Molecular Epidemiology of Rotavirus Diarrhea among Children Aged <5 Years in Nepal: Predominance of Emergent G12 Strains during 2 Years

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Group A rotavirus, a species in the genus Rotavirus within the family Reoviridae, is the single most important etiological agent causing severe diarrhea in infants and young children worldwide [1]. Although rotavirus infects virtually all children by age 3–5 years, the consequence of infection is different in developing and developed countries, because the majority of an estimated 527,000 rotavirus-associated annual deaths occur in developing countries [1]. Currently, live, orally administered rotavirus vaccines are licensed in >100 countries worldwide and are being introduced into the routine childhood immunization schedules of an increasing number of countries [2].

The rotavirus genome consists of 11 segments of double-stranded RNA contained within a triple-layered capsid. The outermost capsid is composed of 2 independent neutralization antigens, VP7 and VP4, which define the G and P serotypes, respectively [3]. Because G and P type-specific immunity is considered to play an important role in protection against disease, the distribution of G and P serotypes of circulating strains

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Emergent G12 Strains in Nepal

Figure 1. Monthly incidence of diarrhea-related hospitalizations in Kanti Children’s Hospital, Kathmandu, Nepal, November 2005–October 2007. The shaded portion of a bar represents cases in which rotavirus was detected, and the unshaded portion represents cases in which rotavirus was not detected. The percentage shown by a small square represents the percentage of rotavirus cases among all diarrhea-related hospitalizations in the designated calendar month.

MATERIALS AND METHODS

Surveillance was conducted at Kanti Children’s Hospital, Kathmandu, Nepal, the largest children’s hospital in the country, with a total of 300 beds, of which 30 were allocated for patients with diarrhea. Informed consent was obtained from the parents of each patient, and the research protocols were reviewed by the ethics committees at the Kanti Children’s Hospital, Kathmandu, Nepal, and at the Liverpool School of Tropical Medicine, Liverpool, United Kingdom.

All children aged <5 years who were admitted with acute gastroenteritis (defined by the passage of ≥3 looser-than-normal stools with or without vomiting during the preceding 24 h) were enrolled after informed consent was obtained from their parents. Demographic data and clinical information were recorded on a standardized questionnaire completed by the study nurse. Major signs and symptoms (diarrhea, vomiting, fever, dehydration, and general level of activity) for each patient were entered. One fecal specimen was collected from each patient, frozen at −20°C and tested once a month by an enzyme-linked immunosorbent assay kit (Rotoclone; Meridian Diagnostics). After transportation of samples to the Department of Medical Microbiology, Liverpool University, molecular characterization into G and P genotypes was performed by reverse-transcription polymerase chain reaction (RT-PCR) as described by Das et al [10] and Gentsch et al [11]. Additionally, genotype G12 was identified by RT-PCR using the forward primer G12F (5′-GTTGTTGTCATGCTGCCAT-3′) and the reverse primer G12R (5′-ATGAATTTTGGTACTGTACT-3′) as described by Pun et al [9].

Rotavirus genomic RNAs were separated on a 10% polyacrylamide gel by electrophoresis for 16 h at a constant current of 8 mA per gel in a Laemmli buffer system using an SE600...
Figure 2. Age distribution of diarrhea-related hospitalizations in Kanti Children’s Hospital, Kathmandu, Nepal, November 2005–October 2007. The shaded portion of a bar represents cases in which rotavirus was detected, and the unshaded portion represents cases in which rotavirus was not detected. The percentage above each bar shows the cumulative percentage of hospitalizations for rotavirus by the end of each of the designated age periods.

Ruby gel apparatus (GE Healthcare Bioscience, formerly Amersham Biosciences) as described elsewhere [12]. A short RNA pattern was defined as a rotavirus genome possessing slower moving 10th and 11th genome segments, whereas a long RNA pattern was defined as a rotavirus genome with faster moving 10th and 11th genome segments [13].

RESULTS

During the 2-year surveillance period from November 2005 through October 2007, a total of 1139 children aged <5 years were admitted to Kanti Children’s Hospital with acute diarrhea. There were a mean of ∼50 diarrhea-related admissions in this hospital each month (Figure 1). Of the 1139 children, 379 (33%) had specimens test positive for rotavirus. In the first year (November 2005–October 2006), 223 (34%) of 666 tested positive, and in the second year (November 2006–October 2007), 156 (33%) of 473 tested positive.

Rotavirus-associated hospitalizations presented a clear seasonal pattern, with more cases occurring in the cool, dry season than in the warm, rainy season, although rotavirus cases could be detected in the off-season (Figure 1). The age distribution of rotavirus-positive patients indicated that only 2.6% of cases occurred in the first 3 months of life. Although 85% of all rotavirus-associated hospitalizations among children aged <5 years occurred between 3 and 24 months of age, the cumulative incidence at the end of the first year of life reached only 55% (Figure 2).

