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Molecular characterization of group A bovine rotavirus in southeastern and central-western Brazil, 2009-2010¹

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ABSTRACT.- Silva F.D.F., Gregori F., Gonçalves A.C.S., Samara S.I. & Buzinaro M.G. 2012. **Molecular characterization of group A bovine rotavirus in southeastern and central-western Brazil.** *Pesquisa Veterinária Brasileira* 32(3):237-242. Departamento de Medicina Veterinária Preventiva e Reprodução Animal, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista "Júlio de Mesquita Filho", Jaboticabal, SP 14884-900, Brazil. E-mail: vete_fer@yahoo.com.br

Rotavirus is an important cause of neonatal diarrhea in humans and several animal species, including calves. A study was conducted to examine 792 fecal samples collected from calves among 65 dairy and beef herds distributed in two of Brazil's major livestock producing regions, aiming to detect the occurrence of rotavirus and perform a molecular characterization of the rotavirus according to G and P genotypes in these regions. A total of 40 (5.05%) samples tested positive for rotavirus by the polyacrylamide gel electrophoresis (PAGE) technique. The molecular characterization was performed by multiplex semi-nested RT-PCR reactions, which indicated that the associations of genotypes circulating in herds in Brazil's southeastern region were G6P[11], G10P[11], G[-]P[5] + [11], G[-]P[6] in the state of São Paulo and G6P[11], G8P[5], G11P[11], G10P[11] in the state of Minas Gerais. In the central-western region, the genotypes G6P[5] + [11], G6P[5], G8P[-], G6P[11], G [-] P[1], G[-] P[11], and G[-] P[5] were detected in the state of Goiás, while the genotypes G6P[5], G8[P11], G6[P11], G8[P1], G8[P5], G6[P1] were circulating in herds in the state of Mato Grosso do Sul. The genotypic diversity of bovine rotavirus found in each region under study underlines the importance of characterizing the circulating samples in order to devise the most effective prophylactic measures.

INDEX TERMS: Rotavirus, diarrhea, genotypes, cattle, Brazil.

RESUMO.- [Caracterização molecular de rotavírus bovino do Grupo A nas regiões Sudeste e Centro-Oeste do Brasil.] Rotavírus é uma importante causa de diarreia neonatal em humanos e várias espécies animais, incluindo bezerros. Foi realizado um estudo a partir de 792 amostras fecais colhidas de bezerros, provenientes de 65 rebanhos

de leite e corte distribuídos em duas das maiores regiões produtoras no Brasil, com o objetivo de se detectar a ocorrência de rotavírus e realizar a sua caracterização molecular quanto aos genótipos G e P nestas regiões. Um total de 40 (5,05%) de amostras testadas foram positivas para rotavírus pela técnica de eletroforese em gel de poliácridamida (PAGE). A caracterização molecular foi realizada através de reações do tipo Multiplex semi-nested RT-PCR demonstrando que as associações de genótipos circulantes em rebanhos da região Sudeste foram G6P[11], G10[P11], G[-]P[5]+[11], G[-]P[6] no Estado de São Paulo e G6P[11], G8P[5], G11P[11], G10P[11] no Estado de Minas Gerais. Na região Centro-Oeste, foram detectados no Estado de Goiás os genótipos G6P[5]+[11], G6P[5], G8P[-], G6P[11], G[-]P[1], G[-]P[11], G[-]P[5] enquanto os genótipos G6P[5], G8[P11], G6[P11], G8[P1], G8[P5], G6[P1] eram circulantes em rebanhos do Estado do Mato Grosso do Sul. A diversidade genotípica de rotavírus bovino encontrada em cada

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região estudada justifica a importância da caracterização das amostras circulantes para medidas profiláticas mais efetivas.

TERMOS DE INDEXAÇÃO: Rotavírus, diarreia, genótipos, bovinos, Brasil.

INTRODUCTION

Neonatal diarrhea is an important cause of morbidity and mortality in newborn calves (Lanz 2008), causing economic losses due to the costs of treatment, diagnostics, and poor growth performance (Constable 2004).

Although numerous etiological agents may be implicated in this clinical symptom (De La Fuente et al. 1998), group A rotavirus is one of the major causes of acute gastroenteritis in infants and in many animal species (Estes & Kapikian 2007).

