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Comparison of IgG Antibody Seroprevalence to Three Pandemic Strains of GII.4 Norovirus in 1999-2000 and 2003-2004

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By

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B.S. in Biology
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Abstract

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By Skyler Brennan

Norovirus is the leading cause of acute gastroenteritis among all age groups worldwide. More specifically, genogroup II, genotype 4 (GII.4) norovirus strains are responsible for the most illness, with a new pandemic strain emerging every few years. The purpose of this study was to examine antibody seroprevalence to GII.4 GB, GII.4 FH, and GII.4 NO in relation to seroprevalence to prior strains and time of sample collection. The pandemic GII.4 strains used in this study are: GII.4 Grimsby (GB), which emerged in 1995, GII.4 Farmington Hills (FH), which emerged in 2002, and GII.4 New Orleans (NO), which emerged in 2009. NHANES serological data from the 1999-2000 and 2003-2004 cycles (Cycle 1 and Cycle 2, respectively) were analyzed (n=2019). Enzyme-linked immunosorbent assays (ELISA) were used to quantify serum anti-norovirus IgG antibodies, with GII.4 GB, GII.4 FH, and GII.4 NO virus-like particles (VLPs) acting as the antigens. The concentration of serum IgG antibodies for each VLP tested was reported as optical density (OD) values. An OD cutpoint of 1.5 for determining seropositivity was chosen based on data from previous norovirus challenge studies. A multivariable logistic regression model, a linear regression model, and one-way ANOVA tests were then used to complete data analysis. The results 1) suggest that 52.49% of GII.4 GB Cycle 1 samples and 54.98% of GII.4 GB Cycle 2 samples are seropositive, 41.45% of GII.4 FH Cycle 1 samples are not seropositive and 61.19% of GII.4 FH Cycle 2 samples are seropositive, and 45.02% of GII.4 NO Cycle 1 samples and 49.13% of GII.4 NO Cycle 2 samples are not seropositive, and 2) show that there is a statistically significant difference in the antibody levels to GII.4 FH between Cycle 1 and Cycle 2 (p-value<0.0001), consistent with the emergence of this strain in 2002. The findings also justify further research of antibody seroprevalence to various GII.4 strains, since norovirus immunology is not well understood.

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Chapter I: Literature Review

Norovirus is the leading cause of acute gastroenteritis worldwide (3, 4, 6, 8, 21). Despite the ubiquitous nature of norovirus, there are several limitations in studying norovirus, which partially explains its vast global presence. The primary trend in norovirus research today is testing potential norovirus vaccines in clinical trials, in addition to studying norovirus immunity and evolution. Great strides have been made in this field of research in the past several years, but the prevalence and public health implications of norovirus demand that even more strides in research need to be made.

With an incubation period of 12-48 hours, symptoms of norovirus infection include nausea, vomiting, diarrhea, and abdominal cramps and typically for one to three days (1). Estimating the total disease burden of norovirus is incredibly difficult for several reasons: many cases are asymptomatic, many mild cases go unreported, and doctors rarely order diagnostics tests for suspected cases (2, 3). A recent review, which analyzed eight studies estimating population-based incidence rates of norovirus in the United States, summarized the prevalence of norovirus in the country. After triangulation of the studies' results, the review concluded that in the US, 56000 to 71000 hospitalizations and 19 to 21 million illnesses occur annually due to norovirus (4). The structure of noroviruses partially explains why it is so widespread. Norovirus is very simple in structure, with two domains contained in the capsid protein: the S and P domains. Additionally, it is a single-strand, non-enveloped RNA virus; non-enveloped viruses are particularly robust and much more difficult to control. In short, the durability of norovirus adds to its dominance, which leads to severe consequences from a public health standpoint.

In addition to being the leading cause of acute gastroenteritis worldwide, norovirus is also the most prevalent enteric disease among all age groups. Norovirus, as with many infectious diseases, is markedly dangerous for the very young and the very old. Most norovirus-related deaths occur in people 65 years and older, while most health care visits relating to norovirus occur in children under 5 years (4). Regardless of age, norovirus infection can originate and spread anywhere, but this is especially the case in crowded environments. Nursing homes, hospitals, cruise ships, and schools are often found to be the source of norovirus outbreaks. One study analyzing 14 laboratory-confirmed outbreaks on 12 cruise ships found that 11 outbreaks were caused by norovirus; another conclusion was that 18% of people on these cruises fell ill (5). In one instance, a cruise ship with an outbreak underwent a full week of intensive cleaning before the next voyage, but another outbreak occurred anyway (5). A review of norovirus outbreaks in long-term health care facilities addressed the challenges of controlling the spread of norovirus in a closed environment (3). This review advised that extreme measures, such as sending sick employees home for two to three days, limiting contact with sick patients, or even closures, are likely the best way to both control an outbreak and minimize financial burden in health care facilities (3).

Although infection and illness can strike year round, norovirus events peak in the winter months, from October to March. In fact, 63% to 73% of norovirus events happen during this time (6). The robust nature of norovirus is enhanced by its multiple modes of transmission. People can acquire norovirus through contaminated food, water, and surfaces, as well as contact with other infected people and fecal-oral contact. One route of norovirus transmission currently being debated in the research community is the viability

of contracting norovirus via airborne transmission. More specifically, researchers are debating if someone can contract norovirus when a person nearby vomits. Most reports thus far are anecdotal (6). However, a recent study at the University of North Carolina published the first data revealing that norovirus can be aerosolized after a vomiting event (6). While the study didn't prove that norovirus can be contracted through airborne transmission, it was an important step in investigating the capabilities of norovirus infection.

