

Epidemiology of Norovirus Infection Among Immunocompromised Patients at a Tertiary Care Research Hospital, 2010–2013

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Background. Noroviruses are a major cause of infectious gastroenteritis worldwide, and viruses can establish persistent infection in immunocompromised individuals. Risk factors and transmission in this population are not fully understood.

Methods. From 2010 through 2013, we conducted a retrospective review among immunocompromised patients (n = 268) enrolled in research studies at the National Institutes of Health Clinical Center and identified a subset of norovirus-positive patients (n = 18) who provided stool specimens for norovirus genotyping analysis.

Results. Norovirus genome was identified by reverse-transcription quantitative polymerase chain reaction in stools of 35 (13%) of the 268 immunocompromised patients tested, and infection prevalence was 21% (11 of 53) in persons with primary immune deficiencies and 12% (20 of 166) among persons with solid tumors or hematologic malignancies. Among 18 patients with norovirus genotyping information, norovirus GII.4 was the most prevalent genotype (14 of 18, 78%). Persistent norovirus infection (≥6 months) was documented in 8 of 18 (44%) individuals. Phylogenetic analysis of the GII.4 capsid protein sequences identified at least 5 now-displaced GII.4 variant lineages, with no evidence of their nosocomial transmission in the Clinical Center.

Conclusions. Norovirus was a leading enteric pathogen identified in this immunocompromised population. Both acute and chronic norovirus infections were observed, and these were likely community-acquired. Continued investigation will further define the role of noroviruses in these patients and inform efforts toward prevention and treatment.

Keywords. epidemiology; immunocompromised; norovirus.

Norovirus is an important cause of gastroenteritis among both adults and children in the United States, and its relative role as a serious diarrheal pathogen in children is increasing in countries where the rotavirus vaccine is routinely administered [1–3]. Noroviruses cause an estimated 800 deaths per year in the United States, there emerging as the second most important cause of mortality due to gastroenteritis after *Clostridium difficile* [4]. Norovirus vaccines are in development, and vaccines based on virus-like particles have shown promise in early clinical trials [5–8]. In addition, a virus-specific monoclonal antibody was able to prevent a homologous norovirus infection in chimpanzees [9, 10].

Noroviruses are nonenveloped icosahedral viruses of 28–30 nm in diameter, and they contain a single-stranded, positive sense ribonucleic acid (RNA) genome [11]. The genome is organized into 3 open reading frames (designated ORF1, ORF2, and ORF3), which encode a nonstructural polyprotein (NS1–7), the major capsid protein (VP1), and a second structural protein (VP2), respectively. Noroviruses are characterized by a high degree of genomic and antigenic diversity and are currently classified into 7 genogroups, GI–GVII, but only genogroups GI, GII, and GIV infect humans. Of these, GI is subdivided into 9 genotypes, whereas GII consists of 22 genotypes [12, 13]. Although most of the genotypes have been described as circulating worldwide with variable incidences, a single genotype, GII.4, is the most prevalent [14, 15]. The reasons for the dominance of this particular genotype are not clear, but it is speculated that a new variant of GII.4 norovirus evolves to escape herd immunity every 3 to 4 years [15–17].

Noroviruses are increasingly recognized as an important cause of gastroenteritis in immunocompromised patients [18–23]. Patients with deficient immune responses resulting from a congenital disorder, immunosuppressive therapy, cancer treatment, or human immunodeficiency virus (HIV) infection may become chronically infected, with prolonged virus shedding and illness. To better understand the epidemiology

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"This manuscript is dedicated to the memory of Dr. Albert Z. Kapikian (1930–2014), who discovered noroviruses in 1972 and dedicated his life to improving the lives of children and adults affected by gastroenteritis. He was our most influential mentor and friend."

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of noroviruses in immunocompromised populations, we studied a cohort of patients referred to the National Institutes of Health (NIH) Clinical Center for treatment. We present a retrospective epidemiologic analysis of this population tested for norovirus at the NIH and provide insight into the molecular epidemiology of the noroviruses in this setting.

MATERIALS AND METHODS

Study Population

Persons referred to the NIH Clinical Center, a 240-bed clinical research hospital, were enrolled in a variety of Institutional Review Board (IRB)-approved research protocols, including those related to immunocompromising conditions, such as immune suppression resulting from cancer therapy, stem cell transplantation (SCT), primary immunodeficiencies (PIDs), and acquired immune deficiencies. Solid organ transplants were not performed at the NIH Clinical Center. Patients presenting with acute gastroenteritis were placed in contact isolation according to hospital protocol, and stool samples were submitted to the NIH Clinical Center. Most patients who tested positive for enteric infectious pathogens remained in contact isolation until symptoms resolved; SCT recipients remained in isolation until follow-up test results were negative.

