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G and P genotyping of bovine group A rotaviruses in faecal samples of diarrhoeic calves by DIG-labelled probes

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The nucleic acid probes specific to genome segments 9 and 4 of bovine group A rotaviruses (BRVs) were developed for detection and determination of the G (G6 and G10) and P (P1, P5 and P11) types in field samples of diarrhoeic bovine calves. The type specific DNA probes were prepared by polymerase chain reaction amplification of hyper divergent regions of VP7 and VP4 genes from three genotypically distinct (G or P types) reference strains of BRV, viz. B223 (G10P11), NCDV (G6P1) and UK (G6P5), labelled with Digoxigenin (DIG). These G and P genotype specific DIG-labelled probes were evaluated for genotyping of rotaviruses of bovine origin directly in faecal samples. Of 308 calf diarrhoeic samples, 138 (44.81 %) were positive for BRV infection in nucleic acid hybridization assay, using VP7 and VP4 gene specific probes independently. Of probe positive samples (138), 112 were typeable by both G and P typing nested probes; 109 samples were typed as P11 (97.32%) and 3 as P1 (2.68%), and 97 as G10 (86.61%) and 15 as G6 (13.39 %). Surprisingly, none of the samples was typed as P5, indicating non-occurrence of P5 genotype among bovine rotaviruses in Haryana and adjoining areas of India. Furthermore, G10P11 was found to be the most prevalent combination among field rotavirus strains examined; whereas, G10P5 and G10P1 combinations were not reported in any of the samples.

Key words: BRV, DIG probes, dot-blot hybridisation, RT-PCR, G and P genotyping
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Introduction

Rotavirus is one of the most important pathogens associated with neonatal diarrhoea in human infants and young ones of most farm animals below three months of age. In developing countries 4-10 million deaths occur every year in children due to acute gastro-enteritis. Of which 8,70,000 deaths are attributed to rotavirus infection alone[1]. Although, information on the prevalence of rotavirus associated diarrhoea is not available on national basis; however, in a 8 years survey of organised animal farms in Haryana, 11-43% incidence of rotavirus associated diarrhoea was reported in calves below one month of age[2-4].

Rotaviruses belong to the family *Reoviridae* under the genus *Rotavirus*. They have segmented double stranded RNA genome, which can be separated into discrete segments by RNA-PAGE. The viral genome is composed of 11 double stranded RNA segments surrounded by three protein shells, i.e. core, inner capsid and outer capsid. The outer capsid consists of two proteins, viz. VP4, a minor component encoded by gene segment 4, and VP7, a glycoprotein encoded by genome segments 7, 8 and 9, depending upon the strain of the virus. The serotypic classification of bovine group A rotaviruses (BRVs) has also been defined on the basis of VP7 specificities by ELISA with VP7 specific monoclonal antibodies (Mabs)[5,6]. The neutralization of specificities of bovine rotaviruses, however, is also dependent on VP4 and the VP4 gene segregates independently from VP7 gene[7]. It has, therefore, been proposed that serotypic classification of rotaviruses should account for both VP4 (P) and VP7 (G) specificities[7-9]. So far, 14 G types and 20 P types have been identified[10]. The scheme of G and P typing has been very helpful in establishing relationship amongst serotypes prevalent in a particular farm or geographical region[11]. The G and P typing may also provide clue about possible origin/source of the new strains causing outbreaks of rotavirus-associated diarrhoea[11].

The major hindrance in generating epidemiological information has been the difficulty in cultivation of rotaviruses in cell culture and lack

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of availability of suitable test for typing of rotaviruses directly in faecal samples. Previous workers have reported the difficulty in preparation of VP4 specific Mabs[12]. During a limited epidemiological study from a single cattle farm near Bangalore, India, the serotype specificities of several isolates of bovine rotavirus, exhibiting identical electropherotypes, could not be determined using a panel of serotyping Mabs specific for G serotypes. In the study, nucleotide and deduced amino acid sequences of the genes encoding the outer capsid proteins VP4 and VP7 were used for G and P genotyping[13]. Therefore, at least for P typing, cDNA probes offer a great promise. In addition, cDNA probes have the potential to detect genomic variants or monotypes, which are not detectable by Mabs[14].

