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Bovine brucellosis vaccine strain S19 detected in calves before vaccination

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ABSTRACT. Bovine brucellosis is a zoonotic disease that has considerable economic impact on the market of products such as meat and milk. As Brazil has one of the largest cattle herds in the world, this disease is a significant agricultural and public health concern. In 2001, Brazil launched the National Program of Control and Eradication of Animal Brucellosis and Tuberculosis. This program included the vaccination of 3-8 month old calves using the smooth and attenuated S19 strain, as well as monitoring with serological tests. There have been reports of excretion and persistence of S19. In this context, the objective of this study was to monitor the persistence of the S19 vaccine strain in blood samples from vaccinated calves. Seroconversion was investigated to evaluate the vaccine immunogenicity. PCR assays of blood and serum were run at at: "day zero" (before vaccination), 1 to 15 days after vaccination and every month until 12 months, totaling 28 collections for each of 10 calves. The vaccine strain was detected throughout the study, even at day zero, before the calves were vaccinated. Sequencing analysis confirmed the presence of the S19 strain. Results from serological tests did not show agglutination in “day zero” samples, meaning the immune response was negative. An immune response was first detected in two samples on the fourth day. One hypothesis that could

explain this positivity before vaccination is that the already vaccinated mothers could have transmitted the vaccine to the calves, as the cows presented excretion of the S19 strain in milk and/or urine. This is the first report of the finding of this bovine brucellosis vaccine strain in the blood of calves before vaccination.

Key words: Brucellosis; S19; Calves; PCR; Sequencing; BAPA

INTRODUCTION

Brucellosis is an infectious contagious disease caused by the bacteria of the genus *Brucella* spp., affecting various species of animals (de Figueiredo et al., 2015), including humans (Hull and Schumaker, 2018). It is considered an emerging and occupational disease and is a potential agent for bioterrorism; consequently, it has caught the attention of health systems (Lawinsky et al., 2010). *Brucella abortus* is the main etiological agent of bovine brucellosis, a disease that affects reproduction, causes orchitis, and a loss of libido and infertility in males (Favero et al., 2008). The main effects in females include abortions, resulting in a reduction in milk production (Chand et al., 2015).

In addition, this disease also causes the devaluation of animal products and results in international barriers, affecting the commercialization of cattle products, leading to great economic losses for the country (Jardim et al., 2006; Dias et al., 2009). Bovine brucellosis is the most common brucellosis in Brazil (Poester et al., 2002).

According to the Brazilian Beef Exporters Association (ABIEC), Brazil has a cattle herd of 213.68 million and the amount of beef exported in 2019 was 2.49 million tons, 12.2% higher than in 2018. Regarding milk production, Brazil produced 33.5 billion liters in 2017 (IBGE, 2017).

Under these circumstances, the National Program of Control and Eradication of Animal Brucellosis and Tuberculosis (PNCEBT) was launched in 2001 by the Ministry of Agriculture, Livestock and Food Supply (MAPA). This program was responsible for implementing measures such as vaccinating calves aged from 3-8 months, using the S19 vaccine strain, as well as tests for diagnosis. These tests include Buffered Acidified Plate Antigen (BAPA), and milk ring tests as triage tests, and 2-mercaptoethanol (2-ME) and Complement Fixation (CF) as confirmatory tests (Brasil, 2006).

The S19 vaccine is the most effective vaccine in preventing bovine brucellosis and it is characterized as a live attenuated strain (Miranda et al., 2013). This strain was isolated from the milk of a Jersey cow and the plaque was forgotten at room temperature for one year. After analysis, the loss of virulence was verified (Brasil, 2006).

Though it has proven effective, this vaccine can infect humans (Osman et al., 2015) and induce abortion in pregnant cows (Sangari et al., 2000). Some studies have demonstrated persistence and excretion of the S19 vaccine strain. This strain has been found in cheese (Miyashiro et al., 2007; Silva et al., 2016; Hérnandez-Carbajan et al., 2018), semen (Júnior et al., 2017; Lourencetti et al., 2018; Junqueira-Junior et al., 2018), urine, and milk of cows up to nine years of age (Pacheco et al., 2012).

False positives in serological tests can occur through cross-reactions with pathogens that present antigens similar to those of *Brucella* spp. (Munoz et al., 2005), as well as in animals that were vaccinated with S19. As S19 is a smooth strain, it expresses the

O chain in its lipopolysaccharide, making it harder to work out which of the animals are actually infected (Sangari et al., 2000). False negative results in serological tests may also occur (Júnor et al., 2017; Junqueira-Junior et al., 2018).

