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ORIGINAL PAPER

Subcutaneous vaccination of pregnant guinea pigs with *Brucella melitensis* Rev.1: a model for the preliminary study on the safety of vaccine candidates against small ruminant brucellosis

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ABSTRACT

Brucella melitensis is the etiological agent of small ruminant brucellosis and abortion is the only noticeable clinical sign in most cases. Vaccination with the attenuated Rev.1 strain is the best option to prevent this clinical manifestation and subsequently control the transmission of the disease. However, colonization of the genital tract in pregnant small ruminants is a common adverse effect observed in this and other brucellosis vaccine strains. Guinea pigs have demonstrated to be an excellent model for testing the immune-protection and efficacy of Rev.1 vaccine, but studies addressing the effects of this vaccine on pregnancy have not been fully explored. The goal of this study was to characterize the effects of subcutaneous inoculation of the *B. melitensis* Rev.1 on pregnant guinea pigs to evaluate the possibility of establishing a suitable laboratory animal model to test and compare the safety on pregnancy of novel vaccine candidates against small ruminant brucellosis. Mid-term pregnant guinea pigs were inoculated subcutaneously with three different concentrations of the Rev.1 strain and euthanized at late-term gestation (>50 days). Blood samples were taken for sero-response before the pregnant guinea pigs were euthanized, and samples for bacteriology and histopathology were collected during necropsy. The Rev.1 strain was more consistently isolated from the spleen, chorioallantoic placentas and fetal organs of animals inoculated with ≥10^7 CFU of Rev.1 than from those inoculated with a lower dose. Histological alterations varied from mild to moderate presence of inflammatory cells in the spleen, mammary gland and pregnant uterus. In conclusion, placental colonization and vertical transmission were observed in pregnant guinea pigs after being inoculated subcutaneously at mid gestation with Rev.1, which is similar to what was reported in pregnant small ruminants vaccinated against brucellosis. Therefore, the pregnant guinea pig would be a useful model to initially asses the safety of vaccine candidates in pregnancy and compare them with the currently available commercial vaccine for brucellosis in small ruminants.

Keywords: *Brucella melitensis*, vaccine, guinea pigs, pregnancy, histopathology, serology.

RESUMEN

Brucella melitensis es el agente etiológico de la brucelosis de los pequeños rumiantes, y el aborto es el signo clínico destacable. La vacunación con la cepa atenuada Rev.1 es la mejor opción para prevenir esa manifestación clínica y controlar la transmisión de la enfermedad. Sin embargo, la colonización del tracto genital es un efecto adverso que se observa tanto en esta como en otras cepas vacunales de Brucella. Los cobayos han demostrado ser un excelente mo-

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delo para evaluar la protección inmune y la eficacia de la vacuna Rev.1, pero no se han llevado a cabo estudios en esta especie para medir la seguridad de la vacuna sobre la preñez. El objetivo de este estudio fue caracterizar los efectos de la inoculación subcutánea (SC) de B. melitensis Rev.1 en cobayas gestantes, con el fin de establecer un modelo animal de laboratorio para evaluar y comparar la seguridad de candidatos vacunales contra la brucelosis de los pequeños rumiantes. Los animales fueron inoculados SC con tres diferentes concentraciones de Rev.1 y sacrificados al final de la gestación (>50 días). Para la necropsia se tomaron muestras para serología, bacteriología e histopatología. La cepa Rev.1 fue aislada de forma más consistente del bazo, membrana corioalantiodea y órganos fetales de aquellos animales inoculados con ≥10^7 UFC de Rev.1 que de los inoculados con una dosis más baja. Las alteraciones histológicas variaron de leve a moderada la presencia de células inflamatorias en el bazo, glándula mamaria y útero gestante. En conclusión, la colonización placentaria y la transmisión vertical fueron observadas en las cobayas preñadas luego de una inoculación subcutánea a mitad de la gestación, similar a lo observado en pequeños rumiantes vacunados contra la brucelosis. Por lo tanto, el modelo de cobaya gestante podria ser útil para evaluar la seguridad de los candidatos vacunales contra la brucelosis durante la preñez antes de testearlas en los hospedadores naturales.

Palabras clave: *Brucella melitensis, vacuna, cobayos, preñez, histopatología, serología.*

INTRODUCTION

Brucella melitensis is the etiological agent of small ruminant brucellosis, although under particular epidemiological situations, goats and sheep may act as occasional hosts for other species of the genus *Brucella* (Rossetti *et al*., 2022). In addition, *B. melitensis*-infected flocks are the origin of cattle outbreak of brucellosis in mixed breeding systems (Muendo *et al*., 2012) and they are responsible for the highest number of human cases due to consumption of unpasteurized milk or milk products, or by direct contact with infected material (Blasco and Molina-Flores, 2011). In spite of being controlled in most first-world countries, the pathogen still produces an extensive negative impact on flocks in low- and middle-income nations, such as the Mediterranean region, the Middle East, Central Asia, Sub-Saharan Africa, and parts of Latin America, where goats and sheep are the major livestock species (Rossetti *et al*., 2017).

