

Severe diarrhea outbreak in beef calves (*Bos indicus*) caused by G6P[11], an emergent genotype of bovine rotavirus group A¹

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ABSTRACT.- Medeiros T.N.S., Lorenzetti E., Alfieri A.F. & Alfieri A.A. 2014. **Severe diarrhea outbreak in beef calves (*Bos indicus*) caused by G6P[11], an emergent genotype of bovine rotavirus group A.** *Pesquisa Veterinária Brasileira* 34(8):717-722. Laboratório de Virologia Animal, Departamento de Medicina Veterinária Preventiva, Universidade Estadual de Londrina, Rodovia Celso Garcia Cid, Campus Universitário, Cx. Postal 10011, Londrina, PR 86057-970, Brazil. E-mail: alfieri@uel.br

The episodes of diarrhea caused by neonatal bovine rotavirus group A (BoRVA) constitute one of the major health problems in the calf rearing worldwide. The main G (VP7) and P (VP4) genotypes of BoRVA strains involved in the etiology of diarrhea in calves are G6P[1], G10P[11], G6P[5], and G8P[1]. However, less frequently, other G and P genotypes have been described in BoRVA strains identified in diarrheic fecal samples of calves. This study describes the identification and molecular characterization of an emerging genotype (G6P[11]) in BoRVA strains involved in the etiology of a diarrhea outbreak in beef calves in a cattle herd of high production in extensive management system. The diarrhea outbreak, which showed high morbidity (60%) and lethality (7%) rates, occurred in calves ($n=384$) Nelore (*Bos indicus*) up to 30-day-old from the State of Mato Grosso do Sul, Brazil. BoRVA was identified in 80% (16/20) of the fecal samples analyzed by polyacrylamide gel electrophoresis (PAGE) technique. In all PAGE-positive fecal samples were amplified products with 1,062-bp and 876-bp in the RT-PCR assays for VP7 (G type) and VP4 (VP8*) (P type) of BoRVA, respectively. The nucleotide sequence analysis of VP7 and VP4 genes of four wild-type BoRVA strains showed G6-III P[11]-III genotype/lineage. The G6P[11] genotype has been described in RVA strains of human and animal hosts, however, in calves this genotype was only identified in some cross-sectional studies and not as a single cause of diarrhea outbreaks in calves with high morbidity and lethality rates as described in this study. The monitoring of the G and P genotypes of BoRVA strains involved in diarrhea outbreaks in calves is important for both animal and public health by allowing the identification of the most frequent genotypes, the characterization of novel genotypes and to identify reassortments with genotypes described in animal and human hosts. The results of this study show the importance of the monitoring of the genotypes of BoRVA strains involved in episodes of bovine neonatal diarrhea as for characterization of frequency of occurrence and pathogenic potential of uncommon genotypes as for monitoring of the emergency of different BoRVA genotypes not included in commercial vaccines.

INDEX TERMS: Calf, enteric infection, rotaviruses, VP7 gene, VP4 gene.

RESUMO.- [Surto de diarreia neonatal em bezerros de corte (*Bos indicus*) ocasionado por um genótipo emergente G6P[11] de rotavírus bovino grupo A.] Os episódios

de diarreia neonatal ocasionados pelo rotavírus bovino grupo A (BoRVA) constituem-se em um dos principais problemas sanitários na criação de bezerros em todo o mundo. Os principais genótipos G (VP7) e P (VP4) de cepas de BoRVA envolvidos na etiologia da diarreia em bezerros são G6P[1], G10P[11], G6P[5] e G8P[1]. No entanto, com menor frequência, outros genótipos G e P têm sido descritos em cepas de BoRVA identificadas em amostras de fezes diarreicas de bezerros. Este estudo descreve a identifica-

