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MICROBIOLOGY

Impact of persistent bovine viral diarrhea virus infection on indicators of innate and adaptive immune function in holstein calves and cows

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ABSTRACT: *We evaluated some indicators of innate and humoral immune response in persistently infected (PI) Holstein calves and cows from 1 to 36 months of age matched with controls from the same herd. The effects were cataloged by grouping animals into the following age groups: <12 months, 13 to 24 months, and 25 to 36 months of age. Blood samples were collected once from each animal to measure total serum protein, haptoglobin, and neutralizing antibodies titers induced by respiratory virus vaccination. Total serum protein (g/dL) was lowest in PI calves younger until 24 months old, while haptoglobin concentration was higher in PI cattle. The serum neutralizing titers against BVDV and BRSV were lower in all PI calves and cattle than in controls. PI cattle have a high serum concentration of haptoglobin, and its possible dysregulated innate immune response appears to impact the efficacy of their adaptative immune responses, resulting in poor vaccine responsiveness.*
Key words: *bovine viral diarrhea virus, persistent infection, neutralizing antibodies titers, respiratory viruses, vaccination.*

Impacto da infecção persistente pelo vírus da diarreia viral bovina sobre indicadores de função imunológica inata e adaptativa em bezerras e vacas holandesas

RESUMO: *O objetivo desta pesquisa foi avaliar alguns indicadores da resposta imune inata e humoral em bezerros a vacas persistentemente infectadas, entre um a 36 meses de idade, pareados com controles oriundos de um mesmo rebanho. As variáveis respostas foram avaliadas agrupando-se os animais nos seguintes grupos etários: < 12 meses, 13 a 24 meses, 25 a 36 meses de idade. Amostras sanguíneas foram coletadas para mensurar as concentrações séricas de proteína, haptoglobina e anticorpos neutralizantes induzidos pela vacinação contra as viroses respiratórias. Os teores de proteína sérica total (g/dL) foram menores nos animais persistentemente infectados (PI) jovens até 24 meses de idade, enquanto que a concentração de haptoglobina foi maior nos animais PI mais velhos (25 a 36 meses). Os títulos de anticorpos neutralizantes contra o BVDV e BRSV foi menor nos animais PIs independentemente da idade, comparado com o grupo controle. Os valores reduzidos ou nulos de anticorpos contra as viroses respiratórias, combinado com a evidência de resposta imune inata desregulada, contribui com a susceptibilidade dos animais PIs para as infecções secundárias.*
Palavras-chave: *anticorpos neutralizantes, vírus respiratórios, vacinação, proteínas de fase aguda.*

INTRODUCTION

Persistent infection (PI) with Bovine Viral Diarrhea Virus (BVDV) has been described as the reliable source of the most expensive infectious disease in the production of cattle (RILEY et al., 2019). Losses are caused by the high frequency of morbidity, mortality, premature voluntary culling, reduced slaughter value, stillbirths, abortion, and other unspecified reproductive losses, veterinary service and treatments costs, the cost of replacement stock, the costs of additional labor, and reduction in milk production (YARNALL & THRUSFIELD, 2017).

PI calves are generated by vertical transmission of non-cytopathic (NCP) BVDV during a window of 40-125 days of gestation. This is before the fetus develops the capacity to mount an adaptive immune response (antibody). The presence of NCP BVDV fetal infection has demonstrated negative effects on immune development and function in both the thymic and splenic tissue before birth. Specifically, the genes for interferon type I related transcriptional

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activators, the process of antigen presentation, and the expected scope and level of T cell surface markers on mononuclear cells and epithelial cells in PI fetal tissues were altered (HOWARD et al., 1992; KNAPEK et al., 2020). The failure to mount immune responses to the virus carried by the PI animal is not generally maintained over a normal lifetime. Many calves succumb to BVDV mediated disease early in life. They frequently become poor doers and are culled, develop secondary disease and are non-responsive to treatment, or die due to mucosal disease caused by mutation of their resident virus to a cytopathic version that causes an innate immune storm in the GI tract with fatal outcome (BOLIN, 1995; FALKENBERG et al., 2018).