Abortion is the characteristic and perhaps the only noticeable clinical sign of brucellosis in goats and sheep (Rossetti *et al*., 2022), and vaccination is the best option to prevent and control this clinical manifestation (Blasco, 1997). The virulence-attenuated strain Rev.1, a *B. melitensis* mutant derived from a streptomycin-dependent variant of the virulent strain 6056 (Herzberg and Elberg, 1953), is the immunogen used worldwide against small ruminant brucellosis. However, due to the affinity that the *Brucella* species has for pregnant uteri, invasion of the genital tract, fetus colonization and abortion are some of the negative side effects retained by this vaccine strain (Alton, 1987; Jimenez de Bagues *et al*., 1989). To date, goats and sheep have been the preferred animal models to test the safety of the *B. melitensis* Rev.1 vaccine on pregnancy (Crowther *et al*., 1977; Jimenez de Bagues *et al*., 1989; Zundel *et al*., 1992; Higgins *et al*., 2017). Using primary hosts to test vaccine safety allows the direct evaluation of the effects under study, but these trials are costly, time-consuming and present significant challenges, including biosafety concerns and the requirement of valid biocontainment facilities, sometimes with undesirable performance. Because of these issues and ethical concerns associated with experimentation in large mammals, laboratory animal species serve as important tools for conducting brucellosis vaccine trials.

Mice (*Mus musculus*) are usually the experimental animal model of choice for evaluating the efficacy of the brucellosis vaccine (Darbandi *et al*., 2022; Grilló *et al*., 2012), due to their ease of handling, their cheapness (purchase, housing and food), their availability, and the existence of vast literature and specific laboratory protocol and reagents (Carvalho *et al*., 2016). However, they have not been extensively used to test the safety of brucellosis vaccines on pregnancy, including the Rev.1 strain (Elizalde-Bielsa *et al*., 2024). Guinea pigs are probably the most susceptible laboratory animal species to *Brucella* infection (Silva *et al*., 2011). They show a very high susceptibility to *B. melitensis* (Braude, 1951; Garcia-Carrillo, 1990), and like the natural hosts, virulent *B. melitensis* causes abortions when inoculated into pregnant females (Hensel *et al*., 2020). Guinea pigs have demonstrated to be an excellent model for testing the immunogenicity and efficacy of the Rev.1 anti-*B. melitensis* vaccine (McCamish and Elberg, 1962; García-Carrillo, 1986; Nicola *et al*., 2014), but the pregnant guinea pig model has not been explored to address the effects of this vaccine strain on pregnancy. The goal of this study was to asses the effects of subcutaneous (SC) inoculation of the vaccine strain *B. melitensis* Rev.1 on pregnant guinea pigs to evaluate the possibility of establishing a suitable laboratory animal model, in order to test and compare the safety on pregnancy of vaccine candidates against small ruminant brucellosis before testing them on natural hosts.

MATERIALS AND METHODS

Bacterial strains, media and culture conditions

The B. melitensis Rev.1 strain was obtained from the commercial vaccine (OCUREV®, CZ Vaccines, Pontevedra, Spain), kindly provided by SENASA (Official Veterinary Service of Argentina). The lyophilized strain was reconstituted according to the manufacturer's instructions and, immediately, 0.1 ml of the suspension was diluted 1:10 in sterile distilled water and cultured in a *Brucella* agar base (BAB, Laboratorio Britania, Buenos Aires, Argentina) plate. After four days of incubation at 37°C, the bacteria were harvested, washed twice in sterile distilled water, and re-suspended in a frozen solution (sterile trypticase soy broth –TSB– containing 15% glycerol). Subsequently, the stock culture was dispensed in 1.5 ml microtubes and stored at -80ºC until use.

When needed, one vial of the Rev.1 strain stock culture was thawed in water bath at 37ºC and cultured in TSB (Laboratorio Britania) at 37ºC under shaking condition (180 rpm) with loose lids. After 48 h, the culture was harvested and washed in sterile distilled water and re-suspended in sterile phosphate saline solution (PBS, pH 7.2). The actual number of viable bacterial/ml was retrospectively obtained by serial dilution in PBS and plated onto BAB for quantitation (Castaño-Zubieta *et al*., 2021). The inoculum was adjusted by dilutions in PBS as appropriate.

Guinea pigs

American short-haired female guinea pigs (*Cavia porcellus*) bred at the INTA animal facilities, weighing between 550 to 650 g, were used. Animal welfare was determined by daily clinical observation of the guinea pigs, their appetite, environmental interaction, body temperature, respiratory rate and stool consistency. The animals received water *ad libitum* and were fed with concentrate and fresh vegetables (spinach, chard, carrots, kale). Barnyards were dry-cleaned three times per week. A week after their arrival, the females mated naturally by introducing one male to every three females in a cage for 17 days (one guinea pig estrus cycle).

At late gestation (>50 days), all the experimental female guinea pigs were euthanized by intramuscular application of 0.5 ml of xylazine (2%) (Richmond, Bs. As., Argentina), followed by intracardiac overdose of sodium pentobarbital (Euthanyle; Brouwer, Bs. As., Argentina). Necropsies were performed to collect the uteri for further assessment of bacterial colonization and microscopic observation of the tissues. Blood for serum was recovered from all the experimental animals before euthanasia. All the animal procedures were approved by the Institutional Animal Care and Use Committee (CICUAE) of CICVyA-INTA under approval number 01/2020.

