

EXCRETION OF *Brucella abortus* VACCINE B19 STRAIN DURING A REPRODUCTIVE CYCLE IN DAIRY COWS

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ABSTRACT

This paper aimed to determine the excretion period of B19 vaccine strain during a complete reproductive cycle (from estrus synchronization, artificial insemination, pregnancy and until 30 days after parturition) of dairy cows from 3 to 9 years old that were previously vaccinated from 3 to 8 months. Three groups were monitored with monthly milk and urine collection during 12 months: G1 with seven cows from 3 to 4 years old; G2 with three cows from 5 to 6 years old; and G3 with four cows from 7 to 9 years old. Urine and milk samples were submitted to bacteriological culture and urine and PCR reactions for detection of *Brucella* spp. and PCR–multiplex for B19 strain identification. Ring test (RT) was also performed in the milk samples, and serum samples were tested by buffered acidified plate antigen test (BAPA). All animals were serologically negative at BAPA and *Brucella* spp. was not isolated from both urine and milk samples. RT revealed 13/210 (6.2%) positive milk samples. PCR reactions detected DNA of *Brucella* spp. in 86/420 (20.5%) samples. In urine it was found a significantly higher frequency (35.2%; 74/210) than in milk (5.7%; 12/210), more frequently from the estrus to 150 days of pregnancy and after parturition (6.7%; 10/150), and from 150 days of pregnancy to parturition (3.4%; 2/60), and they were all identified as B19 strain. In three groups, intermittent excretion of B19 strain was detected mainly in urine samples, which confirmed its multiplication and persistence in cows for until 9 years.

Key words: Bovine brucellosis, vaccination, B19 vaccine, excretion

INTRODUCTION

Brucellosis is a predominantly chronic anthrozoosis, caused by *Brucella abortus* that causes abortion with severe

losses in livestock, with frequently no other apparent symptom. The zoonotic role of the disease is responsible for joints - skeletal system degeneration in humans, with a long treatment period (23).

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Brucellosis still occurs in all Brazilian states, affecting mainly cattle, swine and buffaloes, although it can also affect equine, sheep, goats and dogs (24). The direct losses in livestock caused by brucellosis are associated to low productivity due to abortion and long calving intervals, high rates of animal culling, and decreasing meat and milk production. It still causes international commercial restrictions due to depreciation of herd and its products which affect its competitiveness (1). Prevention of human brucellosis depends on the control and eradication of the disease in the animal herds. Application of preventive vaccine is necessary for programs of animal brucellosis combat.

Aiming to decrease prevalence and incidence of new cases of bovine brucellosis in Brazil, the Ministry of Agriculture implanted the National Program for Control and Eradication of Brucellosis and Tuberculosis (PNCEBT), which determines the vaccination of all bovine females from 3 to 8 mo-old with attenuated live vaccine B19. This vaccine presents low interference with conventional and currently used serological assays after 18 months of vaccination. But, in fact, the excretion and the persistence period of B19 vaccine in vaccinated cows and its effects on the communicant and susceptible hosts have been studied only by means of serodiagnostic assay and bacterial isolation. Meyer and Nelson (1969) (19) detected positive microbiological cultures during three years in the milk of cows vaccinated with B19, and Manthei (1952) (17) observed low persistence of infection by B19 after one year by serological tests and microbiological culture in milk samples of vaccinated cows.

Brucella spp. isolation and identification by bacteriological methods are the definitive diagnosis; however, due to its biological characteristics this methodology can fail when there is reduced bacterial excretion (12) and high contamination of clinical samples (9).

Polymerase chain reaction (PCR) has been tested as an alternative tool for *Brucella* spp. detection in different samples (11, 12, 16, 18, 25, 26). It is fast, highly sensitive and specific

method that is able to detect microorganism DNA from contaminated clinical samples even when low quantities and not viable microorganisms are present.

