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RESEARCH ARTICLE

Evaluation of Passive Immunity Transfer against G6P[1] Rotavirus in Holstein Calves by ELISA

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ABSTRACT

The main strategy to prevent bovine rotavirus group A (RVA) diarrhea in calves is to vaccinate late-term dams aiming to enhance passive immunity transfer of specific immunoglobulins against the virus. This study aimed to evaluate influence of parity in titers of immunoglobulin G (IgG), IgG1 and IgM in serum and colostrum of vaccinated or unvaccinated Holstein cows and in serum of its calves, associated with monitoring for RVA diarrhea in calves. Cows and its calves were allotted into groups according to parity and vaccination (primiparous/multiparous; vaccinated/ unvaccinated) and serum and colostrum samples of cows were taken as well as serum and fecal samples of its calves. Parturition influenced colostral titers of IgG and IgG1, which were higher in multiparous cows, whilst IgM titers were influenced by vaccination, being higher in colostrum of vaccinated dams. Lowest serum titers of IgG and IgG1 were found in calves born to unvaccinated primiparous dams. Eleven calves presented RVA diarrhea, and genotypes G6P[11] and G6P[5] were found in the vaccinated and unvaccinated herds, respectively. Vaccination of dams prolongs humoral immunity in calves and enhances colostrum quality and should be a primary concern in primiparous cows.

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INTRODUCTION

Many pathogens are involved as etiological agents of calf neonatal diarrhea (CND), and bovine rotavirus group A (RVA) deserves especial attention for its epidemiological importance worldwide (Desselberger, 2014; Coura *et al.*, 2015). Rotavirus belongs to the Reoviridae family and viral particle consists of an icosaedric non-enveloped structure, with 32 capsomers and 11 segments of double-stranded RNA protected by a triple capsid. These eleven segments of genome decode at least six structural proteins (VP1-VP4, VP6, and VP7) and six non-structural proteins (NSP1 to NSP6) (Mihalov-Kovács *et al.*, 2015). Antigenic properties of rotavirus are determined by the capsid proteins: viral protein 6 (VP6) determines group specificity and is used to classify this virus in nine groups (A to I), with group A rotavirus usually incriminated in CND. Proteins of the external capsid induce production of specific neutralizing antibodies, and form the basis of a binary classification system, the G (glycosylated) and P (protease-sensitive) genotypes (Estes and Greenberg, 2013).

The most frequent binary combinations of RVA are G6P[1], G6P[5], G10P[11], and G8P[1] (Alkan *et al.*, 2010), with recent reports of genotype G6P[11] in several regions of Brazil, in both epidemiological studies and outbreaks of diarrhea (Silva *et al.*, 2012; Medeiros *et al.*, 2014).

The epidemiology of RVA diarrhea is complex due to co-circulation of different genotypes in each geographical area and due to the occurrence of genetic shifts and reassortments of the virus that might be influenced by regular vaccination of the herd (Fritzen *et al.*, 2019).

In calves, the main strategy to provide immunity against rotavirus is focused in enhancement of lactogenic immunity and passive immunity transfer through vaccination of late-term dams (Cortese, 2009). The aim of vaccination against rotavirus is to preclude severe clinical signs and economical losses due to treatment or death of affected calves, and to provide heterotypic protection to calves, and not to avoid infection (Fritzen *et al.*, 2019).

Silva *et al.* (2008) reported enhancement of specific antibodies against RVA in colostrum and milk through immunization of dams with adjuvant inactivated vaccine, although vaccination did not improve protection of calves against rotavirus diarrhea; and Rousic *et al.* (2000) reported effective passive protection of calves against diarrhea upon challenge with the virus, indicating that the efficacy of vaccine against RVA is variable under field conditions.

Recent reports indicated a genetic distance between reference strains used in vaccines and strains found in the field, which raised concerns about the effectiveness of vaccination in providing adequate immunity against homologous field strains (Dulgheroff *et al.*, 2016; Fritzen *et al.*, 2018). A recent report by Karayel *et al.* (2017) indicated the occurrence of RVA diarrhea in a Turkish herd regularly vaccinated against strain G6P[5] with high morbidity and mortality rates associated with the infection by a heterologous strain (G8P[5]).

The aim of the present study was to determine the influence of parity and vaccination on immunoglobulin kinetics in blood serum of cows immunized or not against RVA in late term; to evaluate the transfer of these antibodies to colostrum, the kinetics of immunoglobulins in lacteal secretions of cows in the first and second days of lactation; to evaluate passive immunity transfer of anti-RVA immunoglobulins to calves, as well as the kinetics of these immunoglobulins in calves' serum in the first 120 days of life.

