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Study of Incidence of Rota Virus in Children under 5 Years of Age with Acute Diarrhoea with Special Reference to Characterization of Rota Virus

Authors
Subha.K[1], Selvi.R[2], Balaji A.V.M[3], Dheepa.D[4]
[1,3]Assistant Professor, Dept.of Microbiology
[2]Professor, Dept.of Microbiology
[4]Specialist Microbiologist, Dept.of Microbiology
Corresponding author
Subha.K
Assistant Professor, Dept.of Microbiology

Abstract
Back Ground: *Diarrhoea is one of the most common causes of morbidity and mortality in children worldwide. Rotavirus disease is the single most important cause of severe gastroenteritis in children throughout the world. The present study was conducted to determine the incidence of Rotavirus in diarrheal cases of children under 5 years of age and characterize rota virus strains circulating in the community.*
Materials and Methods: *A total of 150 children under 5 years of age suffering from acute diarrhoea and 50 controls without diarrhoea were included in the present study. Rota virus was identified from all stool samples with commercially available ELISA kit detecting group specific VP6 antigen for rota virus. ELISA positive samples were subjected for characterization by RNA extraction and Reverse transcriptase Polymerase Chain Reaction.*
Results: *Rota virus was seen significantly in children with diarrhoea. Rota virus incidence was found to be 16% among children under five years of age with diarrhoea. The genotype combination of rota virus identified were common type G1P[8],G2P[4] and uncommon type G10P[untypeable].*
Conclusion: *Continuous and prospective monitoring of circulating strains is necessary to detect any change in the distribution of rotavirus strains which would influence the vaccine based preventive strategies in India.*
Keywords: *Acute diarrhoea, Rota virus, ELISA, Genotyping.*

Introduction

Diarrhoea is one of the most common causes of morbidity and mortality in children worldwide. Diarrhoeal disease continues to be a public health problem especially in developing countries where they were estimated to be responsible for 2.5 million infant deaths per year with an annual mortality rate of 4.9/ per 1000 children and an incidence of 3.2 episodes of diarrhoea per child-year among children under 5 years of age[13]. In India, one third of total paediatric admissions in hospitals were due to diarrhoeal diseases and 17% of all deaths in indoor paediatric patients were diarrhoea related[21]. Diarrhoea was the cause of death in almost 23% of Indian children who die before the age of 5[10].

Although many number of viral , bacterial and parasitic causes of diarrhoea have been identified ,

only a few of the etiologic agents cause the vast majority of diarrhoeal disease in children in the developing world and include *Rotavirus, diarrhoeagenic Escherichia coli, Campylobacter jejunii, Shigella sp, nontyphoidal Salmonella, Giardia lamblia, Cryptosporidium sp* and *Entamoeba histolytica*[24].

Rota viruses are the single most important cause of severe diarrhoeal illness in infants and young children in both developing and developed countries worldwide accounting for 30-50% of these illnesses[19]. Every year, rotavirus gastroenteritis was estimated to cause approximately 527,000(475,000-580,000) deaths globally among children <5 years old[29]. Of about 500,000 annual deaths due to rotavirus worldwide, more than 1,50,000 occur in India[28] and risk of rotavirus disease–associated death by 5 years of age was 1/250 in India[20].

For rota viral infections, epidemiological studies can be used to select areas for vaccine trials depending upon disease burden and to serve as baselines for identification of new strains should they emerge[18].The epidemiology of rota virus in India remains in constant flux. So continuous monitoring of rota virus genotype distribution would be valuable to show up the diversity and changes in the circulating strains.

Hence this study was undertaken to identify the rota viral infections in children under 5 years of age with diarrhoea and from children under 5 years of age presenting to hospital for reasons other than diarrhoeal illness.

