Characterisation of rotaviruses recovered from neonates with symptomatic infection

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An unusually high number of diarrhoeal stools were obtained from neonates at different maternity units in Pretoria during late 1986 and the winter of 1987 and tested for the presence of rotavirus infection. Latex agglutination assay revealed rotavirus in 67% (93/139) of the stools from newborn babies. Gel electrophoresis of the rotavirus genome showed that a genomically stable strain of rotavirus was associated with the diarrhoeal illness in all maternity units investigated. Determination of the VP6 subgroup specificity using monoclonal antibodies demonstrated that all strains were of subgroup II. Examination of the VP4 and VP7 rotavirus genes, which encode for the outer capsid neutralisation proteins of the virus, was conducted by hybridisation analysis using probes directed at the divergent regions of the two genes. These results showed the presence of a VP7 serotype G4 rotavirus strain with a P6 VP4 genotype — the putative 'attenuated' VP4 gene allele — circulating in all maternity units studied.


Although rotavirus-associated diarrhoea has been reported in neonates,1 most studies to date have indicated that the infection is asymptomatic in newborns.2-6 In most of these studies, a single strain of rotavirus was found to be present in the neonatal unit and the viral strains involved showed remarkable persistence over time.7,8 It is currently unknown whether the infection in neonates is attenuated because of host factors (such as maturation of the neonatal intestinal system), the presence of passively acquired antibody, or virulence factors attributable to the different strains of rotavirus recovered from neonatal nurseries.

Two outer capsid proteins of the virion, VP4 and VP7, have been shown to induce neutralising antibodies.9,10 It was recently proposed that the prefix G (for glycoprotein) be used for VP7 and that P (for protease-sensitive protein) be applied to VP4 so that rotavirus classification is based on a binary system.11 To date, nine G serotypes have been identified among human rotaviruses, of which four (G1 - G4) are considered to be epidemiologically important.12

The antigenic diversity of the VP4 protein has been poorly defined; however, molecular studies, involving nucleotide and amino acid sequence analysis, have revealed six distinct VP4 genotypes in human rotaviruses, of which three are epidemiologically important.13 These are derived from the following prototype strains: strain Wa (P8), strain DS-1 (P4) and strain M37 (P6).14

The P8 and P4 genotypes have been detected in children with gastro-enteritis.15-17 The P8 genotype has been reported among strains with G1, G3 and G4 VP7 serotype, while the P4 genotype has been detected exclusively in strains with G2 VP7 serotype.18,19 Conversely, the P6 genotype has been detected in strains exhibiting G1-4 specificity, but which are associated with asymptomatic infection in neonates.20,21

Furthermore, the VP4 gene product has been reported to play a primary role in the virulence characteristics of the rotavirus strain.22 It has also been shown that the VP4 genotype of neonatal rotaviruses is distinct from that recovered from circulating strains in children with gastro-enteritis, but remains conserved among different neonatal strains from various regions of the world.23,24,25 This led to the idea that the VP4 gene allele was responsible for the attenuation of infection in the neonate,26,27 and the suggestion that a neonatal strain may be a good naturally attenuated rotavirus for use as a vaccine candidate.28

In this study, we describe the characterisation of rotaviruses recovered from neonates with symptomatic rotavirus infection in different maternity units in Pretoria.

Materials and methods

Patient specimens

During late 1986 and early 1987, 139 stool specimens were received at Niehaus and Botha Pathology Laboratories in Pretoria from different maternity units in the city environs. The stool specimens were analysed for the presence of rotavirus antigen by a commercial latex agglutination assay (Rota Screen, Mercia Diagnostics, UK).

Virus strains

Well-characterised, tissue culture-adapted rotavirus strains were grown in MA-104 cells. These prototype strains included Wa (VP7 serotype G1 and VP4 genotype P8), DS-1 (G2 and P4), P (G3 and P8), ST-3 (G4 and P6) and strain M37 (G1 and P6). The tissue culture virus was concentrated by centrifugation in a Beckman SW40 rotor for 2 hours, extracted with genetron and then centrifuged through a 30% sucrose gradient in a Beckman SW27 rotor for 2 hours.