The genus rotavirus belongs to the family *Reoviridae* and its genome is composed of 11 segments of double-stranded RNA (dsRNA) that encode six structural proteins (VP1 to VP4, VP6 and VP7) and six non-structural proteins (NSP1 to NSP6) (Greenberg & Estes 2009). These segments are classified serologically into seven different groups or species (A-G) (Estes & Kapikian 2007).

Group A rotavirus is classified as G and P genotypes or serotypes according to the genetic or antigenic characteristics presented by the proteins VP7 and VP4, both located in the virus outer capsid (Dennehy 2008).

Genotype-specific classification is based on the results of reverse transcription reactions followed by the polymerase chain reaction (PCR) or by nucleotide sequence analysis. At least 25 different G genotypes and 33 P genotypes have been described based on molecular differences (Collins et al. 2010, Esona et al. 2010, Abe et al. 2011). Among these genotypes, G6, G8, and G10 are the most commonly found in cattle, associated with P[1], P[5], or P[11] (Fukai et al. 1999, Ghosh et al. 2008), although the occurrence of human rotavirus serotypes G1, G2, and G3, and porcine rotavirus serotype G11 has also been reported (Hussein et al. 1993).

According to Martella et al. (2010), research on genetic variability provides useful data for a better understanding of virus epidemiology, particularly with respect to evidence of interspecies transmission of rotaviruses (Steyer et al. 2008, Matthijnsens et al. 2011), which increases the possibility of the emergence of new strains and unusual genotypic combinations. This type of research also underpins the development and monitoring of effective vaccines.

The purpose of this study was to ascertain the occurrence of rotaviruses in calves of dairy and beef herds in the Brazilian states of São Paulo, Minas Gerais (southeastern region), Mato Grosso do Sul, and Goiás (central-western region), which are two of the country's major beef producing regions, and to characterize the samples testing positive for rotavirus G and P genotypes by RT-PCR (reverse transcription-polymerase chain reaction).

MATERIALS AND METHODS

An analysis was made of 792 fecal samples from 10 to 60-day-old calves with and without symptoms of diarrhea, from 65 dairy and

beef herds. The samples were collected in municipalities located in the states of São Paulo, Mato Grosso do Sul, Goiás, and Minas Gerais from November 2009 to December 2010, and were stored at -20°C until analysis.

To detect rotaviruses, the fecal samples were screened by the polyacrylamide gel electrophoresis (PAGE) technique (Herring et al. 1982) modified by Pereira et al. (1985). Fecal suspensions (v/v; 50%) were prepared with ultra-pure water treated with 0.1% diethyl pyrocarbonate (DEPC water) and were clarified at 10,000 x g/15 min at 4°C, using only the supernatants in the assays.

The polymerase chain reaction (PCR) preceded by reverse-transcription of viral RNA was employed to characterize the bovine rotavirus genotypes G and P, using primers and conditions described by Gouvea et al. (1994a,b). The rotavirus strain NCDV was used as the positive control and ultrapure DEPC-treated water as negative.

Using Trizol reagent (Invitrogen®), the bovine rotavirus RNA was extracted from fecal suspensions clarified according to the manufacturer's instructions.

The cDNA synthesis and the first-round PCR amplification of VP4 and VP7 genes were performed using a SuperScript® One-Step RT-PCR kit (Invitrogen, Life Technologies). Briefly, 5 mL of extracted RNA, previously denatured at 95°C for 5 min, was added to a mixture containing 0.5 mM of each primer Beg9, End9, End9CRW8, End9UK (for G genotypes) or con3 and con2 (for P genotypes). The final volume of 25mL was completed with ultrapure water pretreated with diethyl pyrocarbonate. The cDNA synthesis was performed by incubating the mixture for 30 min at 45°C, followed by a 2-min cycle at 94°C. The resulting cDNA was then amplified in 30 1-min cycles at 94°C, followed by a 2-min cycle at 45°C and a 1-min cycle at 72°C. The final extension was performed at 72°C for 10 min.

