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POLYMERASE CHAIN REACTION FOR CHARACTERIZATION OF VP4 GENE TYPES OF GROUP A BOVINE ROTAVIRUS DIRECTLY IN THE FECES OF NEONATAL DAIRY CALVES

By

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SUMMARY

A PCR (polymerase chain reaction) genotyping assay was applied to identify bovine rotavirus (BRV) gene 4 types (P genotypes, NCDV (P1), UK (P5), and B223 (P11) in the field samples collected from calves with and without diarrhoea. Rotavirus double stranded RNAs (dsRNAs) extracted from the fecal samples were reverse transcribed and amplified by PCR using two nucleotide primers that correspond highly conserved regions of the VP4 gene among rotaviruses. The amplified products (that included the VP8 portion and the connecting peptide of the VP4 gene) were then amplified by PCR int he presence of coctail of specific primers for three reference BRV. The PCR-typing assay was applied to identify BRV P genotypes (P1, P5 and P11) in 73 field samples. These samples were reacted positive for BRV by polyacrylamide gel electrophoresis and monoclonal antibody - based ELISA. The PCR assay was able to identify BRV different P genotypes in 79.4% of the samples. Mix of two P genotypes was effciently detected in the same samples by the applied method. In order to detect all G/P possible combination types among BRV field isolates, the P typing results were compared with the previously reported G-typing assay applied in this study provided a very sensitive method for genotyping of Brv field strains.

INTRODUCTION

Group A bovine rotavirus (BRV) is a major cause of diarrhoea in one - to three-week old calves throughout the world (Woode and Bridger, 1975). The viral genome consists of 11 segments of dsRNA surrounded by a double capsid (Estes and Cohen, 1989). The outer capsid is composed of VP7 glycoprotein encoded by gene segments 7,8 or 9, depending on the virus strain, and VP4 protein encoded by gene segment 4 (Estes and Cohen, 1989). The VP4 and VP7 outer capsid components are responsible for inducing neutralizing antibodies (Estes and Cohen, 1989). The classification of mammalian Group A

rotavirus is primarily based on VP7 (G-types) and VP4 (P-genotypes) and so far, 14 G-types and 19 P-genotyps have been described (Hoshino and Kapikian, 1994; Sereno and Gorziglia, 1994). Based on the use of monoclonal antibodies (Mabs) (Bellinzoni et al., 1989; Snodgrass et al., 1990; Lucchelli et al., 1994; Hussein et al., 1995) or eDNA probes (Parwani et al., 1993; Hussein et al., 1993), at least 7 G-types (G1, G2, G3, G6, G8, G10 and G11) and three P genotypes (P1, P5 and P11) (Mastuda et al., 1990; Snodgrass et al., 1992; Parwani et al., 1993) have been reported among BRV isolates. The use of Mabs in P-typing of BRV has been hampered by the difficulty in preparing VP4-Mabs specific for each P type and the presence of cross reactive epitopes on the VP4 protein (Taniguchi et al., 1990). Previous investigators have used cDNA probes to characterize BRV P types in the fecal samples (Parwani et al., 1993). Although cDNA probes were useful for P typing system, 40% of the BRV-positive field samples were considered untypable (Parwani et al., 1993). The PCR-typing assay to identify BRV p types has been described (Gouvea et al., 1994). Recently, RT-PCR followed by PFLP (restriction fragment length polymorphism) analysis was applied to characterize G and P types of field strains in USA (Chang et al., 1996).

To date, PCR typing assay for BRV VP4 genotypes has only been employed for typing cell culture isolated strains of BRV (Suzuki et al., 1993; Ishizaki et al., 1996) and not for characterizing BRV P genotypes directly in fecal samples. The aim of this study is to apply RT-PCR typing assay in VP4-genotyping of BRV field strains directly in the fecal material and to detect the predominant BRV G/P combination types existing among the field isolates.

MATERIAL AND METHODS

Viruses and cells

Fecal samples were obtained from calves with and without diarrhoea in 12 geographic regions of Quebec between 1992 and 1994, for the detection of group A bovine rotavirus. Seventy three samples were positive for BRV by both polyacrilamide gel electrophoresis (PAGE) and ELISA (Hussein et al., 1995) were used in this study. Three reference strains of BRV (NCDV, UK and B223) were employed as controls and five BRV-negative fecal samples were included as monitors for cross-contamination. The viruses were grown on Rhesus monkey kidney (MA-104) cells in the presence 0.5 Rg of trypsin per ml of medium as described previously (Saif et al., 1988).

