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CHARACTERIZATION OF MIXED INFECTIONS WITH DIFFERENT STRAINS OF BOVINE ROTAVIRUS IN AN OUTBREAK OF DIARRHEA IN DAIRY HERDS IN GOIÁS, BRAZIL

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ABSTRACT

Ten faecal samples of bovine rotavirus from calves less than 30 days old from an outbreak of diarrhea in Hidrolândia, Goiás, Brazil were submitted to serological and molecular characterization, using enzyme immunoassay for subgrouping and serotyping, PAGE for determination of electropherotypes and PCR for genome typing. Nine samples belonged to group A/subgroup I rotavirus and one sample was group A / subgroup non-I/non-II. Four samples were characterized as G10P[11] (B223-like), four samples showed a mixture of two rotavirus strains (G6G10 and P[5]P[11]), one sample was characterized as G6P[11] and one sample was characterized only by G serotyping/genotyping, and did not react with any P primer used. Two electropherotypes were detected and both were present in the same animal. This study demonstrates that two different electropherotypes and/or serotypes of bovine rotavirus can circulate in the same outbreak.

Key words: rotavirus, diarrhea, bovine, genotyping, serotyping

INTRODUCTION

Diarrhea in young calves remains an important worldwide problem in bovine herds and rotavirus is one of the most frequent agent involved in the pathology (27). Rotaviruses are classified in the family *Reoviridae,* genus *Rotavirus*. The viral genome is enclosed in a triple capsid particle and consists of 11 distinct segments of double-stranded (ds) RNA, each of one codifying for, at least, one protein. Three proteins are used for serological characterization of rotaviruses: VP6, the protein of inner capsid, characterizes rotaviruses in groups and subgroups; VP7 and VP4, present in the outer capsid, characterize G and P serotypes and are involved in neutralization of rotavirus (15).

G6 and G10 are the most common serotypes described for bovine rotavirus isolates (12, 18, 19, 20, 27). Subgroup I was identified most frequently. Samples with both subgroup I and subgroup II specificity and samples that did not react with subgroup I or II monoclonal antibodies (mAb), were also detected (7, 9, 17, 18, 29).

Recently, polymerase chain reaction (PCR) technique was described for detection and/or characterization of animal rotaviruses in clinical specimens. PCR primers were prepared for hyperdivergent regions of VP7 and VP4 genes and have been used respectively for rotavirus G and P type characterization in many species. The most frequent rotavirus G genotypes detected in bovine faeces were G[6], G[10] and G[8], although the typically human and swine rotavirus G[1], G[2], G[3] and G[11] were also described. Among the P genotypes, P[1]; P[5] and P[11] were found in bovine isolates (5, 10, 11, 13, 14, 21, 28).

The purpose of this study was to analyze the serological and molecular characterization of bovine rotaviruses recovered from calves, during an outbreak of diarrhea in dairy herds in the district of Hidrolândia, State of Goiás, Brazil.