The PI cattle have a short lifespan (SMIRNOVA et al., 2008); however, they can achieve the reproductive and productive age depending on the health management offered by the dairy farm. Our previous publication described the secondary clinical diseases, milk production, and quality, and reproduction performance in Holstein heifers and cows from a single large commercial herd persistently infected with Bovine Viral Diarrhea Virus Type 2 (BASQUEIRA et al., 2020). The most frequent disease detected in the PI animals was bovine respiratory disease (BRD), especially in adult cows aged from 25 to 36 months (50%). The PI cows had a 1.615 greater chance of developing BRD than the control group (p = 0.012, IC 95% = 1.155–2.259). In addition, PI cattle had a three-times greater chance of developing post-partum disease than the paired control group (IC 95% = 1.348–6.678). It is interesting that the field study was conducted in a single herd that has used a massive vaccination program against BRD associated viruses. So, the effect of different herd and management conditions did not influence the results of this research.

Based on this scenario, the aim of this study provided a snapshot of the impact of BVDV PI on selected components of the innate and adaptive immune systems and to compare the impact on vaccine induced the immune responses. We believed that examining the levels of total protein and haptoglobin in serum, as indicators of differential innate immune activity and priming, in PI and normal cattle within different age windows of the production system will offer clues to what fails in PI cattle. As BVDV is known to be an immune suppressive agent, we compared the development of antibodies in PI and normal cattle in response to a standard vaccine given to the herd. This data may demonstrate the breadth and scope of BVDV induced immune suppression based on whether all adaptive responses are similarly reduced in PI cattle or if they are selectively reduced with respect to only some of the agents examined.

MATERIALS AND METHODS

General characterization of the herd, management and the standard BRD vaccine protocol

All components referred to the work on this herd during the window of time have been summarized in this paper and the full set of data can be assessed in our previously publication (BASQUEIRA et al., 2020). This study was performed on the largest commercial dairy farm in Brazil. Heifer calves were provided a total volume of 5 liters of fresh maternal colostrum (containing at least 50 g/L of IgG, split into two feedings (6 and 18 hours of age)). The calves were vaccinated for pathogens commonly associated with BRD using Cattle Master® GOLD FP 5/L5, Zoetis, Parsippany-Troy Hills, NJ, USA. The first dose was given at 60 days of age and a booster dose of the same vaccine given 30 days later. All the animals were vaccinated twice per year with the same vaccine in April and October.

Ear skin samples were obtained from the dorsal pinna margin of each calf or young heifer (n = 2247), stored in a sterile microtube and frozen at −20°C. Each sample was assessed individually for BVDV using an antigen capture, E^{rns} antigen specific ELISA test (IDEXX BVDV Ag/Serum Plus Test, IDEXX, Westbrook, ME, USA). After 30 days, animals positive at initial screening were retested. This was followed by testing of all live dams and grandams of the animals were identified as PI among the heifers and calves. After the removal of all PI animals identified in this herd, all newborn calves were tested monthly by ear notch sampling. The duration of PI screening lasted about 13 months from September 2015 until October 2016. A total of 26 PI cattle, including 19 calves and heifers, 4 dams, and 3 neonates (born after removal of PI animals from the investigated herd) were found. Twenty-five of the 26 heifers and cows were included in this study. Animals were distributed by age: ≤12 months (n = 8), 13 to 24 months (n = 6) and 25 to 36 months (n = 11). The control group (CT group) was composed of animals free of persistent BVDV (n = 25). They were selected as aged matched pairs for the PI cows and heifers.

Blood samples

Single blood samples were taken from the PI and from age matched controls that were collected via jugular into glass tubes without anticoagulant. For assessment,

serum samples were thawed to allow measurement of haptoglobin, total serum protein, and neutralizing antibody titers against the BRD vaccine antigens.

Serum total protein and haptoglobin

Total protein (g/dL) in serum was estimated using a commercial optical refractometer (range of 0 to 12 g/dL, Model EEQ9042, Astral Scientific, Curitiba, Brazil). The concentration of haptoglobin in serum was measured with a turbidity-based assay previously described by Ramos et al. (2021). The standard curve was based on a serial dilution of serum with a haptoglobin concentration of 59 mg/dL. The results for haptoglobin were based on interpolation of a linear regression of the standard curve for each set of assays. Turbidity was measured as the absorbance at 450 nm using a commercial microplate reader (ELx808, BioTek Instruments, Inc., Winooski, VT, EUA).

