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EVALUATION OF BRUCELLOSIS VACCINES IN BRAZIL

(AVALIAÇÃO DE VACINAS CONTRA BRUCELOSE BOVINA NO BRASIL)

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Para meu marido, Roberto, pelo amor e incentivo.
Para minha mãe, Eliane, Daniel, Caio e Marina pelo amor e apoio.

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RESUMO

O Ministério da Agricultura, Pecuária e Abastecimento lançou em 2001 o Programa Nacional de Controle e Erradicação da Brucelose e Tuberculose (PNCEBT), no qual a vacinação é uma das mais importantes ferramentas contra brucelose. Assim, o presente trabalho foi idealizado para estudar as vacinas aprovadas pelo PNCEBT no Brasil. Neste contexto, o primeiro objetivo foi avaliar o crescimento de amostras de *B. abortus* em meios contendo diferentes agentes inibidores a fim de diferenciar as amostras vacinais B19 e RB51 de amostras-desafio e de outros isolados de campo. Com este estudo, nós concluímos que rifampicina, eritritol e tionina são bons agentes inibidores para tal diferenciação. O segundo objetivo foi avaliar diferentes linhagens de camundongo (CD-1, BALB-c e Suíço) e amostras-desafio (544 e 2308) em testes de imunogenicidade das vacinas B19 e RB51. O experimento demonstrou que ambas amostras-desafio podem ser utilizadas em testes de imunogenicidade de B19 e RB51, bem como as três linhagens de camundongos, porém o uso de BALB-c requer mais estudos para estelecer parâmetros próprios. Esses dois primeiros estudos foram desenvolvidos para padronizar metodologias para se conduzir o terceiro estudo, que objetivou a comparação das propriedades biológicas (imunogenicidade e virulência residual) e perfil gentípico de oito vacinas B19 comercializadas no Brazil com a B19 de referência obtida do USDA. Esse estudo comprovou que todas as vacinas B19 comercializadas no país apresentam propriedades biológicas adequadas quando comparadas à amostra de referência. Além disso, nós concluímos que o grupo de vacinas B19 estudado é geneticamente bastante homogêneo. O último objetivo deste trabalho foi avaliar a liberação de *B. abortus* no leite de vacas vacinadas com RB51. O leite das vacas vacinadas foi testado por cultivo e PCR até 63 dias pós-vacinação e apenas uma amostra de leite foi positiva no PCR, no primeiro dia pós-vacinação. Logo, a eliminação de RB51 no leite parece não representar um problema de saúde pública. No entanto, a pasteurização do leite de vacas recentemente vacinadas com RB51 é altamente recomendada. Concluindo, o Brasil tem ótimas ferramentas para promover o controle da brucelose.

Palavras-chave: brucelose bovina, controle de vacinas, B19, RB51.

ABSTRACT

The Brazilian Ministry of Agriculture, Livestock and Food Supply launched in 2001 the National Program on the Control and Eradication of Brucellosis and Tuberculosis (PNCEBT), in which vaccination is one of the most important tools to control brucellosis. Thus, the present work was idealized to study the vaccines approved by the PNCEBT in Brazil. In this context, the first objective was to evaluate the growth of *B. abortus* strains on media containing different inhibitor agents in order to differentiate the vaccine strains S19 and RB51 from challenge strains and other Brazilian field isolates. With this study, we concluded that rifampicin, erythritol and thionin are good inhibitor agents for this differentiation. The second aim was to evaluate different strains of mice (CD-1, BALB-c and Swiss), and challenge strains (544 and 2308), in the immunogenicity tests of S19 and RB51 vaccines. This experiment demonstrated that both challenge strains can be used in immunogenicity tests of S19 and RB51, as well as the three strains of mice; however the use of BALB-c requires more studies to stablish proper paramenters. These two first studies were carried out to standardize methodologies to conduct the third study that aimed to compare the biological properties (immunogenicity and residual virulence) and genotypic profile of eight S19 vaccines commercialized in Brazil with the reference S19 obtained from USDA. This study comproved that all S19 vaccines commercialized in Brazil show adequate biological properties when compared to the reference vaccine strain. In addition, we concluded that the group of S19 vaccines studied is genetically very homogenous. The last goal of this study was to evaluate the shedding of *B. abortus* in the milk of cows vaccinated with RB51. The milk of vaccinated animals was tested by culture and PCR up to 63 days after vaccination and only one sample of milk was positive in PCR, on the first day after vaccination. Thus, the spread of RB51 by milk seems not to be a public health problem. Nevertheless the pasteurization of the milk from cows recently vaccinated with RB51 is highly recommended. In conclusion, Brazil has great tools to promove brucellosis control.

