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Comparative Prevalence and Molecular Characterization of Group A Rotavirus in Cow Calves of Punjab, India

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ABSTRACT

Group A rotavirus has been recognized as the major etiological agent of diarrhoea in calves, which leads to heavy economic loss to dairy farmers. The present study was done to estimate the prevalence along with genotypic characterization of group A rotavirus circulating in dairy herds of Punjab. A total of 198 faecal samples of cow calves were analyzed from April 2012 to March 2013 in Ludhiana and Bathinda districts of Punjab by ELISA, RNA-PAGE and RT-PCR. G and P genotyping was done by RT-PCR using genotype-specific primer sets targeting VP4 and VP7 genes. RNA PAGE, RT-PCR and ELISA showed a positive prevalence rate of 6.56%, 7.57% and 9.59%. RT-PCR showed perfect agreement with other two diagnostic techniques by Diagnostic Test Agreement. Eight RT- PCR positive samples were further screened by nested multiplex PCR using G and P-genotype specific primers for genotypic characterization. Out of these, two samples exhibited G6 types and rest were untypable. In P typing, four samples showed P[11] type. G6P [11] was the main combination in the present study. This study suggests that the RT PCR is the highly suitable technique for the detection of group A rotavirus. Further investigation is required to find out the untypable strain by designing suitable primers.

Keywords: Group A rotavirus, Prevalence, RNA-PAGE, RT-PCR, ELISA, G and P genotyping.

Group A rotavirus has been recognized as the major etiological agent of diarrhoea in calves and children throughout the world. Rotaviruses are members of the family *Reoviridae*, which is classified into seven different serogroups (A-G) on the basis of the antigen specificity. The viral genome has 11 RNA segments, which encodes six viral structural proteins (VP1- 4, VP6 and VP7) and six non-structural proteins (NSP1-NSP6). Of the seven rotavirus serogroups, group A rotaviruses are known to infect human beings and animals (Broor *et al.,* 2003).

Rotaviruses (RVs) have two independent neutralization antigens on the outer capsid, VP7 and VP4, which establish G and P types, respectively. In extensive genomic studies, 14 P genotypes and 26 G genotypes have been established for rotaviruses of humans and animals (Martella *et al.,* 2006). At least eight G genotypes (G1, G3, G5, G6, G7, G8, G10, G15) and six P genotypes (P[1], P[5], P[11], P[14], P[17] , P[21]) have been described affecting cattle, out of which G6, G8, G10, P[1], P[5] and P[11] are the prevalent genotypes in calf RV strains worldwide (Estes and Kapikian, 2007). However, no detailed information is present on genotypic strains infecting cattle calves in the Punjab state of India.

In India, the prevelence of rotavirus in calf diarrhoea below one month of age ranges between 10 and 52 per cent (Khatter and Pandey, 1986; Singh and Pandey, 1990; Singh *et al*., 1993; Jindal *et al*., 2000; Nataraju *et al*., 2009). In northern India region, Kaur (2011) and Dash *et al*. (2011) reported prevalence rate of group A rotavirus as 15.41% and 16.83% in calves.

As the virus is excreted in large numbers in the faeces, it can be diagnosed easily by electron microscopy which is one of the most specific tests. The other most

widely used methods are ELISA, latex agglutination and polyacrylamide gel electrophoresis (PAGE) (Herring *et al.,* 1982). The detection by PAGE followed by silver staining is a highly specific technique, but lacks sensitivity as a minimum of 3-4 ng of viral RNA is needed for detection (Herring *et al.,* 1981).

Newer techniques like dot blot hybridization using radio labeled cDNA probes, reverse transcriptase polymerase chain reaction (RT-PCR), Real-Time PCR, and automated nucleotide sequencing are now being used as confirmatory methods for detecting rotavirus in stool samples (Wilde *et al.*, 1990; Broor *et al.*, 1995; Husain *et al.*, 1995). A multiplex, semi-nested RT-PCR method is currently being used for identifying G and P serotypes by genotyping (Gentsch *et al.*, 1992).

Continuous typing of Bovine rotavirus circulating strains is essential for a better understanding of the viral epidemiology within a region and the zoonotic transmission, thereby improving the implemented vaccination programs by updating the vaccine strains. The objective of this study was to investigate the prevalence of rotavirus diarrhoea in dairy herds in Punjab, compare the diagnostic efficacy of ELISA, RNA-PAGE and RT-PCR and G and P types of bovine rotavirus.