As mentioned earlier, norovirus is incredibly difficult to control. Several characteristics of norovirus illustrate this, such as its environmental stability, resistance to disinfection, low infectious dose, and short incubation period (3, 7). One characteristic of norovirus that is often studied is its shedding period, which is typically longer than that of other illnesses. Norovirus shedding patterns were described in a study based on 102 stool samples from one hospital outbreak and three nursing home outbreaks (7). After symptomatic and asymptomatic shedding patterns were compared between healthcare workers and patients, both asymptomatic patients and especially symptomatic patients were shown to shed a greater quantity of norovirus for a longer period of time (7). In a retrospective cohort study, 206 stool samples from 77 patients were analyzed to determine length and amount of viral shedding (8). Shedding periods of ten days or more occurred in 20 out of the 77 patient episodes (8). Viral load did not significantly differ between patients of varying ages and immunosuppression statuses (8). Risk factors for norovirus shedding longer than 10 days were also determined, and found to include organ transplantation, immunosuppression, and under 10 years of age (8). The results from both

studies show that an existing illness or immune system deficiencies leads to longer shedding periods, and probably intensifies other symptoms as well.

Laboratory analysis is impeded by the fact that norovirus can't be cultured *in vitro*. However, there are several viable techniques used for norovirus detection, which are compared by cost, sensitivity, and specificity in a recent review of laboratory methods (9). Because of its speed and high sensitivity, the gold standard for norovirus detection is real-time reverse transcription-quantitative PCR (RT-qPCR), which can be used for several types of biological samples as well as food and water samples (9).

Located within the Caliciviridae family, noroviruses can be classified into six genogroups: one through six (GI through GVI). Each genogroup contains a varying number of associated genotypes, which are further divided into strains. Humans are only affected by the genogroups GI, GII, and GIV, with GI and GII causing the most human illnesses. The most prominent type of norovirus is GII.4 (genogroup II, genotype 4). In addition to causing the majority of norovirus outbreaks, every few years, a new pandemic strain of GII.4 emerges and quickly becomes dominant (10). There have been five pandemic GII.4 strains between 1995 and 2009 (10). The first of these strains emerged in the United Kingdom in 1995, called GII.4 Grimsby (a pandemic GII.4 strain is named after the place it was first discovered) (10). The GII.4 Farmington Hills strain supplanted GII.4 Grimsby in 2002, which was then replaced by two pandemic strains (GII.4 Laurens and GII.4 Minerva) in 2006 (10). Then, GII.4 New Orleans emerged in 2009, succeeding the previous 2006 strains (10). It should be noted that while these emergent strains are eventually replaced by other dominant strains at the time, the replaced strains can still circulate and cause infections. Due to the vast presence of GII.4 strains worldwide, it is

imperative that future research focuses on this particular genogroup.

A phylogenetic analysis of GII.4 capsid sequences over 20 years indicated that GII.4 noroviruses are undergoing epochal evolution, meaning that long periods of stasis are punctuated by rapid transitions and emergent strains (11). Furthermore, small changes in the exposed areas of the capsid protein suggest antigenic drift (11). A study on the evolution of pandemic GII.4 strains examined GII.4 Sydney, GII.4 New Orleans, and GII.4 Minerva to determine if antigenic shift had occurred (12). At least two epitopes differed from GII.4 Sydney and the previous epidemic strains, demonstrating the speed with which antigenic drift transpires in GII.4 strains (12). An earlier study by some of the same researchers also discovered clear antigenic differences between GII.4-1987 and GII.4 Minerva (13). An individual's susceptibility to GII.4 infection relies on many things, such as the antigenic properties of the virus and the individual's secretor status.

One factor that helps determine a person's susceptibility to norovirus infection and illness is secretor status. Humans can be categorized as either secretor positive or secretor negative. Secretor status is defined by the type of FUT2 alleles on histo-blood group antigens (HBGAs). The presence of one or more positive FUT2 alleles (+/+ or -/+) indicates a secretor positive status, while the complete absence of positive FUT2 alleles (-/-) indicates a secretor negative status. HBGAs bind to epitopes on noroviruses; in fact, this binding is crucial for the initiation of GI.1 and GII.4 infections. In secretor positive individuals, the H antigen, the precursor for A and B blood group antigens, is released into the bodily fluids, whereas the H antigen in secretor negative individuals stay in the cells. Therefore, those who are secretor negative are highly protected from norovirus infection, including infection from GII.4 strains. However, in one study examining

symptomatic illness from a foodborne outbreak between secretor positive and secretor negative individuals, the analysis of 83 saliva samples yielded not statistically significantly different results (14). Specifically, 38% and 47% of symptomatic participants were secretor positive and secretor negative, respectively (14). A possible explanation given for these contradictory results is the similarity of the outbreak strain to a GI.3 strain known to bind equally to secretor positive and secretor negative saliva (14). While the definition of secretor status is well defined, secretor status as it relates to norovirus immunity is slightly more unclear.

There are several gaps in the norovirus literature, which deters progress in this field of research. For instance, the reservoir for emergent norovirus strains is unclear, as addressed in a recent review (15). Immunocompromised, malnourished, and elderly individuals are all proposed as possible reservoirs (15). The ability of an immunocompromised individual to act as a reservoir highly depends on the extent of immune response; in other words, an intermediate immune response is far more conducive for viral evolution than strong or minimal immune responses (15). Elderly or malnourished individuals are also plausible reservoirs due to their weakened, but not completely decimated immune systems (15).

Another major challenge of eliminating norovirus that is no currently no vaccine for norovirus. This can be attributed to both the inability to culture norovirus and evolution and diversity of norovirus strains. However, there are vaccines being tested in clinical trials that contain virus-like particles (VLPs), which mimic norovirus (16 -19). VLPs are ideal for vaccines in development, as they are non-infectious due to their lack of genetic material but stimulate the immune system similarly to the intact virus (20).

There have been both human challenge studies and animal studies testing the efficacy of VLP vaccines (16-19, 21, 22).

One important piece of literature described results from randomized, double-blinded, placebo-controlled clinical trial testing a bivalent (GI.1 and GII.4) VLP norovirus vaccine. This trial contained a vaccination phase and a challenge phase, in which participants are administered GII.4 and monitored for illness. The researchers, investigating the correlation between prechallenge serum antibody levels and illness, verified that higher serum antibody levels led to a decreased likelihood of illness among placebo recipients (16). Conversely, this correlation was not present among vaccine recipients (16). The results, although contradictory, reveal multi-faceted nature of norovirus immunity. An earlier clinical trial conducted by the same primary researcher assessed the efficacy of a monovalent (GI.1) VLP norovirus vaccine (17). Challenged with GI.1 as opposed to GII.4, there was a significant statistical difference of illness between vaccine and placebo recipients (69% and 37%, respectively) (17).

