ABSTRACT

Fifty-three faecal samples from diarrheic calves were collected from November 2008 to March 2009 and screened by LAT, and polyacrylamide gel electrophoresis (PAGE) to detect the presence of group A rotavirus antigen. Of the 53 samples screened by LAT, 17 (32.08%) tested positive for rotavirus antigen. When the results from the PAGE were compared to those from LAT, the “gold standard” for detection of bovine rotavirus in fecal samples, the sensitivity and specificity were found to be 52.94 and 100%, respectively. Latex agglutination is easy to perform in a short time and does not require expensive equipment or skilled personnel, and the reagents have long shelf lives. These factors make the LAT suitable and highly efficient for use in a clinical laboratory as a rapid screening test for bovine rotavirus.

Keywords: bovine rotavirus, latex agglutination, PAGE

INTRODUCTION

Livestock farming plays an important role in India. The future of any dairy operation depends upon a successful program of raising calves. Incidence of neonatal calf mortality varies from 8.7 to 64 percent throughout world. Among the infectious diseases of calves, neonatal diarrhoea is a matter of major concern, and multiple etiological agents have been involved (Steele et al., 2004; Gumusova et al., 2007). Rotavirus is a main cause of neonatal diarrhoea and has been documented worldwide. It has been reported that diarrhoea in calves from 5-10 days of age is commonly due to rotavirus and infected calves excrete rotavirus in their faeces up to the age of 6 to 8 weeks (Tzipori, 1985; Radostitis, 1986). Group A rotaviruses are morphologically identical but antigenically and electrophoretically distinct from other non-group A rotaviruses (B, C, D, E) (Saif et al., 1988). Group A rotaviruses, belonging to the family Reoviridae, are important viral diarrhoeal agents in children and young animals, including calves, worldwide. These viruses possess eleven segments of double-stranded ribonucleic acid (dsRNA) and two outer capsid proteins, VP4 and VP7, both of which are independently responsible for virus neutralisation (Estes, 2001). Antigenic specificity carried by the VP4 and VP7 proteins is termed P and G genotype/serotype, respectively (Estes and Cohen, 1989). At least, 15 G types and 26 P types have been recognized so far (Kapikian et al., 2001). In India, although the occurrence of BRV-related diarrhoea has been well documented, this paper describes the incidence and electropherotyping of bovine rotavirus in diarrhoeic buffalo and cattle calves.
MATERIALS AND METHODS

A total of 53 faecal samples were collected from nine buffalo calves and 44 cattle calves of 0-8 weeks of age from both organized and unorganized farms in and around the Anand area, including the Livestock Research Station, Anand, Gujarat.

Latex Agglutination Test

An approximately 10% (v/v) suspension of the faecal samples were made by using one ml extraction buffer to 0.1ml of faecal sample in a centrifuge tube. The suspension was centrifuged at 1000Xg for 10 minutes and the supernatant was collected. Two separate drops of the supernatant from each sample were placed, one onto the left black circle, the other onto the right black circle of the test card from the Rotalex kit. The contents of the Rotalex latex reagent vial and the Rotalex control latex reagent vial were mixed by gently rolling the vials between the fingers. A drop of Rotalex latex reagent and Rotalex control reagent was added in left and right circles, respectively, already containing a drop of faecal supernatant. Using clean end of mixing sticks, the two droplets in each circle were mixed carefully trying to cover the full area of the black circle. The test card was tilted and rotated moving the reagents in a circular motion within the circles. It was observed for appearance of latex particles for evidence of agglutination occurring within two minutes.

Extraction of double-stranded ribonucleic acid

A 10% faecal suspension of each sample prepared in phosphate-buffered saline and clarified by centrifugation at 10,000 rpm for 30 minutes. at 4°C was used as the basis for extraction of rotavirus ribonucleic acid (RNA). Viral RNA extraction was done using the phenol chloroform method as described by Herring et al. (1982) with slight modification. In brief, 800 μl of faecal supernatant was treated with 0.1 ml of 10 % sodium dodecyl sulphate (SDS) and 0.1 ml of 2M sodium acetate pH 4.2 (Appendix), followed by incubation at 56°C for one hour in a water bath. An equal volume of tris-saturated phenol: chloroform: isoamylalcohol (25:24:1) mixture was added to the faecal suspension. It was then vortexed and centrifuged at 12,000 rpm for 10 minutes at 4°C. The upper aqueous layer was transferred carefully to another fresh tube without disturbing the interface. The phenol: chloroform: isoamylalcohol extraction was repeated till a clear interface was obtained. The resultant aqueous solution was mixed with an equal volume of chloroform: isoamylalcohol (24:1) and vortexed, and then the mixture was centrifuged again at 12000 rpm for 10 minutes, and the upper clear aqueous phase was transferred to a fresh microcentrifuge tube. To this aqueous solution, a 0.1 volume of 3 M sodium acetate (pH 5.2) was added and vortexed. After adding an equal volume of isopropanol, the eppendorf tube was inverted 4-5 times and left overnight for precipitation at -20°C. The precipitated RNA was pelleted by centrifuging at 12000 rpm for 30 minutes at 4°C. The pellet was then washed with one ml of prechilled 75% ethanol by centrifuging at 12000 rpm for 15 minutes at 4°C and air dried. The pellet was suspended in 20 μl DEPC treated MilliQ water and stored at -20°C till RNA PAGE analysis.

