses.* Viruses, 2010. **2**(3): p. 748-81.
99. Estes, M.K., B.V. Prasad, and R.L. Atmar, *Noroviruses everywhere: has something changed?* Curr Opin Infect Dis, 2006. **19**(5): p. 467-74.
100. Hutson, A.M., R.L. Atmar, and M.K. Estes, *Norovirus disease: changing epidemiology and host susceptibility factors.* Trends Microbiol, 2004. **12**(6): p. 279-87.
101. Prevention, C.f.D.C.a., *Norovirus activity--United States, 2006-2007*, in *MMWR*, C.f.D.C.a. Prevention, Editor. 2007. p. 842-846.
102. Lopman, B.A., et al., *Clinical manifestation of norovirus gastroenteritis in health care settings.* Clin Infect Dis, 2004. **39**(3): p. 318-24.

103. Khan, R.R., et al., *Gastrointestinal norovirus infection associated with exacerbation of inflammatory bowel disease.* J Pediatr Gastroenterol Nutr, 2009. **48**(3): p. 328-33.
104. Kirkwood, C.D. and R. Streitberg, *Calicivirus shedding in children after recovery from diarrhoeal disease.* J Clin Virol, 2008. **43**(3): p. 346-8.
105. Murata, T., et al., *Prolonged norovirus shedding in infants <or=6 months of age with gastroenteritis.* Pediatr Infect Dis J, 2007. **26**(1): p. 46-9.
106. Sakai, Y., et al., *Clinical severity of Norwalk virus and Sapporo virus gastroenteritis in children in Hokkaido, Japan.* Pediatr Infect Dis J, 2001. **20**(9): p. 849-53.
107. Zintz, C., et al., *Prevalence and genetic characterization of caliciviruses among children hospitalized for acute gastroenteritis in the United States.* Infect Genet Evol, 2005. **5**(3): p. 281-90.
108. Payne, D.C., et al., *Norovirus and medically attended gastroenteritis in U.S. children.* N Engl J Med, 2013. **368**(12): p. 1121-30.
109. Lindesmith, L., et al., *Human susceptibility and resistance to Norwalk virus infection.* Nat Med, 2003. **9**(5): p. 548-53.
110. Graham, D.Y., et al., *Norwalk virus infection of volunteers: new insights based on improved assays.* J Infect Dis, 1994. **170**(1): p. 34-43.
111. Johnson, P.C., et al., *Multiple-challenge study of host susceptibility to Norwalk gastroenteritis in US adults.* J Infect Dis, 1990. **161**(1): p. 18-21.
112. Hutson, A.M., et al., *Norwalk virus infection and disease is associated with ABO histo-blood group type.* J Infect Dis, 2002. **185**(9): p. 1335-7.
113. Marionneau, S., et al., *Norwalk virus binds to histo-blood group antigens present on gastroduodenal epithelial cells of secretor individuals.* Gastroenterology, 2002. **122**(7): p. 1967-77.
114. Kelly, R.J., et al., *Sequence and expression of a candidate for the human Secretor blood group alpha(1,2)fucosyltransferase gene (FUT2). Homozygosity for an enzyme-inactivating nonsense mutation commonly correlates with the non-secretor phenotype.* J Biol Chem, 1995. **270**(9): p. 4640-9.
115. Kudo, T., et al., *Molecular genetic analysis of the human Lewis histo-blood group system. II. Secretor gene inactivation by a novel single missense mutation A385T in Japanese nonsecretor individuals.* J Biol Chem, 1996. **271**(16): p. 9830-7.
116. Hakomori, S., *Antigen structure and genetic basis of histo-blood groups A, B and O: their changes associated with human cancer.* Biochim Biophys Acta, 1999. **1473**(1): p. 247-66.
117. Hutson, A.M., et al., *Norwalk virus infection associates with secretor status genotyped from sera.* J Med Virol, 2005. **77**(1): p. 116-20.
118. Bucardo, F., et al., *Asymptomatic norovirus infections in Nicaraguan children and its association with viral properties and histo-blood group antigens.* Pediatr Infect Dis J, 2010. **29**(10): p. 934-9.
119. Choi, J.M., et al., *Atomic resolution structural characterization of recognition of histo-blood group antigens by Norwalk virus.* Proc Natl Acad Sci U S A, 2008. **105**(27): p. 9175-80.
120. Harrington, P.R., et al., *Binding of Norwalk virus-like particles to ABH histo-blood group antigens is blocked by antisera from infected human volunteers or experimentally vaccinated mice.* Journal of Virology, 2002. **76**(23): p. 12335–12343.

