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Research Paper

Different resistance patterns of reference and field strains of *Brucella abortus*

Karina L. Miranda[1], Elaine M. S. Dorneles[1], Fernando P. Poester[1], Paulo S. Martins Filho[2], Rebeca B. Pauletti[1], Andrey P. Lage[1]

[1]Escola de Veterinária, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil.
[2]Laboratório Regional de Apoio Animal, Ministério da Agricultura, Pecuária e Abastecimento, Pedro Leopoldo, MG, Brazil.

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Abstract

The aim of this study was to evaluate the growth of the *B. abortus* reference strains and field isolates on media containing different inhibitor agents. Reference strains were seeded on tryptose agar containing: i-erythritol (1.0 mg/mL), fuchsin (20 μg/mL and 80 μg/mL), thionin (2.5 μg/mL and 10 μg/mL), rifampicin (200 μg/mL) and safranin O (200 μg/mL). Field isolates were tested only on media containing i-erythritol, rifampicin and thionin. Furthermore, each suspension was also inoculated on tryptose agar incubated in air, to test its ability to grow without CO_2. Sensitivity to fuchsin was similar among reference strains evaluated. Growth of S19, 544 and 2308 but not RB51 were inhibited on media containing rifampicin. Medium with safranin O showed no inhibition for RB51, 544 and 2308, but it partially inhibited the S19 growth as well as medium containing i-erythritol. Treatment/control growth ratio for 2308 on tryptose agar containing thionin (2.5 μg/mL) was approximatelly 1.0, whereas S19 and RB51 showed 0.85 and 0.89 ratios, respectively. Growth of 544, S19 and RB51 but not 2308 was completely inhibited on medium with thionin (10 μg/mL). All field strains grew on medium containing i-erythritol, but were completelly inhibited by rifampicin. With exception of A1 (*B. abortus* biovar 3) all field isolates grew on medium with thionin, although some strains showed a treatment/control growth ratio of 0.75-0.80 (10 μg/mL). These results showed that tryptose agar with thionin, i-erythritol or rifampicin could be useful for differentiating vaccine, challenge and field strains of *B. abortus.*

Key words: *B. abortus*, thionin, i-erythritol, rifampicin.

Introduction

Brucellosis is a widespread zoonotic disease, transmitted mainly from ruminants to humans. It is a disease of major public health importance, animal welfare and economic significance worldwide (Corbel *et al.*, 2006). *Brucella* infections may result in significant economic losses due to abortion and the slaughtering of infected animals. Humans are mainly infected through the consumption of contaminated dairy products or by direct contact with infected animals (Corbel *et al.*, 2006). *Brucella* species have also been considered potential biological warfare agents and the organism remains in the list of Centers for Disease Control and Prevention as potential biological warfare agents, category B (Rotz *et al.*, 2002). Brucellosis is widespread in cattle in Brazil, with an uneven distribution of the disease, with areas of the country with very low prevalences and others with high prevalences both in animals and herds (Poester *et al.*, 2002; Chate *et al.*, 2009; Sikusawa *et al.*, 2009). The major strategies of the Programa Nacional de Controle e Erradicação de Brucelose e Tuberculose (PNCEBT) (Brazilian national program on control and eradication of brucellosis and tuberculosis) are the compulsory vaccination of female calves aged 3-8 months with strain 19 (S19) and voluntary vaccination of adult animals with RB51 (Poester *et al.*, 2002; Brasil, 2006; Brasil, 2007).

Send correspondence to A.P. Lage. Universidade Federal de Minas Gerais. Av. Antônio Carlos 6627, Caixa Postal 567, 31270-901 Belo Horizonte, MG, Brazil. E-mail: alage@vet.ufmg.br.

Differentiation among vaccine strains, S19 and RB51, and field strains is important in areas where vaccination is performed due to the possibility of isolation of vaccine strains from milk or other biological samples, as vaginal swabs and lymph nodes. Furthermore, experiments on the evaluation of vaccines must differentiate vaccine from challenge strains. According to the Manual of Standards Diagnostic Tests and Vaccines 2000 (OIE, 2000), the potency of live vaccines could be determined in guinea-pigs or mice, after injection of the test vaccine followed by challenge with a virulent *B. abortus* strain, such as 2308. Afterwards, the animals are killed and the spleen counts for viable *B. abortus* organisms are determined. The protection index relative to the reference preparation is then calculated. In these experiments, not only challenge strains, but vaccine strains can also be recovered, influencing the protection index. Thus, it is necessary to inhibit or to estimate the vaccine strain to be subtracted from the total counts. The differentiation among vaccine strains and field isolates of *B. abortus* is cumbersome. Hence, a biochemical test to identify these strains would be very useful in the routine, particularly for those laboratories with restricted access to molecular techniques.