Aims and Objective of the Study

- To determine the incidence of *Rota virus* infection in children under 5 years of age with acute diarrhoea.
- To examine the age distribution of *Rota virus* infected children.
- To evaluate clinical severity of *Rota virus* infection in children.
- To evaluate the distribution of different genotypes of *Rotaviruses* circulating in the community

Materials and Methods

A total of 150 children, 0-5 years of age were included in the study. The patients were clinically examined and selected for the study after obtaining the history. Children less than 5 years of age with complaints of more than 3 episodes of watery stools with or without mucus and blood for a period less than 2 weeks and not on antibiotics or any laxatives were included in the study. Children above 5 years of age, Children with complaints of diarrhoea more than 2 weeks and children who acquired diarrhoeal illness after hospital admission were excluded from the study.

A total of 50 children aged 5 years or below admitted to the hospital for causes other than diarrhoea were included as controls.

The matching of study and control group was analysed by students 't' test for independent variables and the other variables were analysed by chi square test. The 'z' test of proportions between the groups was performed wherever necessary. The above statistical procedures were performed by the statistical package PASW (Predictive and Analysis Software) statistics -18 so called SPSS. The p values less than 0.05 were considered as significant.

The stool samples were collected in a clean wide mouthed sterile container, labelled properly, transported to the laboratory in ice pack and processed within one to two hours.

Microbiological Analysis

The stool samples were stored in screw capped vials at -70ºc for analysis of rota virus.

Detection and Characterization of Rota Virus

Detection of *Rota Virus*

Rota virus was identified from all stool samples with commercially available ELISA kit detecting group specific VP6 antigen for *Rota virus*.

1. Antigen detection: was done by Enzyme Linked Immunosorbent assay method (ELISA) according to manufacturer's instruction.[RIDASCREEN]

This is a sandwich-type of ELISA. Monoclonal antibodies against a capsid protein of gene 6 (VP6) of the rotaviruses are applied to the surface

of the well in the micro well plate. This is a group-specific antigen which occurs with all *Rotaviruses* which are pathogenic to humans. A suspension of the stool sample which is to be tested together with other monoclonal anti-rotavirus antibodies, which are conjugated with horseradish peroxidase, is pipetted into the well in the micro well plate for incubation. In the presence of rotaviruses, a sandwich complex forms which is made up of the immobilised antibodies, the unattached enzyme-labelled antibodies are removed during a further washing phase. After adding the substrate, the attached enzyme changes the colour of the previously colourless solution in the wells of the microwell plate to blue if the test is positive. On adding the stop reagent, the colour changes from blue to yellow. The extinction is proportional to the concentration of rotavirus antigens present in the sample.

ELISA positive samples were subjected for characterization by RNA extraction and

Reverse transcriptase Polymerase Chain Reaction as standardised by Invitrogen. RNA extraction was done by QIAmp viral RNA extraction method.

Denaturation of ds RNA

40 µl of extracted nucleic acid was added to 0.2 ml PCR tube and ds RNA was denatured at 97°c for 5 min and chilling the tube on ice for 2 min.

Polymerase Chain Reaction

G Typing PCR (VP7)

G Genotyping: First-amplification consensus primers:

VP7 F 5'ATG TAT GGT ATT GAA TAT ACC AC 3' product size 881bp

VP7R 5' AAC TTG CCA CCA TTT TTT CC 3'

Genotyping: 2nd round multiplex PCR

Primer VP7 R

Second amplification Type specific primers

G1 5' CAA GTA CTC AAA TCA ATG ATG G 3' product size 618bp

G2 5' CAA TGA TAT TAA CAC ATT TTC TGT G 3' product size 521 bp

G3 5'ACG AAC TCA ACA CGA GAG G 3' product size 682 bp

G4 5' CGT TTC TGG TGA GGA GTT G 3' product size 452 bp

G5 5' GTC ACA CCA TTT GTA AAT TCG 3' product size 754 bp

G9 5'CTA GAT GTA ACT ACA ACT AC 3' product size 179 bp

G10 5'ATG TCA GAC TAC ARA TAC TGG 3' product size 266 bp

P Typing PCR (VP4)

