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Genotyping of rotavirus of neonatal calves by nested-multiplex PCR in India

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ABSTRACT

A total of 209 diarrhoeic faecal samples were collected from calves in nine diary cattle herds located in different areas in Tamil Nadu state, India. Nine faecal samples positive for group A rotavirus by polyacrylamide gel electrophoresis (RNA-PAGE) followed by silver staining were analyzed by nested-multiplex-reverse transcription polymerase chain reaction (nested-multiplex PCR) to identify the P and G genotypes. Nested-multiplex PCR was carried out using the type-specific primers of common genotypes P[11],G10 and P[5],G6 to identify their prevalence. Three out of nine samples were found to be P[1],G10 genotype, and none of the samples was P[5],G6 genotype. The occurrence of zoonotic important bovine genotype (P[11],G10) was reported in this study.

Key words: calves, diarrhoea, bovine rotavirus, genotype, nested-multiplex PCR

Introduction

Livestock faming plays an important role in the rural development programs in Tamil Nadu, India. The future of any dairy operation depends upon a successful program of raising calves. However, it has been observed that large number of calves die at an early age. 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 rotavirus, members of the genus Rotavirus within the family Reoviridae, are the leading cause of diarrhoea in calves under one month of age throughout the world (SAIF et al., 1994). The viral genome is formed by eleven double stranded RNA (dsRNA) segments, and rotaviruses are classified into seven groups (A to G) based on antigenic differences on the inner capsid VP6 protein (ESTES, 2001).

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The virion of group A rotavirus consists of three concentric layers of protein. There are two independent neutralization antigens on the outer capsid, namely VP7 encoded by gene segment 7, 8 or 9 (depending on the strain) and VP4 encoded by gene segment 4 (ESTES and COHEN, 1989). Group A rotavirus is classified according to its combination of two kinds of serotype, namely G (glycoprotein) serotype determined by VP7 antigenicity and P (protease-sensitive protein) serotype associated with VP4 antigenicity (ESTES and COHEN, 1989). At least 14 G genotypes of rotavirus have been identified in various species of animals and most bovine rotaviruses (BRV) belong to types G6 and G10 (SNODGRASS et al., 1090). Among the 20 P genotypes, P[1], P[5] and P[11] have been established for the VP4 genes of BRV strains (SNODGRASS et al., 1992).

Genotyping has been preferred to serotyping due to its good correlation with serotypes, high sensitivity and use of synthetic reagents (GOUVEA et al., 1990). Since there are many combinations of P and G genotypes in bovine group A rotavirus (BRV), research into the genotyping of BRV is very important for preventive veterinary medicine and, more specifically, for the development of a vaccine. It is also important from the point of view of ecology and public health, because interspecies transmission from cattle to humans and from humans to cattle have been reported. Particularly rotavirus P[11],G10 strains, which are commonly found in cattle had frequently been associated with asymptomatic neonatal infections in Tamil Nadu, India (ITURRIZA GOMARA et al., 2004).

The aim of the present study was to identify the distribution of bovine rotaviruses in Tamil Nadu calves with regard to the combination of P and G types in recent years by using polymerase chain reaction- (PCR) based typing assays.

Materials and methods

Faecal samples. Faecal samples were collected from 209 diarrhoeic calves below 12 weeks of age from various regions in Tamil Nadu, India. All faecal specimens were stored at -20 °C until further use. A 10% suspension of each faecal sample was prepared in phosphate buffered saline (PBS), pH 7.2 and centrifuged at 6,000g for 30 min. at 4 °C. The supernatant was either stored at -20 °C or processed for RNA extraction.

Extraction of rotaviral RNA. The viral dsRNA was extracted from the 10% stool suspension as per the method described by STEELE and ALEXANDER (1987). Briefly, the 10% stool suspension was added to 1\10 volume of 1M sodium acetate containing 1% sodium dodecyl sulphate (SDS) and incubated at 37 °C for 15 min. The mixture was then vortex mixed with one volume of 1:1 Phenol - Chloroform mixture and incubated at 56 °C for 15 min. and centrifuged at 10,000g for 3 min. The aqueous phase thus obtained was added to 1\10 volume of 3M sodium acetate, together with 3 volumes of 70% ethanol to precipitate the RNA. The mixture was kept at -20 °C for 10 min. and centrifuged at

10,000g for 10 min. to pellet RNA. The RNA pellet was air dried and dissolved in 1\20 volume of diethyl pyrocarbonate- (DEPC) treated water.

