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.

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 guanidium isothiocyanate/silica method described by Boom et al. (4). cDNA was generated by reverse transcription in the presence of random primers (hexamers) [Poly(N); 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 GIINoV using a nested RT-PCR. Published oligonucleotide primers
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’ TCC ATC GCC CAC TGG TCG 3’) and GR12 (5’ AGT TGT CAC GAT CTC ATC ACC 3’) were then used to amplify a 126-bp region nested within the first-round PCR product (7). Additionally, a 1,524-bp region of the NoV capsid was amplified using published primers ORF1/2-F1 (5’ CTG AGC ACG TGG GAG GCC G 3’) and GR12 (5’ AGT TGT CAC GAT CTC ATC ACC 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 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 (STATA Corp. 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.

**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 sequenced, and the virus identified, the capsid region of virus in a subset of six samples with diarrhea in western India, while the sequence of 1 sample showed 95% identity to that of a GIIb strain described from Japan. Twenty-seven sequences were identified as belonging to GIb. Of these, the sequences in 26 samples were closest in identity to GIb 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 GIb strain described from Japan. Two other strains, one from a symptomatic neonate and the other from an asymptomatic neonate, were identified as GI genotype 4 (GIb-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 GIb in the RdRp region from both symptomatic and asymptomatic neonates was sequenced, and all viruses were found to belong to the GIb-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 residue, and distended abdomen. Of particular interest is the presence of strains of a single genotype, GII.4, which have been shown to be the dominant strains in young children (3). These findings raise questions regarding the possible preferential infection of neonates in this region with GIIb strains. It is also important to note that exclusive neonatal infections by a unique rotavirus strain, strain G10P[11], have been reported in the same study population (14), raising further questions regarding the determinants of susceptibility to viral infections in the neonatal period.

This study demonstrated the use of RT-PCR as a reliable tool for the detection of NoV in neonates. Although commercial enzyme immunoassays are available for the detection of NoV in human fecal samples, some recent studies have questioned the validity of these assays for testing neonatal stool samples due to high false-positivity rates and a lack of specificity (8, 21). In this study, a subset of samples identified as NoV positive by RT-PCR was confirmed to be positive by sequence analysis. The rates of detection by RT-PCR were much higher than the rate of 10.8% presented in a previous report of NoVs in full-term neonates with acute gastroenteritis from South Korea, determined using enzyme-linked immunosorbent assay (19).

In summary, the present study demonstrates a high prevalence of NoV infections in neonates, a finding that has recently been reported to result in mortality in South Africa. In this report, 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.

REFERENCES


