Original Article

ID: JCDR/2012/3907:2004

Microbiology Section

Rotaviral Diarrhoea in Children: A Comparison of PAGE with ELISA

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ABSTRACT

Background: Rotavirus infects almost all children by the age of five. More than 150,000 annual deaths due to rota virus, occurs in India.

Aims: This study aimed to determine the incidence of Rotavirus infection in children and compare enzyme - linked immunosorbent assay (ELISA) and a modified polyacrylamide gel elctrophoretic (PAGE) analysis in detection of rota virus in stool samples.

Materials and Methods: In this prospective study, a total of 200 stool samples were examined for the presence of Rota virus by ELISA and by a modified PAGE analysis of viral genome. Stool culture was done for common enteric pathogens.

Results: Maximum incidence of rotavirus infection was seen in age group of 6m-24 m(32.3%). Excellent correlation of ELISA and PAGE results was found in194 of 200 (97%) specimens. A total of 51 (25.5%) of them were found to be positive for rotavirus by either methods. The proportion of ELISA +ve PAGE –ve samples 1/200 was lower than the proportion of ELISA-ve PAGE +ve samples (5/200). All 51 rotavirus positive cases did not show infection with bacterial pathogens.

Conclusion: The modified PAGE technique for the detection of viral RNA was found to be rapid, simple, reliable and less expensive technique.

Key Words: Rotavirus, Polyacrylamide gel electrophoresis (PAGE) technique, Enzyme - linked immunosorbent assay (ELISA), Diahorrea

INTRODUCTION

Rotavirus infects almost all children by the age of five, both in the developing and developed countries [1]. Rotavirus is composed by 11 double-stranded RNA segments surrounded by three concentric protein layers. The outer capsid consists of VP7 (a glycoprotein) and VP4 (a protease-sensitive protein) which carry independent neutralization and protective antigens [2]. In temperate climates, rotavirus is most often detected in the winter and rarely in the summer, whereas in the tropics it is found all year round, with less-defined seasonal variation [3]. Of the approximately 600,000 annual deaths due to rotavirus (RV) worldwide, more than 150,000 occur in India. Also, 20 to 30 percent hospitalized cases of diarrhea are due to rotaviruses [4].

Clinically rotavirus gastroenteritis is characterized by profuse diarrhea, mild fever and vomiting leading to mild to severe dehydration. The clinical manifestations of rotavirus diarrhea alone are not sufficiently distinctive to permit diagnosis [1]. The laboratory diagnosis of rotavirus infection is done mainly by ELISA, which require expensive commercial kits and reagents as also expensive instruments. Hence, not many laboratories are able to diagnose rotavirus infection. In view of this we undertook to evaluate the reliability of the Polyacrylamide gel electrophoresis (PAGE) technique as developed by Herring et al [5].

MATERIALS AND METHODS

The study included samples from 200 children belonging to both sexes who attended the pediatric clinics in civil hospital with a complaint of diarrhea.

Inclusion Criteria

- Children not older than 5 years of age group.
- Children with profuse watery diarrhea
- Diarrhoea less than 10 days duration.

Exclusion Criteria

- Children above 5 years.
- Diarrhoea more than 10 days.

Stool samples were investigated for Rota virus by PAGE (Polyacrylamide gel electrophoresis) and ELISA (Enzyme linked immunosorbant assay). ELISA was done on a 10% suspension in PBS by using 'polyclonal' ELISA for the group A rotavirus antigen detection. (Developed at the national institute of virology {NIV} Pune) [6]. PAGE and silver staining technique were performed as per the method of herring et al [5] and Merill et al [7]. Briefly a 0.5ml of 0.1 M sodium acetate solution containing 1 percent sodium dodecyl sulphate and 0.5ml phenol chloroform mixture was added to 100 mg of fecal sample. This was vortexed and centrifuged at 7000rpm for 2 minutes. The aqueous upper layer containing the double stranded RNA was removed for electrophoresis and run on gel of size 14×16cm and 0.75mm thickness with 7 wells. Ten percent polyacrylemide gels with 3 percent stacking gel were used. Each well was loaded with 40µl of RNA extract to which 10 µl of sample buffer containing 0.5 M Tris base, 1 percent bromophenol blue and 20 percent glycerol were added. The running buffer consisted of Tris glycine pH 8.8. Discontinuous electrophoresis was carried out as described by Laemlli at 30 mA for 3 hrs at room temperature [8]. Finally, the double stranded RNA was visualized by silver staining. The gel was gently lifted of the glass and the stacking gel was cutoff and bottom gel was placed in washing solution consisting of 200 ml ethonol (95 %) and acetic acid (5%) and continuously rocked for 25 to 30 minutes. Next washing solution was drained of and 0.011 silver nitrate added for 50 minutes and then drained off. The gel was then briefly rinsed twice with distilled water. Developing solution (NaOH 15 gram, 3.8ml formaldehyde dissolved in 500ml distilled

