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Efficacy of strain RB51 vaccine in heifers against experimental brucellosis

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Abstract

With the goal of providing an additional tool for controlling bovine brucellosis in Brazil and evaluating the full calf dose in adult cattle, the efficacy of the rough *Brucella abortus* strain RB51 vaccine was tested in heifers. Thirty-three females of approximately 24 months of age were divided in two groups: one group ($n = 20$) received the RB51 vaccine and the other group ($n = 13$) were used as non-vaccinated control. Animals in the vaccinated group were split in two sub-groups. One sub-group ($n = 12$) was vaccinated subcutaneously with 1.5×10^{10} colony forming units (CFU) of RB51 at Day 0 of the experiment and the other sub-group ($n = 8$) was vaccinated subcutaneously with 1.6×10^{10} CFU of RB51 at 60 days of gestation (Day 260 of the experiment). All cattle were challenged between 6 and 7 months of pregnancy with 3×10^{8} CFU of the virulent strain 2308 of *B. abortus* by the conjunctival route. Vaccination with RB51 vaccine did not result in the production of any antibodies against the O-side chain of lipopolysaccharide (LPS), as measured by conventional serological tests (rose bengal plate agglutination test (RBPAT), standard tube agglutination test (STAT), and 2-mercaptoethanol test (2ME)). A total of 25% cumulative incidence of abortions was found in the vaccinated group, whereas in the control group the cumulative incidence was 62%. *B. abortus* RB51 was not isolated from any sample, and no abortions were produced by RB51 vaccination of females at 60 days of pregnancy. The results indicate that vaccination with RB51 prevented 59.4% of abortions, 58.6% of cow infections, and 61.0% of fetal infections. The relative risk (RR) revealed that non-vaccinated animals have 2.462 (95% CI 1.029–5.889) times higher risk of aborting than RB51-vaccinated animals.

Keywords: *Brucella abortus* RB51; Vaccination; Heifers; Cattle

1. Introduction

Brucellosis in cattle is caused by *Brucella abortus*, a facultative intracellular bacterium, which can cause abortions and decreased fertility [1], as well as chronic zoonotic infections in humans, including undulant fever, arthritis, and endocarditis [2].

Eradication of brucellosis has been underway in several parts of the world for more than 50 years, and an important component involved in these eradication programs is vaccination of calves (full dose) or cows (reduced dose) with *B. abortus* strain 19 (S19) [3].

Brucella abortus field strains and S19 share antigens of the polysaccharide O-side chain of the cell surface smooth lipopolysaccharide (LPS), which induce an antibody specific response that renders it very difficult to distinguish vaccinated and true infected animals by most serologic tests [4,5]. Other disadvantages of the S19 vaccine include the fact that in some circumstances it can cause abortion in pregnant cows [6] or

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orchitis in bulls [3], and it is pathogenic for human beings [2]. To overcome some of these problems, vaccination with S19 is restricted to female calves between 3 and 8 months of age, although some antibodies may persist in adult animals vaccinated as calves [7].

Another approach to avoid interference with serological diagnosis is by using a vaccine that does not elicit antibodies against the O-side polysaccharide. A mutant vaccine strain with rough characteristics devoid of O-chain named RB51 was obtained by conventional methods. The RB51 was derived from the virulent smooth *B. abortus* 2308 by several passages in media supplemented with sub-inhibitory concentrations of rifampicin [8]. Therefore, this strain differs from other smooth *Brucellae* since it lacks almost all of the LPS O-side chain, consequently antibodies against this immunodominant antigen are not induced by vaccines prepared with strain RB51 when used in calves [9,10] or repeatedly administered to adult cows [11]. It has been determined that *B. abortus* strain RB51 has a *wboA* gene disrupted by an IS*711* insertion element which impairs the synthesis of O-chain [12]. Complementation of RB51 with a functional *wboA* gene indicates that RB51 also contains a second mutation affecting the export of O-chain to the bacterial surface or the coupling of O-chain to the core of the LPS, or both [13,14].