Polyacrylamide gel electrophoresis of viral double-stranded RNA

The rotavirus-positive specimens were analysed by polyacrylamide gel electrophoresis of the genomic double-stranded (ds) RNA as described previously.4 In brief, the ds RNA was extracted from the faecal suspension by de-proteinisation with phenol-chloroform followed by ethanol precipitation. The presence of ds RNA was determined by electrophoresis through 10% polyacrylamide gels at 100 V for 16 - 18 hours. The gels were silver stained using a modification of the methods described previously.29
VP6 subgroup analysis

A solid-phase enzyme immunoassay utilising monoclonal antibodies to the VP6 subgroup I and II epitopes was performed to determine the subgroup specificity of the rotavirus strains recovered. The monoclonal antibodies were developed by Dr Harry Greenberg, Stanford University, and kindly donated to this laboratory. The methods have been described in detail elsewhere.

In brief, the rotavirus were pre-treated with EDTA to strip off the counter capsid of the virion and reveal the inner capsid VP6 protein. These specimens were then added to microtitre plates coated with rabbit anti-rotavirus serum. Each specimen was tested in duplicate and assayed in a standard enzyme-linked immunosorbent assay against the monoclonal antibodies to the subgroup I and II antigenic epitopes.

Preparation of VP4 genotype and VP7 serotype cDNA clones

The VP4 genotype-specific and VP7 serotype-specific cDNA clones used in this study were developed at the Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, and are available in our laboratory. Each cDNA clone corresponds to a type-specific hyperdivergent region of the VP7 gene or of the VP4 gene.

For this study, *Escherichia coli* cells carrying the cDNA inserts were propagated in terrific broth to a high titre. Next, the plasmid clones were extracted and purified from bacterial cells using a DNA purification kit (Magic Minipreps, Promega). The polymerase chain reaction (PCR) technique was used to amplify the VP4- and VP7-specific probes from the purified plasmid DNA. The PCR products were purified using the Magic PCR Prep kit (Promega) and radio-labelling with $\alpha$-dCTP (Amersham) by a random primer-labelling method (Prime-a-gene labelling system, Promega) to yield probes with specific activity greater than 10$^6$ counts per minute.

RNA dot blotting

The ds RNA was dot blotted onto nylon membranes (Hybond-N, Amersham) as previously described. In brief, the ds RNA was denatured by boiling for 5 minutes and immediately chilled on ice. Two microlitres of the RNAs were dotted onto prepared membranes and fixed to the membranes by exposure to ultraviolet light for 5 minutes. The tissue culture-adapted strains with known VP4 and VP7 type were similarly dotted as controls.

Hybridisation

Standard hybridisation analysis of the dot blots was performed. Pre-hybridisation was conducted for approximately 2 hours at the temperature of hybridisation (54°C for the VP4 probes and 52°C for the VP7 probes). Hybridisation was carried out overnight in a hybridisation oven (Hybaid, Teddington, UK). After washing, the filters were removed from the hybridisation bottles, air-dried and covered with plastic wrap before exposure to autoradiographic film for 24-28 hours at -70°C.

**Results**

During the course of the study, 93 of 139 neonates with diarrhoea, located in four different maternity units in Pretoria, were identified to be excreting rotavirus.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was conducted on all the rotavirus-positive specimens, of which 77% (72/93) yielded a visible RNA electrophoretype. A single RNA electrophoretype was identified from 59 of these specimens (for which sufficient material was available) and confirmed to be identical by co-electrophoresis of the RNA (Fig. 1). Three neonates were excreting rotaviruses with a different electrophoretype, which was determined to be the predominant strain circulating in the community at that time.

