A study on the possibility of zoonotic infection in rotaviral diarrhoea among calves and buffalo calves in and around Kolkata, India

S.M. NATARAJU, U.K. CHATTOPADHYAY, T. KRISHNAN*

All India Institute of Hygiene & Public Health, Government of India, Kolkata (India)
*National Institute of Cholera & Enteric Diseases, Kolkata (India)

Abstract. – Group A and group B rotaviruses are important diarrhea causing agents among calves and buffalo calves. Epidemiological studies in Indian calves revealed the predominance of group A rotavirus strains with G6, G8, and G10 specificity and group B rotaviruses. A total of 95 fecal samples were collected from calves and buffalo calves affected with diarrhea from an unorganized cattle farm and two cattle markets in and around Kolkata, in the state of West Bengal of Eastern India. Rotaviruses were detected in 23.15% (22/95) samples by polyacrylamide gel electrophoresis. Of 22 rotavirus positive cases, 10.52% (10/95) samples showed characteristic group A rotavirus-like long type electropherotype (e-type) pattern and 4.21% (4/95) samples showed the characteristic group B rotavirus long type of electropherotype pattern and in 8.42% (8/95) the electropherotype pattern could not be recorded. Out of 22 positive samples, 7 samples of group A rotaviruses were subjected to reverse transcription-polymerase chain reaction, using VP7 generic and genotype [G type] specific primers and 2 of 7 isolates were identified as G10.

Key Words: Group A Rotavirus, Calves, Zoonoses, Kolkata.

Introduction

Rotavirus, a genus within the family Reoviridae, is recognized as the most important viral etiological agent of severe diarrhoeal illness in humans, young animals and birds worldwide. In animals, rotaviruses are major cause of diarrhea in intensively reared farm animals throughout the world. The genome consists of 11 segments of double-stranded RNA. Each of the 11 segments encodes 12 proteins of which 6 are structural and 6 are nonstructural in nature. On the basis of serological tests like immunofluorescence, enzyme linked immunosorbent assay (ELISA) and immune electron microscopy (IEM), rotaviruses are differentiated into 7 groups (A-G). Group A, B and C rotaviruses have been found in both humans and animals, whereas viruses in the groups D, E, F, and G found only in animals till date. In most cases, the electrophoretic pattern for the genome of the Group A rotaviruses is composed of four high molecular weight dsRNA segments (numbered 1-4), two middle sized segments (5 and 6), a distinctive triplet of segments (7 to 9) and two smaller segments (10 and 11). The two structural outer capsid proteins, VP7 (Glycoprotein) and VP4 (Protease sensitive protein) define the G and P serotype/genotype of rotavirus respectively. The VP7 genotype is designated as G-genotype. There are 15 G-genotypes known to date and designation for G-serotype (type determined by neutralization assay) and G-genotype (types determined by amplification of VP7 genes) are identical. VP4 serotype is classified into P (VP4 codes for protease sensitive) serotype. P genotype is designated as a P, followed by assigned number in bracket, designates the P genotype.

Materials and Methods

Sample Collection

A total of 95 fecal samples were collected from calves (55), buffalo calves (40), showing acute diarrhoeal symptoms, by aseptic methods (per rectally using gloved finger) taking care of all precautions so as to avoid contamination of
the samples. Samples were collected from an unorganized cattle farm and two cattle markets in and around Kolkata, in the state of West Bengal of Eastern India. Samples were transported to laboratory in sterile containers by using ice packs. Then samples were stored in the refrigerator at 4°C.

**Extraction of Rotavirus dsRNA from Virus Suspension and Polyacrylamide Gel Electrophoresis**

Fecal suspension was prepared in phosphate buffered saline (1xPBS) and clarified by centrifugation at 3000 rpm for 20 minutes, followed by recentrifugation at 7000 rpm for 20 minutes at 4°C. The supernatant containing virus was carefully aspirated and stored in a fresh sterile 2 ml Eppendorf tube at 4°C as virus suspension. RNA extraction was done by using 50 μl of the viral suspension and to it, 150 μl of 0.1 M sodium acetate buffer with 1% sodium dodecyl sulphate was added. After addition of 200 μl of phenol-chloroform-isooamyl alcohol mixture, the tubes were vortexed vigorously for one minute. The tubes were then centrifuged at 10,000 rpm for 15 minutes at 4°C in a tabletop refrigerated centrifuge to separate the aqueous and phenolic phases. The supernatant containing dsRNA (aqueous phase) was aspirated and extracted RNA was subjected to polyacrylamide gel electrophoresis (PAGE) and silver staining of the gel as per method described by Herring et al3. Then the gel was first examined on a light box to check for the presence of 11 segmented genomic dsRNA of rotaviruses and then photographed using the gel documentation system (BioRad).

