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Molecular Heterogeneity of Human Group A Rotavirus in Rural Bangladesh as Determined by Electrophoresis of Genomic Ribonucleic Acid

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Abstract: Genomic diversity of 248 rotavirus strains from stools collected from patients that sought treatment for diarrhoea in Matlab, Bangladesh, between July 1987 and May 1989, was investigated by analysis of migration patterns of viral genomic ribonucleic acid (RNA) segments by electrophoresis. One hundred and seventy-three gave an electropherotype; 90 were of long electropherotypes ("L") and 83 were short electropherotypes ("S") and after co-electrophoresis, 10 different "L" electropherotypes and 12 different "S" electropherotypes were found. Among "L" electropherotypes, the majority (32 of 90) were identified as L1 followed by L8 (21 of 90) and among "S" electropherotypes, the majority (28 of 83) were S2 followed by S3 (14 of 83). L1 was also found most commonly throughout the study period (12/23 months) as was S2 (11/23 months).

Key words: Rotavirus, Diarrhoea, Ribonucleic acid, Electropherotype, Epidemiology

INTRODUCTION

Human group A rotaviruses (HRV) are recognized as the most important aetiologial agents of acute gastroenteritis among infants and children less than 5 years of age (Cukor and Blacklow, 1984). Since the discovery of rotavirus (RV), epidemiological studies have supported the importance of production of an efficacious vaccine (Flores et al, 1989). Field trials of candidate RV vaccines (Edelman, 1987., Lanata et al, 1989) emphasize the need to study the epidemiology of rotavirus strains in order to formulate an effective RV vaccine. A large number of epidemiological studies have been based on variations in the pattern of RV double stranded RNA (dsRNA) genome segments in polyacrylamide gel electrophoresis (PAGE) (Espejo et al, 1980., Rodger et al, 1981., Brown et al, 1988) and on variation of serotypes.
either determined by monoclonal antibodies (Coulson et al., 1987., Taniguchi et al., 1987) or serotype specific oligonucleotide probes (Sethabutr et al., 1990., Bingnan et al., 1991).

Analysis of the genome pattern by PAGE is relatively easy and rapid. Therefore this technique has become a useful and popular procedure for detection and differentiation of HRV strains in epidemics, mixed infections, of molecular epidemiology studies as well as for detection of atypical rotaviruses (Sethi et al., 1988). Numerous studies have shown that, within a community, a particular electropherotype can be predominant (Rodger et al., 1981; Estes et al., 1984) and different electropherotypes can be predominant in closely separated communities (Unicomb and Bishop, 1989). Although electropherotyping does not permit identification of serotypes and subgroups, it is generally accepted that “Short” electropherotypes belong to serotypes 2 and subgroup 1 (Kalica et al., 1981; Thouless et al., 1982). It is also reported that different electropherotypes can be found among strains of the same serotype and strains with the same electropherotype can be of different serotypes (Gerna et al., 1987).

This study aimed to differentiate HRV strains by electropherotyping in order to study their distribution in a rural area of Bangladesh over a two-year period.

**MATERIALS AND METHODS**

**Specimens**

A total of 248 stool specimens containing RV identified by an ELISA test (Dakopatts, Denmark) were collected from a subset of patients seeking treatment for acute diarrhoea between July 1987 and May 1989 at the Diarrhoeal Treatment Centre in Matlab, a rural area of Bangladesh. The serotyping results from these rotavirus samples is published elsewhere (Bingnan et al., 1991).