The aim of this study was to evaluate the growth of the *B. abortus* reference strains S19, RB51, 544 and 2308, and some field isolates on media containing different inhibitor agents.

Material and Methods

Bacterial strains

B. abortus strain 19, original seed (S19) was obtained from United State Department of Agriculture (USDA, Ames, IA, USA), *B. abortus* strain 2308 was provided by Dr. E. Samartino (INTA - Instituto Nacional de Tecnologia Agropecuaria, Buenos Aires, Argentina), *B. abortus* 544 was obtained from Laboratório Nacional Agropercuário/MG (Ministério da Agricultura Pecuária e Abastecimento - MAPA, Belo Horizonte, MG, Brazil) and *B. abortus* RB51 strain was provided by Dr. G. Schurig (Virginia Tech, Blacksburg, VA, USA). The other strains used in this study were field *B. abortus,* isolated and identified in our laboratory by routine and molecular methods (Alton *et al.*, 1988; LèFleche *et al.*, 2006; Minharro *et al.*, 2013) as *B. abortus* biovar 1 (strains 13A and 13B) biovar 3 (strains A1, A4 and A6), and biovar 6 (17A and 17B) (Minharro *et al.*, 2013).

Before the assays, -80 °C frozen strains were thawed at room temperature, seeded on tryptose agar plates (Difco, Detroit, MI, USA) and incubated at 37 °C in a 5% CO_2, for 48 h. Fresh bacterial growth were harvested in phosphate buffered saline (PBS) (0.01 M, pH 7.2) and adjusted to MacFarland No 3 standard, resulting in a suspension of approximately 1.0 x 10^9 cfu/mL. The required dilutions of fresh suspensions were prepared in PBS before each procedure. From each suspension, six tenfold-dilutions were prepared. Suspensions from RB51 were made in PBS with 0.5% Tween 80 (Sigma, St Louis, MO, USA).

Growth tests

Viable counts of each bacterial suspension of the reference strains (S19, RB51, 544 and 2308) were performed in duplicates by the drop counting method (Miles and Misra, 1938) on tryptose agar, as controls, (Difco, Detroit, MI, USA) and tryptose agar containing the following: i-erythritol (1.0 mg/mL) (Sigma, St Louis, MO, USA); basic fuchsin (20 μg/mL and 80 μg/mL) (Merck, Darmstadt, HE, Germany); thionin (2.5 μg/mL and 10 μg/mL) (Merck, Darmstadt, HE, Germany); rifampicin (200 μg/mL) (Merrell Dow Pharmaceuticals Ltd., Uxbridge, LBHIL, UK); and safranin O (200 μg/mL) (Merck, Darmstadt, HE, Germany).

The plates were incubated at 37 °C for 96 h in 5% CO_2. Furthermore, each suspension was inoculated on two tryptose agar plates, which were incubated at 37 °C for 48 h in air, to test its ability to grow without CO_2. All experiments were repeated three times. The logarithm of the ratio bacterial count of the treatment/bacterial count of the control (growth onto tryptose agar plates in CO_2) for each strain was calculated.

Field isolates were only tested on media containing erythritol (1.0 mg/mL), rifampicin (200 μg/mL) and thionin (10 μg/mL), which were the agents able to inhibit the growth of some reference strains. All tests were done in triplicates.

Results

No difference was found in treatment/control ratio from reference strains grown on tryptose agar with basic fuchsin (20 μg/mL or 80 μg/mL) or tryptose agar incubated in air (Figure 1).

S19 growth was partially inhibited on media containing i-erythritol; there was a 5-log drop from initial inoculum (10^9 to 10^4 cfu/mL). S19, 544 and 2308 growth were inhibited on media containing rifampicin (10^9 to 10^2 cfu/mL), while the rifampicin resistant RB51 was able to grow. Tryptose agar with safranin O showed no inhibition for RB51, 544 and 2308, but S19 growth decreased from 10^9 to 10^8 cfu/mL. Growth in tryptose agar with thionin differed in the two concentrations used. The treatment/control ratio for 2308 in tryptose agar containing thionin 2.5 μg/mL was near 1.0 and S19 and RB51 showed 0.85 and 0.89 ratios, respectively. Growth of 2308 on tryptose agar with thionin 10 μg/mL was not inhibited (treatment/control ratio equal to 0.98), however, S19 and RB51 were completely inhibited (Figure 1).

