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P and G genotyping of bovine rotavirus and its prevalence in neonatal calves

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ABSTRACT

To investigate the epidemical characteristics and genotypic distribution of bovine rotavirus in Punjab, 120 fecal samples were collected from calves exhibiting diarrhea and screened for the presence of rotavirus using RNA-PAGE and RT-PCR. Twenty-three samples were positive by RNA-PAGE having electrophoretic patterns that corresponded to mammalian group A rotaviruses. All the samples were (120) screened by RT-PCR for VP7 and VP4 genes and 16 samples were found to be positive for VP7. Out of these 16 samples, 4 samples were also positive for VP4. These were analyzed further by multiplex semi-nested PCR for G and P genotypes, and it was confirmed that 43.75% (7/16) were the G3 type, while 6.25% (1/16) was the G8 type and 50% (8/16) were dual G3G8 types. P genotyping classified all the samples 100% (4/4) as the P[5] genotype. It may be concluded that most prevalent combination of rotavirus infection in bovine was G3G8P[5] in Punjab.

Key words: bovine rotavirus, RNA-PAGE, RT-PCR, P and G genotyping

Introduction

Rotavirus is the most common cause of severe gastroenteritis in animals (KIM et al., 2012) and predominates among all enteropathogens associated with diarrhea. The economic losses associated with rotavirus in animals are due to increased morbidity, mortality, treatment costs and reduced growth rate (SNODGRASS et al., 1990). It accounts for about 27-36% of infections in neonatal calves worldwide (DHAMA et al., 2009) and infected calves excrete the virus in their feces up to the age of 6 to 8 weeks (TZIPORI, 1985).

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Rotaviruses are non-enveloped viruses of the genus *Rotavirus,* which belong to the family *Reoviridae*. They contain an 11 segmented genome of double stranded RNA that is enclosed within a triple layered capsid protein (ESTES and KAPIKIAN, 2007). The genome encodes six structural (VP1 to VP4, VP6 and VP7) and six non-structural (NSP1 to NSP6) proteins. The structural proteins are located in the core (VP1, VP2, and VP3), inner capsid (VP6) and the outer capsid layer (VP4 and VP7). Both the VP4 and VP7 proteins elicit a serotype specific neutralizing antibodies in the infected host (RAO et al., 2000). Rotaviruses are classified into A-G groups which are antigenically and electrophoretically distinct (ESTES, 2001). Group A rotaviruses are the common cause of diarrhea in calves. These are classified into G (glycoprotein) and P (protease sensitive protein) types, according to their antigenic properties and/or sequence of genes encoding the VP7 and VP4 proteins, respectively (ESTES and KAPIKIAN, 2007). To date, 27 G and 35 P genotypes have been identified in humans and animals (MATTHIJNSSENS et al., 2011).

Genotyping has been preferred over serotyping due to its higher sensitivity and the use of universal reagents (RODRIGUEZ-LIMAS et al., 2009). The segmented nature of the genome makes the virus more prone to mutations and reassortment, leading to the emergence of new genetic variants. As a result, genotyping of bovine rotavirus becomes essential for epidemiological studies (GENTSCH et al., 1996) and improving the existing vaccination programs. So, the knowledge of genotypes prevalence in a particular region is very often of utmost importance. Thus, the present study was conducted with the aim of identifying the prevalence of bovine Rotavirus and its genotypes in calves in Punjab.

Materials and methods

Sample collection. A total of 120 fecal samples were collected from cattle and buffalo calves up to 3 months of age affected with diarrhea, from July 2012 to March 2013. These samples were collected from dairies in and around Ludhiana and from the dairy farm of Guru Angad Dev Veterinary and Animal Sciences University, Punjab. The samples were collected in sterile stool sample containers (Tarsons) and were transported to the laboratory on ice and stored at -20 ^{o}C until processing.

Sample processing. Individual fecal suspension (10%) was prepared in phosphate buffered saline (pH 7.2) and was centrifuged at 12,000 rpm for 30 minutes. The supernatant was separated and stored at -20 ^{o}C.

RNA extraction from the fecal suspension. The viral RNA was extracted using Tri reagent RT (Molecular Research Center, INC.) according to the manufacturer's protocol. The extracted RNA pellet was eluted in 20 µL of nuclease free water (Qiagen) and stored at -80 °C. The purity of the RNA was checked by measuring the OD value at 280 nm wavelength.

RNA Polyacrylamide gel electrophoresis (RNA-PAGE). The extracted RNA was subjected to RNA-PAGE according to the method of ANONYM. (2009), with minor modifications. The staining of the gel was done as described by BASSAM et al. (1991).