The second round of amplification (multiplex semi-nested PCR) consisted of adding 0.5 mL of DNA amplified in the previous step to a solution of 1x PCR buffer II, MgCl₂ (1.5 mM), dNTPs (0.2 mM each); 0.5 mM of each primer (SBeg9, DT6, ET10, HT8, FT5, BT11 for G genotypes or con2, pB223, pGOTT, pNCDV, pOSU, PUK, for P genotypes), and 0.65U of Platinum Taq polymerase (Invitrogen®), added with DEPC water to a final reaction volume of 25 mL. This solution was then heated to 94°C for 2 min, followed by 25 1-min cycles at 94°C, 2-min cycle at 55°C, 1-min cycle at 72°C, and a final extension of 72°C for 10 min.

A total of 8mL of PCR products were analyzed electrophoretically in agarose gel 1.5% stained with 0.5 mg/mL of ethidium bromide and examined under ultraviolet light.

RESULTS

Forty of the samples (5.5%) tested positive for rotaviruses by the PAGE technique. Table 1 lists the G and P genotypes of bovine group A rotavirus identified by RT-PCR. The rotavirus genotypes G and P were fully characterized in 25 (62.5%) of the fecal samples. The VP7 gene could not be identified in 15 (37.5%) samples, while the VP4 gene was identified in 30 (75%) of samples.

With regard to the VP4 gene, four distinct P genotypes were identified (P[1], P[5], P[6] and P[11]). The most frequent genotype was P[11], which was detected in 16 (40%) samples, followed by P[5], P[1] and P[6], which occurred in 13 (32.5%), three (7.5%) and one (2.5%) sample (s), respectively. The VP7 genotypes observed were G6, G8, G10 and G11. Genotype G6 was the most frequent and was identified in 17 (42.5%) samples, followed by genotypes

Table 1. Distribution of G and P genotypes in 40 samples of field strains of bovine group A rotavirus, identified by RT-PCR in fecal material from calves in the states of São Paulo, Minas Gerais, Goiás and Mato Grosso do Sul, Brazil

Genotype	G6	G8	G10	G11	G [NI]	Total
P[1]	1	1	-	-	1	3
P[5]	6	2	-	-	2	10
P[6]	-	-	-	-	1	1
P[11]	8	1	2	1	1	13
P[5]+P[11]	2	-	-	-	1	3
P [ND]	-	1	-	-	9	10
Total	17	5	2	1	15	40

[NI] = Not Identified.

G8, G10 and G11, which were detected in five (12.5%), two (5%) and one (2.5%) sample (s), respectively.

Genotypic associations of P[5]+P[11] and of G6P[5]+P[11] were observed in one and two samples, respectively. Associations of genotypes circulating in herds in São Paulo were G6P[11], G10P[11], G[-] P[5] + [11] and G[-] P[6]. The genotypes found circulating in herds in Minas Gerais were G6P[11], G8P[5], G11P[11], G10P[11], while those identified in Goiás were G6P[5] + [11], G6P[5], G8P[-], G6P[11], G[-]P[1], G[-]P[11], and G[-]P[5]. Genotypes G6P[5], G8 [P11], G6[P11], G8[P1], G8[P5], and G6[P1] were found in herds in Mato Grosso do Sul.

DISCUSSION

Although the genotypes G[6], G[8], G[10] and P[1], P[5] and P[11] are found predominantly in cattle in Brazil (Brito et al. 2000, Alfieri et al. 2004) and in other countries (Falcone et al. 1999, Attar et al. 2002, Garaicoechea et al. 2006), infections in calves caused by group A rotaviruses belonging to other G and P genotypes have also been reported (Abe et al. 2011).

In the state of Goiás, the occurrence of the G6 genotype was found in 50% (9/18) of the samples, followed by genotype G8 in 5.5% (1/18). With regard to the VP4 gene, the following genotypes were detected: P[1] in 5.5% (1/18) of the samples, P[5] in 38.8% (7/18), P[11] in 33.3% (6/18) and associations of P[5] + [11] in 11.11% (2/18), indicating the presence of mixed infections. The most common genotypic combination was G6P[5], which was present in 22.22% (4/18) of the fecal samples. These findings are consistent with those reported by Caruzo et al. (2010), who identified genotype G6 as the most frequent (64.5%), P[1] genotypes in 9.7% and P[11] in 32.2%, and associations of P5 + P11 in a study of 31 samples that tested positive for rotavirus in the state of Goiás, although no G6P[5] genotype was detected.

