ISSN : 2309-3234

TMSS MEDICAL COLLEGE JOURNAL

Volume 04, No. 01, January 2015

An Official Publication of TMSS Medical College, Bogra

TMSS Medical College Journal

January 2015

An Offical Publication
of
TMSS Medical College, Bogra

TMSS Medical College Journal

Vol.3, No. 1, January 2015

An Offical Publication of TMSS Medical College, Bogra, Bangladesh

Published by

Dr. Md. Abdul Mazid
on behalf of the TMSS Medical College

Graphic Designer

Md. Sirajul Islam
TMSS, Bogra

Printed by

TMSS Printing Press
Bogra, Bangladesh

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Comparative Detection of Rotavirus From Stool by Two Diagnostic Tools.

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Abstract

Background: Rotavirus infects almost all children by the age of five. More than 180,000 annual deaths due to rotavirus, occurs in Bangladesh. 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 rotavirus in stool samples. **Materials and Methods:** In this descriptive type cross sectional study, a total of 400 stool samples were examined for the presence of rotavirus by ELISA and by a modified PAGE analysis of viral genome. Stool culture was done for common enteric pathogens. The study was carried out from November 2012 to July 2013 in the department of Microbiology, Mymensingh Medical College, Mymensingh. **Results:** Maximum incidence of rotavirus infection was seen in age group of 6 months - 24 months (67.25%). Excellent correlation of ELISA and PAGE results was found in 365 of 400 (91%) specimens. A total of 125 (31%) of them were found to be positive for rotavirus by either methods. The proportion of ELISA +ve PAGE -ve samples (6/400) was lower than the proportion of ELISA-ve PAGE +ve samples (29/400). All 151 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), Diarrhoea.

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 180,000 occur in Bangladesh. Also, 20 to 30 percent hospitalized cases of diarrhoea are due to rotaviruses[4].

Clinically rotavirus gastroenteritis is characterized by profuse diarrhoea, mild fever and vomiting leading to mild to severe dehydration. The clinical manifestations of rotavirus diarrhoea alone are not sufficiently distinctive to permit diagnosis[1]. The laboratory diagnosis of Rota virus 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

During the period of November 2012 to July 2013, all patients with acute diarrhoea irrespective of sex admitted in Mymensingh Medical College Hospital and S.K hospital, Mymensingh were included in this study. Information about children and their consent were taken from their parents/guardians.

Inclusion Criteria

- Children not older than 5 years of age group.
- Children with profuse watery diarrhoea
- Diarrhoea less than 10 days duration.
- Exclusion Criteria
- Children above 5 years.
- Children with bloody and mucus diarrhoea
- Diarrhoea more than 10 days.

Stool samples were investigated for rotavirus 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} Japan[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 40 µl of RNA extract to which10 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 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 Japan[6].