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ção e caracterização molecular de um genótipo emergente (G6P[11]) em cepas de BoRVA envolvidas na etiologia de um surto de diarreia em bezerros de um rebanho bovino de corte de alta produção em sistema de manejo extensivo. O surto, que apresentou altas taxas de morbidade (60%) e de letalidade (7%), ocorreu em bezerros ($n=384$) da raça Nelore (*Bos indicus*) com até 30 dias de idade, provenientes do estado do Mato Grosso do Sul, Brasil. O BoRVA foi identificado em 80% (16/20) das amostras fecais analisadas pela técnica de eletroforese em gel de poliacrilamida (PAGE). Em todas as amostras fecais PAGE-positivas foi possível a amplificação por RT-PCR de produtos com 1.062 pb e 876 pb referentes aos genes VP7 (G tipo) e VP4 (VP8*) (P tipo), respectivamente, de BoRVA. A análise da sequência de nucleotídeos dos genes VP7 e VP4 de quatro cepas de BoRVA demonstrou a presença do genótipo/linhagem G6-III P[11]-III. O genótipo G6P[11] tem sido descrito em cepas de RVA de hospedeiros humanos e animais. Contudo, em bezerros, este genótipo foi apenas identificado em alguns estudos transversais e não como a única causa de surtos de diarreia em bezerros com altas taxas de morbidade e letalidade como descrito neste estudo. O monitoramento dos genótipos G e P de cepas de BoRVA envolvidos em surtos de diarreia em bezerros é relevante tanto para a saúde animal quanto para a saúde pública por possibilitar a identificação dos genótipos mais frequentes, a caracterização de novos genótipos e por identificar *reassortments* com genótipos descritos em hospedeiros humanos e animais. Os resultados deste estudo demonstram a importância do monitoramento dos genótipos de cepas de BoRVA envolvidas em surtos de diarreia neonatal bovina tanto para a caracterização da frequência de ocorrência e potencial patogênico de genótipos incomuns quanto para o monitoramento da emergência de genótipos distintos daqueles incluídos em vacinas comerciais.

TERMOS DE INDEXAÇÃO: Bezerro, infecção entérica, rotavirose, gene VP7, gene VP4.

INTRODUCTION

Neonatal diarrhea is one of the most important diseases of calves worldwide, causing economic losses to both dairy and beef cattle herds (Holland 1990). Diarrhea in calves is a multi-factorial disease associated with predisposing and determinant factors (environment, management, nutrition, immunology, and microorganisms). There are many microorganisms involved in neonatal calf diarrhea such as bacteria (*Escherichia coli* K99 enteropathogenic), protozoans (*Cryptosporidium* sp. and *Eimeria* sp.), and viruses (rotavirus and coronavirus) (Oliveira Filho et al. 2007, Stipp et al. 2009, Izzo et al. 2011, Blanchard 2012). Bovine group A rotavirus (BoRVA) is one the most important viral agents of neonatal diarrhea in calves worldwide (Barreiros et al. 2004, Alfieri et al. 2006, Badaracco et al. 2012, Midgley et al. 2012). The rotavirus belongs to the *Reoviridae* family and *Rotavirus* genus, and is composed of a triple-layered protein capsid. The genome is formed by 11 double-stranded RNA (dsRNA) segments (Estes & Kapikian 2007). Due to the VP6 protein, which composes the

middle layer capsid, the rotavirus (RV) is classified into eight distinct serogroups (A-H) (Matthijnssens et al. 2012).

The outer layer VP7 and VP4 proteins of rotavirus group A (RVA) strains determine the binary viral classification in G (glycoprotein) and P (protease-sensitive) genotypes, respectively (Estes & Kapikian 2007). Currently, 27 G (VP7) and 37 P (VP4) genotypes of RVA have been described in human and animal hosts (Matthijnssens et al. 2011, Trojnar et al. 2013). The most common combination of G and P genotypes of BoRVA strains isolated from diarrheic calves are G6P[1] (NCDV-Lincoln strain), G10P[11] (B223 strain), G6P[5] (UK strain), and G8P[1] (A5 strain) (Fukai et al. 1999, Gulati et al. 1999, Alfieri et al. 2004, Barreiros et al. 2004). However, with lower frequency other G and P genotypes are sporadically detected in calves and buffalos worldwide such as G6P[3], G10P[3], G8P[7], G6P[7], and G6P[11] (Martella et al. 2003, Manuja et al. 2008, Park et al. 2011).

