ABSTRACT

Determination of the G genotypes of group A bovine rotaviruses from 53 diarrhoeic faecal samples were collected from both organized and unorganized farms in and around the Anand area including the Livestock Research Station, Anand, Gujarat. Rotavirus ribonucleic acid (RNA) was extracted from nine faecal samples of diarrheic calves positive for group A rotavirus by polyacrylamide gel electrophoresis (PAGE) followed by silver staining were analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) to generate the near full length VP7 gene. Only eight samples yielded the desired product. The amplified products were subjected to G-typing by PCR using a cocktail of G6, G8 and G10 typing primers. Thus, among eight RT-PCR positive samples, G10 was the predominant G type (75%) followed by the type G6 (25%). The G8 type was not detected in any of the samples.

Keywords: bovine rotavirus, RNA, RT-PCR

INTRODUCTION

Livestock farming plays an important role in the rural development programs in Gujarat state, India. The future of any dairy operation depends upon a successful program of raising calves. However, it has been observed that a large number of calves die at an early age. The calf crop being the future livestock, diarrhoea affecting the neonates is an important disease in the conditions which affect the herd health and economy of the country (Singh and Singh, 1971).

Group A rotaviruses have been identified worldwide as a major cause of diarrhea in the young of many species, including humans (Bellinzoni et al., 1989). Two rotavirus outer capsid proteins, VP4 and VP7, are independently involved in virus neutralization (Hoshino et al., 1985). Group A rotaviruses are classified into G serotypes on the basis of the outer capsid glycoprotein VP7 (Estes and Cohen, 1989). At least, 15 G types and 26 P types have been recognized so far (Kapikian et al., 2001). Although G types 1, 3, 5-8, 10 and 15 have been described in cattle, only G6, G8 and G10 are the most common group A rotaviruses of cattle (Adah et al., 2003; Garaicoechea et al., 2006). Genotyping has been preferred to serotyping due to its good correlation with serotypes, high sensitivity and use of synthetic reagents (Gouvea et al., 1990). Bovine retroviruses (BRV) are very important for preventive veterinary medicine and more specifically, for the development of a vaccine. They are also important from the point of view of ecology and public health because interspecies

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transmission from cattle to humans and from humans to cattle have been reported (Fukai et al., 1998). Laboratory diagnosis of rotavirus infection in calves has been based on the identification of viral particles, antigens, or nucleic acids in faecal samples. Antigen capture enzyme linked immunosorbent assay (ELISA), latex agglutination (LA) and reverse transcription-PCR (RT-PCR) have become more standard methods for the diagnosis of bovine rotavirus infections in recent years. Latex agglutination test is more rapid than standard ELISAs and is easy to perform (Zvizdic et al., 2004).

The aim of the present study was to identify the distribution of bovine rotaviruses in Gujarat state calves in recent years by using polymerase chain reaction- (PCR) based typing assays.

MATERIALS AND METHODS

A total of 53 faecal samples were collected from 9 buffalo calves and 44 cattle calves of 0-8 weeks of age from both organized and unorganized farms in and around Anand area including Livestock Research Station, Anand, Gujarat.

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). RNA was extracted from 10% (v/v) faecal suspensions using TRI-Reagent (Sigma, USA), following the manufacture’s instructions. To the 250 μl clarified samples, 750 μl of TRI reagent was added, vortexed and incubated for 15-30 minutes at 15-30°C to allow the complete dissociation of nucleoprotein complex. Chloroform (0.2 volume) was added, vortexed and kept at room temperature for 10-15 minutes and centrifuged at 13,000 rpm for 10 minutes at 4°C. The resultant aqueous phase was transferred to other Eppendorf tubes, one ml of isopropanol was added, and the tubes inverted 4-5 times and kept at -20°C overnight. The RNA was pelleted by centrifugation at 13,000 rpm for 10 minutes, and the pellet washed with pre-chilled 70% ethanol. The pellet was air dried, dissolved in nuclease free water (NFW) and stored at -20°C till further use. The RNA thus extracted was used for c- DNA preparation during RT-PCR.