P Genotyping

First amplification consensus primers

con3 5'TGG CTT CGC CAT TTT ATA GAC A3' product size 876 bp

con2 5' ATT TCG GAC CAT TTA TAA CC 3'

P Genotyping: 2nd round multiplex PCR

Primer Con3

Second amplification Type specific primers:

P[8] 5'TCT ACT GGR TTR CAN TGC 3' product size 345 bp

P[4] 5' CTA TTG TTA GAG GTT AGA GTC 3' product size 483 bp

P[6] 5'TGT TGA TTA GTT GGA TTC AA 3' product size 267 bp

P[9] 5'TGA GAC ATG CAA TTG GAC 3' product size 391 bp

P[10] 5'ATC ATA GTT AGT AGT CGG 3' product size 583 bp

P[11] 5' GTA AAC ATC CAG AAT GTG 3' product size 312 bp

Analysis by Gel Electrophoresis: Amplified products were analysed by agarose gel electrophoresis.

Statistical Analysis

The matching of study and control group was analysed by students 't' test for independent variables and the other variables were analysed by chi square test. The 'z' test of proportions between the groups was performed wherever necessary. The above statistical procedures were performed by the statistical package PASW (Predictive and Analysis Software)statistics -18 so called SPSS. The p values less than 0.05 were considered as significant.

Results and Discussion

200 stool samples were collected from children less than 5 years of age which include study group of 150 children with diarrhoea and 50 controls without diarrhoea.

Rota virus antigen detection was done for all samples by ELISA. 24 samples showed positivity. Reverse Transcriptase Polymerase chain Reaction was done for determination of G and P genotype of the *Rota virus* strains.

The results of these tests are as follows:

Rota virus (16%) were identified significantly in the study group and none from the control group.

Table 1: Correlation of clinical severity in rota virus and non rota virus patients

Clinical symptom	Rotavirus diarrhoea patients(n=24)		Non rota diarrhoea patients (n=126)		p value
	No.	%	No.	%	
Fever	16	66.7	77	61.1	p>0.05
≥6 watery stools per day	17	70.8	50	39.7	p<0.01
Vomiting	20	83.3	67	53.2	p<0.001
vomiting ≥4 times per 24 hrs	11	45.8	27	21.4	p<0.05
bloody stools	0.0	0.0	20	15.9	-

A significantly higher proportion of *Rota virus* patients than non rota virus patients were associated with more than 6 watery stools per day (70.8%) and increased bouts of vomiting (45.8%).

Table 2: Distribution of severe dehydration by age group among rota virus and non rota virus diarrhoea patients

Age group (mths)	Rotavirus diarrhoea(n=24)	%	Non rotavirus diarrhoea(n=126)	%	p value
0-6	0.0	0.0	1.0	0.8	-
7-12	4.0	16.7	12	9.5	p>0.05
13-24	3.0	12.5	3.0	2.4	p>0.05
25-60	0.0	0.0	2.0	1.6	-
Total	7.0	29.2	18	14.3	p>0.05

29.2% of rota virus patients were associated with severe dehydration out of which most of them were in 7-12 months age group. 14.3% of non rota virus patients were associated with severe dehydration out of which most of them were in 7-12 months age group. But the difference was not statistically significant.

Table 3: Age wise analysis of group a rota virus infection among children 0-5 years with diarrhoea

Age in mths	Rotavirus patients(n=24)		Non rotavirus patients(n=126)		p value
	No.	%	No.	%	
0-6	2.0	8.3	8.0	6.3	p>0.05
7-12	17	70.8	59	46.8	p<0.05
13-24	4.0	16.7	37	29.4	p>0.05
25-60	1.0	4.2	22	17.5	p<0.05

Overall rota virus infection rate of 16% was found among children with acute diarrhoea. Rota virus positive children were significantly higher in the age group 7-12 months and significantly lesser in the age group 25-60 months.