We defined 2 separate study populations for this investigation. Patients eligible for the retrospective cohort analysis were immunodeficient patients enrolled in any NIH protocol and for whom a norovirus test was ordered. The analysis spanned an 18-month period from September 22, 2011 through March 23, 2013, which corresponded to the establishment of an in-house reverse-transcription (RT) quantitative polymerase chain reaction (PCR) assay for diagnosis of norovirus infection. The retrospective analysis of this cohort was conducted as part of a quality improvement study for the hospital and determined to be exempt from IRB review by the NIH Office of Human Subjects Research Protections. The second study population was defined as norovirus PCR-positive patients from whom informed consent was obtained to perform genetic characterization of viruses in clinical specimens under an IRB-approved NIH protocol for viral pathogens. The genetic characterization study was initiated before the introduction of in-house norovirus testing, when stool samples were sent from the NIH Microbiology Service to a commercial diagnostic laboratory; this study included patients enrolled from April 2010 through November 2013.

Data Collection and Analysis

Demographic, clinical, and laboratory information was abstracted from medical records. The underlying diagnosis was determined based on detailed medical record review and consultation with attending physicians as needed. In addition, medical records were reviewed to identify results for other viral, bacterial, or parasitic pathogens that were identified within

the 30 days before and after norovirus testing. To ensure a representative sample, we have included only tests for which at least half the study population was tested. All analyses were conducted with SAS version 9.2 (SAS Institute Inc., Cary, NC).

Norovirus Reverse Transcription-Quantitative Polymerase Chain Reaction Detection

Stool samples were collected in a sterile container and stored at 4°C before processing. Liquid or formed stool was diluted in S.T.A.R. stool transport and recovery buffer (Roche Diagnostics Corporation, Madison, WI) to create a 10% suspension and then clarified by low-speed centrifugation at 1000 × g for 1 minute. Viral RNA was isolated from 200 µL of the clarified stool supernatant using the NucliSENS easyMAG automated extractor system (BioMerieux, Durham, NC). Norovirus detection was performed using a quantitative real-time TaqMan RT-PCR as described previously [24] with minor modifications in instrumentation and reaction conditions noted below. Primers and probes were designed to detect a broad range of GI and GII noroviruses when combined for use in a multiplex assay. For detection of GI noroviruses, the forward (COG1F) and reverse (COG1R) primers consisted of 5'-CGYGGATGCGNTTYCATGA-3' and 5'-CTTAGACGCCATCATCATTYAC-3', respectively, and probes (RING1a-TP and RING1b-TP) were 5'-FAM-AGATYGCGATCYCCTGTCCA-TAMRA-3' and 5'-FAM-AGATCGCGGTCTCTGTCCA-TAMRA-3'. For detection of GII noroviruses, the forward (COG2F) and reverse (COG2R) primer sequences were 5'-CARGARBCNATGTYAGRTGGATGAG-3' and 5'-TCGACGCCATCTTCATTACA-3', respectively, and the probe (RING2-TP) was 5'-Cy5-TGGGAGGGCGATCGCAATCT-Iowa Black RQ-3'. Real-time PCR was performed on the ABI 7500 instrument (Life Technologies, Carlsbad, CA) in a 20 µL reaction consisting of 1× TaqMan Fast Virus 1-step master mix (Thermo Fisher Scientific, Waltham, MA), 0.9 µM each primer set (both GI and GII), 12.5 µM of each probe (both GI and GII), and 10 µL purified nucleic acid. The reaction mixture was then incubated at 50°C for 5 minutes, 95°C for 20 seconds, followed by 40 cycles at 95°C for 3 seconds and 60°C for 30 seconds. Before extraction, each clarified stool aliquot was spiked with an internal control (pBR322 plasmid DNA) to verify successful recovery of nucleic acid and removal of PCR inhibitors. The internal control in extracted samples was detected by amplification in a separate real-time PCR as described previously [25]. Known norovirus-positive and -negative stools were included in each run.