PCR detection is a fast and accurate method that overcomes the limitations of traditional methodologies (Mohamed et al., 2013). It makes it possible to differentiate between the wild type and the vaccine strain (Sangari and Agüero, 1994; Bricker and Halling, 1995). PCR has been used to detect *Brucella* spp. in various types of samples.

In this context, the objective of this study was to monitor the persistence and immunogenicity of the S19 vaccine strain in the blood samples of vaccinated calves.

MATERIAL AND METHODS

Animal experiments were previously approved by the Ethics Committee for the use of Animals at Universidade Federal de Mato Grosso do Sul (protocol no. 555/2013) and carried out in accordance with guidelines issued by the Brazilian National Council of Animal Experimentation (CONCEA). Ten Brangus breed calves, aged between 6-8 months, were randomly selected for immunization against bovine brucellosis in a private property in the State of Mato Grosso do Sul. In the 12th month the loss of one calf due to loss of the ID tag was reported during sample collection.

A live attenuated S19 vaccine containing 60 billion viable bacteria per dose was used. Two mL of vaccine was administered subcutaneously to the pre-scapular region, according to the manufacturer's instructions.

Collection, preparation, extraction of the samples and Buffered Acidified Plate Antigen

The blood and serum collections were performed using 5mL vacuum tubes with EDTA and without EDTA respectively. The jugular vein was punctured to collect the blood. The samples were collected at "day zero" (before vaccination), 1st to 15th days after the vaccination and monthly until 12 months, totaling 28 collections and 279 samples (because the identification was lost and it wasn't possible to identify one of the calves). The collected blood samples were immediately stored at -20°C until DNA extraction. The tubes without EDTA were kept at room temperature until complete separation of the serum, which was transferred to a 1.5mL tube and stored until its use in the serological test.

The extraction of DNA from the blood was carried out according to the methodology proposed by Araújo et al. (2009), with adaptations. 500 µL of 20% SDS and 2 µL of proteinase K (20 mg/mL) were added into 300 µL of blood, followed by incubation at 65°C for 1 h. 800 µL of chloroform and 350 µL of protein precipitation solution (potassium acetate and glacial acetic acid) were then added, followed by the centrifugation of the mixture at 13.000 rpm for 15 min. The supernatant was collected and the DNA precipitation was performed by adding 1 mL of ice-cold absolute ethanol. Negative controls were performed in all the extractions to ensure non-contamination and no laboratory artifact throughout the process.

Readings at wavelengths of 260 and 280 nm using NanoDrop 2000 equipment (Thermo Fisher Scientific Corporation, USA) were performed to measure the quantity and quality of the samples. All the samples were adjusted to a concentration of 100 ng/uL and

then analyzed using 0.8% agarose gel, stained with SYBR Gold (Invitrogen-Thermo Fisher Scientific Corporation, USA) and further visualized in an ultraviolet translucent.

The Buffered Acidified Plate Antigen (BAPA) serological test was performed in the serum samples until the seroconversion according National Program of Control and Eradication of Animal Brucellosis and Tuberculosis (PNCEBT)

Polymerase Chain Reaction and Sequencing

A pair of primers adapted from the multiplex PCR called Bruce-Ladder (García-Yoldi et al., 2006) were used to identify the *Brucella* spp. The primers selected were BMEII0535f (5 'GCG CAT TCT TCG GTT ATG AA 3') and BMEII0536r (5 'CGC AGG CGA AAA CAG CTA TAA 3'), which amplify a 450 bp fragment related to the bp26 gene encoding an immunodominant antigen (this gene has an 870 bp insert in *B. pinnipedialis* and *B. ceti* species).

The reaction was carried out in a final volume of 20 µL containing: 9.7 µL of ultrapure water; 2 µL of Buffer (10X); 0.6 µL of $MgCl_2$ (50 mM); 0.3 µL of taq DNA polymerase (5 U/µL) (Invitrogen-Thermo Fisher Scientific Corporation, USA); 1 µL of each primer (5 pMol); 0.4 µL of DNTPs (10 mM) (Sigma Aldrich, USA) and 5 µL of DNA (100 ng/uL). The amplification was performed using the Veriti 96-well Thermal Cycler (Applied Biosystems, USA) with the following parameters: initial denaturation at 95ºC for 7 min, followed by 35 cycles at 95ºC for 35 s, hybridization at 54ºC for 45 s and an extension at 72°C for 3 min, followed by a final extension at 72°C for 7 min. All PCR were performed with negative control to ensure non-contamination and no laboratory artifact.

