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The distribution of VP7 (G-) and VP4 (P-) genotypes among 126 rotavirus strains from South Indian children, < 5 years of age and with acute diarrhoea, presenting to a single hospital during the months to November and December, from 1995 to 1998, was studied. Multiplex hemi-nested G- and P-typing polymerase chain reactions determined 101 (80%) G types and 78 (61%) P types, respectively. In order of frequency, the commonest G types were G1, G4, G2, G9, G3, and G8, and P types were P1B[4], P1A[8], and P2A[6] and the most common G:P combinations were G1:P1A[8], G1:P1B[4], G2P1B[4] and G4:P1A[8]. G1, G2, and G4 types were seen in all years. The single G3 isolate was seen in 1998. The single G8 isolate and the 5 G9 isolates were seen in 1997, after a period of heavy rain. Sequence analysis showed that the G8 isolate was related most closely to the bovine strain A5, and the G9 strains were distinct from the nonpathogenic Indian isolate 116E and similar to G9s isolated in Mysore and the United Kingdom described previously. J. Med. Virol. 67:101–105, 2002. © 2002 Wiley-Liss, Inc.

KEY WORDS: rotavirus; genotypes; India

INTRODUCTION

Rotaviruses are the major etiologic agents of gastroenteritis in infants and children worldwide. Rotaviruses are classified into seven different sero-groups, based on the antigenic specificity of the capsid proteins in the virus as well as on the pattern of electrophoretic mobility of the 11 RNA segments of the viral genome [Estes, 1996]. The inner capsid protein VP6 comprises the greatest mass of the particle and this is the protein used to detect and distinguish between viruses of different groups. Of the seven sero-groups, only groups A, B, and C are known to infect humans, and group A viruses are those that cause severe, life-threatening disease in children [Estes, 1996]. For group A viruses, further typing schemes were introduced based on antigenic epitopes on the proteins that form the inner capsid (VP6, subgroups I and II) and the outer capsid, the glycoprotein VP7 (G serotypes), and the protease-sensitive spike protein VP4 (P serotypes) [Hoshino and Kapikian, 1996]. In recent years, reverse transcription-polymerase chain reaction (RT-PCR) has been used in molecular epidemiological studies in both developed and developing countries [Gentsch et al., 1996; Ramachandran et al., 1996; Fruhwirth et al., 2000; Iturriza-Gomara et al., 2000a,b].

It has been recognised that appropriate surveillance of rotavirus strains circulating in the community is an absolute prerequisite before vaccine introduction, to monitor the prevalence of co-circulating rotaviruses and to detect the emergence of new rotavirus strains or reassortants. After the introduction of RT-PCR, this technique has been used in a number of studies to identify G types; studies have shown that G types other than G1–4 are present in certain parts of the world. G5 and G10 have been reported from Brazil, while G8 formed the predominant strains in Malawi in 1997–1998 [Santos et al., 1998; Cunliffe et al., 1999]. G9 strains have been reported from North India and Bangladesh [Ramachandran et al., 1996, Unicomb et al., 1999]. This study reports the use of RT-PCR and electropherotyping to study the circulation of rotaviruses in South India during four consecutive winter seasons.

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MATERIALS AND METHODS

Study Area

This study was carried out during the winter seasons of 1995–1996 to 1998–1999, at the Christian Medical College and Hospital, Vellore, which serves an urban population of 300,000 and a rural population of 100,000. Vellore is situated in South India, 130 km from the coast and has a tropical climate with winter rains due to the northeast monsoon. Meteorological data were obtained from the Regional Meteorological Centre, Chennai.

Specimen Collection

Previous analysis of paediatric hospital records for a 10-year period, 1985–1995, showed that the numbers of children with diarrhoea seen at the hospital was greatest during the months of November and December. Samples were collected prospectively during November and December for 4 consecutive years, 1995–1998. A total of 602 faecal samples obtained from children < 5 years of age, presenting to the hospital with acute diarrhoea, were submitted to the gastrointestinal microbiology laboratory for identification of enteric pathogens. Of these, 126 were positive for rotavirus by electron microscopy, latex agglutination (Meritec, Meridian Diagnostics, Cincinnati, OH), or enzyme-linked immunosorbent assay (ELISA) (IDEIA, Dakopatts, Copenhagen). Clinical details of these patients were obtained by examination of clinical records after identification of rotavirus. An assessment of dehydration was made according to standard methods (Behrman et al., 2000).

