

# Sequential Gastroenteritis Outbreaks in a Single Year Caused by Norovirus Genotypes GII.2 and GII.6 in an Institutional Setting

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**Background.** Norovirus is a leading cause of acute gastroenteritis worldwide. Improved diagnostic capability has been instrumental in the characterization of archival norovirus strains associated with gastroenteritis outbreaks that were investigated decades ago. One such investigation was that of 2 sequential gastroenteritis outbreaks that occurred in 1971 at the former Henryton State Hospital in Maryland. Approximately 40% of the resident population experienced clinical symptoms in both outbreaks, which occurred 11 months apart.

**Methods.** Stored stools and paired sera were re-analyzed to investigate the etiology of the 2 outbreaks.

**Results.** Different norovirus genotypes were identified as the etiological agents responsible for the illnesses, with GII.2 associated with the first outbreak and GII.6 with the second. The viruses were antigenically distinct as determined by analyses of hyperimmune sera raised against the corresponding virus-like particles in animals, as well as paired sera from infected individuals.

**Conclusions.** The observed antigenic differences were consistent with the failure of the GII.2 strain to provide cross-protective immunity to the GII.6 strain a few months later. An understanding of antigenic diversity among norovirus genotypes will be important in the design of norovirus vaccines.

**Keywords.** calicivirus; gastroenteritis; norovirus; outbreak; reinfection.

Norovirus is a leading cause of acute nonbacterial gastroenteritis [1, 2]. The positive-sense norovirus RNA genome is organized into 3 open reading frames (ORFs). ORF1 encodes the viral RNA-dependent RNA polymerase (RdRp) and other nonstructural proteins, ORF2 the major capsid protein (VP1), and ORF3 the minor capsid protein (VP2) [3]. The VP1 is comprised of 2 major domains: the inner shell (S) and the protruding (P) arm, which is further divided into the P1 and P2 subdomains [4]. The hypervariable, surface-exposed P2 subdomain contains major antigenic and histo-blood group antigen (HBGA) binding sites, both playing an integral role in determining susceptibility to infection [5–7]. Upon expression, the VP1 can self-assemble into virus-like particles (VLPs) that are antigenically similar to the native virion [8].

Noroviruses cluster into 7 genogroups (GI–GVII), with GI and GII responsible for most human illnesses [9]. There are presently 9 distinct genotypes within GI and 22 within GII, with

GII.4 as the predominant virus causing epidemics worldwide [9, 10]. Early human volunteer challenge studies suggested that homologous immunity to Norwalk virus (a prototype GI virus) is only short term, with a period of 6 months to 2 years, and that heterologous protective immunity to Hawaii virus (a prototype GII virus) was not induced [11, 12]. A recent mathematical modeling of incidence data suggested that homologous immunity may endure 4 years or longer [13], and a growing number of reports have documented sequential norovirus infections with different genotypes in the same individual [14–16].

Sensitive diagnostic techniques have been instrumental in the characterization of norovirus specimens collected more than 4 decades ago [17]. Two sequential gastroenteritis outbreaks occurred during 1971 at the Henryton State Hospital (now closed) in Marriottsville, Maryland [18], that were characterized by self-limiting nonbacterial acute gastroenteritis of unknown etiology that affected the residential population in January (90 residents) and December (64 residents). Approximately 40% of residents who presented with acute gastroenteritis in the January outbreak experienced disease symptoms again in the December outbreak, suggesting that the first infection did not confer short-term (greater than 1 year) protective immunity to the second infection [18]. The purpose of this study was to identify the presumptive viral pathogens responsible for these sequential outbreaks in an institutional setting to gain insight into the apparent absence of immunity in the second outbreak.

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## METHODS

### Specimens

Samples were collected during regional gastroenteritis outbreak investigations by the National Institutes of Health (NIH) [18], which took place soon after the discovery of the Norwalk virus [19]. Briefly, stool specimens (collected as rectal swabs) with corresponding acute and convalescent serum samples were available for the present study from 1 out of 90 and 55 out of 64 ill individuals residing at the Henryton State Hospital in January and December of 1971, respectively, when the 2 acute nonbacterial gastroenteritis outbreaks occurred (Table 1). A volunteer challenge study designed to investigate the etiology of the first outbreak in January 1971 provided an additional stool specimen from an ill volunteer (17LI), along with serum samples collected 4 days prechallenge and at day 1 (D1), D7, D14, D21, D28, and D57 postchallenge [18]. The ill volunteer was rechallenged 2 months later with the homologous agent and did not develop clinical symptoms of gastroenteritis following rechallenge. A serum sample was collected approximately 2 months following the second challenge (D135). The rectal swab specimens and acute phase sera from the second outbreak in December 1971 were collected 48 hours after the peak incidence of illness, which corresponded to the 10th day after the first case was reported. Based on the available recorded data, 44 of the 55 (80%) stool samples were collected within the first 5 days of infection. Convalescent phase serum day 57 was collected 6 weeks later on January 26, 1972 [18]. Records were not available from individual residents in the chronic care Henryton State Hospital that would inform an underlying condition or that would allow a direct comparison of the clinical features between a first and second gastrointestinal illness in the 2 outbreaks. The low turnover of the patient population and the re-infection rate (approximately 40%) used in this study were those reported from the original outbreak investigation [18].