The primers Eri1 (5 'TTG-GCG-GCA-AGT-CCG-TCG-GT 3') and Eri2 (5 'CCC-AGA-AGC- AAA-CG 3 ') described by Sangari et al. (1994) were used to differentiate the vaccine strain with the wild-type strain. These primers were designed to amplify a gene related to erythritol catabolism, which contained a 1063 bp fragment in the wild strain and a 361 bp in the S19 vaccine strain, due to a 702 bp deletion in the latter. This PCR was performed in the samples that had tested positive for the primers BMEII0535f and BMEII0536r in the "day zero" and the 12th month after vaccination periods, and also in the S19 vaccine strain as positive control, for subsequent sequencing.

The 20 µL final reaction volume contained: 9.5 µL of ultrapure water; 2 µL of Buffer (10X); 0.6 µL of $MgCl_2$ (50 mM); 0.3 µL of taq DNA polymerase (5 U/µL) (Invitrogen-Thermo Fisher Scientific Corporation, USA); 1 µL of each primer (5 pMol); 0.6 µL of DNTPs (10 mM) (Sigma Aldrich, USA) and 5 µL of DNA (100 ng/uL). The amplification was performed using a Veriti 96 Well Thermal Cycler (Applied Biosystems, USA) with the following parameters: initial denaturation at 94°C for 10 min, followed by 35 cycles at 94°C for 1 min, hybridization at 57°C for 2 min, and an extension at 72°C for 2 min, followed by a final extension at 72ºC for 10 min. All PCR were performed with negative control to ensure non-contamination and no laboratory artifact.

The PCR products analysed in a 1.5% agarose gel electrophoresis. The resulting bands of the gel were developed using ethidium bromide in UV light illumination. The reference molecular weight used was 1 kb plus DNA ladder (Invitrogen - Thermo Fisher Scientific Corporation, USA).

To confirm the PCR results, the targeted bands from the "day zero" and the 12th month after vaccination obtained using both primers pair were excised from the agarose gel

and purified. The purification was performed using a homemade protocol by using a 1mL tip with a filter, followed by an addition of sodium acetate and absolute ethanol for the precipitation of the DNA. The DNA was then resuspended in 20 µL of ultrapure water. After purification, the samples from each PCR were placed together forming two pools for sequencing.

The sequencing reactions were performed using a MicroAmp Fast Optical 96-well Reaction Plate with Barcode (Applied Biosystems, USA). Each well contained a final volume amounting to 10 µL consisting of: 0.5 µL of BigDye®Xterminator™, 2 uL of SAM™Solution (Applied Biosystems, EUA), 50 ng of the purified PCR products and 0.64 µL of the respective primer pairs used previously (5 pmol/µL). In a Veriti 96-well thermal cycler (Applied Biosystems), these mixtures were submitted to initial denaturation at 96°C for 1 min, followed by 25 cycles at 96°C for 10 s, hybridization at 50°C for 5 s and an extension at 60°C for 4 min. EDTA and absolute ethanol were added, followed by 15 min at room temperature and centrifugation at 5.700 rpm for 15 min at a temperature of 4°C. After that, 10 µL of Formamide HI-DI was added to the pellet.

A denaturation was performed at 94°C for 3 min in the Veriti 96-well thermal cycler model (Applied Biosystems, USA), and a heat shock was performed by immediate cooling of the samples on ice. The sequencing was performed using ABI 3130XL equipment (Applied Biosystems, USA) based on the Sanger method. All the results were analyzed by BLASTn (Basic Local Alignment Search Tool) and the sequencing obtained with Eri1 and Eri2 it was also aligned with the results from the positive control, S19 vaccine strain, using the software Mega 5.02.

RESULTS AND DISCUSSION

The application of the PCR technique using the pair of primers BMEI0535f and BMEI0536r in the 279 samples (28 collections from each calf over a year) generated positive results throughout the period, including the "day zero" samples. The 450 bp bands showed the presence of genetic material from *Brucella* spp., and the absence of amplification in the negative controls proved that there was no contamination from laboratory artifacts or from manipulation (Figure 1). These results demonstrated that this bacterium remained circulating in the bloodstream throughout the period of study.

