PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 40(2):88-96, February 2020
DOI: 10.1590/1678-5150-PVB-6481

Original Article
Livestock Diseases

Pathogenic potential of *Brucella ovis* field isolates with different genotypic profile and protection provided by the vaccine strain *B. ovis* Δ*abcBA* against *B. ovis* field isolates in mice[1]

Thaynara P. Carvalho[2], Noelly Q. Ribeiro[2], Juliana P.S. Mol[2], Fabíola B. Costa[2], Camila Eckstein[2], Nayara F. Paula[2], Tatiane A. Paixão[3] and Renato L. Santos[2]*

ABSTRACT.- Carvalho T.P., Ribeiro N.Q., Mol J.P.S., Costa F.B., Eckstein C., Paula N.F., Paixão T.A. & Santos R.L. 2020. **Pathogenic potential of *Brucella ovis* field isolates with different genotypic profile and protection provided by the vaccine strain *B. ovis* Δ*abcBA* against *B. ovis* field isolates in mice.** *Pesquisa Veterinária Brasileira 40(2):88-96.* Departamento de Clínica e Cirurgia Veterinária, Escola de Veterinária, Universidade Federal de Minas Gerais, Campus Pampulha, Av. Pres. Antônio Carlos 6627, Belo Horizonte, MG 31270-901, Brazil. E-mail: rls@ufmg.br

Brucella ovis causes economic and reproductive losses in sheep herds. The goal of this study was to characterize infection with *B. ovis* field isolates in a murine model, and to evaluate protection induced by the candidate vaccine strain *B. ovis* Δ*abcBA* in mice challenged with these field isolates. *B. ovis* field strains were able to colonize and cause lesions in the liver and spleen of infected mice. After an initial screening, two strains were selected for further characterization (*B. ovis* 94 AV and *B. ovis* 266 L). Both strains had *in vitro* growth kinetics that was similar to that of the reference strain *B. ovis* ATCC 25840. Vaccination with *B. ovis* Δ*abcBA* encapsulated with 1% alginate was protective against the challenge with field strains, with the following protection indexes: 0.751, 1.736, and 2.746, for mice challenged with *B. ovis* ATCC25840, *B. ovis* 94 AV, and *B. ovis* 266 L, respectively. In conclusion, these results demonstrated that *B. ovis* field strains were capable of infecting and inducing lesions in experimentally infected mice. The attenuated vaccine strain *B. ovis* Δ*abcBA* induced protection in mice challenged with different *B. ovis* field isolates, resulting in higher protection indexes against more pathogenic strains.

INDEX TERMS: Pathogeny, *Brucella ovis*, isolates, genotypic profile, protection, vaccine strain Δ*abcBA*, mice, immunization, field isolated strains, brucellosis.

RESUMO- [Vacina viva atenuada *Brucella ovis* Δ*abcBA* encapsulada protege camundongos frente a desafios de *B. ovis* isoladas de campo.] *Brucella ovis* é responsável por perdas econômicas e reprodutivas em rebanhos ovinos. O objetivo deste trabalho foi caracterizar a infecção com as cepas isoladas de campo de *B. ovis* em modelo murino e avaliar a eficiência vacinal da mutante *B. ovis* Δ*abcAB* para proteção contra desafio com as cepas isoladas de campo. Foram utilizadas sete cepas isoladas de campo foram capazes de colonizar e provocar lesões no fígado e no baço de camundongos após sete dias pós-infecção. Após triagem, duas cepas foram selecionadas para a melhor caracterização (*B. ovis* 94 AV and *B. ovis* 266L). Ambas apresentaram crescimento em placa de cultivo semelhante ao da cepa de referência *B. ovis* ATCC 25840. A vacinação com a cepa de *Brucella ovis* Δ*abcBA* encapsulada com alginato a 1% foi capaz de proteger camundongos desafiados com as cepas isoladas de campo, com os seguintes índices de proteção: 0,751, 1,736 e 2,746, para camundongos desafiados com *B. ovis* ATCC 25840, *B. ovis* 94 AV e *B. ovis* 266 L, respectivamente. Estes resultados demonstraram que as cepas isoladas de campo de *B. ovis* são capazes de infectar e induzir lesão em camundongos experimentalmente infectados. O uso da cepa

[1] Received on August 13, 2019.
Accepted for publication on September 1, 2019.
[2] Departamento de Clínica e Cirurgia Veterinária, Escola de Veterinária, Universidade Federal de Minas Gerais (UFMG), Campus Pampulha, Av. Pres. Antônio Carlos 6627, Belo Horizonte, MG 31270-901, Brazil. *Corresponding author: rls@ufmg.br

[3] Departamento de Patologia Geral, Instituto de Ciências Biológicas, UFMG, Campus Pampulha, Av. Pres. Antônio Carlos 6627, Belo Horizonte, MG 31270-901.

mutante atenuada *B. ovis* Δ*abcBA* para vacinação de fêmeas C57BL/6 desafiados com diferentes cepas de *B. ovis* induziu proteção nos camundongos desafiados com diferentes cepas de *B. ovis*. Deste modo, mostrando-se eficiente na proteção das cepas de campo de *B. ovis*.

TERMOS DE INDEXAÇÃO: Vacina viva, *Brucella ovis*, Δ*abcBA*, camundongos, imunização, brucelose ovina, mutante encapsulada, cepas isoladas de campo.

INTRODUCTION

Brucellosis is a group of infectious diseases caused by facultative, intracellular, Gram-negative coccobacillary bacteria of the genus *Brucella* that affects domestic and wild animals and causes zoonotic infections in man (Olsen et al. 2011). *B. ovis* is considered a non-zoonotic species, and it is responsible for economic and reproductive losses in sheep herds (Poester et al. 2013).

Brucellosis in rams is clinically characterized by unilateral or bilateral granulomatous epididymitis and seminal vesiculitis. These changes result in poor sperm quality with increased defects of the tail of the spermatozoa, presence of inflammatory cells in the ejaculate, and consequent subfertility or infertility (Carvalho Júnior et al. 2012, OIE 2015). In ewes, the disease is usually asymptomatic, but endometritis and, more rarely, abortions, stillbirths, and weak offsprings may be observed (Grilló et al. 1999).

Currently, the vaccine available in some countries for *B. ovis* prevention is the Rev-1 strain, a live attenuated *Brucella melitensis* vaccine (Ridler & West 2011). However, the Rev-1 strain can interfere with serological tests, it can induce abortions when administered to pregnant animals, and it is capable of infecting and causing disease in humans (Blasco & Díaz 1993, Blasco 1997).

Brucella spp., as well as other intracellular bacteria has several strategies to achieve a safe replication niche within the host cell (Gorvel & Moreno 2002). Intracellular survival of *Brucella* spp. requires a functional *virB*-encoded type IV secretion system (T4SS). *Brucella* strains lacking a functional T4SS cannot evade degradation in lysosomes so they do not replicate or survive within the host cell (Celli et al. 2003). A previous study demonstrated that a *B. ovis* specific ABC transporter is required for *B. ovis* survival *in vivo* and evasion from phagosome/lysosome fusion (Silva et al. 2011b, Macedo et al. 2015). Additionally, *B. ovis*-specific ABC transporter is required for normal expression of the *virB*-encoded T4SS since in the absence of this ABC transporter there is a post-transcriptional impairement of expression of *virB*-encoded proteins (Silva et al. 2014). Indeed, *B. ovis* mutant strains lacking a functional *B. ovis*-specific ABC transporter (Silva et al. 2011b) or the *virB*-encoded T4SS (Sá et al. 2012) have similar phenotypes.