Key words: bovine brucellosis, vaccine control, S19, RB51.

Chapter I – Background

1. Introduction

Brucellosis is a highly contagious disease that affects several species of animals and men. Caused by bacteria of the genus *Brucella*, it is considered by the Food and Agriculture Organization (FAO) and World Organization for Animal Health (OIE) one of the most important and widespread diseases in the world (WHO, 1997). In different species of domestic animals it is an important cause of abortion, sometimes followed by temporary or permanent infertility (Thoen et al., 1995). In humans, it causes a disease characterized by undulant fever and, in the chronic phase, endocarditis, arthritis and osteomyelitis (Sauret and Vilissova, 2002, Santos et al., 2005). The genus *Brucella* has six classically described species: *B. abortus*, *B. canis*, *B. melitensis*, *B. neotomae*, *B. ovis* and *B. suis* (Corbel and Brinley-Morgan, 1984). Recently, new species of *Brucella* infecting aquatic mammals (Ross et al., 1994; Corbel and Banai, 2005; Bourg et al., 2007; Foster et al., 2007) and wild common voles (Scholz et al., 2008) have been described.

Brazil has the largest commercial cattle herd in the world, with more than 160 million animals. Livestock farming is .an important pillar of Brazilian economy. With the improvement of the foot and mouth disease (FMD) free zone with vaccination in recent years (Brasil, 2000), the international market for Brazilian products is expanding. However, this market requires safe food products. The main requirements, which are becoming sanitary barriers in the post - FMD are brucellosis and tuberculosis controls.

With the intensification of the brucellosis control, great efforts must be made to overcome the challenge of controlling and eradicating this zoonosis with equal success achieved by recent programs for the eradication of foot and mouth disease and classical swine fever.

The practice of vaccination has contributed enormously to the success of many programs, especially at the stage of controlling the disease. The vaccines that had greater success in the prevention of brucellosis were prepared with live attenuated strains of *Brucella* spp. The quality of the vaccines is essential for the success of the program for control and eradication of brucellosis launched in the country.

Aiming at improving the brucellosis and tuberculosis control in the country and the production of safe food products for the internal and external market, the Ministério da Agricultura, Pecuária e Abastecimento (MAPA - Brazilian Ministry of Agriculture, Livestock and Food Supply) launched in 2001 (Brasil, 2004) the Programa Nacional de Controle e Erradicação da Brucelose e Tuberculose (PNCEBT - National Program on the Control and Eradication of Brucellosis and Tuberculosis). From 2001 on, the major activities of PNCEBT were the establishment of mandatory S19 vaccination program, studies of the prevalence of brucellosis in various States of the country and training of professionals to work on the program.

For the success of the vaccination program, it is necessary good, standardized and high quality vaccines. The S19 vaccines commercialized in Brazil must be approved by the quality control of the producer laboratory before sending to the official control. The Normative Instruction 15 of the Secretaria de Defesa Agropecuária (SDA) of MAPA, rules the quality of brucellosis vaccines in the country and includes: purity, pH, dissociation, humity, viable bacterial counts, negative pressure, thermal stability and analysis of diluents (Brasil, 2004 b).

A recent study showed important data about the control tests applied on S19 vaccines in Brazil by the official control (Caldeira, 2008). In this work it was determined the bacterial counts at approval, at the expiration

date and by the accelerate stability test. The conclusion was that among the S19 vaccines commercialized in Brazil, a high percentage of non approved batches of vaccines with expiration time longer than 12 months was observed. In addition, the author concluded that the accelerate stability test was not useful to estimate bacterial counts at expiration dates.

Despite of the controls carried out either by the producer laboratory or by the official laboratory control, the origin and preservation of the seed strains used for the production of S19 vaccine in Brazil is unknown. It is also unknown among S19 vaccines marketed in Brazil, if there is any difference concerning protection, or if they confer any adequate protection at all.