Norovirus Genotyping and Capsid Sequencing

A total of 28 norovirus-positive stool samples from 18 patients were collected for genotyping. A partial polymerase gene sequence was first obtained using a conventional RT-PCR diagnostic method described previously [26]. In brief, the polymerase gene was amplified using a cocktail of primers

(289hi and 290hijk), and a deoxyribonucleic acid (DNA) amplification of approximately 300 base pairs (bp) was excised from the gel and purified using a QIAGEN spin column (QIAGEN, Germantown, MD). The nucleotide sequence was determined directly from the amplicon using a Big Dye Terminator Cycle Sequencing Ready Reaction Kit, version 3.0 (Applied Biosystems, Carlsbad, CA), and an ABI PRISM 3730 automated DNA sequencer (Applied Biosystems). An online norovirus typing tool was used to provisionally assign the norovirus genogroup [27], followed by capsid gene amplification with primer pairs G1SKF (5'-CTGCCCGAATTYGTAATGA-3') and G1SKR (5'-CCAACCCARCCATTRTACA-3') or G2SKF (5'-CNTGGGAGGGCGATCGCAA-3') and G2SKR (5'-CCRCCNGCATRHCCRTTRTACAT-3') for GI and GII noroviruses, respectively [28]. The capsid gene amplicons (approximately 300–400 bp) were sequenced directly, and the genotype assigned with the online typing tool noted above.

Noroviruses of the GII.4 genotype from 13 patients were selected for further characterization. For some samples, cDNA was generated from the purified viral RNA using the Superscript III First-Strand Synthesis Super Mix (Invitrogen) with either oligo dT or random hexamers (Invitrogen) as primers, followed by PCR with the Elongase System (Invitrogen). In other samples, a one-step RT-PCR was carried out with the Superscript III One Step RT-PCR System (Invitrogen) following the manufacturer's recommendations. Characteristically, an approximately 1600-bp PCR amplicon containing ORF2 sequence was generated with forward primer GIIIF5050 (5'-GGGAGGGCGATCGCAATC-3') corresponding to nucleotides 5049–5065 of GII.4 strain MD145-12 (GenBank accession number AY032605) and reverse primer GII6800RDC32 (5'-STTTCCAGTTCCCATGGGG-3') complementary to nucleotides 6683–6665 of MD145-12. The amplicon was separated in a 1.2% agarose gel, excised, and purified as above. The nucleotide sequence was determined directly from the purified DNA amplicon using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit, version 3.0, and GII.4-specific primers as previously reported [29,30], and the sequencing products were resolved as described above.

Phylogenetic and Structural Analyses

Phylogenetic analysis was performed on the ORF2 nucleotide sequences (23 total) obtained from 13 NIH immunocompromised patients with GII.4 norovirus and the reference strains for each of the major GII.4 variant groups [12]. Sequence alignments were performed with Clustal W and manually edited. The phylogenetic tree was inferred using the Neighbor-Joining reconstruction method and Kimura 2-parameter as a model of nucleotide substitution as implemented in MEGA, version 6 [31]. The statistical significance of the phylogenies constructed was estimated by bootstrap analysis with 1000 pseudo-replicate data sets. The molecular model of the P domain of the VP1 of

Patient A was rendered using the x-ray coordinates from the GII.4 strain VA387 (Protein Data Bank accession number 2OBR) and Chimera 1.8 [32].

Detection of Bacterial and Other Viral Enteric Pathogens in Stool

Bacterial culture (sheep blood agar, MacConkey agar, xylose lysine deoxycholate agar, and *Campylobacter* CVA agar) was used for detection of *Salmonella*, *Shigella*, and *Campylobacter* species following standard methods. The Cepheid Xpert *C difficile* assay was used for detection of toxigenic *C difficile*, and the Premier Adenoclone-Type 40/41 and Premier Rotaclone enzyme immunoassay test kits (Meridian Bioscience, Inc., Cincinnati, OH) were used for the detection of adenovirus serotypes 40 and 41 and rotavirus, respectively. For detection of *Giardia lamblia*, the ProSpecT *Giardia* Microtiter assay (Remel, Inc., Lenexa, KS) was used.

RESULTS

Immunocompromised Study Population Tested for Norovirus as Part of Clinical Care

From September 22, 2011 through March 23, 2013, we identified 281 patients at the NIH Clinical Center with at least 1 valid (non-indeterminate) norovirus test result during the study period. Of these, 13 were excluded from our analysis because their underlying diagnoses were unrelated to primary or secondary immunodeficiency; none of these patients were positive for norovirus. Among the 268 immunocompromised patients, the mean age at the time of the first norovirus test was 42 years. Overall, 168 (63%) were male; of those with race/ethnicity information available, 73% were white and 18% were black/African American. The most common underlying diagnoses of those tested during the study period included those with hematologic malignancies (n = 122; 46%), PIDs (n = 53, 20%), and solid tumors (n = 44, 16%) (Table 1). In the group of 53 patients with PID disorders, the most common diagnoses were chronic granulomatous disease (n = 13; 25%) and GATA2 deficiency [33] (n = 10, 19%).

