Genogroup IIb Norovirus Infections and Association with Enteric Symptoms in a Neonatal Nursery in Southern India

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Noroviruses (NoVs) are increasingly recognized as an important cause of acute gastroenteritis in children worldwide. However, there are limited data on the role of NoVs in neonatal infections and disease. The objectives of the present study were to determine the prevalence of NoVs in neonates with gastrointestinal disease using a case-control study design and to characterize the NoV strains infecting neonates. A total of 309 fecal samples from 161 neonates with gastrointestinal symptoms and 148 asymptomatic controls were screened for genogroup II (GII) NoV using reverse transcription-PCR. A subset of PCR-positive amplicons for the polymerase and capsid regions was sequenced. NoV was detected in 26.2% of samples, with the rate of detection being significantly higher among symptomatic neonates (60/161, 37.2%) than asymptomatic neonates (24/148, 14.1%) (P < 0.001). On the basis of sequencing of 29 strains, a single NoV strain, GIIb, was identified to be the predominant (27/29, 93.1%) cause of neonatal infections. Coinfection with rotavirus was seen in nearly one-third of symptomatic neonates. The study demonstrates a high prevalence of NoV infections in neonates and indicates that coinfection with rotavirus may result in significantly more gastrointestinal disease in this population.

Noroviruses (NoVs) are a leading cause of nonbacterial gastroenteritis among individuals of all age groups worldwide (13). They are nonenveloped, single-stranded RNA viruses belonging to the family Caliciviridae. The 7.7- to 8.0-kb positive-sense single-stranded RNA genome encodes three large open reading frames (ORFs) (16). ORF 1 encodes nonstructural proteins, while the viral structural proteins are encoded by ORFs 2 and 3. NoVs are classified into five distinct genogroups (genogroup I [GI] to GV), of which genogroups I, II, and IV are known to cause human infections. Each genogroup is further subdivided into genotypes. Eight GI genotypes, 17 GII genotypes, and 1 GIV genotype have been identified so far (11, 22).

NoVs are increasingly recognized as the second most important cause of viral gastroenteritis in young children (11). A recent review of reports from both developed and developing countries indicates that the prevalence rates of NoV gastroenteritis in hospitalized children range from 6 to 48%, with the overall median being 14% (9). It has been estimated that NoVs may be responsible for more than 1 million hospitalizations and 200,000 deaths in children <5 years of age in developing countries (17).

There are recent reports of NoV outbreaks in neonatal nurseries associated with clinical presentations such as necrotizing enterocolitis (NEC), diarrhea, vomiting, abdominal distension, and fever (2, 18, 20). However, there are limited data on NoV infections in the neonatal population in nonoutbreak situations (19). The present study was conducted to determine the prevalence rates of GII NoVs among neonates with gastrointestinal (GI) disease and asymptomatic controls using a case-control design and to characterize the strains infecting neonates. This study focused on testing for GII NoVs, as previous hospital- and community-based epidemiological studies in southern India have demonstrated the predominance of the GII genotype and the limited circulation of GI viruses (12). The potential importance of these infections in neonates was highlighted by a recent notification on Promedmail (http://www.promedmail.org/, archive no. 2010521.1689), which reported six norovirus-associated deaths in neonates in Johannesburg, South Africa.

MATERIALS AND METHODS

Study design. The study was carried out at the Christian Medical College, a 2,234-bed tertiary-care hospital in Vellore, southern India, with 60 neonatal beds. A case-control study was carried out with a subset of samples collected for a study on the epidemiology and clinical manifestations of neonatal rotavirus infections (14), in which rotavirus was detected in stool samples using a commercial enzyme immunoassay for the detection of the VP6 antigen (Rotavirus IDEIA; Dako, Ely, United Kingdom). For this study, stool samples from neonates admitted to the nurseries for more than 48 h with symptoms of diarrhea, vomiting, or NEC as well as equal numbers of asymptomatic controls from the same month, wherever available, were screened for NoV GII by reverse transcription-PCR (RT-PCR).

Clinical information. Demographic and clinical information was collected for all neonates enrolled in the study. Information regarding the gestational age, mode of delivery, reason for nursery admission, clinical findings, duration of hospitalization, and progress were collected.

RNA extraction and reverse transcription. Viral RNA was extracted from 20% (wt/vol) fecal suspensions in minimal essential medium (MEM) using the guanidinium isothiocyanate/silica method described by Boom et al. (4). cDNA was generated by reverse transcription in the presence of random primers (hexamers) [Pd(N)6; Pharmacia Biotech, United Kingdom], using 400 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen, Paisley, United Kingdom). The cDNA was stored at -20°C until further testing.

