











## Bovine viral diarrhoea virus and its association with herd characteristics in Brazilian dairy farms

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**ABSTRACT:** Bovine viral diarrhoea virus (BVDV) is a productive and reproductive virus with a high global economic impact on dairy production systems. We investigated the prevalence of BVDV at the herd and individual levels in most dairy-producing regions of Brazil. The frequency of BVDV at the herd level was investigated using quantitative reverse transcription polymerase chain reaction (RT-qPCR) in bulk milk tanks monitored at 289 dairy farms between August 2020 and January 2022. Among these farms, 68 production systems were selected to investigate the prevalence of persistently infected (PI) animals using two antigen-enzyme-linked immunosorbent assays (Ag-ELISAs), at 21-d intervals, from ear-notch samples. In total, 2,902 RT-qPCR and 23,466 Ag-ELISAs were performed. At the herd level, 23.87% (69/289) of dairy farms were considered infected, presenting at least one qPCR test positive. At the individual level, 41.2% (28/68) of the subgroup of selected farms had at least one animal positive in the Ag-ELISA test. Association between tests allowed the classification of farms into the following four categories: level 0, negative for both tests (41.2%, 28/68); level 1, RT-qPCR positive and Ag-ELISA negative (17.6%, 12/68); level 2, RT-qPCR negative and Ag-ELISA positive (13.2%, 9/68); and level 3, positive for both tests (27.9%, 19/68). Multiple correspondence analysis (MCA) suggested a possible association between BVDV positivity and large farms, average daily milk production (herd), breed, and somatic cell counts. The confinement and intensification of animals from different categories, and use of artificial bedding are associated with BVDV infection. Using waste or bulk tank milk to feed calves was also a risk factor for BVDV positivity in RT-qPCR and Ag-ELISA. Despite the use of reproductive vaccines by most producers, their use seems to be associated with BVDV-positive farms. This study presented the epidemiological frequencies of BVDV at the individual and herd levels in the Campos Gerais Paranaense region. The region ranks among the top milk-producing areas in Brazil. Additionally, the association between BVDV tests and farm characteristics indicated the farm risk for BVDV and guides specific control programs.

**Key words:** persistently infected (PI), ear notches, bulk milk tank, RT-qPCR, antigen ELISA.

### Investigação do vírus da diarréia viral bovina associado a características do rebanho em rebanhos leiteiros brasileiros

**RESUMO:** O vírus da diarréia viral bovina (BVDV) é um vírus que afeta a produção e reprodução com alto impacto econômico global nos sistemas de produção de leite. Nosso objetivo foi investigar a prevalência do BVDV em rebanhos e indivíduos na região de maior produção de leite do Brasil. A frequência do BVDV nos rebanhos foi investigada usando reação em cadeia da polimerase com transcrição reversa quantitativa (RT-qPCR) em tanques de leite em 289 fazendas leiteiras, monitoradas entre agosto de 2020 e janeiro de 2022. Dentre esses rebanhos, 68 sistemas de produção foram selecionados para investigar a prevalência de animais persistentemente infectados (PI) usando dois testes imunoenzimáticos para a detecção do antígeno (Ag-ELISAs), em intervalos de 21 dias, a partir de amostras de tecido auricular. No total, foram realizados 2.902 RT-qPCR e 23.466 Ag-ELISAs. Na investigação dos rebanhos, 23,9% (69/289) das propriedades leiteiras foram consideradas infectadas, apresentando pelo menos um teste qPCR positivo. Na pesquisa de animais Pis, 41,2% (28/68) das fazendas selecionadas tiveram pelo menos um animal positivo no teste Ag-ELISA. A associação entre os testes permitiu classificar as propriedades em quatro categorias: nível 0, negativo para ambos os testes (41,2%, 28/68); nível 1, RT-qPCR positivo e Ag-ELISA negativo (17,6%, 12/68); nível 2, RT-qPCR negativo e Ag-ELISA positivo (13,23%, 9/68); e nível 3, positivo para ambos os testes (27,9%, 19/68). A análise de correspondência múltipla (MCA) sugeriu associação entre positividade para BVDV e grandes propriedades, produção média diária de leite do rebanho, raça e contagem de células somáticas. O confinamento e a intensificação de animais de diferentes categorias e o uso de camas artificiais estão associados à infecção pelo BVDV. O uso de leite residual ou a granel também foi um fator de risco para positividade para BVDV em RT-qPCR e Ag-ELISA. Apesar da utilização de vacinas reprodutivas pela maioria dos produtores, a sua utilização parece estar associada a explorações positivas para BVDV. Este estudo apresenta as frequências epidemiológicas do BVDV nos rebanhos e indivíduos de rebanhos da região dos Campos Gerais Paranaense. A região está entre as principais áreas produtoras de leite do Brasil. Além disso, a associação entre os testes de BVDV e as características da fazenda indica o risco da fazenda para o BVDV e orienta programas de controle específicos.

**Palavras-chave:** infecção persistente (PI), biópsia auricular nas orelhas, tanque de leite a granel, RT-qPCR, ELISA antígeno.

## INTRODUCTION

The control of infectious diseases that assist in the production and reproduction of dairy and beef has a significant impact on direct and indirect economic losses. Among the main agents responsible for these losses in cattle is the bovine viral diarrhoea virus (BVDV), which belongs to the Flaviviridae family and pestivirus genus. One of three pestivirus species can cause this disease: BVDV-1, BVDV-2, and HoBi-like virus (EVANS et al., 2018). Losses caused by BVDV infection are estimated to range from US\$ 40–199.50 per naïve dairy cow worldwide (PINIOR et al., 2019).

The impact generated by the BVDV virus is related to its ability to cross the placental barrier and cause a range of problems in the embryo or fetus, including, fetal loss, immunosuppression, teratogenicity, and particularly causing the birth of persistently infected (PI) calves, result of a pregnant female infection by the virus between 40 and 125 d of gestation. The immune system of PI animals recognizes the BVDV as “self,” and persistently infected animals release large viral loads throughout life, keeping the virus in the production system. Moreover, they can present themselves as weak, unprofitable, or even healthy calves without showing signs of the disease. Based on infographics from the Center for Food Safety and Public Health (CFSPH), transiently infected animals (TI) result from the horizontal transmission of BVDV in different ways, such as aerosols, direct contact (reproductive), fomites, and oral routes. The incubation period of BVDV is approximately 6–12 d depending on the viral type, virulence, and load exposure. After infection, these animals excrete a low viral load 3–15 d after infection (EVANS et al., 2018).