The G6P[11] is an emerging RVA genotype that was previously described in diarrheic fecal samples from animals (cattle, lamb, and pig) and humans worldwide (Munford et al. 2007, Martini et al. 2008, Monini et al. 2008, De Grazia et al. 2011, Sherif et al. 2011, Yamamoto et al. 2011, Gazal et al. 2012, Midgley et al. 2012). In Brazilian cattle herds the BoRVA genotype G6P[11] was only identified in diarrheic fecal samples in lower frequency in cross-sectional epidemiological studies (Brito et al. 2000, Alfieri et al. 2004, Barreiros et al. 2004, Buzinaro et al. 2009, Caruzo et al. 2010, Silva et al. 2012). However, there are no reports of the G6P[11] genotype involvement as a single causative agent of diarrheal outbreak in Brazilian beef cattle herds.

The G6 genotype has been divided into five (I-V) distinct lineages. The lineages G6-I, contain human rotavirus (RV) strains, and the G6-IV contain bovine RV strains. The G6-II, G6-III, and G6-V lineages are composed by both human and animal RV strains (Badaracco et al. 2013). The P[11] genotype has been classified in three (I-III) lineages. The lineages P[11]-I and P[11]-II are composed by human RV strains, while the lineage P[11]-III are composed by human and bovine RV strains (Badaracco et al. 2013). This report describes the identification of an emergent BoRVA G6P[11] genotype as cause of a severe neonatal diarrhea outbreak in a beef cattle herd.

MATERIALS AND METHODS

The herd was composed by 2,970 Nelore (*Bos indicus*) cows reared extensively. The reproductive management included a breeding season of 90 days. The diarrhea outbreak, with duration of 20 to 25 days affected 384 calves that were up to 30 days of age, resulting in elevated morbidity (60%) and lethality (7%) rates. All diarrheic calves were medicated with wide spectrum antibiotics, but response to treatment was inconsistent and 27 calves died. Diarrheic fecal samples ($n=20$) were collected diagnosis during September 2011, and were maintained at -20°C until used.

All diarrheic fecal samples were submitted to nucleic acid extraction using a combination of phenol/chloroform/isoamyl alcohol (25:24:1) and silica/guanidinium isothiocyanate methods described by Alfieri et al. (2006). The cell culture adapted BoRVA NCDV strain (G6P[1]) and aliquots of buffer Tris-Ca^{2+} were included in all viral RNA extraction and RT-PCR procedures as positive and negative controls, respectively.

The presence of dsRNA of RVA in the diarrheic fecal samples was evaluated using the 7.5% polyacrylamide gel electrophoresis (PAGE) technique (Pereira et al. 1983) followed by silver staining according to Herring et al. (1982). The fecal samples were submitted to RT-PCR assays using previously described consensus primers for the amplification of VP7 (G type) and VP4/VP8*(P type) of RVA (Gouvea et al. 1990, 1993, Gentsch et al. 1992, Martella et al. 2006).

Four samples (BRA1743, BRA1744, BRA1752, and BRA1758) were selected for nucleotide sequence analysis based on the amount of fecal sample, intensity of dsRNA bands in PAGE, and the RT-PCR results. RT-PCR products of VP7 and VP4 genes were purified using the GFX PCR DNA and Gel Band purification kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and quantified with a Qubit® Fluorometer (Invitrogen Life Technologies, Eugene, OR, USA). The DNA sequences were obtained using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the **ABI3500 Genetic Analyzer sequencer** (Applied Biosystems, Foster City, CA, USA) using the forward and reverse primers. Sequence quality analyses were performed using Phred and CAP3 software and the sequences were accepted if the base quality was ≥ 20 . Multiple and pairwise alignments were realized in MEGA version 5.05 with CLUSTAL W (version 1.4) and the nucleotide identity matrix was constructed using BioEdit software version 7.0.8.0. Phylogenetic trees based on the nucleotide sequences obtained were created using the neighbor-joining method with the Kimura two-parameter model using MEGA 5.05 software. The bootstrap probabilities of each node were calculated with 1,000 replications.