According to several previously published papers, strain RB51 is stable and attenuated when inoculated in mice and guinea pigs [8,15–17]. Heifers vaccinated with RB51 are protected against infection and abortion at levels similar to heifers vaccinated with S19 [9,18]. In addition, RB51 induces a protective cell-mediated immune response against challenge with the virulent strain 2308 [19]. Furthermore, RB51 is safe when inoculated into pregnant females at reduced dose [20], and it is highly attenuated for induction of abortion even when injected intravenously [21].

Strain RB51 has been approved for use as official vaccine in the USA, Chile and Uruguay as a replacement for S19 [22–24], or in conjunction with S19 in Mexico, Paraguay, Venezuela, and some countries of Central America [25–28]. The RB51 vaccine for use in most of the above-mentioned programs has been licensed for subcutaneous vaccination of calves (4–12 months of age) at the full dose of $1.0–3.4 \times 10^{10}$ colony forming units (CFU). In *Brucella*-infected herds the vaccine can be safely used in cows at a reduced dose of 1.0×10^9 CFU [29]. However, the use of the dosage indicated for calves (full dose) in adult or pregnant cows has not been thoroughly studied, in spite of some emerging field information suggesting that higher doses might not cause noticeable increase in abortion rates [30].

Federal regulations through the "Programa Nacional de Controle e Erradicação da Brucelose e Tuberculose—PNCEBT" (National Brucellosis and Tuberculosis Control and Eradication Program) in Brazil, stipulate that one of the strategies for controlling brucellosis in the country is the compulsory vaccination of 3- 8-month-old heifers with S19 in order to avoid persistent antibody titers in routine serologic tests [31,32]. According to the PNCEBT, under certain circumstances, such as heavily infected herds or adult cows that have not been vaccinated as calves, vaccination of bovine females over 8 months of age may be approved with vaccines which do not interfere with standard serological tests, namely the RB51 vaccine [32].

Most studies about the protection induced by RB51 were performed under strictly controlled conditions in mice and/or heifers, and have concluded that animals are protected against moderate challenge, but contradictory results in field experiments under high or moderate challenge appears to indicate that more research is necessary to evaluate the level and duration of immunity under such conditions [33]. Thus, the goals of this study were to evaluate the full dose of the RB51 vaccine, according to the following parameters: (i) efficacy of RB51 vaccination in heifers not previously vaccinated; (ii) detection of antibodies in RB51 vaccinated animals using several serologic tests; and (iii) ability of the RB51 vaccine to induce abortion when used in females at early pregnancy.

2. Material and methods

2.1. Local

The experiment was conducted in an experimental area within the premises of the Brucellosis Laboratory of the "Laboratório Nacional Agropecuário, LANAGRO/MG, Ministério da Agricultura, Pecuária e Abastecimento", Minas Gerais, Brazil. The groups were kept in two separated but adjacent 1 ha paddocks of *Brachiaria decumbens* pasture throughout the experiment. Paddocks were separated by a single fence.

2.2. Animals

Thirty-three crossbreed virgin heifers with approximately 24 months of age were divided in two groups. One group ($n = 20$) received the RB51 vaccine and the other group ($n = 13$) was used as non-vaccinated control. The heifers were fed a balanced diet of corn silage, cottonseed, citrus pulp, and a mineral salt mixture. All heifers were serologically negative in the rose bengal plate agglutination test (RBPAT) for brucellosis [34], and none of them had been previously vaccinated with S19.

2.3. Vacines and vaccinations

Two batches of a commercial RB51 vaccine were used (Professional Biological Company, USA). Prior to and just after vaccination, each batch was evaluated for purity, dissociation, and number of viable cells according to Nielsen and Ewalt [14].