Clinical findings triggered by BVDV infection depend on the viral strain, age, and immunological and reproductive status of the host. Calves and nonpregnant cattle with transitory infections may be asymptomatic or manifest fever, hypoxia, diarrhoea, and decreased milk production, in addition to some systemic changes, such as leukopenia and reduced functional activity of mononuclear and polymorphonuclear immune cells, resulting in a proinflammatory status owing to secondary diseases (BASQUEIRA et al., 2018; CHASE et al., 2015). The most important secondary diseases observed in PI animals were diarrhoea (odds ratio [OR]=1.29, 95% confidence interval [CI] [1.032;1.623]) and bovine disease respiratory (OR=1.615, 95%CI [1.155–2.259]). Fleshing cows had 1.6 greater chances of

developing metritis. The age at first insemination and number of insemination attempts required to establish the first pregnancy were both higher in PI than those in noninfected cattle. PI also results in low milk production and high somatic cell count (SCC) during lactation (BASQUEIRA et al., 2018). Differentiation between PI and TI is possible through diagnostic tests, such as antigen-capture ELISA (GONZALEZ et al., 2014) and real-time polymerase chain reaction (qPCR) (NOBREGA et al., 2023). Triage of a PI animal to a TI animal occurred after the repetition of at least two serial tests, with intervals of 3 weeks between each test with a positive result.

Epidemiological data are required to estimate the economic impact of BVDV and cost-benefit of implementing control programs in production systems (RICHTER et al., 2017). According to BVDV global mapping, Brazil has performed herd testing without any mitigation programs. In South America, the mean prevalence of antibody-positive herds is 93% and 67% in persistently infected and viremic infected animals, respectively. The incidence was 1.2 and 2.3% before and after 2008, respectively (RICHTER et al., 2019). The risk perception of BVDV seems to be higher in Europe, where the antibody positivity in herds has decreased from 57 to 46% and the PI rate decreased from 3.6 to 0.2%, comparing data before and after 2008. According to the EUROPEAN COMMISSION FOR FOOD SAFETY (2023), control programs have been implemented in European countries.

Some countries such as Denmark, Norway, Sweden, and Austria have a free BVDV status that is maintained by a monitoring system plan. The countries chosen to join these programs were based on a combination of cattle population density, BVDV prevalence, farm staff, technical laboratory analyses, and government support.

Brazil ranks third in global milk production and is responsible for the production of 35 billion liters of milk (USDA, 2023). The southeastern and southern regions are concentrated in the main producer states: Minas Gerais, Paraná, Rio Grande do Sul, Santa Catarina, and Goiás (EMBRAPA, 2023). The most productive systems were concentrated in the southern region. The Brazil's top 100 dairy farms ranking, the southern states have the most productive animals, with an average of 37.82 L per animal a day (MILKPOINT, 2023), Castro and Carambei are the Brazilian's most milk-producing municipalities (IBGE, 2022). The Frísia Cooperativa Agroindustrial is responsible for processing the milk produced by 289 regional

producers, distributed in 15 municipalities within a radius of 113 km from the cooperative headquarters, which concentrates around 27,000 milking cows with high genetic potential and produces 882,038 L of milk per day. Owing to the proximity between dairy farms and the intensification of the dairy production systems, in addition to the unknown epidemiological data of BVDV, they submitted a proposal to the “More Healthy Milk Program” financed by the Ministry of Agriculture, Livestock, and Supply, developed in partnership with our research team. The general purpose of this project was to implement an integrated BVDV control program in cooperative dairy farms with three specific aims as follows: 1. To determine regional epidemiological data, 2. Analyze the effectiveness of commercial reproductive vaccines, and 3. To evaluate the biosecurity of dairy farms. This study presents the epidemiological data for this large project.

## MATERIALS AND METHODS

### *Characterization of population and dairy farms*

PI screening at the herd level was performed in 270–290 herds from December 2020 to May 2023 by analyzing the bulk-milk tank. This number represents all dairy farms that cooperated with the Frísia Cooperativa Agroindustrial, and the number of oscillations can be explained by the entry and exit of farm units in the cooperative system. The PI screening was performed on 68 dairy farms chosen through the engagement of producers in other extension programs, and expectation was to follow the culling of PI animals detected during this project. These 68 dairy herds were distributed in nine cities of Paraná State and included for the characterization of region herds like Carambé, Fernandes Pinheiro, Imbituva, Palmeira, Castro, Ponta Grossa, Teixeira Soares, Prudentópolis and Tibagi from Paraná State – Brazil.

Herd composition and characteristics were recorded using a questionnaire containing the following: the number of heads by category, predominant breed, average milk production in L/d/cow, average number of days in milk (DIM), average monthly SCC, number of milking per day, type of housing for each category, type of bedding for lactating cows, type and number of employers (familiar or contracted), individual animal records and description of the types of record, source of liquid diet to fed calves, and quantity of milk offered by young heifers. Moreover, reproductive vaccination protocols were recovered from the herds.

### *Bulk-milk tank analysis*

Bulk-milk tank samples were collected from 270–289 herds to detect BVDV at the herd and individual levels from August 2020 to January 2022. Initially, samples were collected from 290 bulk-milk tanks. Dairy herds were initially sampled quarterly; however, switched to monthly sampling after receiving increased financial support in July 2021. The number of samples from each dairy farm ranged 1–13 because of the entry and exit of producers in the cooperative during the 2 years of the study.

A total of 2,902 tests were conducted during this study. The presence of the BVDV antigen was established using a real-time polymerase chain reaction (RT-qPCR) protocol on all BTM samples with a cycle threshold (Ct) cutoff value of 38, according to the manufacturer’s instructions (IDEXX RealPCR BVDV RNA Test).

Briefly, a sterile bottle of 50 mL was used to harvest bulk-milk samples without preservatives. The milk samples collected were subjected to counter-tests of milk collection on the properties, refrigerated within 48–72 h, to the laboratory for the reverse transcriptase test, followed by RT-qPCR. Initially, milk samples in a conical tube (50 mL) were centrifuged twice for 15 min at  $600 \times g$ , with subsequent disposal of the supernatant and preservation of the cell fraction, which was diluted in a 1:1 buffered saline solution. The ribonucleic acid (RNA) was extracted from 50  $\mu\text{L}$  of the cell suspension, using magnetic separation technology, using a commercial kit (MagMAX CORE Nucleic Acid Purification Kit - Applied Biosystems). The extracted RNA was subjected to RT-qPCR for the detection of specific nucleic acids of BVDV using a commercial kit (Real PCR BVDV RNA Mix, IDEXX), following the manufacturer’s recommendations. The mix was prepared in microtubes, where 10  $\mu\text{L}$  of Mastermix RNA and 10  $\mu\text{L}$  of mix BVD were added, containing primers marked and specific to the BVDV target region (FAMtm probe), as well as internal control (marked with VIC probe). Then, 5  $\mu\text{L}$  of the RNA of the positive controls was added, or 5  $\mu\text{L}$  of purified water for PCR (negative control) and 5  $\mu\text{L}$  of the respective samples at the defined positions. For reverse transcription, a cycle of 15 min at 122 °F (50 °C) was performed, followed by denaturation of 203 °F (95 °C) for 1 min and 45 amplification cycles of 203 °F (95 °C) for 15 s and 140 °F (60 °C) for 30 s.

