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Quantitation of Group A Rotavirus by Real-Time Reverse-Transcription-Polymerase Chain Reaction:

Correlation With Clinical Severity in Children in South India

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Abstract

The epidemiology and pathogenesis of rotaviruses are not completely understood, although recent developments in polymerase chain reaction (PCR) techniques now make it possible to quantify the viral load during an infective episode and investigate its relevance to clinical features of the disease. We studied rotavirus-positive stool samples collected from 10 children without symptoms of gastroenteritis and from 81 children with acute gastroenteritis and in whom the clinical severity of disease was recorded. A semi-quantitative real-time reverse-transcription (RT)-PCR was used to estimate the rotavirus load and to assess its correlation with the Vesikari score for severity of diarrhoea. There was a significant negative correlation ($r = -0.80$, $P < 0.001$) between severity and the PCR cycle at which the PCR amplicons were detectable (crossing point) on the assay, indicating that children with more severe diarrhoea excrete more virus than children with less severe disease.

Keywords

rotavirus; quantitation; real-time polymerase chain reaction

INTRODUCTION

Rotaviral infections occur repeatedly in humans from birth to old age. Infections in young children can result in severe, life-threatening diarrhoea, more commonly in primary infection. Infections in older individuals may be asymptomatic or be associated with mild enteric symptoms, possibly due to increasing cross-protective immunity as a result of repeated infections, although severe infections may also occur in elderly individuals in some settings. The outcome of infection is more serious in developing countries where an estimated 600,000 deaths occur annually and surviving children may fail to thrive [Parashar

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et al., 1998]. These statistics support the global need for an effective vaccine. Unfortunately, the live-attenuated rhesus rotavirus-tetravalent vaccine, the first rotavirus vaccine licensed, was withdrawn from the market due to a temporal association with intussusception [Murphy et al., 2001]. The reason why this vaccine caused intussusception is unclear, since natural rotavirus infection has not been consistently associated with intussusception. This unexpected set-back emphasises the need to understand better rotavirus epidemiology and pathogenesis, including the transmissibility and replication of rotavirus in the gastrointestinal (GI) tract.

Rotaviruses are classified into 7 different serogroups (A-G), based on the antigenic specificity of the middle layer protein of the virus as well as on the pattern of electrophoretic mobility of the 11 RNA segments of the viral genome [Estes, 1996]. Of the seven serogroups, only Groups A, B, and C are known to infect humans, and Group A viruses are more commonly associated with severe, life-threatening disease in children worldwide. In most laboratories, enzyme immunoassays (EIA), which use antibodies specific for Group A-VP6 are used to detect rotaviruses in faecal samples. During diarrhoeal episodes, rotaviruses are usually detectable by EIA for up to 1 week after infection or for prolonged periods in immunocompromised patients. A recent study showed that immunocompetent infants with severe rotavirus diarrhoea may have virus detectable by reverse-transcription-polymerase chain reaction (RT-PCR) for up to 57 days after hospital admission [Richardson et al., 1998], but whether the level of viral replication correlates with the severity of clinical disease has not been studied previously.

The present article describes the evaluation of two commercial EIAs for the detection of rotavirus antigen in faeces and the application of a semi-quantitative real-time RT-PCR method to quantify rotavirus present in the faeces of infected children and the correlation of viral quantitation with clinical severity of diarrhoea as measured by the 20 point scale devised by Ruuska and Vesikari [1990]. A real-time RT-PCR method has been described for Group A rotaviruses recently, but was applied to only two samples from children with acute gastroenteritis, for whom no clinical data were reported [Schwarz et al., 2002].