In vivo inoculation

Experimental design

To determine the behavior of Rev.1 strain in pregnant guinea pigs, the animals from groups A, B and C (three pregnant females in each) were inoculated SC between the third and the fifth week of pregnancy (Hensel *et al*., 2020) with 0.5 ml of inoculum containing 10^8, 10^7 and 10^6 CFU of *B. melitensis* Rev.1, respectively (table 1). The control animals (group D, n = 3) were simultaneously inoculated SC with 0.5 ml of PBS. The experimental animals were euthanized one week (+/- 5 days) before the estimated day of delivery to avoid bacterial dissemination to the environment. Samples for bacteriological detection and histological observation were collected at necropsy.

Serology

Five ml of blood for serum were collected from all the experimental animals by intra-cardiac puncture under sedation. *Brucella*-specific antibodies were determined by buffer plate antigen (BPA) and complement fixation (CF) tests, following international recommendations (Alton *et al*., 1988; OIE, 2018). Briefly, for BPA test (Rosenbusch, Bs. As., Argentina), positive or negative results were determined by the presence or absence of visible agglutination, respectively. A scale was developed to categorize the degree of agglutination as 1) +++ strong, 2) ++ mild, 3) + weak and 4) – no agglutination. CFT was performed in microtiter plates (U bottom) as previously described (Foster *et al*., 2022). A hemolytic reaction of 50% or less at a dilution of 1:4 was considered as the minimum seropositive threshold (i.e., ≥ 20IU/ml).

Bacteriology

At necropsy, samples were taken from the spleen, mammary gland fluid, chorioallantoic placenta, amniotic fluid, and from

Groups	Inoculum (CFU of Rev.1)	Animal #	# Fetus (length)	Average (cm)	Outcome
A	$1x10^8$	1	3 (10 cm)	9.58	Euthanized
		2	4 (10 cm)		Aborted
		3	5 (9 cm)		Euthanized
B	$1x10^7$	1	4 (9 cm)	12.11	Euthanized
		2	3 (14 cm)		Euthanized
		3	2 (14 cm)		Euthanized
C	$1x10^6$	1	1 (13 cm)	11	Euthanized
		2	2 (10 cm)		Euthanized
		3	2 (11 cm)		Euthanized
D	PBS	1	3 (10 cm)	9.79	Euthanized
		2	2 (11 cm)		Euthanized
		3	4 (9 cm)		Euthanized

Table 1. Experimental design and pregnancy outcome in each experimental group.

the spleen, liver, lung and stomach content of the fetuses. For processing, one gram of the mother's spleen, the chorioallantoic placenta, and the liver of the fetus were used; the fetal spleen and the diaphragmatic lobe of the right fetal lung were placed in sterile bags and mechanically homogenized until complete tissue disaggregation. Then, 1 ml of sterile distilled water was added, mixed and placed in a 1.5 ml microtube. One hundred µl of the suspension were cultured on a Farrell's media plate for CFU counting. Simultaneously, a culture swab imbibed with mammary gland content, amniotic liquid and fetal stomach fluid were cultured in Farrell's media by dissemination. All plates were observed daily until colonies appeared, and were maintained at 37ºC for up to 10 days if there was no colony growth. The mothers' spleens were weighed immediately after recovery and before sampling. The following scale was used for evaluating the number of CFU / g of tissue or culture swab: - (negative, no growth), + (1-30 CFU), ++ (31-300 CFU) and +++ (>300 CFU).

Morphological analysis

To assess the histologic changes associated with the inoculation of Rev.1 in pregnant guinea pigs, samples from the mothers' spleens, uteri, mammary glands, chorioallantoic placentas, and from the fetuses' left lungs, spleens and livers were taken at necropsy. The tissues were fixed in 10% neutral buffered formalin and were then routinely processed and embedded in paraffin, sectioned at 5 m, stained with hematoxylin and eosin (H&E), and examined with light microscopy.

Statistical analysis

The CFU (logarithmic transformed) and spleen weight data were subjected to two-way and one-way analysis of variance (ANOVA), respectively. The statistical analyses were performed using the GraphPad Prism 6.0 software (San Diego, CA, USA) and P values < 0.05 were considered significant.

RESULTS

Clinical findings and pregnancy outcome

No adverse side effects were observed after the inoculation of Rev.1 during the course of the study, such as changes in behavior, loss of body weight, or local inflammation in response to the injection in any of the concentrations inoculated. With the exception of a guinea pig inoculated with 10^8 CFU of *B. melitensis* Rev.1 that aborted 21 days p.i., all the other pregnant guinea pigs were euthanized one week before the estimated day of delivery to avoid bacterial dissemination to the environment. The number of gestations was from 1 to 5, with a crown to rump length of 9 to 14 cm (table 1). The length and general features of the intra utero fetuses (same gestation) were uniform, which indicates that no *in utero* fetal death had occurred up to the time of euthanasia.

