Rotavirus G Serotypes and P[4],G Genotypes Identified in Cases of Reinfection Among Children Participating in a Trial with Rhesus-human Reassortant Tetravalent Vaccine (RRV-TV) in Belém, Brazil

by Joana D’Arc P. Mascarenhas, a José Paulo G. Leite, b Yvone B. Gabbay, a Ronald B. Freitas, a Consuelo S. Oliveira, a Taíta A. F. Monteiro, a and Alexandre C. Linhares a

a Instituto Evandro Chagas, Fundação Nacional de Saúde, Belém, Pará, Brazil
b Departamento de Virologia, Instituto Oswaldo Cruz, Rio de Janeiro, RJ, Brazil

Introduction

Group A rotaviruses are the most important agents of severe diarrhea in children and infants worldwide.1 Diarrhea due to rotavirus infection has been associated with classic symptoms, such as diarrhea, vomiting, moderate/high fever and often dehydration. The infections by these agents, may also occur asymptomatically. The number of infections and diarrheal episodes a child experiences is related to the intensity of exposure and to the immune response.2,3

Summary

Group A rotaviruses are the most important agents of severe diarrhea in children and infants worldwide. The aim of present study was to identify rotavirus G serotypes and P[4],G genotypes in cases of reinfection among children who participated in a vaccine trial with the tetravalent rhesus-human reassortant rotavirus vaccine (RRV-TV 4 × 10⁴ pfu/dose) in Belém, Brazil. From July 1990 to June 1992, 540 children received, at their first, third and fifth months of life, oral doses of either vaccine or placebo. A total of 90 rotavirus diarrheal episodes among children who completed the three-dose vaccination schedule were recorded. We studied 11 reinfection rotavirus cases among five children (three female and two male). Fecal specimens were tested by using a enzyme immunoassay (IDEIA™ Rotavirus), followed by EIA with monoclonal antibodies to determine infecting serotypes G1, G2, G3 and G4 and subgroups I and II. The viral dsRNA was extracted and electrophoresed through polyacrylamide gel and then subjected to reverse-transcription-polymerase chain reaction and nested-PCR for the determination of G1, G2, G3, G4, G5 and G9 and P[4], P[6], P[8] and P[9] rotavirus genotypes. A total of 11 cases of reinfection (12 per cent) occurred among five children, three from the placebo group and two from the vaccine group. In four of the cases of reinfection G serotypes and P[4],G genotypes were as follows: for the first and second infections, respectively: (1) G2/P[4],G2 and G1/P[4],G1; (2) G2/P[4],G2 and G2/P[6],G5; (3) G2/P[4],G2 and G1/P[8],G1; and (4) G2/P[8],G1 and G1/P[8],G1. A fifth child had three successive infections caused by serotypes/genotypes G1/P[8],G1, in the first and second infections, and G2/P[4],G2 in the third infection. The common genotypes and unusual genotypes were detected in 8 (73 per cent) and 3 (27 per cent) of the isolates, respectively. With regards to the clinical severity, in two children a score indicated moderate/severe disease in both first and second infections. One child had three successive infections; the first episode was moderate/severe, the second very severe and the third was not available. In contrast, in two other children, the first episode was very severe, and the second episode was moderate/severe in one child and data was not available for the other child. The results obtained in the present investigation underscore the need to broaden our knowledge of the immunity in rotavirus reinfection. This should be useful regarding future rotavirus vaccination strategies in Brazil.

Acknowledgements

This work was supported by OMS, IEC, CGLAB/MS, IOC/FIOCRUZ, and CNPq. The authors wish to thank Dr Jon Gentsch and Dr Roger Glass, members of the WHO/PAHO Rotavirus Collaborating Centre in the Viral Gastroenteritis Section of the Centers for Disease Control and Prevention, for providing specific primers and oligonucleotide probes. We thank our field staff and Mr António de Moura who make this study possible. Correspondence: Dr Joana D’Arc Pereira Mascarenhas, Instituto Evandro Chagas, Fundação Nacional de Saúde (FUNASA), MS, Av. Almirante Barroso, 492, 66000-000, Belém, Brazil. Tel. 00 55 91 214 2106; Fax 00 55 91 214 2005. E-mail <joanamascarenhas@iec.pa.gov.br>.
Several studies indicate that previous infection by rotavirus do not protect against reinfection. Kim et al. have demonstrated that adults with antibodies may often develop asymptomatic reinfections. On the other hand, neonates infected during their first weeks of life seem to be protected against diarrhea associated with rotavirus. Nevertheless, protection against reinfection has not been demonstrated.

In infants and young children, the neutralizing antibodies are directed primarily against the serotype of the infecting rotavirus strain (homotypic response), and develop after primary infection. Some virus serotypes may be more prone to induce heterotypic responses and this will depend on the particular antigenic characteristics of the infecting rotavirus strains, regardless of its serotype.