### *PI screening*

Between January 2020 and January 2022, 68 producers were selected for the PI

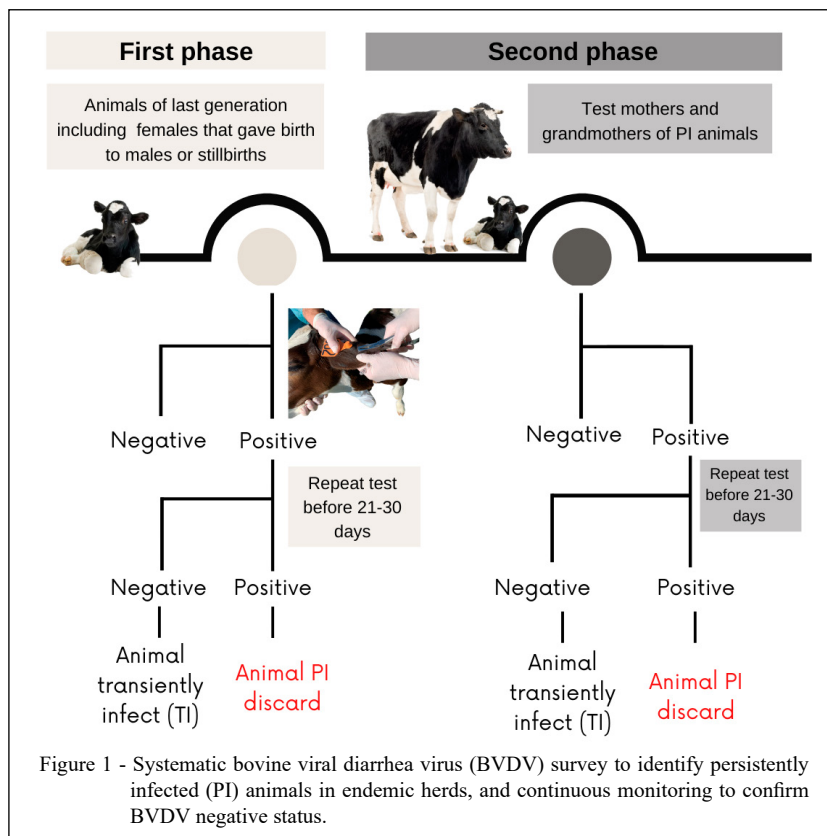
animal screening stage based on their commitment to disposing of the animals after tank sample screening. All producers signed a term agreeing to cull all identified PI during the BVDV project. Researchers and a technical team from Frísia Cooperativa Agroindustrial visited all farms together to enhance the risk perception of BVDV and train the producers and farm collaborators to conduct the PI screening scheme. Ear skin samples (ear notches measuring  $1 \times 0.5$  cm), were obtained from the dorsal pinna margin of each young heifer or calf or cows without descendants using a stainless-steel ear-notching clamp (type V pig). The sample obtained was stored in a sterile microtube (Eppendorf, São Paulo, Brazil), then frozen at  $-4$  °F ( $-20$  °C) until it was processed. Each sample was assessed individually for BVDV using an antigen capture, Erns antigen-specific ELISA test (IDEXX BVDV Ag/Serum Plus Test, IDEXX, Westbrook, ME, USA). Retest of the PI animals after 21–30 d was recommended to distinguish between persistent and transient infections. This recommendation also included the inclusion of PI and grand dams in the target population. After removing all identified PI

animals from the herd, monthly ear-notch sampling was conducted on all newborn calves (Figure 1).

#### Data analysis

Statistical analyses were conducted using the Statistical Analysis System version 9.4 (Institute Inc. NC, USA). The mean, median, standard deviation (SD), standard error of the mean, minimum (min), and maximum (max) values were calculated for continuous variables. Binary and categorical variables were presented as proportions (%).

MCA was used to summarize the associations between BVDV RT-qPCR, Ag-ELISA, and herd characteristics. A combination of BVDV RT-qPCR and Ag-ELISA was used for the MCA analysis. According to this combination, herds were classified into the following four categories: 0, negative for both tests; 1, qPCR positive and ELISA Ag negative; 2, qPCR negative and ELISA Ag positive; and 3, positive in both tests. All other recovered data were transformed into categorical variables. Farm size was determined based on the number of lactating cows as follows: small (1–61 cows), medium (62–201 cows), and large (202–1913 cows). Milk production per cow



was categorized as low (< 28 L/cow), medium (29–36 L/cow), or high (37–44 L/cow). The level of SCC was classified as low, < 155.000/mL; medium, 155.001–251.499/mL; and high, > 251.500 – 550.000/mL.

Multiple correspondence analyses were performed using JMP, a subsidiary of SAS Institute, version 16. MCA is a multivariate method for analyzing multidimensional contingency tables (AMBROGI et al., 2003). MCA creates dimensions using the variance of all observed variables (BVDV tests and herd characteristics) that are presented in descending order of the amount of variation they explain and allows identifying the variables that contribute the most to the creation of the dimension.

## RESULTS AND DISCUSSION

### *Herd characterization*

The herds were composed predominantly of Holstein 66.2% (45/68 herds), and some producers reared Jersey (7.3, 5/68) or both breeds (26.5, 18/68), with an average of 185 lactating cows, an individual daily production of 32 L, daily herd milk production of 6,756 L, and an average of 219,000 SCC/mL. The 68 dairy herds included in this study produced 460,745 L of milk per day, and the average number of DIM was 193. Regarding the young animals, the averages (coefficient of variation) were 27 for pre-weaned calves (1–250), 48 for post-weaned calves (0–600), 35 (0–170) for young heifers aged 9–12 months, and 70 (0–500) for heifers aged 13–24 months. Approximately 15.9% of producers transferred the young postweaning heifers to a specific unit dedicated to heifer raising belonging to the Frísia Cooperative, where they were maintained until they achieved the 60 d before the first parturition. The number of nonlactating cattle (dry cows) was represented as the average of 17 (0–129) animals from 60 to 30 d and 17 (0–170) cows from 31 d to calving. Lactating cows were milked twice (64.7, 44/68) or thrice daily (35.3, 24/68).

The volume of milk offered to pre-weaned calves was  $\geq 6$  L (39.7%, 27/68), and < 6 L was reported in 36.8% of dairy farms (25/68). Approximately 25% did not answer this question (16/68). Individual housing was the predominant system for pre-weaned calves (57/67, 85%), whereas pasture and collective housing systems were the most common systems observed in post weaned young heifers, including heifers aged 9–24 months of age and dry cows.