This paper aimed to determine the excretion period of B19 vaccine strain by PCR during a complete reproductive cycle (from estrus synchronization, artificial insemination, pregnancy and until 30 days after parturition) of dairy cows from three to nine years old that were previously vaccinated at three to eight months.

MATERIALS AND METHODS

Animals and samples

Fourteen Holstein cows from a free brucellosis dairy herd, controlled by sanitary, reproductive and zootechnic management, vaccinated with B19 from 3 to 8 months old were selected to comprise three groups: G1 with seven cows from 3 to 4 years old; G2 with three cows from 5 to 6 years old and G3 with four cows from 7 to 9 years old at the time of the study. Milk and urine samples were collected, during 12 months, representing a whole reproductive cycle, from estrus synchronization and artificial insemination until thirty days after parturition.

Samples were collected at estrus, 18 hours after estrus, 22 days (pregnancy diagnostic), 90, 120, 150, 180, 210, 250, 260, 270 and 280 days of pregnancy, parturition, 15 and 30 days after parturition. Samples were maintained at -20°C until processed.

Indirect diagnosis

All animals were monitored by Buffered Acidified Plate Antigen Test (BAPA) according to Alton *et al.* (1988) (2) along the experiment period. Ring Test (RT) was also performed in the milk samples of all animals.

Microbiological culture for *Brucella* spp.

Milk and urine samples were cultured on sterile petri

dishes containing *Brucella* agar (DIFCO, USA) with 5% of defibrinated sheep blood added with antibiotic suspension composed by 10.000 IU/L of polymixin B, 15.000 IU/L of bacitracin, 0.005 g/L of novobiocin and 0.02 g/L of cicloheximide, incubated at 10% CO₂ atmosphere and aerobiosis conditions at 37°C during 10 days (13). Suspect colonies were identified according to Carter and Chengappa (1991) (7) and Holt *et al.* (1994) (14).

Polymerase chain reaction (PCR) for *Brucella* spp. DNA detection

DNA extraction from milk samples was performed with DNazol (Invitrogen) protocol adapted from Chomczynski (1993) (8) and from urine samples with boiling-phenol extraction methodology adapted from Cortez *et al.* (2001) (9). PCR was achieved with the genus-specific primers B4 and B5 described by Baily *et al.* (1992) (4), that amplify fragments of 223 bp. Amplification analysis was achieved by electrophoresis in a 2% agarose gel with 0,5X TBE buffer (28) under constant voltage of 6-7 V/cm. Gels were stained with ethidium bromide at 0,5 g/mL, and photographed under UV light (300-320 nm) by photo-documentation system (Kodak Digital DC/120 Zoom). As positive control it was used a suspension of standard *B. abortus* strain 544 (ATCC 23488), in a concentration correspondent to scale 8 of MacFairland (2.3 x 10⁹ bact / mL), and ultrapure water as negative control.

Multiplex-PCR

DNA from positive samples of *Brucella* spp. were submitted to multiplex-PCR for differentiating *Brucella* spp. and *B. abortus*, strain B19, using primers *ery 1* and *ery 2* (30). Amplification was carried out according to the protocol described by Bricker and Halling (1995) (6) with Master Mix reagent (Eppendorf®).

Statistical analysis

The proportions of positive samples in the experimental

groups (G1, G2 and G3) and in the different phases were evaluated by chi-square test or Fisher exact test (33) using the Statistical Package for the Social Sciences (SPSS) for Windows software version 13.0 and Epi-info version 6.04, with a significance level of 0.05.

RESULTS

All animals were serologically negative for brucellosis during the experimental period. Milk (n = 210) and urine (n = 210) samples were also negative for *Brucella* spp. in microbiological culture. RT revealed 13/210 (6.2%) of positive samples (Table 1), with 7.6% (8/105) in G1, 2.2% (1/45) in G2 and 6.7% (4/60) in G3, but there was no statistical difference among groups (p > 0.05).