With the advance of international trade, there is a need to harmonize technical requirements and to develop technical levels of quality control of veterinary vaccines for assuring quality, efficacy, potency and safety. Standards are established by relevant laws and directives in each country or region. The World Organization for Animal Health (OIE) hopes that the exchange of information may help to develop international standardization of veterinary vaccines (Makie, 1998).

In addition to the mandatory vaccination of female calves with the S19 vaccine, the PNCEBT also recommends the use of RB51 vaccine in heifers over 8 months of age (Brasil, 2007). The use of RB51 vaccine in Brazil is relatively recent and the routine quality tests for this vaccine are not yet well defined. RB51 vaccine has proven to be protective in many studies (Schurig et al., 1991; Cheville et al., 1993; Uzal et al., 2000; Poester et al., 2006). However, it is a live vaccine that can infect humans, although its level of pathogenicity in humans appears to be less than that of S19 (Ashford et al., 2004). Moreover, it was approved to be used in Brazil as full dose (calf dose) in adult animals, particularly in dairy animals. So, it is necessary to evaluate the possibility of shedding this vaccine strain by the milk of recently vaccinated cows, which could represent a risk to public health.

2. Objectives

The present work was idealized to to study the vaccines approved by the PNCEBT in Brazil. Thus, the objectives were:

1. To evaluate different strains of mice (CD-1, BALB/c and Swiss), and challenging strains (544 and 2308), in the potency test of S19 and RB51 vaccines;

2. To evaluate the growth of *B. abortus* biovar 1 strains on media containing different inhibitor agents in order to differentiate the vaccine strains S19 and RB51 from strain 2308 and other Brazilian field isolates;

3. To compare the biological properties (immunogenicity and residual virulence) and genotypic profile of eight S19 vaccines commercialized in Brazil with the reference S19 obtained from USDA;

4. To evaluate the shedding of *B. abortus* in the milk of cows vaccinated with RB51.

3. Literature review

3.1 *Brucella*

Brucella is an α *Proteobacteria* that causes an infectious disease of mammals that can be transmitted to humans. *Brucella* are small coccobacilli or short rods, Gram-negative, facultative intracellular, nonmotile bacteria. They do not form spores, flagella, pili or true capsula. Six classical species are currently recognized within the genus *Brucella* (*B. melitensis, B. abortus, B. suis, B. neotomae, B.ovis* and *B. canis*) (Corbel and Brinley-Morgan, 1984) and the recent species described infecting aquatic mammals (Ewalt et al., 1994; Ross et al., 1994; Clavareau et al., 1998; Corbel and Banai, 2005; Bourg et al., 2007; Foster et al., 1996; Foster et al., 2007) and wild common voles (Scholz et al., 2008). These species are classified mainly based on the difference in pathogenicity and in host preference. The most pathogenic species worldwide are *B. abortus,* the main agent of bovine brucellosis; *B. melitensis,* responsible for ovine and caprine brucellosis and for the most severe infections in humans; and *B. suis,* responsible for swine brucellosis. These three *Brucella* species may cause abortion and subfertility in their hosts, which results in huge economic losses. *B. ovis* and *B. canis* are responsible for ram epididimitis and canine brucellosis, respectively. *B. neotomae* was only isolated from desert rats (*Neotoma lepida*). *B. melitensis, B. abortus* and *B. suis* are classified into several biovars. Species and biovars are currently identified by phenotypic differential tests based on by A, M and R serotyping, phage typing, dye sensitivity, CO_2 requirement, H_2S production, and metabolic properties (Alton et al., 1988).

Phylogenetically, the genus *Brucella* belongs to the Rhizobaceae group of bacteria (Yanagi and Yamasato, 1993). DNA-DNA hybridization studies revealed a high degree of homology (>90%) between species (Verger et al., 1985, 1987, 2000). *Brucella* spp. constitute a monospecific genus, for which *B. melitensis* has been proposed as the sole representative species (Verger et al., 1985, 1987). The other *Brucella* species would be considered as biovars of *B. melitensis.* However, the classical organization of the genus into species is still preferred, as it is in accordance with the pathogenicity and host preference characteristics of each species.

B. melitensis, B. abortus and *B.suis* strains may occur as either smooth (S) or rough (R) strains. Smooth strains express smooth lipolysaccharide (S-LPS), containing O-chain and rough strains express rough lipoplysaccharide (R-LPS), lacking the O-chain. *B. ovis* and *B. canis* are two naturally R species, expressing thus R-LPS as major surface antigen. The O-chain moiety represents the most exposed antigenic structure of the *Brucella* cell surface and has been shown to be an important protective antigen against S-*Brucella* infection (Cloeckaert et al., 1990).

