High Serum Levels of Norovirus Genotype–Specific Blocking Antibodies Correlate With Protection From Infection in Children

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(See the editorial commentary by Lee and Pang on pages 1691–2.)

Background. Norovirus is a common cause of acute gastroenteritis in children. Serum immunoglobulin G (IgG) antibodies have been implicated in protection against norovirus-associated gastroenteritis, but the level, specificity, and functionality necessary for protection remain to be elucidated.

Methods. Norovirus-specific IgG antibodies to genogroup II (GII)-4-2010 New Orleans (NO), GII-4-1999, GII-12-1998, GI-1-2001, and GI-3-2002 virus-like particles (VLPs) were determined by enzyme-linked immunosorbent assay in serum samples collected from children who presented to the hospital because of acute norovirus gastroenteritis in 2009–2011. The blocking activity of the antibodies was tested in a surrogate neutralization assay.

Results. Most norovirus infections (62.8%) in the study population were caused by a GII-4 NO variant. Children who acquired GII-4 NO infection had a low preexisting type-specific IgG level and blocking activity of the sera, in contrast to children infected with other GII genotypes. Following GII-4 NO infection, genotype-specific seroconversion and a corresponding increase in blocking antibody potential was observed. Although seroconversion to the heterologous GII-4-1999 variant was observed, there was no corresponding increase in the specific blocking antibody titer. There was no concomitant seroconversion against GI VLPs, indicating a highly genogroup-specific antibody response.

Conclusions. High preexisting norovirus genotype–specific serum IgG titers and blocking activity in children indicate protection from norovirus infection in a strain-specific manner.

Keywords. norovirus; antibodies; blocking; protection; children.

Noroviruses (NoVs) are, after rotaviruses, the second most common causative agents of acute gastroenteritis in children [1–5]. In countries with a national rotavirus vaccination program, such as the United States and Finland, NoVs have become the most common cause of acute gastroenteritis in hospitalized children as the incidence of rotavirus infection has decreased [6, 7]. In Finland, the proportion of NoV-associated gastroenteritis cases among all acute gastroenteritis cases seen in a major hospital increased from 24% during the 2 years before introduction of rotavirus vaccine to 37% during the 2 years after the introduction of rotavirus vaccine, owing to a decreased number of rotavirus-associated gastroenteritis episodes during the latter period [7]. Consequently, research and development efforts are underway to create a vaccine that can protect against NoV infection [8, 9].

Most NoV infections in humans are caused by NoVs belonging to genogroup II (GII), with a minority caused by GI. According to recently proposed classification, there are currently 9 GI and 22 GII capsid genotypes [10]. Genotype GII-4 has caused most of the NoV outbreaks and sporadic infections for 2 decades [11, 12]. A novel predominant GII-4 variant emerges every 2–3 years [13, 14] and several mechanisms have been proposed to drive GII-4 escape from herd immunity [13, 15–20]. GII-4 NoV variants have maintained dominance since 1990’s in Finland as well [3]. The
predominant NoV strain during 2009–2011 in Finland and globally was GII-4 New Orleans (NO), even if characteristic genetic diversity coexisted [3, 21].

NoV incidence is the highest in children <2 years of age [6, 22], who also have the least NoV-specific antibodies [22–25]. The prevalence of antibodies to NoV increases rapidly before the age of 5 years, when the majority of the population has been exposed to NoV [22, 24, 26].

Correlates of protection from NoV infection and disease are still poorly understood [27–29]. In adults, both genotype-specific and cross-reactive antibodies, especially within the same genogroup, are generated after NoV infection [30, 31]. Several prospective epidemiological studies have suggested that high NoV genotype–specific serum antibody levels correlate with protection from infection in children [22, 23, 32, 33]. On the other hand, serological studies have demonstrated that even robust and broad genotype-specific preexisting IgG antibodies found in adult sera have not conferred protection from NoV infection [34–36]. More recently, the serum IgG antibodies that block binding of NoV VLPs to histo-blood group antigen (HBGA) ligands on the cell surface have been suggested to correlate with protection against NoV infection [19, 20, 22, 26, 36].

To gain insight into the epidemiologic patterns of sporadic NoV infections and to determine the significance of preexisting immunity to NoVs, we analyzed NoV-specific IgG antibody levels and blocking potential in acute serum from children presenting with NoV-associated acute gastroenteritis. Our results suggest that the magnitude and antibody blockade of GII-4 NO strain–specific serum IgG negatively correlate with susceptibility to GII-4 NO infection.