Brucella spp. DNA was detected in 86/420 (20.5%) of total samples, and all of them were identified as *B. abortus* B19 strain by multiplex-PCR. The excretion was intermittent in urine but persistent for 9 years in cows that had been vaccinated from 3 to 8 months old. Regarding milk samples, 12/210 (5.7%) were positive, with 8.6% samples (9/105) in G1, 2.2% (1/45) in G2 and 3.3% (2/60) in G3. Of the 210 urine samples, 74 (35.2%) were positive, 34.3% (36/105) in G1; 28.9% (13/45) in G2 and 41.7% (25/60) in G3, significantly different when compared to milk samples (p < 0.01).

All animals presented intermittent urinary and milk excretion during the experimental period, independently of the age. But, the DNA detection was more frequent in samples from the estrus until 150 days of pregnancy and post-parturition (6.7%; 10/150), and from 150 days of pregnancy until parturition (3.4%; 2/60).

Considering the frequency of positive results with PCR for detection of DNA of *B. abortus* B19 strain in urine samples, over the total of urine samples collected during the whole reproductive cycle studied it was observed a higher frequency (50 to 54.2%) of positive samples from estrus to 150 days of pregnancy, decreasing (14.3 to 28.6%) between 180 and 280

days of pregnancy and then rising at parturition (41.1 to 53.1%) (Tables 2 and 3). It means that B19 excretion occurred until 150 days of pregnancy and at post-parturition, with a significant difference concerning the intermediary period for

the groups G1 ($p = 0.024$) and G2 ($p = 0.034$) (Tables 2 and 3 and Figure 1). G3 did not present statistical difference, however, when the three group animals were analyzed, a significant difference was observed ($p = 0.0008$).

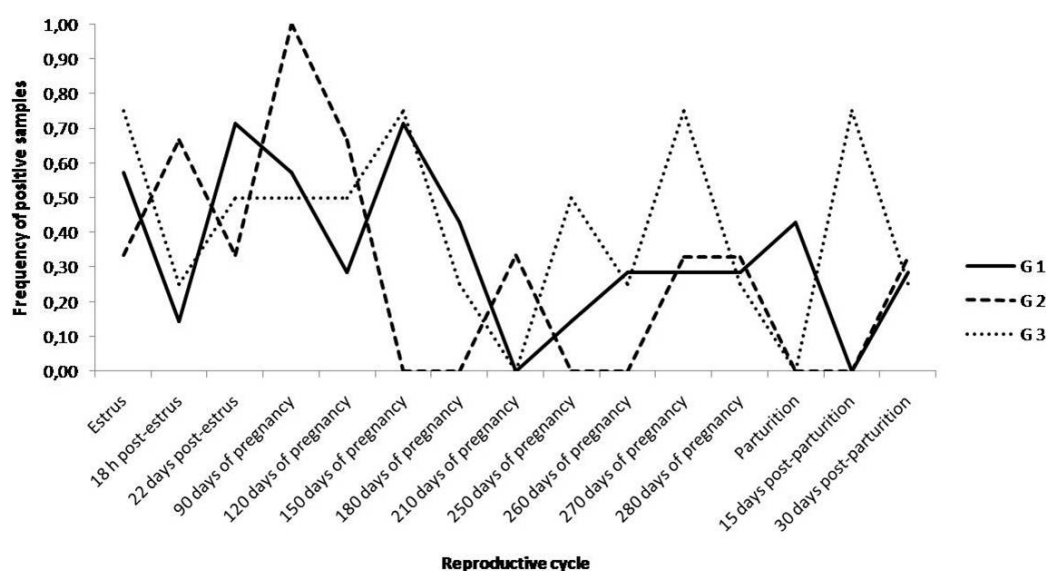


Figure 1. Distribution of B19 strain excretion in urine detected by PCR during a reproductive cycle (estrus, pregnancy and parturition) of cows vaccinated against brucellosis from 3 to 8 month age.

Table 1. Frequency of positive samples in Ring Test in milk and in PCR for detection of *B. abortus* B19 strain in milk and urine from cows vaccinated from 3 to 8 month age.