Extraction of dsRNA and RT-PCR

PRV dsRNA was extracted from fecal samples using the RNaid commercial kit (Bio 101 Inc, LaJolla, Ca, USA) as previously described (Gentsch et al., 1992; Ushijima et al., 1992). The procedure involved dissociating the virus from cells in guanidinium solution, binding the viral dsRNA to glass powder and separating the glass powder from the cell debris by differential centrifugation. The dsRNA was eluted in water and kept at - 20°C until used for the RT-PCR.

Oligonucleotide primers

A Con3 primer, analogous to nucleotides (nt) 11 to 32 (5'TGGCTTCGCTCATTTATAGACA3') and Con2 primer, complementary to nt 868 to 887 (5'ATTTCGGACCATTTATAACC3') were used to amplify an 877 bp fragment of the rotavirus VP4 gene that included the VP8 portion and the connecting peptide. Con2 primer was employed in a second PCR typing assay with a cocktail of primers which included pNCDV, a P1-specific primer complementary to nt 269 to 289 (5' CGAACGCGGGGGTGGTAGTTG 3'), pUK, a P5-specific primer complementary to nt 336 to 354 (5'gccaggtgtcgcatcagag3) and pB223, a P11-specific primer complementary to nt 574 to 594 (5'ggaacgtattctaatccggtg3'). A fragment of about 619 bp was generated for P1, 552 bp for p5 and 314 bp for P11.

P. genotyping by RT-PCR

RT-PCR was performed a modification of previously published methods (Gentsch et al, 1992; Ushijima et al., 1992). Briefly, 5 µ1 of dsRNA was mixed with 3.5 µ1 of dimethylsulfoxide in a microcentrifuge tube, denatured by heating at 95°C for 5 min and immediately cooled on ice. The reverse transcription mixture (50 mM Tris-HCI, pH 8.3, 50 mM KCI, 8mM MgC12, 2.5 mDTT, 500 µM of each dNTP, 20 U of RNA sin (Promega Corporation, Fisher Scientific, Montreal, Que, Bec, Canada), 1 µ M each of the Con3 and Con2 primers and 8 units of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim, Laval Quebec, Canada) was added to the denatured dsRNA and the tubes incubated for 1 hr at 42°C. Five µl of the reverse transcription product was amplified by PCR in the presence of PCR mixture (10mM Tris-HCI, pH 8.3, 50M M KCI, 2mM MgC12, 200 µ M each of dNTP, 0.05% gelatin, 1 µ M each of Con3 and Con2 primers and 2.5 U of Taq polymerase (GIBCO, BRL, Canadian Life Technologies, Inc, Canada). The PCR was performed through 30 cycles of 94° for 1 min, 42°C for 2 min and 72°Ç for 3 min followed by a final incubation for 7 min at 72°C. The second PCR amplification was conducted on the first PCR product after 1:100 dilution in the presence of the same PCR mixture, but containing 1 µ M each of Con2, pNCDV, pUK and pB 223 primers and 2.5 U of Taq polymerase. The second PCR amplification consisted of 25 cycles of 94°C for 1 min, 55°C for 2 min and 72°C for 1 min. Ten µ 1 of each PCR products was loaded onto 2 % agarose gels in Tris borate buffer containing 0.5 µg/ml ethidium bromide. Electrophoresis was conducted for 2 hr at 120 V and the gels were photographed under UV light.

