Atypical Rotavirus Identified from Young Children with Diarrhoea in South Africa

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ABSTRACT

The study was undertaken for antigenic characterization of rotavirus strains isolated from South African children. During July 1996-July 1997, an epidemiological surveillance of rotavirus-associated gastroenteritis was carried out in Pretoria and Gauteng provinces. In total, 1,229 diarrhoeal faecal samples were collected from a pathology laboratory. Of 389 (32%) rotavirus strains detected, most (97%) were group A rotaviruses by serological assay. However, 12 rotavirus strains identified did not apparently carry the common group A-specific antigen as determined by both commercial and monoclonal antibody-based ELISAs. Electrophoretic analysis of the viral RNA genome revealed that these strains shared a common electropherotype and that this resembled the group A rotavirus constellation of RNA segments. Furthermore, the conserved terminal sequences of the group A VP6 and VP7 genes could not be targeted by standard reverse transcriptase-polymerase chain reaction with a routine set of primers to the VP6 or VP7 genes. These strains present an interesting phenotypic variation of the recognized rotaviruses and warrant further characterization.

Key words: Rotavirus; Diarrhoea, Infantile; South Africa

INTRODUCTION

Rotavirus is the most common cause of severe diarrhoea and is responsible for 30-60% of hospitalizations for childhood diarrhoea worldwide (1). Moreover, rotavirus infects children aged 3-5 years in most communities, and different hygienic conditions do not seem to affect its transmissibility (1).

Based on the antigenic and genetic properties, rotaviruses are classified into seven (A to G) serogroups (2). The most common rotaviruses are classified as serogroup A rotaviruses. These viruses share a common and unique group antigen on the VP6 (3), a common RNA profile by polyacrylamide electrophoresis of the viral genome (4), and a unique terminal sequence of the gene segments (4,5).

The VP6 antigen not only provides the group-specificity for these viruses, but can be further differentiated by the presence or absence of two specific subgroup antigenic epitopes. The group A rotaviruses can, thus, be classified into four categories by their reactivity with the VP6 monoclonal antibodies. These include subgroup (SG) I strains, SG II strains, strains with neither SG I nor SG II, or strains reactive to both SG I and SG II. Both the epitopes may exist on all viral strains, and the monoclonal antibodies used merely interact with overlapping conformational determinants on the VP6 (6).

The viral genome consists of 11 segments of double-stranded RNA that can be separated by polyacrylamide gel electrophoresis (PAGE) to yield a constant and characteristic migration profile for each strain (6).
Although the group A rotaviruses share a common constellation of the RNA bands (with a four, two, three, two configuration), great variation is seen amongst the electrophoretic profiles (6).

Before initiating and evaluating national vaccine programmes, it is desirable to determine the burden of rotavirus-associated disease in South Africa and to determine the characteristics of local rotavirus strains. In this study, several rotaviruses were identified which did not show any typical antigenic characteristics. The study was undertaken to characterize these strains further, using both antigenic and molecular techniques.

**MATERIALS AND METHODS**

**Patients**

During July 1996-July 1997, 1,229 diarrhoeal faecal samples were collected from a pathology laboratory in Pretoria, South Africa. The laboratory receives specimens from physicians mainly serving the Gauteng Province, although some specimens were received from the northern and northwest provinces. The samples were collected from children, aged less than three years, with diarrhoea. Over 50% of these children were aged less than 12 months, and 81% were less than 18 months. Specimens from 48 adults with diarrhoea were also included and screened by PAGE.

**Detection of rotavirus antigens**

The presence of rotavirus group A antigen was sought by latex agglutination at the pathology laboratory (RotaScreen, Murex Diagnostics, UK). A commercial enzyme immunoassay (Rotavirus IDEIA, Dako, UK) was used at MEDUNSA for confirming the presence of group A rotavirus antigens in all the 1,229 samples, including those regarded negative by latex agglutination. The enzyme immunoassays are generally considered more sensitive than the latex agglutination assays for the determination of rotavirus antigen in stools (7).

Selected rotavirus strains, including the 12 strains that constitute the subject of this paper, were subjected to analysis with an alternative group A antigen-detection kit (Eldan, International Diagnostic Laboratories, Israel).

**Determination of VP6 subgroup specificity**

The specificity of the subgroup of rotavirus strains was determined by solid-phase enzyme immunoassay using monoclonal antibodies developed and characterized by Greenberg et al. (8,9). The ELISA system uses a group A-specific antibody to confirm the presence of sufficient quantities of the VP6 antigen and monoclonal antibodies specific to SG I (clone 255/60) and SG II (clone 631/9) of the group A rotaviruses. The methods have previously been described (8,10).