Groups	Ring test		Milk PCR		Urine PCR	
	No. of positive samples/total	%	No. of positive samples/total	%	No. of positive samples/total	%
G1	8/105	7.6	9/105	8.6	36/105	34.8
G2	1/45	2.2	1/45	2.2	13/45	28.8
G3	4/60	6.7	2/60	3.3	25/60	41.6
Total	13/210	6.2	12/210	5.7	74/210	35.2

Table 2. Frequency of positive urinary excretion of *B. abortus* B19 strain during a reproductive cycle, detected by PCR, in cows vaccinated with B19 vaccine from 3 to 8 month age.

Groups	Estrus	18h post-estrus	22° day post-estrus	Pregnancy period (days)										Parturition	Post-parturition period (days)	
				90	120	150	180	210	250	260	270	280	15		30	
G1	4/7	1/7	5/7	4/7	2/7	5/7	3/7	0/7	1/7	2/7	2/7	2/7	3/7	0/7	2/7	
G2	1/3	2/3	1/3	3/3	2/3	0/3	0/3	1/3	0/3	0/3	1/3	1/3	0/3	0/3	1/3	
G3	3/4	1/4	2/4	2/4	2/4	3/4	1/4	0/4	2/4	1/4	3/4	1/4	0/4	3/4	1/4	
Total	8/14	4/14	8/14	9/14	6/14	8/14	4/14	1/14	3/14	3/14	6/14	4/14	3/14	3/14	4/14	

Table 3. Frequency of positive urinary excretion of *B. abortus* B19 strain, detected by PCR, according to the different phases of a reproductive cycle in cows vaccinated with B19 vaccine from 3 to 8 month age.

Groups	Estrus to 150 days of pregnancy		Between 180 and 280 days of pregnancy		Parturition	
	No. of positive samples/total	%	No. of positive samples/total	%	No. of positive samples/total	%
G1	21/42	50.0	13/49	26.5	23/56	41.1
G2	9/18	50.0	3/21	14.3	10/24	41.7
G3	13/24	54.2	8/28	28.6	17/32	53.1

DISCUSSION AND CONCLUSION

National Program for Control and Eradication of Brucellosis and Tuberculosis (PNCEBT) in Brazil recommends vaccination of all heifers from 3 to 8 months old with B19 vaccine. This instruction is essential since vaccination will show similar responses and antibodies persistence will last for a short period, that will interfere in the current serodiagnosis for no longer than 18 months (10, 20).

Persistence and fluctuation of serum titers are related to the capacity of the vaccinated animal to eliminate the vaccine microorganism. Bacteriological studies in free brucellosis herds revealed that the antibody titers persistence is due to B19 strain reactivation. The antibody titers variations with no apparent cause can also occur, however, they are not enough to interfere in the serological diagnosis. Concerning this phenomenon, it is hypothesized that stress factors, due to inadequate management practices, would be the reason for this fluctuation (20).

All the 14 cows studied were serologically negative during the monitoring period, and confirmed that it is a brucellosis free herd. The PNCEBT recommends the use of RT, which reveals IgA antibodies in milk adhered to the fat molecules, in order to monitor dairy herds. Only 13 of 210 milk samples responded positively to the RT throughout the experiment. G1 with three to four years old animals presented the highest number of positive samples, but without any association with age or even with the reproductive stage.

In this study, *Brucella* spp. or *B. abortus* B19 strain were

not isolated from urine and milk samples. The failure of *Brucella* spp. isolation could be associated with freezing of samples, which can interfere in the bacteria survival, and also due to the sample contamination by microorganisms less fastidious than *Brucella*, which would compete in the nutrient utilization consuming them rapidly, thus releasing toxic products from their metabolism that change pH of the medium that could prevent *Brucella* survival (7, 9). The expressive frequency of *B. abortus* (86/240; 20.5%) with specific B19 strain primers by Multiplex-PCR carried out in milk and urine samples during the experiment (Table 2), confirms the capacity of persistence of the vaccine strain during 9 years or along the bovine female life even when they were vaccinated early in life. In these conditions, the higher frequency of urinary excretion (74/210; 35.2%) when compared to the milk (12/210, 5.7%), allows to conclude that the organism was viable and able to multiply in the host not only in recognized sites as spleen, mammary gland and lymph nodes, but possibly in the urogenital system, mainly kidneys.