Another human challenge trial testing the bivalent VLP norovirus vaccine focused more on the symptoms of illness experienced by vaccine versus placebo recipients instead of serum antibody response (18). The frequency of mild, moderate, and severe diarrhea differed between vaccine and placebo recipients, although the difference was not statistically different (18). In terms of shedding periods, 22.4% of vaccine recipients and 36.2% of placebo recipients were still shedding norovirus 10 days after the vaccinations (18). A separate clinical trial testing the bivalent VLP norovirus vaccine sought to investigate the effects of vaccine dose and age on immunogenicity (19). It should be established that this particular trial did not include a human challenge stage. Regardless

of the dose size, every participant exhibited a seroresponse to GI.1 after the first vaccination; as for GII.4, 56% of those who received the lowest dose exhibited a seroresponse, while 75%-88% of those who received higher doses exhibited a seroresponse (19). The test of immunogenicity by age group revealed comparable patterns of serum antibody responses to GI.1 and GII.4 among 18-49 year olds, 50-64 year olds, and 65-85 year olds (19). When testing both dose response and age, no substantial increase in serum antibody response was noted after the second administration of the vaccine or placebo (19).

In addition to human challenge trials, studies testing VLP norovirus vaccines have also been conducted on mice and rabbits. A mice study testing multiple monovalent vaccines (GI.1, GI.3, GII.4-1999, GII.4-2010) found very similar serum antibody responses, but widely varying cross-reactive serum antibody responses (21). Cross-reactive serum antibody responses were determined against GI.1, GI.3, GII.4-1999, GII.4-2010, GII.4-2012, and GII.12 (21). Within GI, mice vaccinated with GI.1 VLPs display no cross-reactivity, while mice vaccinated with GI.3 VLPs were able cross-react with GI.1 VLPs (21). Within GII, mice vaccinated with GII.4-1999 VLPs cross reacted with every strain of GII VLPs, while mice vaccinated with GII.4-2010 VLPs only cross-reacted with GII.4-2012 VLPs (21). This last result is most surprising because of the small number of changes in the amino acid sequences of GII.4-2010 VLPs and GII.4-2012 VLPs. In a separate animal study, a novel GII.4 VLP vaccine was created by combining three different GII.4 amino acid sequences to form a consensus GII.4 amino acid sequence (22). This vaccine was designed to generate cross-reactivity among different GII.4 strains, and was tested on rabbits (22). The vaccine produced high serum

antibody responses and cross-reactivity among several strains of GII.4 VLPs, but no cross-reactivity was observed with GI VLPs (22).

Immunity to norovirus is incredibly complicated and not well understood. A review of advances in norovirus immunity shows just how much knowledge has been gained in the past decade (23). Many of these topics, such as adaptive immunity and innate immunity to norovirus have finally been able to be studied due to viable mouse and gnotobiotic pig and calf models (23). The most recent research regarding norovirus immunity involves VLP vaccine trials and studying the association between norovirus and the gut microbiome (23). Furthermore, the frequency of cross reactivity between norovirus strains could possibly play a role in norovirus immunity. Cross-reactivity was discussed earlier in the context of VLP norovirus vaccines, but one study analyzed sera samples from young children infected with GII.4-2009 and examined their cross-reactivity with various GII.4 VLP strains, all without vaccinations (24). ELISA and HBGA blocking assays, with VLPs for GII.4-1999, GII.4-2009, and GII.4-2012, were utilized to analyze the infected sera (24). While no children exhibited cross-reactivity to GII.4-1999, four out of five children exhibited very strong cross-reactivity to GII.4-2012 (24). The results show that children, a population especially susceptible to norovirus infection, are most likely protected from strains closely related to a strain that caused infection or illness (24). Immunity to one strain of norovirus can greatly depend on which specific strains an individual has previously been exposed to.

Prior studies have proposed several theories about the central type of immunity present in humans against norovirus, primarily the role of herd immunity (12, 13). Herd immunity describes the phenomenon in which a few individuals in a population are not

immune to a particular disease, yet are protected because most individuals in that population are immune. One study proposes the notion that herd immunity drives the antigenic drift observed in norovirus (25). Emergent strains of GII.4 quickly dominate because they evade host herd immunity responses; moreover, these emergent strains may be naturally selected based the presence of one or more evolved epitopes that concurrently generate antibody responses against older strains, thereby contributing to herd immunity while thwarting it (25). The complexity of this theory is apparent, especially since there are likely multiples types of immunity involved, but more researchers find it plausible as the knowledge of norovirus immunity expands.

In many studies about antibody response to various GII.4 strains, serum specimens are frequently used for analysis. One study conducted in Valencia, Spain, surveyed immunoglobulin (IgG) antibody seroprevalence against both the P domain and VLPs of GII.4 with enzyme immunoassays (26). Additionally, the researchers examined the association between seroprevalence and age. In total, 434 serum samples were collected and analyzed from healthy individuals ranging from 7 months to 86 years old (26). The results conclude that 429 of the serum samples had antibodies against the GII.4 P domain, and that there was a significant correlation between serum IgG antibody titers against VLPs and the P domain ($r = 0.794$) (26). As far as the association between seroprevalence and age, antibodies against both the P domain and VLPs were detected in approximately 100% of the individuals 11 years of age and older (26). This provides evidence that because of high rates of norovirus infection in young children, they acquire antibodies to GII.4 at early ages. Although children are highly susceptible to norovirus infection, breastfeeding infants could be protected from infection due to large

concentrations of antibodies in breast milk and their mothers' sera.

