Rotavirus Antigenemia in Indian Children with Rotavirus Gastroenteritis and Asymptomatic Infections

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**Background.** Rotavirus gastroenteritis results in significant morbidity and mortality in Indian children. Although there are numerous studies on rotavirus diarrhea, there are few reports on antigenemia and extraintestinal presentations in these populations.

**Methods.** Following screening for rotavirus antigen of stool samples from children with and without acute gastroenteritis with a commercial enzyme immunoassay (EIA), a total of 199 stool and serum sample pairs were identified for additional testing. All EIA-positive stool samples were genotyped, and viral load estimated by real-time reverse-transcriptase polymerase chain reaction (RT-PCR). Serum samples were tested for rotavirus antigen by an in-house EIA, and antigen was quantified by optical density. Scoring of disease severity was performed for all hospitalized children. Data on extra-intestinal presentations were collected if available.

**Results.** Based on screening of stool samples by EIA, the study population could be divided into 3 groups, including 111 children with rotavirus diarrhea, 44 children with diarrhea and no rotavirus detected in stool specimens, and 44 children with asymptomatic rotavirus infection. Antigenemia was significantly higher among children with rotavirus diarrhea (50.4%) than among children with non-rotaviral diarrhea (16%) or asymptomatic infections (2.3%) \((P < .001)\). Low copies of rotavirus were detected by RT-PCR in all 7 children with EIA-negative stool specimens and antigenemia. Presence and levels of rotavirus antigen in serum specimens correlated with stool viral load. Children with antigenemia had significantly more-severe disease but not more extraintestinal presentations than did children without antigenemia.

**Conclusions.** Antigenemia occurs frequently in rotavirus infection and correlates with virus replication in the gut but not with extra-intestinal presentations.
limited data available on antigenemia and extra-intestinal presentations due to rotavirus in India in children with acute gastroenteritis [3, 8]. Antigenemia in asymptomatic infections has not been previously evaluated.

The primary objective of this study was to compare Indian children with diarrhea and asymptomatic rotavirus infection for antigenemia. Paired stool and serum samples and clinical data were used to correlate stool viral load, disease severity, and extra-intestinal symptoms with antigenemia.

METHODS

Study samples. Samples were chosen retrospectively from hospital- and community-based surveillance studies for rotavirus infection and disease performed at the Christian Medical College (Vellore, India). A total of 199 stool and serum sample pairs were identified. Samples could be divided into 3 study groups. Group I comprised children hospitalized with diarrhea with rotavirus detected in stool specimens ($n = 111$), group II included children hospitalized with diarrhea but with no rotavirus detected in stool ($n = 44$), and group III were children with asymptomatic rotavirus infection identified from a community-based birth cohort ($n = 44$). The samples for group III were collected from a cohort of 373 children recruited at birth and followed up for a period of 3 years to understand the natural history of rotavirus infections and disease. Stool samples from all diarrhoeal episodes, as well as surveillance samples collected every 2 weeks, were screened for rotavirus to identify rotavirus diarrhea and asymptomatic infections, respectively. All serum samples were collected either when rotavirus was identified in stool scenarios or during hospitalization for non-rotavirus gastroenteritis. The settings in which these studies were performed have been described previously [13]. The study was performed in accordance with the ethical standards of the Declaration of Helsinki and was approved by the Institutional Review Board of the Christian Medical College (Vellore, India).

Detection and characterization of rotavirus in stool. Stool samples were tested for rotavirus using a commercial enzyme immunoassay (EIA) for the detection of VP6 antigen (Dako Rota IDEIA) according to the manufacturer’s instructions. Viral RNA was extracted from 20% fecal suspensions of EIA-positive samples using guanidine thiocyanate and silica, as described by Boom et al [14]. Complementary DNA (cDNA) was synthesized using random primers (hexamers; Pd(N)6, Pharmacia Biotech) and 400 U of Moloney murine leukemia virus reverse transcriptase (M-MLV RT) (Invitrogen, Life Technologies). The cDNA was used as the template for genotyping in hemi-nested multiplex polymerase chain reaction (PCR) for VP7 and VP4 genes using published oligonucleotide primers and protocols [15–18].

Real-time reverse-transcriptase PCR (RT-PCR) for estimation of stool viral load. Viral RNA was extracted using 20% weighed fecal suspensions using the same procedure as above for the synthesis of cDNA for use in the quantitative real-time RT-PCR. Real-time RT-PCR for estimation of stool viral load was performed using SYBR green dye and primers VP6-F and VP6-R [19] to amplify a 379-bp region of the VP6 gene in a Chromo-4 real-time system (MJ Research/Bio-Rad). Ten-fold serial dilutions of a standard plasmid containing the VP6 amplicon were included in each run to serve as positive controls, as well as for normalization of PCR runs. PCR reactions were set up in 25-μL volumes with 2 μL of standard plasmid or cDNA. Viral load was estimated in terms of the PCR cycle or crossing point (C_{t} value) at which the amplicon could be detected in the real-time PCR assay. The specificity of the PCR product was measured by melting curve analysis where a single peak was expected at the range of 79°C–81°C. All calculations were made using the Opticon Monitor software, version 3.