MATERIALS AND METHODS

Forty eight Holstein cows were allotted in four experimental groups: V1: 12 primiparous, and group V2: 12 multiparous cows immunized according to manufacturer's instructions with a multivalent commercial vaccine against CND containing strains G6P[1] and G10P[11] of inactivated bovine RVA, bovine coronavirus, *Escherichia coli* K99 colibacterin, and type C *Clostridium perfringens* toxoid (immunity against coronavirus, *E. coli* and *C. perfringens* was not evaluated in the present study); group NV1 comprised 12 primiparous cows and group NV2 12 multiparous cows that were not vaccinated.

The animals belonged to different municipalities located in São Paulo state, Brazil, in the years of 2011-2012. Group V herd comprised 1,400 cows in lactation, and group NV comprised 100 cows in lactation. Calves were allotted in groups (C1, C2, NC1, and NC2) according to its dam's distribution.

Blood samples of cows were taken in plain tubes 60 (M -60) and 30 days pre-partum (M -30), and in the day of parturition (M0). Immediately after parturition (M0) and 24 hours after (M1), 15 mL of colostrum were milked from each quarter of the udder. Colostrum management consisted of bottle-feeding calves with their own mother's colostrum until two hours after birth, with a minimum volume of colostrum intake of 4 L/calf.

Blood and fecal samples were taken from calves before colostrum intake (M0) and with 1, 2, 7, 15, 21, 30, 45, 60, 90 and 120 days of life. Throughout the first month of life, whenever calves manifested diarrhea, fecal samples were taken daily, directly from rectum, in individual plastic bags until the calves presented two consecutive days of normal feces. Blood samples were centrifuged at 1,000 x g for 10 minutes for serum separation. Whey from lacteal secretions was obtained as described previously (Rocha *et al.*, 2014). Samples were frozen at -20ºC until analysis.

Determination of titers of isotypes IgG, IgG1, and IgM against RVA in the blood serum and colostrum whey samples was performed by an in-house indirect immunoenzymatic assay as described previously (Silva *et al.*, 2008) with two modifications: viral antigen used was RVA strain G6P[1], and dilutions were performed with skimmed milk denaturated by microwave oven heating until boiling. Volume and dilution of reagents were determined by block titration.

Negative control consisted of serum sample obtained from a newborn calf before colostrum intake. Positive samples consisted of a pool of serum obtained from three group C calves that were positive for rotavirus infection in the first month of life, being subsequently immunized with two IM doses of the same vaccine used in the dams with an interval of four weeks. Blood samples were taken 15, 30 and 45 days after the vaccine booster, and serum was separated as described previously. Ideal dilutions determined by block titration are shown in Table 1.

Mean values for optical densities (OD) of samples were transformed in sample/positive (S/P) values using the following equation: S/P = (Z-X)/(Y-X), where Z (Mean OD of the test sample – blood serum or colostrum whey); X (mean OD of negative control - blood serum); Y (mean OD of positive control – blood serum).

Fecal samples were screened for RVA by polyacrylamide gel electrophoresis (PAGE) according to Herring *et al.* (1982) and modified by Pereira *et al.* (1985), and samples positive for RVA were subjected to RT-PCR and genetic sequencing to characterize the RVA genotypes G and P as described previously (Silva *et al.*, 2015). Data obtained were subject to repeated measures variance analysis (ANOVA) and means were compared with Tukey's test, considered significant at $P<0.05$.

Table 1: Ideal dilutions used in the indirect ELISA to detect anti-rotavirus immunoglobulins in blood serum and colostrum whey samples

Imunorreagents	Ideal dillutions					
	IgG		IgG1		IgM	
	Blood	Colostrum	Blood	Colostrum	Blood	Colostrum
Viral antigen	1:32	1:16	1:16	1:16	1:32	1:16
Serum sample	1:100	1:400	1:25	1:25	1:200	1:800
Conjugates	1:1,000	1:1,000	1:2,500	1:2,500	1:4,000	1:4,000

RESULTS

Immunoglobulins in serum samples of cows: The S/P values of IgG showed no significant difference between M-60 and M-30 in all groups, and decreased significantly between M-30 and the M0 only in groups V1 (-38.4%) and NV2 (-36.2%) (Fig. 1A), although in remaining groups, percentage of reduction was approximately the same (of -33.2% in group V2 and -30.5% in group NV1). The highest S/P values for IgG in all moments evaluated was found in Group NV2, which was approximately twice the value found in the other groups studied.