RESULTS

BoRVA was detected in 80% (16 out 20) of diarrheic fecal samples analyzed by PAGE technique. All samples showed long electrophoretic profile with the 4:2:3:2 distribution patterns of the 11 dsRNA segments. In the RT-PCR assay,

the same 16 PAGE-positive fecal samples amplified 1,062 bp and 876 bp products of VP7 and VP4 genes, respectively.

A 100% of nucleotide (nt) identity was demonstrated between the VP7 and VP4 nt sequences of four wild-type Brazilian BoRVA strains (BRA1743, BRA1744, BRA1752, and BRA1758); these sequences are available in GenBank with the accession numbers KC678693 to KC678700. During the VP7 nt sequence analysis, the four Brazilian wild-type BoRVA strains demonstrated elevated nt identity (95.2 to 96.4%) with human (Hun-3) and bovine (B102) strains, that belong to the G6-III lineage (Fig.1). The VP4 nt sequence analysis of the BoRVA field strains showed high nt identity (95.8 to 96.8%) with GirRV2 (G6P[11]) and B223 (G10P[11]) BoRVA strains, that belong to P[11]-III lineage (Fig.2).

DISCUSSION

The high rate (80%) of BoRVA positive diarrheic fecal samples identified by PAGE technique and RT-PCR assay demonstrated that the neonatal diarrhea outbreak was caused by this virus. Additionally, all fecal samples were still negative for bovine coronavirus by semi-nested-PCR (Takiuchi et al. 2006) and for *Cryptosporidium* spp. by modified Ziehl-Neelsen technique (Henriksen & Pohlenz 1981) (data not shown). Eimeriosis is reported more frequently in calves older than two months of life and the diarrheic calves were refractory to the first generation wide spectrum antibiotic therapy, so the diagnosis of *Eimeria* sp and enteropathogenic bacteria were not included in the present study.

Most studies relating to neonatal calf diarrhea have been cross-sectional investigation with the frequency of BoRVA detection varying from 9.9% (31/331) to 30% (435/1462)