A recent study investigated the amount of IgA GII.4 Minerva antibodies in breast milk and serum samples of 108 breast-feeding mothers (27). Respectively, 75% and 62% of breast milk and serum samples contained IgA antibodies to GII.4 Minerva, and a significant correlation between IgA in breast milk and serum was observed ($r = 0.427$) (27). IgG seroprevalence was also examined, but only in serum samples; approximately 56% of serum samples contained IgG antibodies to GII.4 Minerva (27). Based on the results, IgA antibodies in breast milk are increased by some unknown mechanisms other than diffusion from serum (27). Another insinuation is that breastfeeding mothers and their children are perhaps less likely to develop a norovirus infection.

An additional topic of interest in norovirus immunology is norovirus antibody prevalence in those with travelers' diarrhea, a fairly common occurrence when traveling out of the country. A study of norovirus-associated travelers' diarrhea followed 75 US students that had recently traveled to Mexico (28). Serum samples were collected from each traveler before and during traveling to determine the antibody prevalence against GII.4 VLPs (28). Before traveling, 62 of 75 travelers had immunoglobulin A (IgA) serum antibodies against the VLPs, and all 75 of the travelers had IgG serum antibodies against the VLPs (28). Seroprevalence of both IgA and IgG antibodies before traveling had no effect on the likelihood of contracting GII.4 norovirus infection (28). A primary implication from these results posits that prior norovirus infection does not necessarily protect an individual from reinfection, regardless of whether antibodies are present. The exact role and efficacy of norovirus-specific antibodies in preventing infection is not fully realized yet, but continued research and future advancements could change that

uncertainty.

Although much is known about the characteristics of norovirus and the illnesses and outbreaks it causes, there remain several sizeable gaps in the current literature. These gaps, including vaccine development and immunity, are starting to grow smaller with advancements in methodology. Any discoveries in this field greatly contribute to the betterment of public health, as norovirus is so ubiquitous. Even slightly reducing norovirus infection and illness is a daunting task, but the likelihood of accomplishing that goal increases as the literature becomes more extensive.

Chapter II: Manuscript

Comparison of IgG Antibody Seroprevalence to Three Pandemic Strains of GII.4 Norovirus in 1999-2000 and 2003-2004

By Skyler Brennan

Abstract

Norovirus is the leading cause of acute gastroenteritis among all age groups worldwide. More specifically, genogroup II, genotype 4 (GII.4) norovirus strains are responsible for the most illness, with a new pandemic strain emerging every few years. The purpose of this study was to examine antibody seroprevalence to GII.4 GB, GII.4 FH, and GII.4 NO in relation to seroprevalence to prior strains and time of sample collection. The pandemic GII.4 strains used in this study are: GII.4 Grimsby (GB), which emerged in 1995, GII.4 Farmington Hills (FH), which emerged in 2002, and GII.4 New Orleans (NO), which emerged in 2009. NHANES serological data from the 1999-2000 and 2003-2004 cycles (Cycle 1 and Cycle 2, respectively) were analyzed (n=2019). Enzyme-linked immunosorbent assays (ELISA) were used to quantify serum anti-norovirus IgG antibodies, with GII.4 GB, GII.4 FH, and GII.4 NO virus-like particles (VLPs) acting as the antigens. The concentration of serum IgG antibodies for each VLP tested was reported as optical density (OD) values. An OD cutpoint of 1.5 for determining seropositivity was chosen based on data from previous norovirus challenge studies. A multivariable logistic regression model, a linear regression model, and one-way ANOVA tests were then used to complete data analysis. The results 1) suggest that 52.49% of GII.4 GB Cycle 1 samples and 54.98% of GII.4 GB Cycle 2 samples are seropositive, 41.45% of GII.4 FH Cycle 1 samples are not seropositive and 61.19% of

GII.4 FH Cycle 2 samples are seropositive, and 45.02% of GII.4 NO Cycle 1 samples and 49.13% of GII.4 NO Cycle 2 samples are not seropositive, and 2) show that there is a statistically significant difference in the antibody levels to GII.4 FH between Cycle 1 and Cycle 2 (p-value<0.0001), consistent with the emergence of this strain in 2002. The findings also justify further research of antibody seroprevalence to various GII.4 strains, since norovirus immunology is not well understood.

Introduction

Norovirus, a highly contagious virus, is the leading cause of acute gastroenteritis in all age groups. Norovirus is characterized by a 12-48 hour incubation period and sudden onset of symptoms, such as nausea, diarrhea, vomiting, and abdominal pains. Although symptoms usually subside in one to three days, norovirus is found in stool before symptoms begin and for two or more weeks after the illness ends. The CDC estimates that norovirus leads to 56,000 to 71,000 hospitalizations and 19 and 21 million illnesses every year in the US (4).

Several norovirus characteristics explain why it is so pervasive on both an individual and a population level: environmental stability, a low infectious dose, resistance to disinfection, and very high titers in stool, serum, and emesis (19). Additionally, norovirus employs multiple modes of transmission, including fecal-oral, person-person, and food and waterborne. In fact, most viral food and waterborne outbreaks in developed countries are caused by norovirus (29). Multiple genogroups and strains of norovirus only add to the difficulty of controlling outbreaks.

Norovirus can be classified into six different genogroups (GI-GVI). Only the GI, GII, and GIV genogroups affect humans, with the GI and GII genogroups causing the

most human infections and outbreaks of norovirus (9). Within the GI and GII genogroups, the most common norovirus strain is genogroup II genotype 4, generally written as GII.4. Several pandemic GII.4 norovirus strains have emerged at different times in the last few decades, such as GII.4 Grimsby (GII.4 GB) in 1995, GII.4 Farmington Hills (GII.4 FH) in 2002, and GII.4 New Orleans (GII.4 NO) in 2009 (30). The emergence of pandemic GII.4 strains every few years has been attributed to both epochal evolution and antigenic drift in many studies (11-13). More specifically, epochal evolution explains the sudden structural changes in norovirus after long periods of stasis, and how those sudden changes lead to a new pandemic GII.4 strain to which the population is not immune. Supplanted pandemic GII.4 strains still circulate as the most recent pandemic strain dominates, although the extent has not been clearly defined.