Regarding IgG1, there was no significant difference between M-60 and M-30 in all groups, and decrease from M-30 to M0 was significant in all groups, varying from -58% in group V (subgroups V1 and V2), -48.8% in group NV1, to -38.2% in group NV2 (Fig. 1B). As verified for IgG, group NV2 presented the highest S/P values for IgG1 throughout the experimental period. The S/P values for IgM did not differ between groups (Fig. 1C) and decreased significantly between M-30 and M0 only in the vaccinated dams (-28% in group V1 and -22.5% in group V2).

Immunoglobulins in colostrum samples: In colostrum whey, S/P values for IgG and IgG1 were higher in multiparous cows when compared to primiparous cows in both moments of evaluation (M0 and M1) (Fig. 2). S/P values for IgG decreased in all groups between moments, although not significantly in group V2. Percentage of reduction of S/P values for IgG between moments was of -47.5% (V1), -10.7% (V2), -40.7% (NV1), -28.5% (NV2).

Although not significant, titers of IgG1 were higher in unvaccinated dam's colostrum than in vaccinated dams. Interestingly, S/P values for IgG1 increased 2.5% (V1) and 0.63% (V2) in the vaccinated herd between days 0 and 1, whilst in the unvaccinated herd, values decreased -21.9% (NV1), and -15.8% (NV2).

S/P values for IgM in colostrum whey in M0 were significantly higher in group V when compared to group NV (Fig. 2). Between moments, the only group that did not present a significant decrease in S/P values between M0 and M1 was NV1, although it was evident that this group presented the smallest S/P values for IgM in the comparison between groups.

Immunoglobulins in serum samples of calves: Regarding IgG, between moments, smallest S/P values were found immediately after birth in all groups, followed by a significant increase after colostrum intake. From M1, S/P values for IgG decreased gradually until M90, and increased afterwards, except for group NC2, in which values increased from M15 to M30. Throughout the first month of life and except for the period between 7 and 15 days of age, IgG titers were highest in calves born to multiparous unvaccinated cows and lowest in calves born to primiparous unvaccinated cows.

There was a significant increase in IgG1 titers after colostrum intake in all groups, S/P values decreased gradually until M120 and, in general, group NC2 presented higher S/P values until 60 days of age and smaller values were found in group NC1.

S/P values for IgM were smallest in M0, increased significantly after colostrum intake and then gradually decreased until 30 days of life, followed by another gradual increase, reaching maximum values at M120 in all groups (Fig. 3C).

Fecal analysis: From the 552 fecal samples taken from calves, eleven tested positives for rotavirus and were subjected to RT-PCR. Nine samples were positive for VP4 gene, and seven were positive for VP7 gene; genotype found in group C was G6P[11] and in group NC was G6P[5] (Table 2).

DISCUSSION

Parity number did not influence serum concentration of IgG and IgG1 against RVA in vaccinated dams, and higher titers of IgG and IgG1 were found in unvaccinated multiparous cows, probably due to natural exposure to rotavirus in the environment (Tizard, 2017).

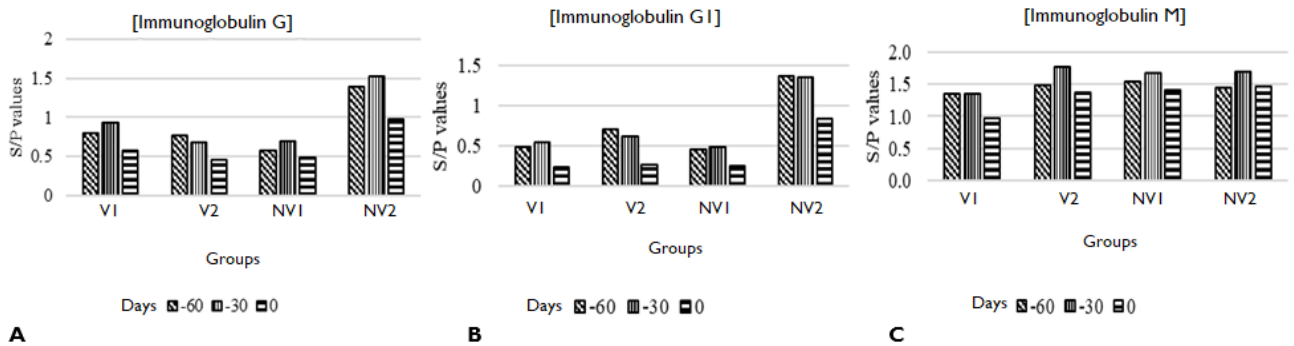

Fig. 1: Mean S/P values of anti-rotavirus immunoglobulins from Holstein cows immunized (Group V) or not (Group NV) against RVA, primiparous (V1, NV1) or multiparous (V2, NV2), before vaccination 60 days pre-partum (-60), at 30 days pre-partum (-30) and in the day of parturition (0). (A) Immunoglobulin G; (B) Immunoglobulin G1; (C) Immunoglobulin M.