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 25mA for 16 h. using 5% stacking and 10% separating polyacrylamide gel. The extracted viral dsRNA was mixed with 0.04% bromophenol blue solution and 1\10 volume (v/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 HERRING et al. (1982). Briefly, the polyacrylamide gel was shaken for 30 min. 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 1 h. in 0.11 M silver nitrate solution. The silver nitrate solution was removed, the gel was washed three times in distilled water and then shaken for 15 m. in a mixture of 0.75 M NaOH and formaldehyde. The gel was washed twice in distilled water, shaken for 5 m. in 5% (v/v) acetic acid solution and the electropherotype was then identified.

Primers. The consensus primers C1 and C2 were synthesised based on the reports of GOUVEA et al. (1990) and type-specific primers for G genotypes employed in this study were synthesized on the basis of the reports of TANIGUCHI et al. (1992) and primers for P genotypes were synthesized on the basis of the reports of ISHIZAKI et al. (1995). The sequences of the primers, their positions in the genomic segment and the prototype viruses from which the sequence taken are presented in Table 1.

Reverse transcription polymerase chain reaction and nested-multiplex PCR. RT-PCR for G gene and nested-multiplex PCR for differentiating G genotypes. G-typing of all the strains was essentially performed as previously described by TANGUCHI et al. (1992). A pair of conversed primers C1 and C2 was used to amplify full-length copies of the rotavirus VP7 gene under PCR conditions of initial denaturation of 94 °C for 5 min. followed by 30 cycles of 94 °C for 1 min., 45 °C for 1.5 min. and 72 °C for 2 min., and a final extension of 72 °C for 10 min.

A 1:100 dilution of the first PCR product was subjected to nested (second) PCR amplification with a mixture of type-specific primers S6 and S10, and a conserved primer C1 with the following PCR conditions: Initial denaturation of 94 °C for 5 min., followed by 30 cycles of 94 °C for 1 min., 45 °C for 1.5 min. and 72 °C for 2 min., and the final extension of 72 °C for 7 min. PCR products were analyzed by electrophoresis on a 1% agarose gel in Tris-acetate-EDTA buffer containing ethidium bromide under UV light.

Reverse transcription polymerase chain reaction and nested-multiplex PCR. RT-PCR for P gene and nested-multiplex PCR for differentiating P genotypes. P-typing of all the strains was carried out by the PCR method described by ISHIZAKI et al. (1995). Briefly, viral dsRNA of isolated strains was used as the template for reverse transcription and first

PCR amplification. A pair of conserved primers PC1 and PC2 was used to amplify partial-length copies of the rotavirus VP4 gene under PCR conditions of initial denaturation of 94 °C for 5 min., followed by 30 cycles of 94 °C for 1 min., 45 °C for 1.5 min. and 72 °C for 2 min., and the final extension of 72 °C for 10 min.

Table 1. Oligonucleotide primers used for G and P genotyping of bovine rotavirus

Primer	Type of PCR	Sequence 5' - 3'	Position	Sense	Strain
G Typing					
C1	RT - PCR	GGT CAC ATC ATA CAA TTC TAA TCT AAG	1039-1062	-	SA 11
C2		GGC TTT AAA AGA GAG AAT TTC CGT CTG G	1-28	+	Wa
S6	Nested-multiplex PCR	GAT TCT ACA CAA GAA CTA GA	481-500	+	NCDV
S10		CTA GAA CAG AAA TAA ACG A	314-332	+	61A
P Typing					
PC1	RT - PCR	AAT GCT TGT GAA TCG TCC CA	1075-1094	-	NCDV
PC2		ATG GCT TCG CTC ATA TAC AGA CAG	10-33	+	UK
PS5	Nested-multiplex PCR	TCA ATT AAA CCA AGA GAA TGT	733-752	+	UK
PS11		ACA TAT TCT CAT CCG GTG CC	577-596	+	A44

A 1:100 dilution of the first PCR product was subjected to nested (second) PCR amplification with a mixture of type-specific primers PS11 and PS5, and a conserved primer PC1 with the following PCR conditions: initial denaturation of 94 °C for 5 min., followed by 30 cycles of 94 °C for 1 min., 45 °C for 1.5 min. and 72 °C for 7 min. PCR products were analyzed by electrophoresis on a 1% agarose gel in Tris-acetate-EDTA buffer containing ethidium bromide under UV light.

Results

In a total of 209 faecal specimens obtained from the diarrhoeal calves, nine specimens were positive for BRV in standard PAGE assays. All nine samples exhibited electropherotype profiles typical of group A mammalian rotavirus, with segments 2, 3 and

4 migrating close together, segments 7, 8 and 9 closely spaced, and segments 10 and 11 as found in long RNA migration patterns. There was no difference in migration pattern by RNA-PAGE.

In the first (RT-PCR) for the identification of G genotype, expected amplicon size of 1062 bp of full-length copies of VP7 (G) gene was obtained in all nine positive field samples (Fig. 1). In the second PCR (nested-multiplex PCR) for differentiating the G genotypes, the expected PCR products of 749 bp size were obtained for the G10 genotype in three field samples and no amplification was noticed for the genotype G6 (Fig. 1).