According to Iturriza-Gomara et al. (2001), these combinations may originate from restructuring or co-infections characterized by genetic mechanisms of evolution, which involve pointwise mutations (drifts), restructuring (shifts, reassortant viruses) or exchange of RNA segments between samples. They may also give rise to rearrangements, which consist of duplications and deletions of nucleotide sequences within a genomic segment (Taniguchi & Urasawa

1995), reinforcing the idea that segments exhibiting VP4 and VP7 genes can segregate naturally and independently.

The most frequent genotype in 33.33% (2/6) of the fecal samples collected from herds in the São Paulo was G6, followed by G10 in 16.66% (1/6). Genotype [P11] occurred in 66.66% (4/6), of the samples, followed by two samples characterized as P[5] and P[6]. The most frequent combination was G6P[11] in 33.33% of fecal samples. These findings are consistent with those of Buzinaro et al. (2009), who performed a molecular characterization of bovine group A rotavirus in São Paulo during 2003 and 2004, and detected a prevalence of the G6 genotype in 61.1%, as well as the occurrence of genotype P[5] P[11] and combinations of P[5] + P[11].

In Mato Grosso do Sul, the G8 genotype was characterized in 37.5% (3/8), with a predominance of G6 in 50% (4/8) and types P[1] in 25% (2 / 80), P[5] in 50% (4/8), and P[11] in 25% (2/8) of analyzed samples. The most frequent combination was G6P[5] in 25% of fecal samples. The combination G8P[11] was also characterized and was considered the most prevalent unusual combination on a farm in Japan where 33 rotavirus samples were isolated and identified (Fukai et al. 1999).

Other researchers have identified strains with the G6 and G10 genotypes in Brazilian cattle herds. Brito (2000) identified G6 as the most common genotype in herds in Goiás, while Alfieri et al. (2004) found that G6 and G8 were the most frequent genotypes in beef and dairy herds in three Brazilian States. Fukay et al. (2002) in Japan and Saravanan et al. (2006) in India also identified G6, G8 and G10 as the most frequent genotypes in cattle.

As for the VP4 gene, the results found in this study can be considered highly unusual when compared with the P strains most frequently reported, P[11] and P[5]. Thus, 40% of the samples were characterized as P[11] and 32.5% as P[5]. Alfieri et al. (2004) reported different results for cattle in the states of Mato Grosso do Sul, São Paulo and Paraná, finding the prevalence of genotype P[5] in 66% of the samples. In beef and dairy herds in Italy, Falcone et al. (1999) detected the genotypes P[1], P[5] and P[11], with a predominance of genotype P[5]. Also, Monini et al. (2008) reported a 65.1% prevalence of P[11], followed by 25% of P[5], and only 2.5% of P-type combinations in Italian herds, but detected no P[1]. In Brazil, Barreiros et al. (2004) also detected P[11] and P[5] genotypes in herds in Mato Grosso do Sul, Paraná and São Paulo.

The most common combinations of VP7/VP4 genes in cattle are G6P[5] (UK-like), G6P[1] (NCDV-like) and G10P[11] (Hussein et al. 1993, Fukai et al. 1999, Ghosh et al. 2008). In this context, Garaicoechea et al. (2006) characterized 60% of the bovine rotavirus group A detected in herds in Argentina as G6P[5]. Alfieri et al. (2004) also reported the G6 P[5] combination as the most prevalent one in 40% of the samples they collected from herds in Brazil's southern, southeastern and central regions, followed by 12% of G6P[1] and 16% of G10P[11]. However, these frequencies were much higher than those found in the present study, which identified 20% as G6P[5], 2.5% as G6P[1] and 5% as G10P[11], despite the smaller number of genotyped samples.

On the other hand, in Ireland, Reidy et al. (2006) identified only 1.9% of their 54 samples as G6P[1] and 1.9% as G10P[11], while Garaicoechea et al. (2006) did not identify the combination G6P[1] and identified only 4.4% as G10P[11].

G6P[11] was the genotype most prevalent in this study, representing 20% of the samples, which is consistent with the findings of Caruzo et al. (2010), who identified 16.2% as G6P[11]. Reidy et al. (2006) and Garaicoechea et al. (2006) also reported this genotype in their studies, albeit at low frequencies (7.4% and 4.4%, respectively).