RESULTS

The PCR employed in this study correctly identified BRV P-genotypes directly from fecal samples as well as the three cell-culture propagated control reference strains (Figure 1). Of the 73 field strains identified as BRV - positive by PAGE and Mab-Elisa, 37 (50.6) were classified as P5, 20 (27.3%) as P11, one (1.2% was P (5+11) and 15 (20.4 %) specimens were negative for the three BRV P-genotypes (Table 1). In order to detect all possible G/P combination types existing among BRV field isolates in

Quebec, the P typing results were compared with G-typing analysis of the same specimens (Hussein et al., 1995; 1996). Of the 73 fecal speciments, 34 (46.5 %) of the specimens were G6P5, 18 (24.6 %) were G10P 11,2 (2.7 %) were G6P11, 2 (2.7 %) were G10P5, one (1.3 %) was G (6 + 10) P (5 + 11), one (1.3 %) G (6 + 10) P5 and 15 (20.5 %) specimens were negative for P1, P5 and P11 genotypes (Table 1). When the PCR buffer was modified to contain 1.5 mM $MgCl_2$ rather than 2mM, P11 strains were detected more readily. Intially 18 specimens were identified as negative, but when the samples were subjected to PCR typing using separate rather than a cocktail of primers, we were able to detect three more samples (two P5 and one P11) to give a total of 37 P5 and 20 P11 and to reduce the number of P-type negative samples from 18 to 15. It should be noted that the VP8 portion of two of the 15 negative samples that identified as G10 virus (Table 1), when sequenced, was found to be P11 type (data not shown).

Table 1: Chracterization of BRV P genotypes in feces of neonatal dairy calv from Quebec using RT-PCR typing assay.

G/ P types	P-typing[a]					
	P 1	P 5	P11	P5 + 11	-	P-typing[4]
G6	-	34	2	-	-	36
G10	-	2	18	-	2[b]	22
G6 + 10	-	1	-	1	-	2
(-)	-	-	-	-	13	13
	-	37	20	1	15	73
Total	(0 %)	(50.6 %)	(27.3 %)	(1.3 %)	(20.5 %)	

[a] G- and P-typing results obtained by using PCR typing assays.
[b] confirmed as P11 type by sequence analysis.

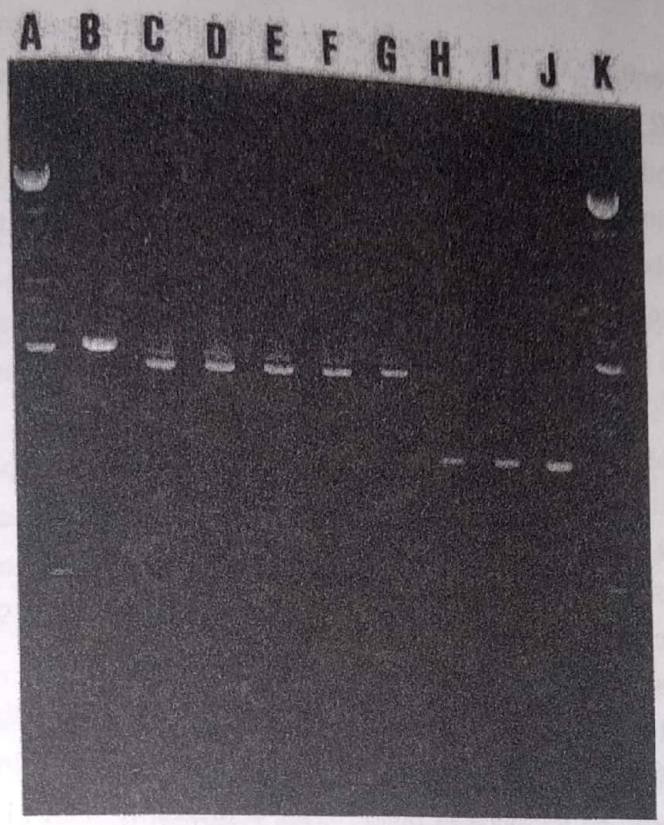

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Figure 1: PCR typing of the amplified VP4 gene from BRV field and reference strains. Lanes A and K represent the 100bp DNA ladder (GIBVE BRL, Canadian Life Technologies, Montreal, Canada). Lane B, band of approximately 619 bp, represents the second PCR typing amplification products from NCDV reference strain (P1-type). Lane C, band of approximately 552 bp, represents the UK reference strain (P5 type). Lanes D to G, represent local field strains characterized from fecal material as P5 type. Lane H , band of approximately 314 bp , represents B223 reference strain (P11-type). Lanes I and J represent local field strains characterized as P11 type.