Reverse transcription polymerase chain reaction (RT-PCR) for full length VP7 gene amplification and semi nested-multiplex PCR for differentiating G genotypes. The RT-PCR reaction was carried out using a One Step RT-PCR Kit (QIAGEN) with a pair of conversed primers, Beg9 and End9 (Table 1), to amplify the full-length VP7 gene of the rotavirus. RT-PCR was performed under the following conditions: reverse transcription at 50 °C for 30 minutes, followed by PCR with initial activation at 95 °C for 15 minutes, followed by 35 cycles of 94 °C for 1 minute, annealing at 48 °C for 45seconds and extension at 72 °C for 1 minute, and a final extension of 72 °C for 10 minutes. The amplified products were analyzed by electrophoresis on 1% agarose, and analyzed and photographed by a gel documentation system (Alphaimager, USA).

Table 1. Primer sequences for VP7 gene

<table>
<tr><td colspan="2">VP7</td><td>Sequence(5′ to 3′)</td><td>References</td></tr>
<tr><td colspan="2">Forward :Beg9</td><td>GGCTTTAAAAGAGAGAATTTCCGTCTGG</td><td rowspan="2">Gouvea et al. (1990)</td></tr>
<tr><td colspan="2">Reverse :End9</td><td>GGTCACATCATACAATTCTAATCTAAG</td></tr>
<tr><td colspan="2"></td><td>Primer sequences for G genotyping</td><td></td></tr>
<tr><td>G3</td><td>GARV-G3</td><td>CTAATTCANCACARGAAG</td><td rowspan="4"></td></tr>
<tr><td>G5</td><td>GARV-G5</td><td>TAGGRTGTTCGACTACAGAC</td></tr>
<tr><td>G6</td><td>GARV-G6</td><td>CAAACGAAATAGCTGATACCGAA</td></tr>
<tr><td>G8</td><td>GARV- G8</td><td>ATGAAGTATAATGCYAATTCAGA</td></tr>
<tr><td>G10</td><td>RVG10</td><td>ATGTCAGACTACARATACTGG</td><td>Iturriza-Gomara et al. (2004)</td></tr>
</table>

For G genotyping, a 1:100 dilution of the above PCR product was subjected to semi nested multiplex PCR amplification with a mixture of the type-specific primers, G3, G5, G6, G8 and G10, with a conserved reverse primer End 9 (Table 1), under the following PCR conditions: 95 °C for 5 minutes; 35 cycles of 95 °C for 45 seconds; 45 °C for 1 minute and 72 °C for 1.5 minutes, and a final extension of 72 °C for 10 minutes. PCR products were analyzed on the Alphaimager system.

RT-PCR for VP4 gene amplification and semi nested -multiplex PCR for differentiating P genotypes. The RT-PCR reaction for VP4 was carried out by using a One Step RT-PCR Kit (Qiagen), with a pair of conversed primers: Bov4Com5 and Bov4Com3 (Table 2).

Table 2. Primer sequences for VP4 gene

VP4		Sequence (5/ to 3/)	Reference
Forward :Bov4Com5		TTCATTATTGGGACGATTCACA	Isegawa et al. (1993)
Reverse :Bov4Com3		CAACCGCAGCTGATATATCATC	
		Primer sequences for P genotyping	
P[1]	GARV-VP4	TTAAATTCATCTCTTAGTTCTC	
P[5]	GARV-VP4	GGCCGCATCGGATAAAGAGTCC	
P[11]	GARV-VP4	TGCCTCATAATATTGTTGGTCT	

For P genotyping, a 1:100 dilution of the above PCR product was subjected to semi nested multiplex PCR amplification with a mixture of type-specific primers: P[1], P[5] and P[11] (Table 2) with a conserved forward primer, Bov4Com 5, under the following conditions: 95 °C for 5 minutes, followed by 35 cycles of 95 °C for 45 seconds, 43 °C for 1 minute and 72 °C for 1.5 minutes and a final extension of 72 °C for 10 minutes.

Results

In the present study, out of 120 fecal samples screened using RNA PAGE, 23 (19.16%) samples were found to be positive for bovine rotavirus. These positive samples had a migration pattern of 4:2:3:2, which is typical of group A mammalian rotavirus, with segments 2, 3 and 4 migrating close together, segments 7, 8 and 9 migrating as a triplet and segments 10 and 11 wider apart .