For adult cattle, free stalls were the most common housing type adopted by producers from Campos Gerais Paranaense, followed by pastures.

The type of bedding is very varied, represented by pastures or pickets (43.7%), sawdust (29.2%), rubber (18.5%), and others (Supp. File S3).

Descriptive results regarding the composition of the herd, zootechnical parameters, housing systems, bedding type, and nutritional plan applied to dairy calves can be assessed using the supplementary material available at <https://doi.org/10.5281/zenodo.10999275>.

Most producers reported implementing vaccination protocols in their health programs (55/69). Seven different commercial brands of reproductive vaccines (A–G) were cited by producers and/or managers, including BVDV and BoHV-1. Most products are inactivated, and only one producer has used modified live vaccines against BVDV. The formulae and intensities of use by farmers are shown in figure 2.

### *BVDV status*

Bulk-milk tank RT-qPCR analysis was negative in 220 dairy herds (220/289; 76.1%) in all tests performed in this study. However, 69 farms had at least one RT-qPCR positive (69/289; 23.9%).

The distribution of farms according to the number of positive RT-qPCR for BVDV between August 2020 and January 2022 is shown in figure 3. A total of 2,903 RT-qPCR from bulk-milk tanks were performed during this research, detecting 97 positive samples (3.3%). The distribution of samples and positivity for BVDV Ag during the study period is shown in table 1.

For the Ag-ELISA test, the number of sampling times ranged from 1 to 25, according to the target populations in each of the 68 dairy farms. A total of 23,466 Ag-ELISA was performed, detecting 692 positive samples (2.9%) from calves, dams, and granddams, including retests between January 2020 and January 2022. One animal died during the study. However, most farms do not follow our recommendation to retest infected animals, and most culled bovines immediately after the emission of the first results from Ag-ELISA. As part of the BVDV identification program, we determined the distribution of positivity for the Ag-ELISA.

Twenty-eight (28/68; 41.2%) dairy farms had at least one Ag-ELISA positivity, whereas 58.8% (40/68) were negative. The geographical distribution of Ag-ELISA positivity is shown in figure 4. The combinations of RT-qPCR and Ag-ELISA results from 68 dairy farms are shown in table 2. Detection of 26 dairy farms was possible by classified at level 0 (negative for both tests); 12 at level 1; 13 at level

Vaccine	Type	Composition	Adjuvant	Farms (n)
A	I	IBR, BVDV, PI3, BRSV, Leptospira pomona, L. wolffii, L. hardjo, L. icterohaemorrhagiae, L. canicola and L. grippotyphosa; Campylobacter fetus fetus, Campylobacter fetus venerealis	Aluminum hydroxide	1
B	MLV	BVD 1 (NP) parental strain KE-9; BVD 2 (NP) parental strain NY-93	Lyophilized: Sucrose Gelatin Potassium hydroxide Acid L-Gl..	22
C	I	BVDV 1 and 2; BoHV-1, PI3, BRSV, Leptospira canicola, L. grippotyphosa, L. hardjo, L. icterohaemorrhagiae and L. pomona	ISCOM (amphigen, cholesterol and Quil A)	
D	I	BVDV 1 and 2; IBR; Leptospira interrogans pomona pomona; Campylobacter fetus subsp. venerealis and Histophilus somni	Aluminum hydroxide	
E	I	BVDV strain NADL; BoHV strain Los Angeles; BRSV and PI3	Oil	
F	I	BoHV-1, BoHV-5; BVDV-1, BVDV-2; Leptospira interrogans serovars: pomona; wolffii; hardjo prajitno; hardjo bovis; tarassovi; icterohaemorrhagiae; canicola and grippotyphosa, Campylobacter fetus subsp. fetus; C. fetus subsp. venerealis;	Aluminum hydroxide	
G	I	Not found	Not found	

I - Inactivated; MLV - Modified Live Virus

Figure 2 - Description and number of citations of each vaccine formulation by producer and/or managers from 68 dairy herds from Campos Gerais Paranaense – Brazil.

Color Legend:  
The grayscale represents the number of farms that use a particular commercial reproductive vaccine composition. The darker the color, the greater the number of farms that use this vaccine. Conversely, the lighter the color, the fewer the number of farms that use this vaccine composition.

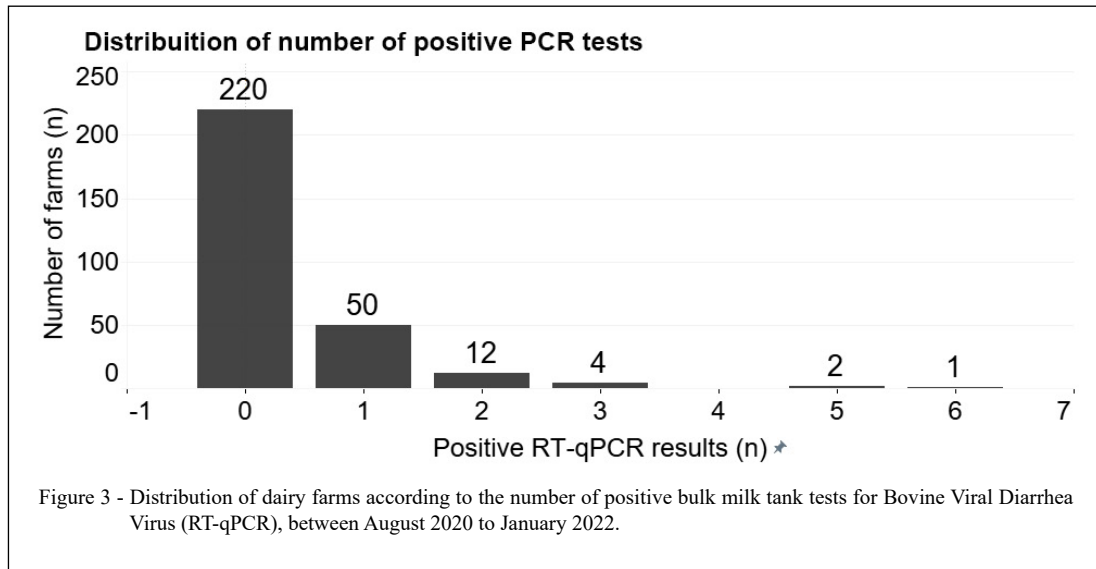
2, detecting half of producers following the PI retest; and 17 at level 3, observing poor adherence to the PI retest recommendation (Table 2).