Table 4: Group A rota virus types detected by RT-PCR(n=12)

No of strains typed by RT-PCR	12	100%
No of strains fully typed	8.0	66.7%
No of strains partially typed	1.0	8.3%
No of strains untypeable	3.0	25%

Out of 24 *rota virus* strains, 12 were subjected to typing by RT PCR. Out of 12 strains, 66.67% were fully typed for G and P genotype,8.33% were partially typed and 25% were not typeable.

Table 5: Distribution of G and genotype of group a rota virus detected among under five children with diarrhoea

Genotype	No of strains	%
G1	6.0	66.7
G2	2.0	22.2
G10	1.0	11.1
Total G genotype typed	9.0	100
P[8]	6.0	75
P[4]	2.0	25
Total P genotype typed	8.0	100

Out of 12 *rota virus* strains, 75% of G genotype and 66.67% of P genotype could be determined. 25% of G genotype and 33.33% of P genotype could not be determined.

The G1 genotype was identified in 66.67% of *rota virus* strains, followed by G2 in 22.22% and G10 in 11.11%. P[8] was identified in 75% of the strains and P[4] in 25% of the strains.

Table 6: Distribution of G and P type combination of group A rota virus strains detected among under five children with diarrhoea

GP type	No of strains	%
G1P[8]	6.0	50
G2P[4]	2.0	25
G10P [untypeable]	1.0	25
Total	9.0	100

G1P [8] was determined in 50% of the strains, followed by G2P [4] in 25% and G10P [untypeable] in 25% of the strains.

Discussion

Acute infectious diarrhoea is one of the major causes of mortality in children, particularly in developing countries like India. *Rota virus* is one among the most common causes of diarrhoea worldwide18, 47 accounting for 134 million episodes/year[12,25,26].

Effective control of rota viral disease in any community depends upon an accurate understanding of disease burden and knowledge of the relative importance of circulating serotypes. This study was conducted to determine *rotavirus* detection and characterization of *rota virus* in children under 5 years of age with acute diarrhoea.

In the present study, *Rota virus* was detected in 16% of the study group. The true incidence of rota virus could be higher than 16% as this data represents only children with diarrhoea requiring medical attention. *Rota virus* was not detected from any one of the control group. This implies that *Rota virus* was significantly associated with diarrhoea. This correlates with the study of M.Aminu et al[14] in which *Rota virus* was not detected in controls.

Rota virus infected patients presented as watery diarrhoea associated with vomiting. A significantly higher proportion of *Rota virus* patients than non rota virus patients were associated with more than 6 watery stools per day(70.8%) and increased bouts of vomiting (45.8%). (Table 1). This study correlates with the study of Khitam Muhsen et al[11] in which rota virus infected children significantly presented with more watery stools (87.1%) and more vomiting(57.6%). This shows that *Rota virus* is associated with more severe illness than other enteropathogens.

In the present study, 29.2% of rota virus patients were associated with severe dehydration out of which most of them were in 7-12 months age group.(Table 2).

This correlates with the study by A.K. Siddique et al[1] in 16% of rotavirus patients presented with severe dehydration and most of them were in 6-11 months age group.

This shows that children in 7-12 months of age group are at higher risk of death due to severe dehydration associated with rota virus infection.

In the present study, rota virus positive children were significantly higher in the age group 7-12 months and significantly lesser in the age group 25-60 months.(Table 3). The present study correlates with the study of P.Saravanan et al[18] , M.John

Albert et al[9] study and A.K.Siddique et al[1] in which majority of rota virus positive patients were

in the age group 7-12 months. This could be because of protective effect of maternal antibodies in infants<6months of age and by the development of natural immunity after successive infections in children >2 years of age. This also indicates that prophylactic measures such as vaccination should be taken in the first few months of life to prevent rota viral infection.

In the present study, 66.7% were fully typed for G and P genotype,8.3% were partially typed and 25% were not typeable(Table 4). This was similar to Vivek jain et al[28]study which has showed 12% of partially or nontypeable strains in India.