ABC transporters have various substrates including polyamines (Igarashi et al. 2001), peptides (Detmers et al. 2001), and amino acids (Hosie & Poole 2001, Danese et al. 2004). *Brucella* spp. genome encodes several ABC transporters, whereas *B. ovis* has 29 pseudogene-forming mutations in coding sequences for ABC-like carrier systems, so *B. ovis* cannot transport some substances such as polyamines, erythritol, and glycine (Jenner et al. 2009). A *B. ovis*-specific genomic island (Tsolis et al. 2009), named BOPI-1 for *B. ovis* pathogenicity island 1 (Silva et al. 2011b), encodes an ABC transporter that is essential for pathogenesis since the *B. ovis* Δ*abcBA* strain is strongly attenuated *in vitro* and *in vivo* so this genomic island has been named BOPI-1 for *B. ovis* pathogenicity island 1 (Silva et al. 2011b). However, the substrates of this particular ABC transporter are still unkown (Silva et al. 2014). In spite of its attenuation, *B. ovis* Δ*abcBA* triggers humoral and cellular immune responses in rams that are indistinguishable from those triggered by the wild-type parental strain (Silva et al. 2013). Therefore, *B. ovis* Δ*abcBA* has been tested as an experimental candidate vaccine strain and provided protection in a mouse model of infection (Silva et al. 2015a). Furthermore, when tested in the natural host, this vaccine strain prevented any clinical sign of disease, macro- and microscopic lesions, and induced sterile immunity in experimentally challenged rams (Silva et al. 2015b).

There is relatively low genetic variability within the genus *Brucella*, which has even supported the proposition of a monospecific genus (Verger et al. 1985). However, there are striking differences in host specificity and pathogenicity among different *Brucella* species (Chain et al. 2005). Therefore, the Multiple-Locus Variable Number Tandem Repeat Analysis (MLVA) has been used as a tool for genetic and epidemiologic characterization of *Brucella* spp. (Whatmore 2009). The analysis of fourteen *B. ovis* Brazilian field isolates demonstrated some degree of genetic diversity (Dorneles et al. 2014). Considering the molecular differences identified by MLVA-16 among *B. ovis* field isolates, the aim of this study was to characterize field isolates of *B. ovis* in a murine model of infection and to evaluate the efficiency of the *B. ovis* Δ*abcBA* vaccine strain to protect mice challenged with *B. ovis* field isolates.

MATERIALS AND METHODS

Bacterial strains. As detailed in Table 1, this study included seven field *Brucella ovis* strains isolated from semen of naturally infected rams, which have been previously genotypically characterized by MLVA-16 (Dorneles et al. 2014), the reference wild-type strain *B. ovis* ATCC 25840, and the candidate vaccine strain *B. ovis* Δ*abcBA* (Silva et al. 2011b, 2015a, 2015b). Bacteria were grown on the tryptic soy agar (TSA) plates supplemented with 1% hemoglobin, for 3 days at 37°C with 5% CO_2. For the vaccine strain (*B. ovis* Δ*abcBA*), TSA was supplemented with 1% hemoglobin and 100 µg/mL of kanamycin. Bacteria were suspended in phosphate-buffered saline (PBS) (pH 7.4) and bacterial concentration was estimated by spectrophotometry (Smart Spec, Bio-Rad, Hercules, CA) at the optical density of 600nm (OD 600).

Animals. The experimental protocol used in this study has been approved by the Animal Experimentation Ethics Committee at the Universidade Federal de Minas Gerais (CEUA-UFMG protocols 41/2018 and 107/2015). Mice were maintained in cages under controlled temperature and humidity (25°C, 70%), fed commercial feed and water *ad libitum*. Mice were intraperitoneally infected with 1 x 10^6 colony forming units (CFU) of *B. ovis* suspended in 100µL of sterile PBS. Euthanasia was performed at 1, 7, or 30 days post-infection (dpi).

***In vitro* growth of *Brucella ovis* ATCC 25840 and field isolated strains.** *In vitro* growth of *B. ovis* ATCC 25840 and field isolates (94 AV and 266L) was evaluated on solid media as follows: bacterial suspensions were prepared in PBS to a concentration of 10^3 CFU/mL. 100µL of each suspension were then plated on TSA medium with 1% hemoglobin and without antibiotics. Plates were incubated at 37°C in 5% CO_2 and at 0, 12, 24, 48, 72, 96, and 120 hours post-inoculation colonies were harvested and suspended in either 1mL (0 to 48h)

Table 1. *Brucella* strains used in this study

Strain	City	Country	Year of isolation
B. ovis ATCC 25840	—	Australia	1960
B. ovis Δ*abcBA*	—	—	2011
B. ovis 94 AV	Livramento/MS	Brazil	1995
B. ovis 266 L	Livramento/MS	Brazil	1995
B. ovis 0204	Uruguaiana/MS	Brazil	1997
B. ovis 286 L	Livramento/MS	Brazil	1995
B. ovis 252 L	Livramento/MS	Brazil	1995
B. ovis 100 V	Livramento/MS	Brazil	1995
B. ovis 203 L	Livramento/MS	Brazil	1995

or 2mL (72 to 120h) of sterile PBS. Bacterial suspensions were serially diluted (10-fold dilutions) and plated on TSA plus 1% hemoglobin by using the drop plate method. The experiment was performed in duplicate, and the total numbers of CFU per milliliter were determined at each time point.

***In vitro* infection of RAW 264.7 murine macrophages.** The murine macrophage cell line RAW 264.7 was cultured in RPMI medium (Gibco; Invitrogen) supplemented with 10% fetal bovine serum (FBS). Cells were seeded in 96-well culture plates (5×10^5 macrophages/well) and incubated at 37°C with 5% CO_2. Macrophages were infected with the *B. ovis* ATCC 25840 or field isolates (*B. ovis* 94 AV or 266 L) at a multiplicity of infection (MOI) of 100. Plates were centrifuged at 1,000 × *g* for 5min at 15°C and incubated at 37°C for 30min. Macrophages were washed once with sterile PBS and then incubated at 37°C for 1h with RPMI solution supplemented with 10% FBS and 50µg/mL of gentamicin (Invitrogen, São Paulo, Brazil). Next, each well was washed once with sterile PBS, and macrophages were lysed with sterile distilled water for 20min at 0, 4, 24, and 48hpi. Intracellular bacteria recovered from lysed macrophages were serially diluted (10-fold dilutions) in PBS and plated in duplicate on TSA medium with 1% hemoglobin for 3-6 days of incubation at 37°C with 5% CO_2 for CFU counting. Two independent experiments were performed in triplicates.