Genotyping

Genotyping of the rotaviral isolates was carried out as described previously [Iturriza-Gomara et al., 1999]. Briefly, viral RNA was extracted from 200 μl of the 10% faecal suspensions according to the method of Boom et al. [1991] and eluted in 50 μl of RNase-free sterile distilled water containing 40 U of ribonuclease inhibitor (RNasin, Promega, Madison, WI). Complementary DNA (cDNA) was generated from 20 μl of the extracted RNA by reverse transcription (RT) in the presence of random primers (hexamers; PdN6, Pharmacia Biotech), using 200 U of M-MuL V reserve transcriptase (FPLC-pure, cloned M-MuL V; Life Technologies, Gaithersburg, MD). The resulting cDNA was the template for both VP7- and VP4-specific typing PCR, using the oligonucleotide primers described by Gouveia et al. [1990] and Gentsch et al. [1992], respectively. Primers Beg9/End9 were used for the first-round VP7-PCRs to amplify the entire VP7 gene. The second-round G-typing PCR included primers specific for G1, G2, G3, and G4, G8, and G9 genotypes [Iturriza-Gomara et al., 1999]. Primers Con2/Con3 were used for the first round PCRs to amplify a 876-bp fragment of the VP4 gene. The second-round P-typing PCR included primers specific for genotypes P[8], P[4], P[9], and P[10] and the consensus primer Con3 [Iturriza-Gomara et al., 1999].

Electropherotyping

RNA extraction and electropherotyping was carried out as described previously for all strains that were negative in the G- and P-typing PCR and for unusual strains (Brown et al., 1988).

Sequencing

A subset of the VP7 and VP4 amplicons were sequenced using an automated sequencer. The first round PCR amplicons were purified using a commercial spin column method (QIAquick PCR purification kit, Qiagen), cloned into the TA vector (TopoTA kit, Invitrogen). Transformation into bacterial cells and growth on agar plates were performed according to manufacturer’s instructions. For sequencing, approximately 75 ng of purified DNA was sequenced in both directions with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin–Elmer Applied Biosystems, Foster City, CA). Extension products of the sequencing reaction were purified by ethanol precipitation to remove excess dye terminators. Sequence data was imported into the DNASTar package (DNASTar Ltd, London, UK) for alignment using the MegAlign program. The nucleotide sequence for the VP7 gene of the G8 strain has been deposited in GenBank and assigned the accession number AF 317424.

RESULTS

Sample Collection and Clinical Features

A total of 126 rotaviral strains were obtained from 602 children with diarrhoea during the winter seasons of 1995–1996 to 1998–1999 (year 1–20, year 2–41, year 3–42, year 4–23). Rotaviruses were identified in 18–24% of samples examined each year, but in the first year of the study, and in November and December, fewer samples were received. Examination of hospital records showed that in 1995, the number of children presenting with diarrhoea was similar to that in the 5 preceding years but all did not have samples sent to the laboratory. However, in the 1998–1999 winter season, the peak incidence of paediatric diarrhoea occurred in January and February, and not in November and December, as in previous years (data not shown).

The age and sex distribution and the clinical symptoms of the 126 patients with rotaviral diarrhoea are shown in Table I. The mean age of children with rotaviral diarrhoea was 19.5 months (range 3 to 60 months, standard deviation 13.6), the median for the number of stools was 5 (range 3–11). Dehydration was seen only in children < 2 years of age. Clinical grading of dehydration was mild in eight children, moderate in five children, and severe in two children. Five children required intravenous fluid replacement; the others were treated with oral rehydration therapy. There was no mortality. There was no correlation between
the genotype of the rotavirus isolated and the age of patients seen, the number of stools, or the degree of dehydration.

### Genotyping

Of 126 rotaviral strains, 101 (80%) could be genotyped in the G-typing PCR (Table II). Of these, mixed infection were seen in 10 patients, with dual infections with G1,G4, G2,G4, G1,G9, and G4,G9. Mixed infections were seen in five patients in 1997, and the remaining five in other years. In all years, G1 strains were seen most frequently, comprising 39.6% of all strains (range 29–46%) in the 4 years of the study. G4 strains were 23.8% of the total (range 13–27%). G2 strains formed 19% of the total, responsible for 9–22% of rotaviral infections. The five G9 strains and the single G8 strain were seen in December 1997, after Vellore had received heavy rainfall in November (247.9 mm, range 16.3–247.9 mm between 1994 and 1999, average 122.1 mm). The single G3 strain was identified in December 1998.

The commonest G:P combinations seen were G2P1B[4], G1:P1A[8], G4:P1A[8], and G1:P1B[4]. A large proportion of G1 (20/50, 40%) and G4 (15/30, 50%) strains were not P-typable by RT-PCR (Table III).