Moyo et al study[16] showed 6.1% of nontypeable strains and 6% of partially typeable strains in Tanzania. In the present study , 75% of G genotype and 66.67% of P genotype could be determined. 25% of G genotype and 33.33% of P genotype could not be determined.(Table 5) . This correlates with the review study of N.A.Cunliffe et al[17] in which 26% of G genotype and 32% of P genotype could not be typeable. In a review study by Ali M.Kheyami et al[3] , 11-31.3% of *rota virus* strains could not be typeable for G type. In a study by Zuccotti et al[30] ,19.8 % of rota virus strains could not be typeable for P type. The nontypeability of strains may be due to the emergence of new strains. Since *rotaviruses* genetically mutate, it is to be expected that sometimes RT-PCR methodologies are unable to identify all types. Another reason is that the strain untypeable could be unrecognised which needs further investigation as stated by M.Aminu et al[14].

In the present study, G1 genotype was identified in 66.7% of rota virus strains being the most predominant type followed by G2 in 22.2% and G10 in 11.1%. (Table 5). This correlates with the study of Malek MA et al[15] in which G1 was the predominant type (80%). Ryuichi Uchida et al[23] study showed predominance of G1 type (77%) among children with rotavirus diarrhoea. Also studies done by Zuccotti et al[30] , Aminu.M et al[14] , Ali M.Kheyami et al[2], N.A.Cunliffe et al[17] showed predominance of G1 type in rota virus strains.

Other than the globally common genotypes G1 and G2, G10 genotype was also seen in the present study. G10 genotype has been reported in the study by Gagandeep Kang et al[7] in children with diarrhoea in south India. But previous studies by Dunn et al[5] and Sukumaran M et al[27] have reported G10 type in asymptomatic newborn infants. This implies that G10 genotype is increasingly being associated with symptomatic diarrhoea. G10 has also been reported in the study by Zuccotti et al[30]. In the present study, P[8] was the predominant P type (75%) followed by P[4] 25%. (Table 5) .This correlates with the study by Buesa J et al1[2](77.1% of P[8]) and Zuccotti et al[30] in which only p[8] and p[4] types were seen with predominance of p[8]. The studies by Ryuichi Uchida et al[23] , Malek MA et al[15] and Moyo et al[16] showed predominance of P[8] type of about 80-100%.

In the present study, G1P[8] was determined as the most common combination of G and P type(50%), followed by G2P[4] and G10P [untypeable]. (Table6). G1P[8] andG2P[4] have been described as globally common genotypes in a review study done by Gentsch et al[8]. This study correlates with Khitam Muhsen et al[11] study which has

shown 49.1% of G1P[8] combination and Buesa J et al[3] study in which 42.7% of G1P[8] combination was found and Foster et al[6] study has shown 40.3% of G1P[8] combination. Ryuichi Uchida et al[23] study have also reported predominance of G1P[8] combination(70%).

Conclusion

In the present study, *Rota virus* incidence was found to be 16% among children under five years of age with diarrhoea. Majority of them were in the age group of 7 to 12 months associated with severe dehydration and protective effect of breast feeding was not found against rota virus infection in this age group. The genotype combination of *Rota virus* identified were G1P[8],G2P[4] and G10P[untypeable]. Thus the present study shows significant association of *Rota virus* in children

under five years of age with acute diarrhoea and the prevalence of uncommon genotype G10.

The significant association of *Rota virus* in childhood diarrhoea along with clinical severity suggests consideration of a rota virus vaccine in the childhood immunization program. In developing countries like India, exposure to an environment contaminated with human and animal faeces and close contact with animals in the domestic environment are factors enabling viral reassortment and the emergence of new strains.

Most of the *Rota virus* strains in circulation appear to be like those of common genotypes G1-4 which are components of current vaccine. But the non typeable strains pose an unknown antigenic challenge to the current vaccine Rotateq. Hence continuous and prospective monitoring of circulating strains in a large scale study will detect any change in the distribution of rotavirus strains which would influence the vaccine based preventive strategies in India.

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