MATERIALS AND METHODS

Study Material

A total of 666 children <15 years old presenting with gastroenteritis were enrolled in a prospective etiological study at Tamperere University Hospital [3, 7] from the beginning of September 2009 to the end of May 2011. The first NoV-positive cases were detected at the end of December 2009, with screening detecting NoV in 111 patients with gastroenteritis (16.7%) during the study period. During December 2009–April 2011, acute-phase serum samples were collected from 43 NoV-positive children and paired convalescent-phase serum samples were collected from 6 children positive for GII-4-2010 NO.

Eligible subjects were ≤15 years of age who were either visiting the hospital outpatient clinic or admitted to a hospital ward with acute gastroenteritis. The study protocol and informed consent forms had been approved by the Ethics Committee of the Pirkanmaa Hospital District, and informed consent was given by the parents or guardians of eligible children before samples were collected. NoV infection was confirmed in stool samples by reverse-transcription polymerase chain reaction as previously reported [3, 7]. Acute-phase serum samples were collected within a week after disease onset, and convalescent-phase serum samples were obtained 2–6 weeks after the onset of disease. The sera were stored at −20°C before analysis.

Antigens

Five NoV capsid–derived virus-like particles (VLPs) belonging to GI and GII were used as coating antigens in enzyme-linked immunosorbent assays (ELISAs). The VLPs were produced using a baculovirus–insect cell expression system and purified by gradient ultracentrifugation as previously described [8, 37]. The selected genotypes were GII-4-2010 NO (GenBank accession number GU445325), GII-4-1999 (AF080551), GII-12-1998 (AJ277618), GI-1-2001 (AY502016.1), and GI-3-2002 (AF414403). Protein concentration, purity, integrity, morphology, and in vitro antigenicity were determined as described earlier [8, 37, 38].

Serum Antibody ELISA

Sera were tested for total IgG against NoV VLPs in ELISA as previously described [8, 22, 39], with some modifications. In brief, 96-well half-area polystyrene plates (Corning, Corning, NY) were coated with 0.5 µg/mL of GII-4 NO, GII-4-1999, GII-12, GI-3, or GI-1 VLPs in phosphate-buffered saline. The serum samples were added to the plates blocked with 5% milk at 2-fold serial dilutions, starting at 1:100. One known positive human serum sample and 1 known negative human serum sample were included on every plate as controls. Bound antibody was detected with horseradish peroxidase–conjugated anti-human IgG (Invitrogen), followed by reaction with 0.4 mg/mL o-phenylenediamine dihydrochloride substrate (FAST-OPD, Sigma-Aldrich). The OD was measured in a microplate reader (Victor2 1420, PerkinElmer) at 490 nm. Background signal from the blank wells (ie, wells without serum) was subtracted from all of the OD readings on a plate. A positive reaction was defined as an OD that was 3 standard deviations above the mean OD for negative control serum and at least 0.200. End point titers were defined as the reciprocal of the final serum dilution giving a mean OD_{490 nm} ≥0.200 after background subtraction. Seroconversion was considered to have occurred if the convalescent-phase serum sample had a ≥4-fold greater titer than the acute-phase serum sample. Antibody values were log transformed to establish geometric mean titers (GMTs) for analysis.

Serum Antibody Blocking Assay

Blocking of GII-4 NO-2010 and GII-4-1999 VLPs binding to HBGA carbohydrates in secretor-positive human saliva type A was performed as previously described for mouse antisera with slight modifications [40]. In brief, 2-fold serial dilutions of sera ranging from 1:25 to 1:204 800 were pre-incubated with 0.1 µg/mL of GII-4 NO or GII-4-1999 VLPs at 37°C for 1 hour, followed by a 1.5-hour incubation at 37°C on 96-well plate coated with human saliva type A. VLP binding was detected by GII-4
NO– or GII-4-1999–specific mouse sera (a 1:800 dilution) followed by goat anti-mouse IgG–horseradish peroxidase (Sigma) and Sigma Fast-OPD substrate. The blocking index was defined as the percentage of VLPs binding in the presence of serum, compared with the maximum binding in the absence of serum, using the following equation: 100 – [OD for wells with serum/OD for wells without serum] × 100. The titer at which 90% of binding was blocked (BT90) was determined for each sample, defined as the reciprocal of the lowest serum dilution that blocked at least 90% of VLPs binding to HBGA. An arbitrary BT90 of 12.5 was assigned to BT90 of <25 for statistical analyses.