MATERIAL AND METHODS

Sample collection and extraction of rotavirus dsRNA from faecal sample

A total of 198 faecal samples were collected from diarrhoeal and non-diarrhoeal cow calves below three months of age from organized and unorganized farms of Ludhiana and Bathinda districts of Punjab during the period of April 2012 to March 2013. Out of 198, 190 samples were diarrhoeal cases (96 males and 94 females), while eight samples (seven males and one female) were collected from apparently healthy calves using standard sampling protocols.

Detection of group A rotavirus from faecal samples

Three methods *viz*., RNA-PAGE, ELISA and RT-PCR were standardized for the detection of group A rotavirus. All the positive samples by RT-PCR were further subjected to genotypic characterization for G and P typing by seminested PCR typing assay.

Detection of group A rotavirus by ELISA

All the faecal samples were screened for the presence of rotavirus antigen by ELISA as described by the kit manufacturer ('Bio-X EASY-DIGEST 4 ELISA KIT' Product ID: BIO K 151/2). The optical densities were read at 450 nm in microplate reader (Nano quant Infinite M 200 PRO).

Detection of group A rotaviral dsRNA by RNA-PAGE

Trizol extraction method (TRIzol$^{(R)}$ reagent Ambion) as per the manufacturer's protocol was used for extraction of dsRNA of rotavirus from faecal samples. The isolated RNA was then used for further downstream applications. The RNA extracted from the field faecal sample was subjected to ribonucleic acid-polyacrylamide gel electrophoresis (RNA-PAGE) with concentration of separating gel of 7.5% and stacking gel of 5% at 150 V. The gel was silver stained and photographed (Herring *et al*., 1982).

Detection of group A rotavirus by Reverse transcription-polymerase chain reaction

The RNA was subjected to two step reverse transcriptase PCR with VP7 gene specific primers (Forward GAT CCG AAT GGT TGT GTA ATC CAA T Reverse AAT TCG CTA CGT TTT CTC TTG G) as per cycling conditions described by Husain *et al.* (1995). The resultant PCR products (304 bp) was analysed by agarose gel electrophoresis and digitally recorded by gel documentation system (G:BOX, SYNGENE).

Characterization of group A rotavirus

The positive isolates obtained by RT-PCR were subjected to G and P genotyping as per the details given below.

G genotyping

The cDNA was synthesized and amplified with VP7 gene specific primers Beg9 End9UK as per published literature (Gouvea *et al.*, 1990; Gouvea *et al.*, 1993). Briefly, a 1062 bp (full-length) gene segment nine, encoding the VP7

glycoprotein of bovine group A rotaviruses was amplified followed by two semi-nested PCRs using G type specific primers (Gouvea *et al.*, 1990; Gouvea *et al.*, 1994a).

P genotyping

For P genotyping, the cDNA was synthesized and amplified using VP4 gene specific primers Con3Con2 as per the method of Gentsch *et al.* (1992). The 876 bp PCR product of VP4 encoding gene of bovine group A rotavirus was amplified followed by two semi-nested PCRs using P type specific primers (Gentsch *et al.,* 1992; Gouvea *et al.,* 1994b).

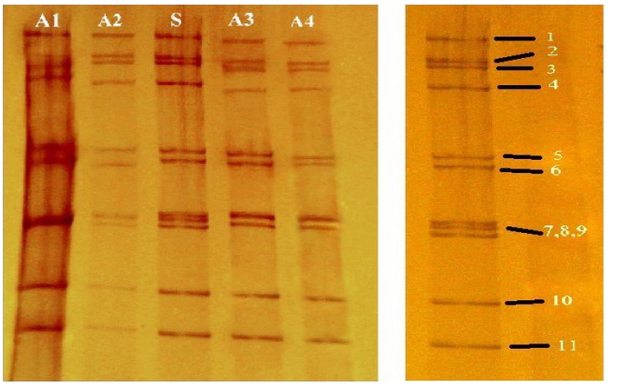
Statistical analysis

Comparative statistical analysis of the detection methods used for group A rotavirus, namely RNA-PAGE, RT-PCR, and ELISA was performed using Diagnostic test agreement employing a software Win Episcope 2.0 version. The comparative statistical analysis was performed to ascertain the best technique for detection of group A rotavirus. The kappa value was measured to find agreement between positive and negative results of these the three detection techniques at 95 % confidence level. The confirmation was done on the basis of kappa value, with the kappa value less than zero indicating no agreement, 0-0.20 as slight, 0.21-0.40 as fair, 0.41-0.60 as moderate, 0.61-0.80 as substantial, and 0.81-1 as almost perfect agreement. The Chi-squared test was applied using Epi Info 7 statistical calculator (Sullivan *et al.,* 2009) to determine association of the disease prevalence with male or female calves.