![Fig. 1. Polyacrylamide gel electrophoresis of the ds RNA genome of rotaviruses recovered from newborn babies in four neonatal nurseries in Pretoria (A, B, C and D) showing the conserved RNA electrophoretype.](image-url)
Analysis of the VP6 subgroup

All rotavirus strains which could be typed by the monoclonal antibodies were found to carry the subgroup II-specific epitope (Table I).

Table I. Preliminary characterisation of rotavirus isolates from symptomatic neonates using polyacrylamide electrophoresis and subgroup — specific monoclonal antibodies

<table>
<thead>
<tr>
<th>Maternity unit</th>
<th>RNA electrophoresis</th>
<th>VP6 subgroup</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LN</td>
<td>LA</td>
</tr>
<tr>
<td>Unit A</td>
<td>27</td>
<td>23</td>
</tr>
<tr>
<td>Unit B</td>
<td>18</td>
<td>14</td>
</tr>
<tr>
<td>Unit C</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>Unit D</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>62</td>
<td>51</td>
</tr>
</tbody>
</table>

LN and LA define two distinct long RNA electrophoretypes.

Hybridisation with the probes

Selected strains, showing the presence of high quantities of RNA on polyacrylamide gel electrophoresis, were further examined by hybridisation to VP4- and VP7-specific probes directed to the four most common VP7 serotypes and three most common VP4 genotypes. As seen in Table I, all typeable rotavirus strains with the common electrophoretype hybridised to a VP7 serotype G4 probe and to the VP4 genotype P6 probe. The three strains with a different electrophoretype were confirmed to be a different strain of rotavirus as they hybridised to the VP7 serotype G1 probe and the VP4 genotype P8 probe.

Table II. Characterisation of rotavirus isolates from symptomatic neonates by hybridisation with VP4- and VP7-specific probes

<table>
<thead>
<tr>
<th>Maternity unit</th>
<th>VP7 specificity</th>
<th>VP4 specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G1</td>
<td>G2</td>
</tr>
<tr>
<td>Unit A</td>
<td>27</td>
<td>2</td>
</tr>
<tr>
<td>Unit B</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>Unit C</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>Unit D</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>62</td>
<td>3</td>
</tr>
</tbody>
</table>

Discussion

Rotavirus infection has proved to be endemic and asymptomatic in neonatal units in South Africa, as it has in other parts of the world. The question still arises, however, whether the asymptomatic outcome of neonatal infection is due to host- or virus-related factors. Hoshino and colleagues speculated that neonatal host factors may help to select for less virulent strains of rotavirus that are better able to tolerate and survive in the gut of the newborn. However, because of the obvious difficulties of investigating the neonate host, most studies to date have centred on examining characteristics of the viruses recovered from asymptomatic neonatal rotavirus infection. The identification of rotavirus recovered from neonates with asymptomatic infection in separate hospitals in Pretoria provided the opportunity to characterise these strains further.

A conserved rotavirus strain was observed to be circulating in the neonatal units included in this study, and is representative of the ‘endemic’ rotavirus strain often seen in this context. The strain was shown to be conserved via analysis of the viral genome by polyacrylamide gel electrophoresis, as well as by examination of several antigenic and genetic markers of the virus. The ‘endemic nursery’ rotavirus strain circulating in the neonatal units of Pretoria was shown to be a VP7 serotype G4, VP4 genotype P6 virus with a constant long RNA electrophoretype and subgroup II VP6.

During the same period, we examined stools from neonates without evidence of diarrhoea in two of the four maternity units. We identified 3 rotavirus-positive stools and characterised the strains by the same procedures described above. The same G4/P6:SGII strain with identical RNA electrophoretype was identified (our own unpublished data). This indicates that the same rotavirus strain is apparently associated with both the normal asymptomatic infection in newborn babies and the symptomatic rotavirus infection described in this paper.