**Polyacrylamide gel electrophoresis**

Viral RNA was extracted from 10% faecal suspensions by phenol-chloroform treatment and ethanol precipitation as described previously (11). The RNA was analyzed overnight in 10% polyacrylamide gel slabs with 3% stacking gel at 100 V at room temperature, and the RNA segments were visualized by silver staining (11). Both EIA-positive and negative faecal samples were analyzed by PAGE to investigate the presence of non-group A rotaviruses which would yield a different RNA profile and which would not be detected by the ELISA assays used.

**Electron microscopy**

Direct-staining electron microscopy was performed on selected rotavirus samples. The faecal samples were cleared by low-speed centrifugation before they were ultracentrifuged at a speed of 35,000 rpm for two hours. The resultant pellets were stained with 3% phosphotungstate acid and viewed at a magnification of 40,000x as described previously (12).

**Reverse transcription-polymerase chain reaction**

Viral dsRNA was extracted from faeces as described above and were purified using the RNAid kit (Bio101, Inc., California, USA). The dsRNA was used as a template for reverse transcription (RT) with AMV reverse transcriptase (Promega, Madison, WI) to produce copy DNA of the VP6 and VP7 genes.

The VP6 gene was reverse-transcribed using a primer targeted to the terminal sequences of the VP6 gene. The reaction was performed at 43 °C for 30 minutes in presence of dNTPs and AMV buffer. The VP6 cDNA was subsequently amplified by PCR using primers complementary to the terminal sequences of the gene. These primers were 5'-GGCTTTTTAAACGAAGTCTTC-3' at the 5' end of the VP6 gene and 5'-GGTCACATCCCTCTCCTACT-3' at the 3' terminus of the gene (13). The polymerase chain reaction (PCR) amplification was performed in 30 cycles at 95 °C for one minute, 48 °C for two minutes, and 72 °C for three minutes. The PCR products were visualized in 1.2% agarose gel stained with ethidium bromide.

The VP7 gene was reverse-transcribed in the same manner using an oligonucleotide primer to the terminal
sequences of the serotype-specific gene that is conserved amongst the group A rotaviruses (14). The PCR amplification was performed using the same cycle reactions in a Perkin Elmer DNA Thermal Cycler. The primers that are directed to the terminal sequences of the VP7 gene were described by Gouvea et al. (14) and included all four VP7 terminal primers: pBeg and psBeg for the 5’ end and pRVG9 and pEnd for the 3’ end of the gene.

In both the sets of reactions, standard positive and negative controls were included. These comprised the known group A rotavirus strains which were included to ensure that the reverse transcription and the PCR worked.

RESULTS

Detection of group A antigen

Rotavirus group A antigen was detected in 389 (32%) of the 1,229 faecal samples, using the Dako Rotavirus IDEIA, which was more sensitive than the latex agglutination assays. The rotavirus-positive specimens were predominantly from young children, and the mean age of the excretors was 12 months. Furthermore, a seasonal pattern to the shedding of rotavirus was confirmed during the cool, dry months as is reported elsewhere in this area (Sebata. Personal communication). Two of the adult stool samples were also positive for rotavirus antigen.

Twelve specimens that seemed to contain rotavirus by PAGE (see below) but failed to react with the Dako Rotavirus IDEIA were investigated further with an alternative ELISA for the group A antigen. None of the selected specimens reacted in the Eldan ELISA system either, indicating a general lack of reactivity with group A-specific antibody. These specimens had already tested negative in the latex agglutination assay at the pathology laboratory. The specimens were received from different physicians and hospitals in the region.

Subgroup specificity

SG II rotaviruses predominated in 52% of the 322 specimens tested. SG I strains occurred in 25%, and two strains were determined to have a non-SG I or II specificity as they reacted with the group A-specific antibody but showed no reactivity with either SG-specific monoclonal antibody. Eight strains reacted equally well with both SG I- and SG II-specific monoclonal antibodies. The remaining specimens did not react with the group A-specific monoclonal antibody, indicating the low levels of virus in the stool.

The 12 antigenically-distinct strains were confirmed to lack any reactivity with the VP6 antigenic epitopes recognized by monoclonal antibodies to the group A, SG I or SG II epitopes and did not react in this ELISA either.