**Detection of group A rotavirus (RV)**

A 10% (w/v) phosphate buffered saline (PBS), pH 7.2, stool extract was prepared from thawed stool specimens and tested for the presence of group A rotavirus using a commercial ELISA kit (Dakopatts, Denmark) according to the manufacturer’s instructions. Briefly, 96-well microtiter plates (Nunc, Denmark) were coated with anti-human rotavirus sera or negative control sera (normal rabbit serum), both prepared in rabbit, at a dilution of 1/50 in 0.05 M carbonate buffer (pH 9.6). Following 1 h incubation at room temperature (RT), the plates were washed with PBS with 0.05% Tween-20 (PBST). Each stool extract was added to two wells, one coated with antisera and the other with negative control sera and incubated at RT for 1 h then washed with PBST. Horseradish peroxidase (HRP) conjugated anti-trotavirus antibody diluted at 1/250 in PBST-2% bovine serum albumin (BSA) was added to wells and incubated for 1 h at RT. Substrate (tetramethyl benzidine, TMB) was added to all wells and incubated for 10 min at RT, then 1 M sulphuric acid was added and absorbance was read in a spectrophotometer (Titertek, Multiskan) at 450 nm. A sample was considered positive if the $A_{450}$ of positive well was 0.1 absorbance units greater than $A_{450}$ in the negative well.
Polyacrylamide gel electrophoresis (PAGE) of viral RNA

Viral RNA was extracted according to the methods of Herring et al. (1982). Briefly, 450 μl of 10% (w/v) PBS-stool extract was mixed with 50 μl of 1 M sodium acetate buffer containing 1% sodium dodecyl sulphate (SDS), pH 5.0, and 500 μl of phenol: chloroform: isoamyl alcohol (25:24:1), vortexed for 1 min and centrifuged at 10,000 rpm for 4 min at RT. The RNA containing upper phase was extracted further with an equal volume of phenol-chloroform-isoamyl alcohol.

The extracted RNA was analysed by PAGE following the methods of Laemmli (1970) using 10%, 0.75 mm thick polyacrylamide slab gels with 3% stacker gels and using the discontinuous buffer system without SDS. A 60 μl aliquot of viral RNA and sample buffer was loaded onto gels and electrophoresis was performed at 100V for 16 h at RT. After electrophoresis, the gels were stained with silver nitrate as described by Herring et al. (1982).

Co-electrophoresis was performed in order to distinguish subtle differences in migration of viral dsRNA bands. Equal volumes of RNA from different stool samples were mixed and electrophoresed with the separate RNA containing specimens in flanking lanes of the mixture.

RESULTS

Isolates showing slight differences in genome segment migration were coelectrophoresed (Fig. 3) before their identity was established. After extensive comparative analysis of isolates by co-electrophoresis of viral RNAs, 22 different electropherotypes were established of which 10 belonged to “L” which were designated as L1 through L10 (Fig. 1) and 12 belonged to “S” which were designated as S1 through S12 (Fig. 2).

Results of electrophoresis of dsRNA of 248 RV ELISA positive faecal specimens are shown in table 1. RNA patterns were detected in 173 (69.76%) of the 248 specimens; 90 (52.02%) were identified as “L” and 83 (47.98%) as “S” electropherotypes. Seventy-five specimens did not display any PAGE patterns or displayed very faint bands. Of the “L” and “S” electropherotypes, L1 and S2 were identified as predominant types (Fig 4). Of the “L” electropherotypes, 1 specimen had an extra band located between segments 8 and 9 and was identified as L4 (Fig. 1).

The 22 electropherotypes found were examined using the classification scheme proposed by Lourenco et al. (1981) which divides the 11 RNA segments into four groups: RNA group I includes gene segments 1-4; group II includes gene segments 5 and 6; group III includes gene segments 7-9 and group IV includes gene segments 10 and 11. In both “L” and “S” electropherotypes, considerable variations in gene segments found in all the four RNA groups (Fig. 3). Variations were observed in RNA group I and III in “L” electropherotypes but there was almost no variation in RNA group II among different “L” electropherotypes (Table 2). However, electropherotic variations were observed in all the RNA groups of “S” electropherotypes (Table 3).
Fig. 1. Ten different electropherotypes identified among 90 long ("L") patterns, designated as L1 through L10. Two L3 (lane L3) and 2 L6 (lane L6) electropherotypes were found to be identical by co-electrophoresis. Genomic segments are indicated on the left of the figure. SA11 represent the control "L" electropherotype.

