Investigation of the Environment and of Mothers in Transmission of Rotavirus Infections in the Neonatal Nursery

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A distinct feature of neonatal rotavirus infection is the association of unusual strains that appear to be prevalent only in neonatal units and persist for long periods of time. The main aims of this study were to determine if rotavirus can be detected on environmental surfaces in the neonatal nursery and whether the infection occurs in mothers of infected and uninfected neonates. Thirty rotavirus positive neonates and an equal number of negative neonates were enrolled in this study. Stool samples from 15 mothers in each group and environmental swabs collected from the bed and surfaces around neonates were tested for rotavirus using single round and nested PCR for the VP6 gene. Rotavirus could be detected in environmental swabs using single round PCR for VP6 gene in 40% of neonates positive for rotavirus antigen by enzyme immunoassay (EIA) and 33.3% of EIA negative neonates. The detection rate was almost 100% using the nested VP6 PCR. Rotavirus was detected in maternal samples only if the nested VP6 PCR was used, with no significant difference between rates of rotavirus detection in maternal fecal samples of infected and uninfected neonates (p-0.4). Sequence analysis of nested VP6 amplicons from two environmental swabs revealed them to be closest in identity to G10P[11], the most common genotype causing infections in neonates in this setting. Interestingly, sequences of amplicons from maternal stool samples did not cluster with G10P[11] or other VP6 subgroup I strains but showed clustering with human strains of VP6 subgroup II.  

KEY WORDS: rotavirus; neonates; nursery; environment

INTRODUCTION
Group A rotaviruses are the most common cause of acute gastroenteritis in infants and young children worldwide. Rotaviruses are double stranded (ds) RNA viruses comprising a genus within the family Reoviridae. The mature virus particles are about 70 nm in diameter and possess a triple layered protein capsid. The genome consists of 11 segments of ds RNA coding for 6 structural viral proteins and 6 nonstructural proteins. Typing schemes for description of rotavirus strains are based on the outer capsid proteins VP7 (G typing) and VP4 (P typing). At least 15 different G types and 27 different P types have been described to date [Estes, 2001; Kapikian et al., 2001; Khamrin et al., 2007]. Studies have shown that rotavirus infections in neonates differ both clinically and epidemiologically from infections in older children. Clinically, rotavirus disease in older children is characterized by severe dehydrating diarrhea, vomiting and fever [Kapikian et al., 2001]. In contrast, neonatal rotavirus infections appear to be predominantly asymptomatic in most settings [Sukumaran et al., 1992; Dunn et al., 1993; Cicirello et al., 1994]. Host features such as physiological immaturity of the neonatal gut, role of maternal antibodies and virulence characteristics of the unique neonatal strains are believed to play a role in the asymptomatic infection seen in neonates [Haffejee, 1991]. Although symptomatic infections in neonates have been described, they may not always present with diarrhea and dehydration but may manifest as bloody mucoid stools, abdominal distension and feed intolerance [Sharma et al., 2002; Iturriza Gomara et al., 2004]. A unique feature of neonatal rotavirus infection is the association of unusual strains that appear to be endemic to neonatal nurseries and maternity wards in some settings [Flores et al., 1988; Vial et al., 1988]. Single rotavirus strains have been shown to persist for long periods of time, contrasting with the strain diversity
that is described in older children [Steele et al., 2002]. The most common VP4 genotype detected in neonatal infections is P[6], seen in combination with G1, G2, G3, G8 or G9 VP7 genotypes [Cicirello et al., 1994; Cunliffe et al., 2002]. In India, neonatal rotavirus infections with G9P[11] and G10P[11] genotypes have been described [Dunn et al., 1993; Cicirello et al., 1994; Iturriza Gomara et al., 2004].

Symptomatic and asymptomatic neonatal rotavirus infection with the G10P[11] strain has been previously described in Vellore [Iturriza Gomara et al., 2004]. This strain has remained the only dominant strain in the neonatal nurseries for almost a decade, supporting other reports of single rotavirus strains circulating in neonatal units for long periods of time [Flores et al., 1988; Steele et al., 2002].

The remarkable stability of a single strain in a specific setting such as a neonatal nursery for such long periods of time is intriguing. Two factors that could play a role in the continued detection of the G10P[11] strain may be (i) persistence of the virus in the nursery environment or (ii) transmission from infected individuals handling the neonates. The aim of this study was to use molecular techniques to assess the role of environmental surfaces and rotavirus infection in primary care givers (mothers) in neonatal rotavirus infections.