121. Huang, P., et al., *Norovirus and histo-blood group antigens: demonstration of a wide spectrum of strain specificities and classification of two major binding groups among multiple binding patterns.* J Virol, 2005. **79**(11): p. 6714-22.
122. Nordgren, J., et al., *Norovirus gastroenteritis outbreak with a secretor-independent susceptibility pattern, Sweden.* Emerg Infect Dis, 2010. **16**(1): p. 81-7.
123. Takanashi, S., et al., *Characterization of emerging GII.g/GII.12 noroviruses from a gastroenteritis outbreak in the United States in 2010.* J Clin Microbiol, 2011. **49**(9): p. 3234-44.
124. Hutson, A.M., et al., *Norwalk virus-like particle hemagglutination by binding to h histo-blood group antigens.* J Virol, 2003. **77**(1): p. 405-15.
125. Czakó, R., et al., *Serum hemagglutination inhibition activity correlates with protection from gastroenteritis in persons infected with Norwalk virus.* Clin Vaccine Immunol, 2012. **19**(2): p. 284-7.
126. Hierholzer, J.C. and M.T. Suggs, *Standardized viral hemagglutination and hemagglutination-inhibition tests. I. Standardization of erythrocyte suspensions.* Appl Microbiol, 1969. **18**(5): p. 816-23.
127. Cheetham, S., et al., *Binding patterns of human norovirus-like particles to buccal and intestinal tissues of gnotobiotic pigs in relation to A/H histo-blood group antigen expression.* J Virol, 2007. **81**(7): p. 3535-44.
128. Tan, M., et al., *Outbreak studies of a GII-3 and a GII-4 norovirus revealed an association between HBGA phenotypes and viral infection.* J Med Virol, 2008. **80**(7): p. 1296-301.
129. Tan, M. and X. Jiang, *Norovirus gastroenteritis, carbohydrate receptors, and animal models.* PLoS Pathog, 2010. **6**(8): p. e1000983.
130. Tan, M. and X. Jiang, *Norovirus and its histo-blood group antigen receptors: an answer to a historical puzzle.* Trends Microbiol, 2005. **13**(6): p. 285-93.
131. Tan, M., R.S. Hegde, and X. Jiang, *The P domain of norovirus capsid protein forms dimer and binds to histo-blood group antigen receptors.* J Virol, 2004. **78**(12): p. 6233-42.
132. Patel, M.M., et al., *Noroviruses: a comprehensive review.* J Clin Virol, 2009. **44**(1): p. 1-8.
133. Rockx, B., et al., *Characterization of the homo- and heterotypic immune responses after natural norovirus infection.* J Med Virol, 2005. **77**(3): p. 439-46.
134. Reeck, A., et al., *Serological correlate of protection against norovirus-induced gastroenteritis.* J Infect Dis, 2010. **202**(8): p. 1212-8.
135. Moe, C.L., et al., *Diagnosis of norwalk virus infection by indirect enzyme immunoassay detection of salivary antibodies to recombinant norwalk virus antigen.* Clin Diagn Lab Immunol, 2004. **11**(6): p. 1028-34.
136. Larsson, M.M., et al., *Antibody prevalence and titer to norovirus (genogroup II) correlate with secretor (FUT2) but not with ABO phenotype or Lewis (FUT3) genotype.* J Infect Dis, 2006. **194**(10): p. 1422-7.
137. Iritani, N., et al., *Humoral immune responses against norovirus infections of children.* J Med Virol, 2007. **79**(8): p. 1187-93.
138. Harrington, P.R., et al., *Norovirus capture with histo-blood group antigens reveals novel virus-ligand interactions.* Journal of Virology, 2004. **78**(6): p. 3035-3045.

139. Lindesmith, L.C., et al., *Heterotypic humoral and cellular immune responses following Norwalk virus infection.* J Virol, 2010. **84**(4): p. 1800-15.
140. Swanstrom, J., et al., *Characterization of blockade antibody responses in GII.2.1976 Snow Mountain virus-infected subjects.* J Virol, 2014. **88**(2): p. 829-37.
141. Leon, J.S., et al., *Randomized, double-blinded clinical trial for human norovirus inactivation in oysters by high hydrostatic pressure processing.* Appl Environ Microbiol, 2011. **77**(15): p. 5476-82.
142. Monroe, S.S., et al., *Detection of antibody to recombinant Norwalk virus antigen in specimens from outbreaks of gastroenteritis.* J Clin Microbiol, 1993. **31**(11): p. 2866-2872.
143. *Methods in Molecular Biology, The ELISA Guidebook*. 2009: Humana Press.
144. Azevedo, M., et al., *Infection by Helicobacter pylori expressing the BabA adhesin is influenced by the secretor phenotype.* J Pathol, 2008. **215**(3): p. 308-16.
145. Patel, M.M., et al., *Systematic literature review of role of noroviruses in sporadic gastroenteritis.* Emerg Infect Dis, 2008. **14**(8): p. 1224-31.
146. Kirby, A.E., et al., *Disease course and viral shedding in experimental Norwalk virus and Snow Mountain virus infection.* J Med Virol, 2014.
147. Atmar, R.L., et al., *Norovirus vaccine against experimental human Norwalk Virus illness.* N Engl J Med, 2011. **365**(23): p. 2178-87.
148. Atmar, R.L., et al., *Norwalk virus shedding after experimental human infection.* Emerg Infect Dis, 2008. **14**(10): p. 1553-7.
149. Okhuysen, P.C., et al., *Viral shedding and fecal IgA response after Norwalk virus infection.* J Infect Dis, 1995. **171**(3): p. 566-9.