Age (months)	ELISA + ve only	PAGE + ve only	ELISA & PAGE +ve	Rota Virus Positive Group		Rota Virus Negative Group		Grand Total
				No.	%	No.	%	
< 6	–	–	2	2	28.6	5	71.4	7
6 - 12	1	2	22	25	30.6	56	69.1	81
13 - 24	–	1	19	20	34.5	38	65.5	58
25 - 36	–	2	–	2	11.1	16	88.9	18
37 - 48	–	–	1	1	5.6	17	94.4	18
49 - 60	–	–	1	1	5.6	17	94.4	18
Total	1	5	45	51	25.5	149	74.5	200

[Table /Fig-1]: Incidence of Rotavirus in Children of different age Groups.

water) was added for 5 to 10 minutes. This was replaced with stopping solution namely 5% acetic acid for 5 min and examined for the eleven bands. Total time for PAGE and silver staining was approximately 5 hours which included 15 min for RNA extraction, 3h for run and 2h for staining.

In each run a control strain i.e., SA-11 (Simian rotavirus strain) was run which was obtained from NIV Pune.

Culture of stool samples were done to know the association of common enteric pathogen with rotavirus positive cases by using standard culture techniques [9].

RESULTS

During the study 51 out of 200 samples (25.5%) were positive for rotavirus infection by either PAGE or ELISA methods. Children belonging to the study group were in relation to their ages in months as <6 months, 6-12 m, 13- 24 m, 25-36 m, 37-48 m, and ≥49 m [Table/Fig-1]. Maximum incidence of rotavirus infection was seen in age group of 6 m-24 m (32.3%), whereas age groups <6 and >24 months showed an incidence of 9.8%. The study shows a statistically significant difference (Z = 4.27, P = 0.001) in the incidence of rotavirus infection between the age groups 6-24 months and < 6 months and >24 months. The youngest patient found to be positive for rotavirus infection in this was 4 months old and the oldest was 60 months (5 years).

Out of a total of 51 children showing evidence of rotavirus infection, 34 were male and 17 were female giving a ratio of 2:1 with male predominance.

Rotavirus positive samples were found throughout the study period from November to July, except in the month of July where no cases were detected. Maximum incidence of rotavirus positive samples was noted in January (28%) and February (28%). The incidence showed a declining trend between March to June i.e. from 12 % to 2 % [TableFig-2].

All the 200 samples were separately subjected to ELISA and PAGE. A total of 51 (25.5%) of them were found to be positive for rotavirus by either methods. 46(23%) samples were shown positive by ELISA method alone. Where as, in PAGE 50 (25%) samples were positive. Among 45 samples which were positive by both ELISA and PAGE methods, 31 showed long and 14 short electrophoretypes.

Excellent correlation of ELISA and PAGE results was found in 194 of 200 (97%) specimens. 45 (22.5%) were positive and 149(74.5%) were negative for rotavirus as shown by both the methods. Remaining 6(3%) samples showed conflicting results between ELISA and PAGE. Among these 5(2.5%) ELISA negative samples

Month	Total ELISA + ve	Total PAGE + ve	ELISA & PAGE + ve	Total cases (200)
November	2	2	2	9
December	3	5	3	18
January	13	14	13	37
Febuary	12	14	12	21
March	6	6	6	21
April	4	4	4	25
May	5	4	4	39
June	1	1	1	10
July	–	–	–	20

[Table/Fig-2]: Seasonal distribution of Rotavirus Positive cases.

were clearly shown to be rotavirus positive by a single PAGE test. This demonstrates sensitivity of PAGE over the ELISA method. Only 1(0.5%) sample with positive ELISA result was shown to be PAGE negative. There was a perceptible though statistically non significant (p= 0.07) difference between the proportion of ELISA +, PAGE – samples (1/200) and ELISA–, PAGE+ (5/200). Fifty one samples were found positive by at least one method. 46 ELISA positive and 50 PAGE positive. Hence, relative sensitivity of PAGE and ELISA were 98% and 90 % respectively. This suggests that PAGE is more sensitive than ELISA.

Analysis of RNA pattern: A total of 50-rotavirus positive sample by PAGE were studied for their RNA migration pattern on poly acrylamide gel. The migration patterns were classified as long and short; the 'long' RNA pattern recognized by faster migration of gene segments 10 and 11 and 'short' pattern in which there was slower migration of gene segments 10 and 11. The migration pattern of SA11 virus (Simian virus) was documented as a control strain. Control strain SA11 migrates down with 9 distinct bands. Gene segments 3, 4, 8 and 9 co migrate [10]. There were 31 long electrophoretypes and 14 short electrophoretype observed in our study. Analysis of RNA pattern in 5 of the ELISA negative, but PAGE positive samples showed 4 long electrophoretypes and 1 Short electrophoretype.

Associated enteric pathogens: All 51 of the rotavirus positive cases did not show simultaneous infection with bacterial pathogens. Whereas in the remaining (n=149) in whom rotavirus couldn't be demonstrated by both ELISA and PAGE method, pathogenic bacteria were isolated in 37 samples out of 149 samples with an isolation rate of 24.83%. Among the bacterial pathogens isolated, E. coli were isolated in 22 samples (59.46%), Vibrio cholera in 13 (35. 14%) and Klebsiella in 2 of the samples (5.40%). No shigella or salmonella were detected.