The assessment of genetic variability by hybridization, including Northern blot, dot-blot and liquid assays[15], has been an alternative approach. Most Northern blot and liquid hybridization assays have utilised cDNA or ssRNA probes synthesised from all 11 segments in a single hybridization reaction[16,17] and thus limit the amount of segment specific information available from the test. The cDNA probes synthesised from cloned individual genome segment of interest have been used in dot-blot assays[15] but the presence of conserved sequences between serotypes of rotaviruses resulted in a background signal. This makes it necessary to quantify accurately the input of target RNA, a difficult process when dealing with nanogram level in faecal extracts containing other species of nucleic acid. On the other hand, the polymerase chain reaction (PCR) technique has provided powerful tool for both the detection[18] and typing[19,20] of rotaviruses directly from faecal samples. However, the disadvantages of PCR include risk of generating false positive through cross contamination and purification steps to remove the inhibitory substances from faecal extracts greatly reduce the sensitivity of the assay[21]. In order to overcome these problems and to understand the epidemiology of BRV infection, the present study was undertaken to determine the prevalence of G and P types in faecal samples of diarrhoeic calves using non-radioactive DIG-labelled probes.

Materials and Methods

Reference Strains

Reference strains used in the study were B223 (G10P11), NCDV (G6P1) and UK (G6P5). These reference strains were grown in MA 104 cell line.

Collection of Faecal Samples from Cow and Buffalo Calves

Faecal samples were collected from 308 diarrhoeic bovine calves below one month of age, from different animal farms in Haryana and adjoining areas. The faecal samples were stored at -20°C till processed for extraction of the viral RNA.

Extraction of Viral RNA from Faecal Samples

A 10% faecal sample was made in phosphate buffered saline (PBS, *p*H 7.2) and centrifuged at 10,000 × g for 15 min to remove coarse particles and cellular debris. The clarified supernatant (faecal extract) was stored at -20°C till further use. The faecal extract was screened by ELISA to determine the positivity of samples for rotaviruses. The viral dsRNA was extracted from faecal extracts and supernatant of cell culture grown reference rotaviruses by phenol chloroform extraction method[22] with some modifications. Finally, the pellet was suspended in 30 µl of nuclease free water and stored at -20°C till further use. The RNA was also extracted from uninfected MA104 cells, bluetongue virus and *Salmonella typhimurium* using the same procedure and used as controls in the assays.

RNA-Polyacrylamide Gel Electrophoresis (RNA-PAGE)

RNA extracted from all the diarrhoeic faecal samples was tested by RNA-PAGE and silver staining for presence of rotavirus and their electrophoretic migration patterns by the resolution of 11ds RNA genomic segments in polyacrylamide gel[23].

RT-PCR Amplification of VP4 and VP7 Genes

RT-PCR was used to produce full length BRV VP7 and partial length VP4 gene fragments. cDNA was synthesised by using single tube two enzymes system for RT-PCR provided in the Access RT-PCR kit (Promega Corp., USA). The RT-PCR mixture containing 5 X AMV/Tfl reaction buffer, dNTP (10 m*M*, each), $MgSO_4$ (25 m*M*), 20 units RNAsin (Promega Corp., USA), 100 ng of VP4 primer con 2 (nt 868-887 -ve sense) and con 3 (nt11-32 +ve sense) as described earlier[24] and VP7 gene specific full length primers (nt 50-71 and complementary to nt 1038-1060), AMV reverse transcriptase (5 units/µl) and Tfl DNA polymerase (5 units/µl). First strand cDNA synthesis was obtained by incubating the reaction mixture at 48°C for 45 min. Thirty amplification cycles were conducted, with each cycle having denaturation temperature of 94°C for 1 min, annealing at 45°C for 2 min and extension at 72°C for

2 min followed by final extension at 72°C for 7 min. The full-length VP7 gene specific and partial-length VP4 gene specific PCR products were analysed on 1% agarose gel using standard protocols[25]. The full-length and partial-length PCR products were further used as templates for generating G and P type specific fragments, respectively. The nested PCR was performed with VP4 gene specific typing primers nt 269-289 (NCDV, P1), nt 336-354 (UK, P5) and nt 574-594 (B223, P11) along with the common primer con2. The type specific PCR amplified fragments were analysed on 1% agarose gel. Similarly, VP7 gene specific G6 and G10 type specific PCR products were obtained by using nested primers for G6 (complementary to nt 315-336) and G10 (complementary to nt 671-692) along with common generic primer.