Genotyping by RT-PCR successfully assigned 91% of 379 rotavirus-positive specimens into G and P types, leaving 9.2% of specimens untypeable for either G or P type (6.3%) or both (2.9%). The most prevalent G type during the study period was G12 (158 strains [42%]), accounting for 50% of all rotavirus-positive specimens in 2005–2006 and 29% in 2006–2007 (Table 1), followed by G1 (26%) in 2005–2006 and G9 (28%) in 2006–2007. The relative frequency of G2 increased from 12% in 2005–2006 to 20% in 2006–2007. A far greater increase was observed for G9, whose relative frequency of 7% in 2005–2006 increased to 28% in 2006–2007, a 4-fold increase in percentage (Table 1).

The most prevalent P type was P[8] (158 strains [42%]), followed by P[6] (134 [35%]) and P[4] (59 [16%]). Although this order did not change between the 2 seasons, the difference in the relative frequencies of each P type was greater in 2006–2007 than in 2005–2006 (Table 2). When G and P types were combined, genotype G12P[6] was the most prevalent, accounting for 34% in 2005–2006 and 24% in 2006–2007 (Table 3). This was followed by G1P[8] (23%) in 2005–2006 and G2P[4] (20%) and G9P[8] (17%) in 2006–2007 (Table 3). The prevalence of genotype G9 strains, which were mostly G9P[6], increased from 7% in 2005–2006 to 28% in 2006–2007. Similarly, the detection rate of genotype G2 increased from 12% in 2005–2006 to 20% in 2006–2007. Thus, the predominance of G12 over other genotypes in the second year was less marked than in the first year (Table 1).

When the relationships between the RNA patterns and the major combinations of G and P genotypes were examined, it was found that strains carrying G12P[6] and G12P[8], as well as G9P[6] and G9P[8], exclusively possessed long RNA patterns and that strains carrying G2P[4] exclusively possessed short RNA patterns. In addition, there were a few unusual combinations of G and P genotypes, including 2 G9P[4] strains possessing long RNA patterns and 2 G9P[4] strains possessing a short RNA pattern. Furthermore, there was 1 G2P[8] strain with a long RNA pattern and 2 G2P[6] strains, 1 of which had a long RNA pattern. The other G2P[6] strain, however, showed no identifiable RNA pattern.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>2005–2006&lt;sup&gt;a&lt;/sup&gt;</th>
<th>2006–2007&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 223)</td>
<td>(n = 156)</td>
</tr>
<tr>
<td>G1</td>
<td>57 (26)</td>
<td>21 (13)</td>
</tr>
<tr>
<td>G2</td>
<td>26 (12)</td>
<td>31 (20)</td>
</tr>
<tr>
<td>G3</td>
<td>4 (2)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>G9</td>
<td>15 (7)</td>
<td>43 (28)</td>
</tr>
<tr>
<td>G12</td>
<td>112 (50)</td>
<td>46 (29)</td>
</tr>
<tr>
<td>Nontypeable</td>
<td>9 (4)</td>
<td>14 (9)</td>
</tr>
</tbody>
</table>

<sup>a</sup> November 2005–October 2006.<br><sup>b</sup> November 2006–October 2007.
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Table 2. Distribution of P Types among Rotavirus-Positive Specimens Collected from Hospitalized Children Aged <5 Years, Kathmandu, Nepal, 2005–2007

<table>
<thead>
<tr>
<th>Genotype</th>
<th>2005–2006a (n = 223)</th>
<th>2006–2007b (n = 156)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P[4]</td>
<td>22 (10)</td>
<td>37 (24)</td>
</tr>
<tr>
<td>P[6]</td>
<td>83 (37)</td>
<td>51 (33)</td>
</tr>
<tr>
<td>P[8]</td>
<td>104 (47)</td>
<td>54 (35)</td>
</tr>
<tr>
<td>Mixed P types</td>
<td>4 (2)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Nontypeable</td>
<td>10 (4)</td>
<td>13 (8)</td>
</tr>
</tbody>
</table>