#### Multiple correspondence analysis between risk factors

MCA of farm characteristics, RT-qPCR, and ELISA antigen positivity are shown in figure 5. The first dimension of the analysis accounted for a very large proportion (96.6%) of the inertia or variation in the original data. Correspondence analysis can be read at three levels: along the x-axis from left to right and from top to bottom, and the association between the predictor and outcome variables. An association between positive results in both BVDV tests and large-size farms, the Holstein breed, medium production, extremes of SCC (low and high), and the adoption of reproductive vaccination protocols are observed from the left to right (x-axis). The farm characteristics associated with a negative BVDV test for BVDV are presented on the right side of the x-axis. Negative farms were associated with small and medium herds, low production, the raising of Holstein and/or Jersey breeds, the absence of reproductive vaccination protocols, and medium SCC. High production is in the middle of the x-axis and is probably not associated with the results of the BVDV tests.

MCA between BVDV herd levels (from 0 to 3) and dairy farm characteristics is shown in figure 6. The two main dimensions were named based on the variables that contributed the most. The first dimension accounted for 59.5% of the variance, whereas the second accounted for 35.5%. Herd level 0 (negative for both tests) was on the left side of the x-axis and was associated with low milk production, small herds composed of Jersey or Holstein and Jersey breeds, and absence of vaccination. On the right side of the x-axis, it is possible to observe the association between positive results for BVDV tests at the herd and individual levels and large-size farms, high production, Holstein breed, use of BVDV vaccines, and low SCC.

MCA between the BVDV tests and some characteristics of heifer-raising systems is shown in figure 7. Dimension 1 contributed 96.5% of the variability between BVDV-positive and -negative herds. In the preweaned calves category, the use of individual housing, waste milk, or bulk-milk tank and a liquid diet volume of > 6 L/day. Conversely, calves raised in tropical calf housing, Argentine calf housing, or calf feeders, feeding with milk replacer or natural sucking in the dams, and < 6 L of milk per day were correlated with negative BVDV tests. For the postweaning category (until 9 months of age), young heifers raised in pastures and other systems



were correlated with negative results for BVDV tests, whereas calves maintained in individual cages, collective cages, free-stalls, and compost barns were on the right x-axis correlated with infected herds and animals. After 9 months, young heifers raised in free-stall or compost barns seemed to be correlated with BVDV-positive tests. Similar results were observed for heifers from 13 to 24 months of age, with a correlation between positive tests and compost barn, free-stall, and free-stall with pasture access as housing types.

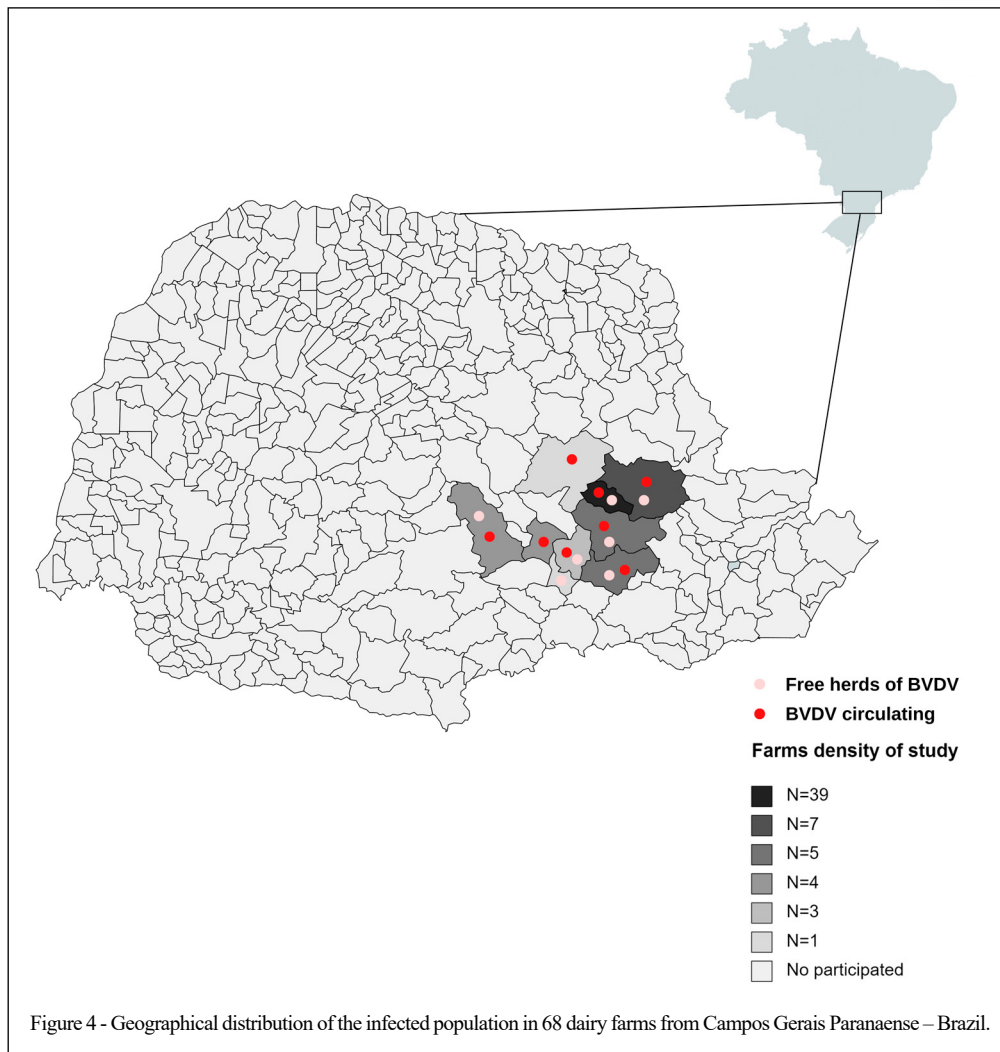
MCA between the BVDV tests and some characteristics of the nonlactating and lactating cow categories is shown in figure 8. Dimension 1 contributed 89.3% of the variability. The extensive

rearing system in pastures and pickets for dry, prepartum, and lactating cows is associated with negative tests for BVDV, whereas the confinement of lactating or nonlactating cows, in addition to the use of sand, sawdust, and rubber bedding, is associated with positive tests.

The present study represents important research showing the integration of public universities, the dairy industry, and Brazilian regulatory agencies. As the largest milk producer, the dairy production system in Campos Gerais, Paranaense, has specific characteristics. Campos Gerais Paranaense has a high land value, with a predominance of herds adopting intensive production systems for lactating cows with high genetic

Table 1 - Distribution and positivity results in the RT-qPCR from bulk milk tank samples harvested from August 2020 and January 2022.

Dates	Total of samples	Positive Samples	%
1/8/2020	267	14	5.2
13/10/2020	267	12	4.5
1/1/2021	267	2	0.7
1/3/2021	240	28	11.7
1/6/2021	235	4	1.7
1/7/2021	254	8	3.1
1/8/2021	253	3	1.2
1/9/2021	184	6	3.3
2/10/2021	252	3	1.2
3/11/2021	169	5	2.9
1/12/2021	259	10	3.9
2/1/2022	256	2	0.8
Total	2,903	97	3.3



pressure to select high-producer cows, which results in a high risk of infectious diseases, sub-estimated by the low-risk perception and implementation of BVDV biosecurity practices.