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

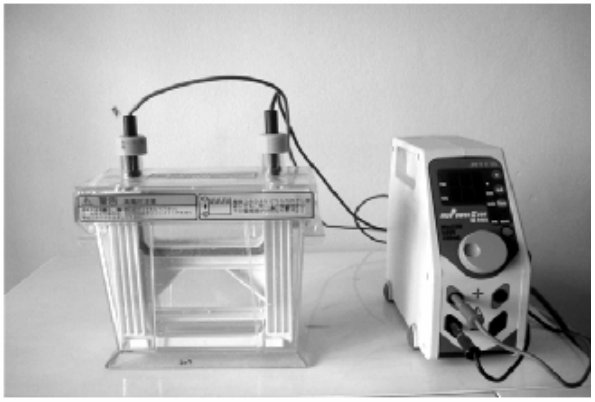

Figure : Polyacrylamide gel electrophoresis Chamber

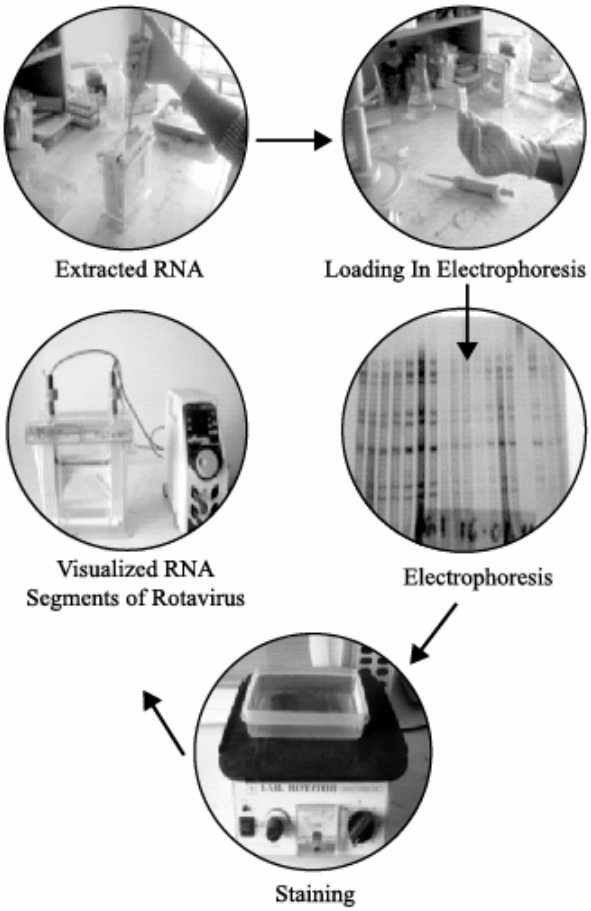

Figure: Pictorial presentation of major steps of PAGE

Photograph of polyacrylamide gel electrophoresis of rotavirus showing segments(11) of RNA genome.

Table 1: Incidence of Rota virus in children of different age groups

Age (Month)	ELISA +VE ONLY (%)	PAGE +VE ONLY (%)	ELISA& PAGE +VE (%)	Both Negative (%)	GRAND TOTAL (%)
6	4	2	7	1	14
6 - 12	30	84	44	4	162
13 - 24	25	49	37	5	116
25 - 36	13	21	23	-	57
37 - 48	–	10	4	2	16
49 - 60	–	2	10	23	35
Total	72(18)	168(42)	125(31)	35(9)	400(100)

Table 2: Seasonal distribution of Rota virus positive cases

Month	Total ELISA (+ ve)	Total PAGE (+ ve)	ELISA & PAGE (+ ve)	Total cases (400)
November	6	4	16	28
December	7	17	11	36
January	21	10	32	64
February	18	24	28	72
March	12	31	12	62
April	12	21	14	50
May	15	28	4	48
June	9	6	7	28
July	–	–	–	12

Results

During the study, correlation of ELISA and PAGE results was found in 365 of 400 (91%) specimens. And in the study 125 out of 400 samples (31%) 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 [Table1]. Maximum incidence of rotavirus infection was seen in age group of 6 m-24 m (67.50%), whereas age groups <6 and >24 months showed an incidence of 23.75%. 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).

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 February (18%) and January (16%). The incidence showed a declining trend between March to June i.e. from 12 % to 5 % [Table 2].

The proportion of ELISA +ve PAGE -ve samples 6/400 was lower than the proportion of ELISA-ve

PAGE +ve samples (29/400). Hence, relative sensitivity of PAGE and ELISA were 98% and 92 % respectively. This suggests that PAGE is more sensitive than ELISA.

All 151 rotavirus positive cases did not show infection with bacterial pathogens.

Discussion

During the current study 125 out of 400 samples (31%) 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 31% of all diarrhea cases being associated with rotavirus falls within the range of values reported by workers from Bangladesh. 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 (67.50%), whereas other children <6 and >24 months accounted for only in 23.75%. Many investigators from different parts of Bangladesh 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].

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 Bangladesh 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.

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[2]. 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 365 (91%) of the 400 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 35 (9%) 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[25,26].

On the other hand, 6 of the samples which gave positive results by PAGE method were negative by ELISA test. These 6 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. 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.

Acknowledgement

We are grateful to Professor Nobumichi Kobayashi, Sapporo Medical University of Japan for providing with us equipment of PAGE and technical assistance for this study.

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