### DISCUSSION

The characteristics of rotavirus diarrhea among children aged <5 years in Nepal included marked seasonal variation, with peaks occurring in the cooler and drier months of the year (Figure 1). By contrast, hospitalizations for diarrhea due to causes other than rotavirus showed no such distinct seasonality (Figure 1). As to the age distribution of patients, only 55% of rotavirus-associated hospitalizations occurred among children in the first year of life in Nepal, an unusual finding in poor countries, where children with rotavirus diarrhea tend to be hospitalized at younger ages than children in higher-income countries. For example, ~80% of rotavirus-related hospitalizations involve children aged <1 year in India and Myanmar, whereas only 30%–40% of hospitalizations involve that age group in Korea, Hong Kong, Taiwan, and Japan [8]. Whether there are a large number of rotavirus-associated hospitalizations early in life has an important implication regarding the impact of rotavirus vaccine. Because the first vaccine dose should be administered to infants aged 6–12 weeks [14], the full vaccination schedule must be finished before or shortly after age 6 months [14], and the protective efficacy of incomplete vaccination is not established, infants are considered to be less well protected by the vaccine if they are exposed to rotavirus infection before they finish the vaccination schedule. Therefore, the smaller the number of children who experience rotavirus gastroenteritis early in their life, the more advantageous the vaccine. Thus, the fact that only 2.6% of rotavirus infections occurred in the first 3 months of age predicts a higher impact of the vaccine in Nepal.

Although this study is a continuation of 2 preceding studies in the same hospital to describe the distribution of G and P types of rotavirus strains circulating in Nepal [7, 9], there are 3 important observations that merit particular mention. First, this study identified, for the first time, G12 strains as the most prevalent genotype, accounting for one-half of the circulating rotavirus strains detected in one of the year-long survey periods. Furthermore, frequent detection of G12 rotavirus strains was an observation consistent with the preceding studies, whereas detection of other G genotypes changed more markedly over time (Figure 3). As to the relative frequency of G types throughout the 2003–2007 study periods, G12 was the highest, with a weighted average of 33%, followed by G1, with a weighted average of 27%. This observation provided further evidence of the notion that G12 strains should no longer be considered unusual or rare strains but that they exhibit a capacity to spread among children just like human rotavirus strains of other G types. Among the G12 strains identified, G12 was in combination with P[6] in 76% of strains, with P[8] in 24% of strains, and with P[4] rarely (1 strain). The dominance of G12P[6] over G12P[8] strains was consistent over the 4-year observation period that included 2 preceding studies, with a ratio of 1.4:1 in 2003–2004 and 7.6:1 in 2006–2007 (overall ratio for the four 4-year period, 2.9:1). Thus, multiple strains of G12P[6], G12P[8], and G12P[4] circulated during the 4-year period from September 2003 through October 2007, confirming and extending the previous hypothesis that extensive genomic diversity exists among G12 strains in Nepal, which was predicted from the observation that at least 5 electropherotypes of G12 strains were detected in a single year [7]. However, it was not possible to detect any definite diversification of G12 strains during the 4-year period, which leaves unanswered the question of whether the identification of G12 strains represented a true recent emergence or, alternatively, the recognition of an established serotype.

Second, despite the overall predominance of G12 strains, there were a total of 13 different G and P genotype combinations observed during the 2-year study period, of which G12P[6], G1P[8], G2P[4], and G9P[8] together accounted for...
~75% of the rotavirus-positive specimens (Table 3). Although the relative frequency of each of the major G and P combinations of rotavirus strains circulating in Nepal varied in the 2 studies that immediately preceded the current study, G12P[6], G12P[8], G1P[8], G2P[4], and G9P[6] rotavirus were all detected, suggesting that there existed a large genetic pool of rotavirus genotypes in this country.

Third, a G4 strain was not detected in this study or in 2 preceding studies. Similarly, there were very few G3 strains in this study, accounting for only 1% of rotavirus strains detected. This observation was consistent with the results from the preceding studies, in which 0% and 6% of rotaviruses detected were type G3 in the periods 2003–2004 and 2004–2005, respectively [7, 9]. The scarcity of G3 strains in Nepal may be contrasted with results of other studies in China and Japan, where there were dramatic increases in G3 strains from 2000 to 2006 [15, 16].

Although the initial identification of the previously novel genotype G12 was traced back to the detection of a G12P[4] strain with a long RNA pattern (strain L26) in the Philippines in 1987 [17], global spread of G12 strains, particularly in combination with either P[8] or P[6], occurred during the past decade, and the spread of G12 appears to have accelerated further in recent years [18–23]. Given the number of G12 strains detected and their genetic diversity in terms of electrophoretotypes, nucleotide sequences, and the association with a variety of P types, the introduction of G12 strains in the Ganges River region of the Indian subcontinent is speculated to be much earlier than that in other geographic regions, such as Saudi Arabia, where only a single electropherotype was identified among G12P[8] strains [24].

Of rotavirus-positive specimens, 6% remained untypeable for G type, 6% for P type, and 9% for both G type and P type. Possible explanations include too few virus particles with intact RNA in the stool specimens, the viruses not being recognized by the primer sets, and the viruses not belonging to genotypes included in the primer set.

In conclusion, the results obtained in this study disclosed an unusually high prevalence of G12 rotavirus strains in Nepal and also underscore the importance of closely monitoring the long-term trend of the distribution of genotypes circulating in any county where rotavirus vaccine introduction is being considered.

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