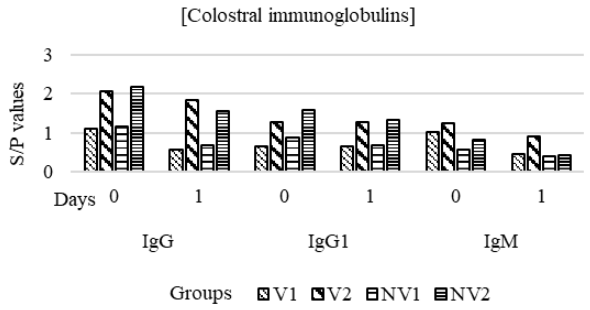

Fig. 2: Mean S/P values of immunoglobulins against rotavirus in lacteal secretions of Holstein cows immunized (Group V) or not (Group NV) against RVA, primiparous (V1, NV1) or multiparous (V2, NV2), immediately after parturition (0) and in the second day of lactation (1).

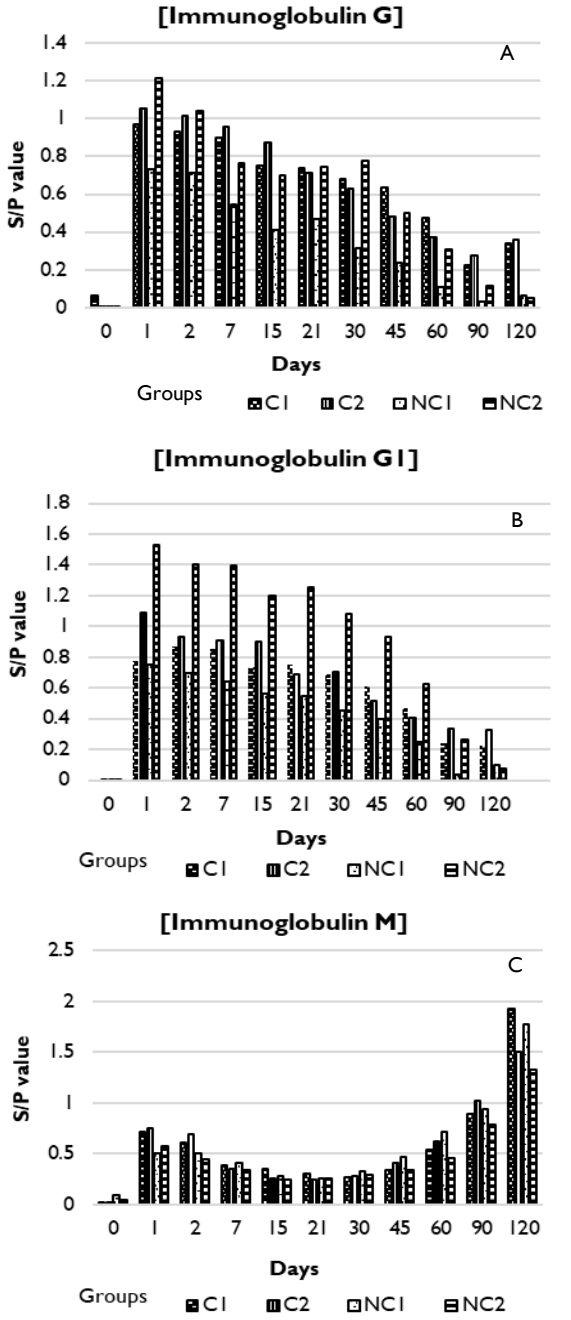

Fig. 3: Mean values of immunoglobulin G (A), IgG1 (B) and IgM (C) against rotavirus in blood serum of Holstein calves born to vaccinated (group C) and unvaccinated dams (group NC) against RVA, primiparous (groups C1 and NC1) and multiparous (C2 and NC2), before colostrum intake (0) and with 1, 2, 7, 15, 21, 30, 45, 60, 120 days of life.