All samples have been stored in the NIH Laboratory of Infectious Diseases. An exemption for the analysis of archival specimens in the repository was obtained from the NIH Office of Human Subjects Research and Protection.

### Viral RNA Extraction and Genotyping

Viral RNA was extracted from stool in rectal swab samples with the MagMAX Viral RNA Isolation Kit (Life Technologies Corp., Carlsbad, CA) and amplified in a reverse-transcription polymerase chain reaction (RT-PCR) using universal primers (290 and 289) that target the norovirus RdRp region [20]. The amplicons were sequenced using a Big Dye terminator cycle sequencing ready reaction kit (Applied Biosystems), and the norovirus typing tool was used to assign genotype [10, 21]. The near full-length genome sequences were determined as described previously [22]. In addition, the authentic 5'- and 3'-end genomic sequences were verified using the Roche 5'/3' RACE Kit, 2nd Generation (Roche Diagnostics Corporation, Indianapolis, IN) according to the manufacturer's protocol.

### Phylogenetic Analyses

Phylogenetic analyses of ORF2 were performed as previously described using the neighbor-joining and Tajima-Nei methods [22] in the Molecular Evolutionary Genetics Analysis program, version 7 [23].

### Structural Modeling

The capsid monomer from each Henryton virus (GII.2 and GII.6) was modeled upon a known GII.4 VP1 structure (3SEJ) with I-TASSER [24]. The electrostatic potential of amino acid residues on the surface of the resulting protruding domain model was calculated with UCSF Chimera [25].

### Expression and Purification of VLPs

To express VLPs, the consensus ORF2 gene of the norovirus strain GII/Hu/US/1971/GII.2/HenrytonSP17 was amplified and cloned into the pENTR Gateway vector (Invitrogen, Grand Island, NY). Recombination with baculovirus DNA was performed using the Baculodirect kit, as recommended by the manufacturer (Invitrogen). The consensus ORF2 gene of the norovirus strain GII/Hu/US/1971/GII.6/HenrytonSP24 was synthesized with flanking SalI and NotI restriction enzyme sites (New England Biolabs) and cloned into the corresponding sites of the pFastBac vector. Recombination with baculovirus DNA was performed using the Bac-to-Bac system (Invitrogen).

**Table 1. Features of 2 Acute Infectious Nonbacterial Gastroenteritis Outbreaks at the Henryton State Hospital, Maryland, in 1971 and Samples Available for this Study**

Month of Outbreak	Ill Individuals/Total Residents (%)	Stools Available/Ill Individuals (%)	Number Stools Positive for Norovirus by RT-PCR (%)	Norovirus Capsid Genotype	Number Paired Sera Available (Day Collected <sup>a</sup> )
January	90/367 (25)	1 <sup>b</sup> /90 (1)	1/1	GII.2 (1/1)	1 (d13 & d109)
December	64 <sup>c</sup> /382 (17)	55/64 (86)	38/55 (69)	GII.6 (38/38)	55 (d10 & d57)

Abbreviation: RT-PCR, reverse-transcription polymerase chain reaction.

<sup>a</sup>Acute or convalescent serum collection day (d) corresponds to the number of days after the first case was reported for each outbreak.

<sup>b</sup>The stool from this resident was administered to an adult volunteer who developed illness following oral challenge (18). Our present study reports that the ill volunteer shed GII.2 norovirus and developed a serologic response to the challenge strain.

<sup>c</sup>Twenty-six (41%) of these 64 individuals presenting gastroenteritis symptoms also reported illness in the January outbreak (18).

A baculovirus stock for each strain was generated, and the purification and expression of VLPs representing the GII.2 and GII.6 viruses were carried out as previously described [15]. The protein concentration of each VLP was determined with a commercial Bradford assay kit (Pierce, Rockford, IL).

#### Generation of Hyperimmune Sera in Guinea Pigs

Hyperimmune sera specific to the Henryton GII.2 and GII.6 VLPs were produced in guinea pigs under an approved National Institute of Allergy and Infectious Diseases Institutional Animal Care and Use Committee animal study protocol [7]. Animal work was conducted according to the institution's guidelines for animal use and followed the guidelines and basic principles in the United States Public Health Service Policy on the Humane Care and Use of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals by certified staff in an Association for Assessment and Accreditation of Laboratory Animal Care International–accredited facility on the NIH campus in Bethesda, Maryland.

#### Enzyme-Linked Immunosorbent Assay

Norovirus GII.2 and GII.6 Henryton VLPs were used as antigens in an enzyme-linked immunosorbent assay (ELISA) to measure antibody titers, as described previously [15]. Sera were subjected to serial dilution, and binding of antibodies to the VLPs was detected with either antihuman immunoglobulin G (IgG) conjugated with horseradish peroxidase or guinea pig anti–guinea pig IgG conjugated with horseradish peroxidase. ELISA titers were calculated using the reciprocal of the highest serum dilution that yielded a positive signal, defined as an optical density absorbance value at 405 nm (OD<sub>405</sub>) of  $\geq 0.2$ .