Ruminants and swines are heavily infected in many areas of the world, and wildlife is not exempt of brucellosis, thus acting as a potential risk for humans and cattle. *Brucella* spp. differ in their host preference, physiological abilities and cell surface structural characteristics (Alton, 1988). Those affecting livestock are *B. abortus* (cattle), *B. melitensis* (sheep and goats), *B. suis* (swine), and *B. ovis* (sheep).

3.2 Bovine Brucellosis

Brucellosis in cattle is usually caused by biovars of *Brucella abortus.* In areas where cattle are kept in close association with sheep or goats, infection can also be caused by *B. melitensis.* Occasionally, *B. suis* may cause infection in the mammary gland of cattle, but it has not been reported to cause abortion (Ewalt et al., 1997).

The disease is usually asymptomatic in non pregnant females. Following infection with

B. abortus or *B. melitensis*, pregnant adult females develop placentitis usually resulting in abortion between the fifth and ninth month of pregnancy. Even in the absence of abortion, profuse excretion of the organism occurs in the placenta, fetal fluids and vaginal discharges. The mammary gland and associated lymph nodes may also be infected, and organisms may be excreted in the milk. Xavier et al. (2009) demonstrated in a study of pathology, immunohistochemestry, and bacteriology of tissues and milk of experimentally infected cows, that *B. abortus* was detected in milk and mammary tissues of many animals. *B.* abortus-induced mastitis has been described (Emminger and Schalm, 1943, Xavier et al., 2009) and it has been demonstrated that an infected cow that develops mastitis has a high risk of shedding the pathogen in the milk, which may be destined to human consumption (Xavier et al., 2009). Subsequent pregnancies are usually carried to term, but uterine and mammary infection recurs, with reduced numbers of organisms in uterine discharge products and milk. In acute infections, the organism is present in several lymph nodes. Adult male cattle may develop orchitis and brucellosis may be a cause of infertility in both sexes. Carpal hygromas, are common manifestations of brucellosis in some tropical countries and may be the only obvious evidence of infection in a herd. The hygroma fluids are often infected with *Brucella* (OIE, 2004)

Bovine brucellosis is considered a serious animal and public health problem. It is a worldwide zoonosis and its prevalence is higher in developing countries and lower in developed ones, where it is in some countries almost eradicated (Crawford et al., 1990; Young, 1995). Being a zoonosis, almost every human case has an animal origin, thus the eradication of the disease is primarily a veterinary responsibility (Nicoletti, 2002). Countries like the United States of America, which have implemented severe control measures for more than five decades, such as the slaughter of infected animals and vaccination, the number of human brucellosis cases has greatly reduced (Ragan, 2002).

The economic importance attributed to bovine brucellosis is based on direct losses caused by abortions, stillbirths, early disposal and birth of weak calves, death of animals, weight loss, decreased milk production, ,disposal of high genetic value animals and carcasses condemnations (Thoen et al., 1995). It is estimated that an infected animal looses 10 to 25% of their productive efficiency. Another frequent consequence that occurs is the loss of credibility of the property, even after the eradication of the disease. The regional and national economies are also affected once the international trading rules require that animals and animal products must be originated from countries or areas free of infection.

The detailed knowledge of the pathogenesis and epidemiology of brucellosis is crucial for the success of control and eradication programs. Large quantities of *B. abortus* are discharged into the environment during parturition or abortion. After the intake by a susceptible animal, the bacteria invade the oral mucosa and the regional lymph nodes. After a brief bacteremia, *Brucella* spp. can be found in the uterus, placenta, udder and regional lymph nodes. The most common clinical sign in cattle is abortion in the final term of gestation. In the period between exposure and seroconversion infected animals may present negative serological results. In general cows do not transmit the infection except during the time of delivery or abortion. Generally within 30 days after delivery or abortion, cows do not transmit the infection and remain in this condition until the next gestation (Enright, 1990; Ragan, 2002).