Studies conducted in Belém by Linhares, et al., Freitas, et al. and Oliveira, et al. have shown cases of reinfection among children involving only rotavirus G serotypes. In addition, Arias, et al. studied the immune response to rotavirus in children in Belém, Brazil with serologically defined primary infection and reinfection, and correlated with the G serotype of the infecting virus. In the present work we characterized cases of reinfection by rotavirus G serotypes and [P],G genotypes among children who were part of a tetravalent rhesus-human reassortant rotavirus (RRV-TV) or placebo study carried out in Belém, Brazil.

Patients and Methods

A total of 90 rotavirus diarrheal episodes were recorded during a vaccine trial with a reassortant tetravalent rotavirus (RRV-TV 4 × 10^9 pfu/dose) in Belém, Brazil. In this study, 540 children received, at their first, third and fifth months of life, oral doses of vaccine or placebo and were followed for 2 years to determine the vaccine efficacy.

In the present investigation we studied 11 cases of rotavirus infection among five children (three female and two male) who were reininfected throughout the study.

The severity of each episode of diarrhea was evaluated using a modified 20-point scoring system (CS), as proposed by Flores, et al. The parameters evaluated were: duration and frequency of diarrhea and vomiting, temperature, dehydration, and need of treatment. A score of 0–8 was defined as indicating mild disease, a score of 9–14 moderate-to-severe disease, and a score of more than 14, very severe disease.

Enzyme immunoassays to detect rotavirus group A and for G serotyping and subgrouping

All stool specimens were tested using a commercial enzyme immunoassay (IDEIA™). Rotavirus code No. K6020, Copenhagen, Denmark) for group A rotavirus antigen, following the manufacturer’s recommendations.

The positive samples were tested by EIA using monoclonal antibodies to determine the subgroups (I, II) and serotypes (G1, G2, G3, and G4), essentially as described by Taniguchi, et al. These monoclonals were kindly provided by Dr Shozo Urasawa, Department of Hygiene and Epidemiology, Sapporo Medical College, Sapporo, Japan.

Viral dsRNA was extracted, as described previously, followed by polyacrylamide gel electrophoresis (PAGE) of dsRNA, using a buffer system, as described by Pereira, et al. The gel was stained by silver nitrate, and the electrophoretic pattern was stained as described by Herring, et al.

Reverse transcription-polymerase chain reaction and nested PCR for P and G typing

The reverse transcription-polymerase chain reaction (RT-PCR) was carried out as originally described by Gouveia, et al. and Gentsch, et al. with modifications introduced by Leite, et al. using a mixture of consensus primers 9con1/9con2 (G genotype) or 4con3/4con2 (P genotype). The nested PCR was performed using a mixture of specific primers to G genotyping as follows: G1, G2, G3, G4 and G5 and G9; or for P genotyping, P[8], P[4], P[6] and P[9]. All amplicons were subsequently subjected to electrophoresis on 1 per cent agarose gel in TBE buffer containing ethidium bromide (0.5 µg/ml).

Southern hybridization and chemiluminescent detection

Southern hybridization and chemiluminescent detection with genotype-specific products was performed according to the protocols and reagents recommended by Boehringer Mannheim Corp as described by Leite, et al. for G genotypes and Ramachandran, et al. for P genotypes.

Results

Subgroups, electropherotypes, G serotypes, and P,[G] genotypes of rotavirus group A

A total of 90 diarrhoeic stool specimens were positive for group A rotavirus (Linhares, et al. 1996), including 11 samples (12 per cent), from five children who developed reinfection. Three cases involved placebo recipients, and two vaccine recipients only. Four cases included children with two successive rotavirus infections (cases B, C, D and E), and one, with three consecutive infections (Case A) (Table 1).

It was possible to determine the subgroups in 7/11 (64 per cent) isolates. Among the four cases with two successive infections, results were as follows: for the first and second infections, respectively: (1) I/II
It was possible to determine the electropherotypes, G serotypes and P[,G genotypes in all isolates. In the four cases of two infections, short and long electropherotypes were noted for the first and second infections, respectively. The child with three infections excreted rotaviruses with long electropherotypes in the first and second infections, and a short pattern could be observed during the third infection.

G serotypes and P[,G genotypes were characterized as follows: case (A) G1/P[8],G1 specificities in the first and second infections and G2/P[4],G2 in the third infection; (B) G2/P[4],G2 and G1/P[4],G1; (C) G2/P[4],G2 and G2/P[6],G5; (D) G2/P[4],G2 and G1/P[8],G1; and (E) G2/P[8],G1 and G1/P[8],G1 (Table 1, Figs 1 and 2).