#### Hemagglutination Inhibition Assay

Hemagglutination inhibition (HAI) assays for the detection of norovirus-specific antibodies were performed as described [26] with B+ red blood cells (RBCs) from a healthy adult donor. HAI titers were calculated as the reciprocal of the highest dilution of serum that inhibited hemagglutination of RBCs by the target norovirus VLP.

#### Fucosyl Transferase Gene 2 Genotyping

DNA was extracted from serum (200  $\mu$ L) using the DNeasy Blood & Tissue Kit (Qiagen) and amplified in an RT-PCR reaction using a primer pair that proved to be optimal in the reaction: forward 5'-GATGGAGGAGGAATACCGCCAC-3' [27] and reverse 5'-GGCTGCCTCTGGCTTAAAG-3' [28], which target position 428 of the *FUT2* gene. Human growth hormone–specific gene primers were used as an internal PCR control to produce a 428-bp amplicon [28, 29]. Amplicons were sequenced, and the *FUT2* genotype was assigned by analysis of the sequencing chromatogram for each individual; a single G peak at position 428 of the *FUT2* gene signified a homozygous secretor, a mixed population of 2 peaks (G and A) signified a heterozygous secretor, and a single A peak signified a homozygous nonsecretor.

## RESULTS

### Association of Noroviruses With the Henryton Hospital Outbreaks

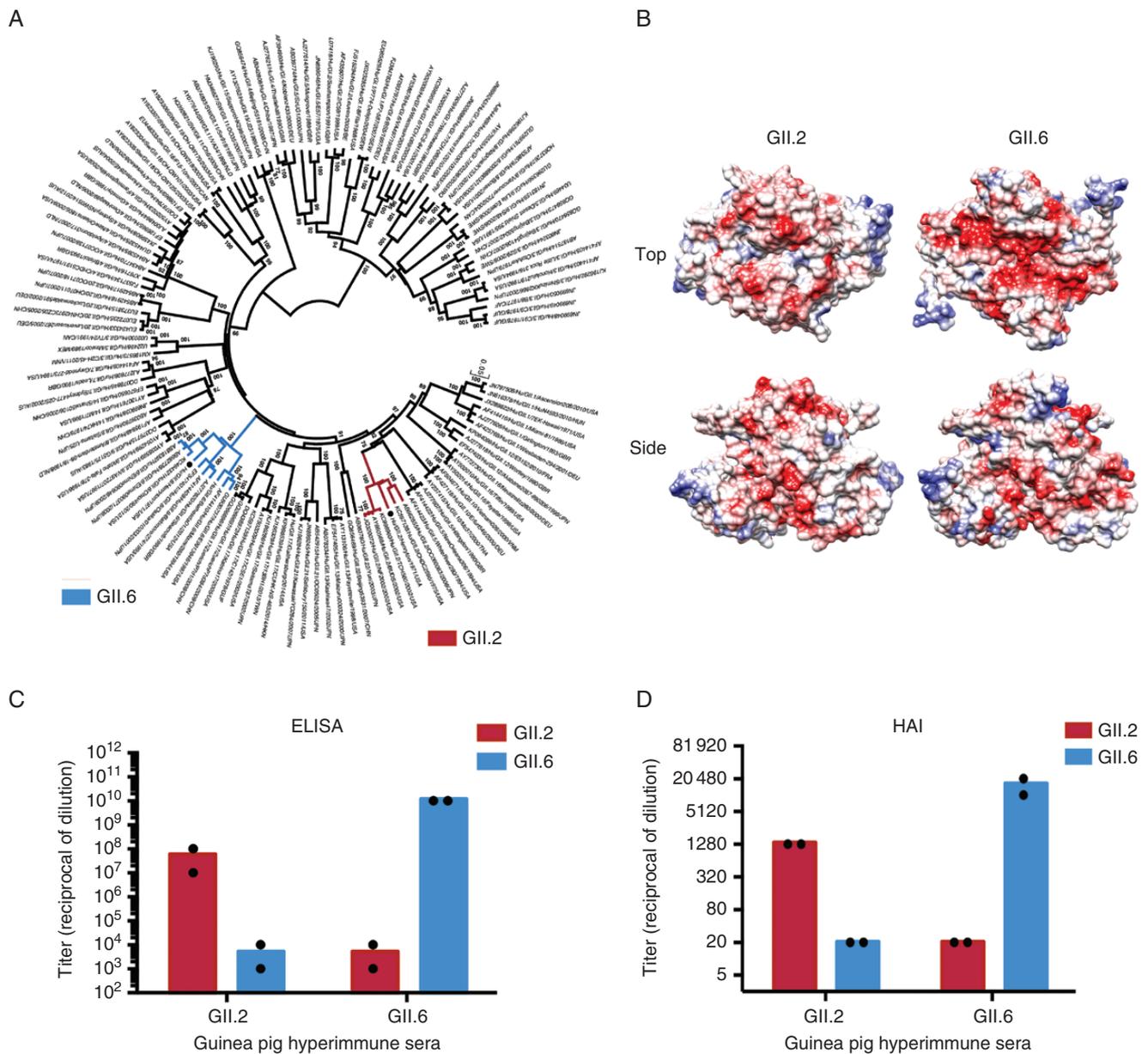
To establish the etiology of the 2 sequential outbreaks of gastroenteritis that occurred at the Henryton Hospital in January and December of 1971, the 56 available stool specimens obtained from residents experiencing gastroenteritis were analyzed for the presence of norovirus by a diagnostic RT-PCR that targets the RdRp (Table 1). Norovirus was detected in 39/56 (70%) of the stool specimens from ill residents, and amplicon sequences matched 2 distinct genotypes; a GII.2 norovirus was associated with the January outbreak (88% nucleotide identity match with GII/Hu/US/1975/GII.2/SnowMountain), and a GII.6 norovirus was associated with the December outbreak (92.1% nucleotide identity match with GII/Hu/JP/2009/GII.6/OH09014). An adult volunteer received an oral challenge with a stool filtrate derived from a resident in the January outbreak and developed gastroenteritis [18]. The GII.2 norovirus detected in the volunteer's stool was nearly identical to that of the resident.