MATERIALS AND METHODS

Study Hospital and Setting

The study was carried out in the neonatal nurseries of the Christian Medical College, a 2,200 bed tertiary care hospital in Vellore, southern India with 60 neonatal beds. There are approximately 250 admissions in the neonatal nurseries each month, of whom about 50% stay for longer than 48 hr. The nursery has three levels of care for newborns—I, II, and III, providing early intervention for high risk babies and cardio-respiratory support to very sick newborns. The neonates are moved to different levels or to different beds within each level based on the type of care required. Strict aseptic techniques with emphasis on prevention of infection are followed in the nursery. The nursing staffs are specially trained in handling neonates, with one staff nurse for the care of every 3–5 neonates, based on the level of care required. A 20-bedded area is provided adjacent to the nursery, for mothers of sick babies to stay once discharged from the postnatal wards. The mothers are taught hygienic practices like hand washing before handling the baby and are encouraged to feed the babies themselves, usually once in 2 hr. Babies who cannot be directly breast fed are given expressed breast milk (EBM) by nasogastric tube or using an autoclaved container with spout called “paladai.” The EBM is kept in a sterile ounce glass till just before feeding. Individual paladai are used for each child and a sterile one is taken for every feed.

Strict cleaning procedures are followed in the nursery. The acrylic fiber walls of the cradles are wiped with liquid soap every morning while 7% lysol is used for cleaning the beds. All cradles are cleaned with 7% lysol between babies. All linen is changed every morning and whenever soiled. No invasive procedures or feeding is carried out during cleaning.

Study Design

This study was carried out from February to June 2007. During this period, stool samples were collected from 157 neonates as part of an ongoing study on molecular surveillance for rotavirus disease and strains. Of these, 30 consecutive neonates positive for rotavirus by enzyme immunoassay (EIA) and an equal number of negative neonates who fulfilled the following inclusion criteria were included in this study. A neonate was included if (i) the mother consented to participate in the study and provided a stool sample for testing for rotavirus and (ii) the neonate had not been moved to a different bed in the previous 24 hr before surface swabs were taken. Clinical data such as gestational age, mode of delivery, reason for nursery admission, clinical findings and progress were recorded for all neonates enrolled in the study. The demographic characteristics of neonates in both groups are given in Table I.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Rotavirus EIA –ve (n = 30)</th>
<th>Rotavirus EIA +ve (n = 30)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Male</td>
<td>11</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>19</td>
<td>10</td>
</tr>
<tr>
<td>Gestational age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preterm</td>
<td>23</td>
<td>23</td>
<td>0.8</td>
</tr>
<tr>
<td>Term</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Small for gestational age</td>
<td>22</td>
<td>20</td>
<td>0.8</td>
</tr>
<tr>
<td>Mode of delivery</td>
<td>NVD</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>LSCS</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>Assisted (breech presentation/forceps)</td>
<td>2</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Mean (SD) birth weight in kg</td>
<td>1.78 (0.69)</td>
<td>1.99 (0.72)</td>
<td>0.26</td>
</tr>
<tr>
<td>Median age of neonate at sample collection (IQR)</td>
<td>4 (3–6.75)</td>
<td>4 (3–5.75)</td>
<td>0.4</td>
</tr>
<tr>
<td>Mother’s sample collection—median days post-child birth (IQR)</td>
<td>6 (5–8.75)</td>
<td>6 (5–7.75)</td>
<td>0.5</td>
</tr>
<tr>
<td>Neonates with gastrointestinal symptoms</td>
<td>7</td>
<td>4</td>
<td>0.5</td>
</tr>
<tr>
<td>Number of neonates breast fed</td>
<td>30</td>
<td>30</td>
<td></td>
</tr>
</tbody>
</table>

The study was approved by the Research Committee of the Christian Medical College, Vellore, India.

**Sample Collection**

Three sets of samples were collected for each neonate enrolled in the study—stool sample from neonate, stool sample from neonate’s mother and environmental swabs. The sample collection and testing algorithm is shown in Figure 1.