At Day 0 of the experiment, heifers in the vaccinated group were divided in two sub-groups: 12 heifers were vaccinated at Day 0 of the experiment and the remaining 8

heifers were vaccinated at the 60th day of gestation (260th day of the experiment), with a 2 mL dose, by subcutaneous route, according to the manufacturer's recommendations. The heifers of the control group received 2 mL of sterile saline solution. The heifers were observed for 2 h post vaccination for immediate hypersensitivity reactions.

2.4. Breeding protocol

The heifers were bred by artificial insemination after estrous synchronization [35]. The hormonal protocol consisted of intravaginal implants of progesterone (CIDR, Pharmacia Animal Health, USA) applied at the 160th day of the experiment. The implants were withdrawn 9 days later, and 0.5 mg of cloprostenol (Ciosin, Coopers, Brazil) was injected in each heifer. Artificial insemination was performed during the following estrous, which were observed from 1 to 4 days after implant withdrawn. Twenty-two heifers returned to estrous after first insemination and were reinseminated. Thirty-five days after artificial insemination the pregnancy was confirmed by ultrasonography. Thereafter, heifers were monitored all day long for detection of either return to estrous or abortion.

2.5. Experimental challenge

All animals were challenged with the virulent *B. abortus* strain 2308.[1] After growth in triptose agar (Difco, USA) for 48 h in 5% CO_2, a saline suspension of strain 2308 was prepared and adjusted to a concentration of 3.0×10^8 CFU/mL by spectrophotometry (E205 D, CELM, São Paulo, Brazil), which was confirmed by plate counts [29]. Animals were exposed to the challenge strain between 6 and 7 months of pregnancy by conjunctival instillation of 50 μL/eye (100 μL/heifer) of the strain 2308 suspension, resulting in a 3.0×10^7 CFU challenge per heifer, according to Cheville et al. [36].

2.6. Serologic testing

Blood samples were collected at 30 days before vaccination, in the day of vaccination and at the following days after vaccination: 15, 21, 30, 150, 270, 300, 360, and 380 (day of challenge). Sera were centrifuged, separated in aliquots, and stored at −20 °C. After the challenge, all heifers were bled at Days 15, 30, 60, and at necropsy. Sera were assayed for detection of anti-*B. abortus* antibodies by the rose bengal plate agglutination test, the standard tube agglutination test (STAT), and 2-mercaptoethanol test (2ME). Results were expressed as positive or negative for the RBPAT and complete agglutination at 1:25 dilution or more in the 2ME was considered positive, according to the recommendations of the PNCEBT [32].

2.7. Bacteriologic studies

Abortion was defined as premature expulsion from the uterus of a non-viable fetus born more than 15 days before the predicted date of delivery [37]. Aborted fetuses and newborn calves were collected or sacrificed at late about 12 h after abortion or calving, to avoid increasing environmental contamination. Cows that aborted or delivered normal calves were immediately euthanized. All animals were euthanized by electrocution after intravenous administration of 15–20 mg/kg of xylazine (Coopazine, Coopers, Brazil) [38]. This experimental protocol was approved by the Ethics Committee in Animal Experimentation of the Universidade Federal de Minas Gerais (CETEA—UFMG, Protocol 028/05). Specimens of parotid, retropharyngeal, prescapular, supramammary, internal iliac, and bronchial lymph nodes, mammary gland, lung, spleen, liver, milk, vaginal swab, and placentome were collected from cows. Specimens of bronchial lymph nodes, lung, abomasal content, spleen, liver, and rectal swab were collected from aborted fetuses and calves. All samples were stored at −20 °C until cultured. Thawed tissues were macerated in a stomacher (Seward Medical, UK). All macerated samples were plated on triptose agar supplemented with antibiotics (Farrell's medium—*Brucella Selective Supplement* SR83, Oxoid, UK), and incubated at 37 °C under 5% CO_2 atmosphere for 12 days. All isolates were identified by routine methods [8,34,39], and stored at −70 °C in a cryoprotector medium prepared with peptone broth and glycerol as described by Jones et al. [40].