Detection of rotavirus antigen in serum. Rotavirus antigen was detected in serum using a protocol modified from Blutt et al [2]. Ninety-six-well microtiter plates were coated with 100 μL of a mouse monoclonal anti-VP6 antibody (kindly provided by Sue Crawford of the Baylor College of Medicine, Houston, Texas). Serum samples were treated at room temperature for 20 min with 0.5 M ethyleneediaminetetraacetic acid and tested undiluted for VP6 antigen. Hyperimmune rabbit anti-rotavirus immunoglobulin G (IgG) was used as a detector antibody, followed by horseradish peroxidase–conjugated goat anti-rabbit IgG (Sigma-Aldrich). The plates were read at 450 nm after addition of tetramethyl benzidine (TMB) substrate. Rotavirus strain WC3 lysate and rotavirus antigen negative serum were included in each assay as positive and negative controls, respectively. Assay cutoff values were calculated as mean optical density (OD) of negative controls plus 3 standard deviations (SDs) from the mean. All samples were tested in duplicate. A sample with a mean OD above the cutoff value was considered to have positive results. Levels of antigenemia were expressed as adjusted mean OD of test sample at 450 nm multiplied by 1000 U after normalization for interassay variability against the mean OD value for the negative control serum sample from all assays.

Collection of clinical data. Detailed clinical information on onset and duration of diarrhea and vomiting, fever, and dehydration were collected for all hospitalized children. The severity of disease was assessed using the 20-point Vesikari scoring system [20]. Data on other clinical presentations apart from gastroenteritis was recorded if available.

Statistical analysis. Proportions were compared between groups using χ² test or Fisher’s exact test, as appropriate. Continuous variables were compared between groups either using
independent 2-sample \( t \) tests or Mann-Whitney \( U \) test. Spearman’s rank correlation was used to assess the relationship between OD and C(t) values. All \( P \) values are 2-sided and were calculated using Stata software (Stata Corp).

RESULTS

Demographic characteristics. A total of 199 children had specimens tested in this study. The mean ages of children hospitalized with and without rotavirus diarrhea were 10.9 and 12.6 months, respectively. Children in both groups required hospitalization for a mean duration of 2.8 days. All asymptomatic infections were identified from surveillance stool samples collected during the follow-up of a community-based birth cohort. Children with asymptomatic rotavirus infection were older, with a mean age of 17.6 months. There was no significant difference in ratio of male to female children between the 3 study groups.

Rotavirus antigenemia in children. Rotavirus VP6 antigen was detected in the serum samples of 56 (50.4%) of 111 children with rotavirus diarrhea, 7 (16%) of 44 diarrheal samples from children with no rotavirus detected in stool by EIA, and 1 (2.3%) of 44 children with an asymptomatic infection. Antigenemia was significantly higher among children with rotavirus diarrhea than it was among children with non-rotaviral diarrhea or asymptomatic infections (\( P < .001 \)). Levels of rotavirus antigenemia in the 3 study groups are shown in Figure 1.

Correlation of antigenemia with stool viral load. Real-time RT-PCR for measurement of stool viral load was performed for 121 children whose stool samples were positive for rotavirus by EIA. This included 79 children hospitalized with rotavirus diarrhea and 42 children with asymptomatic rotavirus infection in the community. In addition, stool samples from 7 children from the non-rotaviral diarrhea group who were antigenemia positive were also tested by RT-PCR. The mean viral load of children with rotavirus diarrhea (C[t], 18.0) was significantly higher than that for children with asymptomatic infection (C[t], 23.8; \( P < .001 \)). Stool samples from all 7 children who were EIA negative but had results that were positive for antigen in serum tested positive for rotavirus by real-time RT-PCR and contained low copies of rotavirus (mean C[t], 28.0).