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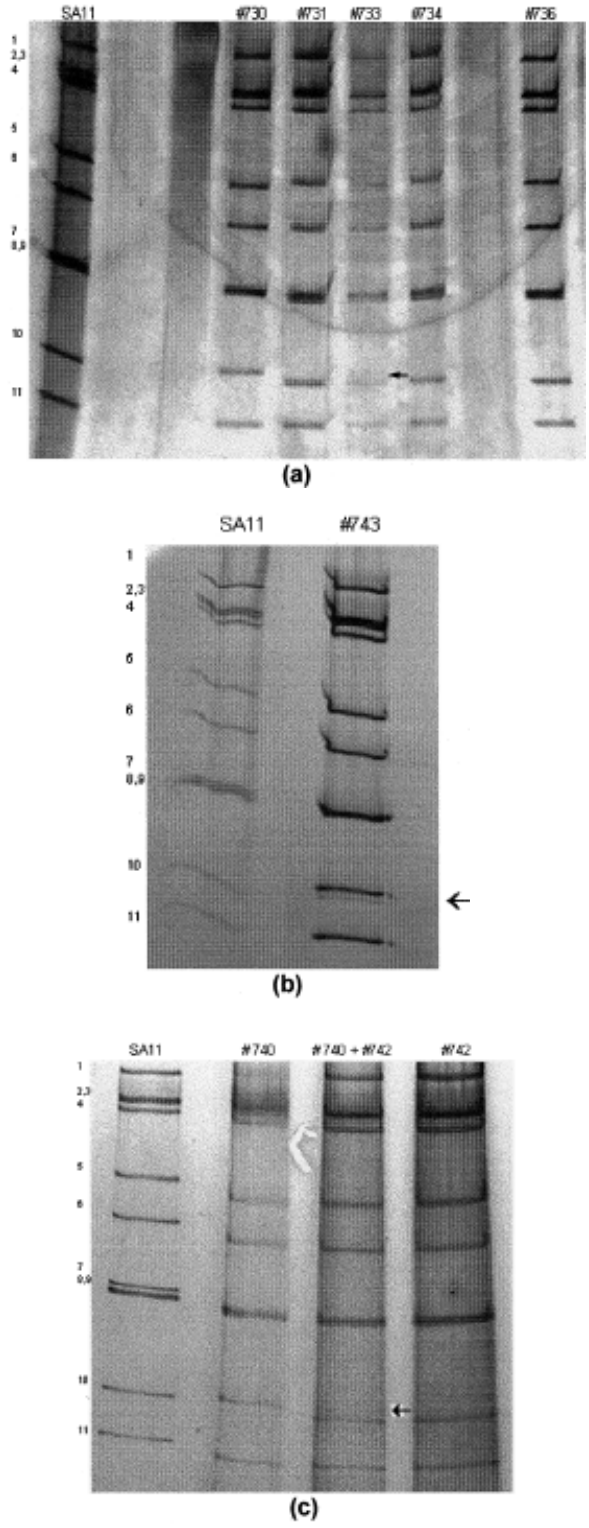

Figure 1: Electrophoretic patterns of dsRNA of bovine rotavirus. **(a)**: Reference strain (SA11) and samples #730 (type Y); #731, #734 and #736 (type X); #733 (mixed electropherotypes, X/Y). **(b)**: Sample #743, with mixed electropherotypes (X/Y). **(c)**: Co-electrophoresis of samples #740 (X) and #742 (Y), showing differences in segment migration. Arrows indicate extra segments.

MATERIALS AND METHODS

Samples. Sixteen fecal specimens were obtained from calves in four dairy herds, in Hidrolândia District, Goiás State, Brazil with the purpose to investigate an outbreak of diarrhea. All samples were from calves less than 30 days old. The samples were tested for group A rotaviruses by enzyme immunoassay for rotavirus and adenovirus (EIARA-Fiocruz) and polyacrylamide gel electrophoresis (PAGE) as previously described (23, 24). Cell culture adapted strains of bovine rotavirus strains NCDV(G6P[1]), UK(G6P[5]) B223(G10P[11]), kindly supplied by Dr. D. R. Snodgrass (Moredun Reseach Institute, Scotland), were used as bovine rotavirus controls.

mAb-ELISA for subgrouping and G serotyping. Rotavirus samples were submitted to monoclonal enzyme immunoassay (mAb-ELISA) with a technique adapted from Pereira *et al.* (24). Briefly, mAbs against group A rotavirus, subgroup I (255/60) and II (631/9) (Dr. H. B. Greenberg), serotypes G6 and G10 (Dr. D. R. Snodgrass) and serotypes G1, G2, G3 and G4 (Serotec-RotaMA), were used as capture antibodies in polystyrene microtiter plates (Nunc). Samples were diluted in PBS/T/BSA/EDTA (PBS 0,01M pH 7.4/ Tween 20 0.05%/ BSA 1%/ EDTA 0.1M) for group and subgroup determination. Samples for serotyping analysis were diluted with PBS/T/BSA (PBS 0.01M pH 7.4; Tween 20 0.05%/ BSA 1%). Group A rotavirus, subgroup I (SA11), subgroup II (an human sample previously tested), G6 (UK) and G10 (B223) were used as positive controls. Between each ELISA procedures steps, microplates were washed 5 times with PBS/T. The following steps were done as described in the original technique (24) with guinea-pig anti-rotavirus serum, peroxidase-conjugated rabbit anti-guinea-pig IgG and ortho-phenylene diamine as substrate.

Electrophoretic characterization of dsRNA. dsRNA was extracted from fecal samples, analyzed by PAGE and the genome segments were visualized by silver staining (23). Samples with electropherotypes that showed differences in segment migration were analyzed by co-electrophoresis.