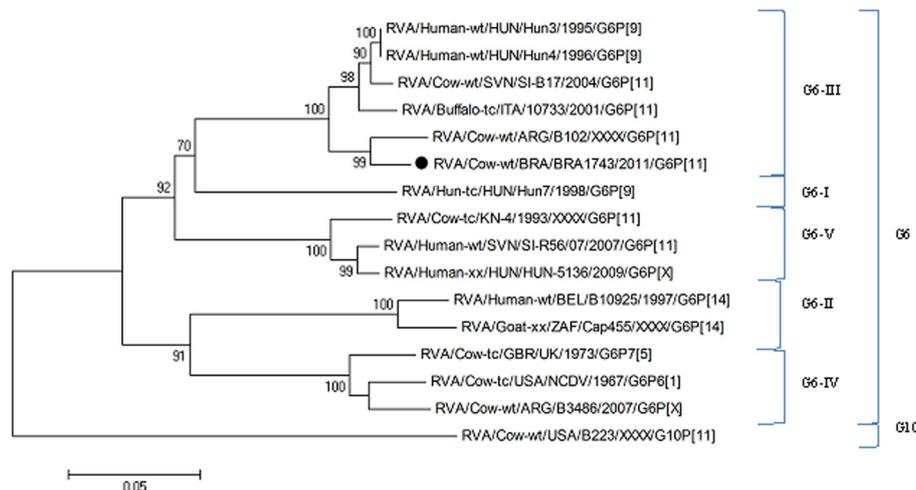


Fig.1. Phylogenetic tree with 928 bp amplicon (nt 75-1,002) of the VP7 gene with Brazilian G6 genotype BoRVA strains. The tree was constructed using neighbor-joining and Kimura two-parameter as a nucleotide substitution model. The numbers adjacent to the nodes represent the percentage of bootstrap support (1,000 replicates) for the clusters. Bootstrap values less than 70% are not shown. The Brazilian G6-III strain is marked with a filled circle. GenBank accession numbers: B102 (DQ631815), 10733 (AY281360), SI-B17 (JX094039), Hun3 (AJ487831), Hun4 (AJ487833), Hun7 (AJ488134), KN-4 (D12710), SI-R56 (JX094033), NCDV (M12394), UK (X00896), B10925 (EF554120), Cap455 (AY128708), B3486 (KC895754), HUN-5136 (HQ315855), BRA1743 (KC678693). The G10 genotype BoRVA (B223 strain) was used as outgroup (X57852).

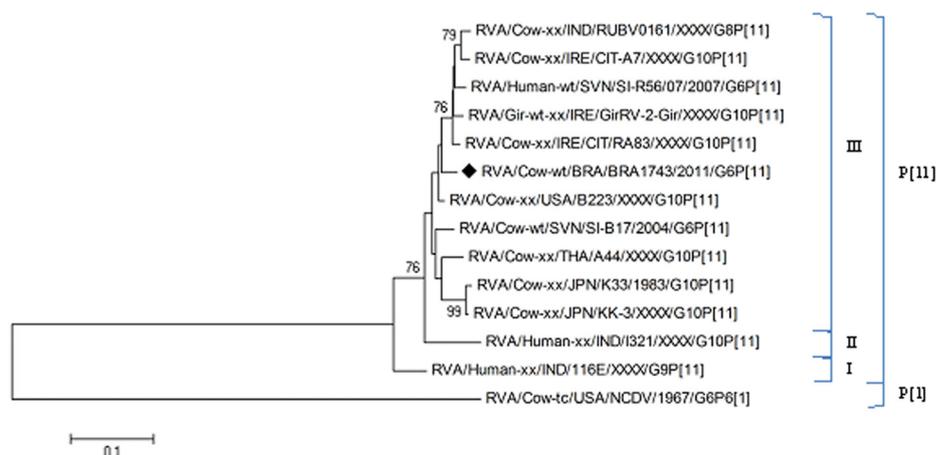


Fig.2. Phylogenetic tree with 700 bp amplicon (nt 190-889) of the VP4 gene with Brazilian P[11] genotype BoRVA strains. The tree was constructed using neighbor-joining and Kimura two-parameter as a nucleotide substitution model. The numbers adjacent to the nodes represent the percentage of bootstrap support (1,000 replicates) for the clusters. Bootstrap values less than 70% are not shown. The Brazilian P[11] strain is marked with a filled diamond. GenBank accession numbers: K33 (D13393), KK-3 (D14367), A44 (D13392), 116E (L07934), I321 (L07657), SI-B17 (JX402794), B223 (D13394), CIT/RA83 (EU164421), GirRV-2-Gir (EU548033), SI-R56 (JX094031), RUBV0161 (EF200558), CIT-A7 (GQ414747), BRA1743 (KC678697). The P[1] genotype BoRVA (NCDV strain) was used as outgroup (AB119636).

(Barreiros et al. 2004, Caruzo et al. 2010, Badaraco et al. 2012). In a longitudinal study, the BoRVA was detected in 11% (11/100) of fecal samples analyzed (Oliveira Filho et al. 2007). Brito et al. (2000) described an outbreak of neonatal diarrhea in a dairy cattle herd from midwest Brazil where the G10P[11] (B223-like strain), a common genotype of BoRVA was found in 68.75% (11/16) of the fecal samples evaluated.

The P[11] genotype is frequently related to BoRVA strains in association with the G10 genotype (B223 prototype strain) (Beg et al. 2010). The G6P[11] is not the most commonly combination described in cattle herds worldwide. However, this emergent BoRVA genotype has already been diagnosed in surveys done in calves from Europe and Argentina, and sheep in India (Monini et al. 2008, Gazal et al. 2012, Midgley et al. 2012, Badaracco et al. 2013). Moreover, the G6P[11] genotype has been identified in RVA strains from human hosts from Africa (Sherif et al. 2011), Asia (Yamamoto et al. 2011), Europe (De Grazia et al. 2011, Steyer et al. 2013), Oceania (Chandrasekhar et al. 2010), and America (Brazil) (Munford et al. 2007, Martini et al. 2008). Moreover, cattle can be a reservoir of rotavirus G6P[11] genotype for human hosts (Martella et al. 2010).