### Sequence Analyses and Phylogenetic Relationships

The full-length genome sequence was determined for each outbreak virus. The January GII.2 and December GII.6 norovirus genomes were 7536 and 7553 nucleotides in length, respectively, with an overall identity of 64.9%. Phylogenetic comparisons of the norovirus capsid regions (encoded in ORF2) with reference GI and GII sequences showed that each Henryton norovirus clustered closely with a specific genotype, GII.2 (highlighted in rust), or GII.6 (highlighted in blue) (Figure 1A). The GII.6 viruses form 3 genetic clusters, designated here as A, B, and C. The Henryton GII.6 norovirus grouped within cluster C, which includes viruses detected in the United States and Japan during 2008–2012 [22, 30]. The Henryton GII.2 norovirus grouped with the single cluster of the GII.2 genotype represented by the prototype Snow Mountain virus, which has remained highly conserved for approximately 40 years [22]. Alignment of the VP1 amino acid sequences revealed that the GII.2 and GII.6 Henryton viruses differed by 28.3% (157/554), with the capsid P domain having the greatest amino acid variation (36.6%; 120/328) and the S domain having the least (16.4%; 37/226). The Henryton outbreak viruses were designated as GII/Hu/US/1971/GII.2/HenrytonSP17 and GII/Hu/US/1971/GII.6/HenrytonSP24 and assigned the GenBank accession numbers MF405169 and KY424345, respectively.

### Antigenic Comparison of the Henryton Noroviruses

Electrostatic modeling of each Henryton norovirus capsid monomer showed an overall similarity in the surface charge distribution, but with a notable difference in the exposed surface of the P2 subdomain (Figure 1B). To explore antigenic differences, VLPs representing the Henryton GII.2 and GII.6 outbreak viruses were generated and VLP-specific hyperimmune serum was raised in guinea pigs. The antigenic relationship between the 2 capsids was examined by ELISA (Figure 1C)



**Figure 1.** Characterization of the GII.2 and GII.6 norovirus strains responsible for sequential gastroenteritis outbreaks at the Henrynton State Hospital in 1971. (A) Phylogenetic analysis of the Henrynton GII.2 and GII.6 ORF2 nucleotide sequences (indicated with solid circles) in comparison with representative strains from the GII.2 (rust) and GII.6 (blue) genotypes. (B) Electrostatic potentials on the interaction surfaces of the protruding domain of each capsid monomer. White, red, and blue indicate neutral, negative, and positive charges, respectively. (C) Hyperimmune sera raised against each Henrynton virus-like particle was used to assess antigenic differences by enzyme-linked immunosorbent assay and (D) hemagglutination inhibition assay. Black solid circles on the bars represent individual guinea pig values, assays conducted in duplicate.

and HAI (Figure 1D) assays. In both assays, the homologous titer against the immunizing VLP was higher ( $\geq 5$ -logs) than that against the heterologous VLP, consistent with antigenic differences between the 2 viruses.

#### FUT2 Secretor Status Genotyping

An association between the FUT2 gene allele sequence at 428 and susceptibility to norovirus infection was investigated (Table 2). Of the 55 residents with serum samples available for genotyping analysis in the December GII.6 outbreak, the majority (73%) were identified as secretors. Evidence for norovirus

infection, as determined by the identification of GII.6 norovirus RNA in stool, was found in 38 (69%) of these 55 individuals. Of these 38, 30 (79%) were identified as secretors and 8 (21%) as nonsecretors. The adult volunteer who developed illness following challenge with the GII.2 norovirus from the January outbreak was genotyped as a nonsecretor (data not shown).