2.8. Statistical analysis

The relative risk (RR) was used as a measure of association between exposure (non-vaccinated group) and the cumulative incidence of abortion, infection of the cow and infection of the fetus. Confidence intervals of this risk ratio were calculated using the logarithmic approximation [41,42].

Vaccine efficacy was estimated in the form of an attributable fraction in the exposed group [(RR − 1)/RR], where the "non-vaccinated group" is the exposed group or risk factor positive. It can be interpreted as the proportion of cases that would have occurred in the vaccinated group should the vaccine had not been used [42,43] call it the preventable fraction, which, in this example, represents the fraction of the caseload under no exposure (no vaccination) that could be prevented by exposure (vaccination).

3. Results

Counting of viable cells in the vaccines at Day 0 (before and after vaccination) and at Day 260 (60 days of gestation)

[1] Kindly supplied by Dr. Luís Ernesto Samartino—Instituto Nacional de Tecnología Agropecuaria, Centro de Ciencias Veterinarias y Agronomicas, Instituto de Patobiologia, Las Cabañas y Los Reseros, (1712) Villa Udaondo, Castelar, Argentina.

Table 1
Serological response of RB51-vaccinated and non-vaccinated cattle after challenge with *B. abortus* strain 2308

Days after challenge	RBPAT[a]		STAT[b]		2ME[c]	
	RB51 ($n=20$)	Control ($n=13$)	RB51 ($n=20$)	Control ($n=13$)	RB51 ($n=20$)	Control ($n=13$)
0	0	0	0	0	0	0
15	2	0	1	0	0	0
30	11	11	4	2	4	2
60[d]	14	7	14	7	8	5
N[e]	15	12	9	10	11	10

[a] RBPAT, rose bengal plate agglutination test.
[b] STAT, standard tube agglutination test.
[c] 2ME, 2-mercaptoethanol test.
[d] Two cows of RB51 group and six of control group aborted and were necropsied before Day 60 after challenge.
[e] Blood samples collected at the day animals were necropsied.

of the experiment resulted in counts of 1.5×10^{10} and 1.6×10^{10} CFU/dose, respectively. Counts that were carried out immediately after vaccination did not differ from those performed before vaccination. The vaccine was pure and 100% rough in all tests.

Sera from all animals of RB51 vaccinated and control groups did not show anti-*Brucella* antibodies on Days −30, 0, 15, and monthly thereafter until the day of challenge, as shown by RBPAT screening. After challenge with strain 2308, all sera were positive to at least one serologic test, with a peak of antibodies between 30 and 60 days after challenge, and higher numbers of positive heifers observed when the blood samples were obtained soon after abortions or parturitions (Table 1). All heifers of the control group that aborted or delivered premature weak calves were positive to RBPAT with high titers in STAT and 2ME, with the exception of one cow that delivered a premature weak calf. This cow had a positive reaction to RBPAT, a weak reaction in STAT (25), and a negative result in the 2ME. Five heifers of the vaccinated group, which had normal parturitions, reacted positively to the RBPAT, with titers of ≥50 and ≥25 to STAT and 2ME, respectively.

The results of biochemical tests (catalase, oxidase, citrate, nitrate reduction, urease), acriflavin agglutination, agglutination to anti-*Brucella* smooth and rough sera and crystal violet staining of the colonies, classified all isolates as smooth *B. abortus*, including the isolates from two infected cows vaccinated with RB51 early in pregnancy. None of isolates was classified as *B. abortus* RB51.