Reverse transcription - polymerase chain reaction (RT-PCR). Semi-nested RT-PCR for G and P genotyping was conducted as previously described (10,11) with modifications in the extraction step. Rotavirus dsRNA was extracted with Trizol® Reagent (Gibco BRL), precipitated with isopropanol for 10 minutes, and resuspended in 75 µl water with 0,1% diethyl pyrocarbonate. 5 µl of dsRNA was added to microcentrifuge tubes containing 3 µl of dimethyl sulfoxide, denatured at 97°C in boiling water for 5 minutes and cooled on ice water-bath for 5 minutes. The denatured dsRNA was used as template for RT-PCR as described for G and P genotyping (10,11).

RESULTS

In this study, eleven samples of bovine rotaviruses were detected from sixteen faecal samples of diarrheic calves less than one month old in dairy herds. Ten out of eleven have been identified according to their electrophoretic, serological and genotypic characteristics. One sample (#736), belonging to group A by EIERA and X electropherotype by PAGE, was not tested by the other techniques, due to lack of sufficient faecal material. The results are summarized in Table 1.

Nine samples belong to group A rotavirus, subgroup I, and one sample (#742) did not react with any subgroup I or II mAbs. Electrophoretic characterization showed two different electropherotypes (X and Y); both were detected in the same sample and also in the two farms (samples #733, #738 and #743). The differences observed in segment migration between different samples were confirmed by co-electrophoresis (Fig. 1).

Serologic and RT-PCR genotyping characterization showed four samples (#731, #739, #740 and #742) as G10P[11] (B223-like). Sample #734 has been characterized as type G10 (B223-like) by mAb-ELISA and PCR, but it did not react with any P primers. One sample (#738) clearly showed mixture of two rotavirus strains, detected by all techniques, having G and P characteristics of both UK and B223 strains. Three other samples (#732, #733 and #743) were considered also as mixtures of two strains, detected by some, but not all, techniques. One sample (#730) was characterized as G6 by mAb-ELISA, but not by PCR. It was also characterized as P[11] by PCR. Sample #742 was G10P[11] by PCR, but not by mAb-ELISA. Some RT-PCR findings are shown in Figs. 2 and 3. Samples did not react with G1, G2, G3 and G4 mAbs.

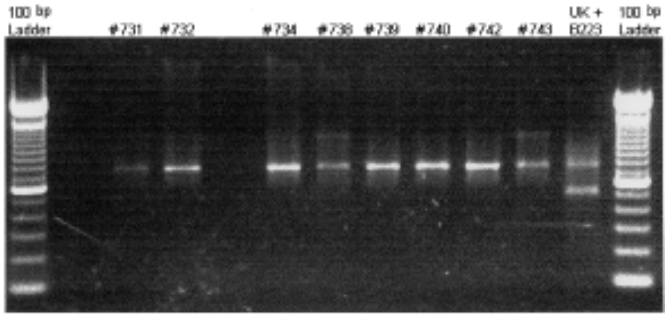

Figure 2: Results of some nested RT-PCR of bovine rotavirus samples with G primers. Samples #731, #732, #734, #738, #739, #740, #742 and #743 (G10). G type UK (G6) and B223 (G10) used as standards. Ladder: 100 bp marker with highlighted 600 bp segment (Gibco BRL).

DISCUSSION

In this study we characterized Brazilian bovine rotavirus strains detected in calves less than one month old with acute diarrhea, in two farms. All samples were identified as belonging to group A and showed the typical electrophoretic pattern. This study showed two electropherotypes (X and Y) among rotavirus identified in calves, both occurring in the same farm and also in the same animal. The differences among the electropherotypes occurred in segments 5 and 10 of dsRNA genome. Differences in the migration of dsRNA of rotavirus were described by several authors (4, 29). Theil and McCloskey (29) pointed out that when fecal specimens were collected from two or more different calves, within the same herd, at the same time, only one genome electropherotype was detected; different genome

Table 1. Serological and molecular characterization of bovine rotavirus samples, from diarrheic calves from an outbreak in two dairy herds in Hidrolândia, Goiás, Brazil.