Due to both genetic diversity and the inability to culture in the laboratory, the immunology of norovirus is not well understood. However, serum samples can be analyzed for antibodies specific to norovirus strains. Numerous studies have utilized serum samples to measure antibodies to norovirus, all of which demonstrate that higher serum antibody levels result in a decreased likelihood of norovirus infection and illness (16, 19, 21, 22, 26-28). The amount of antibodies to previous pandemic GII.4 strains after another develops is currently unknown, but this knowledge could help further research towards developing a norovirus vaccine.

The goal of this study was to model the antibody response to GII.4 NO based on antibody response to prior pandemic GII.4 strains (GII.4 GB and GII.4 FH), age, and time of data collection. The change in antibody response to GII.4 GB, GII.4 FH, and GII.4 NO over time was also determined. These aims were accomplished using

serological data from the 1999-2000 and 2003-2004 cycles of the National Health and Nutrition Examination Survey (NHANES).

Methods

Study Design

For this study, the National Health and Nutrition Examination Survey (NHANES) database was used. A program of the National Center for Health Statistics (NCHS) and the Centers for Disease Control and Prevention (CDC), the NHANES is a population-based cross-sectional study representative of the general US population. Approximately 5,000 participants are surveyed per year. NHANES data is categorized into cycles, or two-year periods. Participants who complete the NHANES consent to both an interview and a physical examination. The survey collects extensive demographic, medical, dietary, and health-related information from each participant, including biological samples.

NHANES Serum Samples

Stored NHANES serum samples were from the 1999-2000 cycle or the 2003-2004 survey cycle; GII.4 GB emerged before both cycles (in 1995), GII.4 FH emerged between the two cycles (in 2002), and GII.4 NO emerged after both cycles (in 2009). Only samples from participants between the ages 16 and 49 were analyzed to prevent confounding by age and immunocompromising conditions. Analyzing samples from participants between the ages of 16 and 49 was additionally rationalized because those in this age group have a more varied and higher number of social contacts, which increases the likelihood of secondary infections. Overall, a 1/3 subsample of NHANES serum samples matching the ages and cycles of interest was obtained, with a total of 2152 serum

samples. The sub-sample was selected such that it maintained national representativeness based on the US census at the time of collection.

Exposure Assessment: GII.4 VLP Panel

Enzyme-linked immunosorbent assays (ELISA) were used to identify and calculate the serum IgG antibodies, with various VLPs acting as the antigen for the assay. The tested VLPs were GII.4 GB, GII.4 FH, and GII.4 NO, all of which were created in a baculovirus expression system. Serum samples were diluted 1:50 before being added to the 96-well plates coated with the selected VLPs. There was a positive and negative IgG control and each sample was tested in duplicate for each VLP. After appropriate incubation and washing, the plates were read by a spectrophotometer and microplate reader to determine the optical density value (OD). A detailed protocol of the ELISAs that detect anti-norovirus serum IgG antibodies can be found in full in an earlier study (31). All samples were thawed and analyzed in 2014 and 2015.

Outcome Assessment: Measurement of VLP-Specific Serum IgG

The concentration of serum IgG antibodies for each VLP tested was ascertained by measuring OD values, which range from 0 to 3. A higher OD value corresponds to a higher concentration of antibodies. OD values less than zero were replaced with 0.001, while OD values greater than 3 were replaced with 3.5.

Determination of Seroprevalence Cutpoint

Serum anti-norovirus IgG results from uninfected participants from previous human challenge studies of Norwalk virus (GI.1) were used to determine the appropriate OD cutpoint (32, 33). Serum IgG results were adjusted to match the dilution of NHANES serum samples (Table 2, grouped into deciles and the 90^{th} percentile was used to define

the cutpoint, 1.5. In other words, OD values below 1.5 were considered seronegative, while OD values above 1.5 were considered seropositive. The cutpoint of 1.5 approximately represents the 83rd percentile of OD values in uninfected participants (Table 2).

Statistical Analysis

Box plots were made to examine the distribution of the OD data by GII.4 strain and NHANES cycle. Linear regression was used to assess the association between antibody response to GII.4 NO and antibody response to GII.4 GB and GII.4 FH, adjusted for age and cycle (n=1872). Antibody response to GII.4 NO, GII.4 GB, and GII.4 FH were log-transformed for normality. One-way ANOVA tests were conducted to determine if there was a difference in OD values by cycle and VLP (n=2019). Two-sided P-values <0.05 were considered statistically significant. All statistical analyses were performed using SAS 9.4.

Results

Analysis of Serological Data from NHANES Cycles 1999-2000 and 2003-2004

Initially, 2151 serum samples were tested for antibodies to GII.4 GB, 2127 were tested for GII.4 FH, and 7017 were tested for GII.4 NO (Figure 1). After removing entries with missing data and merging the data, a total of 2019 serum samples were available for analysis (Figure 1).

Seropositivity and Distribution of OD Values by Pandemic GII.4 Strain and NHANES Cycle

Table 1 describes the seroprevalence of serum samples based on pandemic GII.4 strain and NHANES cycle. In Cycle 1, the percentage of seropositive (>1.5) samples for

GII.4 GB, GII.4 FH, and GII. NO are 52.49%, 41.45%, and 45.02%, respectively. In Cycle 2, the percentage of seropositive (>1.5) samples for GII.4 GB, GII.4 FH, and GII. NO are 54.98%, 61.19%, and 49.13%, respectively. In Cycle 1, the percentage of highly seropositive (>3) samples for GII.4 GB, GII.4 FH, and GII. NO are 32.36%, 3.27%, and 4.33%, respectively. In Cycle 2, the percentage of highly seropositive (>3) samples for GII.4 GB, GII.4 FH, and GII. NO are 27.85%, 13.52%, and 6.48%, respectively.