RNA-PAGE. The extracted viral dsRNA was analyzed by PAGE, which was performed according to the method of Laemmli (1970) with minor modifications. Briefly, PAGE was performed at 100 V for 5-6 h using 5% stacking and 8%
separating polyacrylamide gel. The extracted viral dsRNA was mixed with 0.25% w/v bromophenol blue solution and 40% w/v of sucrose and loaded in wells to perform PAGE.

**Silver staining.** Silver staining of the polyacrylamide gel was performed according to the method of Svensson et al. (1986). Briefly, the polyacrylamide gel was shaken for 30 minutes in a mixture of 10% (v/v) ethanol and 0.5% (v/v) acetic acid. The mixture was removed and the gel was shaken for 30 minutes in 0.1 M silver nitrate solution. The silver nitrate solution was removed, and the gel was washed three times in distilled water and then shaken for 15 minutes in a mixture of 0.75 M NaOH and formaldehyde. The gel was washed twice in distilled water, and shaken for 5 minutes in 5% (v/v) acetic acid solution, and the electropherotype was then identified.

**RESULTS**

Of a total of 53 diarrhoeic samples tested for rotavirus, 17 (32.08%) were found positive for rotavirus by the LA test as indicated by clear agglutination of latex particles in test samples. Out of nine buffalo calves tested, 7 (77.78%) were found positive for rotavirus. Similarly, 10 cattle calves out of 44 tested were positive for rotavirus with a 22.73% prevalence.

During the study, diarrhoeic samples collected from buffalo and cow calves were divided into two groups each to find the susceptibility of the animals according to age and sex. The results showed that female calves (38.46%) were more susceptible than the male calves (25.93%), as 10 out of 26 females calves and seven out of 27 male calves were positive (Table 1).

Agewise, seven, five, four and one samples were positive out of 20, 16, 12 and five samples collected from age groups of 0-2, 2-4, 4-6 and 6-8 weeks, respectively, yielding agewise incidences of 35.00, 31.25, 33.33 and 20.00 percent (Table 2).

Out of 53 faecal samples tested, nine (16.98%) samples were found positive for rotavirus by RNA-PAGE. PAGE of genomic RNA obtained from the nine positive faecal samples showed a well-defined and reproducible pattern of 11 segments. All the nine electropherotypes belonged to a long genome electropherotype pattern as per Tam et al. (1986). Further analysis of PAGE was done on the basis of migration and co-migration of 11 segments in the I, II, III and IV regions and were analysed as per Tam et al. (1986) and Rasool et al. (1989). The subgroup analysis revealed that all the nine positive samples belonged to the II subgroup as they revealed separate 4-2-3-2 patterns with a long migration pattern of the 10th and 11th segments. Of these, two samples (B4 and B29) from buffalo calves (belonging to the same farm) yielded the G pattern (distinct and separate 1, 2, 3 and 4 segments and co-migration of segments 7, 8 and 9 was designated as G), while the remaining seven showed the F pattern (Co migration of 2 and 3 and of 7, 8 and 9 as F). Of the seven the IIF subgroups, one was from a buffalo calf and all the six positive samples from cattle calves yielded IIF subgroup pattern (Table 3).

**Relative sensitivity and specificity of LA and PAGE**

Diagnosis of rotavirus infection is conventionally made by detection of the virus, viral RNA segments or viral antigen in the faeces. A variety of tests are available for this purpose. In the present study, relative sensitivity and specificity of LA and RNA-PAGE were compared using 53 faecal samples from the diarrhoeic bovine calves.
Table 1. Species-wise prevalence of rotavirus.