The concentration of circulating *B. abortus* B19 strain was probably reduced to be achieved by bacteriological culture as well as for inducing serological response along the life of the vaccinated animal, thus it was not revealed by the usual diagnostic tests, and these results agree with the observations described by Manthei (1952) (16) and Meyer and Nelson (1969) (19) that detected the short period of B19 persistence of infection by means of microbiological culture. In this study, PCR was a fundamental tool for this elucidation.

The intermittent excretion of B19 strain in milk and urine

has occurred during a whole reproductive cycle, from the estrus synchronization, artificial insemination, pregnancy and parturition of all animals. Although the mechanism of B19 strain infection is similar to that of *B. abortus*, it is not fully understood which substances can stimulate its multiplication in the host organism. During the estrous cycle, a sequence of hormonal events results in greater resistance of the female reproductive tract to injuries and/or contamination by pathogens. If other substances act in order to enhance the multiplication of *Brucella* spp., such as steroid hormones, especially estradiol, since the great majority of abortions due to brucellosis occurs in the last months of pregnancy (3, 21, 27); in this experiment this has not been verified. The estradiol 17 β concentrations increases considerably during the last week of pregnancy in cows, while progesterone concentrations slightly decreases at the same period, and then abruptly decrease close to the parturition; at this moment, a peak of the estrogen takes place for a new ovulation (32). In case of estrogen to stimulate the proliferation of B19 strain during the bovine female life, the higher frequency of excretion should be limited to the postpartum and the beginning of a new ovulatory cycle, during the estrus, until about 40 days of pregnancy when the placentation occurs, with production of progesterone.

It was observed that the higher frequency of positive samples occurred from estrus (estrogen phase) to 150 days of pregnancy (progesterone phase); decreasing between 180 and 280 days of pregnancy and then rising at parturition (decreasing of progesterone followed by estrogen peak), so it was not demonstrated a dependency on the hormonal period. There was no difference among the three animal groups, so there was no association with the animal age and the reproductive cycle phase (Table 2).

The erythritol metabolism is the great difference between virulent *B. abortus* and B19 strain (29). In the vaccinal strain, *ery* gene (702 bp) is deleted, and this would be the explanation for its sensitivity to erythritol, and make it unable to survive in its presence. Erythritol is a polyalcohol found in the pregnant

uterus, which has its maximum concentration around 150 days of pregnancy when achieve a stable level until the parturition proximity (15).

Since B19 is sensitive to high erythritol concentration, this strain disappears from circulation in this period, returning after parturition (24). As exposed in Table 2, B19 excretion occurred mainly until 150 days of pregnancy, with a marked decrease in the period from 180 to 270 days of pregnancy, returning to increase with the parturition proximity. The persistence of some positive results in the intermediary period of the reproductive cycle after 150 days of pregnancy until the parturition can be due to different concentrations of circulating erythritol. Some B19 strains can be tolerant to certain erythritol concentrations and maybe this is one of the causes of persistent infection (5, 31).

The impact of the presence of B19 strain in the environment, the possibility of its transmission between cattle, especially to bulls, or even to other animal species, communicant and susceptible hosts including man, still need more studies. Miyashiro *et al.* (2007) (20) reported the detection of *Brucella* spp. DNA by multiplex-PCR in 37/192 fresh cheese; 30 were classified as been B19 strain, showing the real risk to public health.

In the veterinary medicine practice, the B19 vaccine handling requires very special attention to its zoonotic risk, even during management of vaccinated animals that can be potential renal carriers, as well as to the consumption of raw milk and its sub-products provided from B19 vaccinated animals.

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