Vaccine experiments. Encapsulation of the *B. ovis* Δ*abcBA* vaccine strain was performed as previously described (Silva et al. 2015a). Briefly, 2 x 10^{10} CFU of *B. ovis* Δ*abcBA* were resuspended in 2mL of 1% alginate solution (Sigma-Aldrich) and dripped in 10mL of polymerization solution (0.5 mM $CaCl_2$), using a 0.23mm x 4mm needle, followed by homogenization for 15 minutes. Capsules were washed twice with 10mM MOPS solution with 0.85% NaCl (pH 7.4) for 5min. Capsules were then shaken with 0.05% alginate solution for 5min. The encapsulated vaccine strain was subcutaneously inoculated in fifteen female C57BL/6 mice with a final dose of 1 x 10^8 CFU per mouse. Other fifteen mice were inoculated with sterile PBS by the same route. The size of the capsules has previously been described by Silva et al. (2015a). Four weeks after immunization, mice were intraperitoneally challenged with 10^6 CFU of wild-type *B. ovis* (ATCC 25840) or fields strains (*B. ovis* 94 AV and 266 L). Two weeks later, mice were euthanized, and samples of liver and spleen were aseptically collected, weighed, and homogenized in 2mL of sterile PBS. Serial 10-fold dilutions of the homogenates were plated for the CFU counting. Briefly, organs were homogenized in sterile PBS and plated on the TSA plates with 1% hemoglobin. Bacterial colonies were counted at 3-6 days after plating.

Histopathology. Liver, spleen, superficial cervical lymph node, and the subcutaneous site of vaccination were sampled, fixed by immersion in 10% buffered formalin for 24 hours, and embedded in paraffin. Four µm tissue sections were stained with hematoxylin and eosin. Lesions (inflammation and necrosis) were scored from 0 to 3, being 0-absent, 1-mild, 2-moderate, and 3-severe, with a total score ranging from 0 to 6.

Statistical analysis. Statistical analyses were performed using the Graph Pad Prism version 5.0 software. CFU values were logarithmically transformed prior to analysis of variance (ANOVA). Means were compared by the Tukey's test. Histopathological scores were compared using the non-parametric Mann-Whitney test.

RESULTS

Brucella ovis field isolates were capable of infecting mice

Infectivity of field isolates was assessed in BALB/c mice (n=3 per group) that were inoculated with 10^6 CFU of each *Brucella ovis* strain (100 V, 203 L, 266 L, 204, 286 L, 252 L, and 94 AV). At 7 days post-infection, strains 94 AV and 252 L had higher numbers of CFU/g in the spleen when compared to the other strains ($p<0.05$), with differences of more than one log of CFU (Fig.1A). In the liver, *B. ovis* 94 AV was recovered in higher numbers when compared to other strains (Fig.1B). The spleen and liver (Fig.1C,D) from all infected mice had multifocal microgranulomas characterized by a histiocytic and neutrophilic inflammatory infiltrate with epithelioid macrophages. There were no significant differences in histopathology scores between different strains (data not shown). These results indicate that all *B. ovis* field isolates were capable of colonizing and cause lesions in the liver and spleen of BALB/c mice.

Brucella ovis field isolates had variable growth kinetics in RAW 264.7 cells

Considering our initial results (Fig.1), two strains were selected for further characterization: *B. ovis* 94 AV, which was recoverd in higher numbers in the liver and spleen; and *B. ovis* 266 L, which had a phenotype similar to the other isolates. Initially, *in vitro* growth of *B. ovis* field isolates was compared to that of the reference strain *B. ovis* ATCC 25840 in TSA medium with 1% hemoglobin. Both field isolates had *in vitro* growth curves similar to the reference stain. All strains had an exponential growth phase between 24 and 72h of incubation at 37°C with 5% CO_2, and then entered the stationary growth phase (Fig.2A). The kinetics of intracellular growth of these strains was then assessed by infecting RAW 264.7 murine macrophage cells. At 0 and 4 hours after infection, significantly higher CFU numbers of *B. ovis* ATCC 25840 were recovered when compared to the field isolates ($p<0.05$), with more than