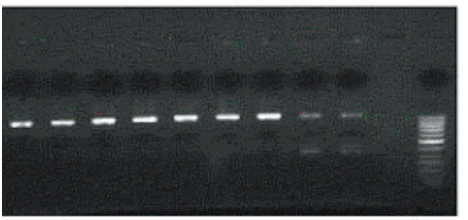

Fig. 1. Agarose gel (1%) electrophoresis for demonstration of RT-PCR product (1062 bp) of partial-length VP7 gene. Lanes 1-9: Amplified product of VP7 (G) gene of rotavirus positive field isolates. Lane 10: Negative control. Lane 11: 100 bp DNA Ladder.

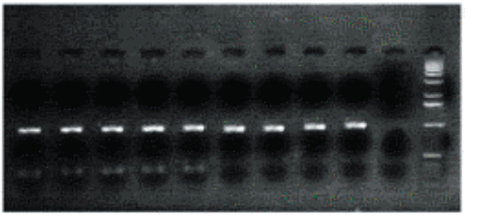

Fig. 2. Agarose gel (1%) electrophoresis for demonstration of RT-PCR product (1042 bp) of partial-length VP4 gene. Lane 1-9: Amplified product of VP4 (P) gene of rotavirus. Positive field isolates. Lane 10: Negative control. Lane 11: 1 kb DNA Extension Ladder.

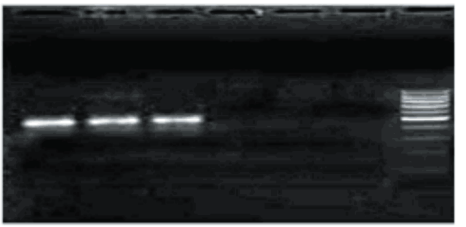

Fig. 3. Agarose gel (1%) electrophoresis of nested-multiplex PCR product for differentiating P genotypes. Lanes 1-3: VP4 positive samples showing amplification of P[11] genotype. Lane 1: ERV 18. Lane 2: VRV 03. Lane 3: ERV 19. Lanes 4-6: VP4 positive samples showing no amplification for P[11] & P[5] genotypes. Lane 7: 100 bp DNA Ladder.

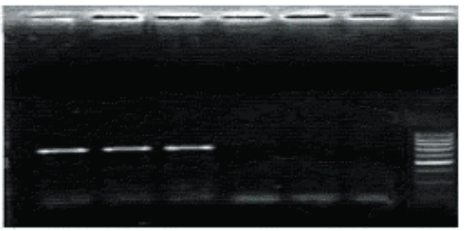

Fig. 4. Agarose gel (1%) electrophoresis of nested-multiplex PCR product for differentiating G genotypes. Lanes 1-3: VP7 positive samples showing amplification of . G10 genotype. Lane 1: ERV 18. Lane 2: VRV 03. Lane 3: ERV 19. Lanes 4-6: VP7 positive samples showing no amplification for G10 & G6 genotypes. Lane 7: 100 bp DNA Ladder.

In the first PCR (RT-PCR) for the identification of P genotype, expected amplicon size of 1042 bp of partial-length copies of VP4 (P) gene was obtained in all the nine positive field samples (Fig. 2). In the second PCR (nested-multiplex PCR) for differentiating the P genotypes, the expected PCR products of 515 bp size was obtained for the P[11] genotype in three field samples and no amplification was noticed for the genotype P[5] (Fig. 3).

The G and P genotypes of only three samples identified using the type-specific primers of commonly found bovine genotypes were P[11],G10 and these accounted for three samples. Prevalence of zoonotic important bovine rotavirus genotype (P[11],G10) in Tamil Nadu was identified in this study.

Discussion

The genome electropherotyping by polyacrylamide gel followed by silver staining was a highly sensitive method that had been undertaken in this study for detection of rotavirus as reported by KHATTER and PANDEY (1986). In this study, the dsRNA obtained by direct extraction of faeces on PAGE revealed the characteristic 11 segmented electropherotyping migration pattern in all the nine field samples positive by SS-PAGE. On electrophoresis followed by silver staining, 11 bands of dsRNA genome appearing in the gels were grouped into 4 classes in 4:2:3:2 pattern based on their electrophoretic mobility.

In the present study, consensus primers were used to amplify the partial-length copies of the rotavirus VP4 gene which yielded the expected amplicon size of 1042 bp and full-length copies of VP7 gene, which yielded 1062 bp product in all the nine field samples, which in turn was used as a template in nested-multiplex PCR to identify all the possible G and P genotype present in bovine rotavirus strains as reported by ALFIERI et al. (2004).