All field strains were able to grow normally on tryptose agar containing i-erythritol 1.0 mg/mL, likewise *B. abortus* 2308 and RB51 (Figure 2). All field strains were totally inhibited on media containing rifampicin, where RB51 was the only strain able to grow. On media containing thionin (10 μg/mL) the reference strain 544 and the field strain A1, *B. abortus* biovar 3, were totally inhibited like the vaccine

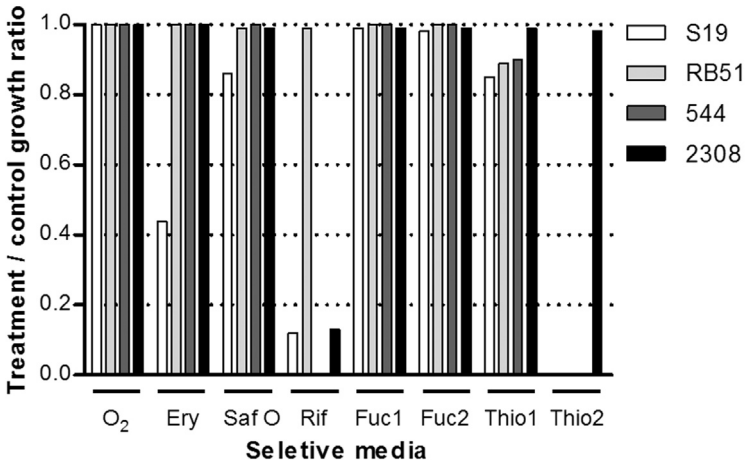

Figure 1 - Growth patterns of *B. abortus* strains S19, RB51, 544 and 2308 on different selective media or atmospheric condition. The growth ratio on tryptose agar plates in 5% CO_2 and on tryptose agar plates on air (O_2) or containing i-erythritol (1.0 mg/mL) (Ery), safranin O (200 μg/mL) (Saf O), rifampicin (200 μg/mL) (Rif), fuchsin [20 μg/mL (Fuc1) and 80 μg/mL (Fuc2)] or thionin [2.5 μg/mL (Thio1) and 10 μg/mL (Thio2)] was calculated for the reference *Brucella* strains.

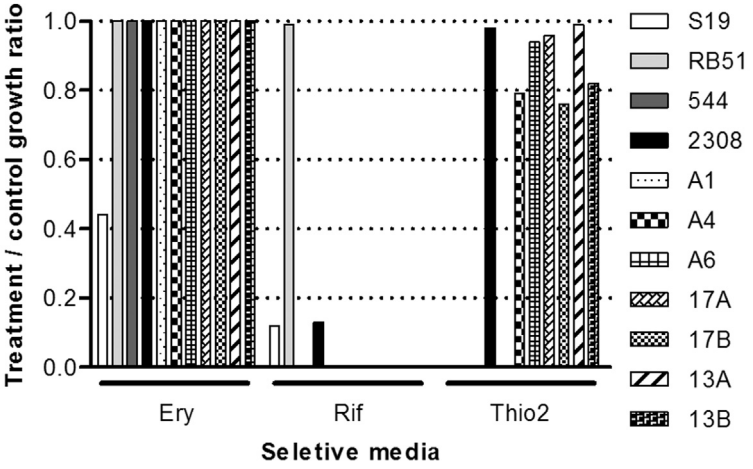

Figure 2 - Growth patterns of *B. abortus* S19, RB51, 544, 2308 and field strains (A1, A4, A6, 17A, 17B, 13A, and 13B) on different selective media. The growth ratio on tryptose agar plates and on tryptose agar plates containing i-erythritol (1.0 mg/mL) (Ery), rifampicin (200 μg/mL) (Rif) or thionin (10 μg/mL) (Thio2) was calculated for all studied *B. abortus* strains.

strains, S19 and RB51. However, *B. abortus* biovar 3, strains A4 and A6, were able to grow with only a small inhibition. All field strains, with the exception of A1, could grow on agar tryptose with thionin (10 μg/mL); strains A4, 17B and 13B showed a treatment/control ratio of 0.75-0.81, which represents a small inhibition.

Discussion

The results of the present study showed that the use of tryptose agar with thionin (10 μg/mL), i-erythritol (1.0 mg/mL) or rifampicin (200 μg/mL) could be useful in the differentiation among vaccine, challenge and field strains of *B. abortus*. Although several molecular biology techniques have been used as additional tools in the identification and characterization of *Brucella* spp., such as the techniques based on PCR (MLVA, Multiplex-PCR) or sequencing (MLST) (Bricker and Halling, 1995; LèFleche *et al.*, 2006; Whatmore *et al.*, 2007), many laboratories lack adequate facilities and equipments to perform these molecular tests, and base their characterization and identification of *Brucella* spp isolates solely on phenotypic tests. Futhermore, differentiation among *B. abortus* strains by the growth inhibition tests continue to be very useful, specially to differentiate vaccine strains from field isolates, which is required in routine vaccine evaluations (Miranda *et al.*, 2013).