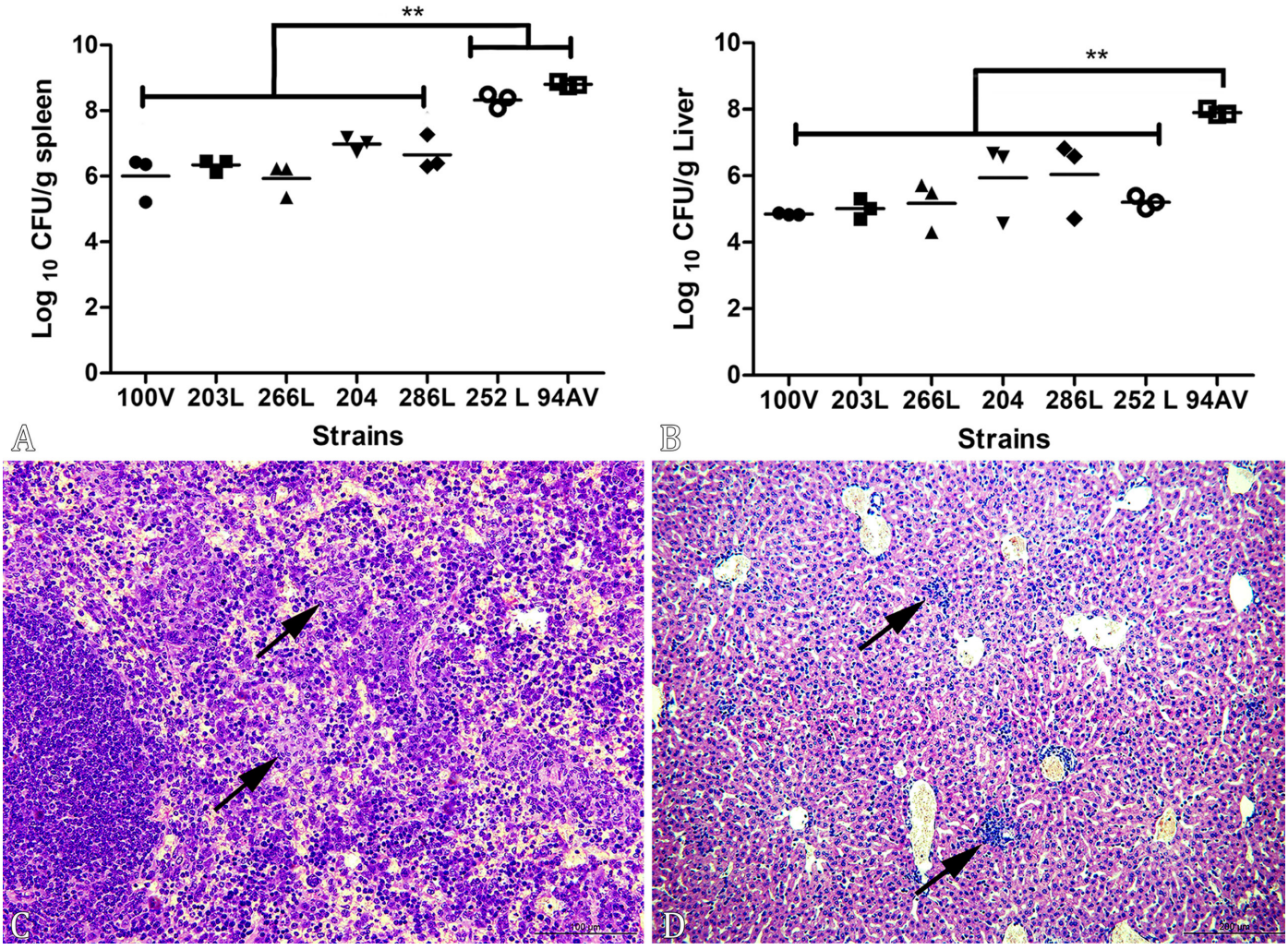

Fig.1. Experimental infection of BALB/c mice with different field isolated strains of *Brucella ovis*. Mice were infected with 1×10^6CFU/mouse from different field strains (100 V, 203, 266 L, 204, 252 L, 286 L, and 94 AV). Each data point represents the number of CFU/g recovered from the (**A**) spleen and (**B**) liver of each mouse at 7dpi. The line in each group indicates the mean. All data were logarithmically transformed prior to ANOVA, and the means were compared by the Tukey's test. Statistically significant differences are indicated by asterisks (** $p<0.01$). All infected mice developed microgranulomas (arrows) in the (**C**) spleen and (**D**) liver. (**C**,**D**) HE, bar = 100μm, bar = 200μm.

one log difference at time 0, indicating that the reference strain *B. ovis* ATCC 25840 had higher levels of internalization in RAW cells when compared to field strains (Fig.2B). At 24 hours after inoculation, the opposite was observed with significantly higher CFU numbers of field isolates recovered from the intracellular compartment of macrophages (approximately one log difference) when compared to the reference strain ($p<0.05$), demonstrating that the reference strain underwent a decrease in its intracellular population before it started growing intracellularly, whereas the field isolates, although less invasive, grew steadily from the beginning of the time course. At 48 hours after inoculation, both field isolates were recovered in higher numbers when compared to the previous time points indicating they were all able to survive and grow intracellularly in RAW cells (Fig.2B). These results clearly demonstrated a different kinetics of internalization and intracellular survival between the reference strain and field isolates. Although less invasive, field isolates were able to start multiplying intracellularly at very early time points, when compared to the reference strain, which had an initial decline before start multiplying within macrophages (Fig.2B).

Colonization of spleen and liver of mice infected with *Brucella ovis* field isolates

Considering the differences in intracellular growth, we investigated the kinetics of infection of *B. ovis* 94 AV and 266 L in the mouse model. BALB/c mice (n=5 per group) were intraperitoneally infected with 10^6 CFU/mice of the reference strain *B. ovis* ATCC 25840 or the two field isolates. Mice were sampled at 1, 7, and 30dpi. At 1dpi, bacterial loads in the spleen and liver were significantly higher (nearly 2 log difference) in mice infected with the reference strain ($p<0.05$), when compared to both field isolates (94 AV and 266 L). At 7dpi, all strains had similar bacterial loads in the spleen

(Fig.3A), whereas *B. ovis* 266 L was recovered in lower numbers from the liver when compared to the reference strain (Fig.3B). At 30dpi, mice challenged with *B. ovis* 266 L had higher bacterial loads in the spleen and liver ($p<0.05$) when compared to mice infected with the other field isolate (94 AV) and the reference strain (Fig.3A,B).

Histological changes were similar in the liver and spleen from mice infected with different strains. At 1dpi, there were no inflammatory changes, whereas at 7 and 30dpi there were moderate multifocal microgranulomas in the liver and spleen. There were no significant differences in histopathology scores attributed to histological lesions in the spleens and livers from mice infected with different strains.

Immunization with encapsulated *Brucella ovis* Δ*abcBA* induces protection of experimentally challenged mice with field isolates

Previous studies demonstrated that the attenuated mutant strain *B. ovis* Δ*abcBA* induces protection in mice and in rams (Silva et al. 2015a, 2015b). Here we assessed whether vaccination with *B. ovis* Δ*abcBA* protects mice challenged with field isolated *B. ovis* strains, which is relevant since all previous studies evaluated protection against the reference strain, and in here we demonstrated differences in the kinetics of intracellular and *in vivo* infection and growth when comparing field isolates with the reference strain. As expected, immunized mice had significant reduction in bacterial loads in the liver and spleen when compared to non-immunized mice ($p<0.001$). Protection indexes are described in Table 2. Interestingly, protection indexes were higher in mice challenged with field strains, particularly mice challenged with *B. ovis* 266 L, with reductions in splenic bacterial loads close to 1, 2 or 3 logs of CFU in mice challenged with *B. ovis* ATCC 25840, *B. ovis* 94 AV, or *B. ovis* 266 L, respectively.

Bacterial colonization in the liver was also significantly lower in vaccinated mice, with decreases in bacterial loads in the range of 0.5, 1, and 1 for mice challenged with *B. ovis* ATCC 25840, *B. ovis* 94 AV, and *B. ovis* 266 L, respectively.

Immunized mice challenged with different *B. ovis* strains did not develop splenomegaly, while non-immunized mice developed evident splenomegaly after infection. In the liver of all non-immunized mice, there were multifocal coalescent firm white nodular lesions of approximately 0.1 to 0.4cm in diameter. In contrast, immunized mice developed less

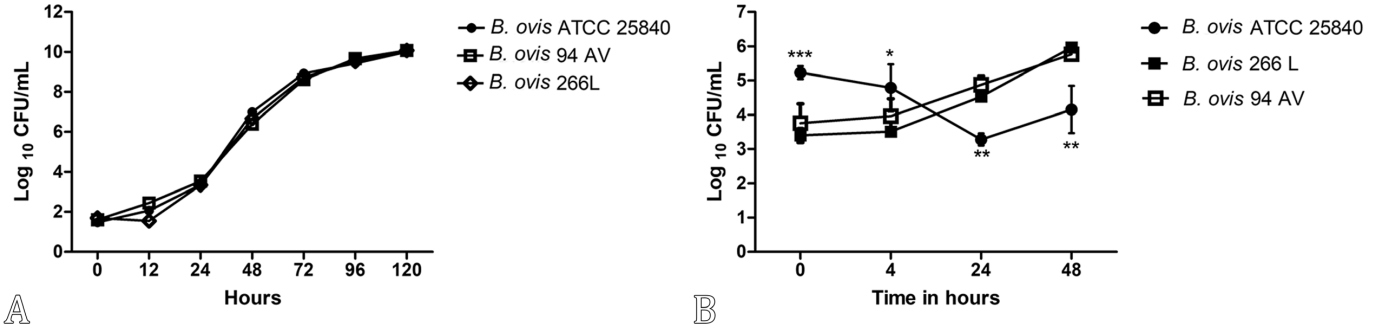

Fig.2. (**A**) *In vitro* growth curve of different *Brucella ovis* strains. *B. ovis* ATCC 25840, 94 AV, and 266 L were grown on TSA plates with 1% hemoglobin at 37ºC with 5% CO_2. (**B**) *In vitro* infection of murine macrophage cell line RAW 264.7 (MOI 100) with the reference *B. ovis* strain (ATCC 25840) or field isolates (94 AV and 266 L). Macrophages were grown in 96-well plates and infected with *B. ovis* strains with a MOI of 1:100. Intracellular bacteria were recovered at 0, 4, 24, and 48hpi. Time zero represents the number of CFUs of bacteria after 1 hour of incubation with gentamycin. Data points represent the mean and standard deviation of two independent experiments performed in triplicates. Data were logarithmically transformedprior to ANOVA, and means were compared by the Tukey's test. Statistically significant differences are indicated by asterisks (* $p<0.05$; ** $p<0.01$).