There was a significant correlation between stool viral load and detection of antigenemia in children. The mean (± SD) stool viral load of children with rotavirus antigenemia was 17.5 ± 7.1 and was significantly higher than the viral load in children whose serum samples were negative for rotavirus (mean C[t] ± SD, 23.2 ± 6.7; \( P < .001 \)). There was a significant negative correlation between C(t) value of the stool samples and the antigenemia level in serum samples (\( r = -0.41; P < .001 \) indicating that higher stool viral load correlates with higher levels of antigen detected in serum. It was interesting to note that, of the 7 children with antigenemia and EIA-negative, RT-PCR–positive stools specimens, 6 samples with very low viral loads (mean C[t], 29.8) had antigenemia levels just above the cutoff value (mean value, 200 U), whereas 1 sample with a higher viral load (C[t], 19.8) had high levels of antigenemia (>2000 U). Testing additional dilutions of serum samples was not required for quantitating antigenemia, because the OD values of >95% of the test samples were well below

Figure 1. Levels of rotavirus antigenemia among the 3 study groups. Serum antigenemia levels were expressed as 1000 times the adjusted mean absorbance value of test sample at 450 nm. The median and interquartile range (IQR) of antigenemia levels are given below each group. The cutoff value was calculated as the mean value of negative controls plus 3 standard deviations from the mean. OD, optical density.
Antigenemia and time of serum collection. Time of serum collection was examined as a factor affecting detection of antigenemia in children. Among children hospitalized with rotavirus diarrhea, rates of antigenemia were higher in serum samples collected within 48 h of hospitalization (52%) than in serum samples collected >48 h after hospitalization (43%; \(P = .5\)). However, correlation of stool viral load with levels of antigenemia was significantly affected by time of serum collection. A significant negative correlation between stool C(t) value and antigenemia level in serum \(r = -0.52; \ P < .001\), Figure 2A) was seen in serum samples collected within 48 h of hospitalization than in serum samples collected >48 h after infection \(r = -0.01; \ P = .68\), Figure 2B).

Severity of disease and systemic illness. Severity of rotavirus gastroenteritis was compared between children with and without antigenemia. Children with antigenemia had more severe disease than did children whose serum samples were negative for rotavirus. The median Vesikari score for children with rotavirus antigenemia was 10 (interquartile range [IQR], 9–11) and was significantly higher than the score of 8 (IQR, 0–10) for children without antigenemia (\(P < .001\)).

Data on extraintestinal presentations, such as respiratory infections and seizures, were available for 83 hospitalized children. Interestingly, the number of children with other clinical symptoms apart from gastroenteritis was lower in children with antigenemia (7 [21%] of 34) than in children without circulating antigen (18 [37%] of 49) \(P = .12\). Lower respiratory tract infections including bronchiolitis and pneumonia were seen in 15 children, including 3 children with antigenemia. Upper respiratory tract infections were seen in 3 children, whereas seizures were described in 7 children, including 2 with rotavirus antigenemia. There was no difference in degree of fever between both groups.

Other factors in rotavirus antigenemia. The age of children and infecting genotype were analyzed to identify other factors that could affect antigenemia in children. Rates of antigenemia did not vary significantly between those with infection due to common rotavirus genotypes G1P[8], G2P[4], and
Table 1. Rotavirus Antigenemia in Children Infected with Different Genotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of cases (n = 118)</th>
<th>No. of cases of rotavirus diarrhea (n = 89)</th>
<th>No. of cases of asymptomatic infection (n = 48)</th>
<th>No. (%) of cases with antigenemia (n = 53)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1P[8]</td>
<td>24</td>
<td>11</td>
<td>13</td>
<td>7 (29)</td>
<td>.30</td>
</tr>
<tr>
<td>G2P[4]</td>
<td>33</td>
<td>23</td>
<td>10</td>
<td>14 (42)</td>
<td>.61</td>
</tr>
<tr>
<td>G9P[8]</td>
<td>15</td>
<td>14</td>
<td>1</td>
<td>8 (53)</td>
<td>.21</td>
</tr>
<tr>
<td>Other genotype*</td>
<td>27</td>
<td>12</td>
<td>15</td>
<td>10 (37)</td>
<td>.84</td>
</tr>
<tr>
<td>Partial typing (G or P typed)</td>
<td>19</td>
<td>19</td>
<td>0</td>
<td>11 (58)</td>
<td>.07</td>
</tr>
<tr>
<td>Mixed infections</td>
<td>19</td>
<td>10</td>
<td>9</td>
<td>3 (16)</td>
<td>.02</td>
</tr>
</tbody>
</table>

* Other genotypes include G10P[11], G10P[8], G12P[6], G12P[8], G1P[4], G1P[6], G2P[8], and G9P[4].

G9P[8], although the number of children with antigenemia was significantly lower among those with infections due to multiple genotypes, compared with among those with infections due to single genotypes (Table 1; P = .02).

Comparison of rates of antigenemia across different age groups showed that antigenemia was seen in 50% of children <1 year of age, 53% of children between 12 and 24 months of age, and 20% of children >2 years of age (Table 2). However, it must be noted that the number of children hospitalized with rotavirus diarrhea >2 years of age was very low. The only case of antigenemia seen among individuals with asymptomatic infections in the community was in a 27-month-old child. There was no history of any diarrhea in the child in the month preceding or in the month after the detection of rotavirus during surveillance.