Serological responses in pregnant guinea pigs

The agglutination test (BPA) results showed that all the treated guinea pigs presented a strong agglutination reaction, except for one animal inoculated with 10^6 CFU of Rev.1 that presented a mild agglutination reaction (table 2). When the serum samples were analyzed by CFT, the guinea pigs inoculated with 10^8 (group A) and 10^7 CFU (group B) of Rev.1 showed a level of 1,000 international units per ml of complement fixation (CFIU/ml). In group C (10^6 CFU), 2 animals presented 31 CFIU and the other animal was negative (<20 CFUI/ml). The control animals were all negative to both serological tests (table 2). Taken together, these results would indicate that the lower the dose of the inoculum, the longer the time required for a strong humoral immune response.

Group (inoculum concentration)	Animal #	Serology	
		BPA	CFT (IU)
A (10^8 CFU of Rev.1)	1	+++	>1702
	2	+++	>1702
	3	+++	>1702
B (10^7 CFU of Rev.1)	1	+++	>1702
	2	+++	>1702
	3	+++	>1702
C (10^6 CFU of Rev.1)	1	+++	40
	2	++	40
	3	+++	Neg
D (PBS, control)	1	-	Neg
	2	-	Neg
	3	-	Neg

Table 2. Anti-*Brucella* immune response in serum samples of pregnant guinea pigs immunized with *B. melitensis* Rev.1, or inoculated with PBS (controls), determined by buffered plate antigen (BPA) and complement fixation test (CFT), 4 weeks post inoculation. For BPA: +++ = strong, ++ = mild, + = weak, and – no agglutination. CFT positive ≥ 20 international units per ml (IU/ml).

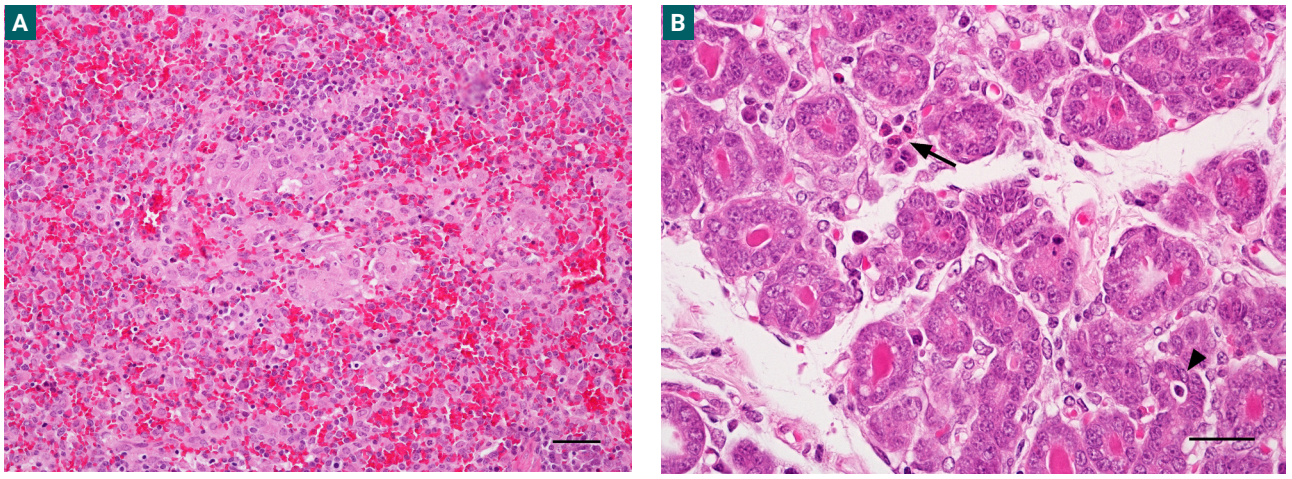

Figure 1. Outstanding histological images observed in spleens and mammary glands of pregnant guinea pigs SC inoculated with *B. melitensis* Rev.1, four weeks p.i. (A) Infiltration of epithelioid macrophages in the red pulp of spleen (H&E, 20x, bar = 100µm); (B) Moderate number of neutrophils in the interstitium (arrow) and in the lumen of the acinus (arrowhead) of the mammary gland (H&E, 40x, bar = 50µm).

Bacteriological and histopathological analysis of tissues from pregnant guinea pigs

The *B. melitensis* Rev.1 was isolated from the spleen of all the treated guinea pigs, in direct relationship between the concentration of the inoculum and the number of CFU isolated, i.e., the higher the inoculum concentration, the higher the number of CFU of Rev.1 isolated from spleens (group A > group B > group C) (table 3). As expected, no *Brucella* was isolated from the spleens of the control animals.

Weight gain and spleen enlargement were observed randomly in inoculated animals, and there was no significant difference among the average spleen weight in the inoculated groups, regardless of the inoculum concentration used (10^8 = 1.92 g, 10^7 = 2.09 g, and 10^6 =2.08 g; P > 0.05; table 3). In contrast, the average spleen weight of the control group was 1.43 g, but the moderate differences with the average spleen weight of the inoculated groups were not significant (P > 0.05). Histological images of spleens from pregnant guinea pigs inoculated with Rev.1 four weeks p.i. were characterized by a mild to moderate inflammatory infiltrate in the red pulp of histiocytes and epithelioid macrophages with fewer lymphoplasmacytic cells and congestion (figure 1 A). Uninfected controls had no histologic lesions in spleens. The degrees of histological alterations in the spleen did not correlate with the inoculum concentration, spleen bacterial load (CFU count) or spleen weight (table 3). In addition to enlargement, the only grossly visible alteration in spleens was a superficial abscess in a single animal inoculated with 10^6 CFU of Rev.1.