Polyacrylamide gel electrophoresis

Five different RNA profiles, typical of the group A rotavirus migration constellation, were observed in 354 rotavirus-positive specimens subjected to PAGE. Three of these profiles were long-patterns and comprised 50% of the RNA profiles observed. Two short RNA profiles were seen, comprising 25% of the electropherotypes observed.

Twelve strains were identified which, although did not react with three different ELISA systems and a latex-agglutination assay for the group A antigen, revealed the presence of viral dsRNA in an electrophoretic constellation–typical of the group A rotaviruses with a long RNA profile (Fig. 1).

![Fig. 1](image-url) RNA electropherotypes, typical of group A rotaviruses, exhibited by antigenically distinct, non-group A rotaviruses. Lane A, C, and D represent atypical rotaviruses; lane B is a negative control; and lane E represents a typical group A rotavirus strain. The double-stranded RNA segments are numbered from largest to smallest and present the typical group A rotavirus constellation of 4, 2, 3 and 2 segment groupings.
Electron microscopy

Morphologically typical rotavirus particles were observed in five of the 12 samples by negative stain electron microscopy (EM) (Fig. 2), although many specimens seemed laden with bacteria.

Amplification of VP6 gene

The RT-PCR amplification of antigenically typical group A rotaviruses was achieved with the oligonucleotide primers directed to the VP6 gene terminal sequences. However, no amplification product could be shown for any of the 12 ‘atypical’ strains, suggesting that the target sequences on the termini of the VP6 gene were either missing or altered in these 12 specimens.

Amplification of VP7 gene

The RT-PCR amplification of the VP7 serotype-specific gene, which is routinely performed in this laboratory, was also repeatedly attempted on the 12 strains. No amplification product was obtained (data not shown).

DISCUSSION

From this study, several conclusions could be drawn with regard to rotavirus infection in South African children. Rotavirus was confirmed as an important cause of acute infantile diarrhoea in this area and was associated with 32% of diarrhoeas in children aged less than three years. Rotavirus infection occurred in children aged less than two years (95%), and most cases were found during the cool and dry months of the year. Furthermore, a diversity of rotavirus strains, as predicted by the RNA profile, was observed to be circulating in nature in this region as shown previously (15).

However, interestingly, 12 rotavirus strains were identified, exhibiting distinct, unusual characteristics. These atypical or antigenically-distinct rotaviruses presented an interesting phenotypic variation of the recognized rotavirus serogroups and were studied further. These rotaviruses lacked the common group A antigen as indicated by the lack of reactivity with several antigenic assays, including three commercial assays (two EIAs and a latex-agglutination assay). Interestingly, these three assays use polyclonal antibodies raised to different group A rotavirus antigens which include a bovine group A rotavirus (Dako IDEIA), a simian group A rotavirus (Eldan ELISA), and a human rotavirus antigen (Murex LX).

However, the viruses exhibited a typical group A rotavirus electropherotype, with the constellation of RNA segments in a 4, 2, 3, 2 pattern, which identifies the group A rotaviruses (3,6). Rotaviruses bearing this same phenotype were described previously in South Africa (11). It may be possible that the group A antigen is masked by antibody in the stool specimens, although two factors make this unlikely. Firstly, the EM micrographs do not indicate that the virus is coated with antibody (Fig. 2). Secondly, the treatment of the stool suspensions with ethylene diamine tetra acetic acid, during the VP6 subgroup antibody ELISA, would strip any coating antibody off the particles and should have yielded a result with this assay.

It was earlier speculated that the PAGE–positive, ELISA-negative specimens for group A rotaviruses may be a specific strain circulating in limited numbers in a defined population (11). The strains all occurred in 1985 and in young children attending Ga-Rankuwa Hospital. However, in this study, 10 of the 12 specimens occurred in infants aged less than 12 months and originating in the Pretoria city environs. Two of the 12 were of African ethnic origin, while the rest were of European descent. The remaining two specimens were from a three-month-old infant in Brakpan, outside Pretoria and a 46-year old man from Potchefstroom, approximately 180 km from Pretoria.
Furthermore, atypical rotaviruses, exhibiting the group A antigen but showing a non-typical group A RNA electrophoretype, have also been described in South African neonates (16). It may be possible that, if antigenic group A rotaviruses can display a unique non-group A RNA electrophorotype, the converse may also apply: group A rotaviruses which are not antigenically similar can display the traditional group A RNA electrophorotype.

These rotavirus strains represent an interesting variation of the recognized rotavirus serogroups. What remains to be determined is whether the current group A rotavirus vaccine candidates under development will be effective in preventing severe illness attributed to these and other atypical rotavirus strains.

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