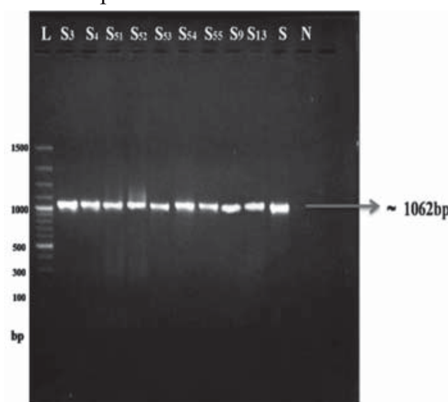

Fig. 1. PCR amplification of VP7 gene of bovine Rotavirus. Lane L - 100 base pair plus DNA ladder (Thermo scientific, 0.5 µg/µL), Lane S3, S4, S51, S52, S53, S54, S55, S9, S13 - Positive samples, Lane S - Positive control, Lane N - Negative control.

In the RT-PCR for the identification of the VP7 gene, an amplicon size of the 1062 base pair was obtained in 16 (13.3%) of the 120 fecal samples (Fig. 1). Analysis of these

16 positive samples, using semi nested-multiplex PCR for identifying the G genotypes, revealed PCR products of 812 base pair and 590 base pair, indicating the presence of G3 and G8 genotypes respectively (Fig. 2). Out of the 16 samples typed for G genotypes, G3 were observed in 7 samples (43.75%), G8 in 1 (6.25%), while 8 samples (50%) had mixed G genotypes i.e. both G3 and G8. However, there was an absence of genotypes G5, G6 and G10.

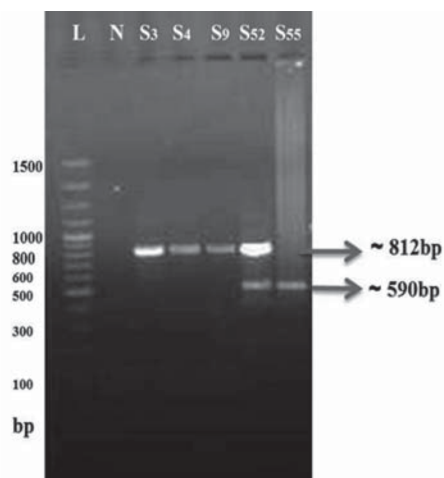

Fig. 2. PCR based G genotyping showing G3, G3G8 and G8 type. Lane L - 100 base pair plus DNA ladder (Thermo scientific, 0.5 µg/µL), Lane N - Negative control, Lane S3, S4, S9 - Positive for G3, Lane S52 - Positive for G3 and G8, Lane S55 - Positive for G8.

In the RT-PCR conducted for the identification of VP4 gene, an amplicon size of 863 base pair was obtained in 4 (3.33%) of the 120 fecal samples (Fig. 3). Analysis of these four VP4 gene positive samples by semi nested-multiplex PCR to identify the P genotypes, revealed PCR products of 661 base pair size, indicating the presence of P[5] genotypes (100%) (Fig. 4) and no amplification was obtained for the genotypes P[1] and P[11].

The overall results revealed that 4 samples were typeable for both G and P genotypes, while the remaining 12 samples were positive for the VP7 gene only. Out of the 4 typeable samples, the following genotype combinations were obtained: G3P[5] in one and G3G8P[5] in 3 samples.

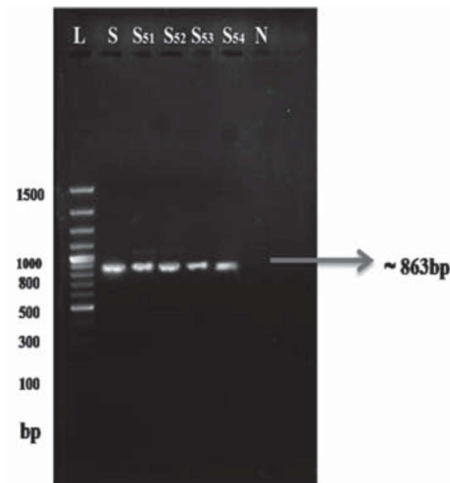

Fig. 3. PCR amplification of VP4 gene of bovine Rotavirus. Lane L - 100 base pair plus DNA ladder (Thermo scientific, 0.5 µg/µL), Lane S - Positive control, Lane S51, S52, S53, S54 - Positive samples, Lane N - Negative control.