The challenge 2308 strain was isolated from several tissues and other kind of samples at necropsy (Table 2). Importantly, colonization, measure as the number of *B. abortus*-

Table 2
Summary of *B. abortus* isolation from culture-positive heifers after challenge with strain 2308 in the RB51-vaccinated and non-vaccinated control groups

Specimen	RB51 ($n=7/20$)		Control ($n=11/13$)		Total ($n=18$)	
	N[a]	%[b]	*N*	%	*N*	%
Cows						
Retropharyngeal LN	2	28	7	64	9	50
Parotid LN	3	43	9	82	12	67
Bronchial LN	1	14	1	9	2	11
Prescapular LN	2	28	5	45	7	39
Internal iliac LN	3	43	5	45	8	44
Supramammary LN	3	43	6	54	9	50
Liver	1	14	0	0	1	6
Spleen	0	0	2	18	2	11
Mammary gland	2	28	5	45	7	39
Placentome	7	100	11	100	18	100
Vaginal swab	6	86	11	100	17	94
Milk	4	57	10	91	14	78
Fetuses[c]						
Bronchial LN	4	57	7	64	11	61
Lung	4	57	9	82	13	72
Liver	4	57	4	36	8	44
Spleen	5	72	8	73	14	78
Abomasal fluid	4	57	4	36	8	44
Rectal swab	6	86	6	54	12	67

[a] *N*, number of animals which *B. abortus* was isolated from that site.
[b] Percentage of *B. abortus* isolation from the tissue in culture-positive animals.
[c] Fetuses, includes aborted fetuses, weak and live calves.

Table 3
Abortion and parturition of RB51-vaccinated and non-vaccinated cattle after challenge with *B. abortus* strain 2308

Group	Abortion		Parturition		Total
	N[a]	%	N[b]	%	
RB51	5	25	15	75	20
Control	8[c]	62	5	38	13

[a] Number of cows that aborted.
[b] Number of cows that delivered a viable calf.
[c] Including three premature weak calves, delivered 15 days before the expected day of parturition or earlier.

positive samples per heifer, was more intense in the non-vaccinated control group when compared to the vaccinated group ($\chi^2 = 4.07$, $P = 0.04$). In both groups, the frequency of *B. abortus* isolation was higher in placentomes, vaginal swabs, and milk samples, whereas in the aborted fetuses, premature weak calves or infected live calves, higher frequency isolation occurred in the lung, spleen, and rectal swabs (Table 2).

For the purpose of analysis in the present study, premature weak calves, delivered 15 days before the expected date of parturition or earlier, were considered as abortions [37]. In the vaccinated group, five cows aborted with a total of 25% cumulative incidence of abortions. One cow vaccinated at the 60th day of pregnancy aborted at Day 225 of gestation (59 days before predicted date of delivery), and the isolate was characterized as a smooth strain of *B. abortus*. In the control group, eight cows aborted (three delivered premature weak calves), resulting in a cumulative incidence of abortions of 62%. The number of cows that aborted or delivered normal calves is shown in Table 3.

The cumulative incidence of infections in the vaccinated group was 35% whereas in the non-vaccinated group the cumulative incidence of infections was 85%. The number of *B. abortus* isolates after abortion or parturition of cows challenged with strain 2308 and the number of *B. abortus* isolates from aborted fetuses or calves are shown in Table 4.

Table 5 summarizes the attributable fraction in the exposed and the relative risk of controls versus RB51 vaccinated animals. The relative risk revealed that non-vaccinated animals had 2.462 (95% CI 1.029–5.889) times higher risk of aborting than RB51-vaccinated animals. If the attributable fractions are interpreted as preventable fractions [43], the present results indicate that vaccination with RB51 prevented 59.4% of abortions, 58.6% of cow infections and 61.0% of fetal infections.

4. Discussion

In 2001, the Brazilian Ministry of Agriculture (MAPA), the federal animal health authority, revised the existing regulations on the control of bovine brucellosis, which were based mainly on voluntary vaccination of heifers, diagnostics at herd level, and test and voluntary slaughter of reactors but were never fully implemented, deciding to establish a new program [31]. The new National Program is based on: (1) compulsory vaccination of heifers aged 3–8 months with S19; (2) voluntary accreditation of free herds, in accordance with international standards; (3) voluntary monitoring of beef herds based on a periodic sampling scheme; (4) regulatory tests for breeding stock prior to interstate movement and to entrance into livestock fairs/exhibitions; (5) compulsory slaughter of cattle testing positive, in approved abattoirs; (6) standardization of testing procedures through short courses for accredited veterinarians [44]. Among the proposed strategies, the compulsory vaccination of heifers from 3 to 8 months of age with the live S19 vaccine plays an important role. Heifers vaccinated at the recommended age are not eligible for serologic testing up to 24-month-old to avoid interference with antibodies induced by the smooth S19 vaccine. The program also envisages the strategic use of the non-agglutinogenic vaccines in adult animals that were not vaccinated as calves with S19 or in affected herds after the culling of infected animals [32].