The G and P genotypes were confirmed by Southern hybridization, with specific probes by genotypes G1, G2, G5 and P[4], P[6] and P[8] of rotavirus (data not shown).

The common and unusual genotypes were detected in 8/11 (73 per cent) and 3/11 (27 per cent) of isolates, respectively. The common genotypes more often detected were serotypes and genotypes G1/P[8],G1 and G2/P[4],G2. However, unusual serotypes/genotypes such as G1/P[4],G1, G2/P[6],G5 and G2/I/Short/P[8],G1 were detected in the second infections involving two children in the placebo group (cases B and C) and in one case in the first infection in the vaccine group (case E). The G5 genotype was characterized on a molecular basis (nested-PCR and

### Table 1: Characterization of subgroups, electropherotypes, G serotypes and P[,G genotypes of group A rotavirus in reinfection cases

<table>
<thead>
<tr>
<th>Case</th>
<th>Infection</th>
<th>Age in months</th>
<th>Sex</th>
<th>Group</th>
<th>Subgroup</th>
<th>Electropherotype</th>
<th>G serotype</th>
<th>P[,G genotype</th>
<th>Clinical severity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>First</td>
<td>4</td>
<td>F</td>
<td>Placebo</td>
<td>II</td>
<td>Long</td>
<td>G1</td>
<td>P[8],G1</td>
<td>Moderate/severe</td>
</tr>
<tr>
<td></td>
<td>Second</td>
<td>11</td>
<td></td>
<td>Placebo</td>
<td>II</td>
<td>Long</td>
<td>G1</td>
<td>P[8],G1</td>
<td>Very severe</td>
</tr>
<tr>
<td></td>
<td>Third</td>
<td>23</td>
<td></td>
<td>Placebo</td>
<td></td>
<td>Short</td>
<td>G2</td>
<td>P[4],G2</td>
<td>NA</td>
</tr>
<tr>
<td>B</td>
<td>First</td>
<td>3</td>
<td>F</td>
<td>Placebo</td>
<td>I</td>
<td>Short</td>
<td>G2</td>
<td>P[4],G2</td>
<td>Very severe</td>
</tr>
<tr>
<td></td>
<td>Second</td>
<td>16</td>
<td></td>
<td>Placebo</td>
<td>II</td>
<td>Long</td>
<td>G1</td>
<td>P[4],G1</td>
<td>NA</td>
</tr>
<tr>
<td>C</td>
<td>First</td>
<td>21</td>
<td>F</td>
<td>Placebo</td>
<td>b</td>
<td>Short</td>
<td>G2</td>
<td>P[4],G2</td>
<td>Moderate/severe</td>
</tr>
<tr>
<td></td>
<td>Second</td>
<td>24</td>
<td></td>
<td>Placebo</td>
<td></td>
<td>Long</td>
<td>G2</td>
<td>P[6],G5</td>
<td>Moderate/severe</td>
</tr>
<tr>
<td>D</td>
<td>First</td>
<td>8</td>
<td>M</td>
<td>Vaccine</td>
<td>I</td>
<td>Short</td>
<td>G2</td>
<td>P[4],G2</td>
<td>Very severe</td>
</tr>
<tr>
<td></td>
<td>Second</td>
<td>21</td>
<td></td>
<td>Vaccine</td>
<td></td>
<td>Long</td>
<td>G1</td>
<td>P[8],G1</td>
<td>Moderate/severe</td>
</tr>
<tr>
<td>E</td>
<td>First</td>
<td>8</td>
<td>M</td>
<td>Vaccine</td>
<td>I</td>
<td>Short</td>
<td>G2</td>
<td>P[8],G1</td>
<td>Moderate/severe</td>
</tr>
<tr>
<td></td>
<td>Second</td>
<td>13</td>
<td></td>
<td>Vaccine</td>
<td>II</td>
<td>Long</td>
<td>G1</td>
<td>P[8],G1</td>
<td>Moderate/severe</td>
</tr>
</tbody>
</table>

* Non-reactive (lack of reaction with any of the subgroups).
* Multiple reaction (multiple reaction with both subgroups).

NA, not available.

![FIG. 1. Nested-PCR to determine rotavirus G genotypes. Lanes: MW, 123 pb molecular weight marker (GIBCO-BRL Laboratories Gaithersburg, MD); 5 and 6, genotype G1; 7 and 8, genotype G2; 9, negative control of Nested-PCR.](image1)

![FIG. 2. Nested-PCR to determine rotavirus P genotypes. Lanes: MW, 123 pb molecular weight marker (GIBCO-BRL Laboratories Gaithersburg, MD); 1 and 2, genotype P[6]; 3, genotype P[4]; 4, genotype P[8]; 5, negative control of Nested-PCR.](image2)
Clinical and epidemiological aspects of reinfections

Clinical severity was determined in 9/11 (82 per cent) of cases. Cases ranged from moderate/severe to very severe disease. In two children moderate/severe was identified in both first and second infections (cases C and E). In case D, very severe disease was noted in the first episode and moderate/severe diarrhea in the second episode. In case A, the first episode was moderate/severe, the second very severe and the third episode could not be characterized. In cases B and D the first episode was very severe and the second episode was moderate/severe in case D and was not available in case B.