One stool sample was collected from every neonate enrolled in the study and sent to the laboratory on the same day for testing. All samples were screened for rotavirus using an enzyme immunoassay (EIA) for detection of VP6 antigen (Rota IDEIA, Dako Ltd., Ely, UK) according to the manufacturer’s instructions. The babies were classified into EIA positive and EIA negative groups according to the immunoassay results. Stool samples were then collected from mothers of enrolled neonates, usually within 24–48 hr of enrollment in the study and stored at -70°C till testing.

For the testing of nursery surfaces, swabs were taken from the bedside of all enrolled neonates. The experimental protocol for testing of environmental swabs was standardized using spiking experiments with rotavirus positive stool suspension (data not shown). The methodology for collection and processing of swabs was modified from a published protocol [Gallimore et al., 2006]. Three swabs were collected per child. One sterile cotton swab dipped in minimum essential medium (MEM) was used to sample the acrylic walls of the cradle and two sterile dry cotton swabs were used to swab the bedclothes and clothes in which the neonate was wrapped. All the swabs were then placed in 3 ml MEM and transported to the laboratory. The swabs were then transferred to tubes containing 1 ml L6 buffer containing guanidine thiocyanate and incubated at room temperature for 1 hr. Following incubation, the supernatant was transferred into fresh tubes and stored at -70°C till testing (fraction referred to as GTC).

**RNA Extraction and cDNA Synthesis**

Viral RNA was extracted from 20% fecal suspensions and from 200 μl of both MED and GTC fractions of the environmental swabs using the guanidine thiocyanate and silica based method [Boom et al., 1990]. RNA extraction was carried out using random primers (hexamers; Pd(N)6, Pharmacia Biotech, Amersham, UK) and 400 units of Moloney murine leukemia virus reverse transcriptase (M-MLV RT) (Invitrogen, Life Technologies, Paisley, UK).

**PCR for Detection of Rotavirus**

Rotavirus was detected using a single round PCR for the amplification of a 380 bp region of VP6 gene using the primers VP6-F/VP6-R. In order to improve sensitivity, all samples that were negative in the single round PCR were tested with a nested PCR using primers VP6NF (5′-GAT GCA ACC AGC TTA GAG CTG-3′) and VP6NR (5′-GAT TCA CAA ACT GCA GA-3′). Cycling conditions for the nested PCR were: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 42°C for 30 sec, and extension at 72°C for 30 sec, and a final extension of 72°C for 5 min [Gallimore et al., 2006]. Water blanks and negative controls were included in each experiment to rule out any laboratory contamination. The sensitivity of the single round and nested PCR was compared by testing amplification of 10-fold serial dilutions up to 10^-11 of cDNA from two rotavirus positive stool samples.

**Characterization of Rotavirus Strains**

All neonate stool samples that were positive for the single round VP6 PCR were characterized further by VP7 and VP4 genotyping using published oligonucleotide primers and methods [Gouvea et al., 1990; Cicirello et al., 1994; Iturriza-Gomara et al., 1999, 2004; Banerjee et al., 2006]. Genotyping was also carried out for environmental swabs and maternal stool samples from a subset of...
10 neonates, using specific priming reverse transcription for the VP7 and VP4 genes, since random priming did not generate sufficient cDNA for genotyping.

**Sequencing**

VP6 nested PCR amplicons of environmental swabs from two neonatal stool samples (NES1689 and NES1700) and two maternal samples (NM1693 and NM1700) chosen at random were purified and sequenced with ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). Sequences were resolved in the automated DNA sequencer, the ABI PRISM 310 Genetic Analyzer (Applied Biosystems), and electropherograms were analyzed using the sequencing analysis software (version 1.01, Applied Biosystems). The nucleotide sequences were aligned with VP6 gene sequences of selected rotavirus strains corresponding to different VP6 and VP7 types available in GenBank. Multiple alignments and phylogenetic analysis were performed using the Clustal W and neighbor joining algorithms of the BioEdit software package (version 7.0.5.3). Phylogenetic trees were drawn using Phylodraw (version 0.8) and Tree View (version 1.6.6) programs.

**Statistical Analysis**

The data was analyzed using Epi Info 2002. Chi square, Fisher’s exact tests, Mann–Whitney U-test and Student’s t-test were performed to determine the significance of differences observed between groups.