RESULTS AND DISCUSSION

Detection and comparative prevalence of group A rotavirus

All 198 faecal samples of cow calves below three month of age were screened for group A rotavirus by RNA-PAGE, ELISA and RT-PCR. The non-diarrhoeal samples (eight) were found negative for rotavirus by all of the three tests. The RNA-PAGE revealed group A rotavirus in 6.56% (13/198) cow calves. All the 13 RNA PAGE positive samples showed a typical 4:2:3:2 "long" electropherogram migration pattern (Fig. 1). The results are in accordance with the findings of Malik *et al.* (2013) who revealed a prevalence of 6.45% using similar technique. Likewise Kaur (2011) and Udaykar *et al.* (2013) reported prevalence rate of 4.89% and 4.30% respectively, along with "long" electropherogram migration pattern in most of bovine samples.



(A) **(B)**

Fig. 1: RNA-PAGE showing electrophoretic migration (long pattern) of bovine group A rotavirus genome. **(A)** Lane S - Positive control; Lane A1, A2, A3, A4 - Positive diarrhoea samples. **(B)** Shows 11 segments of the ds RNA of group A rotavirus, separated on RNA-PAGE.

Prevalence of group A rotavirus in bovine calves by RT-PCR was 7.57% (15/198 (Fig. 2). Other studies report slightly higher prevalence ranging from 7.81% to 11.23% from different parts of the India (Basera *et al.,* 2010; Kaur 2011; Mondal *et al.,* 2013).

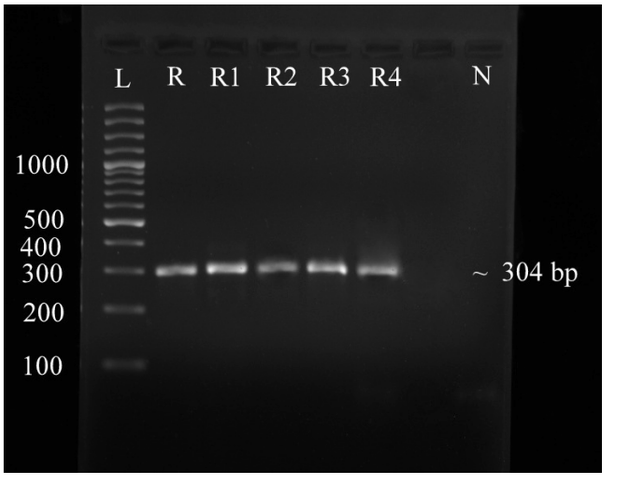


Fig. 2: RT-PCR amplification of conserved part of VP7 gene of

bovine group A rotavirus with ~304 bp amplicon. From left to right: Lane L - 1000 bp plus DNA ladder (Thermo scientific, 0.5 ug/ul); Lane R - Positive control; Lane R1, R2, R3, R4 - Positive diarrhoeal samples; Lane N - Negative control.

On screening of all faecal samples by ELISA a positivity of group A rotavirus antigen was detected 9.59%, with 19 samples positive out of a total of 198. In previous studies conducted in North India by employing ELISA, Manuja *et al*. (2008); Kaur (2011); Rai *et al.* (2011) reported prevalence rate of 4.61%, 15.41% and 15.68% , respectively, among diarrhoeic bovine calves.

All three techniques RNA-PAGE, RT-PCR and ELISA were compared on the basis of their positive and negative results by a statistical method namely Diagnostic test agreement. The RT-PCR showed perfect agreement with RNA-PAGE and ELISA with a kappa value 0.923 and 0.871 respectively, at 95% confidence level (Table 1-3). The analysis of data reveals that RT-PCR is more promising technique than ELISA and RNA-PAGE for the detection of group A rotavirus as only a minute quantity of RNA is needed. Other studies also indicated that PCR is the best confirmatory detection technique for group A rotavirus in faecal samples (Wilde *et al.*, 1990; Husain *et al.*, 1995; Slovis *et al.*, 2014). The detection by PAGE followed by silver staining is a highly specific technique, but lacks sensitivity as a minimum of 3-4 ng of viral RNA is needed for detection (Herring *et al.,* 1981).