DISCUSSION

To date, primer-specific PCR typing assay has only been employed for typing BRV field strains isolated in cell cultures (Suzuki et al., 1993; Ishizaki et al., 1996) and not for characterizing BRV P-types directly in fecal samples. The sensitivity of the PCR typing assay depends on the formulation of primers, the reaction condition and the presence of inhibitors in the samples. The PCR assay used in this study was sensitive enough to dentify 79.4 % (58/73) of the rotaviruses in fecal samples as P-genotypes. PCR typing assay can therefore provide the basis for a rapid and sensitive screening and simultaneous typing of BRV directly from fecal samples.

The number of P-genotype negative strains reported in this study (20.4%) and in a previous epidemiological survey utilizing cDNA probes (40.8 %) (Parwani et al., 1993) may be due to the sensitivity of the procedures, or alternatively, the presence of other P-genotypes and subtypes among the bovine population. Likewise, sequence

variation may also hamper the type-specific primers used in the PCR typing assay. Recently, the use of RT-PCR and RFLP to characterize G-and P-types of BRV in rotavirus-positive fecal samles from calves has been described (Chang et al., 1996), but the method requires a larger quantity of the amplified gene than is necessay for PCR typing assay in which there is no need to see a band of the RT-PCR amplified product to be utilized in the second PCR typing assay in. Moreover, the technique requires many enzymes that always cleave at a specific site, if there is a sequence variation, especially inthe third base of a codon, these enzyme would be ineffective. It would be siutable to utilize the RFLP analysis in characterization of the BRV untypable samples. In a further study to verify the presence of other non-bovine P-types among the 13 bovine P-type-negative viruses, a PCR typing assay using primers specific for porcine rotaviruses (Gouvea et al., 1994) was employed (data not shown), None of the 13 viruses could be typed. Specific primers to other types of rotaviruses, such as human rotavirus, might therefore be needed to identify more rotavirus field strains in he bovine species.

To date, three P-genotypes (P1, P5, and P11) of bovine rotavirus have been described (Matsuda et al., 1990, Parwani et al., 1993). The predominance of P5, followed by P11 were reported in studies using nested PCR (Suzuki et al., 1993; Ishizaki et al., 1996), cDNA probes (Parwani et al., 1993) and recently RT-PCR and REFLP (Chang et al., 1996). We report in this study the identification of BRV P-genotypes in 73 field strains obtained from dairy herds in Quebec. The typing results show similar findings to the aforementioned studies, with P5 strains being the most prevalent (50.6 %) followed by P11 strains (27.3 %). BRV G6P1 strain, which is commonly used in vacines in the USA (Chang et al., 1996), but not in Quebec, was not detected among the field strains we examined.

Other investigators reported the frequencies of G/P-types of BRV either in the fecal samples or in the cell culture isolated strains (Parwani et al., 1993; Suzuki et al., 1993; Chang et al., 1996; Ishizaki et al., 1996). In Japan, Suzuki et al., (1993) demonstrated that BRV G6P5 was the most prevalent strain in the field samples (42.5 %), followed by G6P11 (17.5 %), G6P1 (10 %), G10P5 (10 %) and G10P11 (7.5 %), However, in another study in Japan, Ishizaki et al. (1996) reported that, out of 76 BRV isolated in cell culture from fecal samples, G6P5 was predominant at 60.5 %, followed by G10P11 (17.1), G6P11 (9..2) and G10P5 (6.6 %). In the USA, Chang et al. (1996) reported that the predominant BRV strains were G6P5 at 46.7 % followed by G10P11 (12.8 %), G10P5 (7 %) and G6P11 (7 %). In this study, BRV G6P5 strain was the most common G/P combination type in the field (46.5 %); followed by G10P11 (24.6 %); G6P11 (2.7 %); G10P5 (2.7 %), G6 + 10 P5 + 11 (1.2 %) and G6 + 10P5 (1.3 %). The existence of multiple G and P combination and mixtures of viruses among bovine species indicate that a rate of reassortment occurs during natural infection (Gouvea and Brantly., 1995). This study shows that the most prevalent BRV P-type in Quebec is

P5 like others in the United states (Parwani et al., 1993; Chang et al., 1996), Great Britian (Redmond et al., 1992), Japan (Suzuki et al., 1993; Ishizaki et al., 1996) and Germany (Brussow et al., 1994). Studies on the distribution of G-and P-types within field strains of BRV are important for development of rotavirus vaccines.

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