**RESULTS**

**Rotavirus Infection in Neonates**

A total of 30 rotavirus EIA positive and 30 EIA negative neonates were enrolled in the study. Gastrointestinal symptoms were seen in 18% of the neonates. Clinical presentations include loose stools (3), vomiting (1), feed intolerance (3), and necrotizing enterocolitis (4).

RNA extraction was carried out for samples from all 60 neonates. The cDNA were tested by single round VP6 PCR, and if negative, then tested by the nested VP6 PCR. All EIA positive and 5 samples that were EIA negative were identified as rotavirus positive by the single round VP6 PCR. Of the remaining 25 samples, 18 samples tested positive using the nested VP6 PCR.

Hemi nested multiplex PCR for identification of G and P types was carried out for all samples that were tested positive in the single round PCR. G10P[11] was the most common genotype detected in 28/35 or 80% of the samples. Samples that were not typed as G10P[11] were identified as either G10 with an untypable P type (11.4%) or were untypable (8.6%). No other genotype was identified in the neonate stool samples.

**Rotavirus Detection in Environmental Surface Samples**

A total of 22 samples (36.7%) were positive for rotavirus using single round PCR for VP6 gene. These include 12 samples from rotavirus EIA positive neonates and 10 samples from RV EIA negative neonates. Twenty samples tested positive for rotavirus in the GTC fraction (33.3%) while 9 MED samples were positive (15.0%). Both GTC and MED were positive for 7 samples (11.7%). Of the remaining 38 samples, 36 samples (94.7%) were positive for rotavirus using the nested VP6 PCR. The two negative samples were from rotavirus negative neonates.

**Rotavirus in Maternal Stool Samples**

Samples from mothers of 15 rotavirus EIA positive neonates and 15 rotavirus EIA negative neonates chosen at random were tested for the presence of rotavirus. Single round and nested VP6 PCRs were carried out. None of the mothers’ samples tested positive for VP6 gene in the first round PCR. However, 12 of 15 samples from mothers of rotavirus positive neonates (80%) and 9 of 15 samples from mothers of rotavirus negative neonates (60%) tested positive by the nested VP6 PCR, the difference was not significant (p=0.4).

**Sensitivity of Nested VP6 PCR**

The sensitivity of nested VP6 PCR was assessed using 10-fold serial dilutions of cDNA. The nested VP6 PCR resulted in a 100- to 1,000-fold increase in the limit of detection as shown in Figure 2.

**Sequencing of Viral Strains**

Genotyping of samples from the environment and mothers could not be carried out even after specific priming, possibly due to a low viral load. Nested VP6 amplicons of 2 environmental surface swabs (NES1689 and NES1700) chosen at random were sequenced. Phylogenetic analysis of nucleotide sequence revealed closest identity (97.5%) to I321, a G10P[11] isolate from Bangalore, India as well as other G10P[11] isolates from Vellore (99%) (Fig. 3).

Nested VP6 amplicons from the mother of a rotavirus positive neonate (NM1693) and a rotavirus negative neonate (NM1700) were also sequenced and showed only a low level of identity with I321 or other G10P[11] isolates from Vellore (71% and 72%, respectively). Subsequent phylogenetic analysis of the NM isolates with VP6 gene sequences of selected rotavirus strains corresponding to different VP6 subgroups showed that these clustered with human rotaviruses belonging to subgroup II rather than with VP6 subgroup I strains such as G10P[11] (Fig. 4).

**DISCUSSION**

One of the primary reasons for the persistence of a single rotavirus strain in a neonatal nursery could be their genetic stability, in comparison with high mutation rates in other RNA viruses. In one study, examination of 19 strains from asymptomatic neonates over a 4-year period revealed only 5 nucleotide substitutions in the VP7 gene, none of which led to a
significant amino acid substitution [Flores et al., 1988]. Similarly, studies in South Africa have shown the detection of G4P[6] strains in the neonatal units for a 10-year period [Steele et al., 2002]. In Vellore, G10P[11] rotavirus infection has been described since 1999 [Iturriza Gomara et al., 2004].