Samples	Farm	EGPA	mAb-ELISA			RT-PCR	
#			Group	SG[1]	G serotypes	G genotypes	P genotypes
730	A	Y	A	I	6	n[G]	[11]
731	A	X	A	I	10	[10]	[11]
732	A	X	A	I	6/10	[10]	n[P]
733	A	X/Y	A	I	6/10	[10]	[11][5]
734	A	X	A	I	10	[10]	n[P]
736	B	X	A	NT[2]	NT*	NT*	NT*
738	B	X/Y	A	I	6/10	[6][10]	[5][11]
739	A	X	A	I	10	[10]	[11]
740	A	X	A	I	NT*	[10]	[11]
742	A	Y	A	nInII[3]	nG	[10]	[11]
743	A	X/Y	A	I	10	[10]	[5][11]

1. Subgroup
2. NT= not tested
3. Subgroup non-I/non-II

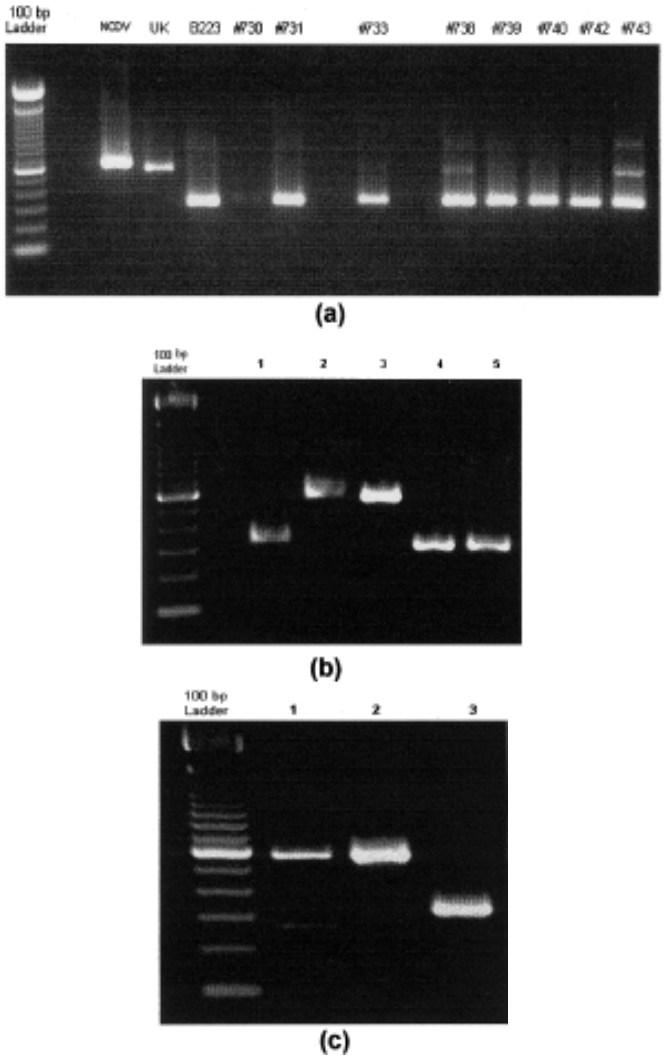

Figure 3: Results of some nested RT-PCR of bovine rotavirus samples with P primers. **(a)**: P types NCDV (P[1]), UK (P[5]), B223 (P[11]) used as standards; samples #730, #731, #739, #740 and #742 - P[11]; #733, #738 and #743 - mixed P[5] and P[11]. **(b)**: nested RT-PCR conducted with separate primers. Lane 1- #743 with primer P11; lane 2 #743 with primer P5; lane 3- standard UK (P[5]); lane 4- standard B223 (P[11]); lane 5- #738 with primer P11. **(c)**: nested RT-PCR conducted with separate primers. Lane 1- #738 with primer P5; lane 2 standard UK (P[5]); lane 3 standard B223 (P[11]). Ladder 100 bp marker with highlighted 600 bp segment (Gibco BRL).

electropherotypes were detected in the same herd, when fecal specimens were obtained over longer periods of time. Bellinzoni *et al.* (1) described a single electropherotype present in samples, in an outbreak of diarrhea caused by one serotype. Data in the present study disagree with both authors: samples characterized as G[10] by RT-PCR showed two different electropherotypes, X (#731, #734, #739 and #740) and Y (#742).

MAb-EIA for subgroup characterization showed all samples with subgroup I specificity, but one sample (#742) did not react with subgroup I or II mAbs, although reacted strongly with mAb for group A. Studies conducted in many countries showed that subgroup I is predominant for bovine rotaviruses (7, 9, 17, 18, 29). In Germany, one sample from a three days old calf did not react with subgroup I or II mAbs. The authors considered that sample as a natural interspecies transmission of rotavirus because the strain was characterized as an avianlike group A rotavirus (5). The sample in this study that did not react with subgroup mAbs, in contrast, has G and P genotype as B223-like bovine rotavirus. Lopez *et al.* (16) pointed out that a single amino acid mutation in the VP6 protein is sufficient to change the subgroup specificity. In case of samples without subgroup I or II specificity, the mutation could prevent the capacity of the mAb to interact with VP6 subgroup epitopes.