Serum BRD viral neutralizing titers

Virus neutralizing antibody titers were assessed between 4 and 5 weeks following the booster vaccination given to the heifers and cows. The titers for BVDV-1 and BoHV-1 were done following the standard protocol as described in the OIE manual (2016), as adapted by Laboratory of Virus Diseases of Cattle (LVB) at the Instituto Biológico (São Paulo, Brazil). Titers for BRSV and BPIV-3 were determined using the procedures described by Samal et al. (1993) and protocols from the Code of Federal Regulations (2005), respectively. The target antigens used in these assays were strain NADL (type 1a CP), BoHV-1 (Los Angeles, TCC-VR 188; USA), BRSV (ATCC VR-1485) and BPI3-V (ATCC VR-281). The geometric mean value of neutralizing activity was calculated using $\log_2$ titers of four replicates per dilution (REED & MUENCH, 1938).

Statistical analysis

Statistical analyzes were performed using the SPSS 19.0 program (IBM Corp. Released 2011, IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp.). All datasets were tested for normal distribution using the Shapiro-Wilk test. Some datasets were not normally distributed, thus we subjected them to a log10 transformation, a square root transformation, or an inverse transformation of the value measured, in an attempt to obtain a normal distribution for these variables. The remaining non-normally distributed datasets or those that were not based on continuous measurements were assessed using the Kruskal-Wallis test.

Parametric data were assessed using the mean and standard deviation of each group for comparison. Variables that were non-parametric were assessed using the median, minimum and maximum values. Differences between PI (persistently infected) and NI (non-PI) paired controls were evaluated using the unpaired Student's T test (for continuous variables) or the Mann-Whitney U test (for non-normal or discontinuous measures).

The comparison of parametric variables seeking to compare changes with age ranges within each experimental group was analyzed using an unpaired one-way ANOVA. Significance differences between ages in these series were further subjected to the Bonferroni post-hoc test. The comparison between ages within each experimental group was analyzed using methods for non-parametric data comparison by application of the Kruskal-Wallis test.

RESULTS AND DISCUSSION

In this study, we attempted to offer a snapshot of some indicators of innate immune activity that are the consequences of being PI at the three important phases in the production scheme for dairy cattle and an assessment of how being PI impacts viral vaccine responses during the same phases. The study was facilitated by the complete screening of a herd that showed characteristics of having a long-term problem with PI cattle among the members of their herd. This screening and identification were previously reported by our team in Basqueira et al (2020). This herd presented the unusual opportunity to conduct this study as it had a large enough number of cattle in each of the three important phases (early heifer development $\leq$ 12 months of age (n=8), transitional heifer development to first pregnancy of 13 to 24 months of age (n=6) and representing milking cows of 25 to 36 months age (n=11)). Age distribution of PI was not fully expected as the general rule of thumb is that persistently infected animals most often die or are removed from the herd within the first year (TAYLOR et al., 1997), and the most proportion of PI identified in this research had more than a year of age (17/25, 68%). The farm did not adopt any BVDV control management before the PI screening reported in this manuscript, such as the remotion of weak calves or neonates presenting malformation or congenital problems.

The means and standard deviations for total protein and haptoglobin in serum are shown in Table 1. The concentration of total protein (g/dL) in serum was higher for CT (6.99 ± 0.66) than PI animals (6.53 ± 0.93) when analyzed as a total dataset (global, p=0.021). When the production stage was considered,

the youngest CT heifers and heifers in transition to first pregnancy had higher serum protein values than PI heifers in the same stage (p=0.022 and p=0.008, respectively). When serum protein was assessed using an unpaired one-way ANOVA, similar values were observed for both young PI heifers and those at the next stage (12-14 months old). PI cows had a slight, but not significant, increase in serum protein levels. For the CT cattle the lowest values for serum protein were found in the youngest heifers. The values increased numerically, but not significantly as the heifers aged (13-24 months) and again for the cows (25-36 months). While the difference was progressive with age in CT cattle, it was not a significant increase.