Statistical Analyses
The Fisher exact test was used to determine the statistical difference in antibody quantity between the group infected with GII-4 NO and the group infected with other NoV GII genotypes, as well as between different age groups. The Spearman rank correlation coefficient was used to examine the differences between preexisting antibody titer, blocking titer BT90 and infection acquired. Statistical analyses were performed using IBM SPSS Statistics (SPSS, Chicago, IL) version 20.0. Statistical significance was defined as a $P$ value of <.05.

RESULTS

NoV Infection and Association With Age
All age groups were represented among the enrolled patients with gastroenteritis. The proportion of young children (age, <2 years) was slightly higher among the NoV-positive cases (75.6%), compared with all enrolled patients with gastroenteritis.

![Figure 1](image1.png)

**Figure 1.** Norovirus (NoV) infections and age distribution. A, Distribution of enrolled subjects presenting with gastroenteritis (GE) of any cause and those presenting with NoV–associated gastroenteritis (NoV GE), stratified according to age group. B, The number of subjects, by age, presenting with each infecting NoV genotype detected in the study population. Abbreviations: GII, genogroup II; NO, New Orleans.

![Figure 2](image2.png)

**Figure 2.** Norovirus (NoV) genotype-specific preexisting serum immunoglobulin G (IgG) antibody titers. A, Genotype-specific IgG responses, by age group: <1 year ($n = 18$), 1–2 years ($n = 14$), and >2 years ($n = 11$). B, Genotype-specific IgG titers for children with and children without NoV genogroup II (GII)-4 New Orleans (NO) infection. Bars represent $\log_{10}$ geometric mean titers with 95% confidence intervals. Statistical differences between any 2 experimental groups were determined by a Fisher exact test, and a $P$ value of $\leq .05$ was considered to be statistically significant.
(58%; Figure 1A). The age distribution in the group of NoV-positive patients with acute serum samples collected was similar to that in the group of all NoV-positives patients (data not shown).

Of 43 children presenting with acute gastroenteritis due to NoV, 27 (62.8%) experienced GII-4 NO infection, whereas 16 (37.2%) were infected with other G11 genotypes (Figure 1B).

Preexisting NoV Genotype–Specific Antibody Responses
Preexisting serum IgG levels against 5 NoV genotypes—GII-4-1999, GII-4 NO-2010, GII-12-1998, GI-1-2001, and GI-3-2002—were analyzed in 43 sera collected within a week after the onset of acute gastroenteritis symptoms. NoV VLP type–specific serum GMTs stratified according to donor age (≤1 year, 1–2 years, and > 2 years) are shown in Figure 2A. Significant differences between different age groups for GII-4 NO– and GII-4-1999–specific antibody titers were observed (P < .05). Overall, 55.6% of <1-year-old children and 35.7% of 1–2-year-old children had a low preexisting serum end point titer (≤800) against all tested NoV genotypes, whereas all of the children >2 years of age had high NoV–specific antibody titers (end point titer, ≥3200) at least against one of the NoV genotypes (data not shown).

Children infected with GII-4 NO had significantly lower levels of preexisting GII-4 NO–specific IgG, compared with children infected with other GII genotypes (P < .05; Figure 2B).

Figure 3. Norovirus (NoV) genotype–specific immunoglobulin G (IgG) titration curves in acute-phase serum specimens. Sera from children with (n = 27; gray lines) and children without (n = 16; black lines) genogroup II (GII)-4 New Orleans (NO) infection were titrated with 2-fold serial dilutions, and IgG antibodies were analyzed against GII-4 NO (A), GII-4 (B), GII-12 (C), GI-1 (D), and GI-3 (E) genotypes. The cutoff for a positive response was an OD_{400 nm} of ≥0.200.
Acute sera from all GII-4 NO–infected patients recognized genotype GII-4 NO VLP but at a low titer (GMT, 958). On the contrary, patients infected with non–GII-4 NO had significantly higher preexisting GII-4 NO–specific titers (GMT, 13958). Concomitantly, when ancestor GII-4-1999 VLP–specific reactivity was analyzed, the trend was similar to the GII-4 NO VLP seroresponses (Figure 2B), with patients with GII-4 NO infection having a lower response than patients without GII-4 NO infection (GMT, 910 vs 51 200; P = .061). In general, moderate serum levels of IgG antibody to GII-12, GI-1, and GI-3 NoV VLPs were detected (GMT, < 100, and 61.9% of these children had a BT$_{90}$ of ~25. On the contrary, 56.3% of children without GII-4 NO infection had a high preexisting GII-4 NO–specific IgG end point titer (>51 200) and a BT$_{90}$ of >200. NoV VLP type–specific IgG antibody titration curves for each patient’s acute sera are shown in Figure 3A–E. A majority of GII-4 NO–infected patients (74.1%) had a GII-4 NO–specific end point titer of ≤1600 (Figure 3A), and all end point titers were ≤12 800. Corresponding low end point titers were determined for GII-4-1999 (Figure 3B), GII-12 (Figure 3C), GI-1 (Figure 3D), and GI-3 (Figure 3E) in GII-4 NO–infected patients’ sera. To the contrary, 56.3% of patients without GII-4 NO infection had high GII-4 NO–specific end point titers (≥51 200; Figure 3A), and 12.5% had high GII-4-1999–specific titers (Figure 3B). No difference in GII-12, GI-1, and GI-3 specific titers in acute-phase sera was detected between children with and those without GII-4 NO infection (Figure 3C–E).