<table>
<thead>
<tr>
<th>Species</th>
<th>Samples tested</th>
<th>Rotavirus +ve</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffalo</td>
<td>09</td>
<td>07</td>
<td>77.78</td>
</tr>
<tr>
<td>Cattle</td>
<td>44</td>
<td>10</td>
<td>22.73</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>53</strong></td>
<td><strong>17</strong></td>
<td><strong>32.08</strong></td>
</tr>
</tbody>
</table>

Table 2. Age- and Sex-wise prevalence of rotavirus by LA.

<table>
<thead>
<tr>
<th>Age (Weeks)</th>
<th>Male calves screened</th>
<th>+ve by LA</th>
<th>Incidence (%)</th>
<th>Female calves screened</th>
<th>+ve by LA</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-2</td>
<td>11</td>
<td>04</td>
<td>36.36</td>
<td>09</td>
<td>03</td>
<td>33.33</td>
</tr>
<tr>
<td>2-4</td>
<td>08</td>
<td>02</td>
<td>25.00</td>
<td>08</td>
<td>03</td>
<td>37.50</td>
</tr>
<tr>
<td>4-6</td>
<td>04</td>
<td>-</td>
<td>-</td>
<td>08</td>
<td>04</td>
<td>50.00</td>
</tr>
<tr>
<td>6-8</td>
<td>04</td>
<td>01</td>
<td>25.00</td>
<td>01</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>27</strong></td>
<td><strong>07</strong></td>
<td><strong>25.93</strong></td>
<td><strong>26</strong></td>
<td><strong>10</strong></td>
<td><strong>38.46</strong></td>
</tr>
</tbody>
</table>

Table 3. Group, subgroup and electropherotypes of bovine rotaviruses.

<table>
<thead>
<tr>
<th>Source of Sample</th>
<th>Sample no.</th>
<th>Group as suggested by Parwani et al. (1995)</th>
<th>Sub group as suggested by Tam et al. (1986) and Rasool et al. (1989)</th>
<th>Long/ short electropherotype as suggested by Tam et al. (1986)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle calf</td>
<td>C2</td>
<td>Group A</td>
<td>IIF</td>
<td>Long</td>
</tr>
<tr>
<td>Cattle calf</td>
<td>C3</td>
<td>Group A</td>
<td>IIF</td>
<td>Long</td>
</tr>
<tr>
<td>Buffalo calf</td>
<td>B4</td>
<td>Group A</td>
<td>IIG</td>
<td>Long</td>
</tr>
<tr>
<td>Cattle Calf</td>
<td>C10</td>
<td>Group A</td>
<td>IIF</td>
<td>Long</td>
</tr>
<tr>
<td>,,</td>
<td>C16</td>
<td>Group A</td>
<td>IIF</td>
<td>Long</td>
</tr>
<tr>
<td>,,</td>
<td>C20</td>
<td>Group A</td>
<td>IIF</td>
<td>Long</td>
</tr>
<tr>
<td>,,</td>
<td>C21</td>
<td>Group A</td>
<td>IIF</td>
<td>Long</td>
</tr>
<tr>
<td>Buffalo calf</td>
<td>B29</td>
<td>Group A</td>
<td>IIG</td>
<td>Long</td>
</tr>
<tr>
<td>,,</td>
<td>B31</td>
<td>Group A</td>
<td>IIF</td>
<td>Long</td>
</tr>
</tbody>
</table>
As LA detected a higher number of samples positive than PAGE, sensitivity and specificity of PAGE considering LA as reference test were calculated as per Samad et al. (1994). Out of 53 samples, LA detected 17 samples positive and 36 negative for BRV, while PAGE detected nine and 44 samples as positive and negative, respectively. Eight samples negative by PAGE were positive by LA, while none of the samples negative by LA was positive by PAGE. Thus, the relative sensitivity and specificity of PAGE to LA were 52.94 percent and 100 percent, respectively. Overall agreement between the two tests was 83.02 percent (Table 4).

**DISCUSSION**

Out of nine buffalo calves tested, seven (77.78%) were found positive for rotavirus. Similarly, 10 cattle calves out of 44 tested were positive for rotavirus with a 22.73% prevalence. The results were in contrast with Singh et al., 1985, who showed overall prevalence of bovine rotavirus in cattle and buffalo calves as 46.29 and 25.65 percent, respectively. The higher percentage recorded in buffalo calves could be due to relatively smaller sample size in the present study. Overall incidence of rotavirus found in the present study was similar the studies by Herbst et al. (1986) and Erdogan et al. (2003), who observed 32.07% and 31.00% incidences, respectively. Other reports in India showed the lower prevalence of rotavirus (3% by Gandhi, 1992; and 6.12% by Vaugh, 2009).