MATERIALS AND METHODS

Evaluation of Rotavirus Detection EIA

Two different commercial assays, Rotavirus screen EIA (Cat. No. Rota 003, Microimmune Ltd., Brentford, UK) and Ridascreen Rotavirus (Cat. No. C 0901, R-Biopharm GmbH, Darmstadt, Germany) both of which use monoclonal antibodies against Group A rotavirus VP6, were compared to the Rota IDEIA (Cat. No. K6020, Dako Ltd., Ely, UK), which was used as the screening test and uses polyclonal antibodies against Group A rotaviruses. A total of 100 rotavirus-positive and 50 rotavirus-negative samples were tested, including 56 rotavirus positive samples from hospitalised children with acute diarrhoea, 44 rotavirus positive samples from a neonatal nursery, 26 pathogen negative samples collected during a community surveillance study. Twenty samples, positive for bacterial and parasitic pathogens and 4 samples from children with bloody diarrhoea in which no pathogen was identified, were obtained from the Departments of Clinical Microbiology and Pathology, Christian Medical College, Vellore, and had been submitted for the investigation of diarrhoea. The neonatal samples were collected from symptomatic and asymptomatic neonates admitted to the nursery, and were used for EIA evaluation alone, since the clinical severity was not known. All assays were carried out according to the manufacturers' instructions.

Rotaviral Load Study Samples

A total of 91 rotavirus positive children were recruited between January 2002 and July 2003. Fifty-five children required admission to hospital for acute watery diarrhoea and 11 were seen at a hospital outpatient clinic with symptoms of gastroenteritis. The samples from the 55 children admitted to hospital were also evaluated by all 3 EIAs. All other rotavirus positive samples were selected after screening with the Microimmune EIA. A further 25 samples were collected from the community and were investigated during a study of childhood diarrhoea: 15 were symptomatic cases and 10 asymptomatic. The samples were collected as soon as possible after the onset of symptoms or during the screening of asymptomatic children.

Assessment of Severity

Clinical severity of disease was assessed by examination of the child and interview of the mother by the study paediatrician using the 20 point scale of Vesikari [Ruuska and Vesikari, 1990] which is based on the frequency and severity of diarrhoea, episodes of vomiting, associated fever and dehydration.

RNA Extraction and RT-PCR

Nucleic acid was extracted from 200 μl of 10% (100 μl of stool and 900 μl of phosphate buffered saline, pH 7.2) rotavirus-positive faecal suspensions using the guanidinium isothiocyanate-silica nucleic acid extraction method [Boom et al., 1991]. RT was carried out using random priming with hexamers (Amersham-Pharmacia Biotech, Amersham, UK). The cDNA was used in the VP6-specific PCR using the LightCycler™ (Roche, Mannheim, Germany) real-time PCR thermal cycler as previously described [Iturriza Gomara et al., 2002]. The primers VP6-F (sense) (5′ GACGGVGCRACTACATGGT 3′) (nucleotides [nt] 747-766) and VP6-R (antisense) (5′ GTCCAATTCATNCCTGGTGG 3′) (nt 1126-1106) amplified a 379-bp region (nt 747-1126) of the *VP6* gene. The cDNA was also used to G and P genotype using multiplex hemi-nested PCRs that have been described previously [Iturriza-Gomara et al., 1999; Kang et al., 2002].

SYBR green I (Roche, Molecular Biochemicals) was used in the amplification reaction to monitor PCR product accumulation in each cycle and for quantitation, as there is a direct relationship between the number of PCR cycles that are required to detect a template and the number of template molecules present initially in the sample. Serial 10-fold dilutions of a plasmid containing the 379 bp VP6 insert derived from a clinical specimen were used for calibration using the LightCycler™ software and for normalisation between PCR runs (Fig. 1). The crossing point, defined as the PCR cycle at which the amplification signal intersects the log linear region, was used as a direct correlate for the amount of rotavirus RNA present in the clinical samples.

The specificity of the reactions was determined by melting curve analysis of the amplicons.

Statistical Methods

Linear regression was used to quantify the association between crossing point and total Vesikari score or individual components of Vesikari score and to adjust for age, and G- and P-type where appropriate. A possible extra-linear association was assessed by introducing a term for square of the Vesikari score. Correlations were assessed using Spearman's rank correlation coefficient. Vesikari score was compared among G- and P-types using the Kruskal–Wallis test.