The level of serum protein is somewhat lower in PI cattle, particularly before 24 months of age, as demonstrated in this study. Piccinini et al. (2006) investigated the serum protein eletrophoresis in PI and uninfected cattle. They reported that uninfected cattle had higher concentrations of β2-globulin than the PI cattle. This reflected the generally higher levels of total serum protein and antibodies in the uninfected cattle.

In general, the level of haptoglobin (mg/dL) in the serum was higher for PI than for uninfected group (NI) cattle across all ages examined; however, the observed difference was only significant for adult cows in the milking stage (Table 1, p=0.017). Haptoglobin levels did not vary significantly by age for PI or uninfected group (NI) cattle and were similar for the younger heifers in both groups, with a general decline in level for both groups as they entered the milking phase of the production system.

The initiation of the innate immune response occurs by the interaction between Pattern Recognition Receptors (PRRs) on the innate immune cells (especially macrophages) and molecules frequently found in pathogens (the so-called Pathogen-Associated Molecular Patterns—PAMPs), or molecules released by damaged cells (the Damage-Associated Molecular Patterns—DAMPs). This interaction triggers the production of proinflammatory cytokines, mainly IL-1, IL-6, and TNF-α, that initiate the innate immune response, and its substances are responsible to increase the synthesis

Table 1 - Indicators of innate and adaptative response measured in persistently infected (PI, n= 25) and paired control (CT, n=25) Holstein heifers and cows.

Variables	Groups	All ages	Age (months)		
			<12 (n=8)	13-24 (n=6)	25-36 (n=11)
BVDV	PI	0 (0 -7.0)	0 (0-0)	0 (0-7.0)	0 (0-4.0)
	CT	7.0 (0 -11.0)	8.0 (3.0-10.0)	7.0 (0-11.0)	5.0 (0-10.0)
	P	0.000	0.000	0.013	0.026
BRSV	PI	0 (0 – 6.0)	0 (0-6.0)	0 (0-3.0)	0 (0-3.0)
	CT	5.0 (2.0 -9.0)	6.0 (4.0-8.0)	5.0 (2.0-7.0)	5.0 (4.0-9.0)
	P	0.000	0.001	0.002	0.003
BPIV-3	PI	6.0 (0 – 9.0)	7 (4.0-9.0)^{a}	4.0 (0-8.0)^{b}	6.0 (3.0-8.0)^{ab}
	CT	8.0 (0 -9.0)	9 (4.0-9.0)^{a}	4.0 (0-8.0)^{b}	9.0 (2.0-9.0)^{a}
	P	0.146	0.108	0.874	0.145
BoHV-1	PI	5.0 (0 – 9.0)	6.0 (3.0-9.0)^{a}	4.0 (1.0-9.0)^{ab}	2.0 (0-7.0)^{b}
	CT	8.0 (3.0 – 10.0)	8.0 (7.0-10.0)	9.0 (3.0-9.0)	9.0 (5.0-9.0)
	P	0.000	0.006	0.031	0.012
Haptoglobin	PI	1.44±0.34	1.51±0.22^{ab}	1.70±0.44^{a}	1.24±0.25^{b}
	CT	1.26±0.43	1.42±0.32^{a}	1.65±0.51^{a}	0.97±0.24^{b}
	P	0.121	0.512	0.891	0.017
Total Serum Protein	PI	6.53±0.93	5.56±0.44^{b}	6.57±0,10^{b}	7.21±0.42^{a}
	CT	6.99±0.66	6.29±0.59^{b}	7.20±0.14^{a}	7.41±0.39^{a}
	P	0.021	0.022	0.008	0.278