The specificities of the selected primers used in the present study for the G- typing assays have been evaluated previously (Isegawa et al., 1993). The upstream generic primer on the VP7 gene and three specific G-typing primers (Table 1) were used in a second round of PCR amplification for the characterization of the G6, G8, and G10 serotypes.

RT-PCR of full-length VP7 and partial-length VP4 genes

Single RNA preparations were centrifuged at 13,000 rpm for 10 minutes at 4°C, and the pellets were washed with 70% ethanol. The ethanol was removed, and the pellets were dissolved in 30 μl of RNase-free water and used as templates for RT-PCR amplification. A total of 2 μl of genomic dsRNA was transferred to a 0.6-ml Eppendorf tube with 1.5 μl (15pmol) of each of the generic G primers and 11.0 μl of nuclease free water (NFW), denatured at 96°C for 5 minutes, and immediately chilled on ice. The denatured dsRNA was then added to the reaction mixture, consisting of 2 μl
of a deoxynucleoside triphosphate (dNTP) mixture (each dNTP at a concentration of 0.8 mM), 1 μl of Ribolock (10 U/μl), 1 μl of reverse transcriptase from MMLV-RT (200 U/μl) and 5.0 μl of 5X RT buffer in a final volume of 25 μl. The sample was preincubated in a thermal cycler (model 480; Perkin-Elmer Europe B.V., Monza, Italy) at 42°C for 60 minutes. The first round of amplification of full-length VP7 consisted of 35 cycles of 1 minute at 94°C, 1 minute at 58°C, and 1 minute at 72°C, followed by a final incubation at 72°C for 10 minutes.

**Determination of G genotype**

RT-PCR products were submitted to a second round of amplification by a modification of a previously described method. An aliquot of 2 μl of (150ng) DNA products was added to a reaction mixture consisting of 12.5 μl of 2X PCR Master Mix, 1 μl (10 pmol) of VP7 upstream primer, 1 μl (10 pmol) of each primer specific for the G6, G8, and 2 μl (20 pmol) of G10 genotypes, nuclease free water to a final volume of 25 μl. sample was preincubated at 94°C for 5 minutes to allow activation of the AmpliTaq Gold. The second round of amplification consisted of 30 cycles of 1 minute at 94°C, 2 minutes at 56°C, and 1 minute at 72°C, followed by a final incubation at 72°C for 10 minutes.

**Analysis and detection of products.** PCR products were analyzed on a 1.5% agarose gel containing ethidium bromide (0.5 mg/ml) in Tris-borate-EDTA buffer.

**RESULTS**

During this study, all the nine PAGE positive faecal samples of calves were subjected to RT PCR assay. PCR was used to amplify the the BRV VP7 gene to identify specific genotypes of the BRV strains. The simplified procedure of RNA extraction and the use of a second amplification which allowed the simultaneous detection of different genotypes were adopted.

As shown in Plate1, full length (1062bp) amplification of VP7 gene of BRV was obtained after cDNA synthesis by reverse transcription using Bov9Com5 and Bov9Com3 primers. Out of nine samples, eight samples (88.89%) yielded a specific amplicon of 1062bp (Plate 1).

**Table 1.** Oligo-primers used in RT-PCR and G typing of BRV.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences (5’-3’)</th>
<th>Location</th>
<th>Expected product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bov9Com5</td>
<td>GGCTTTAAAAGAGAGAAATTCCGTGG</td>
<td>1-28</td>
<td>1062 bp</td>
</tr>
<tr>
<td>Bov9Com3</td>
<td>GGTCACATCATACAAACTCTAATCT</td>
<td>1039-1062</td>
<td></td>
</tr>
<tr>
<td>G6</td>
<td>CTAGTTCCCTGTGTAAGATC</td>
<td>499-481</td>
<td>500 bp</td>
</tr>
<tr>
<td>G8</td>
<td>CGGTTCGGATTAGACAC</td>
<td>273-256</td>
<td>274 bp</td>
</tr>
<tr>
<td>G10</td>
<td>TTCAGCCGTTGGCACTTC</td>
<td>714-697</td>
<td>715 bp</td>
</tr>
</tbody>
</table>
Plate 1. Full length BRV VP7 gene amplification by RT-PCR.
Lanes 1, 4: showing negative control
Lanes 2, 5, 6, 7: showing 1062 bp products
Lane 3: -1 kb plus DNA ladder