Norovirus was identified in 35 (13%) of the 268 immunocompromised patients (Table 1). Among these 35 patients who tested positive, 20 (57%) reported an acute onset of disease, with 17 (85%) of these illnesses occurring during the winter months of November–March. Six of these patients reported similar gastrointestinal illnesses among family members, although family history information was not systematically collected. The most common presenting symptoms were diarrhea (95%) and vomiting (55%). Among those without an acute onset noted at the time of testing, 9 (27%) presented with a history of chronic diarrhea, 8 of whom had a PID and 1 of whom had HIV/acquired immune deficiency syndrome (AIDS). Patients who tested positive for norovirus had a mean of 2.4 positive tests (range, 1–11). Among the 17 with more than 1 positive test, the median interval between first and last positive tests during the study period was 46 days

Table 1. Prevalence of Norovirus and Other Pathogens Identified at the Time of Norovirus Testing^a

| Pathogen | Hematologic Malignancies (n = 122) | | Solid Tumor (n = 44) | | Primary Immune Deficiency (n = 53) | | Other Hematologic (n = 26) | | HIV/AIDS (n = 13) | | Autoimmune/Autoinflammatory (n = 7) | | Total | |
|------------------------------|------------------------------------|-----------|----------------------|-----------|------------------------------------|-----------|----------------------------|-----------|-------------------|-----------|-------------------------------------|-----------|-------|-----------|
| | n ^b | n pos (%) | n | n pos (%) | n | n pos (%) | n | n pos (%) | n | n pos (%) | n | n pos (%) | n | n pos (%) |
| Norovirus | 122 | 13 (11) | 44 | 7 (16) | 53 | 11 (21) | 26 | 1 (4) | 13 | 3 (23) | 7 | 0 | 268 | 35 (13) |
| <i>Clostridium difficile</i> | 119 | 13 (11.9) | 43 | 10 (23) | 42 | 3 (7.1) | 26 | 6 (23) | 12 | 1 (8.3) | 6 | 1 (17) | 251 | 39 (16) |
| <i>Campylobacter</i> | 122 | 2 (1.8) | 40 | 1 (2.5) | 43 | 0 | 25 | 0 | 12 | 1 (8.3) | 6 | 0 | 243 | 4 (1.6) |
| <i>Salmonella</i> | 114 | 0 | 40 | 7 (18) | 43 | 0 | 25 | 0 | 12 | 0 | 6 | 0 | 243 | 7 (2.9) |
| <i>Shigella</i> | 114 | 0 | 40 | 0 | 43 | 0 | 25 | 0 | 12 | 0 | 6 | 0 | 243 | 0 |
| Adenovirus | 89 | 2 (2.2) | 26 | 1 (3.8) | 33 | 0 | 22 | 2 | 8 | 0 | 5 | 0 | 184 | 5 (2.7) |
| Rotavirus | 92 | 0 | 30 | 0 | 37 | 0 | 22 | 0 | 9 | 0 | 5 | 1 (20) | 197 | 1 (0.05) |
| <i>Giardia</i> | 101 | 0 | 37 | 0 | 40 | 1 (2.5) | 24 | 1 (4.2) | 13 | 1 (7.7) | 6 | 0 | 223 | 3 (1.3) |

Abbreviations: AIDS, acquired immunodeficiency syndrome; HIV, human immunodeficiency virus; pos, positive.

^a Concurrent pathogens defined as testing done within 30 days before or after norovirus test.

^b n: the first "n" refers to the number tested for each pathogen.

(range, 5–413 days). Among the 20 patients with acute onset, 10 were admitted to the clinical center and 6 were tested for norovirus within 3 days of admission (median, 1.5 days; range, 0–23 days). Of the remaining 15 without an acute onset, 7 were tested for norovirus following an admission to the Clinical Center, 2 within 3 days (median, 5 days; range, 1–284 days).

The prevalence of norovirus shedding varied by diagnosis. Among those with PID, norovirus was relatively common, with 11 (21%) of 53 testing positive. For the 166 patients with solid tumors or hematologic malignancies, 20 (12%) were positive, and among those with HIV/AIDS, 3 (23%) of 7 were positive (Table 1). Overall, 11 (31%) of the 35 with norovirus infection were SCT recipients.

The distribution of blood group in the norovirus-positive cohort was as follows: O, 38%; A, 28%; B, 22%; and AB, 12%. No apparent correlation was observed between blood group and infection with a particular norovirus genotype (Table 2). The secretor status, which determines the phenotypic expression of histo-blood group antigen carbohydrates on the intestinal epithelium [34], was not available.

At the time of initial norovirus testing, most patients were receiving treatment for an underlying condition, which included steroids (14; 40%), T-cell immunomodulators (10; 29%), and therapeutic monoclonal antibodies (3; 9%). Of the 9 norovirus-positive patients who had received intravenous immune globulin in the month before testing, all had a PID.