For the establishment of BVDV status at the herd and individual levels, the following two different

diagnosis strategies were included to conduct the “Integrated Control Program against BVDV”: 1. RT-qPCR from milk bulk tank to screening herd status; and 2. Ag-ELISA for the detection of PI at individual-level.

A total of 2,903 RT-qPCR from bulk milk tanks were performed during this study, detecting

Table 2 - Distribution of herds according to their respective results for PCR and antigen ELISA tests.

	PCR -	PCR +	Total
ELISA -	26 (level 0)	13 (level I)	39
ELISA +	12 (level II)	17 (level III)	29
Retest ELISA -	3 *	1 **	4
Retest ELISA +	3 *	1 **	4

Legend: -: Negative; +: positive; \*Six farms tested negative for PCR, but they were not retested using ELISA \*\*Fifteen farms were PCR positive and did not realize retest ELISA.



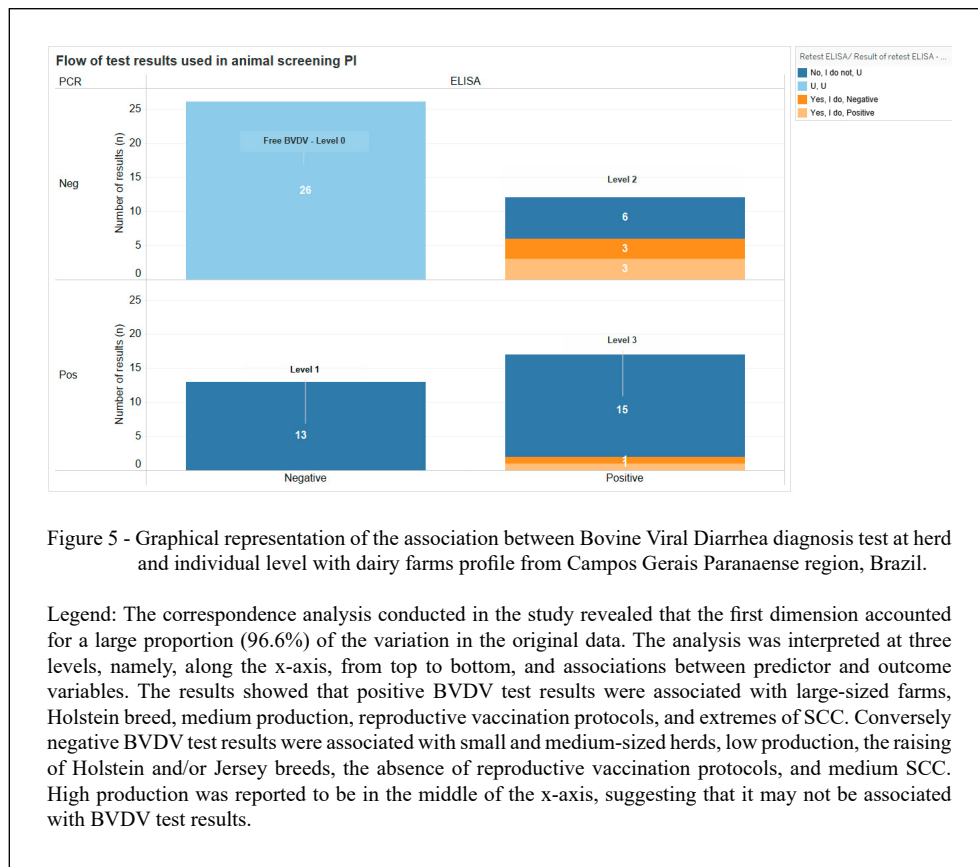


Figure 5 - Graphical representation of the association between Bovine Viral Diarrhoea diagnosis test at herd and individual level with dairy farms profile from Campos Gerais Paranaense region, Brazil.

Legend: The correspondence analysis conducted in the study revealed that the first dimension accounted for a large proportion (96.6%) of the variation in the original data. The analysis was interpreted at three levels, namely, along the x-axis, from top to bottom, and associations between predictor and outcome variables. The results showed that positive BVDV test results were associated with large-sized farms, Holstein breed, medium production, reproductive vaccination protocols, and extremes of SCC. Conversely negative BVDV test results were associated with small and medium-sized herds, low production, the raising of Holstein and/or Jersey breeds, the absence of reproductive vaccination protocols, and medium SCC. High production was reported to be in the middle of the x-axis, suggesting that it may not be associated with BVDV test results.

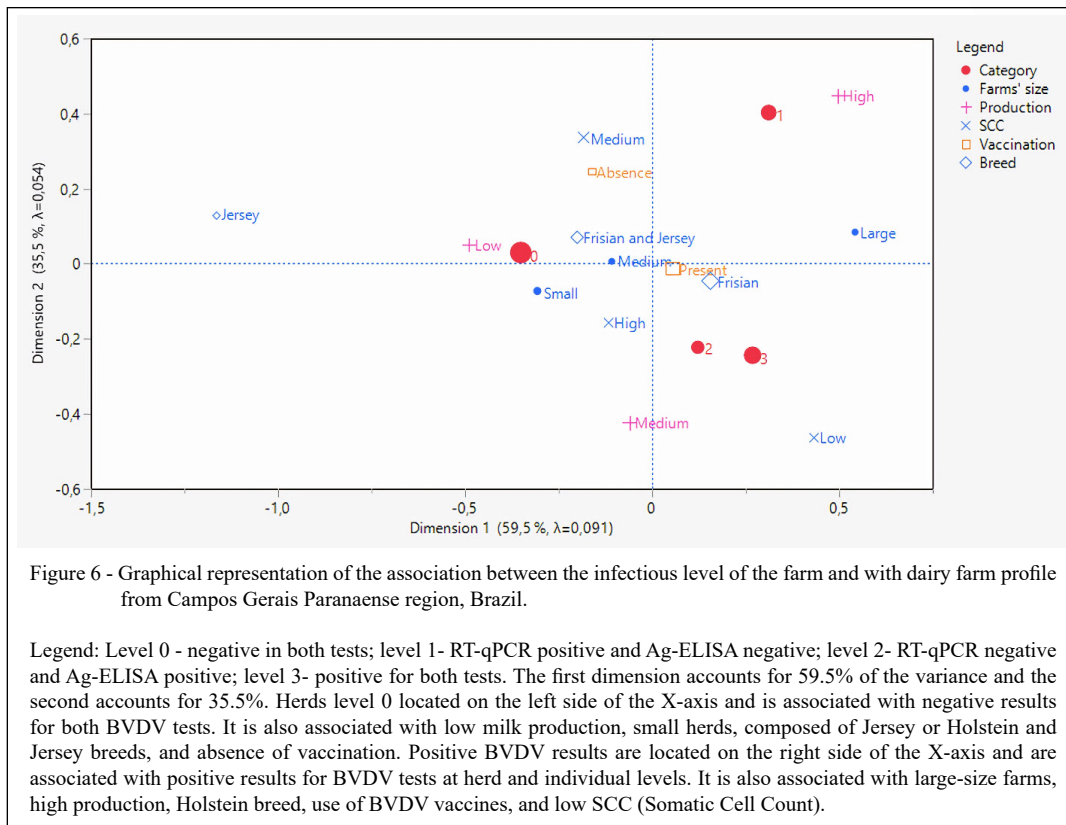
150 positive samples (5.17%). Sixty-nine among a total of 289 dairy farms (23.9%) had at least one RT-qPCR positive result from bulk-milk tank analysis. RT-qPCR offers high sensitivity, making it suitable for testing specimens with potentially low quantities of viruses, such as bulk milk. Additionally, this technique enables sample pooling and detection of viral RNA independent of specific antibodies (HILBE et al., 2007; SANDVIK, 2005).