Table 2: Results of polymerase chain-reaction preceded by retro-transcription (RT-PCR) and genetic sequencing in fecal samples of Holstein calves born to vaccinated (group C) and unvaccinated dams (group NC) against RVA, primiparous (groups C1 and NC1) and multiparous (groups C2 and NC2)

Group	Age (days)	Fecal consistency	Results of genetic sequencing
C1	15	Normal[§]	G6P[11]
C2	10	Diarrhea[*]	G6P[11]
	11	Diarrhea[†]	G6P[11]
	15	Diarrhea[*]	G6P[11]
	18	Diarrhea[*]/normal[§]	G6P[11]
NC1	14	Diarrhea[*]	G6P[5]
NC2	11	Diarrhea[*]	-
	30	Diarrhea[*]	G6P[5]

[§]Fecal score: 1 (normal feces); [*]2 (semiliquid feces); [†]3 (liquid feces).

Contrary to the findings of Crouch, Oliver and Francis (2001), that reported a significant increase in serum titers of immunoglobulins against RVA in vaccinated cows when compared to unvaccinated cows, the expected increase in S/P values for IgG and IgG1 after administration of anti-RVA vaccine was identified only in primiparous cows. Vaccination protocol was performed according to the vaccine label and special care was taken to avoid potential causes of vaccine failure. It is possible that the repeated exposure of multiparous cows to the agent in the environment associated with vaccination in the previous gestations represented a factor that reduced the immunological stimulus of the vaccine for these individuals (Tizard, 2017).

Although it was not possible to test the production of antibody titers against strain G10P[11] – also present in the commercial vaccine used – the results clearly show a more pronounced response of the unvaccinated cows against the strain tested, as compared to the vaccinated cows, maybe as a result of the heterotypic protection incited by the strain circulating in the unvaccinated herd, G6P[5].

The transfer of immunoglobulins from bloodstream to colostrum during colostrogenesis led to the significant decrease of serum S/P values of IgG1 found in all groups in M0 when compared to M-30, as IgG1 is the main class of immunoglobulin actively transported in mammary secretory tissue, while most of the IgM present in colostrum is derived from local production in the mammary secretory tissue (Barrington and Parish, 2001), which might explain why the S/P values for IgM did not decrease between M-30 and M0 as much as IgG1 values.

It is important to point out that in current literature, cows are vaccinated with a certain strain of RVA and all the tests for vaccine response evaluation aim the detection of this strain, however, under field conditions cows are exposed to the natural challenge with RVA present in the environment, which means that causes of vaccine failure include the lack of sufficient antigenic stimulation for the induction of antibody production, the interference of administration of multiple vaccines in the same moment, as well as interference of immunoglobulins produced as a result of natural exposure to antigens (Cortese *et al.*, 2011).

Few experiments were performed to evaluate RVA vaccine response under field conditions (Lu *et al.*, 1994; Kohara and Tsunemitsu, 2000; Silva *et al.*, 2008). Although experimental conditions favor the analysis of aspects of the immune response without the interference

of factors that cannot be controlled, they tend to produce conditions that do not correspond to the multiplicity of factors that influence humoral immune response. The establishment of immunity to pathogens is complex and depends on the interaction of many factors, as stated above (Tizard, 2017).

Multiparous cows presented higher levels of IgG and IgG1 against RVA in colostrum whey when compared to primiparous cows, and this difference is explained by the full development of the mammary tissue. In addition, as reported by Cozzi *et al*. (2011), older cows also present higher concentrations of total protein and globulins in blood serum. The continuous exposure of animals to antigens from the environment leads to an increase in the variety and concentrations of serum antibodies (Rocha *et al*., 2019).

Decrease of colostrum IgG in M1 compared to M0 was more evident in primiparous than in multiparous dams. As a result, higher levels of immunoglobulins will be available in the intestinal lumen of calves born to multiparous cows after the second colostrum feeding, maintaining a more efficient protection of the intestinal lumen against rotavirus infection (Parreño *et al*., 2004), which constitutes one of the main strategies to reduce the severity of RVA infection and, since the continuous intake of colostrum by calves throughout the first week of life is an important strategy to protect against diarrhea, colostrum of vaccinated cows proved to be adequate for this purpose, as in the vaccinated group there was an increase of IgG1 S/P values when comparing M0 and M1. Nonetheless, factors as management of animals and infection pressure in the farms are also determinants of the incidence of RVA infection in dairy herds.