The box plots illustrating the distribution of OD values by pandemic GII.4 strain and NHANES cycle are shown in Figure 2. The distribution of GII.4 GB data is highly right-skewed for both NHANES cycles, while the distributions of GII.4 FH and GII.4 NO data are relatively symmetrical for both NHANES cycles. Also, the interquartile ranges of Cycle 1 and Cycle 2 for GII.4 FH vary widely, the interquartile ranges for GII.4 NO vary slightly, and the interquartile ranges for GII.4 GB vary narrowly.

Linear Regression Model for GII.4 NO Reactivity

The results of the linear regression model for GII.4 NO reactivity are described in Table 3. For every one unit increase in the OD to the log of GII.4 FH, the OD to GII.4 NO increases by 0.827 units (p-value<0.0001). The OD to GII.4 NO increases by 0.151 units for every one unit increase in the OD to the log of GII.4 GB (p-value<0.0001). For every one year increase in age, the OD to GII.4 NO increases by 0.004 units (p-value=0.004).

Comparison of Antibody Response to Pandemic GII.4 Strains Between NHANES Cycle 1 (1999-2000) and Cycle 2 (2003-2004)

One-way ANOVA tests were completed to illustrate the change in antibodies to each pandemic GII.4 strain from Cycle 1 to Cycle 2 (Tables 4-6). The results show there

is no statistically significant difference in the amount of antibodies to GII.4 GB between Cycle 1 and Cycle 2 (p-value=0.3778) (Table 4). However, there is a statistically significant difference in the amount of antibodies to GII.4 FH and GII.4 NO between Cycle 1 and Cycle 2 (p-values<0.0001) (Table 5-6).

Discussion

The results of this study suggest that after the emergence of a pandemic GII.4 strain, there is a substantial increase in seropositivity towards that strain. These findings are consistent with the following hypotheses: the majority of GII.4 GB Cycle 1 and Cycle 2 samples are seropositive (52.49% vs. 54.98%), the majority of GII.4 FH Cycle 1 samples are not seropositive and the majority of GII.4 FH Cycle 2 samples are seropositive (41.45% vs. 61.19%), and majority of GII.4 NO Cycle 1 and Cycle 2 samples are not seropositive (45.02% vs. 49.13%) (Table 1). The confirmation of the initial hypotheses justifies investigation of these associations in further studies.

Previous literature has addressed potential mechanisms behind changes in antibody levels to pandemic GII.4 strains over time. A 2009 study examined the difference in antibody binding before and after the emergence of GII.4 FH, using two variations of GII.4 VLPs (one prior to the GII.4 FH pandemic, called GII.4v0, and one after, called GII.4v2) and monoclonal antibody (mAb) response (34). The results demonstrated that the anti-GII.4v0 mAbs only recognized a conformational epitope, while the anti-GII.4v2 mAbs only recognized a partially conformational epitope (34). This explains how given the rapid transformation of norovirus epitopes, antibody response to one GII.4 strain will change depending on the dominance of that strain and others. Similarly, other studies have remarked on the importance of GII.4 FH as a turning

in the antigenicity of GII.4 noroviruses (10, 25). A study found that anti-GII.4 1987 mAbs reacted to GII.4 1987, GB, and FH VLPs, but did not react to any GII.4 VLPs associated with strains emerging after 2002 (25). However, in this study, the reactivity to GII.4 NO was higher than expected, with the percentage of seropositive samples close to 50% in both Cycles 1 and 2 (Table 1). Furthermore, the one-way ANOVA test showed that there is a statistically significant difference in the amount of antibodies to GII.4 NO between Cycle 1 and Cycle 2, when it was expected that there would be no statistically significant difference (Table 6). These surprising results could be because of structural similarities between GII.4 NO and GII.4 FH, which could produce an antibody response to GII.4 NO. It's also possible that there are structural similarities between GII.4 NO and a minor GII.4 strain that emerged around the time GII.4 FH emerged. Another prior study determined that GII.4 FH is antigenically distinct from previous strains GII.4 GB and GII.4 Minerva (10). The discrepancy between GII.4 FH and prior GII.4 strains confirms the presence of antigenic variation among GII.4 noroviruses, which in turn confirms the changes in antibodies observed in this study's one-way ANOVA test of antibodies to GII.4 FH in Cycle 1 and Cycle 2. The structural distinction of GII.4 FH from earlier pandemic strains also gives more credit to the idea that GII.4 FH and the subsequently emerging GII.4 NO could have structural similarities. Overall, the literature supports the biological plausibility of varying antibody responses due to evolutionary mechanisms that impact every new successive GII.4 strain.

Earlier studies haven't directly addressed the effect of age and year of data collection on antibody response to different pandemic GII.4 strains, but some have researched related topics. For instance, a study conducted in Korea concluded that among

346 serum samples from participants from birth to over 70 years old, seroprevalence to GII.4 FH was 94.5%; additionally, seroprevalence among participants over 20 years old was over 80% (35). The fact that all serum samples were collected between 2005 and 2006 explains the high seroprevalence to GII.4 FH, as it emerged in 2002. Another study conducted in Valencia, Spain investigated antibody seroprevalence to GII.4 strains circulating between 2008 and 2011 (26). Of the 434 serum samples analyzed, GII.4 NO was the most commonly detected GII.4 strain at 35.5%, which is unsurprising as GII.4 NO was the only pandemic strain that emerged during the study period (26). The antibody seroprevalence to GII.4 Alpeldoorn, which first emerged in 2007, showed that antibody titers increased with age, and that seroprevalence statistically significantly differs between participants younger and older than 30 years (p-value<0.05) (26). The results of the study in Valencia, Spain coincide with this study's findings on the association between age and antibody seroprevalence to GII.4, because age was found to statistically significantly increase reactivity to GII.4 NO (p-value$=0.004$).

Strengths and Limitations

The primary strength of this study is the robustness of the NHANES database, which provided an extensive variety of information for a very large sample size. In addition to being robust, the NHANES data was representative of the entire country. However, a limitation of this study was that the NHANES data was severely right-censored. Antibody responses could therefore be drastically different between individuals, since it isn't known when participants last contracted norovirus. Additionally, the NHANES data was cross-sectional, so the changes in GII.4 antibody levels in individuals over time cannot be determined.