BVDV: Bovine Viral Diarrhea Virus; BRSV: Bovine Respiratory Syncytial Virus; BPIV-3- Bovine Parainfluenza Virus type 3; BoHV-1: Bovine Herpesvirus type 1. Titers of antibodies (log_2) are presenting as median (minimum-maximum) compared between groups and within treatments using the Mann-Whitney U Test and Kruskal-Wallis assessment, respectively. Statistical diferences were declared if $P \leq 0.05$. Different letters in the same line means statistical differences between age´s. Other variables are presented as mean (± standard deviation) compared between PI and CT using the Student's T Test. Significance was declared when $P < 0.05$. Different superscript letters within the same line indicate a statistical difference based on age as assessed using a one-way unpaired ANOVA and Bonferroni post-hoc test.

and secretion of the acute phase protein production by hepatocytes. Haptoglobin is the main acute phase protein used to screen inflammation in ruminants, and has an important role in protecting against infection (ECKERSALL; BELL, 2010). The primary function of Hp is to bind free hemoglobin in the blood. The Hp-hemoglobin binding reduces the availability of the heme residue and its iron from bacterial use; and therefore, Hp has an indirect antibacterial activity (ECKERSALL, 2008).

A high level of haptoglobin appears to suggest a dysregulated innate immune response in PI cattle may be associated with a poor vaccine response observed in PI animals. Pro-inflammatory cytokines recruit and/or activate APC, including macrophages, monocytes, and dendritic cells (DCs), at the site of inflammation, then enhancing antigen presentation capacity, and migration to lymphoid tissue where the DCs interact with T and B cells to initiate and develop the adaptative immune response (BACCILI et al., 2019).

There are few studies evaluating the innate immune response in PI animals. Weng et al. (2015) reported the expression of TLR-7, IFN-α, and IFN-β mRNA was downregulated in PI cattle, between other differences. The TLRs, including TLR-7, act as initial sensors of RNA viral infection and trigger signal transduction cascades that induce the expression of type I IFN. So, the dysregulation of the innate immune response can be an evasion mechanism developed by BVDV.

The vaccine induced neutralizing titers is documented in Table 1. It is clear that CT cattle mounted a much more robust antibody response to the vaccine antigens: BVDV type 1, BRSV, and BoHV-1 than did PI cattle. However, the overall analysis indicated that being a PI animal did not completely block vaccine response, as the responses to BPIV-3 in the vaccine were never significantly different between CT and PI cattle in this study. PI heifers and cows in this study appeared to completely fail to mount an antibody response to BVDV or BRSV and a much weaker response to BoHV-1 than the CT animals under the same vaccine protocol and timing.

No clear enhancement in vaccine antibody response was observed in either the CT or PI animals over the course of maturing in the production management system of this farm. The responses to BVDV and BRSV at each production stage were not different when assessed by ANOVA in either cohort of cattle. The boHV-1 response appeared to decline with age in PI cattle, but this was not significant due to the variation in response among the PI heifers and cows. There was not a pattern of antibody response to the vaccine for either CT or PI cattle among the three stages of the production system in this study.

The differential vaccine responses of PI and control cattle in this study were very interesting. The vaccine responses to BVDV and BRSV were essentially absent in the PI cattle in this study. No response over the entire production cycle to either vaccine antigen was observed here. In contrast, the response by the control cattle was typical and clearly more robust against both of these vaccine antigens. This indicates that PI cattle failed to recognize, or at least had significantly dampened adaptive response, to the vaccine BVDV antigens (both type 1 and 2 were present in the vaccine) and to the BRSV antigen in the vaccine. It would not be assumed that PI cattle should be unable to recognize and respond to BRSV. It is possible that the poor BRSV response was in part due to the poor inflammatory management we observed at all ages in these cattle.

BoHV-1 SN titers were significantly different for PI and the controls. The SN titers were generally 3-5 dilutions higher for the controls. While there is little evidence that circulating antibodies provide significant protection against acute BoHV-1 disease, a higher antibody response is often a good indicator of good vaccine response. In vaccines that can induce cell-mediated immunity (CMI), higher titers may represent an indication of a response that will protect against BoHV-1 (LEVINGS & ROTH, 2013). Thus, it appears that PI heifers and cows may be likely to be more susceptible to BoHV-1 infection throughout the production cycle from early heifer development to their time in the milking herd. Further, if this is true, the immune suppression associated with BoHV-1 infection may complicate the management of disease in PI cattle. This may be an additional factor in their high mortality.