Preparation of DIG-Labelled Probes

All the VP7 and VP4 gene specific full-length, partial-length and type specific nested PCR products were purified on agarose gel, the specific bands were cut and the DNA was eluted by using ethidium bromide minus spin columns (Sigma). The purified PCR products thus obtained were used to produce probes by random primer extension method to incorporate DIG11-dUTP (Boehringer Mannheim Corp., Germany) with DIG DNA labelling and detection kit as per the manufacturer's instructions. The probes were purified and quantified as per the manufacturer's instructions. The entire DIG-labelled probes were stored at –20°C until used.

Dot-Blot Hybridization

The dot-blot hybridization assay was performed using homologous rotavirus nucleic acid of reference strains extracted by GIT lysis method[26]. The viral nucleic acids, PCR products of VP4 gene and negative controls (bluetongue dsRNA, DNA from *Salmonella* spp. and nucleic acid from uninfected MA104 cell culture) were dotted onto the Zeta probe nylon membranes. After UV cross-linking of the blotted membrane, it was incubated for 60 min in pre-hybridization solution at 60°C in water bath. The DIG-labelled DNA probe was denatured in boiling water bath for 10 min and then cooled rapidly on ice. Subsequently, the denatured probe was added to the prewarmed, prehybridization solution and mixed well. The hybridization was carried out overnight at 60°C using 5ng/ml of the probe. The membrane was washed twice in sample 2 X SSC with 0.1% SDS at room temperature to remove unbound probe. Finally the membrane was washed twice for 15 min each in 0.1 X SSC and 0.1% SDS at 68°C with constant agitation to remove non-specifically bound probe**.** The hybridized probe on the membranes was immunologically detected with anti-DIGoxigenin-AP, Fab fragments and then visualised with the colorimetric substrate NBT/X-phosphate buffer as per the detection protocols of the kit (Boehringer Mannheim Corp., Germany). The membranes were kept in dark without shaking for development of colour. The sensitivity and specificity of probes in dot-blot hybridization assay were determined by hybridizing RNA of each strain from which the corresponding probe was derived.

Results

RNA-PAGE Analysis

Of 308 faecal samples from diarrhoeic bovine calves, 74 (24.03%) were found positive for BRV by RNA-PAGE analysis. All the faecal rotaviruses represented long electropherotypic migration pattern (4:2:3:2) typical of bovine rotaviruses (Fig. 1). The close migration of segments 7, 8 and 9 as a triplet is characteristic of group A rotaviruses irrespective of host species[27]. All the samples, detected PAGE positive, were also positive by ELISA (data not shown).

Dot-Blot Hybridization and Genotyping by Nested Probes

Nucleic acid dot-blot hybridization assay, employing hybridization of DIG-labelled VP4 and VP7 genes specific DNA probes from strains B223, NCDV and UK onto viral dsRNA from homologous

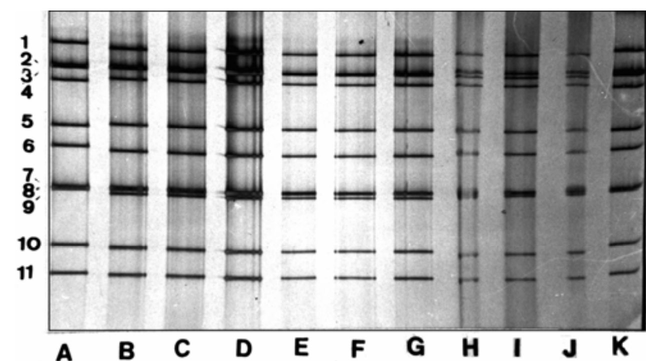

Fig. 1—RNA-polyacrylamide gel electrophoresis of faecal rotaviruses. Lanes A-K represent long electropherotypes of bovine rotaviruses from faecal samples. Numbers 1-11 indicate 11 dsRNA genomic segments typical of bovine group A rotaviruses. The close migration of segments 7, 8 and 9 as a triplet is characteristic of group A rotaviruses.