Table 4
Isolation of *B. abortus* from RB51-vaccinated and non-vaccinated heifers after challenge with *B. abortus* strain 2308

Group	Isolates from cows		Total	Isolates from fetuses or calves		Total
	Positive (%)	Negative (%)		Positive (%)	Negative (%)	
RB51	7(35)	13(65)	20	6(30)	14(70)	20
Control	11(85)	2(15)	13	10(77)	3(23)	13

All isolates were smooth *B. abortus*.

Table 5
Attributable fraction in the exposed (non-vaccinated) group and relative risk of abortions and infections of cows and fetuses in non-vaccinated vs. RB51-vaccinated groups

RB51-vaccinated group	Attributable fraction in the non-vaccinated group	Relative risk	Confidence interval 95%[a]
Abortion	0.594	2.462	1.029–5.889
Cow infection	0.586	2.418	1.274–4.588
Fetal infection	0.610	2.564	1.232–5.335

[a] Logarithmic approximation ([41], [42]).

Strain 19 remains the most widespread vaccine against brucellosis in cattle worldwide because it is safe, immunogenic, and easy to produce [3]. However, the persistence of antibodies following S19 vaccination, which are serologically indistinguishable from those induced by virulent strains, has led to the slaughter of a large number of non-infected cattle [5]. In Brazil, the use of S19 is not allowed in adult cows that have not been vaccinated as calves, thus delaying the time for building up adequate herd immunity at the national level.

Eradication of brucellosis involves a series of approaches. Among the most important are: individual identification of animals, control of cattle movements and trade, high vaccination coverage, and the monitoring of herd status by serology. Control and eradication might be partially achieved by using the best vaccines available or combining vaccines with a view to securing high levels of herd immunity with minimum serological interference. This need prompted us to evaluate a commercial RB51 vaccine, on local cattle, mainly zebu or zebu croosbreds, focusing on the presence of post-vaccinal antibodies, degree of protection, and ability to induce abortion in cows, employing a full calf dose on adult heifers.

The results of serology using the tests suggested by PNCEBT [32], namely RBPAT, STAT, and 2ME, were negative from Day 0 (day of vaccination) through Day 380 after vaccination (day of challenge), confirming the absence of interference of RB51 vaccination with the serological diagnostic tests for bovine brucellosis. As the antibodies detected by those tests are directed to the O-side chain of smooth *Brucellae*, the lack of antibody response in RB51 vaccinates is most likely due to the absence of O-chain in RB51 [8–10,19,36]. After challenging with *B. abortus* strain 2308 all animals gave positive results in the serological tests used. These results were expected since the O-side chain is the main antigen in smooth *Brucellae*. This is an important desirable feature of the RB51 vaccine that greatly favors the differentiation of RB51 vaccinates from naturally infected animals [20].

Evaluation of brucellosis vaccines in the natural hosts are normally performed by using standardized virulent challenge protocols in strictly controlled experiments, with animals kept isolated, where clinical, bacteriological and serological findings can be more adequately evaluated [45]. In the present study, the animals in each group remained together throughout the time span of the experiment, consequently receiving a greater challenge due to the close contact with aborted materials. This fact might partly explain the 25% of abortions and 35% of infections observed in the vaccinated group.