The most common copathogen identified at the time of initial norovirus testing was *C difficile*, which was identified in 39 (16%) of the 251 immunocompromised patients tested (Table 1). Of the 32 patients positive for norovirus who were also tested for *C difficile*, 3 (9.4%) had both pathogens. Among 29 patients positive for norovirus and also tested for *Campylobacter*, none were positive for both. Most patients tested for norovirus had no other enteric pathogen identified (Table 1).

Norovirus Characterization

One goal of this study was to establish whether an endemic nosocomial norovirus strain was being transmitted in the Clinical Center that could place immunocompromised patients at risk for norovirus infection during inpatient and outpatient visits. Between April 2010 and February 2013, stool samples were collected from 18 norovirus-positive patients (designated here as A through R) with a number of underlying conditions (Table 2), 15 of whom were also included in the epidemiologic analysis. Norovirus RNA was extracted from stool, and virus-specific RT-PCR amplicons were sequenced. The capsid genotype was assigned using the norovirus online typing tool [27]. Norovirus GII.4 was the most common genotype detected (14 of 18), comprising 78% of the samples, followed by GII.3 (1 of 18, 5%), GII.2 (1 of 18, 5%), GII.1 (1 of 18, 5%), and GI.3 (1 of 18, 5%) (Table 2). The duration of shedding, as determined by

Table 2. Immunodeficiency Diagnosis and Genotype Among Patients With Stool Samples Collected for Sequencing

| Patient | Diagnosis | ABO Blood Group | Duration Norovirus Shedding (Days) ^a | Stool Collection Date | Stool Designation | Norovirus Genotype (GII.4 Variant) | GenBank Number |
|---------|-------------------------------------|-----------------|---|-----------------------|-------------------|------------------------------------|----------------|
| A | PID/Hyper IgM, stem cell transplant | A | 939 | 4/1/2010 | A.1 | GII.4 (Apeldoorn 2007) | KX608847 |
| | | | | 6/15/2010 | A.2 | GII.4 (Apeldoorn 2007) | KX608848 |
| | | | | 12/20/2011 | A.5 | GII.4 (Apeldoorn 2007) | KX608849 |
| | | | | 10/20/2012 | A.13 | GII.4 (Apeldoorn 2007) | KX608850 |
| B | HIV/leukoencephalopathy/CD4 = 0 | AB | 3 | 6/22/2010 | B.2 | GII.4 (Den Haag 2006b) | KX608851 |
| | | | | 6/23/2010 | B.3 | GII.4 (Den Haag 2006b) | KX608852 |
| | | | | 6/24/2010 | B.4 | GII.4 (Den Haag 2006b) | KX608853 |
| C | Leukemia/ALL | O | 5 | 2/4/2011 | C.2 | GII.4 (Den Haag 2006b) | KX608854 |
| D | HIV/carcinoma CD4 = 8 | O | 205 | 6/14/2011 | D.1 | GII.4 (New Orleans 2009) | KX608855 |
| E | PID/SCID | A | 554 | 11/22/2011 | E.1 | GII.4 (New Orleans 2009) | KX608856 |
| F | Hodgkin's lymphoma | B | 1 | 12/20/2011 | F.1 | GII.4 (New Orleans 2009) | KX608857 |
| G | PID/CVID | O | 1 | 12/15/2011 | G.1 | GII.4 (Osaka 2007) | KX608858 |
| H | PID/CVID | AB | 641 | 2/17/2012 | H.1 | GII.4 (New Orleans 2009) | KX608859 |
| | | | | 7/19/2012 | H.3 | GII.4 (New Orleans 2009) | KX608860 |
| | | | | 7/24/2012 | H.4 | GII.4 (New Orleans 2009) | KX608861 |
| | | | | 9/5/2012 | H.5 | GII.4 (New Orleans 2009) | KX608862 |
| | | | | 9/18/2012 | H.6 | GII.4 (New Orleans 2009) | KX608863 |
| I | Leukemia/ALL | A | 417 | 2/18/2012 | I.1 | GII.4 (New Orleans 2009) | KX608864 |
| J | PID/Hyper IgE | A | 1 | 2/22/2012 | J.1 | GII.4 (New Orleans 2009) | KX608865 |
| K | Solid tumor | A | 1 | 2/28/2012 | K.1 | GII.4 (New Orleans 2009) | KX608866 |
| L | Aplastic anemia | B | 34 | 4/6/2012 | L.2 | GII.4 (Den Haag 2006b) | KX608867 |
| M | PID/IRAK4 deficiency | AB | 385 | 4/2/2012 | M.1 | GII.4 (not assigned) | KX608868 |
| | | | | 7/14/2012 | M.4 | GII.4 (not assigned) | KX608869 |
| O | PID/PLAID | O | 304 | 5/6/2011 | O.1 | GII.4 (not assigned) | KX608870 |
| N | PID/SCID | AB | 16 | 3/10/2011 | N.2 | GII.3 | KX608871 |
| P | PID/SCID | B | 677 | 7/22/2011 | P.1 | GII.2 | KX608872 |
| Q | Lymphoproliferative disorder | B | 1 | 11/21/2011 | Q.1 | GII.1 | KX608873 |
| R | Leukemia/CLL | O | 1 | 2/3/2012 | R.1 | GI.3 | KX608874 |