### Sequencing

Representative strains from each G and P type, and all infrequent strains (G9, G8, G3, and P2A[6]), were sequenced. Sequence analysis showed that the G9 strains were 98% similar at the nucleotide level and 99% similar at the amino acid level to strains obtained from Mysore in 1998–1999 [Iturriza-Gomara et al., 2000a]. The single G8 strain, 99-19774, was 96% identical at the nucleotide level and 97% identical at the amino acid level to the bovine strain A5 [Taniguchi et al., 1991]. This strain appeared to be related more closely to A5 than to MP409, the only other human G8 strain reported from India, to which it had 90% similarity at the nucleotide level and 96% similarity at the amino acid level [Jagannath et al., 2000].

### Electropherotyping

In 14/126 (11%) samples positive originally for rotavirus by the assays used, no amplification was seen with either G- or P-typing PCR. The RNA from these samples was re-extracted and the electropherotype patterns determined. In eight strains, the electropherotypes were short; in one strain, it was long. RNA was not obtained on re-extraction of the remaining six samples, possibly due to initial false-positive results in the screening tests for rotavirus, or loss during storage of the original specimen for 6 months at 4°C. The electropherotypes of five G1 strains, five G4 strains, and the single G3 strain were long. The RNA patterns of three of the five G2s were short, while two had long electropherotypes. The RNA patterns from the five G9 and the single G8 strain were all short.

### DISCUSSION

Of 126 children brought to hospital with diarrhea, 60% were male (Table I). This is not statistically significant, although there is a perception that male children who are ill are more likely to be brought for medical attention. Only 9 (7%) children had diarrhea of > 5 days duration. In this study, 73 (58%) of the children with acute diarrhea were < 2 years old, but all children with clinical dehydration were in this age group (Table I). This is similar to findings in Venezuela, where 86% of dehydration occurred in children < 2 years of age [Perez-Schael et al., 1999]. Children aged < 2 years also had a significantly greater number of stools than occurred in older children \( (P = 0.05) \) Table I.

Our study examined the patterns of G and P distribution over the rotavirus “high season” for a period of 4 years and found that there are distinct shifts from year to year, which may be associated with climatic changes. Overall, the patterns of rotaviral circulation in Vellore appear to be different from those described from other parts of India. A previous study on children with acute diarrhea from Delhi, identified G1P1A[8] and G2P1B[4] as the predominant types [Husain et al., 1996]. While these were seen in our study, G4P1A[8] were as frequent. This is similar to previous data from Vellore during the early 1980s [Brown et al., 1988].

Some unusual strains were obtained during the course of this study. G1P1B[4] has been previously described as an infrequent strain from India [Husain et al., 1996] and other countries [Bon et al., 1999] but

| Age (mo) | n | M | F | Duration of | No. of | Dehydration |
|---------|---|---|---| diarrhea (days) | stools/ | <5 | ≥5 |
| < 6 | 12 | 6 | 6 | 5 | 6 | 1 | 2 | 10 | 3 |
| 6–12 | 33 | 21 | 12 | 16 | 15 | 2 | 2 | 31 | 8 |
| 13–24 | 28 | 16 | 12 | 9 | 19 | 0 | 4 | 24 | 4 |
| 25–36 | 23 | 16 | 7 | 6 | 15 | 2 | 12 | 11 | 0 |
| 37–48 | 18 | 8 | 10 | 3 | 12 | 3 | 12 | 6 | 0 |
| 49–60 | 12 | 8 | 4 | 6 | 5 | 1 | 9 | 3 | 0 |
| Total | 126 | 75 | 51 | 45 | 72 | 9 | 41 | 85 | 15 |

M, male; F, female.
was found to occur in all 4 years in Vellore and to be one of the commonest G:P combinations detected. These comprised 44% of the P-typed G1 strains and 11% of the total rotavirus strains obtained. This is similar to a recent report from Argentina, where this combination was seen in 14% of isolates [Arguelles et al., 2000]. It is possible that these strains may have arisen as a result of reassortment among human strains.