A total of 23,466 Ag-ELISAs were performed during PI screening, and 692 positive tests (2.9%) were obtained. Twenty-eight (28/68; 41.2%) dairy farms had at least one Ag-ELISA result during the investigation period, while 58.8% (40/68) of the dairy farms showed negative BVDV. According to ASSUNÇÃO et al. (2022), ELISA was able to detect approximately 37.7–49.4% of seropositive animals in cattle herds in the states of Pará and Minas Gerais. The PI prevalence in our study was not reported because most producers culled positive animals immediately after the first positive Ag-ELISA test, and only eight farms (11.8%, 8/68) performed the PI

screening following the recommendation described in the Materials and Methods of this study. According to RICHTER et al. (2017), individual BVDV infection status, rather than PI prevalence, has been reported in most studies.

The combination of RT-qPCR and Ag-ELISA results from 26 dairy farms were classified as level 0 (negative for both tests), 12 as level 1 (RT-qPCR positive and ELISA Ag negative), 13 as level 2 (negative RT-qPCR and positive for Ag-ELISA), and 17 as level 3 (positive in both tests). The results of RT-qPCR in bulk-milk tanks depend on the surveillance of PI until adulthood and productive age, since the RT-qPCR technique has been used to detect whether BVDV-infected (PI and transiently infected) animals are lactating cows (HOE & RUEGG, 2006). This can explain some inconsistencies between the results from the diagnostic test strategies used in our study. Among the 68 dairy farms, 13 were classified as level 2 (negative by RT-qPCR and positive by the PI test).

Researchers (FREITAS et al., 2021) also used the PI screening protocol adopted in our study.



They reported 37.5% of positive herds from 40 dairy herds distributed across 10 municipalities in the state of Paraná, which presented a PI prevalence of 0.3–8.9% in 6,465 female Holstein Friesian dairy cattle. Another study conducted in the Paraná State reported a regional PI prevalence of 0.3% among 692 dairy cattle tested (DEZEN et al., 2013). In our study, 41.17% of dairy farms had at least one Ag-ELISA positivity while 58.8% were BVDV-free. However, the general prevalence of PI in herds could not be determined because of the nonadoption of Ag-ELISA retesting by producers. The positive characteristic of our study is its large sample size, which shows the endemic characteristics of BVDV in herds from Campos Gerais, Paranaense.

The risk factors for BVDV reported in the literature include herd characteristics, such as geographical location, reproductive management, and the adoption of biosecurity measures (external and internal) by the farmers (WERID et al., 2023; VAN ROON et al., 2020; FERNANDES et al., 2016; ALMEIDA et al., 2013).

MCA showed positive and negative results for the bulk milk tank and Ag-ELISA

on the same side of the x-axis, demonstrating an association between the tests. A positive correlation in the MCA was observed between positive results on large farms and high production in herds with a predominance of Holstein cows. In contrast, negative tests correlated with small and medium herds and low production, composed of Holstein and Jersey breeds. The most frequent risk factor associated with BVDV infection is herd size (ZIRRA-SHALLANGWA et al., 2022) because of high population density and intensive production systems (VAN ROON et al., 2020; FERNANDES et al., 2016; ALMEIDA et al., 2013).

The correlation between BVDV tests and SCC is difficult to interpret. High SCC is in the middle of the x-axis of the MCA graph, showing that this characteristic is independent of the BVDV herd status. Studies have shown a higher SCC in herds that are positive for BVDV. In a previous study conducted by our team, PI dairy cows showed high SCC and low milk production during lactation (BASQUEIRA et al., 2018). Many other factors, which were not investigated in this study probably affected SCC antigen levels and mastitis control.

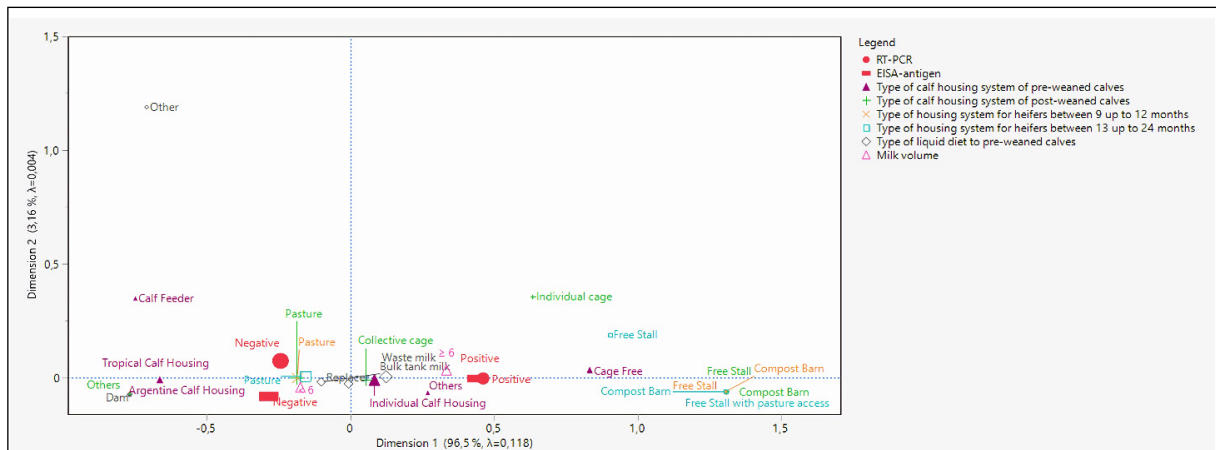


Figure 7 - Graphical representation of the correlations between BVDV tests with the type of young cattle housing (variables).