DISCUSSION

Antigenemia has been described in 43%–75% of children with rotavirus gastroenteritis [2, 4–9, 21]. This study confirms previous reports of the presence of rotavirus antigens in the blood stream of children with rotavirus gastroenteritis, with antigen detected in ~50% of children hospitalized with rotavirus diarrhea and in 1 (2.3%) of 44 children with asymptomatic rotavirus infection.

Stool viral load can be considered as a surrogate measure of virus replication in the gut. We have previously shown that severity of diarrhea correlates with levels of virus excreted in stool [22]. In this study, the presence and levels of rotavirus antigen in serum correlated with stool viral load. Clinically, children with antigenemia presented with more-severe disease. Surprisingly, the proportion of children with extraintestinal presentations was not higher among those with antigenemia, which indicates that escape of the virus into the blood stream may be part of the normal course of infection with rotavirus but may not adversely affect other sites in the body. Although correlation between histopathological and functional changes in the intestine and diarrheal severity have not been clearly established, the detection of antigenemia in children with higher stool viral loads and severe disease raises the possibility of greater damage to the intestinal epithelium leading to entry of virus into the circulatory system.

The time of serum collection after infection has been reported as a critical factor for detection of antigenemia in children [9]. For children in the hospital-based study, the mean time for serum collection after hospitalization was 1.6 days. There was a trend that did not achieve statistical significance in rates of antigenemia between serum collected before and after 48 h. However, correlation of levels of antigenemia with stool viral load was significantly affected by time of serum collection. When samples were collected 48 h after infection, no correlation with stool viral load was seen (Figure 2).

Although the children in the birth cohort were intensively followed up, the mean duration for serum collection was 7 days after identification of rotavirus infection. The longer time interval between infection and serum collection, taken together with the low stool viral load in individuals with asymptomatic infections, could be reasons for the low rates of antigenemia associated with asymptomatic rotavirus infection. Children with asymptomatic infection were also older than children hospitalized with diarrhea. It is possible that older children may have developed greater immunity to the virus and are able to restrict infection to the gut, but in the absence of antibody data, it is not possible to infer whether rotavirus antigenemia occurs more often in children previously unexposed to rotavirus.

Table 2. Age of Rotavirus Infection and Antigenemia

<table>
<thead>
<tr>
<th>Age, months</th>
<th>Rotavirus diarrhea</th>
<th>Asymptomatic infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All cases</td>
<td>Cases with antigenemia</td>
</tr>
<tr>
<td>0–12</td>
<td>76/111 (68)</td>
<td>39/76 (61)</td>
</tr>
<tr>
<td>13–24</td>
<td>30/111 (27)</td>
<td>16/30 (53)</td>
</tr>
<tr>
<td>&gt;24</td>
<td>5/111 (5)</td>
<td>1/5 (20)</td>
</tr>
<tr>
<td>Total</td>
<td>111</td>
<td>56/111 (50)</td>
</tr>
</tbody>
</table>

NOTE. Data are proportion (%) of cases.
Rotavirus antigenemia and viremia have been previously reported in 1 study each from India [3, 8]. In the previous study on antigenemia, only low-to-moderate correlation with stool viral load was seen, and antigenemia was significantly associated with infection with G1 serotype [8]. The findings of the present study differed from the previous report in greater correlation of antigenemia levels with stool viral load and a lack of correlation with any specific rotavirus genotype. Testing for rotavirus viremia was not performed in this study. Detection of viremia has been shown to be directly related to detection of antigen in serum specimens [2]. However, technical difficulties in the culture of human rotaviruses from serum samples, particularly because of the presence of inhibitors in human serum, are also well established [10]. Although RNA can be detected by methods such as RT-PCR, this would not provide confirmatory evidence for the presence of infectious virus in the circulation.

The presence of antigenemia and viremia in rotavirus infection has been established in recent years, resulting in reevaluation of our understanding of the tissue and cellular tropism of the virus [10]. Through a combination of animal models and clinical studies, it is important to continue to elucidate the role of antigenemia and viremia in virus pathogenesis and systemic illness. Even if the proportion of children with systemic complications is low, the substantial burden of rotavirus in India would mean that a large number of children would be affected. These studies are particularly relevant in the current context of introduction of live oral rotavirus vaccines worldwide. Introduction of safe and effective rotavirus vaccines will be of highest benefit in countries, such as India, that have the highest disease burden. It is currently unknown whether any vaccines result in antigenemia, or whether it would be of any consequence. These data illustrate the high rates of antigenemia and their correlation with disease severity and viral load, and they indicate the need for a clear understanding of levels of antigenemia and the extent of extraintestinal presentation during natural infection, disease, and vaccination.

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