**Reverse Transcription (RT) for VP7 Gene**

Extraction of high quality viral dsRNA from viral suspension was done by using QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the instructions of the manufacturers. The pure RNA was then subjected to RT-PCR. For RT-PCR, at first dsRNA was incubated at 56°C for 5 minutes. A mixture of 8 μl of the extracted dsRNA with 2.5 μl of dimethyl sulfoxide, 1 μl of VP7 antisense (VP7R) and 1 μl of VP7 sense primers (VP7F) (Table I) were added to a 0.5 ml microcentrifuge tube, vortexed and centrifuged properly. The samples were then incubated at 98°C for 5 mins and then immediately cooled on ice and used as template for the RT reaction. Next 12.5 μl of cooled template was added to RT mix consisting of 4 μl of 5x First strand buffer [250 mM Tris-HCl (pH 8.3 at room temp.), 375 mM KCl, 15 mM MgCl2], 2 μl of 0.1 (mM) dithiothreitol (DTT) and 1 μl of deoxynucleoside triphosphate (dNTP) mix (dATP, dCTP, dGTP and dTTP). The above mixture was mixed properly by vortexing and then pulsed down. Then finally 0.5 μl (50 U) Superscript II Reverse transcriptase was added to the above mix. The thermal cycler was programmed to operate at required temperature and timings for RT reaction for reverse transcription of RNA. The mixture was incubated at 25°C for 10 mins, 42°C for 50 mins, 70°C for 10 mins and kept on hold at 4°C in the Thermal Cycler.

**Amplification of VP7 Gene**

For amplification of VP7 gene, forward (VP7F) and reverse (VP7R) primers, were used in the first round PCR to generate the 881 bp partial length VP7 gene product. For each sample, 5 μl of cDNA was added to a 0.5 ml PCR tube containing 2.5 μl of 10XPCR buffer [200 mM Tris-HCl, (pH 8.3), 500 mM KCl, 0.75 μl MgCl2 2H2O (50 mM), 0.5 μl dNTP mixture [0.2 mM dATP, dACTP, dGTP, dTTP]; 1 μl of primer (VP7R), 1 μl of forward primer (VP7F) and 14 μl sterile triple distilled water. Above mixture was vortexed properly and given a small spin. Then subsequently 0.25 μl of Taq polymerase was added to each reaction tube. The tubes were placed in a thermal cycler and the PCR reaction was cycled 35 times maintaining the proper conditions.

**G Typing**

The VP7 generic primer and different pools of specific primers (Table I) for G1, G2, G3, G4, G8, G9, and G10 are forward primers whereas VP7R was a reverse primer. An aliquot of 2 μl of VP7 PCR product was added to a reaction mixture consisting of 0.75 μl of MgCl2, 2.5 μl of 10xPCR buffer 1 μl of each dNTP, 0.5 μl (10 pmol/μl) of each primer 0.25 μl of Taq Polymerase and triple distilled water were added to a final volume of 25 μl. The second round of amplification consisting of 25 cycles was carried out maintaining the proper conditions in the thermal cycler.

**Results**

The incidence of rotavirus was 27.3% (15/55) and 17.5% (7/40) for calves and buffalo calves re-
spectively. Age group of calves and buffalo calves was under one month, most of them were between 10-15 days old. Among calves, male calves showed higher incidence 32% (8/25) and among buffalo calves male buffalo calves showed higher positivity 5/23 (21.74%). Among the sites of sample collection, both cattle markets showed almost same rate of incidence 29.42% (5/17) and 30.76% (16/52) whereas organized farms showed less incidence 3.85% (1/26). Of 22 rotavirus positive cases 10 (10.52%) samples showed characteristic group A rotavirus-like long type electropherotype (e-type) pattern in 7% PAGE. In course of this study, 4 different e-types of group A rotavirus were detected. The 4 different types of migration patterns were Group A (long) 1,2-3,4 5,6 7-8,9 10,11; group A (Short) 1,2,3,4 5,6 7-8,9 10,11 (Table II, Figure 1). During the course of study, group A rotavirus electropherotype L3 (12.72%, 7/55) and L1 (5%, 2/40) was observed more often in calves and buffalo calves respectively. Only one buffalo calf showed group A rotavirus electropherotype S1 (2.5%, 1/40). (Table II, Figure 2). Of 22 rotavirus positive samples 2 (2.10%) samples showed the characteristic group B rotavirus long type of electropherotype pattern. In this study 2 different types of group B rotavirus electropherotypes were detected and the 2 different types of migration patterns were group B (long) 1,2,3,4 5,6 7-8,9 10,11 and group B (long) 1,2,3,4 5,6 7-8,9 10,11 (Table II, Figure 2). Group B rotavirus positivity in calves with electropherotype LI and buffalo calves with electropherotype LII was 1.82% (1/55) and 2.5% (1/40) respectively.