RESULTS

Evaluation of Rotavirus Detection EIA

The comparison of 150 samples from children with acute diarrhoea, neonates, and controls tested by the three immunoassays used for screening is shown in Table I. The Dako IDEIA for rotavirus gave one false-positive test with a sample that contained *Shigella dysenteriae*, but was negative for rotavirus by four other tests (Table I). Forty-four samples from symptomatic and asymptomatic neonates were also tested in all three assays, with 43 positive by all three tests. One neonatal sample was positive by the Dako IDEIA, but negative by the other two tests.

Rotaviral Load Study

The 66 children presenting to hospital with diarrhoea due to rotavirus had a mean age of 12.1 months (SD 6.7). Of these, a total of 52 (79%) children presented with fever >37°C, 27 (41%) with dehydration, and 59 (89%) with vomiting. Diarrhoeal episodes had lasted >4 days in 30% of children at the time of presentation. Sixty-three children were treated with intravenous rehydration followed by oral rehydration and three received only oral rehydration. One child did not require clinically intravenous rehydration, but was treated because of the parents' anxiety. In eight children, in addition to the symptoms of gastroenteritis, other systemic problems were also recorded. These were lower respiratory infections in seven children and a hemolytic uremic syndrome in one child. The children from the community survey were younger than those presenting to hospital: mean age of symptomatics 3.7 months (SD 3.0); mean age of asymptomatics 3.2 months (SD 3.0). All children recruited into the study were immunocompetent.

Rotaviruses of diverse G- and P-type combinations were found infecting these children, G9P[8] and G2P[4] were two most frequently found G- and P-type combinations. For the first time, G10 strains, in association with P[4] or P[11] were seen in children with acute gastroenteritis. Multiple types indicative of coinfection with two different strains were also identified (data not shown).

The scoring of the severity of disease by means of the 20 point Vesikari scale rated the symptomatic children between 3 and 15 (median 10). Asymptomatic children were assigned severity scores of 0. Vesikari score was not associated with age or with rotavirus G- or P-type (data not shown).

Reproducibility of the VP6 real-time PCR was confirmed through inclusion of a serial dilution of a control plasmid in all the PCR runs (Fig. 1), and the inclusion of repeated test samples selected at random in each PCR run. The linear regression calculated from the results of the 10-fold dilutions of the control plasmid gave r-values of 0.99-1.00, and gradients between −3.557 and −3.131. Crossing points ranged from 6.035 to 42.35, median 15.3 and did not correlate with age after adjusting for the source of sample (i.e., hospital or community, $P = 0.76$). However, the crossing point was strongly associated with total Vesikari score ($r = -0.80$, $P < 0.001$). Samples from the community survey had both higher crossing point, and were from children with milder disease and the correlation with crossing point and severity was evident in sub-group analysis of the samples from hospitalised children ($r = -0.52$, $P < 0.001$), and samples from children seen at a hospital outpatient clinic ($r = -0.56$, $P < 0.001$). The regression equation of crossing point on Vesikari score was $Y = 36.9 - 1.97X$ (Fig. 2), and this association was unchanged after adjustment for age, or G- and P-type. There was no evidence of extra-linear association ($P = 0.52$). Grouping the data broadly according to severity, there was a clear difference in the mean PCR crossing points between asymptomatic children (mean crossing point 35.7 [95% confidence interval (CI): 32.5-39.0], children with low severity scores (3-9; mean crossing point 26.8 [95% CI:

24.6-29.0]), and children with high severity scores (10-15; mean crossing point 11.7 [95% CI: 10.5-12.9]) (Fig. 3). In an analysis of crossing point and individual components of the Vesikari score among hospitalised children, significant negative associations were found with scores for the maximum number of stools in 24 hr ($P < 0.001$), maximum number of times vomited in 24 hr ($P = 0.001$), and dehydration ($P = 0.02$), but not with duration of diarrhoea, duration of vomiting, or fever ($P > 0.05$).