The BoRVA G6P[11] genotype was previously described in Brazilian cattle herds. Some cross-sectional epidemiological surveys for the determination of the most frequent BoRVA genotypes done in stored fecal samples have identified the combination G6P[11] in detection rates that varied from 2% (1/34) to 34.3% (12/35) of the samples analyzed (Alfieri et al. 2004, Barreiros et al. 2004, Buzinaro et al. 2009, Caruzo et al. 2010, Silva et al. 2012). However, this is the first report of a severe neonatal diarrhea outbreak caused by a single infection of the G6P[11] genotype in Nelore calves (*Bos indicus*). Additionally, the BoRVA genotype

G6P[11] identified in this diarrheal outbreak belongs to the G6-III lineage that contains human and bovine RV strains, distant from the most usual G6-IV lineage of bovine RVA strains that includes the G6P[5] (UK strain) and G6P[1] (NCDV-Lincoln strain) prototypes.

Neonatal diarrhea is the main important disease in calf worldwide (Holland 1990). However, two characteristics that predominate in Brazilian beef cattle herds make the diarrhea outbreaks are not so frequent. The extensive management system used within the country decreases the transmission risk of enteropathogenic microorganisms and Nelore (*Bos indicus*) cattle, the most prevalent breed in the country, is characterized by rusticity when compared with *Bos taurus* or crossbreed calves.

Some aspect that might increase the risk of neonatal diarrhea outbreak is the reproductive management adopted throughout Brazilian beef cattle herds with a breeding season of 90-120 days. The concentration of birth increases the number of susceptible animals favoring the transmission of enteric pathogens. In this situation, calf sanitary management should include risk analysis of neonatal diarrhea and the adoption of measures for control and prophylaxis of the infections. Therefore, two conditions of sanitary management may have contributed to the severity, morbidity, and mortality of neonatal diarrhea outbreak observed in this beef cattle herd. First, the large number of susceptible animals concentrated in a calving area, which is an impacting risk factor. Second, the non-adoption of a neonatal diarrhea immunoprophylaxis programs with cow vaccination. These two conditions (management x immunity) predispose the occurrence of neonatal diarrhea that in association with the presence of this emergent strain of BoRVA for which the herd immunity was probably low or inexistence, contributed to the severity of the outbreak.

Around the world, the monitoring of the G and P genotypes of RVA strains involved in diarrhoea outbreaks is important for animal and public health. The identification of a new G and/or P genotypes or the uncommon combination of G and P types in reassortment strains has great impact on the effectiveness of vaccination programs against rotavirus implemented in both human and livestock animals such as cattle and pigs. The G6P[11] is an uncommon combination of the G and P genotypes in BoRVA strains and their detection as single genotype involved in a severe diarrhoea outbreak in beef calves reared extensively serve as an alert to the importance of this emergent BoRVA genotype for the health of calves. Since cattle can be a reservoir of G6P[11] strains for human hosts this study might be of public health concern. Although this survey is one of the few studies in the literature that has accompanied all the outbreak, with marked features of high morbidity and mortality in cattle infected with rotavirus G6P[11] in Brazil.

CONCLUSION

Only with the molecular characterization of BoRVA strains will be possible to determine the most frequent genotypes as well as to monitor the emergence of genotypes not included in commercial vaccines, and to evaluate the pathogenic potential of uncommon BoRVA genotypes involved in calf diarrhoea outbreak. With the use of molecular tools this study allowed the first identification of an emerging genotype, G6P[11], of BoRVA involved in the etiology of a neonatal diarrhoea outbreak in a Brazilian beef cattle herd.

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