In conclusion, the findings, taken in context with findings for previous studies, imply that antibody seropositivity is low before a particular GII.4 strain's emergence, and then increases dramatically afterwards, leading to heightened immunity towards that pandemic strain. More studies will need to be conducted, as this study is one of the first to examine antibody seroprevalence to different pandemic GII.4 strains in relation to a specific strain and time of sample collection.

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Tables

Table 1. Percentage of seropositive serum samples based on pre-determined seroprevalence cutpoints

Variable	n	> 1.5	> 3
Cycle 1	924		
GII.4 GB	--	52.49%	32.36%
GII.4 FH	--	41.45%	3.27%
GII.4 NO	--	45.02%	4.33%
Cycle 2	1095		
GII.4 GB	--	54.98%	27.85%
GII.4 FH	--	61.19%	13.52%
GII.4 NO	--	49.13%	6.48%

Abbreviations: M, mean; SD, standard deviation

Table 2. Serum IgG antibody seroprevalence to Norwalk virus (GI.1) measured in raw and adjusted OD by decile (based on data from previous studies (31, 32))

Decile	Raw OD	Adjusted OD
1	0.0375	0.15
2	0.0515	0.206
3	0.0695	0.278
4	0.0835	0.334
5	0.1045	0.418
6	0.1645	0.658
7	0.2425	0.97
8	0.3615	1.446
9	0.4205	1.682
10	0.6015	2.406

Table 3. Linear regression model for antibody response to GII.4 NO, based on the log of antibody response to GII.4 GB and GII.4 FH, age, and NHANES cycle (n=2019)

Variables	β	SE	P-value
log GII.4 FH	0.827	0.012	< 0.0001
log GII.4 GB	0.151	0.011	< 0.0001
Age	0.004	0.001	0.004
Cycle	-0.109	0.030	0.0003

Abbreviation: SE, standard error

Table 4. One-Way Analysis of Variance (ANOVA) of antibody response to GII.4 GB by NHANES cycle (n=2019)

	Sum of Squares	df	Mean Square	F	P-value
Between Groups	1.199	1	1.199	0.78	0.3778
Within Groups	3106.722	2017	1.540		
Total	3107.920	2018			

Table 5. One-Way ANOVA of antibody response to GII.4 FH by NHANES cycle (n=2019)

	Sum of Squares	df	Mean Square	F	P-value
Between Groups	139.884	1	139.884	153.790	< 0.0001
Within Groups	1834.568	2017	0.910		
Total	1974.452	2018			

Table 6. One-Way ANOVA of antibody response to GII.4 NO by NHANES cycle (n=2019)

	Sum of Squares	df	Mean Square	F	P-value
Between Groups	19.464	1	19.464	22.38	< 0.0001
Within Groups	1754.260	2017	0.870		
Total	1773.724	2018			

Figures

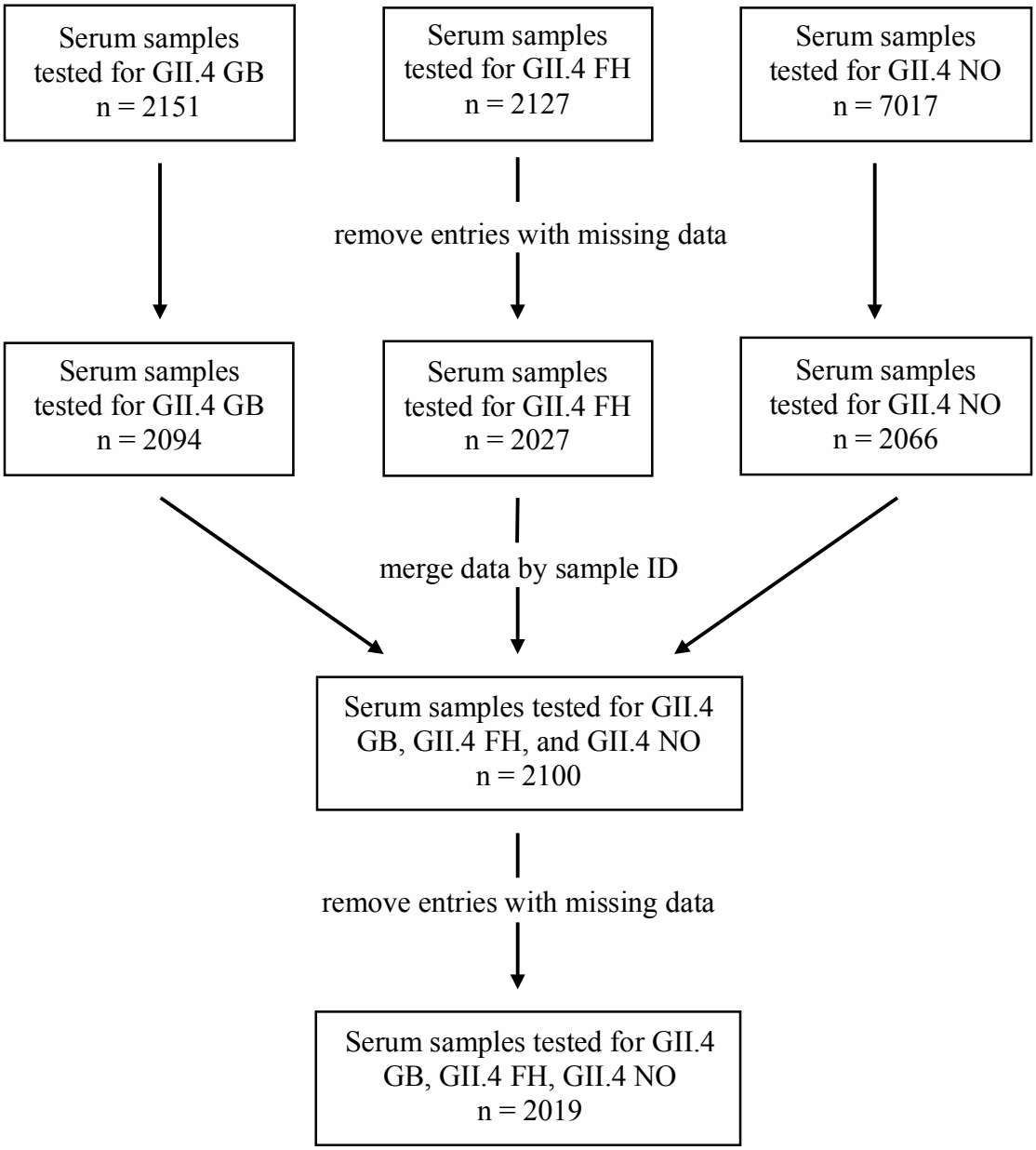

Figure 1. Flowchart describing combined serum sample data from NHANES cycles 1999-2000 and 2003-2004