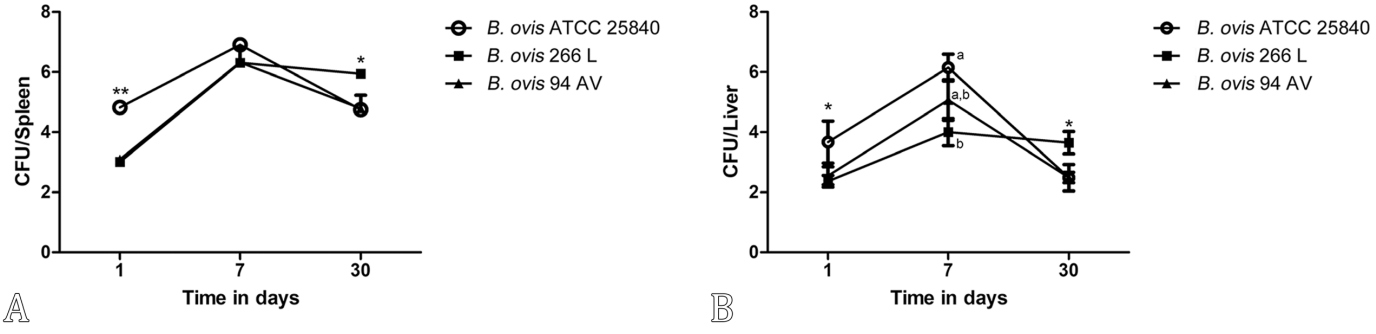

Fig.3. Kinetics of *Brucella ovis* infection in BALB/c mice. Mice were intraperitoneally infected with 1×10^6CFU of *B. ovis* ATCC 25840, 94 AV, or 266 L. Samples of (**A**) spleen and (**B**) liver were collected for bacterial counts at 1, 7, and 30dpi. Each data point represents the mean and standard deviation (n=5). Data were logarithmically transformedprior to ANOVA, and means were compared by the Tukey's test. Statistically significant differences are indicated by asterisks (* $p<0.05$; ** $p<0.01$). Differences between the groups in the liver at 7dpi are indicated by different letters ($p<0.05$).

severe lesions (Fig.4). Histologically, non-immunized mice had a moderate to severe, multifocal, inflammatory infiltrate composed of epithelioid macrophages and neutrophils with mild accumulation of fibrin in the marginal zone and red pulp in the spleen, characterizing a moderate to severe, multifocal pyogranulomatous splenitis. Immunized mice had milder similar microscopic lesions. Histopathology scores were significantly lower in immunized mice when compared to non-immunized controls ($p<0.01$). Histological changes in the liver of nonimmunized mice were characterized by a mild to moderate, multifocal, randomly distributed, inflammatory infiltrated composed of epithelioid macrophages, neutrophils, and lymphocytes, associated with moderate multifocal necrosis and thrombosis. Immunized mice developed only a few mild

Table 2. Protection indexes induced by *Brucella ovis* Δ*abcBA* encapsulated with alginate in C57BL/6 mice experimentally challenged with diferente strains of *B. ovis*

Challenge strain (1x 10^6 per mouse)	CFU/spleen immunized mice	CFU/spleen non-immunized mice	Protection index
B. ovis ATCC 25840	4.668 ± 0.383	5.419 ± 0.219	0.751*
B. ovis 266 L	3.786 ± 0.276	6.532 ± 0.649	2.746**
B. ovis 94 AV	4.846 ± 0.599	6.596 ± 0.355	1.736**

* Statistically significant difference ($p<0.05$), ** statistically significant difference ($p<0.01$)