Detection and characterization of norovirus strains. The cDNA was used as the template for the detection of the RNA-dependent RNA polymerase gene (RdRp) of GII NoV using a nested RT-PCR. Published oligonucleotide primers...
TABLE 1. Comparison of demographic characteristics between symptomatic and asymptomatic neonates

<table>
<thead>
<tr>
<th>Variable</th>
<th>Symptomatic (n = 161)</th>
<th>Asymptomatic (n = 148)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preterm</td>
<td>73 (48)</td>
<td>78 (52.7)</td>
<td>0.173</td>
</tr>
<tr>
<td>Term</td>
<td>87 (56)</td>
<td>68 (45.9)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>93 (53)</td>
<td>84 (56.8)</td>
<td>0.858</td>
</tr>
<tr>
<td>Female</td>
<td>68 (52)</td>
<td>64 (43.2)</td>
<td></td>
</tr>
<tr>
<td>Mode of delivery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lower-segment cesarean</td>
<td>67 (41.6)</td>
<td>81 (54.7)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Assisted</td>
<td>13 (8.1)</td>
<td>16 (10.8)</td>
<td></td>
</tr>
<tr>
<td>Normal vaginal</td>
<td>81 (50.3)</td>
<td>51 (34.5)</td>
<td></td>
</tr>
</tbody>
</table>

GR21 (5′ ACC ATT AAT GAG GGA CTA CC 3′) and GR22 (5′ GCT GTC AGT TTC TCT GGG TC 3′) were used to amplify a 203-bp fragment of the NoV GII RNA polymerase gene in the first-round PCR. Primers SR46 (5′ AGT TTC TCT GGG TC 3′) and GR21 (5′ ACC ATT AAT GAG GGA CTA CC 3′) were used to amplify a 126-bp region nested within the first-round PCR product (7). In addition, a 1,524-bp region of the NoV capsid gene was amplified using published primers ORF1/2-F1 (5′ CTG AGC ACG TGG GAG GCC G 3′) and ORF1/2-F2 (5′ AAC ATT AAT GAG GGA CTA CC 3′) for a subset of positive samples (1). Strain characterization was carried out by sequencing the second-round PCR amplicons of the RdRp region and the capsid region for subsets of positive samples.

Sequence analysis. Sequencing of amplicons was carried out using an ABI Prism Big Dye Terminator cycle sequencing ready reaction kit (Applied Biosystems). The sequences were resolved in an automated DNA sequencer (ABI Prism 310 genetic analyzer; Applied Biosystems), and electropherograms were analyzed using sequencing analysis software (Finch TV, version 1.4.0). Multiple alignments and phylogenetic analysis of the nucleotide sequences were performed using the BioEdit software package (version 7.0.5.3). Dendrograms were constructed using the MEGA program (version 4.0), and genetic lineages were inferred by the neighbor-joining algorithm using 1,000 pseudoreplicates. Strains were characterized on the basis of >90% homology at the nucleotide level with published sequences from strains within a given genotype available in GenBank.

Statistical analysis. The data were analyzed using STATA 10.0 (STATACorp, TX, 1984-2009). Differences in predictor variables between the symptomatic and asymptomatic neonates were compared using χ² test or Fisher’s exact test for categorical variables and two-tailed t test for continuous variables.

Nucleotide sequence accession numbers. The RdRp nucleotide sequences from the present study have been deposited in GenBank, and the accession numbers are GU120361 to GU120389.

RESULTS

Stool samples from 161 neonates with GI symptoms, including 70 cases with loose stools, 29 cases with vomiting, and 62 cases with NEC, and 148 asymptomatic controls were screened for GII NoV by nested RT-PCR. Analysis of demographic data showed no significant differences in the gestational ages or the male/female distributions between the two groups of neonates. However, significantly more neonates born by normal vaginal delivery than neonates born by lower-segment cesarean delivery or assisted delivery presented with gastrointestinal symptoms (Table 1).

NoV was detected in 81 (26.2%) of the 309 samples tested in the study. The rate of detection of NoV was significantly higher among the symptomatic neonates (60, 37.2%) than among the asymptomatic neonates (24, 14.1%) (χ² test, P < 0.001). However, analysis of individual clinical presentations showed no significant differences in the proportion of cases with loose stools or NEC among NoV-positive neonates in comparison with that among NoV-negative neonates. In contrast, a significantly higher proportion of NoV-negative neonates (19.8%) than NoV-positive neonates (5.7%) presented with vomiting (P < 0.001).