#### Serological Response of an Adult Volunteer Challenged With the GII.2 Outbreak Virus

The GII.2 and GII.6 Henrynton VLPs were utilized as antigens in an ELISA to detect the virus-specific serum antibody response

**Table 2. FUT2 Secretor Genotype in Henryton State Hospital Residents and Norovirus Infection**

	Total Number of FUT2-Genotyped <sup>a</sup> Residents (%) (n = 55)	No. Norovirus Positive <sup>b</sup> (%) (n = 38)	No. Norovirus Negative (%) (n = 17)
<b>Secretors</b>			
SeSe	12 (22)	7 (18)	5 (29)
Sese428	28 (51)	23 (61)	5 (29)
Subtotal	40 (73)	30 (79)	10 (59)
<b>Nonsecretors</b>			
se428se428	15 (27)	8 (21)	7 (41)

<sup>a</sup>G428A mutation site in FUT2 gene.

<sup>b</sup>Positive is defined as the detection of norovirus RNA in stool.

of the adult volunteer (17LI) challenged with the Henryton GII.2 virus. The homologous GII.2-specific and heterologous GII.6-specific serum IgG titers were 12 800 (Figure 2A) at the time of the initial challenge, when the volunteer developed illness. After the initial challenge, GII.2-specific serum IgG titers peaked to a titer of 819 200 (the heterologous response to GII.6 was lower) and steadily decreased over time but did not return to prechallenge levels (Figure 2A). The homologous serum IgG titer 5 days prior to rechallenge with the GII.2 outbreak virus (that occurred at day 62 after the first challenge) was 204 800, 16-fold higher than the titer at the time of the first challenge. The volunteer did not develop illness, consistent with the development of short-term homologous immunity after the first challenge.

Certain norovirus VLPs display hemagglutination activity with human RBCs, and an increase in antibodies that inhibit this activity (HAI) can be detected in individuals following norovirus infection or human experimental challenge [26]. Moreover, HAI has been used as a surrogate neutralization assay [26]. We examined the HAI titers of volunteer 17LI at the time of the first and second challenge with the homologous GII.2 virus. The homologous serum HAI titer at the time of the first challenge (pre) when illness occurred was 40 (Figure 3A). A 64-fold increase in the titer of GII.2-specific HAI antibodies was detected at day 14 after the first challenge (Figure 3A), and the HAI titer at the time of the second challenge when illness did not occur was approximately 1280. A heterologous GII.6 HAI response developed following the GII.2 challenge, but lower titers (160) were observed (Figure 3A).

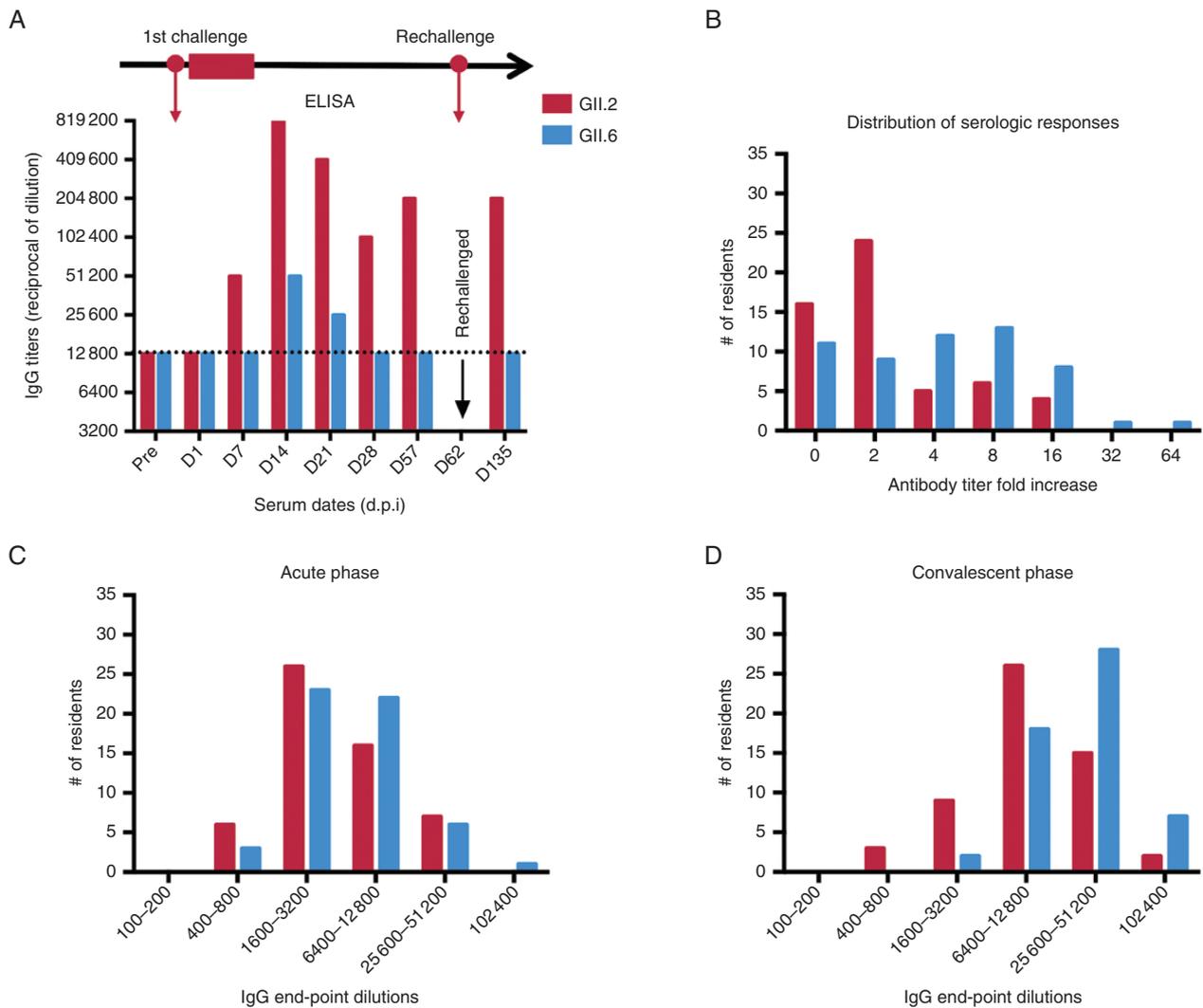
#### Serologic Responses of Henryton Residents During the 1971 Outbreaks