DISCUSSION

Rotavirus gastroenteritis continues to be the single most important cause of dehydration in young children. In this study 67/204 (33%) of children who presented to hospital with acute diarrhoea requiring rehydration, tested positive for rotavirus. The availability of EIA, in easy to use formats, allows laboratories to test for rotaviruses. The comparison of EIA tests in this study shows that the available tests have comparable sensitivity and specificity but unfortunately these tests are not used widely in tropical developing countries.

We have reported previously the unusual diversity of rotaviruses in Vellore during the period from 1996 to 1999 [Kang et al., 2002]. In 2002, we saw a marked increase in the proportion of G9 strains, from a maximum of 11% in 1997-1998 to 47% in 2002. G1 rotaviruses accounted for 40% of the strains characterised during 1996-1999, but constituted only 16% of the strains in 2002. G10P[11] strains have been described previously as asymptomatic neonatal strains, reported from Bangalore [Dunn et al., 1993], but have not been shown in acute diarrhoea prior to this report. It is interesting to note that G9 strains were described originally as asymptomatic neonatal strains in India [Das et al., 1994], and were subsequently recognised to cause disease in children worldwide.

In developing countries, exposure to an environment contaminated with human and animal faeces, and close contact with animals in the domestic environment are factors that are likely to promote mixed infections and inter-species transmission of strains, enabling viral reassortment and the emergence of new strains. The emergence of new strains may, in part, be responsible for the severe disease seen in children in developing countries, and the concomitant increased need for medical attention. However, although new strains were identified in our study, the severity of disease could not be associated with any particular G- or P-type. The relatively low numbers of samples within any particular genotype did not allow rigorous statistical analyses.

Our analysis demonstrated that severity of diarrhoea as determined by the Vesikari score is significantly and negatively associated with the crossing point of the semi-quantitative real-time PCR ($P < 0.001$), indicating that children with severe diarrhoea excrete more virus than children with less severe disease. Part of this association was due to a significant association with the number of diarrhoeal stools ($P < 0.001$), suggesting that increased stool frequency may reflect higher viral loads. Increased excretion may have been expected earlier in illness, but there was no significant association with the duration of diarrhoea in this study, possibly because of inevitable delays before children presented to health care. Although data on the number and consistency of stools was available from nursing records, the total volume of each stool was not recorded. In future studies, it would be useful to assess the total stool volume or dry stool weight for each child in order to accurately measure the total amount of virus excreted.

The development of amplification techniques has revolutionised the ability to detect viruses both quantitatively and qualitatively, and to study viral load in relation to disease progression. In the context of rotaviral infections, the ability to quantify VP6 accurately from cDNA, with appropriate internal controls, will enable the study of the natural history of

rotaviral infection by evaluating sequential samples from patients. In conjunction with viral genotyping and assessment of pre-existing antibodies, VP6 quantitation will permit analysis of strain-specific disease severity, and help to determine whether newly introduced strains produce more severe disease than strains which have been previously circulating in the community. In addition, accurate determination of excretion by asymptomatic subjects and measurement of contamination in environmental samples will lead to an understanding of the requirements for transmission in different settings such as households, food preparation areas, and water supply systems.

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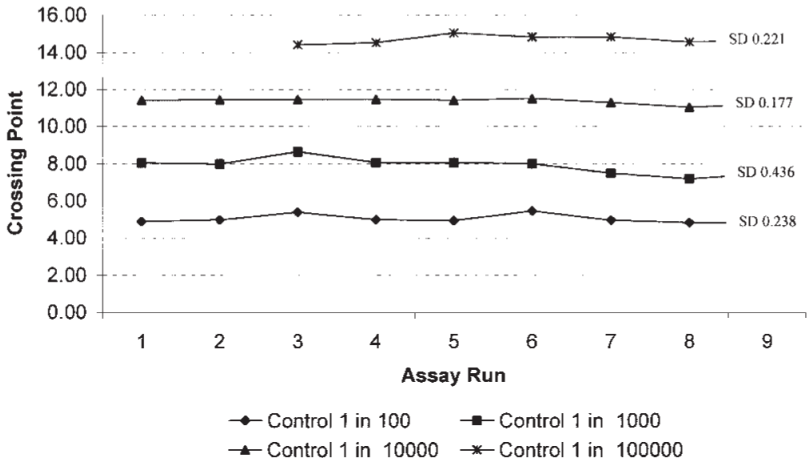

Fig. 1.