Two potential modes of infection in newborns could be transmission of the virus from the environment in a closed setting or infection from individuals handling the neonates. The presence of rotavirus in hospital surfaces has been documented in several studies [Soule et al., 1999; Gallimore et al., 2006]. Studies in other settings have also shown that asymptomatic adults may provide a vehicle for the transmission of rotavirus [Horst and Kohlhase, 1986; Barnes et al., 2003]. The study described in this paper was thus designed to identify if either or both these factors could play a role in the persistence of G10P[11] infections in the nurseries, and indicates that persistent contamination of the environment is likely to a major role in transmission of rotavirus infection.

The environment around at least a third of neonates had sufficient virus to be detected by a single round PCR and almost all samples were positive by nested PCR.

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**Fig. 2.** Sensitivity of nested VP6 PCR in comparison to single round VP6 PCR. The upper panel has amplicons from the nested VP6 PCR while the lower panel has amplicons from single round VP6 PCR. Lane 1: marker, lanes 2–13: dilutions of cDNA from neat to $10^{-11}$.

**Fig. 3.** Phylogenetic dendrogram constructed using neighbor joining method for partial VP6 nucleotide sequence of rotavirus isolates from surface swabs from the nursery and VP6 sequences of rotavirus strains belonging to various G genotypes available in GenBank. The samples are identified by their origin, name and G genotype. NES1889 and NES1700 (●) from surface swabs in neonatal nurseries cluster closest to G10P[11] strains from Vellore and Bangalore. The circle represents other G10P[11] isolates from Vellore (unpublished sequences).
There were no significant differences in the levels of rotavirus detection in the environment surrounding infected and uninfected neonates, indicating widespread contamination in the nursery. That environmental contamination is mainly due to the persistence of a single strain is evidenced by sequence analysis of the VP6 amplicons, showing them to be almost identical to I321 and other G10P11 strains from India, which cause the majority of neonatal infections in southern India.

There have been few reports of rotavirus detection in mothers. Most of these studies were carried out when molecular techniques for characterization of viral strains were not extensively used [Brussieux et al., 1985; Garbag-Chenon et al., 1985]. One study using RT-PCR showed rotavirus detection in 26% of pregnant women [Zhong et al., 1997]. In this study, rotavirus could not be detected in mothers by single round PCR but could be detected in 70% of mothers using the nested VP6 PCR. Sequence analysis showed that rotavirus infection in these mothers was caused by a different VP6 subgroup from that of G10P[11] strains, which are bovine-human reassortant viruses belonging to VP6 subgroup I [Iturriza-Gomara et al., 2004]. There were no significant differences in the levels of rotavirus detection between mothers of infected and uninfected neonates. This, taken together with the differences in strains infecting neonates and their mothers indicates that maternal rotavirus infection may not play a role in transmission of neonatal infections.

The ability of the virus to remain on the environmental surfaces may play a major role in its transmissibility. Based on a 4-year study on the epidemiology and clinical manifestations of rotavirus infection in the neonatal nurseries where rotavirus was detected in 44% of neonates tested, it was hypothesized that G10P[11] rotavirus may be a highly transmissible, but possibly less virulent virus strain than other genotypes, thereby causing widespread infection but limited disease in a susceptible population (unpublished data). In this study, 16.6% of neonate stool samples that were negative for rotavirus by EIA were identified as positive by single round VP6 PCR. This indicates that rotaviral infections in neonatal nurseries may be far higher than previously estimated.

The data shows that there was no difference in rotavirus positivity between mothers and the environment of infected and uninfected neonates. Strains from mothers did not resemble the strains in their children while environmental strains found in the nursery did resemble those found in infected children. This suggests that while mothers play no role in transmitting rotavirus infection to neonates, the environment is a source of infection possibly because of resistance to cleaning measures in use. In addition, given the high level of environmental contamination, the fact that not all neonates were infected supports the possibility of host resistance to infection mediated by maternal antibody, gut receptor expression or other factors.

Standard protocols for cleanliness, sanitation and asepsis defined by the Hospital Infection Control Committee are followed in the hospital. Rigorous cleaning practices are adhered to and considerable importance is placed on prevention and transmission of nosocomial infections. Despite the excellent infection control practices and well trained staff in the neonatology unit, low levels of rotavirus were detected in the nursery environment. This indicates the need for more rigorous cleaning practices and/or use of different disinfection protocols other than those currently in place to be able to eliminate or control rotaviral infections in neonatal nurseries in developing country settings.

REFERENCES
Environmental Role in Neonatal Rotavirus Transmission