Table 1: Diagnostic test agreement between RNA-PAGE and ELISA

1 \ 2	(+ve)	(-ve)	Confidence level	Confidence interval	Kappa value
(+ve)	13	0	95%	0.660 to 0.933	0.797
(-ve)	6	179			

RNA-PAGE (1) and ELISA (2)

Table 2: Diagnostic test agreement between RNA-PAGE and RT-PCR

1 \ 2	(+ve)	(-ve)	Confidence level	Confidence interval	Kappa value
(+ve)	13	0	95%	0.784 to 1.062	0.923
(-ve)	2	183			

RNA-PAGE (1) and RT-PCR (2)

Table 3: Diagnostic test agreement between RT-PCR and ELISA

1 \ 2	(+ve)	(-ve)	Confidence level	Confidence interval	Kappa value
(+ve)	15	0	95%	0.733 to 1.010	0.871
(-ve)	4	179			

RT-PCR (1) and ELISA (2)

Season, age and gender-wise prevalence in cow calves

The prevalence of rotaviral diarrhoea among cow calves showed a seasonal variation. Out of a total of 58 samples screened during the winter period (November to February), 11 were positive for group A rotavirus, which accounted for 18.96% of the total cases. A prevalence of 3.79% was recorded in the months of July to October, corresponding to rainy season; followed by 61 samples collected in hot and dry summer season between March and June, with 1.64% prevalence. In the present study, the peak incidence of rotaviral gastroenteritis was recorded between a day old to one months of age, with a positivity of 12.04% (10/83). Only five animals were found positive in the age group of 1-3 months, with a positivity of 4.34%. Thus, as the age increased susceptibility decreased. Similarly Minakshi *et al.* (2005); Dash *et al.* (2011) and Udaykar *et al.* (2013) reported that the susceptibility of bovine calves to rotavirus infection decreases with age, probably due to loss of receptors on enterocytes.

Current research showed that the males were more susceptible to group A rotavirus infection as compared to female calves. A higher prevalence of 8.73% (9/103) was associated with male calves, while a prevalence of 6.31% (6/95) was recorded in female calves, however the difference was non-significant (Chi-square = 0.41; p = 0.52). The possible reason for slightly high prevalence could be due to the managemental practices as in most of the dairy farms female calves are better looked after than male calves. Similar studies by Sharma (2004); Dash *et al.* (2011) and Gantasala (2013) reported that males were more prone to rotavirus disease.

Molecular characterization of group A rotavirus in cow calves

Out of 15 positive samples eight samples were screened by nested PCR for molecular characterization due to the

non-availability of sufficient quantity of faecal sample in the remaining samples. Out of eight samples, four samples were successfully amplified with both VP7 and VP4 gene based primers (Fig. 3 and Fig. 4), while rest of the samples (n = 4) could not be amplified. Out of these four samples two samples showed G6 (Fig. 5) and rest of two were untypable. In P typing all four samples showed P[11] type (Fig. 6). G6P[11] as the main combination depicted, with G6 and P[11] were the main predominate types.

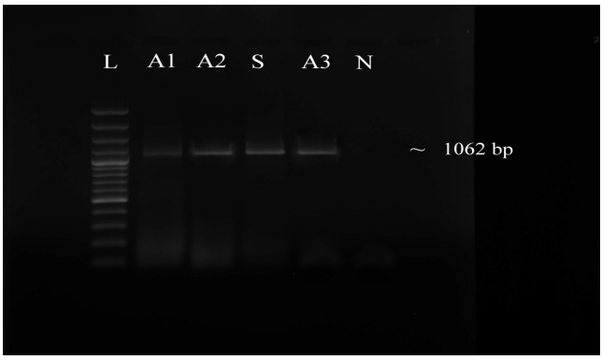


Fig. 3: PCR amplification of VP7 gene of bovine group A rotavirus with ~ 1062 bp amplicon. From left to right: Lane L - 1000 bp plus DNA ladder (Thermo scientific, 0.5 µg/ µl); Lane A1, A2, A3- Positive calf diarrhoea samples; Lane S - Positive control; Lane N -Negative control