**Acute-Phase Serum Blocking Antibodies**

Individual acute serum samples were also analyzed for the potential to block the binding of GII-4 NO VLP to saliva type A, and blocking activity was compared to preexisting GII-4 NO–specific IgG end point titers (Figure 4). A positive correlation was detected between the antibody levels and BT$_{90}$ (Spearman rank correlation coefficient [r], 0.477; P < .01). All GII-4 NO–infected children had a GII-4 NO–specific end point titer of <12 800 and a BT$_{90}$ of ≤100, and 61.9% of these children had a BT$_{90}$ of <25. On the contrary, 56.3% of children without GII-4 NO infection had a high preexisting GII-4 NO–specific IgG end point titer (>51 200) and a BT$_{90}$ of >200.

**Convalescent-Phase Sera Antibody Responses**

Paired sera from 6 patients infected with GII-4 NO were tested for seroconversion against each of the NoV VLPs (Table 1). Acute-phase serum samples were collected 2–6 days (median, 3.5 days) and convalescent-phase serum samples 18–29 days (median, 26 days) after onset of acute gastroenteritis. All patients seroconverted to GII-4 NO, 4 seroconverted to GII-4-1999, 1 seroconverted to GII-12, and 0 seroconverted to GI-1 and GI-3. GII-4 NO infections induced high type-specific serum IgG responses, as well as a cross-reactive response to the GII-4-1999 strain. Serum antibody levels against more-distinct GI-1 and GI-3 NoV VLPs remained similar after infection, indicating low cross-reactivity between GI and GII NoVs. Blocking of GII-4 NO and GII-4-1999 VLP binding to saliva type A by the 6 convalescent-phase serum samples was also tested and compared to genotype-specific IgG titers (Figure 5A–F).

**Table 1. End Point Titers Against Different Norovirus Genotypes in Paired Acute-Phase (I) and Convalescent-Phase (II) Serum Specimens**

<table>
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<tr>
<th>Donor</th>
<th>Age, mo</th>
<th>Disease Onset to Sampling, d$^a$</th>
<th>GII-4 NO</th>
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$^a$ Time from the onset of acute gastroenteritis to collection of the convalescent-phase serum specimen.

$^b$ Convalescent-phase serum specimens in which seroconversion was detected.
GII-4 NO–specific blocking index of each subject increased along with the IgG titer following GII-4 NO infection. The youngest, a 3-month-old patient (Figure 5E), had a weaker IgG response after infection, compared with other patients (Figure 5A–D and 5F), but both seroconversion and a 16-fold increase in blocking titer were observed (acute-phase serum $\text{BT}_{90}$, 12.5; convalescent-phase serum $\text{BT}_{90}$, 200). The period between the onset of the disease and the collection of convalescent-phase serum specimen was 7–11 days shorter for this patient than for the others (Table 1). However, despite seroconversion to GII-4-1999 in 4 subjects, no corresponding increase in GII-4-1999–specific blocking titer was observed (data not shown).