Fig. 2. Twelve different electropherotypes found among 83 short ("S") patterns, designated S1 through S12. Genomic segments are indicated on the left of the figure. SA11 represent the control "L" electropherotype.
Fig. 3. Demonstration of long ("L") (Fig. 3A) and short ("S") (Fig. 3B) electropherotypes by co-electrophoresis of similar and different types. Samples having similar electropherotypes were run singly and in mixtures. Genomic segments are indicated on the left of the figure. SA11 represent the control "L" electropherotype. The electropherotype or electropherotype mixtures are indicated at the top of the figure.
Table 1. Frequencies of detection of PAGE patterns from 173 PAGE positive strains
from patients seeking treatment for diarrhoea in Matlab, Bangladesh

<table>
<thead>
<tr>
<th>Pattern code</th>
<th>No. +ve</th>
<th>Pattern code</th>
<th>No. +ve</th>
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<tbody>
<tr>
<td>L1</td>
<td>32</td>
<td>S1</td>
<td>12</td>
</tr>
<tr>
<td>L2</td>
<td>3</td>
<td>S2</td>
<td>28</td>
</tr>
<tr>
<td>L3</td>
<td>11</td>
<td>S3</td>
<td>14</td>
</tr>
<tr>
<td>L4</td>
<td>2*</td>
<td>S4</td>
<td>7</td>
</tr>
<tr>
<td>L5</td>
<td>9</td>
<td>S5</td>
<td>3</td>
</tr>
<tr>
<td>L6</td>
<td>3</td>
<td>S6</td>
<td>6</td>
</tr>
<tr>
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<td>2</td>
<td>S7</td>
<td>2</td>
</tr>
<tr>
<td>L8</td>
<td>21</td>
<td>S8</td>
<td>3</td>
</tr>
<tr>
<td>L9</td>
<td>4</td>
<td>S9</td>
<td>2</td>
</tr>
<tr>
<td>L10</td>
<td>3</td>
<td>S10</td>
<td>1</td>
</tr>
<tr>
<td>L11</td>
<td>3</td>
<td>S11</td>
<td></td>
</tr>
<tr>
<td>L12</td>
<td>2</td>
<td>S12</td>
<td>75</td>
</tr>
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</table>

*One had an extra band

Fig. 4. Occurrence of rotavirus "L" and "S" electropherotypes from July 1987 to May 1989.

Numbers at the bottom represent the number of samples PAGE tested/number of samples positive for rotavirus for each month.
Table 2. Segment variations among different RNA groups of 10 “L” electropherotypes as determined by
coelectrophoresis of genomic RNA

<table>
<thead>
<tr>
<th>RNA segment group*</th>
<th>L7+4</th>
<th>L4+10</th>
<th>L10+9</th>
<th>L9+8</th>
<th>L8+6</th>
<th>L6+5</th>
<th>L5+3</th>
<th>L3+2</th>
<th>L2+1</th>
<th>Total no. of variations</th>
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<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>8/9</td>
</tr>
<tr>
<td>Group II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>1/9</td>
</tr>
<tr>
<td>Group III</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>7/9</td>
</tr>
<tr>
<td>Group IV</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3/9</td>
</tr>
</tbody>
</table>

*Grouping of RNA segments according to Lourenco et al. (1981)

Table 3. Segment variations among different RNA groups of 12 “S” electropherotypes as determined by
coelectrophoresis of genomic RNA

<table>
<thead>
<tr>
<th>RNA segment group*</th>
<th>S1+2</th>
<th>S2+3</th>
<th>S3+4</th>
<th>S4+6</th>
<th>S6+7</th>
<th>S7+8</th>
<th>S8+10</th>
<th>S10+11</th>
<th>S11+12</th>
<th>S12+5</th>
<th>S5+9</th>
<th>Total no. of variations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7/11</td>
<td></td>
</tr>
<tr>
<td>Group II</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>6/11</td>
<td></td>
</tr>
<tr>
<td>Group III</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>4/11</td>
<td></td>
</tr>
<tr>
<td>Group IV</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>6/11</td>
<td></td>
</tr>
</tbody>
</table>