Only 1 paired serum set was available from a resident who developed illness in the January 1971 GII.2 outbreak (Table 1), with the acute serum collected 13 days after the first reported episode of gastroenteritis in the hospital and the convalescent serum collected 109 days later. Although an anamnestic response resulting from previous exposure to norovirus was likely already in progress, the resident showed a 4-fold antibody response against the homologous GII.2 outbreak virus by

both ELISA (titer change from 12 800 to 51 200) and HAI (titer change from 160 to 640). Of note, there was no change in the HAI titer of 40 against GII.6 VLPs.

Fifty-five paired acute and convalescent sera were available from ill residents in the December 1971 GII.6 norovirus outbreak (Table 1), and we tested them against both VLP antigens by ELISA and HAI. It should be noted that the acute sera in the second outbreak were collected only once at day 10 following the first report of illness, potentially allowing time for the initiation of an anamnestic or primary adaptive response in some individuals. First, we measured the antibodies by ELISA (Figure 2). Analysis of the acute phase sera showed that there were no seronegative individuals and that the majority of the residents 48/55 (87%) possessed IgG antibody titers between 1600 and 12 800 against both Henryton antigens at the time of the December outbreak (Figure 2C). The IgG titer distribution in convalescent phase serum showed a shift to higher titers, as 35/55 (64%) of the residents developed a serologic response to the homologous GII.6 virus (Figure 2D), with end-point titers ranging between 6400 and 51 200 for the majority of individuals (Figure 2C). A serologic response in 17/55 (31%) of the residents was observed against the heterologous GII.2 virus as well. As noted above, a homologous GII.2 serum ELISA titer of 12 800 did not correlate with protection in volunteer 17LI at the time of initial challenge with the GII.2 Henryton norovirus. Likewise, 33/48 (69%) residents exhibited a homologous GII.6 serum titer of up to 12 800 at the time of the GII.6 outbreak, suggesting that this ELISA titer may not reflect immunity to GII.6. A serological response ( $\geq 4$ -fold increase) was detected in 35/55 (64%) residents against homologous GII.6 VLPs, in contrast to 15/55 (27%) against heterologous GII.2 VLPs (Figure 2B). Of the residents exhibiting a heterologous GII.2 serological response, 11/15 (73%) were positive for GII.6 virus shedding in the second outbreak. However, it was noteworthy that 2-fold increases did occur in more than half of the residents against the GII.2 virus, possibly reflecting an anamnestic response related to infection during the first outbreak. Considering both 4-fold and 2-fold increases combined, 49/55 (89%) developed a serologic response to the homologous GII.6 outbreak virus, as determined by ELISA.

The 55-paired sera collected during the GII.6-associated December outbreak were tested next against both VLPs in an HAI assay (Figure 3). The distribution of homologous GII.6 HAI titers in the acute phase sera showed that approximately 25/55 (45%) had titers  $\leq 40$  (Figure 3C). Of these, 18/25 (72%) shed GII.6 norovirus in stool. Of the remaining 30 residents with homologous acute phase HAI titers  $> 40$ , 20 (67%) were infected with GII.6 norovirus, as determined by detection of the virus in stool. Interestingly, the distribution of HAI titers against the heterologous GII.2 was higher, with 49/55 (89%) residents having titers  $\geq 80$  in their acute phase sera, likely reflecting infection with the first outbreak virus (Figure 3C). The majority of residents (53/55) displayed HAI end-point titers  $\geq 80$  in their convalescent phase



**Figure 2.** Serologic responses as measured by enzyme-linked immunosorbent assay (ELISA). (A) An adult volunteer (17L1) was challenged with the GII.2 Henrynton norovirus, and serum was collected prior to challenge (pre) and at several time points (within days 1–135) postchallenge. Serum antibodies were assayed against homologous GII.2 (rust) or heterologous GII.6 (blue) virus-like particles (VLPs) by an ELISA that detects immunoglobulin G (IgG)-specific antibodies. (B) Distribution of serological fold rises in paired sera against the Henrynton GII.6 (blue) and GII.2 (rust) VLP antigens among the 55 Henrynton residents in the GII.6 outbreak. (C) Distribution of the acute phase IgG titers of 55 Henrynton residents during the GII.6-associated outbreak against homologous Henrynton GII.6 (blue) and heterologous GII.2 (rust) VLP antigens. (D) Distribution of convalescent phase IgG titers of the 55 Henrynton residents against the homologous Henrynton GII.6 (blue) and heterologous GII.2 (rust) VLP antigens.