(Note: Patients B, C, and N were enrolled in a viral pathogens protocol before the start of the retrospective review summarized in Table 1.)

Abbreviations: ALL, acute lymphocytic leukemia; CLL, chronic lymphocytic leukemia; CVID, common variable immune deficiency; HIV, human immunodeficiency virus; Ig, immunoglobulin; IRAK4, interleukin-1 receptor-associated kinase-4; NIH, National Institutes of Health; PID, primary immunodeficiency; PLAID, plaid syndrome (phospholipase C-gamma 2-associated antibody deficiency and immune dysregulation); SCID, severe combined immune deficiency.

^a Duration of norovirus shedding based on data from clinician-ordered tests sent to NIH microbiology laboratory during study period. The precise start and end dates for each infection were not known.

the consistent detection of norovirus genome in the available stool samples collected over the time period of this study, is noted in Table 2. Sequencing analysis confirmed the presence of a single genotype within each patient during periods of prolonged shedding.

Norovirus Evolution in Immunocompromised Patients

Twenty-three stool samples from 13 patients with GII.4 norovirus were selected for further sequencing of the major capsid gene (Figure 1). Norovirus GII.4 has been the predominant global genotype for several decades, and new GII.4 variants (named according to an early location where first detected) emerge periodically to displace the predominant GII.4 strain associated with disease [35]. Comparison of the NIH GII.4 viruses with GII.4 variant reference strains showed that most of the viruses, with the exception of the viruses from Patient M and Patient O (data not shown) clustered with an established variant group, including Den Haag 2006b, Osaka 2007, Apeldoorn

2007, and New Orleans 2009 (Figure 1A). The Sydney 2012 GII.4 variant (designated on the dendrogram as Hu/GII.4/NSW0514/AU/2012/JX459908), first identified in Australia in March 2012 [36] and responsible for nearly half the gastroenteritis outbreaks in the United States by October 2012 [37], was not detected.

Several noroviruses clustered within the same GII.4 variant group. The relatedness of the strains at the nucleotide and amino acid level and possible transmission patterns were examined (Figure 1B). Specifically, the noroviruses detected in patients D, E, F, H, I, J, and K all clustered within the New Orleans 2009 group. The highest identity in pairwise comparisons in the New Orleans cluster was observed among the sequential virus samples of Patient H. The second closest relationship in the New Orleans 2009 cluster was between the viruses from Patients F and I, with only 10 nucleotide differences in the ORF2 gene. No common link with regard to the timing of inpatient or outpatient visits or to room location was