The deduced amino acid sequence of the G8 strain was most similar to the bovine isolate A5 from Thailand [Taniguchi et al., 1991]. Another G8 strain has been recently described from India. This isolate, MP409, has the P type 6[1], a long electropherotype and subgroup 1 specificity; it has been proposed that it represents an example of interspecies transmission [Jagannath et al., 2000]. Our strain, 99-19774, had a short electropherotype and was a P1A[8], and thus differed from the G8 strains isolated in Malawi, which were P2A[6] [Cunliffe et al., 1999]. The VP7 gene of this virus was cloned and resembled A5 more closely than MP409 (nt similarity of 96% and 90%, respectively). It is possible that this virus arose as a reassortant between human and bovine strains. Antibodies to G8 and other bovine-related G types have been described earlier from Pune in central India [Kelkar et al., 1996]. Bovine-related G10 strains from asymptomatic neonates have also been described from Bangalore [Dunn et al., 1993]. The five G9 sequences were 98% similar at the nucleotide level to 1998 isolates of G9 from Mysore [Iturriza-Gomara et al., 2000a], although these were isolated 1 year earlier. G9 rotaviruses have not been reported previously from South India, although, in northern India, both G9P[6] and G9P[11] strains have been reported in symptomatic and asymptomatic infections [Das et al., 1994; Ramachandran et al., 1996]; of these 116E, a G9P[11] strain has been proposed as a vaccine candidate. However, the G9 strains isolated in this study were similar to those isolated from Mysore in 1998 [Iturriza-Gomara et al., 2000a] and distinct from 116E. With the apparent emergence of G9 strains in different parts of the world during the 1990s [Ramachandran et al., 1996; Unicomb et al., 1999; Cubitt et al., 2000], a retrospective surveillance of collections isolated earlier than 1990 is required, to determine whether this is due to radical changes in rotavirus circulation during the past decade, or to the use of more discriminating molecular tools to type viral isolates.

It is interesting to note that six mixed infections, the G8 and G9 strains were seen in a single season, after a period of unusually heavy rainfall. Previous studies in this town have shown contamination of water supplies and subsequent outbreaks of infectious enteric disease after such climatic conditions [Ramakrishna et al., 1996]. The possibility of contamination by animal faeces as a mode of transmission cannot be ruled out. In nine strains, neither G nor P types could be determined by the primers used, although RNA was seen in silver-stained gels. This raises the possibility of other unusual strains of group A circulating in this area. Since a previous study identified four group C-related viral isolates from Vellore [Brown et al., 1988] and group B rotavirus has been reported from Calcutta [Sen et al., 2000], from which a large proportion of the patient population at Vellore is derived, it is possible that other groups of rotavirus may also be circulating in this area. The inability to type these strains could also be due to an accumulation of point mutations at common G- and P-type specific primer binding sites (Iturriza-Gomara et al., 2000b).

The isolation of unusual combinations of genotypes and electropherotypes provides evidence for the formation of reassortant strains, which could potentially infect a naive population. G2 strains with long RNA patterns, proposed to be reassortants, have been previously reported from Asia and Africa [Ahmed et al., 1989, Krishnan et al., 1994, Nakata et al., 1999]. Even with the more classical strains, variations in circulation require constant monitoring in order to form an epidemiological basis for formulating appropriate

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**TABLE II. Rotaviral G Types Identified by RT-PCR During Four Consecutive Winters in South India**

<table>
<thead>
<tr>
<th>Period</th>
<th>n</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>0</th>
<th>0</th>
<th>0</th>
<th>Mixed</th>
<th>G not typed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1995–1996</td>
<td>20</td>
<td>9</td>
<td>4</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>10</td>
<td>4</td>
<td>20%</td>
</tr>
<tr>
<td>1996–1997</td>
<td>41</td>
<td>12</td>
<td>9</td>
<td>0</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>6</td>
<td>14%</td>
</tr>
<tr>
<td>1997–1998</td>
<td>42</td>
<td>18</td>
<td>9</td>
<td>0</td>
<td>11</td>
<td>1</td>
<td>5</td>
<td>6</td>
<td>4</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>1998–1999</td>
<td>23</td>
<td>11</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>7</td>
<td>30%</td>
</tr>
<tr>
<td>Total</td>
<td>126</td>
<td>50</td>
<td>24</td>
<td>1</td>
<td>30</td>
<td>1</td>
<td>5</td>
<td>10</td>
<td>8</td>
<td>25</td>
<td>20%</td>
</tr>
</tbody>
</table>

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**TABLE III. Genotyping of Rotaviruses From South Indian Children With Acute Diarrhoea**

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td>G1</td>
<td>15</td>
<td>14</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>G2</td>
<td>0</td>
<td>16</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>G3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G4</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G8</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G9</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Uncertain&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7</td>
<td>2</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>39</td>
<td>32</td>
<td>7</td>
<td>48</td>
</tr>
</tbody>
</table>

<sup>a</sup>Polymerase chain reaction product detected only in first round of amplification.

<sup>b</sup>G genotype not obtained.
vaccines. The results of this study illustrate the complex epidemiology of rotavirus infections, where the diversity of circulating strains may be affected by climatic changes and environmental conditions. Further molecular epidemiological studies in developing countries could elucidate the mechanisms of intra- and interspecies transmission, as well as the identification of reassortant viruses, data that are essential to formulate control measures to prevent the spread and occurrence of rotavirus disease.

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