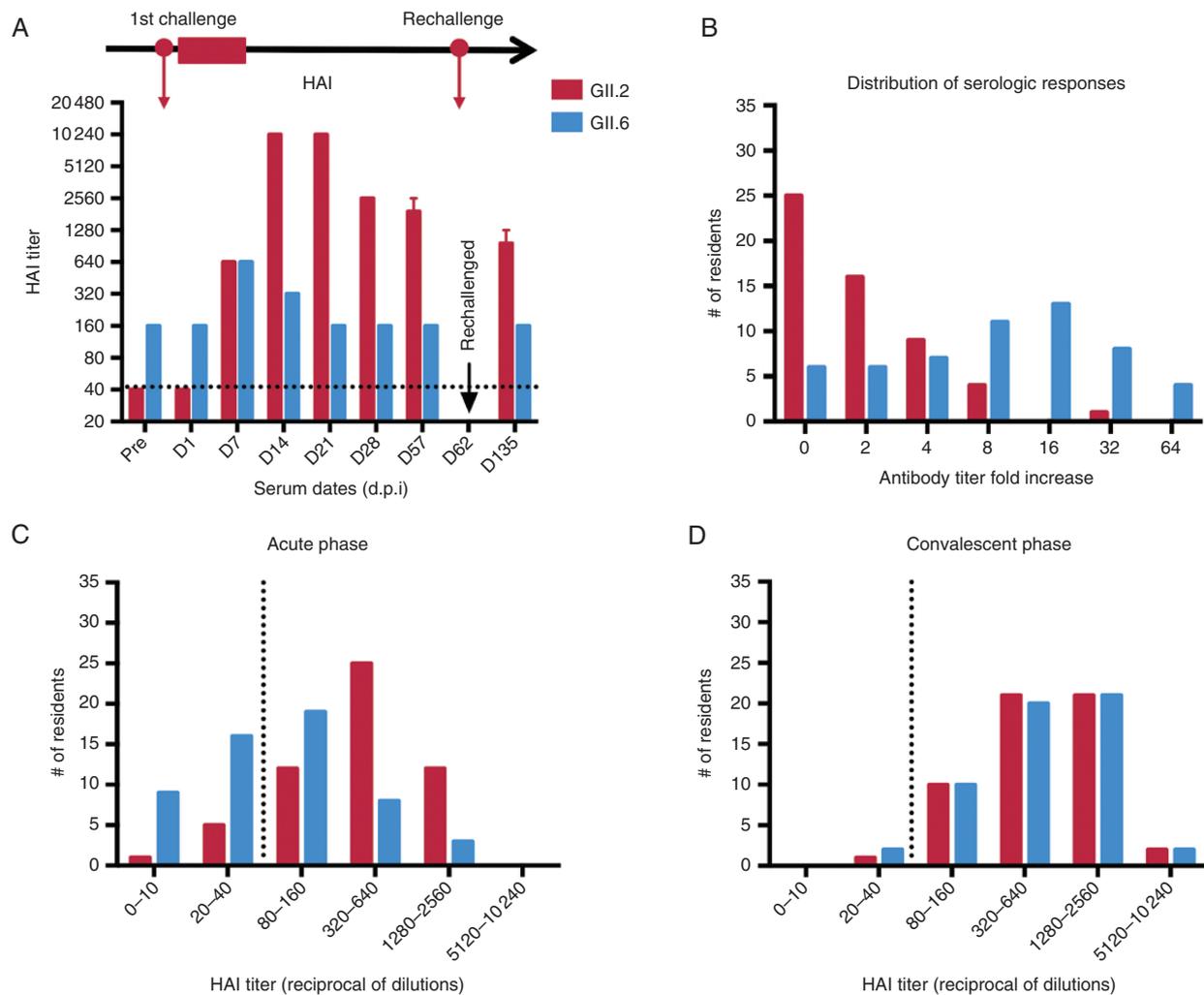
serum against both VLPs (Figure 3D). In 43/55 (78%) residents, a  $\geq 4$ -fold increase in GII.6 antibody titer was detected between acute and convalescent phase serum, indicative of a GII.6 infection (Figure 3B). Norovirus (GII.6) was detected in the stools of 77% (33/43) of these residents. A cross-reactive GII.2 serological response was detected in 25% (14/55) of the residents exposed to the GII.6 virus. Of the 14 residents who responded serologically to both GII.6 and GII.2 VLPs as determined by the HAI assay, 12 (86%) were positive for GII.6 norovirus shedding.