The Rev.1 strain was also isolated in three (one from group A –A2- and two from group C –C1 and C3-) out of 7 samples processed from the treated animals (two samples from the treated animals were not processed), in concentration of <30 CFU / culture swab (table 4). The samples from PBS-inoculated guinea pigs were negative. The low level of colonization of the mammary gland correlates with a mild interstitial infiltration of histiocytic and plasmatic cells, regardless of the concentration of the inoculum (figure 1 B). Neutrophils were observed in the lumen of the acinus in few samples (figure 1 B). The mammary gland samples from the control group presented no lesions.

These results showed that Rev.1 has the ability to colonize and develop histopathological changes in the spleen and mammary gland of pregnant guinea pigs.

Placenta invasion and vertical transmission

Rev.1 colonized chorioallantoic placentas after its inoculation in pregnant guinea pigs, although at different levels according to the inoculum concentration. Thus, all the placentas from pregnant guinea pigs inoculated with 10^7 and 10^8 CFU of Rev.1 were colonized. Contrary, only two out of three placentas of the guinea pigs inoculated with 10^6 were colonized, although at lower concentrations (table 4). Concordantly, the *B. melitensis* Rev.1 was isolated from most of the amniotic fluid of those allantoic sacs in which *Brucella* was isolated from the placenta. No Rev.1 isolation was reported from placentas o amniotic fluid in PBS inoculated group (table 4).

The histopathological analysis of the pregnant uteri indicated that, with one exception, all of the guinea pigs inoculated with the *B. melitensis* Rev.1, regardless of the inoculum concentration received, presented mild endometritis and myometritis with the presence of inflammatory cells, mainly histiocytes and lymphocytes. The exception was a pregnant guinea pig inoculated with 10^7 CFU of Rev.1 (B3) which had severe myometritis, characterized by intense neutrophils, lymphocytes and macrophages infiltration (figure 2 A). Regarding the placenta, no lesions were observed in any of the Rev.1 or PBS inoculated guinea pig, with the exception of the placenta of the guinea pig with severe endometritis and myometritis (B3), which presented focuses of coagulative necrosis and arterial thrombi (figure 2 B).

Vertical transmission is the ability of a pathogen to colonize the fetus from an infected mother via transplacental route. This study showed that the Rev.1 strain is able to cross the placenta and infect the fetuses, being the number of infected gestations directly related to the inoculum concentration (table 4). The *B. melitensis* Rev.1 was randomly isolated from the lung, liver, spleen and abomasum content of the fetuses from inoculated groups, but not from the tissue samples processed from con-

Group (inoculum concentration)	Animal #	Spleen weight (g)	Average (SD) spleen weight (g)	Brucella isolation (CFU/g)	Main histological findings
A (10^8)	1	1.80		+++	Moderate epithelioid cells infiltration and congestion
	2	1.82	1.92 (±0.2)	++	Mild histiocytic infiltration
	3	2.15		++	Not evaluated
B (10^7)	1	1.28		++	Lymphoid hyperplasia and mild histiocytic infiltration in red pulp
	2	1.27	2.09 (±1.41)	+	Lymphoid hyperplasia and moderate lymphocytic infiltration
	3	3.72		++	Moderate epithelioid cells infiltration and congestion
C (10^6)	1	2.06		++	Mild epithelioid cells infiltration
	2	2.65	2.08 (±0.56)	+	Moderate epithelioid and lymphocytic cells infiltration
	3	1.54		+	Lymphoid hyperplasia
D (PBS, control)	1	1.39		-	No microscopic changes
	2	1.35	1.43 (±0.11)	-	
	3	1.56		-	

Table 3. Absolute and average (±SD) weight, number of CFU isolated and microscopic lesions of spleens of pregnant guinea pigs inoculated SC with 3 different doses of *B. melitensis* Rev.1. To assess the number of CFU isolated / g of spleen, the following scale was used: - (negative, no growth), + (1-30 CFU), ++ (31-300 CFU) and +++ (>300 CFU). No significant differences were observed in spleen weight between groups (P > 0.05).

Group	Animal #	Mammary gland	Amniotic fluid	Chorioallantoic placenta	Fetuses			
					Spleen	Liver	Lung	St Fluid
A (10^8)	1	-	+	+	+	-	-	-
	2	+	++	+++	+	+	++	+++
	3	NP	+++	+++	+	+	++	+
B (10^7)	1	-	-	+	-	-	-	-
	2	NP	+	+++	+	++	+	-
	3	-	++	+++	+	-	-	-
C (10^6)	1	+	-	-	-	-	-	-
	2	-	++	++	++	+	++	+
	3	+	-	+	-	-	-	-
D (Control)	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-

Table 4. Number of CFU isolated from mammary gland, placenta, amniotic fluid or fetus tissues samples taken from pregnant guinea pigs inoculated SC with 3 different doses of *B. melitensis* Rev.1. To assess the number of CFU isolated / g of tissue (mammary gland, placenta, spleen, liver, lung) or culture swab (amniotic and stomach fluid), the following scale was used: - (negative, no growth), + (1-30 UFC), ++ (31-300 UFC) and +++ (>300 UFC). The positive results indicate that at least one of the samples analyzed was positive for isolation. NP = not processed.