In the present study we observed 5% of G6P[5] + P[11] combinations, suggesting the presence of mixed infections, but at lower frequencies than those reported by Reidy et al. (2006) and Swiatek et al. (2009), who found mixed combinations of G6P[5] + P[11] in 11% and 15.4% of their samples, respectively. Therefore, the presence of more than one type of VP4 or VP7 gene in samples suggests mixed infections in a host, which can facilitate the occurrence of reassortants during replication of the virus, resulting in a new and diverse rotavirus population involving the exchange of one or more segments during replication of the virus, resulting in a new and diverse rotavirus population.

In the state of Minas Gerais, G6P[11] combinations were detected in 25% (2/8), G10P[11] in 12.5% (1/8), G8P[5] in 12.5% (1/8), and G11P[11] in 12.5% (1/8) of analyzed samples.

A G11 rotavirus strain was first isolated from pigs in several regions of Mexico in 1983 (Ruiz et al. 1988), and was later identified in the United States and Venezuela (Ciarlet et al. 1994, Rosen et al. 1994). Hussein et al.'s study (1993) confirmed the existence of the G11 genotype in samples of bovine rotavirus and other uncommon strains in the United States. The participation of genotype G11, which is typically considered porcine and has also been identified in humans (Bányai et al. 2009, Rahman et al. 2005, Shim et al. 2011), was also observed in the present work. This finding can be considered of major epidemiological significance in view of the scanty reports of this genotype in Brazilian cattle, and may be a result of sharing of the same environment by cattle and pigs. On the other hand, Gregori et al. 2009 also observed the presence of predominantly bovine genotypes for the molecular characterization of fecal samples from pigs in São Paulo state, suggesting a potential cross-infection of the virus.

G and P genotypes were not identified in 9 (22.5%) of the samples by semi-nested RT-PCR. In addition, the VP7 gene was not identified in 6 (15%) samples, but the VP4 gene was identified in 30 (75%) samples.

Several authors have reported unsuccessful molecular characterization of rotavirus samples, including Fukai et al. (2002), who identified genotype G in 76% of samples and P in 68.3% of bovine samples subjected to PCR, and Alfieri (1999), who identified genotypes in 96% of the samples he subjected to PCR.

Indistinct genotypic results by RT-PCR may be caused by RNA extracted directly from fecal samples, which could cause the co-precipitation of unspecific inhibitory substan-

ces present in the samples, which interfere in the early stages of PCR amplification, inhibiting the denaturation and annealing of primers, as described by Gouvea et al. 1990. Other possible causative factors are the long-term storage of samples, which may decrease the amount of target viral genomes (Rådström et al. 2002, Shay Fout et al. 2003); the virus in question may belong to a different genotype from the ones for which the primers used in the reactions were intended (Winiarczyk; Gradzki, 2002); or even the presence of non-group A rotaviruses circulating in the regions under study.

Today, knowledge about group A rotavirus genotypes/serotypes is essential to establish mechanisms for adequate epidemiological surveillance and to control infections in the species most commonly affected. The commercially available inactivated vaccines against rotavirus contain strains that are prevalent in the regions where they are produced (the U.S.A and Argentina), such as NCDV-Lincoln (G6P[1]), UK (G6P[5]) and B223 (G10P[11]). The immune response of animals to vaccination is more directed to the vaccine strain, facilitating the occurrence of infections caused by circulating strains that differ from those contained in the vaccine (Conner et al. 1994).

Furthermore, despite maternal vaccination, rotavirus is still detected in the feces of calves with diarrhea (Barreiros et al. 2004). This has led to the emergence of antigenic variants and an increase in mixed infections due to the formation of recombinants, giving rise to new viral strains (Lu et al. 2004, Dennehy, 2008). This situation emphasizes the increasing importance of knowledge about the genotypes prevalent in regions for a more effective response to vaccination.

CONCLUSIONS

The molecular analyses performed in this study indicate that most of the samples of rotaviruses circulating in cattle herds in the Brazilian states of São Paulo, Mato Grosso do Sul, Goiás and Minas Gerais present genotypes consistent with those described in the literature for Brazil and in other countries.

However, the occurrence of a typical porcine genotype was detected, suggesting a possible cross-infection. This finding calls for a more in-depth investigation, particularly the addition of epidemiological and genetic sequencing data of the VP4 and VP7 encoding genes, such as other regions of the genome. Ongoing research to monitor the genotypes in cattle herds is essential for the improvement of specific prophylactic measures.

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