*Grouping of RNA segments according to Lourenco et al. (1981)

Seasonal distribution of electropherotypes

Samples were examined from each month from 1987 to 1989 with the exception of October, 1987. “L” and “S” electropherotypes were found to co-circulate (Fig. 4) and L1 was the predominant long type (present for 12 of 23 months). Patterns L1, L3, L5 and L9 were found intermittently throughout the study period, L8 was found after May 1988, whereas the remaining “L” patterns were found in low numbers. Among “S” electropherotype, S2 was the predominant type (present for 11 of 23 months) and S1 and S2 were found throughout the study period. S4 and S6 were found in 1988 only and S3 was found after May 1988. The remaining “S” patterns were found in small numbers (Fig. 4).


DISCUSSION

This study did not attempt to study RV prevalence but to examine the variation of RV strains by selecting a subset of RV positive samples. Reports from different parts of the
world have indicated electropherotyping as a potential tool for studying the molecular epidemiology of HRV infections (Espejo et al., 1980; Dimitrov et al., 1984).

In our study, over a 23 month period, 22 different electropherotypes were found. Limited data from Bangladesh are available (Haider et al., 1985) involving 40 rotavirus positive samples from which 6 different electropherotypes were identified without performing co-electrophoresis. We analysed 248 faecal specimens by PAGE and observed typical electrophoretic patterns of RV RNA in 173 samples. This rate of detection (69.75%) is similar to that found by other investigators (Brown et al., 1988, Sethi et al., 1988, Unicomb and Bishop, 1989). All HRV isolates analysed in our study had either “L” or “S” electropherotype pattern as determined by the relative mobility of gene segments 10 and 11 by PAGE. Seventy-five (30.2%) specimens were ELISA positive but did not display any RNA pattern in PAGE due to insufficient intact RNA in the specimens. Segment variations have been found among different RNA groups of “L” and “S” electropherotypes. Among different “L” electropherotypes, variations occurred mostly in group I and III RNA segments, whereas variations were found in all four RNA groups among different “S” electropherotypes. The mechanisms of generating extensive genomic diversity among HRV strains are not known but it is thought that genetic reassortment can occur (Matsuno et al., 1988; Ward et al., 1988), as well as genetic rearrangement either naturally or due to imposition of pressure by the host immune system (Biryahwaho et al., 1987).

In the present study, two electropherotypes, L1 and S2, were predominant and were found throughout the study period along with L3, L5, L9 and S1. Numerous other patterns were present for limited times with both “L” and “S” electropherotypes co-circulating. The observation that certain electropherotypes are persistent during the study period with several less common types occurring at varying intervals (Fig. 4) indicates a constantly changing population of rotaviruses in the community of Matlab area of Bangladesh. The results presented here confirm and extend those reported by Rodger et al. (1981) and Dimitrov et al. (1984) but contrast with the result of Haider et al. (1985) from Bangladesh who detected “S” electropherotypes only in one year of three year study period examining only 40 strains.

In this study, a subset of samples from Matlab area was analysed and found that both “L” and “S” electropherotypes were present almost in equal numbers. Since the “S” and “L” electropherotypes correlate with subgroup 1 and 2 rotavirus (Kalica et al., 1981; Thouless et al., 1982), it can be concluded that both subgroups 1 and 2 are present at all times.

One specimen with an L4 electropherotype showed an extra band between segments 8 and 9. When this strain was grown in a monkey cell line, it failed to produce the extra band and showed only 11 bands (data not shown). The reason for such an extra band may be the presence of a subset of RVs with the band migrating at a different rate due to a secondary structure being eliminated or created.

The epidemiology of RV infection is better understood through serotype determination; however, electropherotyping is helpful in identifying and differentiating HRV strains, detection of mixed infections, tracing of atypical strains as well as monitoring the distribution and spread of RV infection in the community of developing countries like Bangladesh.
ACKNOWLEDGEMENTS

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