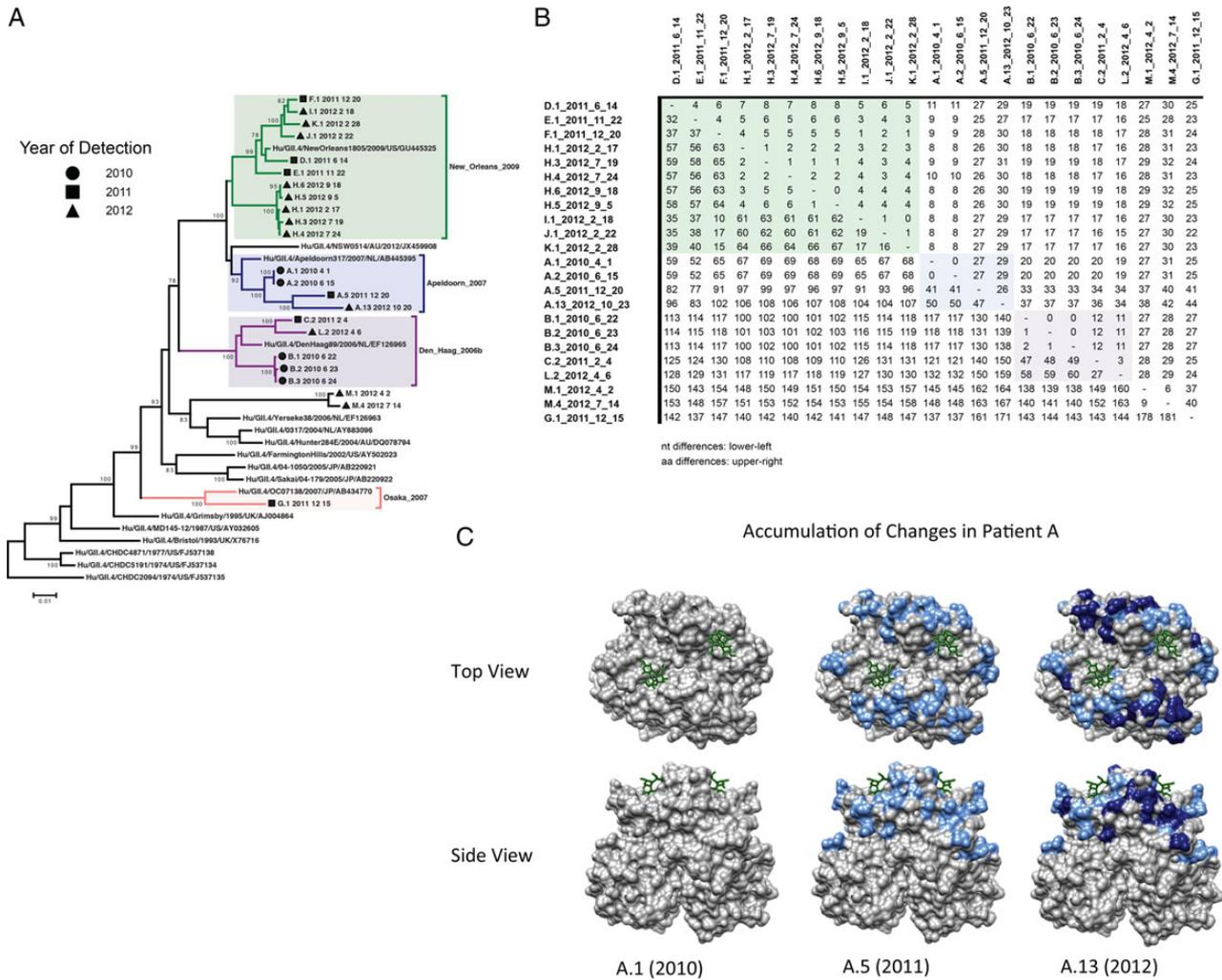


Figure 1. Diversity and evolution of GII.4 strains detected in immunocompromised patients undergoing care at the National Institutes of Health (NIH) Clinical Center. (A) Phylogenetic analysis of the major capsid protein VP1 gene of selected GII.4 strains showing the clustering of NIH strains within the different GII.4 variants. The phylogenetic tree was inferred using the neighbor-joining reconstruction method and Kimura 2-parameter as a model of nucleotide substitution. The statistical significance was calculated using bootstrap (1000 pseudo-replicates). The GII.4 viruses detected in NIH patients are indicated based on the year of detection. At least 1 strain from each recognized GII.4 variant was included in the analysis [12]. Two pandemic variants (New_Orleans_2009 and Sydney_2012) were circulating during the period of this study. The Sydney_2012 is represented by the strain Hu/GII.4/NSW0514/AU/2012/JX459908. Phylogenetic clusters that included NIH strains detected within a single GII.4 variant are indicated by different colors. (B) Matrix showing nucleotide (lower-left) and amino acid (upper right) differences among the GII.4 viruses detected in patients from the NIH Clinical Center. (C) Structural model of the P domain showing the location of amino acid substitutions that occurred in a patient persistently infected (Patient A). Substitutions detected in 2011 (when compared with the first sample, 2010) are indicated in light blue, and those in 2012 in dark blue. The interacting site of the norovirus capsid with histo-blood group antigen (HBGA) carbohydrate (represented in green) is indicated.

identified. Another of the closest genetic relationships (27 nucleotide differences) was between the ORF2 of noroviruses in the Den Haag 2006b cluster that were associated with infection in Patients C and L. Again, no common link in exposure could be identified based on medical records review.

Chronic norovirus infections were present in several patients. Patient A (who had PID and received immune globulin therapy) had the most prolonged shedding of norovirus (939 days or 2.6 years) (Table 2). We modeled the evolution of the GII.4 capsid sequence (an Apeldoorn 2007 variant) over time. As noted for other immunocompromised individuals, the majority of

amino acid substitutions occurred in the surface exposed Protruding (P) domain of the capsid [38, 39]. The mechanisms responsible for evolution in the major antigenic and receptor binding sites of the viral capsid will require further investigation.