strain, yielded specific results for detection and G and P genotyping of their respective types (Figs 2 & 3). When VP4 and VP7 genomic probes were used for detection of BRV infection in the diarrhoeic faecal samples, 138 (44.81%) samples were positive (Figs 4 & 5). Of probes positive samples, 112 were typeable by both G and P typing nested probes. On nucleic acid hybridization with P1, P5 and P11 type specific nested probes, 109 samples were typed as P11 (97.32%) and 3 were typed as P1 (2.68%) (Fig. 6). Surprisingly, none of the rotavirus positive faecal samples, except reference strain (UK), yielded P5 genotype. Interestingly, only P11 genotype was recorded in the dairy farms located in Hisar district of Haryana. Whereas, both P1 and P11 were detected in the samples collected from Ambala (Haryana) and Meerut dairy farms (Uttar Pradesh).

The samples that were used for P genotyping were also used for G genotyping. Of 112 samples, 97 (86.61%) were positive for G10, whereas 15 (13.39 %) samples were typed as G6. Thus, the G10 genotype was the most predominant genotype prevalent among bovine calves. Further, the most prevalent G and P genotypic combinations among bovine calves were G10P11 (86.61%), followed by G6P11 (10.71%) and G6P1 (2.68%). However, G10P1 or G10P5 combinations were not observed in any of the samples.

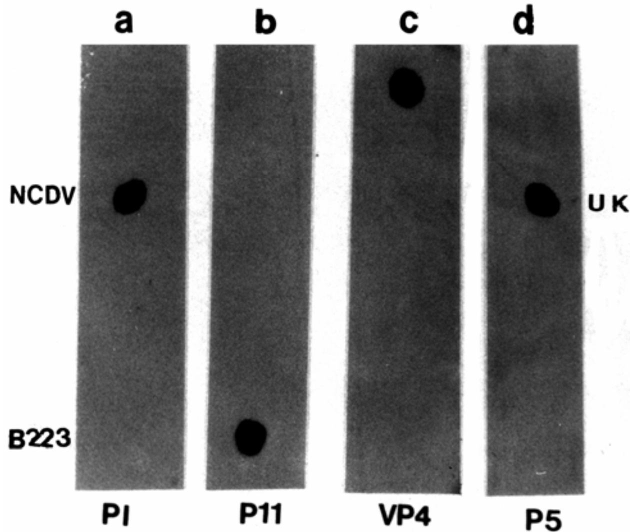

Fig. 2—Detection and P genotyping of reference strains of bovine rotavirus by DIG-labelled probes. DIG-labelled gene 4 (VP4) probes from reference strains NCDV, UK, and B223 hybridized to viral dsRNA from homologous bovine strains of their respective P types. Membrane a: P1 type; b: P11 type; d: P5 type; c: Detection of homologous dsRNA by partial length VP4 genomic probe.

Discussion

In the present investigation, the genotypic classification of cell culture adapted reference strains with known G and P types, viz. B223 (G10P11), NCDV (G6P1) and UK (G6P5), was confirmed by hybridization analysis with DIG-labelled G and P genotype specific probes (also confirmed by RT-PCR; data not shown). Further, hybridization assay of bovine rotaviruses in faecal samples by VP7 full length or VP4 partial length DIG-labelled DNA probe yielded 44.81% diarrhoeic calf faecal samples as positive for rotaviruses infection, whereas only 24.03% faecal samples were positive by RNA-PAGE.