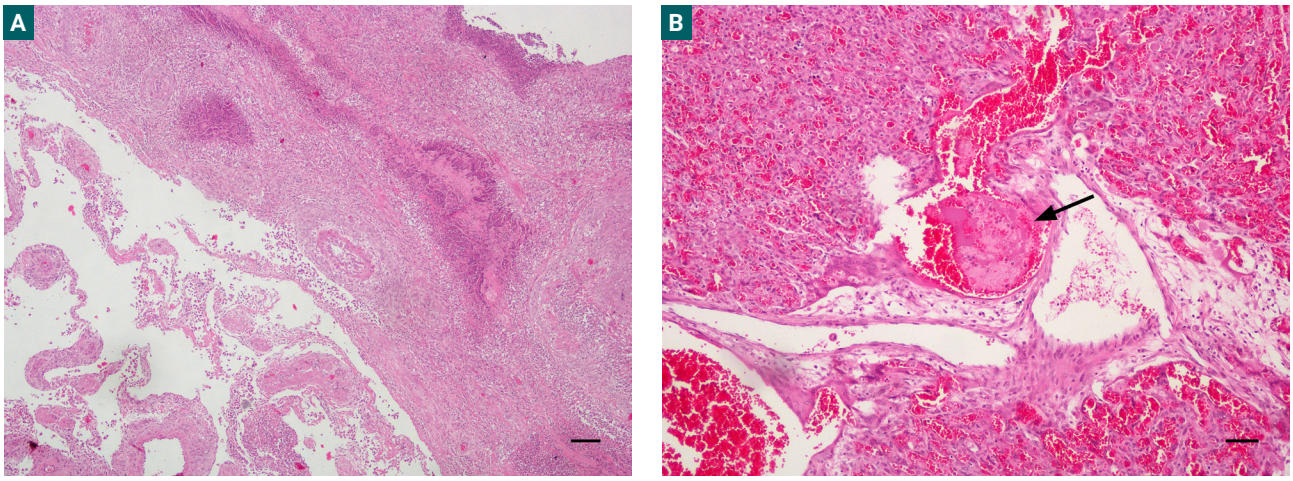

Figure 2. Highlighted microscopic images in uterus and placentas of pregnant guinea pigs SC inoculated with *B. melitensis* Rev.1, four weeks p.i. (A) Severe endometritis and myometritis characterized by intense neutrophils, lymphocytes and macrophages infiltration, and necrotic focus in the myometrium (H&E, 4x, bar = 250µm); (B) Thrombus in placental artery (arrow) (H&E, 10x, bar = 100µm).

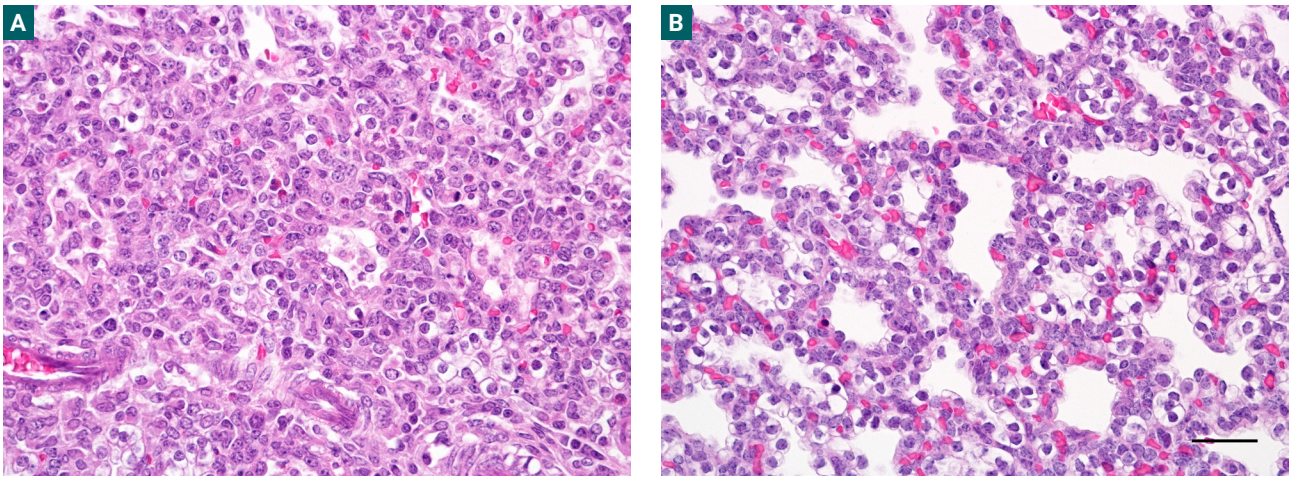

Figure 3. Histological images of post-mortem processed lung fetus. (A) Mononuclear cells infiltration into the lung interstitium of a fetus whose mother had been inoculated with *B. melitensis* Rev.1 during pregnancy; (B) Lung of fetal guinea pig from control group (H&E, 40x, bar = 50µm).

trol animals. No gross or histologic lesions were detected in any of the fetal tissues examined (lungs, livers and spleens) in any group, except for a mononuclear cells infiltration in the lung interstitium in a fetus whose mother was inoculated with 10^6 CFU of B. *melitensis* Rev.1 (figure 3).