The PCR using the pair of primers ERI1 and ERI2 was performed in the 19 samples collected on the "day zero" and on the 12th month after vaccination, to identify the differences between the wild type and the vaccine strain. The 361 bp bands confirmed the presence of the vaccine strain. The presence of wild strain would have only been demonstrated with the presence of bands at 1063 bp. These data showed that the S19 strains were circulating in the bloodstream for one year; this strain was already present in the calves before vaccination.

The sequencing was carried out using the same 19 samples (day zero and 12th month after vaccination) to identify the amplified fragments using the primers BMEI0535f and BMEI0536r (450 pb) and Eri1 and Eri2 (361pb). The comparison between the results of the sequencing to the NCBI database using the BLASTn search program was performed using Megablast (*Highly similar sequences),* confirming our earlier results. *Brucella* spp. presented a total score of 67.6, an e-value of 1e-07 and a 90.38% similarity to the "*Brucella abortus* RB51 – AHVLA strain RB51chromossome I, complete sequence" (access

CP046720.1). The total score of the sequence obtained from the Eri1 and Eri2 PCR was 374, with an e-value of 2e-99 and 100% identity with "*B. abortus* S19 chromosome 2, complete sequence" (access CP000888.1). In addition, the alignment between the sequences from the samples and the positive control of the vaccine strain using Mega-X software, once again, confirmed the presence of the S19 vaccine strain (Figure Supplementary 1 and Figure Supplementary 2).

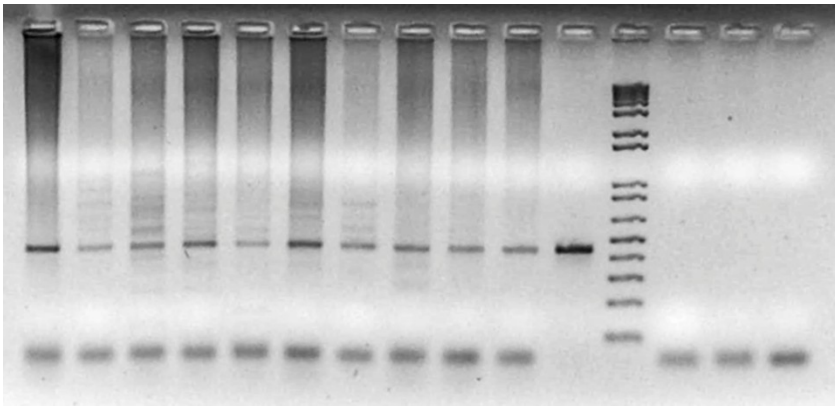

Figure 1. Amplification of a 450 bp fragment using the pair of primers BMEII0535f/BMEII0536r to detect genus *Brucella* spp. 1.5% agarose gel stained with ethidium bromide. Application of 20 µL of PCR using the pair of primers BMEII0535f and BMEII0536r in the 6th month samples. Lines 1-10: all the 6th month samples from calves, line 11: positive control, line 12: reference molecular weight 1 kb plus DNA ladder (Invitrogen - Thermo Fisher Scientific Corporation, USA), line 13: negative control, and lines 14-15: negative control of extraction.

Serological results using the Buffered Acidified Plate Antigen (BAPA) showed no immune response in the “day zero” samples, which was demonstrated by the absence of agglutination. The samples remained negative until the third day after vaccination. Two positive samples were observed on the fourth day, increasing to eight on the fifth day and to nine on the sixth day. From the seventh day after vaccination all the samples were positive.

DISCUSSION

Vaccinations are crucial to the National Program for Control and Eradication of Animal Brucellosis and Tuberculosis (PNCEBT). An effective vaccine induces protective immunity without causing pathogenicity (Sadanand, 2011). The vaccination with S19 induces a strong serological response that persists for a few months and reduces significantly over time. However, some animals can maintain this induction longer (Blankenheim et al., 2015). PNCEBT recommends performing serological tests only when the animal reaches 24 months of age (Brasil, 2006).

The possibility of false positives in serological tests has been investigated and discussed in great detail. Junqueira Junior et al. (2017) highlighted the importance to consider the occurrence of false negative results. Out of 27 samples of bovine semen, two animals with negative serological tests presented the wild strain after analysis by PCR, demonstrating how effective this technique is at detecting genetic material of *Brucella* spp.