It is well established in the literature that protection in brucellosis is measured by a significant decrease in abortions or birth of weak calves, and a significant decrease in colonization of vaccinated cattle when compared to non-vaccinated controls after challenge [47]. Therefore, the isolation of the challenge strain from different specimens was used as criterion for brucellosis infection, as it is widely recognized to be the most definitive criterion for measuring the effect of brucellosis vaccination. This study demonstrated that vaccinated heifers had lower rates of abortion and lower rates of infection than the non-vaccinated controls. The higher frequency of *B. abortus* 2308 isolation from the placentomes, vaginal swabs and milk, and from fetal lungs, spleens and rectal swabs (Table 2) were expected as these are the most frequently infected tissues in *B. abortus* infected animals [9].

A large number of studies have been conducted to evaluate the resistance conferred by S19 in cattle, either in controlled or field conditions. The most important aspects of interest of these trials are doses, routes of vaccinations, effects of age, challenge dose, effects of revaccination, length of immunity, and degree of protection. The overall results indicate that approximately 65–75% of S19 calf vaccinated cattle will have complete protection against most kinds of exposure to brucellosis, and the remaining 25–35% will have variable degree of protection [46]. The protective effect of RB51 vaccine in cattle has been evaluated in several experiments and the results were similar to that obtained with S19 [9,36].

Protection against abortions indicates fewer direct losses for farmers. In addition, protection against infection indicates less risk of dissemination of the bacteria to other animals and men. In this study, the RB51 vaccine protected 75% of cows against abortion, while in the control group only 38% of the animals had normal parturition (Table 3). Protection against infection was 65 and 70% in cows and calves or fetuses, respectively (Table 4). These results are consistent with those reported by Cheville et al. [18], who found that 87% of heifers vaccinated with RB51 at different ages were protected against infection, while 60% of animals in the control group were infected. Animals in that study [18] were housed individually in biosafety level 3 facilities, differently from animals in this study which were raised together by group, and this may also have influenced the slight differences noted between this study and Cheville et al. data [18].

In vaccine trials, an ideal situation is obtained when over 99% of the challenged controls become infected [33]. Unfortunately, the setting of a challenge dose to achieve such an infection level in control group is frequently not feasible because it may result in over-challenging the experimental cattle, making it difficult to draw appropriate conclusions [46].

Generally, the effect of vaccination has been measured by indirect indexes of protection rates. In this study, the attributable fraction in the non-vaccinated group, and the relative risk of contracting the disease were used for evaluating protection. The attributable fractions for abortions, infection of cows, and infection of fetuses (Table 5) are consistent with other authors' findings and showed a significant impact of vaccination upon the three parameters. Elzer et al. [47] reported that two animals were infected and three aborted out of 10 animals in the vaccinated group, whereas eight animals were infected and seven aborted out of 10 control animals. Hence, this experiment yielded an attributable frac-

tion of 57% against abortion, whereas Olsen [48] found 31% against infection and 54% against abortion.

It is not well established whether pregnant heifers should be vaccinated. Some authors suggest that administration of full dosage during pregnancy should be avoided since it may cause fetal losses [49]. On the other hand, others claim that the full RB51 dosage practically does not cause abortions in pregnant heifers [50]. Moreover, the use of reduced dose (10^9 CFU) in pregnant cattle, in spite of being considered too low to induce effective immunity for some authors [33], has proved safe and a mean to greatly enhance resistance to *Brucella* infection. Furthermore, this reduced dose is not associated with abortions, placentitis, or birth of weak calves [20,29,51]. In the present study, no rough *Brucella* was isolated from the heifers vaccinated during early pregnancy, which indicates that it is safe, not causing abortions when used at 60 days of pregnancy.

The data presented show that the RB51 vaccine induced protective immunity against challenge with virulent *B. abortus*, did not interfere with conventional serological tests, and did not produce abortion or infection when used at full dose in heifers at early pregnancy. Therefore, RB51 vaccine could be a useful alternative to PNCEBT, with potential for strategic vaccination of cattle older than 8 months that were not vaccinated with S19, and for vaccination of cows in outbreaks in conjunction with elimination of infected cattle.

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