The growth inhibition of S19 on media containing i-erythritol was incomplete, although a 5-log drop from initial inoculum was observed (Figure 1). The stability of this characteristic was confirmed by the results of the three ex-

periments. This partial growth inhibition of i-erythritol, at 1.0 mg/mL or 2.0 mg/mL, was already reported by S19 field isolates in United Kingdom (Whatmore *et al.*, 2007). However, it was demonstrated that this inhibitor agent can be very useful in differentiating S19 from the challenge strains, 544 or 2308, in immunogenicity studies and also in the differentiation of field isolates.

B. melitensis, *B. abortus* and *B. suis* identification at biovar level is currently performed by four main tests: carbon dioxide requirement, production of hydrogen sulphide, dye (thionin and basic fuchsin) sensitivity, and agglutination with monospecific A and M antisera (Alton *et al.*, 1988; OIE, 2009). *B. abortus* biovar 1, 2, 3 and 4 requires CO_2 for growth, however, in some cases these strains can usually require CO_2 only on primary isolations. This feature was found among all reference strains of *B. abortus* biovar 1 tested, as they were able to grow in air (S19, RB51, 544 and 2308) (Figure 1). Thus, CO_2 dependence must be carefully used, because although CO_2 requirement is an important differential feature among *B. abortus* biovars, this requirement is not always stable (Alton *et al.*, 1988; OIE, 2009).

Media containing thionin (20 μg/mL) can inhibit the growth of *B. abortus* biovar 1, 2 and 4, but not 3, 5, 6 and 9 (Alton *et al.*, 1988). This concentration was not tested, but in a lower concentration (10 μg/mL), A1, a *B. abortus* biovar 3 field strain, was completely inhibited. This atypical behavior in dye sensibility tests of field isolates have been reported by other research papers for both *B. abortus* and *B. melitensis* isolates (Garcia *et al.*, 1988; Corbel, 1991). Other finding was that the *B. abortus* reference strain 544 was not able to grow on media containing thionin 10 μg/mL, while the reference strain 2308, which was also *B. abortus* biovar 1, could grow normally. Thus, the use of media containing thionin (10 μg/mL) in immunogenicity tests for S19 or RB51 will only be useful if the challenge strain used is *B. abortus* 2308.

Moreover, the present results confirmed that strain RB51 is resistant to rifampicin, while all other strains tested had their growth inhibited on media containing this antibiotic. These results endorse rifampicin-resistance as an important trait to differentiate this vaccine strain from other *B. abortus* strains. However, for potency tests of RB51 in animal model, the use of an agent that inhibits the growth of RB51 but does not inhibit the growth of the challenge strain would be desirable. This can be accomplished by the use of media containing thionin (10 μg/mL) if the challenge strain is *B. abortus* 2308, that can grow on this condition. Thus, the lack of suitable media for its differentiation precludes *B. abortus* 544 to be used as challenge strain in potency tests of RB51.

The Manual of Diagnostic Tests and Vaccines for Terrestrial Animals - OIE (OIE, 2009) suggests the use of CO_2-dependent *B. abortus* strain 544 as challenge strain in studies of immunogenicity of S19 vaccine in mice. So, the plates for counting of challenge strain in target organs should be incubated in a 10% CO_2 atmosphere and in air. This would solve the problem of immunogenicity tests where the growth of vaccine strain together to the challenge strain can give a biased estimation of protection. However, CO_2 requirement is not always stable, subculture provides the opportunity for the development of mutants that are CO_2 independent (Alton *et al.*, 1988). Thus, the challenge strain must be checked for this characteristic before use. *B. aboruts* strain 544 used in our laboratory is not CO_2-dependent, and hence this phenotype cannot be used to differentiate challenge and vaccine strain in studies of immunogenicity of *B. abortus* vaccines in the mouse model.

In summary, the overall results suggest that differentiation between S19 and challenge strains 544 and 2308 can be achieved by the use of i-erythritol (1 mg/mL). Media containing thionin (10 μg/mL) can differentiate between S19 or RB51 and *B. abortus* 2308. Furthermore, RB51 can be differentiated from challenge strains by growth on rifampicin (200 μg/mL). For CO_2-dependent *B. abortus* strain 544, differentiation from vaccine strains can be attained by the lack of growth on plates incubated in air.

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