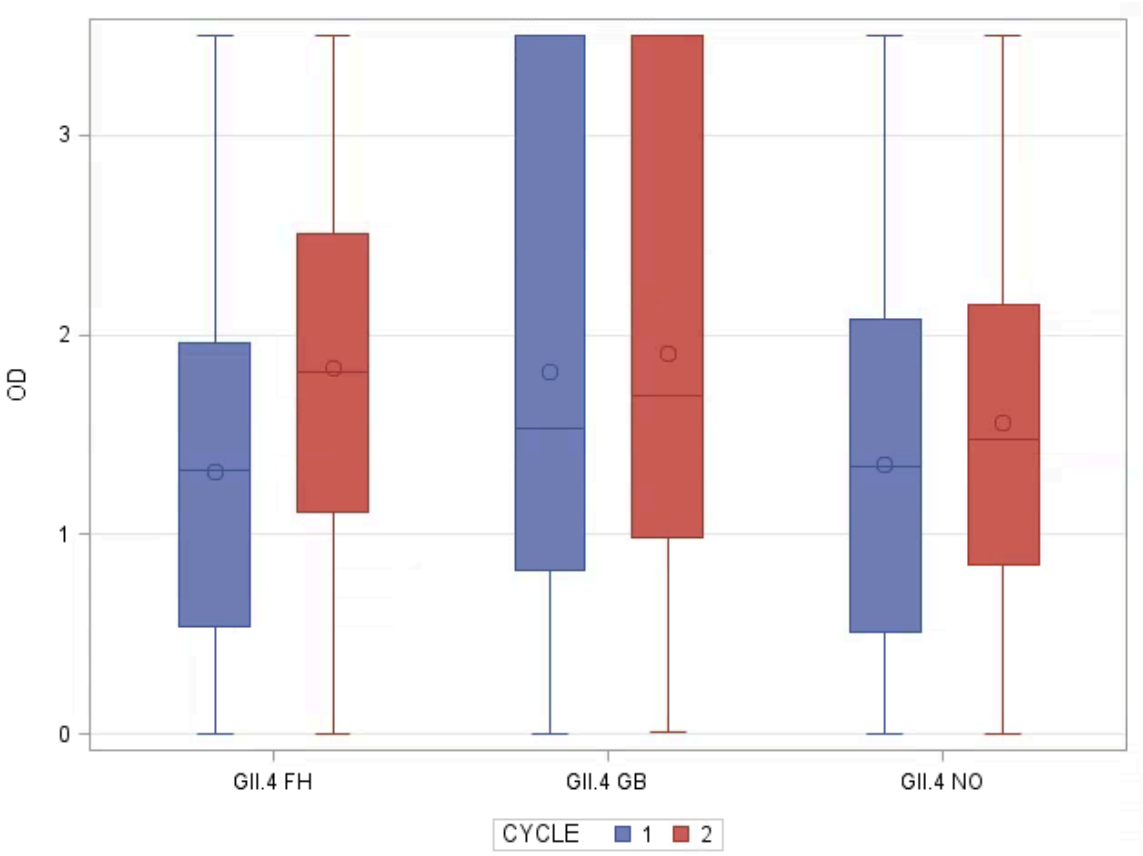

Figure 2. Box plot of OD values categorized by pandemic GII.4 strain and NHANES cycle (Total n=2019; Cycle 1 n=924; Cycle 2 n=1095)

Chapter III: Public Health Implications, Possible Future Directions

The public health implications of norovirus infection are numerous and extensive, given its vast global presence. The findings from this study can contribute to multiple areas of norovirus prevention and research, including vaccine development, evolution of pandemic GII.4 strains, and immunology.

Currently, there is no vaccine for norovirus, although several are in various stages of development. The mapping of antibody response to pandemic GII.4 strains at different points in time can help researchers determine how often a new vaccine needs to be distributed, in addition to how a vaccine's composition should change with each iteration. As far as the evolution of norovirus, the results can assist others in ascertaining how fast norovirus is mutating. Similarity between strains also indicates the extent of evolution that has occurred, which can be detected through comparing antibody response to different GII.4 strains. If antibody levels to a prior strain increases along with a more recent strain, then the strains have similar epitopes, ultimately revealing that there has been no appreciable change in the evolution of norovirus. The findings can also improve predictions for when the next pandemic GII.4 strain emerges by following the decline of antibodies to the previous strain. Overall, this study offers new information that could herald new advancements in norovirus research, particularly in the way of immunology to pandemic GII.4 strains.

There are some aspects that could be altered if this study were ever repeated. For instance, conducting this experiment as a prospective cohort study would offer results that clearly show how antibody levels change within individuals, rather than a cyclical cross-sectional study with different participants every two years. Since NHANES data is

publicly available, divulgence of identifying characteristics is forbidden. It would be interesting to observe how ethnicity and location affect antibody response to successive pandemic GII.4 strains. These potential changes could perhaps provide more insight on the circulation of norovirus both on an individual level and a population level.

Norovirus presents a lot of public health issues, as it is incredibly infectious and frequently causes outbreaks. That, paired with the inability to culture norovirus and the lack of an effective vaccine, makes controlling norovirus a much more difficult public health task. However, great strides are being made in this field of research, which will hopefully become tangible accomplishments in the near future.