The first infections clustered within the first 4 months of age and reinfections were observed from 11 months onwards. The smallest interval between two infections in the same child was 3 months (case C, placebo group) and the largest was 13 months (cases B and D, vaccine group).

Discussion

Sequential infections by rotavirus involving the G homologous G1, P[8] and P[4] suggests that homotypic protection can be incomplete. In the present study of five cases of reinfection, two involved the homologous P[8],G1 (cases A and E) and, in one, the P[4] (case B), was involved. Velázquez, et al.22 identified reinfection by rotavirus in 56 per cent of cases based on viral excretion involving asymptomatic and symptomatic cases. These results are in contrast with the present study, where reinfections were noted in 12 per cent of the situations, i.e. in a four-fold lower rate. Arias, et al.8 observed both homotypic and heterotypic patterns of response in primary infection. Overall, 92 per cent of the 25 children studied developed neutralizing antibodies to the serotype G1, strain Wa, in the primary infection regardless of the serotype of the infecting virus. On the other hand, we tested only for the presence of the infecting strain, when the majority of the reinfections were caused by heterologous P[G] genotypes, suggesting that protection is mostly serotype-specific.

As already demonstrated, the infection does not elicit complete protective immunity, but subsequent infections tend to be less severe. Velázquez, et al.22 have shown that natural rotavirus infection confers protection against subsequent infections. This protection increases with each new infection and reduces the severity of the diarrhea. In the present study, in two children the severity of diarrhea was the same in both first and second infections. One child had three successive infections; the first episode was moderate/severe, the second very severe, and the third was not available. In contrast, in two other children, the first episode was very severe and the second was moderate/severe in one child and data was not available for the other child.

Bishop,23 on the other hand, reported that severe symptoms during reinfection are associated with P genotype differing from that of primary infection. Our study shows that in three situations P genotype differed from that of first infection (cases A, C and D). In case C the clinical severity was moderate/severe in both infections. The case A was very severe in the second infection but data were not available for the third infection. The case D was very severe during the first episode and moderate/severe in the later episode.

With regards to the serotypes, studies report a partial protection with occurrence of reinfections involving either the same or different rotavirus serotypes, suggesting a short-term immunity against the disease.8,15,25 Previous studies conducted in Belém by Linhares, et al.,9 Freitas, et al.26 and Oliveira, et al.11 also demonstrated the occurrence of successive infections caused by either the same or different G serotypes. In the present investigation only one case was caused by the same G and P genotypes. The four other cases were caused by different G or P type.

Mascarenhas, et al.,25 in a reanalysis of 54 rotavirus-positive stool samples obtained during a longitudinal study conducted in Belém, Brazil identified two cases of reinfection involving P genotypes of rotavirus as P[8+4]G1. In the present study no case of reinfection involving mixed P genotypes was identified.

The occurrence of G5 genotype was already recorded in Belém, Brazil, involving four children, three of them in the placebo group and one in the vaccine group.26 One of these children was included in the present evaluation (case C). This child had rotavirus diarrhea by genotype P[4],G2 at 21 months of life, followed by another infection at 24 months of age by genotype P[6],G5. This genotype is unusual, not only because of the detection of G5, but also for its association with P[6], a P genotype known to infect neonates without causing diarrhea.

Although the natural infection by rotavirus seems not to confer protection against subsequent infections, reports indicate that first infection may protect for at least 1 year. A study conducted in Cincinnati, Ohio, USA, by Bernstein, et al.27 showed that children suffering from natural symptomatic or asymptomatic rotavirus infection during the first year of life were protected against reinfection in the second year. These results are in contrast with our data since four children (cases A, B, D and E) had first infections within their first year of life, but were not protected against reinfection during the second year. Furthermore, child C had first and second infections within the second year with a 3-month interval.
Several studies involving cases of reinfection by rotavirus relate to the G serotypes only. In a broader context, the present investigation specifies which subgroups, electropherotypes, G serotypes and P\[\],G genotypes are involved in reinfection cases.

The results obtained in the present investigation underscore the need to broaden our knowledge about immunity in rotavirus reinfection. This should be useful regarding future rotavirus vaccination strategies in Brazil, as well as in assessing the possible interference of rotavirus vaccine on the prevailing serotypes/genotypes all over the country.

References