Table I. List of primers used to amplify the VP7 gene.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Name r of prime</th>
<th>Sequences</th>
<th>Primer position</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>VP7F[+]</td>
<td>5’ ATG TAT GGT ATT GAA TAT ACC AC 3’</td>
<td>51-71</td>
<td>881bp</td>
</tr>
<tr>
<td>2</td>
<td>VP7R[-]</td>
<td>5’ AAC TTG CCA CCA TTT TTT CC 3’</td>
<td>914-932</td>
<td>881bp</td>
</tr>
<tr>
<td>3</td>
<td>G1[+]</td>
<td>5’ CAA GTA CTC AAA TCA ATG ATG G 3’</td>
<td>314-335</td>
<td>618bp</td>
</tr>
<tr>
<td>4</td>
<td>G2[+]</td>
<td>5’ CAA TGA TAT TAA CAC ATG TTT TTT CC 3’</td>
<td>411-434</td>
<td>521bp</td>
</tr>
<tr>
<td>5</td>
<td>G3[+]</td>
<td>5’ ACG AAC TCA ACA CGA GAG G 3’</td>
<td>250-269</td>
<td>682bp</td>
</tr>
<tr>
<td>6</td>
<td>G4[+]</td>
<td>5’ CGT TTC TGG TGA GGA GTT G 3’</td>
<td>480-498</td>
<td>452bp</td>
</tr>
<tr>
<td>7</td>
<td>G8[+]</td>
<td>5’ GTC ACA CCA TTT GTA AAT TCG 3’</td>
<td>178-198</td>
<td>754bp</td>
</tr>
<tr>
<td>8</td>
<td>G9[+]</td>
<td>5’ CTT GAT GTG ACT AYA AAT AC 3’</td>
<td>757-776</td>
<td>179bp</td>
</tr>
<tr>
<td>9</td>
<td>G10[+]</td>
<td>5’ ATG TCA GAC TAC ARA TAC TGG 3’</td>
<td>666-687</td>
<td>266bp</td>
</tr>
</tbody>
</table>

Table II. Different electrotypes of rotavirus A & rotavirus B detected in fecal samples of calves and buffalo calves in and around Kolkata, during the study.

<table>
<thead>
<tr>
<th>Kind of animal</th>
<th>No. of samples</th>
<th>No. of Electropherotype (%)</th>
<th>ND**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Rotavirus A</td>
<td>Rotavirus B</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L1</td>
<td>L2</td>
</tr>
<tr>
<td>Calves</td>
<td>55</td>
<td>1 (1.82%)</td>
<td>1 (1.82%)</td>
</tr>
<tr>
<td>Buffalo Calves</td>
<td>40</td>
<td>2 (5.0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Total</td>
<td>95</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Different long electropherotype are denominated L1, L2, L3, LI, & LII and short electropherotype S1; **Rotavirus positive undetermined electropherotype.
(Table II, Figure 2). 8 samples showed undetermined electropherotype rotavirus positivity pattern (Table II). Out of 22 positive samples 7 were selected for the further molecular characterization, two samples exhibited a 266 bp product corresponding to G10 specificity (Figure 3).

**Discussion**

In this study, age of positive calves was between 8-15 days and matched the observation by other group of workers who had reported age group as minimum 1 day to maximum of 30 days. Though previous studies in cattle in the markets of the city of Kolkata, reported the prevalence of group B rotavirus from calves in and around Kolkata, the present study is reporting high incidence of the group A rotavirus in calves and buffalo calves. The previous studies from cow and buffalo calves in an organized farm reported five distinct electropherotypes with all short electropherotypes patterns except the single long electropherotype observed in a buffalo calf. In the present study, 5 different patterns of long

![Figure 1](image1.png)

**Figure 1.** Gel electrophoresis showing the characteristic genomic double stranded RNA migration pattern of Group A bovine rotavirus circulating in and around Kolkata during the study.

![Figure 2](image2.png)

**Figure 2.** Gel electrophoresis --VV1119 LI- characteristic genomic double stranded RNA migration pattern of Group B bovine rotavirus circulating in and around Kolkata during the study.

![Figure 3](image3.png)

**Figure 3.** The RT-PCR product of the bovine rotavirus isolates. Lane 1 represents the 1 KB plus molecularweight marker (Invitrogen, USA). Lane 2 and 3 represents the amplified VP7 gene (266 bp).
type rotavirus have been observed with one short e-type pattern. Moreover, both rotavirus A and rotavirus B e-type patterns have been found among the rotavirus positive cases. Group A BRV was reported by group of workers\(^7\) with highest 62.5\% (250/400) of the total reported cases of diarrhea. In current study, group A rotavirus incidence is (20/95), whereas group B rotavirus is 2/95. Group B rotavirus incidence has also been referred by several researchers\(^8\) with different rate of prevalence. In the present study on G typing of rotaviruses 2 G10 rotaviruses were observed. Many group of workers reported G10 as a highly prevalent strain circulating in bovines. Our study agrees with the other researches\(^11\). It is seen that G6, G8, G10 are the common G types found in bovines\(^13\) but this study it was found that G 10 rotavirus was seen in the selected positive samples after RT PCR. Various hypotheses previously supported that BRVs may cross the host species barrier and circulate among neonates or adults and can cause zoonotic transmission and can infect human beings. We observed that G10 genotype of rotavirus is prevalent in Kolkata during the period of this study and thereby it needs more molecular work to prove the possible zoonotic transmission.

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References