Recently, modified PCR methods such as nested-multiplex PCR had been developed to type rotaviruses. The nested-multiplex PCR was used in this study for genotyping by using a second stage PCR routinely and using new type-specific primers for genotypes as previously described by ISHIZAKI et al. (1996). This technique had been used throughout the world for the group A rotavirus in strains obtained directly from extracts and/or cell culture by ALFIERI et al. (2004).

P and G genotype associations most commonly identified in cattle were P[5],G6, P[11],G10 and P[1],G6, as reported by SNODGRASS et al. (1990). Hence, in this study we attempted to discover the associations of P[5],G6 and P[11],G10 which were very common genotypes observed in bovine rotavirus, as reported by ISHIZAKI et al. (1996).

In this study, nested-multiplex PCR for differentiating P genotypes showed predominance of P[11] genotype, three out of nine rotavirus-positive field samples in Tamil Nadu and the occurrence of P[5] was not observed, while differentiating G genotypes showed that G10 was more prevalent, three out of nine rotavirus positive field samples in Tamil Nadu and the occurrence of G6 was not noticed. The most prevalent

combination of bovine group A rotavirus was found to be P[11],G10 in Tamil Nadu (3 out of 9 rotavirus-positive samples) and none of the samples showed the combination of P[5],G6 as reported by GULATI et al. (1999). The type-specific of very common and zoonotic important genotypes alone were used in this study. Hence, some other genotypes may be present in other rotavirus-positive field samples.

These findings were contrary to the distribution of G and P genotypes of group A BRV in the global scenario. In Japan, FUKAI et al. (1998) reported that 89.3% of BRV isolates belonged to P[5],G6 and only 3.6% were P[11],G10 genotype. In Brazil, ALFIERI et al. (2004) reported that 40% of isolates were P[5],G6, and that only 16% were P[11],G10 genotype. In our study, the common genotype may not be as prevalent as reported by previous workers. Among the Indian bovine population, GULATI et al. (1999) reported that 83% of bovine isolates were only P[11],G10 genotype. Our research findings indicating P[11], G10, the most common genotype in Tamil Nadu, was in agreement with the findings of GULATI et al. (1999).

Of the nine positive rotavirus samples, five were from calves aged less than 4 weeks, and the remainder from calves above 4 weeks of age. For the relative frequencies of the G and P types of the Tamil Nadu BRV, the most conspicuous finding was that 3 out of 9 BRVs carried P[11] VP4s and G10 VP7s, and that three out of nine bovine rotavirus positive samples showed the combination of P[11]G10 rotavirus strains. In the P[11],G10 rotavirus, two rotavirus samples were from calves less than 4 weeks of age and one from a calf older than 4 weeks. Although interpretation of this study was limited by a small sample size and sample collection only in restricted regions in Tamil Nadu, 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 summer 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).

The P[11],G10 genotype strain was an important genotype of group A BRV because of its zoonotic transmission from humans to cattle and also from cattle to humans, as reported by ITURRIZA-GOMARA et al. (2004) in Tamil Nadu. In this study, we describe the detection of bovine P[11],G10 genotype strains in Tamil Nadu which may be recognized as an important contributor to the diversity of rotaviruses found in human infections. The close interaction of the majority of the Indian population, particularly that of the Tamil Nadu population with cattle, makes possible the transmission of rotavirus from cattle to children. In conclusion, nested-multiplex PCR, which was an alternative to serotyping for identification and typing of rotavirus genotypes, had facilitated the studies on the occurrence and distribution of individual P and G genotypes of the bovine population in Tamil Nadu, in which, P[11],G10, a zoonotic important bovine genotype, was found to be present.

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SAŽETAK

Ukupno je bilo prikupljeno 209 uzoraka proljeva teladi s devet farmi mliječnih krava na različitim područjima pokrajine Tamil Nadu u Indiji. Devet uzoraka pozitivnih na skupinu A rotavirusa pretragom poliakrilamid gel elektroforezom uz naknadno bojenje srebrom bilo je analizirano mnogostruko ugniježđenom lančanom reakcijom polimerazom (PCR) uz prethodnu reverznu transkripciju radi identifikacije genotipa P i G. Mnogostruko ugniježđena PCR provedena je upotrebom tipski specifičnih početnica skupnog genotipa P[11],G10 i P[5],G6 radi određivanja njihove prevalencije. Za tri od devet uzoraka ustanovljeno je da su pripadali genotipu P[11]G10. Nijedan uzorak nije pripadao genotipu P[5],G6. Opisana je pojava genotipa rotavirusa goveda (P[11],G10) važnog kao uzročnika zoonoze.

Ključne riječi: telad, proljev, goveđi rotavirus, genotip, mnogostruko ugniježđena lančana reakcija polimerazom