DISCUSSION

We describe the prevalence of norovirus infection among immunocompromised individuals referred to the NIH Clinical Center. In our cohort, norovirus and *C difficile* were the most commonly detected enteric pathogens, with prevalence rates

of 13% and 16%, respectively. Our findings are consistent with studies conducted in similar populations: among solid organ and hematopoietic stem cell transplant recipients, norovirus was identified in 25 (22%) of 116 pediatric patients and was the most common enteric pathogen identified [18]. In a cohort of 62 children with primary immune deficiencies, norovirus was the most common pathogen identified, found in up to 17.7% of patients systematically screened [19]. Likewise, Echenique et al [23] reported that norovirus and *C difficile* were the predominant enteric pathogens found in solid organ transplant recipients. As the ability to detect enteric pathogens improves with the availability of sensitive multiplex diagnostic assays, data concerning the risk factors, prevalence, and relative disease burden of norovirus in immunocompromised hosts will further define high-risk populations.

Noroviruses are known to establish persistent infections in immunocompromised individuals, and chronically infected patients could be a reservoir for the emergence of new norovirus strains over prolonged periods [40]. Moreover, nosocomial transmission and outbreaks have been linked to immunodeficient patients [41–43]. Although several patients in our study showed evidence of chronic infection with the same virus genotype over sustained periods, we did not find evidence of transmission of these specific strains within the NIH Clinical Center. The reason for this may be 2-fold: first, noroviruses may acquire mutations over time that affect their transmission and virulence; and second, rigorous infection control measures, including contact isolation, have been shown to prevent spread in the hospital [44]. An epidemiologic investigation in a pediatric tertiary care hospital attributed the absence of endemic enteric viruses in their setting to rigorous infection control [45]. Although some norovirus transmission may have occurred among the non-immunodeficient patients not tested for norovirus, we think this is unlikely given that all non-immunodeficient patients tested but excluded from our analysis were negative for norovirus, and no norovirus outbreaks were detected during the study period. Future studies with systematic screening for norovirus would provide a broader view of norovirus shedding and transmission across all patient populations in this setting. The risk that persistent norovirus shedding poses for spread to susceptible hosts is an important area of future investigation that will be facilitated by cell culture systems and animal models that can assay norovirus infectivity in clinical samples.

Our phylogenetic analysis of the GII.4 noroviruses showed genetic clustering of a specific norovirus strain within each patient, but not among patients, indicating that an endemic GII.4 norovirus strain was not present in the hospital. The seasonal pattern of virus detection, with a peak in the winter months (November–March), is consistent with the known seasonality of norovirus, the “winter vomiting disease” [2]. These data

suggest that patients are likely exposed to norovirus in the community during the outbreak season when the risk of infection is highest in the general population. Once exposed, immunocompromised individuals vary in their ability to clear virus infection, and the virus may become chronic. Our data showed that patients maintained the same virus strain (albeit evolving) when chronically infected, which is consistent with several studies that have shown persistence of the same virus strain in an individual over time [38, 46]. Considering that formerly dominant GII.4 variants were detected in our cohort, it is possible that a number of these patients were infected years ago. Clearance and reinfection with a new norovirus has been reported in immunocompromised patients [18], so continued monitoring of our patient cohort with molecular tools will be needed to better define the extent of this risk.

The primary limitation of our study is that patients included in this retrospective cohort analysis represent a selected group of patients screened for norovirus and other gastrointestinal pathogens based on physician discretion, rather than through a systematic prospective approach. Nonetheless, the similarity of the norovirus prevalence found in our cohort to that for other immunocompromised groups studied, in both representative and nonsystematic samples, indicates that these estimates are likely to represent the true burden of norovirus in this population. Patients screened for norovirus are more likely to be those with clinically significant acute or persistent diarrhea, so the prevalence shown here is likely to represent clinically significant diarrhea. However, the causal relationship of norovirus with diarrhea in immunocompromised individuals needs further study because gut homeostasis is profoundly affected in many of these conditions and diarrhea is common. In addition, future studies with systematic follow-up among defined cohorts of immunocompromised persons will be useful in identifying factors related to persistence and clearance of norovirus infections.

CONCLUSIONS

Based on the findings in this and other similar studies that norovirus and *C difficile* are the most common etiologic agents of diarrhea in immunocompromised individuals, we concur with Echenique et al [23] that screening for etiologic agents of diarrhea should at a minimum include targeting these 2 major pathogens. Increased use of multiplex panels in symptomatic patients should improve diagnosis and treatment of persistent diarrhea in this immunocompromised population. Finally, the search for promising norovirus therapies continues, and their efficacy in immunocompromised patients will be important in their evaluation [47]. Vaccines that reduce norovirus circulation in the general population should impact norovirus transmission dynamics: the risk for exposure and chronic infection in patients with underlying immune deficiencies will be reduced.

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