Conclusions and Public Health Implications

Noroviruses are the most common cause of epidemic, viral gastroenteritis and are a significant global health concern, especially in developing countries [4, 27, 28]. A systematic review of literature by Patel et al., found that norovirus infections are estimated to cause over 1 million hospitalizations and up to 200,000 deaths among children under the age of 5 in developing countries [132, 145]. The limited knowledge currently available on the immune response to norovirus infection hinders the ability to reduce the morbidity and mortality associated with this pathogen.

Currently, an effective norovirus vaccine does not exist, and it is a logical next step in preventing the morbidity and mortality caused by norovirus infection. However, the lack of understanding of the immune response to infection remains a major obstacle to vaccine development. Another obstacle to development is that an effective vaccine needs to provide protective immunity against a hefty list of norovirus strains that continues to grow. Development and evaluation of the multitude of potential vaccine candidates requires tools to measure vaccine efficacy.

The use of serum HAI assays in norovirus research is of particular interest because it has been shown to be a correlate of protection from illness [6]. Czakò et al., was able to reflect the practical use of this assay by measuring serum HAI titers as a predictor of protection against illness from norovirus [6]. However, this relationship was not clearly seen in this study.

Serum HAI assays can also serve as a potential alternative to the HBGA blocking assay that is widely used as a correlate of protection against illness due to norovirus. The HBGA blocking assay that is currently used is highly sensitive, expensive and includes

several technical challenges [125, 134, 139]. The assay used in this study was relatively inexpensive and timely, and yielded valuable results to facilitate immunologic research. However, obtaining appropriate red blood cells for use in this assay is limited as donors of known secretor status are not commercially available.

Further exploration and work is necessary with serum HAI assay and its role in understanding the dynamic course of norovirus infection. Factors of norovirus infection, like viral shedding characteristics from asymptomatic, infected individuals, pose serious public health implications due to the highly virulent nature of norovirus. The correlation found between HAI titer and viral shedding among the Snow Mountain study group is of particular interest as this is the first study to demonstrate the relationship.

Although this study could not find an association between presentation of clinical illness and serum HAI titer, Czakò et al., produced promising results with their serum HAI activity study [125]. From the findings of their study, it is reasonable to hypothesize that serum HAI titer can be used as a measurable correlate of protection against norovirus associated disease. Finally, from the results of this study, it is evident that the serum HAI assay is a hopeful alternative to the current research methods of immune response to norovirus infection.

References

1. Glass, R.I., U.D. Parashar, and M.K. Estes, *Norovirus gastroenteritis.* N Engl J Med, 2009. **361**(18): p. 1776-85.
2. Greenberg, H.B., et al., *Role of Norwalk virus in outbreaks of nonbacterial gastroenteritis.* J Infect Dis, 1979. **139**(5): p. 564-8.
3. Parks, C.G., et al., *Genomic diversity of "Norwalk like viruses" (NLVs): pediatric infections in a Brazilian shantytown.* J Med Virol, 1999. **58**(4): p. 426-34.
4. Patel, M.M., et al., *Noroviruses: a comprehensive review.* J Clin Virol, 2009. **44**(1): p. 1-8.
5. Patel, M.M., et al., *Systematic literature review of role of noroviruses in sporadic gastroenteritis.* Emerg Infect Dis, 2008. **14**(8): p. 1224-31.
6. Czakó, R., et al., *Serum hemagglutination inhibition activity correlates with protection from gastroenteritis in persons infected with Norwalk virus.* Clin Vaccine Immunol, 2012. **19**(2): p. 284-7.
7. Reeck, A., et al., *Serological correlate of protection against norovirus-induced gastroenteritis.* J Infect Dis, 2010. **202**(8): p. 1212-8.
8. Lindesmith, L.C., et al., *Heterotypic humoral and cellular immune responses following Norwalk virus infection.* J Virol, 2010. **84**(4): p. 1800-15.