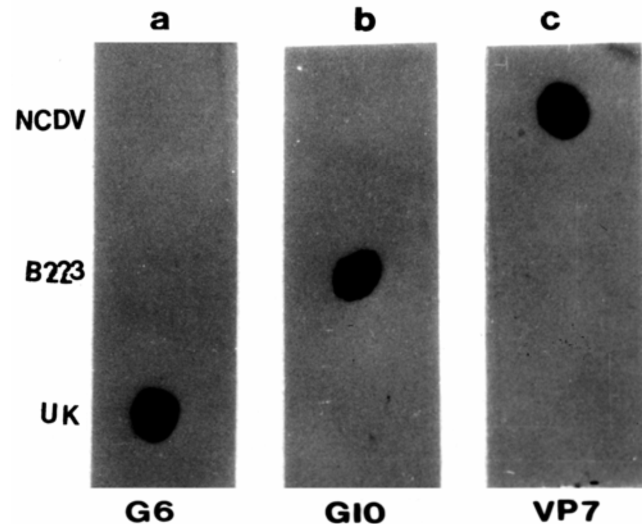

Fig. 3—Detection and G genotyping of reference strains of bovine rotavirus by DIG-labelled probes. DIG-labelled gene 9 (VP7) probes from reference strains UK, B223 and NCDV hybridized to viral dsRNA from homologous strains of their respective G types. Membrane a: G6 type; b: G10 type; c: Detection of homologous dsRNA by full length VP7 genomic probe

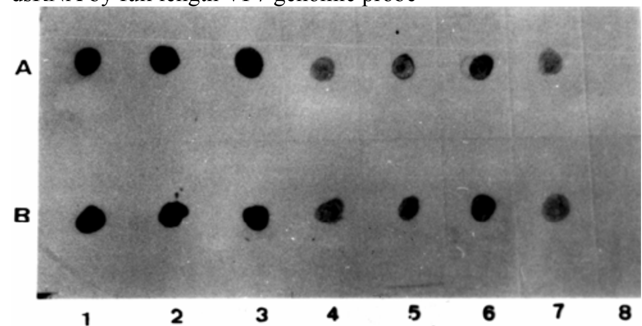

Fig. 4—Detection of rotavirus in nucleic acid extracted from faecal samples and reference strains using slot blot hybridization assay with DIG-labelled VP4 gene probe. Column A- 1: Positive control (NCDV strain); 2-7: Bovine faecal samples exhibiting hybridization signals; 8: Cell culture control (MA104). Column B- 1-7: Bovine faecal samples exhibiting hybridization signals; 8: heterologous control (dsRNA, BTV).

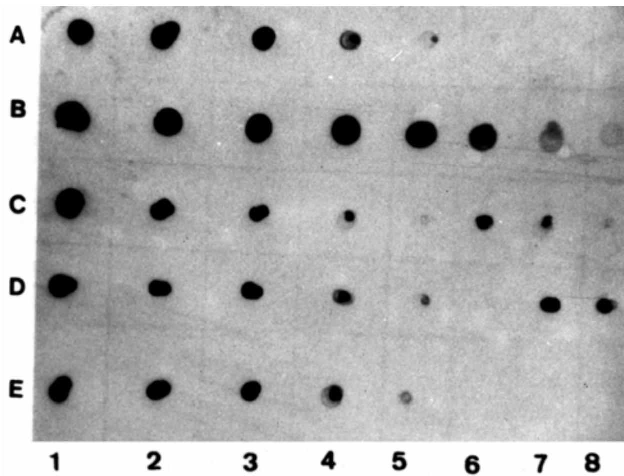

Fig. 5—Detection of rotavirus in nucleic acid extracted from faecal samples and reference strains by dot-blot hybridization assay using DIG-labelled gene 9 probe. Row A—1: Positive control (NCDV); 2-8: RNA from bovine faecal samples. Row B and D— 1-8: RNA from bovine faecal samples. Row E—1-6: RNA from faecal samples; 7 & 8: Uninfected cell culture (MA 104) and Heterologous (Bluetongue ds RNA) nucleic acid control.