In this study, coinfection with rotavirus was seen in 55 samples, with 46 of the samples being from symptomatic neonates and 9 being from asymptomatic controls (P < 0.001). There were significantly more cases of loose stools among neonates with rotavirus-norovirus coinfection than among neonates with only norovirus infection (P < 0.05), while there were significantly more cases of NEC among neonates with NoV infection than among those with a rotavirus-norovirus coinfection (P < 0.05) (Table 2). A significant association with vomiting was observed among rotavirus-norovirus-coinfected neonates compared with that among neonates with only norovirus infection (P < 0.05).

Strain characterization. Sequencing of NoV-positive PCR amplicons from the RdRp region was carried out for samples from 14 neonates with NEC and 15 asymptomatic controls. Twenty-seven sequences were identified as belonging to GIIb. Of these, the sequences in 26 samples were closest in identity to GIIb strain sequences (96% to 99%) described from children with diarrhea in western India, while the sequence of 1 sample showed 95% identity to that of a GIIb strain described from Japan. Two other strains, one from a symptomatic neonate and the other from an asymptomatic neonate, were identified as GII genotype 4 (GII.4) and had 96% identity to published sequences from Spain and France (5). To further characterize the virus identified, the capsid region of virus in a subset of six samples identified as GIIb in the RdRp region from both symptomatic and asymptomatic neonates was sequenced, and all viruses were found to belong to the GII.4 genotype (Fig. 1).

DISCUSSION

With increasing evidence for the role of NoV as an important gastrointestinal pathogen in children and recent reports of nosocomial outbreaks and mortality in neonatal nurseries, it is imperative to study the prevalence of NoV infections in neonates. In this study, NoV was detected in stool samples from 26.2% of neonates, and a significantly higher prevalence rate
was seen among symptomatic neonates, indicating an association with gastrointestinal disease. Sequencing of the RdRp region revealed that GIIb with a capsid belonging to the GII.4 genotype was the predominant strain in this population. To the best of our knowledge, this is the first report characterizing NoV strains from symptomatic and asymptomatic neonatal infections, since the recent report from Johannesburg of neonatal deaths did not provide sequence-confirmed data.

The recent reports of NoV infections in neonates has described a wide variety of clinical courses, including bloody stools, vomiting, necrotizing enterocolitis, increased gastric remider, and distended abdomen. Of particular interest is the demonstration of NoV in association with necrotizing enterocolitis. To determine the association of NoV with gastrointestinal symptoms, we chose to study samples from infants presenting with classical NoV symptoms, such as diarrhea and vomiting, as well as recently described symptoms, such as necrotizing enterocolitis. While an overall significant association of NoV infections with gastrointestinal symptoms was seen, a single NoV strain caused 90% of infections, and it appears that coinfection with rotavirus may result in significantly more cases of other types of gastrointestinal disease in this population.

A surprising finding from this study was the coinfection of rotavirus with GIIb strains. It is also important to note that exclusive neonatal infections by GIIb strains have recently been reported to result in mortality in South Africa. In this study, a single NoV strain caused >90% of infections, and coinfection with rotavirus resulted in significantly more cases with gastrointestinal symptoms. Multiple viral infections in a highly susceptible population such as neonates raise interesting questions regarding nursery transmission, susceptibility to infection, and clinical significance. Quantitative studies using real-time RT-PCR may be useful to determine the relative loads of both pathogens among those with a coinfection, and further molecular and immunologic studies will be needed to understand transmission and susceptibility.

FIG. 1. Phylogenetic dendrogram constructed by the neighbor-joining method based on the nucleotide sequence of the gene encoding the capsid region. Bootstrap values for 1,000 pseudoreplicates are shown. Samples from the present study are boxed. The reference sequences (GenBank accession numbers) used are Hawaii virus, 1971/US GII.1 (U07611), Hildingdon/90/UK GII.5 (A2277667), Chestertonfield/434/1997/US GII.2 (AY054300), Melksham/1995/UK (X81879), Snow Mountain/1976/US GII.2 (U70059), Leeds/90/UK GII.7 (A227768), Amsterdam/91-18/1996/NET GII.8 (AF195848), Lordsdale virus/1995/UK GII.4 (X86557), Guangzhou/NVgz01/CHN GII.4 (DQ569797), Oxford/B5S22/2003/UK GII.4 (AY587985), Hunter 284E/90/UK GII.4 (DQ078814), Grimsby/1995/UK GII.4 (AY054300), Cambertwell/101922/94/AUS GII.4 (AF145896), and Bristol/1993/UK GII.4 (X76716).

REFERENCES