The P6 genotype is borne by the viral strains commonly identified in newborn infants in neonatal wards worldwide. These ‘nursery’ strains are shed asymptomatically and share a highly conserved VP4 (P6) gene which is postulated to account for their avirulent phenotypes. However, several such ‘asymptomatic’ or ‘nursery’ strains have been identified in children with gastroenteritis, including symptomatic neonates at Ga-Renkhuwa Hospital. During the same period, we detected the P6 genotype in 50 of 62 specimens from neonates with rotavirus-associated diarrhoea in Pretoria. The association of rotavirus strains bearing the P6 genotype with diarrhoea in neonates has important implications for the strategy of developing a rotavirus vaccine based on the ‘naturally attenuated nursery’ strain.

In the remaining 3 cases, a different rotavirus strain was identified by electrophoresis which was shown by examination of all four markers to be the circulating strain of rotavirus in the community. These babies were likely to have been infected by an adult contact who had had contact with other young children and was carrying the infection subclinically. The possibility of co-infection with other diarrhoeal agents was excluded in the majority of these cases, as full bacteriological and parasitological studies were conducted in conjunction with the rotavirus studies.

In vivo reassortment of the segmented rotavirus genome has been demonstrated in the laboratory and in field studies. However, the genetic stability of rotaviruses is probably optimal in the neonatal nursery because of the closed nature of the environment. This should reduce the possibility of genetic reassortment and optimise the maintenance of a single conserved strain of rotavirus which is adapted to the prevailing conditions in the nursery. However, the potential introduction of rotavirus circulating in the community poses a continual threat, as was indicated by the different strain recovered from 3 babies. Furthermore, antigenic drift among rotaviruses is well described and may be a mechanism whereby changes to the P6 VP4 gene result in changes in pathogenesis of the virus. We are at present conducting sequence analysis of these rotavirus strains to try to answer this question.
We would like to thank Errol Gove and Peter Meewes, Niehaus and Botha Pathology Laboratories, for the kind donations of specimens. I would also like to thank Dr Jorge Flores, Laboratory of Infectious Diseases, NIAID, NIH, Bethesda, Maryland, for donating the clones and Dr Harry Greenberg, Stanford University, Palo Alto, California, for donating the monoclonal antibodies. This study was supported in part by grants from the Poliomyelitis Research Foundation and the South African Medical Research Council.

REFERENCES


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Atlando-axial rotatory fixation

M. Lukhele

OBJECTIVES. To analyse the causes of atlanto-axial rotatory fixation (AARF) and discuss the diagnosis and treatment.

DESIGN. Retrospective case studies.

SETTING. Medical University of Southern Africa — Ga-Rankuwa referral hospital.

PATIENTS. A total of 10 patients admitted to and treated in the Department of Orthopaedics, Ga-Rankuwa Hospital, between July 1989 and June 1993.

OUTCOME MEASURE. Dynamic computed tomography (CT) scan.

RESULTS. Upper respiratory tract infection and trauma were the commonest causes of AARF. There was a delay in diagnosis ranging between 4 weeks and 2 years 6 months. Clinical and radiological reduction was obtained by gradual skeletal traction in 6 patients. Two patients had improvement of the torticollis but still had subluxation on the CT scan. In 1 patient no reduction was obtained on occipitocervical fusion and transoral decompression was necessary. In 1 case the parents refused any form of treatment. There was no recurrence in the 7 patients followed up (minimum 6 months).

CONCLUSION. This study shows that AARF is often diagnosed late. The patients diagnosed early responded well to skeletal traction followed by external support. In patients diagnosed late the AARF could not be reduced completely and needed surgical fusion. If untreated, condition can be complicated by tetraparesis.

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Torticollis is a not uncommon condition in children. There are many causes, including soft tissue pathology, bone pathology and intradural pathology. Atlanto-axial rotary subluxation is one of the commonest causes of torticollis. Many of these subluxations are easily correctable. However, a few may be difficult to reduce. Fielding and Hawkins' coined the term 'atlanto-axial rotary fixation' (AARF) for those subluxations that are difficult to reduce. The purpose of this paper is to review this condition in our patients and to emphasise early diagnosis and proper management to prevent late complications.