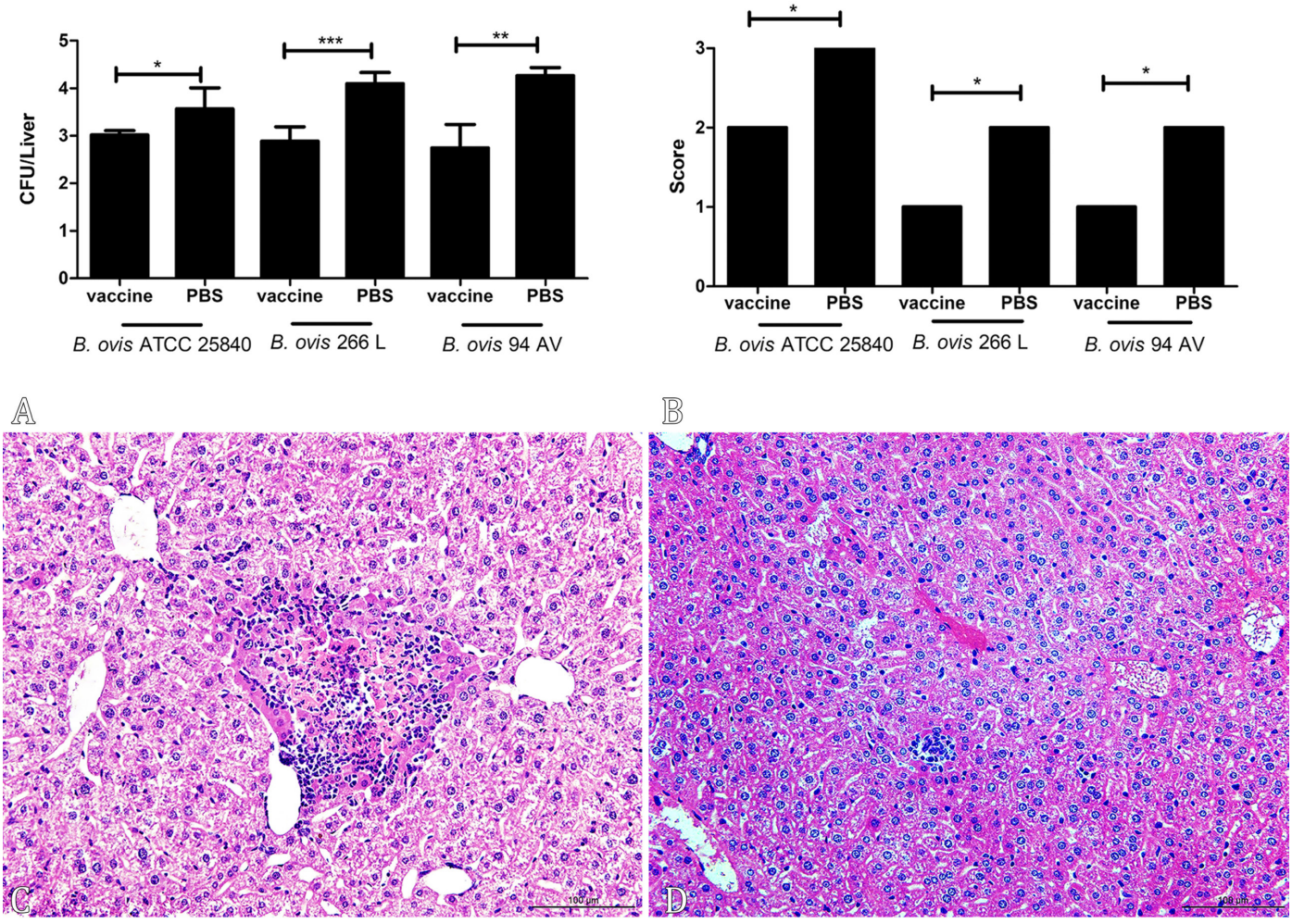

Fig.4. Protection induced by the vaccine strain *Brucella ovis* Δ*abcBA* encapsulated with alginate in C57BL/6 mice experimentally challenged with diferente *B. ovis* strains. (**A**) Number of of *B. ovis* CFU recovered from the liver. Each column represents the mean and standard deviation (n=5). Raw data were logarithmically transformed prior to ANOVA, and means were compared by Tukey's test. Significant differences between bacterial strains are indicated by asterisks (* $p<0.05$; ** $p<0.01$; *** $p<0.001$). (**B**) Score for lesions in the liver of mice. Means were compared by the Kruskal-Wallis nonparametric test. Representative histological changes in the liver of (**C**) non-immunized mice with with extensive microgranulomas associated with necrosis; or (**D**) immunized mice with very mild changes. Mice challenged with *B. ovis* 266 L. (**C**,**D**) HE, bar = 100µm.

microgranulomas in the liver (Fig.4). Histopathology scores for hepatic lesions in groups immunized with encapsulated *B. ovis* Δ*abcBA* were significantly lower when compared to non-immunized mice ($p<0.01$) regardless of the challenge strain (Fig.4).

Vaccination sites were initially swollen, but this change regressed significantly until the day of euthanasia. Histopathologically, there were small granulomas at the site of vaccination (data not shown).

DISCUSSION

This study characterized *in vivo* and *in vitro* behavior of *Brucella ovis* field isolates. There were clear differences in pathogenic potential among *B. ovis* field isolates based on intracellular growth as well as *in vivo* infection in the mouse model. This is a relevant finding considering the fact that *Brucella* spp. have little genetic variability (Tsolis 2002). Interestingly, protection indexes induced by the candidate vaccine strain *B. ovis* Δ*abcBA* were higher for strains with higher virulence. These results indicate that the vaccine strain protects against different strains of *B. ovis*, and protection is even more evident against more pathogenic strains, demonstrating a robust immunogenicity of this experimental vaccine formulation.

Variable pathogenicity among field isolates should not be considered unexpected since *Brucella,* like other bacteria, is able to undergo spontaneous mutations or metabolic adaptations depending on the environmental conditions to which it is exposed, including temperature, humidity, host cell defense, and intracellular environment. Minimal genomic mutations may result in major phenotypic changes affecting survival and virulence of bacteria. For instance, the vaccine strain *B. abortus* S19, isolated in 1923 from the milk of a Jersey cow (Buck 1930), that after being accidentally left out at room temperature for one year spontaneously developed an attenuated phenotype (Graves 1943). Importantly, there were no previous studies comparing the pathogenicity of *B. ovis* strains with different genotypes based on MLVA-16 (Dorneles et al. 2014).

All seven *B. ovis* strains included in this study were directly isolated from the semen of naturally infected rams (Dorneles et al. 2014), indicating that these rams likely had clinical changes and were sources of infection to other sheep within their herds (Burgess 1982). Although there is no information regarding clinical signs associated with these isolates, MLVA16 demonstrated different genotypes (Dorneles et al. 2014). Therefore, in order to assess possible differences in pathogenic potential of these strains, we used the mouse, which has been extensively employed as an infection model for *Brucella* spp. (Silva et al. 2011a) being a suitable model for *B. ovis* infection (Silva et al. 2011b). In this study, mice infected with 10^6 CFU of *B. ovis* field isolates (100 V, 203 L, 266 L, 204, 286 L, 252 L, and 94 AV) became experimentally infected. All strains were capable of causing lesions in the liver and spleen at 7dpi, although there were significant differences in their ability to multiply intracellularly and colonize and survive *in vivo*.

B. ovis strains included in this study were isolated directly from the natural host (Dorneles et al. 2014), where the bacteria face harsh intracellular conditions including exposure to reactive oxygen species, low pH, and low nutrient levels. *Brucella* spp. can adapt to the intracellular environment upon activation of expression of certain virulence factors (Kohler et al. 2002). Different strains of a given *Brucella* species may exhibit different intracellular kinetics (Harmon et al. 1988, Kohler et al. 2002, Silva et al. 2014). This may explain the differences in intracellular and *in vivo* survival and multiplication observed in this study, whereas *in vitro* growth on solid medium was remarkably similar among these isolates. Adaptation and attenuation of *Brucella* reference strains handled frequently in the laboratory conditions to *in vitro* and *in vivo* models is described (Bosseray 1991, Grilló et al. 2012). Although it may warrant different phenotype of reference strain from field isolates, it does not explain *in vivo* difference between field isolates.

BALB/c and C57BL/6 are suitable models for *B. ovis* infection since they develop a systemic infection that results in lesions in the liver and spleen. BALB/c mice are more susceptible to *B. ovis* than C57BL/6 mice, and under experimental conditions, mice do not develop *B. ovis*-induced genital lesions as observed in the natural host, which makes the mouse a useful model of infection although they do not mimic the natural disease (Silva et al. 2011b). In general, the mouse model is useful for comparing different strains. In this study, strains 94 AV and 266 L were able to colonize the spleen and liver and persist for up to 30 dpi. Virulent *Brucella* strains are capable of colonizing the liver and spleen of mice and persist for a long period (Silva et al. 2011b, Grilló et al. 2012). Our results indicate that the two field isolates tested were fully virulent since they were capable to establish systemic infection and persist in the mouse, and survive intracellularly in cultured macrophages. Importantly, the field strain *B. ovis* 266 L had the best fitness both intracellularly in cultured macrophages as well as *in vivo* in mice.

Recent studies have demonstrated that the candidate vaccine strain *B. ovis* Δ*abcBA* induces a protection in mice (Silva et al. 2015a), while it promotes sterile immunity in experimentally challenged rams (Silva et al. 2015b). In mice, higher protection indexes were induced by the vaccine strain *B. ovis* Δ*abcBA* in C57BL/6 mice when compared to BALB/c mice (Silva et al. 2015a). This study demonstrated that the vaccine strain *B. ovis* Δ*abcBA* also provided protection for mice challenged with different field isolates. Interestingly, the highest protection index was observed in the group challenged with strain 266 L, which had the best adaptation to intracellular survival in cultured macrophages and *in vivo* colonization and persistence. Importantly, vaccinal protection was not restricted to lower colonization by the virulent strain, but also by prevention of lesions since histopathology scores were lower in the vaccinated mice. These results are quite encouraging since the vaccine strain performed even better when vaccinated mice were challenged with more pathogenic field strains, which likely activate virulence factors more efficiently for adaptation to the host (Kohler et al. 2002). These results also support the dogma in brucellosis vaccinology that live vaccines are more efficient compared to the other vaccine categories (Carvalho et al. 2016). Considering the efficiency of this vaccine strain under experimental conditions, and the absence of a commercially available and specific *B. ovis* vaccine, this vaccinal protocol may potentially be an efficient tool for preventing reproductive losses caused by *B. ovis* (Carvalho Júnior et al. 2012, Poester et al. 2013). Furthermore, unlike the *B. melitensis* Rev1 vaccine strain used in several

countries, *B. ovis* Δ*abcBA* does not have zoonotic potential, thus eliminating the occupational risks due to accidental human vaccine exposure (Xavier et al. 2009).