**DISCUSSION**

In the present study, we determined preexisting NoV-specific IgG antibody levels against 5 different NoV genotypes—GII-4-1999, GII-4-2010 NO, GII-12-1998, GI-1-2002, and GI-3-2001—in sera from Finnish children who had acquired NoV infection. Most of the NoV-infected patients studied here were <2 years of age, in accordance with previous reports.
demonstrating that the incidence of NoV-associated gastroenteritis peaks in children from 6 to 23 months of age [25, 26, 41–43]. Antibodies against all NoV genotypes were detected in acute-phase sera, but IgG levels against GII-4 NO were generally the highest, especially among children >2 years old. Norovirus variant GII-4 NO dominated since 2009 in Finland [2, 3] and worldwide [21]. No GI infections among study population were detected, which is congruent with the low prevalence of GI infections reported in Finland [2, 3] and in accordance with the low end point serum titers to GI-3 and GI-1 NoV we observed.

The main objective of the present study was to assess correlation of preexisting serum IgG levels and/or antibody-blocking potential and susceptibility to infection with the predominant GII-4 NO genotype. Significant difference in preexisting GII-4 NO-specific antibody responses was observed when IgG titers of the patients who acquired GII-4 NO infection were compared to IgG titers of patients who acquired other GI genotype infection. Of 16 patients without GII-4 NO infection, 9 had very high preexisting GII-4 NO IgG levels (end point titer, >51 200; GMT, 94 809), whereas all 27 GII-4 NO-infected patients had acute serum GII-4 NO-specific end point titers of <50 000 (GMT, 958). High GII-4 NO-specific titers detected only in acute-phase serum from patients without GII-4 NO infection probably reflect the magnitude of the genotype-specific IgG titer that is required to confer protection from natural infection.

A response pattern similar to that seen for GII-4 NO genotype, was observed with the serum IgG titers to the ancestor GII-4-1999 strain. The GII-4-1999-specific response measured was likely contributed by cross-reactive IgG antibodies between GII-4 NO and GII-4-1999 VLPs, which share high sequence homology (93% capsid protein identity, by the Protein Basic Local Alignment Search Tool). Earlier serological studies have also demonstrated cross-reactivity within the genogroup, even after primary infection, in children [44] and adults [30, 31]. When end point titers against GII-12, GI-1, and GI-3 in acute-phase sera were compared, no difference was observed between patients with and those without GII-4 NO infection. The relatively low end point titers against these genotypes are likely due to less extensive circulation in the population and lower cross-reactivity with the predominant GII-4 variant [3, 22, 45].

Furthermore, the ability of acute-phase serum to block binding of GII-4 NO VLPs to HBGA ligand correlated with homologous type-specific IgG titers. The analysis of paired sera from the study subjects further confirmed positive correlation between the GII-4 NO-specific serum IgG level and homologous blocking potential of the serum. When recently acquired IgG antibody was assessed from convalescent-phase sera, it was observed that all patients seroconverted after GII-4 NO infection, and concurrently, there was 16–256-fold increases in the GII-4 NO-specific BT g90, compared with acute-phase sera. Taken together, our results show a correlation between a low acute-phase serum GII-4 NO-specific IgG titer (end point titer, ≤12 800) and a low antibody-blocking potential (BT g90, ≤100) with a high susceptibility to GII-4 NO infection. Congruently, a high specific IgG end point titer and a considerable blocking potential appeared to protect from GII-4 NO infection. In contrast to our results, there are publications showing that high preexisting serum NoV-specific IgG levels measured by ELISA have not been protective from virus challenge in adults [35, 36]. The discrepancies can be partly explained by the fact that not all antibodies detected by ELISA are functional in blocking the binding of the virus to the receptor and thus preventing the infection. We also observed that a high preexisting GII-4 NO antibody titer has not been able to protect children from infection with other circulating GI genotypes—GII.7/GII.6, GII.e/GII.4, GII.b, and GII.g/GII.12—indicating the relevance of strain-specific immunity in protection from NoV infection.

Sera collected from children for prospective epidemiological study give valuable information for elucidating correlates of protection in young populations by defining the magnitude and functionality of preexisting and recently acquired NoV-specific immune responses. Data presented here indicate a positive correlation between high preexisting genotype-specific serum IgG titers and blockade of VLP binding to HBGA receptors to protection from infection, addressing the relevance of serum IgG responses in children. Our results are in concordance with those of earlier studies [22, 23, 32, 33] showing that a low preexisting antibody titer indicates higher risk of developing NoV infection in the near future in children. Our data support the prevailing understanding that induction of broad, high titer serum IgG levels with good blocking potential are necessary for protection from NoV infections.

Notes
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Potential conflicts of interest. All authors: No reported conflicts.

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1762 • JID 2014;210 (1 December) • Malm et al