The chronicity of infection results from the ability of *Brucella* to survive reactive oxygen intermediate and nitric oxide killing in host phagocytes, following which they activate bacterial genes in response to the acidic phagosome environment, prevent

phagolysosomal fusion by remodeling the intracellular compartment, and subsequently replicate intracellularly. The crucial component of immunity that results in survival of the host and thus maintenance of this chronic infective state is interferon-γ (IFN-y). Production of IFN-γ results from the ability of *Brucella* components, including lipid A, to interact with Toll-like receptors for the production of IL-12 and TNF-α. Although CD4 and CD8 T cells are clearly involved in the production of IFN-γ, and CD8 T cells may be cytotoxic, a role for NK cells and cytotoxicity in protective immunity to brucellosis has not been substantiated experimentally. Moreover, antibodies have been shown to have a limited role in passive transfer studies (Baldwin and Goenka, 2006).

3.3 Zoonotic aspects of brucellosis

Brucellosis is a widespread zoonosis mainly transmitted from cattle, sheep, goats, pigs and camels through direct contact with infected placenta, fetuses or uterine secretions, or through consumption of contaminated raw products (especially unpasteurized milk and soft cheese). The infection is acquired by different ways. Ingestion is the most frequent due to the consumption of unpasteurized milk. The infection by direct contact is more frequent among farmers, veterinarians, farm workers, slaughter works, etc. where the bacteria may invades through damaged skin or by mucosal epithelium of the conjunctiva and lachrymal ducts. Other forms of invasion include inhaling of dried substances from infected animals such dust, wool in the corral or in the laboratory by sprays. Accidental inoculation can also occur when handling syringes with live germs, especially during vaccination (Aguilera, 2005). Moreover, human brucellosis is a severe and debilitating disease requiring a prolonged antibiotic treatment and often leaving permanent and disabling sequelae (Ariza, 1999).

Brucella spp. are classified within Risk III group, because it is potentially transmitted by aerosols and in some cases are able to cause lethal infection. Brucellosis is one of the most easily acquired laboratory infections (Al-Aska and Chagla, 1989; Novielo et al., 2004), and strict safety precautions should be observed when handling cultures and heavily infected samples, such as products of abortion. Laboratory manipulation of live cultures or contaminated material from infected animals is hazardous and must be done under containment level 3 or higher (OIE, 2004).

In endemic areas, human brucellosis has serious public health consequences. In countries where eradication in animals (through vaccination and/or elimination of infected animals) is not feasible, prevention of human infection is primarily based on sanitary education, food-safety measures, occupational hygiene and laboratory safety. In most countries, brucellosis is a notifiable disease.

Human brucellosis may present an acute or insidious onset, with continued, intermittent or irregular fever of variable duration, profuse sweating, fatigue, anorexia, weight loss, headache, arthralgia and generalized aching. Abscess formation is a rare complication. *Brucella* spp. endocarditis and neurobrucellosis may be cause of death (Young, 1995).

Treatment is usually done with doxycycline and streptomycin/gentamicin or doxycyclin and rifampicin. Optimal treatment in pregnant women, neonates and children is trimetoprim/sulfamethoxazole combined with an aminoglycoside (streptomycin, gentamycin) or rifampicin (Solera et al., 1997).

The prevention of human cases needs education to avoid consuming unpasteurized milk and milk derivatives, barrier precautions for professionals at risk (butchers, farmers, slaughterers, and veterinarians), careful handling and disposal

of afterbirths, especially in cases of abortion. Serological testing of animals; immunization of herds may be envisaged with elimination of infected herds. Brucellosis is a true zoonosis and the control of the disease in humans is largely a veterinary responsibility (Nicoletti, 2005).

3.4 Anti-bovine brucellosis vaccines

The most successful method for prevention and control of brucellosis in animals is vaccination. It has been a critical component of most brucellosis control or eradication programs throughout the world. Even when prevalence of brucellosis is low, stop vaccination with reliance only on test and removal of infected animals can be associated with resurgence of human and/or livestock brucellosis (Olsen and Stoffregen, 2005).

The development of an efficacious vaccine for brucellosis has been a challenge for scientists for many years. Despite the availability of good smooth live vaccines like S19 for cattle and Rev1 for small ruminants and a further rough attenuated strain, RB51 for cattle, the search for improved vaccines has never ended (Cutler at al., 2005). The reasons for this are, in part due to the remaining virulence of the live vaccines for humans (Hoover et al., 2004), the residual abortifacient potential of smooth vaccines for pregnant animals (Blasco, 1997) and their interference with conventional serological tests (Schurig et al., 2002). It is generally agreed that all of the available vaccines are only efficacious in specific hosts, and cross-protection is not readily achieved (Cutler et al., 1998).