DISCUSSION

During the current study 51 out of 200 samples (25.5%) were positive for rotavirus infection by either PAGE or ELISA methods. The available data highlights the importance of rotavirus as a cause of diarrhea in children, which is severe enough to deserve specialized care. The observed proportion of 25.5% of all diarrhea cases being associated with rotavirus falls within the range of values reported by workers from India. The reported positivity varies from 10.5% to 70.7% [4,11,12]. The positivity rates also vary between various settings, i.e. hospitalizations, symptomatic and asymptomatic infections and nosocomial infections [13]. In this study majority of children who showed evidence of rotavirus infection belonged to the age group of 6 months to 24 months(32.3%), whereas other children <6 and >24 months accounted for only in 9.8%. Many investigators from different parts of India expressed their similar views about more prevalence of rotavirus infection occurring in the age group of 6-24 months [4,14,15,16]. It appeared that infants below 4 months of age were initially protected to some extent by maternal antibodies against severe diarrhoea due to rotavirus [4]. The greater risks of infants and young children in the interim period of 6 to 12 months with declined levels of maternal antibodies to rotavirus infection have been documented [4].

Sex distribution of rotavirus positive children in our study showed a Male: female ratio of 2:1. Similar male predominance in the percentage incidence of rotavirus infection was reported by some of the authors [2,17].

Analysis of seasonal variation pertaining to rotavirus revealed that cooler months had increased rate of rotavirus associated diarrhea than the hotter months. Similar observations were made by some reports from India and other countries [4,18,19,20]. It has been observed that temperature influences the stability of human and animal rotavirus that contributes to the efficient transmission of the human rota virus [4]. Moreover the influence of low relative humidity in the home has been suggested as a facilitating factor for the survival of rotaviruses on surface. This is suggestive of the indirect but important influence of meteorological factors on the complex epidemiology of human rotavirus infection [4].

In our study we did not find simultaneous infection with bacterial pathogens in rotavirus positive cases. Some of the authors [14,21] showed an association of bacterial pathogens with rotavirus positive cases. Various enteropathogens isolated in their study were *E coli, Salmonella, Shigella* and *V. cholera* and the isolation of these bacterial pathogens was higher in rota virus negative cases. This finding co relates with our study. *E coli, V cholera* and *Klebsiella sps* were the bacterial isolates in our rota virus negative cases i.e., in 37 of 149 cases.

The earliest technique used to diagnose Rotavirus infection was direct electron microscopy. The identification of virus is done based on morphology. Hence, it is 100% specific. It suffers from low sensitivity being able to detect only about 100,000,000 particles / ml [22]. Immune electron microscopy (IEM) increased the sensitivity of electron microscopy by a factor of 100, detecting about 1,000,000 particles/ml. The principle disadvantages are the need for electron microscope, and very careful titration to determine the optimum ratio of antigen and antibody and prozone phenomenon [23]. Isolation of rota virus has a sensitivity of about 500 infectious particles/ml [24]. This level of sensitivity is reached by ELISA with much less labour.

In our study a complete concordance of ELISA and PAGE results were observed in 194 (97%) of the 200 tested specimens. This finding closely correlates with the findings of other authors who found a 96.7% to 97.14% [10,25,26] concordance results between ELISA and PAGE methods.

The remaining 6 (3%) samples showed conflicting results. In a lone sample in which the O.D value of ELISA test was 0.195, this value was almost at the cutoff level, the possibility of this sample being positive by ELISA test is doubtful. Negative result of the same sample in PAGE method is difficult to explain, the possibility of presence of lot of empty virus particles or due to low concentration of viral RNA in the fecal specimen and insufficient extraction of viral RNA could be possible.

On the other hand, 5 of the samples which gave positive results by PAGE method were negative by ELISA test. These 5 samples had a typical 4-2-3-2 RNA pattern. The reason for their being ELISA negative thus remains unexplained, however blocking factors [27] or the presence of inhibitory substance [28] in stools might have been responsible. The samples containing predominantly complete particles can also give false negative results [29]. Since, the group antigen is not exposed. Earlier studies [30,31] have also reported PAGE to be the most sensitive technique although some are of view that it is laborious procedure. How ever, the PAGE system used in this study was very simple to perform and the results were available on the same day. The main requirement was of trained personnel and proper standardization of the technique. Most reports states that the greatest advantage of PAGE and silver stain method are its lack of ambiguity and the fact that it provides information about viral electropherotypes. More over it generated epidemiological data regarding the circulation of strains in the community.

CONCLUSION

The modified PAGE system was thus found to be reliable, rapid, no expensive reagents were required and simple enough to establish in small laboratories, in which facilities and budgets are limited. Locally available reagents from HI media were used. A locally produced slab gel electrophoresis system with power pack was the only equipment required. This method could be used for the routine diagnosis of rotavirus infection in the laboratory.

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FINANCIAL OR OTHER COMPETING INTERESTS:

None.

Date Of Submission: **Dec 05, 2011**
Date Of Peer Review: **Feb 07, 2012**
Date Of Acceptance: **Feb 20, 2012**
Date Of Publishing: **Apr 15, 2012**