CONCLUSION

In conclusion, there were significant differences in pathogenicity among *Brucella ovis* field isolates. Importantly, the *B. ovis* Δ*abcBA* vaccine strain induced protection against field isolates with protections indexes that were higher for mice challenged with more pathogenic strains.

Acknowledgements.- Work in RLS lab is supported by "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq), "Fundação de Amparo a Pesquisa do Estado de Minas Gerais" (FAPEMIG), and "Coordenação de Aperfeiçoamento de Pessoal de Nível Superior" (CAPES), Brazil. T.A.P. and R.L.S. have fellowships from CNPq.

Conflict of interest statement.- The authors have no conflicts of interest to declare.

REFERENCES

Blasco J.M. 1997. A review of the use of *B. melitensis* Rev 1 vaccine in adult sheep and goats. Prev. Vet. Med. 31(3-4):275-283. <http://dx.doi.org/10.1016/S0167-5877(96)01110-5> <PMid:9234451>

Blasco J.M. & Díaz R. 1993. *Brucella melitensis* Rev 1 vaccine as a cause of human brucellosis. Lancet 342(8874):805. <http://dx.doi.org/10.1016/0140-6736(93)91571-3> <PMid:8103891>

Bosseray N. 1991. *Brucella melitensis* Rev 1 living attenuated vaccine: stability of markers, residual virulence and immunogenicity in mice. Biologicals 19(4):355-363. <http://dx.doi.org/10.1016/S1045-1056(05)80025-9> <PMid:1797046>

Buck J.M. 1930. Studies on vaccination during calfhood to prevent bovine infectious abortion. J. Agric. Res. 41(9):667-689.

Burgess G.W. 1982. Ovine contagious epididymitis: a review. Vet. Microbiol. 7(6):551-575. <http://dx.doi.org/10.1016/0378-1135(82)90049-9> <PMid:6762755>

Carvalho Júnior C.A., Moustacas V.S., Xavier M.N., Costa E.A., Costa L.F., Silva T.M.A., Paixão T.A., Borges A.M., Gouveia A.M.G. & Santos R.L. 2012. Andrological, pathologic, morphometric, and ultrasonographic findings in rams experimentally infected with *Brucella ovis.* Small Rumin. Res. 102(2/3):213-222. <http://dx.doi.org/10.1016/j.smallrumres.2011.08.004>

Carvalho T.F., Haddad J.P., Paixão T.A. & Santos R.L. 2016. Meta-Analysis and advancement of brucellosis vaccinology. PLos One 11(11):1-28. <http://dx.doi.org/10.1371/journal.pone.0166582> <PMid:27846274>

Celli J., de Chastellier C., Franchini D.M., Pizarro-Cerda J., Moreno E. & Gorvel J.P. 2003. *Brucella* evades macrophage killing via VirB-dependent sustained interactions with the endoplasmic reticulum. J. Exp. Med. 198(4):545-556. <http://dx.doi.org/10.1084/jem.20030088> <PMid:12925673>

Chain P.S.G., Comerci D.J., Tolmasky M.E., Larimer F.W., Malfatti S.A., Vergez L.M., Aguero F., Land M.L., Ugalde R.A. & Garcia E. 2005. Whole-genome analyses of speciation events in pathogenic *Brucellae.* Infect. Immun. 73(12):8353-8361. <http://dx.doi.org/10.1128/IAI.73.12.8353-8361.2005> <PMid:16299333>

Danese I., Haine V., Delrue R., Lestrate P., Stevaux O., Mertens P., Paquet J.Y., Godfroid J., De Bolle X. & Letesson J.J. 2004. The Ton system, an ABC transporter, and a universally conserved GTPase are involved in iron utilization by *Brucella melitensis* 16M. Infect. Immun. 72(10):5783-5790. <http://dx.doi.org/10.1128/IAI.72.10.5783-5790.2004> <PMid:15385478>

Detmers F.J.M., Lanfermeijer F.C. & Poolman B. 2001. Peptides and ATP binding cassette peptide transportes. Res. Microbiol. 152(3-4):245-258. <http://dx.doi.org/10.1016/S0923-2508(01)01196-2> <PMid:11421272>

Dorneles E.M.S., Freire G.N., Dasso M.G., Poester F.P. & Lage A.P. 2014. Genetic diversity of *Brucella ovis* isolates from Rio Grande do Sul, Brazil, by MLVA16. BMC Res. Notes 7(1):447. <http://dx.doi.org/10.1186/1756-0500-7-447> <PMid:25015223>

Graves R.R. 1943. The story of John M. Buck's and Matilda's contribution to the cattle industry. J. Am. Vet. Med. Assoc. 102:193-195.

Grilló M.J., Marín C.M., Barberán M. & Blasco J.M. 1999. Experimental *Brucella ovis* infections in pregnant ewes. Vet. Rec. 144(20):555-558. <http://dx.doi.org/10.1136/vr.144.20.555> <PMid:10371013>

Grilló M.J., Blasco J.M., Gorvel J.P., Moriyón I. & Moreno E. 2012. What we have to learn from bruccelosis in the mouse model? Vet. Res. 43(1):1-72. <http://dx.doi.org/10.1186/1297-9716-43-29> <PMid:22500859>

Gorvel J.P. & Moreno E. 2002. *Brucella* intracellular life: from invasion to intracellular multiplication. Vet. Microbiol. 90(1-4):281-297. <http://dx.doi.org/10.1016/S0378-1135(02)00214-6> <PMid:12414149>

Harmon B.G., Adams L.G. & Frey M. 1988. Survival of rough and smooth strains of *Brucella abortus* in bovine mammary gland macrophages. Am. J. Vet. Res. 49(7):1092-1097. <PMid:3138931>

Hosie A.H.F. & Poole P.S. 2001. Bacterial ABC transporters of amino acids. Res. Microbiol. 152(3-4):259-270. <http://dx.doi.org/10.1016/S0923-2508(01)01197-4> <PMid:11421273>

Igarashi K., Ito K. & Kashiwagi K. 2001. Polyamine uptake systems in *Escherichia coli.* Res. Microbiol. 152(3-4):271-278. <http://dx.doi.org/10.1016/S0923-2508(01)01198-6> <PMid:11421274>

Jenner D.C., Dassa E., Whatmore A.M. & Atkins H.S. 2009. ATP binding cassette systems of *Brucella.* Comp. Funct. Genomics 2009:1-17. <http://dx.doi.org/10.1155/2009/354649> <PMid:20169092>