## DISCUSSION

Norovirus-associated gastroenteritis outbreaks are a major concern in closed institutional settings, where the potential for person-to-person transmission is high [1, 18]. Herd immunity

would provide an important source of protection against recurrent norovirus episodes in this setting [31, 32]. We analyzed specimens from 2 sequential outbreaks that occurred 11 months apart in the same institution; a GII.2 norovirus caused a January outbreak, followed by a GII.6 outbreak in December. Although the 2 outbreak viruses both belonged to Genogroup II and shared a common ancestral lineage, our data indicate that the viruses have evolved as antigenically distinct genotypes. This observation is consistent with the lack of observed cross-protection in the residential hospital population between the GII.2 and GII.6 outbreak viruses and our analysis of virus-specific antibody responses in immunized animals and infected individuals.

A comparison of hyperimmune sera raised against VLPs from the GII.2 and GII.6 Henrynton noroviruses provided



**Figure 3.** Serologic responses as measured by hemagglutination inhibition assay (HAI). (A) Serum antibodies from volunteer 17LI were assayed against homologous GII.2 (rust) or heterologous GII.6 (blue) virus-like particles (VLPs) by HAI, which functions as a surrogate neutralization test. (B) Distribution of serological fold rises against the Henryton GII.6 (blue) and GII.2 (rust) VLP antigens among the 55 Henryton residents in the GII.6 outbreak. (C) Distribution of the acute phase IgG titers of 55 Henryton residents during the GII.6-associated outbreak against homologous Henryton GII.6 (blue) and heterologous GII.2 (rust) VLP antigens by HAI. (D) Distribution of convalescent phase IgG titers of the 55 Henryton residents against the homologous Henryton GII.6 (blue) and heterologous GII.2 (rust) VLP antigens by HAI. The dotted line in (C) and (D) corresponds to the  $\geq 40$  HAI titer that has been proposed as a correlate of protection against symptomatic gastroenteritis with the GI.1 prototype Norwalk virus [26].

direct evidence that the viruses were antigenically distinct. Cross-reactive antibodies were also detected, albeit at lower levels. The relatively higher levels of preexisting GII.2-specific HAI antibodies in the Henryton population at the time of the second GII.6 outbreak (the majority with GII.2 HAI titers  $\geq 80$ ) and the absence of cross-protection further supported the antigenic diversity of these viruses. However, serological correlates of immunity and the role of serotypes remain difficult to define [12, 33, 34], and our clinical records in this study were incomplete. Surrogate neutralization assays, such as HAI, have been used to establish potential correlates, such as proposed protective HAI titer of 40 for GI.1 Norwalk virus [26]. The adult volunteer (17LI) who underwent challenge with the GII.2 Henryton virus was not protected with a preexisting homologous HAI titer of 40, suggesting that serological correlates of resistance to illness may vary, depending on norovirus genotype and the assays employed [35].

Our data are consistent with recent reports that reinfection with noroviruses from different genotypes occurs often in children [16, 22, 36] and that norovirus reinfections can occur within a single year in both children and adults [14, 15]. Similar to our previous report that infection with 2 different GII genotypes (GII.4 followed by GII.6) could occur within a single year in a young child [15], we show here that yet another GII genotype pairing (GII.2 followed by GII.6) could cause sequential outbreaks in adults within a single year. In addition to the antigenic differences that were observed between the 2 Henryton outbreak viruses, we explored the role of the FUT2 gene, first associated with host resistance to the GI.1 Norwalk virus [34, 37, 38]. This genetic marker may not be applicable to all norovirus genotypes [27, 39]. One of 9 ill volunteers challenged with the GII.2 Snow Mountain norovirus was identified as secretor negative [40], and the ill volunteer challenged with the Snow

Mountain-like Henryton GII.2 norovirus in this study was also secretor negative. Approximately 21% of the Henryton residents who shed GII.6 norovirus in stool and who developed antibodies against GII.6 were secretor negative, indicating that a functional fucosyl transferase enzyme (as determined by the FUT2 genotype at position 428) was not essential to confer susceptibility to this GII.6 norovirus. The role of HBGA carbohydrates in the binding of these noroviruses to host cells in both secretor and nonsecretor individuals will require additional investigation.

In summary, our study characterized the etiological agents, both of which are the oldest viruses known to date within their respective genotypes, responsible for sequential illnesses at a state hospital in 1971. The immunological responses of residents exposed to the 2 different norovirus genotypes suggest the absence of cross-reactive immunity, independent of preexisting norovirus antibodies from previous exposure history. The most likely interpretation of our data is that GII.2 and GII.6 viruses are antigenically distinct and likely represent distinct serotypes. Moreover, GII.2 and GII.6 belong to different “immunotypes,” our recently proposed system that defines clusters of antigenically related genotypes [22]. Continued analyses of antigenic diversity will be important in the development of broadly protective norovirus vaccines.

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**Potential conflicts of interest.** All authors: no reported conflicts of interest.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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