In this study, female calves (38.46%) were found more susceptible than the male calves (25.93%). This accords with the study by Kusumakar (2006), in which female buffalo calves were reported more susceptible (25.00%) than male calves (21.00%). However, the result was in contrast to Sharma (2004), who observed higher susceptibility of male bovine calves (42.85%) to BRV in comparison to females calves (28.20%).

Kapoor (1988) and Lyoo et al. (1989) reported higher prevalence of rotavirus infection in diarrhoeic cattle calves and buffalo calves under the age group of 4-14 days. Kaushick et al. (1983) and Shah (1989) reported higher prevalence of rotavirus in buffalo and cow calves of 4-8 weeks of age. In the present study, all the positive samples except one were from the calves below six weeks of age. None of the male calves in the age group of 4-6 weeks were positive, while four out of eight female calves in the same age group were positive. This discrepancy might be due to the different locations of these calves.

The prevalence rate observed in our study (16.98%) was in agreement with earlier reports from Haryana in which prevalence rates of 11-43% by RNA-PAGE were reported (Singh and Pandey, 1990; Chauhan and Singh, 1993; Grover et al., 1998). Three (33.33%) out of nine samples from buffalo calves and six (13.64%) out of 44 samples from cattle calves were positive. Earlier, Sharma (2004) reported 40 percent and 34 percent and

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Table 4. Sensitivity and specificity of PAGE with LA for detection of BRV.

<table>
<thead>
<tr>
<th>Test</th>
<th>LA Positive</th>
<th>LA Negative</th>
<th>Total</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Overall Agreement (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAGE</td>
<td>9</td>
<td>0</td>
<td>9</td>
<td>52.94</td>
<td>100</td>
<td>83.02</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>36</td>
<td>44</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td>36</td>
<td>53</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Kusumakar (2006) reported 23.16 percent and 21.43 percent incidences of bovine group A rotavirus by PAGE in cattle and buffaloes, respectively. The relatively higher incidence by PAGE in buffalo calves might be due to the smaller sample size in present study. However, incidence in cattle calves can be considered reflecting a true picture of the selected population due to the optimal sample size.

Out of nine, two samples (B4 and B29) from buffalo calves (belonging to same farm) yielded the G pattern (distinct and separate 1, 2, 3 and 4 segments and co migration of segments 7, 8 and 9 was designated as G), while the remaining seven showed the F pattern (Co migration of 2 and 3 and of 7, 8 and 9 as F). Of the seven IIF subgroups, one was from a buffalo calf, and all the six positive samples from cattle calves yielded the IIF subgroup pattern (Table 3). Rasool et al. (1989) reported that the IIC pattern of electropherotype was predominate, followed by the IIG electropherotype during a 10-year study, while Dash (2008) found that the IIG pattern of electropherotype was predominate, followed by the IIC and IIF patterns of electropherotype. The usefulness of RNA-PAGE for detection and/or characterization of bovine rotavirus has been documented by various workers (Hammami et al., 1990; Gulati et al., 1995; Jindal et al., 2000; Fodha et al., 2005; Sharma et al., 2008).

Plate 1. Electropherotype of bovine rotavirus.
Lane 1, 2 shows Co migration of 2, 3 and 7, 8 and 9 segments designed as F pattern.
Lane 4 shows Co migration of 7, 8 and 9 segments designed as G pattern.
Lane 3, 5 and 6 are negative.
In our study, the relative sensitivity and specificity of PAGE to LA were 52.94 percent and 100 percent, respectively. Overall agreement between the two tests was 83.02 percent. Hammami et al. (1990) found LA to be more sensitive than PAGE in detecting BRV. Garcia Sanchez et al. (1993) found 93.33 percent agreement between PAGE and LA for BRV diagnosis. Beer et al. (1997) compared electron microscopy, PAGE and LA for the detection of bovine rotavirus in faeces and found that LA test was slightly more sensitive than PAGE and EM. Nussbaum et al. (1999) found that QLAT had 92.5% sensitivity and 96.8% specificity with PAGE for detection of rotavirus in faeces of calves.

Thus, a unified interpretation of the experimental findings from the present study indicates prevalence of BRV infection in cattle and buffalo calves of this area, with 32.08% incidence as ascertained by the LA test. Female calves and calves under six weeks of age appeared to be susceptible with calves under two weeks of age showing comparatively higher incidences of BRV. Electropherotyping of the local BRV by RNA-PAGE revealed Group A rotaviruses with long electropherotype, the majority of them falling into subgroup IIF, VP7 based RT-PCR using Heminested approach by G-type specific primers resulted in identification of G10 and G6 genotypes with G10 type predominating in this area. RNA-PAGE was less (52.94%) sensitive than LA in detecting BRV from faecal samples.

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