Kohler S., Foulongne V., Ouahrani-Bettache S., Bourg G., Teyssier J., Ramuz M. & Liautard J.P. 2002. The analysis of the intramacrophagic virulome of *Brucella suis* deciphers the environment encountered by the pathogen inside the macrophage host cell. Proc. Natl. Acad. Sci. 99(24):15711-15716. <http://dx.doi.org/10.1073/pnas.232454299> <PMid:12438693>

Macedo A.A., Silva A.P., Mol J.P., Costa L.F., Garcia L.N., Araújo M.S., Martins Filho O.A., Paixão T.A. & Santos R.L. 2015. The *abcEDCBA*-encoded ABC transporter and the *virB* operon-encoded type IV secretion system of *Brucella ovis* are critical for intracellular trafficking and survival in ovine monocyte-derived macrophages. PLoS One 10(9):1-23. <http://dx.doi.org/10.1371/journal.pone.0138131> <PMid:26366863>

OIE. 2015. Ovine epididymitis (*Brucella ovis*), p.1467-1479. In: Ibid (Ed), Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. OIE, Paris. Available at <http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.07.07_OVINE_EPID.pdf> Access on January 17, 2020.

Olsen S.C., Thoen C.O. & Cheville N.F. 2011. Brucella, p.429-438. In: Gyles C.L., Prescott F.J., Songer G.J. & Thoen C.O. (Eds), Pathogenesis of Bacterial Infections in Animals. Wiley-blackwell, Iowa.

Poester F.P., Samartino L.E. & Santos R.L. 2013. Pathogenesis and pathobiology of brucellosis in livestock. Rev. Sci. Tech. 32(1):105-115. <http://dx.doi.org/10.20506/rst.32.1.2193> <PMid:23837369>

Ridler A.L. & West D.M. 2011. Control of *Brucella ovis* infection in sheep. Vet. Clin. N. Am. Food Anim. Pract. 27(1):61-66. <http://dx.doi.org/10.1016/j.cvfa.2010.10.013> <PMid:21215890>

Sá J.C., Silva T.M., Costa E.A., Silva A.P., Tsolis R.M., Paixão T.A., Carvalho Neta A.V. & Santos R.L. 2012. The *virB*-encoded type IV secretion system is critical for establishment of infection and persistence of *Brucella ovis* infection in mice. Vet. Microbiol. 159(1-2):130-140. <http://dx.doi.org/10.1016/j.vetmic.2012.03.029> <PMid:22483850>

Silva A.P.C., Macêdo A.A., Silva T.M., Ximenes L.C., Brandão H.M., Paixão T.A. & Santos R.L. 2015a. Protection provided by an encapsulated live attenuated

Δ*abcBA* strain of *Brucella ovis* against experimental challenge in a murine model. Clin. Vaccine Immunol. 22(7):789-797. <http://dx.doi.org/10.1128/CVI.00191-15> <PMid:25947146>

Silva A.P.C., Macêdo A.A., Costa L.F., Rocha C.E., Garcia L.N., Farias J.R., Gomes P.P., Teixeira G.C., Fonseca K.W., Maia A.R., Neves G.G., Romão E.L., Silva T.M., Mol J.P., Oliveira R.M., Araújo M.S., Nascimento E.F., Martins-Filho O.A., Brandão H.M., Paixão T.A. & Santos R.L. 2015b. Encapsulated *Brucella ovis* lacking a putative ATP-binding cassette transporter (Δ*abcBA*) protects against wild type *Brucella ovis* in Rams. PLoS One 10(8):1-23. <http://dx.doi.org/10.1371/journal.pone.0136865> <PMid:26317399>

Silva T.M.A., Costa E.A., Paixão T.A., Tsolis R.M. & Santos R.L. 2011a. Laboratory animal models for brucellosis research. J. Biomed. Biotechnol. 2011:518323. <http://dx.doi.org/10.1155/2011/518323> <PMid:21403904>

Silva T.M.A., Paixão T.A., Costa E.A., Xavier M.N., Sá J.C., Moustacas V.S., den Hartigh A.B., Carvalho Neta A.V., Oliveira S.C., Tsolis R. & Santos R.L. 2011b. Putative ATP-Binding cassette transporter is essential for *Brucella ovis* pathogenesis in mice. Infect. Immun. 79(4):1706-1717. <http://dx.doi.org/10.1128/IAI.01109-10> <PMid:21300772>

Silva A.P., Macêdo A.A., Costa L.F., Turchetti A.P., Bull V., Pessoa M.S., Araújo M.S., Nascimento E.F., Martins-Filho O.A., Paixão T.A. & Santos R.L. 2013. *Brucella ovis* lacking a species-specific putative ATP-binding cassette transporter is attenuated but immunogenic in rams. Vet. Microbiol. 167(3-4):546-553. <http://dx.doi.org/10.1016/j.vetmic.2013.09.003> <PMid:24075357>

Silva T.M.A., Mol J.P.S., Winter M.G., Atluri V., Xavier M.N., Pires S.F., Paixão T.A., Andrade H.M., Santos R.L. & Tsolis R.M. 2014. The predicted ABC transporter AbcEDCBA is required for type IV secretion system expression and lysosomal evasion by *Brucella ovis.* Plos One 9(12):1-27. <http://dx.doi.org/10.1371/journal.pone.0114532> <PMid:25474545>

Tsolis R.M. 2002. Comparative genome analysis of the alpha-proteobacteria: relationships between plant and animal pathogens and host specificity. Proc. Natl. Acad. Sci. USA 99(20):12503-12505. <http://dx.doi.org/10.1073/pnas.212508599> <PMid:12271145>

Tsolis R.M., Seshadri R., Santos R.L., Sangari F.J., Lobo J.M., de Jong M.F., Ren Q., Myers G., Brinkac L.M., Nelson W.C., Deboy R.T., Angiuoli S., Khouri H., Dimitrov G., Robinson J.R., Mulligan S., Walker R.L., Elzer P.E., Hassan K.A. & Paulsen I.T. 2009. Genome degradation in *Brucella ovis* corresponds with narrowing of its host range and tissue tropism. PLoS One 4(5):1-28. <http://dx.doi.org/10.1371/journal.pone.0005519> <PMid:19436743>

Verger J.M., Grimont F., Grimont P.A.D. & Grayon M. 1985. *Brucella*, a monospecific genus as shown by deoxyribonucleic acid hybridization. Int. J. Syst. Evol. Microbiol. 35(3):292-295.

Whatmore A.M. 2009. Current understanding of the genetic diversity of *Brucella*, an expanding genus of zoonotic pathogens. Infect. Genet. Evol. 9(6):1168-1184. <http://dx.doi.org/10.1016/j.meegid.2009.07.001> <PMid:19628055>

Xavier M.N., Paixão T.A., Poester F.P., Lage A.P. & Santos R.L. 2009. Pathological, Immunohistochemical and Bacteriological Study of Tissues and Milk of Cows and Fetuses Experimentally Infected with *Brucella abortus.* J. Comp. Pathol. 140(2-3):149-15. <http://dx.doi.org/10.1016/j.jcpa.2008.10.004> <PMid:19111839>