Legend: The figure indicates that dimension 1 determining 96.5% of the variability of the variation in the original data. For pre-weaned calves, the use of individual housing, waste milk or bulk tank milk, and liquid diet volume of more than six liters per day is associated with BVDV-positive tests. Conversely, calves reared in tropical calf housing, Argentine calf housing, or Calf feeder, feeding with milk replacer or natural sucking in the dams, and receiving less than six liters of milk per day are correlated with negative BVDV tests. For the post-weaning category (until 9 months of age), young heifers raised in pasture and other systems are correlated with negative results for BVDV tests. In contrast, calves kept in individual cages, collective cages, free-stall, and compost barn are correlated with infected herds and animals. After nine months, young heifers raised in free-stall or compost barns seem to be correlated with BVDV-positive tests. Similar results were observed for heifers from 13 up to 24 months of age, observing a correlation between positive tests and compost barn and free-stall with pasture access as a housing type.

Considering the classification of herds at the level, the farms defined as level 0 (negative for both tests) and 1 (RT-qPCR positive and ELISA Ag negative) are on the same side of the x-axis of the MCA graph, showing similar herd characteristics. This could indicate false-positive RT-qPCR results from the bulk-milk tank. In contrast, herd levels 2 (RT-qPCR negative and ELISA Ag positive) and 3 were similar (positive for both tests). As presented previously, level 2 could be explained by the high mortality of PI at < 1 year of age, which excludes the contribution of these animals to the bulk-milk tank (SMIRNOVA et al., 2008).

MCA shows an association between negative BVDV tests and the adoption of an extensive system for lactating dairy cows and preweaned calves (tropical calf housing system). In contrast, positive BVDV tests are associated with the adoption of compost barns as a housing system for post-weaned calves and heifers aged 13–24 months, in addition to the adoption of free stalls and sawdust or rubber bedding for lactating cows. The reported high prevalence of BVDV in cattle raised in confined housing and extensive raising systems act as protective factors against the disease (AMELUNG et al., 2018).

High animal population density has been reported as a risk factor for infectious diseases (VAN

ROON et al., 2020; FERNANDES et al., 2016; ALMEIDA et al., 2013). In our study, the mean number of animals per dairy herd system was 373, indicating a balance between the number of nonlactating young animals (< 24 months of age,  $n=182$ ), lactating dairy cows (184), and dry cows (16.77). Herds with more than 100 animals are susceptible to BVDV infection, and the composition of the herd can also contribute to a positive dairy production system status because of the higher prevalence in young animals than that in adult animals (WERID et al., 2023).

Most producers implement vaccination protocols in their health programs. However, there is an opportunity for 14 among 69 producers, who do not include reproductive vaccines in their sanitary calendar. Seven different commercial brands of reproductive vaccines were cited by producers, most of which were manufactured by multinational industries. In our study, most producers reported the use of inactivated vaccines composed mainly of aluminum hydroxide as an adjuvant. Only one farm answered the adoption of modified live vaccines in the reproductive vaccination protocol. The MCA analysis presented controversial results regarding the association between positive BVDV tests and the adoption of reproductive vaccines. Large farms have used reproductive vaccines with a higher frequency;

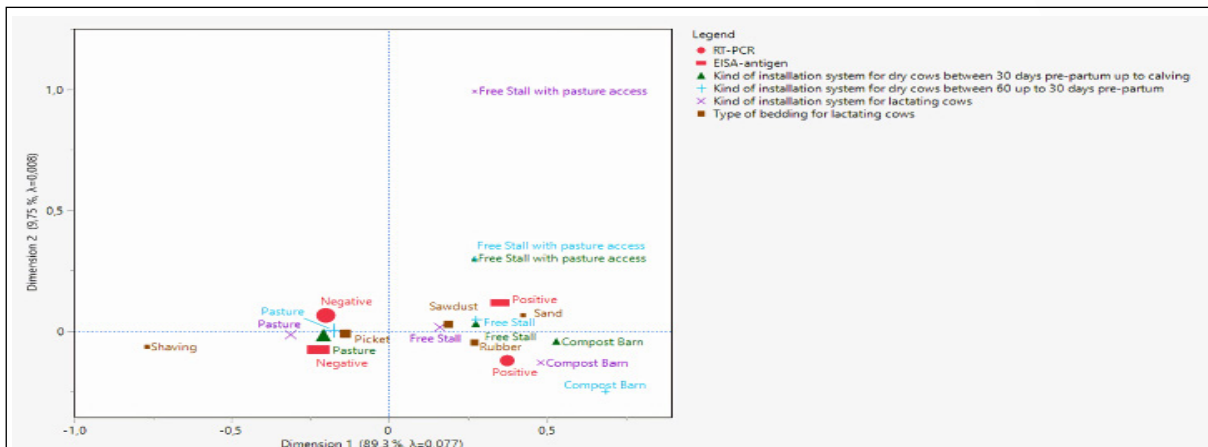


Figure 8 - Graphical representation of the correlations between BVDV tests with the type of adult cattle housing (variables).

Legend: The first dimension is responsible for 89.3% of the observed variability. The extensive raising system in pastures and pickets for dry cows, pre-partum cows, and lactating cows is associated with lower rates of positive Bovine Viral Diarrhea Virus (BVDV) tests. Confinement of cows, especially lactating or non-lactating cows, is associated with higher rates of positive BVDV tests. Moreover, the use of sand, sawdust, and rubber bedding is also linked to higher rates of BVDV.

however, this practice is associated with herds or animals positive for BVDV. This highlighted the effectiveness of the available formulations chosen by producers and technicians. Based on these results, we conducted a parallel study to evaluate the effectiveness of 10 BVDV commercial vaccines available in Brazil. This study is part of a series of subprojects developed in this huge research in partnership with the Frisia Cooperative. Previous Brazilian publications have highlighted the low effectiveness of reproductive vaccines against BVDV. In general, the BoHV-1 response appears to be good (FREITAS et al., 2021).

BVDV epidemiological data are essential for designing specific control programs and can incentivize federal regulatory rules. In our study, an infection rate of 2.8% was detected in the Campos Gerais Paranaense region; however, the PI determination was compromised by the conservative profile of producers.

## CONCLUSION

This study provided the epidemiological frequencies of BVDV at the herd and individual levels in the Campos Gerais Paranaense region, which tops the ranking of milk-producing municipalities in Brazil. Additionally, the association between BVDV tests and farm characteristics indicated the farm risk for BVDV and guides specific control programs.

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## DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. 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.

## AUTHOR'S CONTRIBUTIONS

All authors contributed equally.

## BIOETHICS AND BIOSECURITY COMMITTEE APPROVAL

The Ethics Committee on Animal Use of the Faculdade de Medicina Veterinária e Zootecnia of the Universidade de São Paulo (CEUA/FMVZ) approved this research (protocol number: 1288180520) and humans (Platform Brazil number: 37108020.2.1001.5390).

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://doi.org/10.5281/zenodo.10999275>.

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