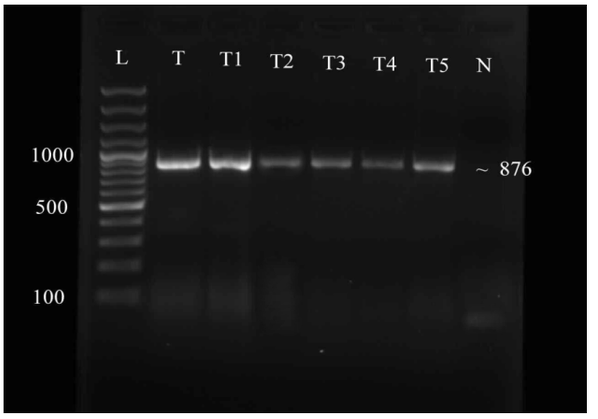


Fig. 4: PCR amplification of VP4 gene of bovine group A rotavirus with ~ 876 bp amplicon. From left to right: Lane L - 1000 bp plus DNA ladder; Lane T1, T2, T3, T4, T5-Positive calf diarrhoea samples; Lane T - Positive control; Lane N - Negative control

In support to our study, Manuja *et al.* (2010) who conducted molecular characterization of bovine group A rotavirus in North India, also observed G6, G10 and P[11] genotypes as the main circulating among calves. The findings of our study are also in consistent with Matthijnssens *et al.* (2008), who detected G6 genotype as the most frequently detected G type in cattle, followed by G10, G8, and G15. Furthermore, Kaur (2011) detected 37% untypeable strains and rest of 63% samples showed G6 (64.7%) followed by G3 (17.6%), G8 (11.8% each) and G10 (5.89%) among calves in North India. The most common P type among diarrhoeic calves was P[11], accounting for 92.3%, followed by P[3] (7.7%). Among the G-P combinations; G6P[11] was the most prevalent (63.6%).

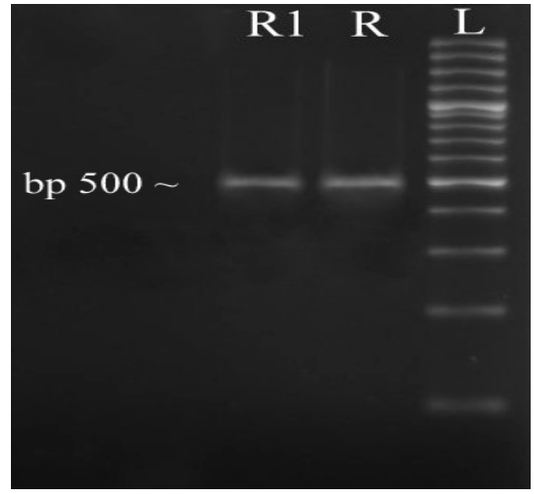


Fig. 5: PCR based G genotyping showing G6 with ~500 bp. From Right to Left: Lane L - 1000 bp plus DNA ladder; Lane R & R1 - Positive for G6 type

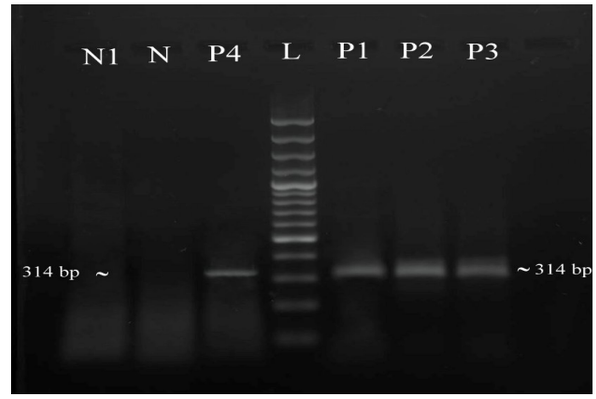


Fig. 6: PCR based P genotyping showing P[11] with ~314 bp. Lane L - 1000 bp plus DNA ladder; Lane P1, P2, P3, P4 - Positive for P[11] type; Lane N,N1 - Negative control

CONCLUSION

From the above study it can be concluded that RT PCR is the highly suitable technique for the detection of group A rotavirus. RT-PCR showed a 7.57% prevalence rate in cow calves. G6 and P[11] were the predominant strains circulating in dairy herds of Punjab, along with untypable strains, and requires further investigation by designing suitable primers.

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