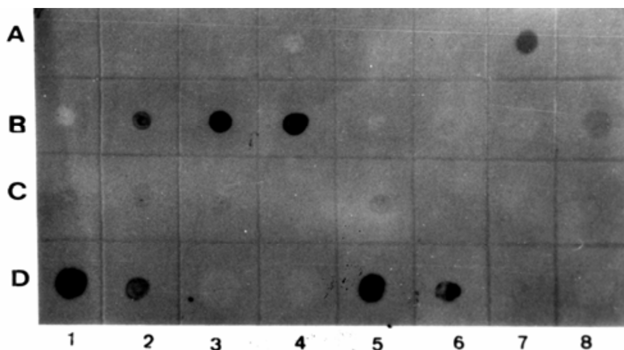

Fig. 6—P11 genotyping of bovine faecal samples by DIG-labelled nested probes. Column A-C: RNA of faecal samples showing hybridization signals with P11 probe. Column D —1: Positive control (B223 strain); 2-6: Faecal RNA; 7 & 8: Cell culture and heterologous nucleic acid controls

Earlier, the dot-blot hybridization has also been reported 10 to 100 times more sensitive than confirmatory ELISA[28]. Pande & Pandey[29] used DIG-labelled VP7 gene specific probe for detection of bovine rotaviruses and hybridization assay was found 1000 times more sensitive than PAGE. Husain *et al*[30] reported almost no difference in terms of sensitivity when typing by nested-PCR was compared with internal primers using serotype specific radio-labelled oligonucleotide probes. Moreover, the chances of carry over contamination in the later approach were almost negligible. In the present study, each probe was specific as it hybridized specifically to target DNA or RNA from rotaviruses of homologous genotype and no cross hybridization was observed with heterologous nucleic acids (Bluetongue virus and *S. typhimurium*).

Many workers have used ^{32}P labelled probes for G and P typing of human and bovine rotaviruses[31-34]. However, non-radiolabelled probes have only been recently used for detection and G and P typing of group A rotaviruses of animal and human origin. The very first DIG-labelled non-radioactive PCR derived cDNA probe was used to type G6 genotype in bovine field samples[35]. Subsequently, these non-radioactive probes were also used for G typing of stool samples of diarrhoeic children[36] and P genotyping to discriminate between the different alleles[37-40]. DIG-labelled non-radioactive typing probes, corresponding to the variable regions in VP4 and VP7 cognate genes of bovine prototype strains, were of G6 and G10 genotypes (2 G type specific), and P1, P5 and P11 genotypes (3 P type specific). In a study conducted outside India, both G10 and G6 were recorded predominant G types of group A rotaviruses of bovine origin[6]. In our studies, however, G10 was recorded the most predominant G type, followed by G6. These findings are in conformity with the studies conducted in Sweden and Thailand where G10 has also been reported to be the major genotype among cattle herds[41,42]. While, in countries like Argentina, China, Japan, UK and USA, the genotype G6 has been reported the most dominating G type, followed by G10[6,34,43-48]. Further, P11 was reported the most predominant P type, in the present study, followed by P1 and surprisingly P5 type was absent. The complete absence of P5 types indicates that this particular genotype is not circulating at all among diarrhoeic bovine calves in Haryana and adjoining areas of India. On the contrary, the P typing reports in other countries, such as USA and Japan, demonstrate that P5 is the most prevalent type, followed by P1 and P11 among diarrhoeic calves[34,46,47,49,50]. In the present study, the most prevalent G and P genotypic combination reported among bovine calves was G10P11 (74.64%), followed by G6P11 (18.11%) and G6P1 (7.25%); whereas, G10P1 or G10P5 combinations were not observed. However, in other countries, G6P5 has been reported the most predominant combination, followed by G6P11 and G6P1 or G10P5; whereas, combination G10P11 was least frequently found[34,46,47,49].

The non-radioactive nucleic acid based hybridization method, used in the present study, was found suitable for the analysis of large number of samples and thus could provide a cost-effective tool for use in epidemiological studies. Being non-radioactive, these probes are safe in use. Further, the versatility of the nucleic acid hybridization assay could be increased by incorporation of probes against less common G or P types. The DIG-labelled probes could be stored for extended period of time. In addition, any isolates of unusual or novel G or P types could be used to generate additional probes, which could then be screened against available animal or human G and P types.

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