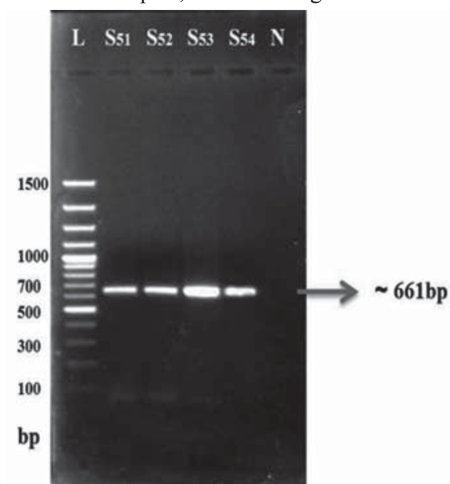

Fig. 4. PCR based P genotyping, showing P[5] type. Lane L - 100 base pair plus DNA ladder (Thermo scientific, 0.5 µg/µL), Lane S51, S52, S53, S54 - Positive for P[5] type, Lane N - Negative control.

Discussion

In the present study, the results obtained by RNA PAGE are comparable to the results of DASH et al. (2011) who detected rotavirus in 16.83% diarrheic calves from Mathura, India, and also with that of GHOSH et al. (2007) who detected rotavirus in 22% diarrheic bovine samples from West Bengal. The electropherotype profile obtained in the present study is typical of group A mammalian rotavirus. It is known from earlier studies that group A rotaviruses are the major cause of diarrhea in calves all over the world (OKADA and MATSUMOTO 2002; SARAVANAN et al., 2006).

The results obtained in the present study for RT-PCR for the identification of G genotype is in complete agreement with the results of MALIK et al. (2012), in which they found 52.9% of G3 and 47% of mixed G types in the samples. The occurrence of G8 in 6.25% samples in the present study is well in accordance with the reports of BEG et al. (2010), who reported the occurrence of G8 in 9.67% from Srinagar. In a similar study conducted by FUKAI et al. (1999) a prevalence of 4.7% by G8 genotype of the bovine group A rotavirus was reported.

Similarly, the results obtained for the RT-PCR for the identification of P genotype in the present study are in accordance with the results reported earlier by REIDY et al. (2006). REIDY et al. (2006) reported that P[5] accounted for 77.8% and P[11] for 9.3% in their study in Ireland. In an another study conducted, CASHMAN et al. (2010) also reported that P[5] was most commonly detected (41.9%) followed by P[11] (28.6%), P[5+11] (28.6%) and then P[1] (0.9%).

The presence of mixed genotypes in the samples screened in the present study are also in agreement with those of ALFIERI et al. (2004) and GHOSH et al. (2007) who also observed mixed infections with more than one type of bovine group A rotavirus in calves.

In this study PAGE yielded higher (19.16%) prevalence as compared to RT-PCR (13.3%) thus underlining the benefit of simultaneous use of at least two tests for improved diagnostic potential**.** Thus, PAGE can be considered more sensitive than RT-PCR. The above results are in corroboration with the study of MANUJA et al. (2008) in which 4.61% prevalence was observed for bovine rotavirus by employing PAGE, but 3.29% by employing RT- PCR, therefore advocating use of PAGE for surveillance studies.

Thus, it can be concluded from the above study that G3 alone or in combination with G8 is the predominant genotype among bovine populations in Punjab, India. It could also be concluded that the most prevalent combination of rotavirus infection in bovine was G3G8P[5] in Punjab.

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SAŽETAK

Radi istraživanja epizootioloških obilježja i proširenosti genotipova goveđeg rotavirusa u Punjabu, prikupljeno je bilo 120 uzoraka izmeta teladi s proljevom te pretraženo na prisutnost rotavirusa RNA-poliakrilamid-gel-elekroforezom (RNA-PAGE) i lančanom reakcijom polimerazom uz prethodnu reverznu transkripciju (RT-PCR). Od toga su 23 uzorka bila pozitivna pretragom RNA-PAGE s elektroforetskim uzorkom koji je odgovarao skupini A rotavirusa sisavaca. Svih 120 uzoraka izmeta bilo je pretraženo RT-PCR-om na prisutnost gena za proteine VP7 i VP4. Ustanovljeno je 16 pozitivnih za VP7. Od tih 16, četiri su bila pozitivna i za VP4. Ti su dodatno bili pretraženi višestrukom poluugniježđenom lančanom reakcijom polimerazom za genotipove G i P. Utvrđeno je da je 43,75% (7/16) pripadalo genotipu G3, dok je 6,25% (1/16) bilo genotipa G8, a 50% (8/16) pripadalo je i G3 i G8 genotipu. Svi su uzorci (4/4) na osnovi P genotipizacije pripadali genotipu P[5]. Može se zaključiti da je infekcija goveda u Punjabu najčešće uzrokovana kombinacijom rotavirusa G3, G8 i P[5].

Ključne riječi: goveđi rotavirus, RNA-PAGE, RT-PCR, P i G genotipizacija