Molecular characterization of group A bovine rotavirus in southeastern and central-western Brazil, 2009-2010

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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], G8P[11], G6P[11], G8P[1], G8P[5], G6P[1] 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.
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 polycrylamide 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/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 DPEC-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 Bg9, End9, End9CRW8, End9UK (for G genotypes) or con3 and con2 (for P genotypes). The final volume of 25 mL 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 cDNA 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 (Sb9eg9, DT6, ET10, HT8, FTS, BT11 for G genotypes or con2, pB223, pGOTT, pNCDV, pSUJ, PUK, for P genotypes), and 0.65U of Platinum Taq polymerase (Invitrogen®), added with DPEC 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 8 mL 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 G8, G10 and G11.


**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**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>G6</th>
<th>G8</th>
<th>G10</th>
<th>G11</th>
<th>[NI]</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>P[1]</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>P[5]</td>
<td>6</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>P[6]</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>P[11]</td>
<td>8</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td>P[ND]</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>15</td>
<td>40</td>
</tr>
</tbody>
</table>

[NI] = Not Identified.

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


**DISCUSSION**

Although the genotypes G[6], G[8], G[10] and P[1], P[5] and P[11] are found predominately 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. (2009), 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 restructurings or co-infections characterized by genetic mechanisms of evolution, which involve pointwise mutations (drifts), restructurings (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.


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 / 80) 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 Saravanaan 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[5] (NDV-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 Garairochea 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 Caruso et al. (2010), who identified 16.2% as G6P[11]. Reidy et al. (2006) and Garairochea 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.


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 (Garriot 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 substances 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; Grzadzki, 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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**REFERENCES**