DISCUSSION

In this study, we demonstrated that Rev.1 inoculated SC in guinea pigs at midterm gestation induces a humoral immune response, while invading and colonizing the placenta, mammary gland and fetuses via vertical transmission, which is similar to that observed in pregnant natural hosts inoculated with this vaccine (Jimenez de Bagues *et al*., 1989; Higgins *et al*., 2017). The susceptibility of pregnant guinea pigs to *Brucella* strains was initially demonstrated for *B. abortus* infection after parenteral inoculation (Bosseray and Diaz, 1974; Samartino *et al*., 1994) and, more recently, to *B. melitensis* via intratracheal challenge (Hensel *et al*., 2020). Likewise, the abortifacient capacity of *B. abortus* vaccine strains such as S19 and RB51 (Thomas *et al*., 1981; Yazdi *et al*., 2009) was also observed in 33% of the pregnant guinea pigs after being inoculated SC with $1x10^{11}$ CFU of each strain (Samartino *et al*., 1996a). This would be the first time that the safety of Rev.1 has been evaluated in pregnant guinea pigs. Abortion, the main adverse effect after Rev.1 immunization, was not observed in this trial, with the exception of one animal, most probably because the assay was finalized early to avoid environmental dissemination of *Brucella*.

In control and eradication programs of brucellosis in small ruminants, the Rev.1 is inoculated by conjunctival instillation to

a concentration of 1-2x10^9 CFU. Ocular instillation not only confers similar immune protection than that induced by subcutaneous vaccination, but also reduces some of the disadvantages that occur when the vaccine is administered subcutaneously (Blasco and Molina-Flores, 2011). In any case, in this trial, the inoculum was administered SC because it is the preferable route for practical purposes and favors the comparison with other vaccines against brucellosis since it is the route initially chosen when novel vaccine developments are tested (Carvalho *et al*., 2016). Regarding the concentration of the inoculum, three different doses were tested. The doses chosen to evaluate the clinical and pathological consequences in guinea pigs pregnancy were 10^8, 10^7 and 10^6 CFU of *B. melitensis* Rev.1, which are 1/10, 1/100 and 1/1,000 of that used in vaccination campaigns against brucellosis in small ruminants, respectively. It is broadly known that the official dose of the vaccine generates an abortion outbreak when goats and ewes are immunized between the second and fourth month of gestation *(Jimenez de Bagues et al*., 1989; Blasco, 1997). Thus the 10^7 CFU dose maintains the CFU vaccine / live weight ratio between the target population of the vaccine and the laboratory animal species tested (10^9 CFU / 60 to 80 kg -small ruminants- vs. 10^7 CFU / 0.6-0.8 kg —guinea pigs—). In addition, 10^6 and 10^7 CFU of Rev.1 have showed to be protective against challenge with virulent *Brucella* in guinea pigs without adverse effects (García-Carrillo, 1986; Nicola *et al*., 2014), and 10^6 has been indicated as a recommended dose for Rev.1 vaccine potency test assays in guinea pigs (Alton, 1990). A dose higher than those used in this study (10^9 CFU) had been similar to that used to vaccinate small ruminants and, consequently, it would have colonized the genital tract and generated an abortion outbreak in the inoculated guinea pigs. Conversely, a lower initial concentration of Rev.1 (≤ 10^5 CFU) may not have enough time to reach a critical number to infect and colonize the genital tract (Alton, 1969).

Contrary to what have been reported by McCamish and Elberg (1962), no clinical evidence of adverse vaccine reaction was observed in guinea pigs following subcutaneous immunization with Rev.1, including local inflammation at the site of inoculation, loss of appetite or lethargy. The length and general characteristics of fetuses from the same gestation were not affected either. The differences in the length of the fetuses between different pregnancies could be due to the different gestational ages at the time of euthanasia, since the estrus was not synchronized and, therefore, some females became pregnant earlier than others, or by the number of fetus/pregnancy since, the greater the number of fetus, the smaller the size of each fetus.

The humoral immune response elicited by Rev.1 in pregnant guinea pigs 4 weeks p.i. was found to be consistent with that observed in the same animal species as well as in natural hosts by other reports. In those publications, the highest level of humoral immune response was reached between 14 and 45 days p.i. (McCamish and Elberg, 1962; Alton, 1969; Fensterbank *et al*., 1985; Castaño-Zubieta *et al*., 2021) even with lower doses of Rev.1. In the study reported here, the animals immunized with the lowest dose (group C; 10^6) had the lowest humoral immune response, with a medium to high agglutinating response, and low or no complement activation activity. The low level of anti-*Brucella* specific antibodies in animals inoculated with a lower inoculum concentration was most probably due to the delay in the initial immune response that did not occur until the bacteria had reached a critical number at the site of infection necessary to spread.