It was striking that the BPIV-3 vaccine response was not different for PI and control cattle. Therefore, being PI was not sufficient to block all vaccine induced immunity. The PI cattle in this study were shown to mount a viral vaccine response. It appears, that the limits in inflammatory management were not severe enough to derail PI-3 antibody development. It is possible that BPIV-3 antigen processing and antigen activation requires either a different set of tissue-based signals or has a different level of amplification in the secondary lymphoid tissue than the other antigens. In this case, PI status may not have a strong enough effect to eliminate the response.

The mechanisms of tolerance and immune responsiveness in PI have been carefully investigated only relative to BVDV antigen response (BROWNLIE

et al., 1987). No publications examining a similar response for other respiratory viruses are available. In our study, cattle persistently infected with BVDV type 2 did not produce specific antibodies against BVDV-1 after vaccination using a commercial vaccine formulation containing inactivated BVDV type 1 and type 2.

There are some limitations in our study development in concomitance with a PI screening process in the largest dairy herd from Brazil. The events reported here occurred in a short period due to the fast PI culling made unfeasible the evaluation of the innate and adaptative response more broadly, which requires working with fresh whole blood in a short time after harvesting samples. Also, the conditions of the conduction of this study is a real farm routine not allowing the evaluation of the serological status of the herd prior to vaccination. However previous publication for our team developed in the same dairy herd shows an endemic status of this herd for BVDV, BoHV-1, BRSV, and BPI3 (SILVA et al., 2018; GOMES et al., 2019; SILVA et al., 2020). Another evidence of the natural circulation of respiratory viruses is the profile presented in this study by the control group. The presence of prior immunity, in addition to the intensive vaccination program, proves the immunotolerant status of PI not only for BVDV, but also for other respiratory viruses.

Fulton et al. (2003) have reported the effect of PI on vaccine response. Seronegative persistently infected (PI) calves infected with a BVDV subtype 1b virus were vaccinated with four modified live virus BVDV containing vaccines, each with a different BVDV viral antigen set, and with *Mannheimia haemolytica* bacterin-toxoid. They reported humoral immune response only in those calves receiving vaccines containing the heterologous type 1a viral antigens. The PI BVDV 1b calves did produce increased *Mannheimia haemolytica* antibodies after vaccination. This was in contrast to BVDV negative control receiving the same *Mannheimia haemolytica* vaccine. These findings support the finding of general poor vaccination response we reported in this paper presentation.

This research suggested that the imbalance in communication between innate and adaptative immune compartments in PI cattle is likely to decrease the resistance to secondary infection in the PI. Basqueira et al. (2020) previously detailed the appearance of clinical disease, poor reproductive function and reduced level of growth and production in PI from this herd. In this study, the PI cattle had a 1.29 higher odds ratio for diarrhea than control heifers (p=0.001, IC95%=1.032-1.623) and 1.615 greater chance of developing Bovine Respiratory Disease (BRD) (p=0.012, IC95%=1.155-2.259). Somatic cell counts (SCC) were higher in PI cows than in control cows across lactation. PI cattle also had a higher incidence of disease, produced less milk, high SCC, and poorer reproductive performance than the controls.

CONCLUSION

PI cattle have a high serum concentration of haptoglobin, and its possible dysregulated innate immune response appear to impact the efficacy of their adaptative immune responses, resulting in poor vaccine responsiveness. Thus, the cost of vaccines for PI cattle is not well justified according to our data. Finally, it appears that being PI represent a high-risk to the herd not only of the main source of BVDV for inducing acute infection, but also as they are compromised even if they survive to become members of the milking herd. So, it is clear that the economic losses can be prevented with the implementation of early screening for and removal of PI calves.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest for this article. The founding sponsors had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

Conceptualization: VG and NSB, Data acquisition: VG, NSB and KNS. Design of methodology and data analysis: VG, NSB and EMP. VG, SAP and DJH prepared the draft of the manuscript. All authors critically revised the manuscript and approved of the final version.

BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

This study was conducted and approved by the Ethics Committee for Animal Use of the School of Veterinary Medicine and Animal Science (Protocol number 5131190216).

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