Studies conducted with bovine rotavirus recovered worldwide showed that serotype G6 and G10 predominate in cattle, although samples with specificity to serotypes G1, G2, G3, G7, G8 and G11 can also be found (1, 3, 6, 18, 25, 27, 13, 22). Four samples (#731, #734, #739 and #743) were characterized as serotype G10 (B223-like) and one sample (#730) was characterized as serotype G6 (NCDV or UK-like). The remaining strains were not G6 or G10 specific by mAb-ELISA and did not react with G1, G2, G3 or G4 mAbs. Serological untypability of some samples was found in other studies and may due to the lack of double-shelled virus particles in the samples (27).

Bovine rotaviruses have been characterized as G6P[1] (NCDV-like), G6P[5] (UK-like) and G10P[11] (B223-like) by RT-PCR. This study detected four samples (#731, #739, #740 and #742) with G[10]P[11] specificity. Suzuki *et al.* (28) found in Japan, 42.5% of samples G6P[5], 17.5% of samples G6P[11], 10% of samples G6P[1], 10% of samples G10P[5] and 7.5% of samples G10P[11] among bovine rotavirus isolated in cell culture. In the present work, four out of ten samples were G10P[11]. Brüssow *et al.* (6) found high percentage of samples with P[5] in combination with G[6] or G[10], but not any combination with P[1]. Parwani *et al.* (22) found P[5] and P[11] genotyping among most field strains of bovine rotavirus, but a few samples showed P[1] (NCDV-like) specificity. Gouvea *et al.* (11) detected only P[5] and P[11] among most fecal specimens obtained from calves, results similar to this results.

In this study, four samples showed evidence of mixture of two strains (G6G10 and P[5]P[11]). The mixture in sample #738 was detected in all tests used. Three other samples (#732, #733 and #743) could also be described as mixed infections, although they did not react in all tests. Suzuki *et al.* (28) described a mixture of two different electropherotypes and serotypes in the same sample collected during an eight years survey in Japan, but no one occurred during the same outbreak. In the present study, mixed samples were found in the same farm, at the same time. Other authors described outbreaks of diarrhea due to rotavirus in cattle, man and pigs, and in all of them, only one

serotype and/or electropherotype was detected (2, 22, 26). Similar to these findings, Gatti *et al*. (8), in Brazil, detected rotaviruses in eleven out of thirteen faecal specimens of piglets from an outbreak with three different electrophoretic profiles. In five samples, there were more than one band in the regions of segments 4 and 5, probably because the animals were infected with more than one virus type.

The presence of various combinations of G and P serotypes among field isolates of bovine rotavirus suggests that genetic reassortments frequently occurred between viral strains with genes encoding different G and P serotypes (28). In the present work we showed that this possibility exists in Brazil, as we found several samples with two different strains of rotavirus, as shown by several techniques. Currently available bovine rotavirus vaccine strain (NCDV, G6P[1]) may not be ideal for rotavirus prevention in Brazil as we did not find any P[1] sample.

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RESUMO

Caracterização de infecções mistas por diferentes cepas de rotavirus bovino em um surto de diarréia em Goiás, Brasil

Dez amostras fecais de rotavírus de bovinos de menos de 30 dias de idade, provenientes de um surto de diarréia no município de Hidrolândia, Goiás, Brasil foram submetidas à caracterização sorológica e molecular. Nove amostras foram caracterizadas como grupo A/subgrupo I e uma amostra foi grupo A/ subgrupo não-I/não-II. Quatro amostras foram caracterizadas como G10P[11] (B-223-like), quatro apresentaram mistura de rotavirus (G6G10 e P[5]P[11]), uma amostra foi caracterizada como G6P[11] e uma amostra foi caracterizada apenas como G10, não sendo caracterizada para genotipo P. Foram detectados dois eletroferótipos diferentes, ambos presentes no mesmo animal. Este estudo demonstra que em surtos de diarréia, podem estar presentes dois eletroferótipos e/ou sorotipos/genotipos de rotavírus de bovinos.

Palavras-chave: rotavírus, diarréia, bovino, genotipagem, sorotipagem

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