VP6 real-time polymerase chain reaction (PCR) inter-test variation: PCR crossing points of a serial dilution of the VP6 control plasmid obtained in nine independent PCR runs.

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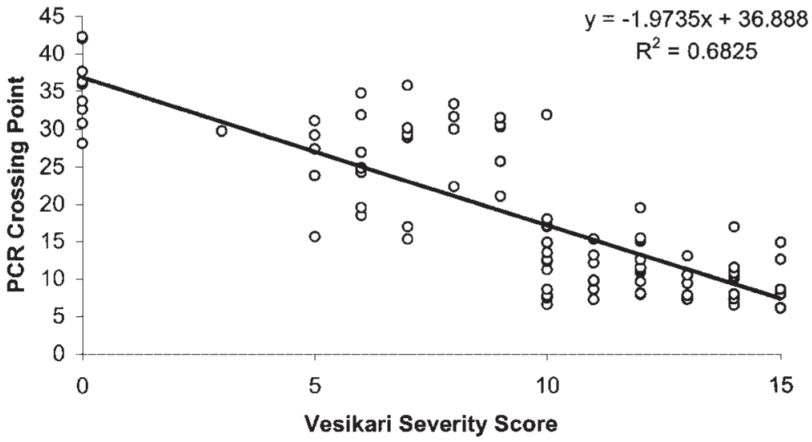

Fig. 2.
Plotting of the normalised VP6 real-time PCR crossing point versus Vesikari score, and linear regression analysis.

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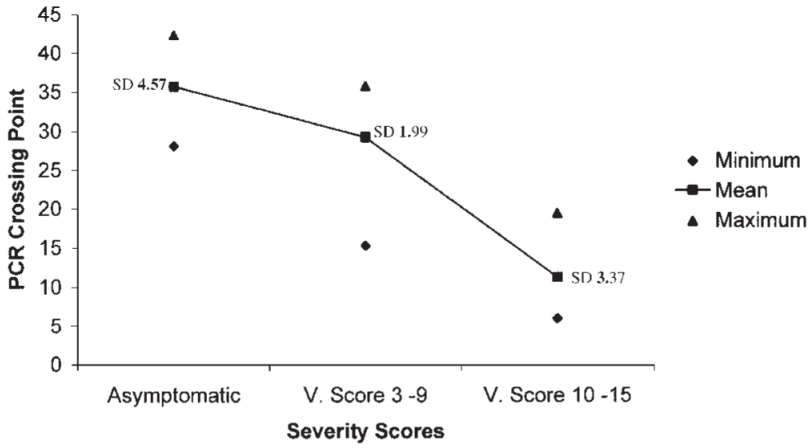

Fig. 3.

VP6 real-time PCR crossing points (mean and range) in rotavirus-positive asymptomatic children, children with low severity scores, and children with high severity scores.

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TABLE I

Comparison of Three Commercially Available Enzyme Immunoassays (EIA) for Detection of Group A Rotavirus

Samples	n	Dako IDEIA[a]	Microimmune[b]	RIDA Screen[c]
Rotavirus positive				
Acute diarrhoea	56	56	56	56
Neonatal infection	44	44	43	43
Rotavirus negative				
Other pathogens	20	1[d]	0	0
Negative-all pathogens	26	0	0	0
Bloody diarrhoea	4	0	0	0

[a] Dako IDEIA rotavirus: solid phase sandwich EIA using polyclonal antibodies.

[b] Microimmune rotavirus screen: solid phase sandwich EIA using monoclonal antibodies.

[c] RIDASCREEN rotavirus: solid phase sandwich EIA using monoclonal antibodies.

[d] One sample was positive in the Dako IDEIA, but negative in the Microimmune rotavirus screen and the RIDA screen. It was also negative by electron microscopy and RNA electrophoresis.