Plate 2. VP7 based G genotyping of BRV by heminested PCR.
Lane 1: Negative control
Lanes 2, 4, 5, 8, 9: Showing the type G10 (715 bp product)
Lanes 3, 6: Showing the type G6 (500 bp product)
Lane 7: 100 bp DNA ladder
Genotyping of circulating BRV strains was done by heminested PCR of 1062bp amplification product of the VP7 gene. This was achieved by using a common forward primer Bov9Com5 and a pool of typing (reverse) primers (G6, G8 and G10) as suggested by Falcone et al. (1999). The expected size of the amplified products from G6, G8 and G10 primers were 500bp, 274 bp and 715bp, respectively. Two samples yielded the products of 500bp and six samples yielded 715bp products indicating prevalence of G6 and G10 types (Plate 2). Thus, among eight RT-PCR positive samples, G10 was the predominant G type (75%) followed by the type G6 (25%). The G8 type was not detected in any of the samples.

Bovine G8 strains have been reported to be rare in some areas while they are detected more frequently in other reports (Reidy et al., 2006). In this study, no sample was found to be positive for the type G8. A bovine G8 strain was first identified in Scotland (Snodgrass et al., 1990) and then North America (Parwani et al., 1993), Thailand (Taniguchi et al., 1991) and Japan (Fukai et al., 1998, 1999). Reports have indicated that G8 is one of the less common serotypes among bovine rotaviruses. However, it could be considered a diarrhoeal pathogen detected with lower frequency in calves.

**DISCUSSION**

The serotype specificities of field isolates and, consequently, the availability of reliable diagnostic tools have, nowadays, become of primary relevance to the development of appropriate systems of epidemiological surveillance and control of BRV infection.

In this context, the limitations of existing serological assays are well recognized, while molecular techniques, such as PCR assays, have become widely accepted as the assay of choice for the fast and complete characterization of field isolates (Taniguchi et al., 1993). As further proof of these points, in the present study, PCR was used to amplify the BRV VP7 directly from the feces of infected calves and to identify specific genotypes of the BRV strains. The simplified procedure of RNA extraction and the use of a second amplification, which allowed the simultaneous detection of different genotypes, resulted in increased sensitivity and in the rapid characterization of the isolates in a large number of field samples.

The higher prevalence of the G10 genotype found in our study is in agreement with reports from many parts of the world (de Verdier Klingenberg et al., 1999; Reidy et al., 2006). Among the Indian bovine population, Balvinder et al. (2008), Wani et al. (2004), Minakshi (1999) and Gulati et al. (1999) have also reported G10 to be the predominant genotype of bovine rotavirus.

These results were in contrast with the distribution of BRV genotypes elsewhere in the world, where low prevalence rate of the G10 genotype and higher prevalence of the G6 genotype have been reported by Snodgrass et al. (1990), Chang et al. (1996), Falcone et al. (1999), Alfieri et al. (2004), Pisaneli et al. (2005), Reidy et al. (2006), Mayameii et al. (2007), Monini et al. (2008) and Howe et al. (2008). Thus, these findings suggest that the G6 and the G10 types, predominance may be restricted as per geographical location, and as far as India is concerned, G10 type appears to be predominant. The present finding was also the first time a study conducted in Gujarat state endorsed similar studies undertaken in other parts of the country. Although interpretation of this study was limited by the small sample size and sample
collection only in restricted regions in Gujarat, this finding was in sharp contrast to the distribution of BRV genotypes elsewhere in the world. The minimum number of rotavirus-positive samples obtained in our study may be due to seasonal distribution, as reported by Davidson et al. (1975), since most samples were collected during winter months. The minimal rotavirus infection may also be due to presence of colostral antibodies, which protect the calves from exposure to rotaviral infection, as reported by Paul and Lyoo (1993).

In conclusion, heminested-multiplex PCR, which was an alternative to genotyping for identification and typing of rotavirus genotypes, facilitated the studies on the occurrence and distribution of G genotypes of the bovine population in Gujarat state.

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