Calves born to primiparous unvaccinated cows are probably at higher risk of developing clinical signs due to RVA infection when compared to calves born to multiparous cows, either vaccinated or unvaccinated. According to Parreño *et al*. (2004), lower serum concentrations of anti-RVA antibodies delay the development of active immunity in calves, and also are insufficient to protect calves against infection and clinical disease, and these facts, associated with a high incidence of RVA infection in cattle, indicate that calves born to unvaccinated dams are highly susceptible to diarrhea caused by rotavirus. Vaccination probably increased levels of IgM produced as a result of the primary immune response, as this response was not evident in unvaccinated cows (Tizard, 2017).

Due to placental barrier, calves are born hypogammaglobulinemic (Barrington and Parish, 2001), which was shown by S/P values extremely low in all groups before colostrum intake. As a result of passive immunity transfer, there was a significant increase in S/P values after colostrum intake, and thus, the smallest S/P values for IgG and IgG1, although not significant, were found in calves born to unvaccinated primiparous cows, except for IgM, which presented more pronounced differences between vaccinated and unvaccinated groups and not by parturition. This dynamic reflected the findings of cows' colostrum.

Crouch *et al*. (2001) and Parreño *et al*. (2004), verified that calves that received colostrum from vaccinated dams presented higher concentrations of immunoglobulins than calves from unvaccinated dams, but the number of lactations of the cows used in the experiment was not assessed. In the present study, this finding was true only when evaluating calves born to primiparous cows, since calves born to vaccinated multiparous cows did not present higher S/P values of IgG and IgG1 than calves born to multiparous unvaccinated cows. Nonetheless, natural exposure to RVA apparently was more efficient in increasing S/P values for the strain of RVA tested in multiparous cows, as shown by the results found in groups C2 and NC2.

S/P values for IgG and IgG1 increased in both groups after colostrum intake, followed by a gradual decrease until M120 due to the normal catabolism of colostral immunoglobulins (Barrington and Parish, 2001). S/P values for IgM gradually increased after 30 days of age, indicating the development of a primary immune response after natural infection with RVA (Tizard, 2017), which was proved by the presence of diarrheic animals positive for RVA in both groups. The initiation of the secondary immune response represented by an increase in S/P values for IgG and IgG1 was not evident in the period evaluated.

Clinically, vaccination of cows and passive immunity transfer of immunoglobulins were not capable of protecting calves against RVA infection, since 5/8 calves positive for RVA belonged to the vaccinated herd. The aim of vaccination against RVA is to avoid severe clinical manifestations due to infection (Parreño *et al.*, 2004); the strain of RVA isolated in this farm – G6P[11] – was different from the strains in the vaccine, yet, the clinical signs observed in these calves were mild, and none of the affected animals died due to infection, which might indicate that vaccination is an important management procedure and its implementation should be evaluated carefully. Also, the strains present in the vaccine were not found in the farms, indicating that there was protection against infection with homologous strains of RVA (Fritzen *et al.*, 2018).

The genotype of RVA found in the vaccinated herd (G6P[11]) might be a result of genomic reassortment induced as an adaptation to herd immunity provided by consecutive years of regular vaccination (Steele *et al.*, 2004), and helps explain, associated with the higher pressure of infection that results from the higher degree of exposure to the virus, the higher incidence of RVA diarrhea in the vaccinated herd, as compared to the nonvaccinated herd.

The difference between naturally acquired immunity through antigen exposure and the immunity developed in face of vaccination with an inactivated virus might be relevant. Apparently, our results suggest that natural exposure to RVA provides a more pronounced immunoglobulin response than vaccination with inactivated virus.

Colostrogenesis resulted in a significant decrease in serum levels of IgG and IgG1 in vaccinated and unvaccinated herds, but not for IgM in unvaccinated cows. Vaccination may aid in the improvement of colostrum quality, especially regarding IgG1. Also, parity influenced colostral titers of specific immunoglobulins, which were higher in multiparous cows.

From the fecal samples taken, 1.99% tested positive for rotavirus and the genotype found in the vaccinated

herd was G6P[11], whilst in the unvaccinated herd was G6P[5]. Although S/P values were higher in serum of C2 and NC2 calves, these were also the subgroups with more calves affected by RVA diarrhea. Vaccination should be a special concern in primiparous dams, as calves born to primiparous cows present smaller S/P values for immunoglobulins.

Authors contribution: TGR, DGS, MGB, and JJF conceived and designed the study. TGR, KRS, and FDFS executed the experiment and analyzed the samples. TGR, AAA, HJM, FG, and LFZ analyzed the data and critically reviewed the manuscript.

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