After bacteremia, *Brucella* remains in cells of the reticuloendothelial system (RES) and induces splenomegaly in human and laboratory animal models due to an inflammatory response (Grilló *et al*., 2012). Based on that, many authors suggest that spleen enlargement and weight increase is a good indicator for brucellosis infection and it may replace bacteriology in experimental animal models (Braude and Spink, 1951; Thornton and Muskett, 1972; Garcia-Carrillo and Trenchi, 1974; Nicola *et al*., 2014). In this study, weight gain and spleen enlargement were randomly observed in the inoculated animals (table 3), and there was no significant difference between the mean spleen weights in the inoculated and control groups. Previous publications reported that the basal size and weight of the guinea pigs' spleens show great variability (Garcia-Carrillo, 1977) and vary in animals inoculated with Rev.1 (McCamish and Elberg, 1962; Alton *et al*., 1988) or virulent strains (Braude, 1951). Accordingly, but difficult to explain, in this study, a large dispersion was observed in the weight of the spleens of two animals inoculated with 10^7 CFU of Rev.1 (group B) in comparison with the weight of the spleens of the other inoculated animals (table 3). Interestingly, these two spleens were lighter than the spleens of the control animals (group D), even when the Rev.1 was re-isolated and the histological lesions were observed. Histological images of all the spleens of the guinea pigs inoculated with Rev.1 were characterized by a mild to moderate inflammatory cells infiltration of the red pulp, which was comparable to images informed by others in similar circumstances (McCamish and Elberg, 1962), but less severe than the histologic appearance of the spleens infected with virulent *B. melitensis* strains (Braude, 1951; Hensel *et al*., 2019).

It is well documented that the attraction of Brucella to placentas increases during the late stage of gestation (Samartino and Enright, 1996b), which is consistent with the abortions in the last trimester of pregnancy. However, placental colonization and vertical transmission are not always linked to abortion *(*Tobias *et al., 1993;* Hensel *et al*., 2020); that is more related to the moment of pregnancy, the inoculum concentration and the passage of time after inoculation. In goats and sheep, Rev.1 induces abortion 40 to 60 days after vaccination and when it is inoculated between the second and fourth month of gestation (Blasco, 1997). The trophoblasts are probably not mature enough to allow intracellular growth of Rev.1 during the first month of pregnancy; and, when inoculated in the last month, the strain does not have sufficient time to colonize the placenta and induce abortion. In this study, pregnant guinea pigs were inoculated at mid-term gestation (20-35 days of pregnancy), a period in which the placenta of this animal species had previously been shown to be permissive of colonization and, therefore, susceptible to abortion by brucellae (Samartino *et al*., 1996a; Hensel *et al*., 2020). Even when no abortions were observed, except in one experimental animal inoculated with 10^8 CFU of Rev.1 (group A), the strain colonized the chorioallantoic placenta and was able to cross it. The lack of abortions, as well as the absence of lesions in the placenta and fetal organs, that are observed in goats and sheep immunized with Rev.1 (Jimenez de Bagues *et al*., 1989; Mazlan *et al*., 2021), can be mainly attributed to the experimental animals being euthanized 5 to 13 days before the estimated date of delivery. These differences in the gestational age may have affected the degree of susceptibility to *Brucella* infection and, consequently, the number of colonized fetal tissues or the isolated burden (table 4), as was also noted in other publications (Bosseray and Diaz, 1974). In addition, the lower level and number of colonized placentas and fetuses in animals inoculated with 10^6 CFU of

Rev.1 (group C) could be because, the lower the concentration of the inoculum, the longer it takes to establish an infection with clinical consequences (Alton, 1969).

Brucella spp. also has an affinity for udders It is eliminated through milk or colostrum, with consequences such as environmental contamination and potential risk of infection of dairy workers, consumers of unpasteurized dairy products and other susceptible animals residing in the same area, as well as vertical transmission to litters *(Meador et al*., 1989). Therefore, the potential shedding of the vaccine strain in milk and colostrum is an important parameter that needs to be evaluated with caution during *Brucella* vaccine developments. In this study, we demonstrated that Rev.1 can be present in colostrum and potentially be excreted. These data are in concordance with previous reports in which they isolated Rev.1 in milk or colostrum of small ruminants after being vaccinated during pregnancy (Jimenez de Bagues *et al*., 1989; Higgins *et al*., 2017). The low number of mammary glands in which Rev.1 was detected (3 out of 7 samples processed; 43%) may be due to the few samples tested (7 samples), the small sample size (one culture swab / sample), because *Brucella* spreads intermittently from each mammary gland (Morgan and McDiarmid, 1960), or all of the above. In any case, this study showed that *B. melitensis* Rev.1 is able to colonize the mammary glands of the guinea pig.

In conclusion, the mid-term pregnant guinea pigs inoculated subcutaneously with *B. melitensis* Rev.1 reproduced the undesirable side effects reported in pregnant small ruminants immunized with this vaccine strain, such as placental colonization, vertical transmission and affinity for udders. This model may be useful as a hypothesis-driven model to assess the safety of the modifications in Rev.1 formulation or dose concentration in pregnancy before testing them in natural hosts. Furthermore, it could eventually be extended to initially evaluate and compare the safety and vertical transmission of new brucellosis vaccine candidates in pregnant animals.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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