In addition, the same research group detected 14 positive samples for the vaccine strain by PCR, of which five also contained the DNA of wild-type *B. abortus*. Similar results were obtained by Lourencetti et al. (2018) who identified 68 semen samples contaminated with S19 from a total of 100 by using PCR, however no serum sample from the same cattle was positive when using the Rose Bengal test (RBT). According to Júnior et al. (2017), bovine brucellosis diagnosis based only in serological tests probably underestimates infection caused by *B. abortus* in bulls. The authors submitted 335 serum and semen samples to serological tests and PCR analyses, and identified five bulls positive in the conventional RBT, (2-ME), semen plasma agglutination (SPA), modified 2-ME, microbiological culture in Farrell media, and the detection of *B. abortus* strain S19 by PCR. Two bulls presented negative results in all the serodiagnosis tests but were positive by PCR for *B. abortus* field strain. Therefore, the authors indicated the inclusion of molecular methods to improve the diagnosis in bovine bulls. The data from these studies were similar to our results, which presented negative BAPA tests in the "day zero" serum samples, even with the presence of the S19 strain detected by PCR and confirmed by sequencing.

Although vaccination is restricted to females, the presence of S19 in semen can be explained through the wrong vaccination of males (Júnior et al., 2017) or contamination of the environment by the vaccinated cows. The cows could infect the environment with S19 through excretion and therefore contaminate the males (Júnior et al., 2017; Junqueira Junior et al., 2018). This contamination of the environment by the S19 may also explain the findings from a study conducted in North Carolina. This study detected S19 in 10% of 80 wild pigs that were in an area with no cattle since 1970 (Stoffegen et al., 2007).

Studies were carried out to evaluate longer excretion periods of the vaccine strain in vaccinated cows between the ages of 3-9 years, in a complete reproductive cycle. The results showed that the excretion mainly occurs up until the 150th day of pregnancy, during and after labor. This was confirmed by analyzing the milk and urine of the cows, with the latter being the main source of excretion (Pacheco et al., 2012). Miyashiro et al. (2007) evaluated 192 illegally produced cheeses from the states of São Paulo and Minas Gerais, and found that 37 of them tested positive for the genus *Brucella* spp., 30 out of the 37 (81.08%), presented S19, demonstrating that this strain can be excreted in milk. The presence of S19 in cheese was also reported by Silva et al (2016) and Hérnández-Carbajal et al (2018).

The contamination of humans by S19 vaccine was confirmed by the isolation of this strain in the blood samples of the milkers (Osman et al., 2015). The transmission of a live strain used as a vaccine was evaluated by Kojouri and Gholami (2009). PCR analyses were carried out using blood samples from vaccinated sheep (n = 10) with the live vaccine strain Rev1, and from non-vaccinated sheep (n = 5). The live vaccine strain Rev1 is used for immunization against *B. melitensis*. On the 14th day after vaccination, 40% of the sheep in the non-vaccinated group showed positive PCR results, and serological tests showed that both groups (vaccinated and unvaccinated) presented antibodies against *Brucella* spp.

The main known difference between the wild-type strain and the S19 vaccine strain is the metabolism of erythritol. The latter does not contain the enzyme D-erythrose-1-phosphate dehydrogenase, which is essential for the catabolism of this polyalcohol. This results in the accumulation of intermediate toxic products (D-erythrose 1-phosphate) and a reduction of the ATP, which inhibits the growth of S19 (Sperry and Robertson, 1975). On the other hand, erythritol could be responsible for the growth and presence of *B. abortus* in

the placenta, and in other fetal tissues of pregnant cows (Smith et a., 1962). In addition, some mutant strains of S19 presented tolerance to erythritol and can cause a miscarriage and / or persistence when inoculated with the administration of the S19 strain vaccine (Sangari et al., 1996).

The persistent excretion of the S19 vaccine strain may explain the results obtained with the calves in this study. The presence of this strain in calves before being vaccinated could be the result of contact with their mothers, which already presented the vaccine strains in their urine and / or milk. This fact along with the ability of the S19 strain to contaminate the environment, as well as the capacity of vaccine strains to be transmitted between the animals can explain our findings.

This is the first study that showed a bovine brucellosis vaccine strain presented in the blood of calves before vaccination. Further study is essential to understand the presence of S19 vaccine and how it leads to the contamination of the environment, its possible transmission capacity between cattle and other species, including humans, as well as the epidemiological importance of this process. Furthermore, the use of only serological tests could have underestimated the infection or presence of *Brucella* spp. The molecular techniques are growing within the scope of testing and both methods could be used together to improve the diagnosis of brucellosis.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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