The characteristics of some anti-brucellosis vaccines are described. In this review we present information only on the vaccines that are/were actually used (S19, 45/20 and RB51), and those under study or in development will not be mentioned.

B. abortus strain 19 has been the most widely used vaccine for prevention of bovine brucellosis for more than six decades. This strain was first described by Buck in 1930. It was originally isolated from milk of a Jersey cow as a virulent strain in 1923, but after being kept in the laboratory at room temperature for over a year, was found to have become attenuated (Buck, 1930) and able to induce protective immunity in cattle. Strain 19 is an attenuated smooth organism, normally unable to grow in the presence of erythritol (Jones et al., 1965). As a smooth strain, it induces serologic responses on most brucellosis surveillance tests that cannot be differentiated from antibody responses caused by infection with field strains. The low and stable pathogenicity, the relatively high immunogenicity and antigenicity are characteristics of this strain that have contributed to its use as a vaccine. The attenuation, its culture characteristics and biological stability do not change after several passages in guinea pigs or by intravenous passage in pregnant female cattle (Mingle et al., 1941).

The efficacy of this vaccine depends on a number of variables such as age at vaccination, dose, route and prevalence of brucellosis (Nicoletti et al., 1990). The length of protection conferred by S19 is estimated to be around seven years (Nicoletti et al., 1990; Schurig et al., 2002). The infection with this vaccine strain usually persists for few weeks, but in rare cases it can persist for more than two years (Meyer and Nelson, 1967).

The live attenuated S19 strain is considered the best vaccine available for the prophylaxis of brucellosis in cattle. However, one of the disadvantages of this vaccine includes the fact that in some circumstances it can cause abortion in pregnant animals (Corner and Alton, 1981) or orchitis in males (Nicoletti, 1990), and is pathogenic for humans (Young, 1995).

The 45/20 is a vaccine prepared with heat-killed 45/20 *B. abortus* with oil adjuvants (McEwen and Samuel, 1955). The smooth strain 45/0 was firstly isolated from a cow in 1922 and a rough derivative was obtained after 20 passages in guinea pigs; this derivative called strain 45/20, was able to protect guinea pigs and cattle from *Brucella* infection (McEwen and Priesley, 1938; McEwen, 1940). However, when used as a live vaccine, strain 45/20 was not stable and tended to revert to the smooth, virulent form, thereby defeating the purpose of using live rough strains. Because of this, it was used as a bacterin incorporated in adjuvants usually based on water in oil emulsions. Strain 45/20 did not cause abortions when used as bacterin. Batch to batch variations occurred and no satisfactory means of controlling this was available. The variability of the reported protection, together with unpredictable serological effects and the occurrence of severe local reactions at the site of vaccine injection in some animals, eventually prompted the discontinuation of the strain 45/20 vaccination (Schurig et al., 2002).

The strain RB51 is a stable, rifampicin-resistant, rough mutant, derived from smooth *B. abortus* strain 2308, which lacks the O-chain, or contains an insufficient amount of this molecule to induce the production of O-chain specific antibodies in immunized animals, overcoming the serologic problems. It was derived by repeated passages of strain 2308 on trypticase soy agar supplemented with varying concentrations of rifampicin and/or penicillin. Its roughness is very stable after multiple passages *in vitro* and *in vivo* through various species of animals (Schurig et al., 1991).

This strain is attenuated as indicated by studies carried out in mice, guinea pigs, goats and cattle, from all of which it is cleared in a relatively short period of time, usually less than 14 weeks (Olsen et al., 1999). Furthermore, it has no, or highly reduced, abortifacient characteristics (Schurig et al., 1991, Palmer et al., 1996, Roop et al., 1991). When used in single vaccination protocols the protection in cattle is similar to that induced by strain 19 (Cheville et al., 1992; 1996), According to Poester et al. (2006), the protection conferred in adult cows by RB51 against infection is 65%, while Cheville et al. (1996) encountered a protection of 87%.

RB51 carries an IS711 element spontaneously inserted into *wboA* (putatively coding for a glycosiltransferase), but carries additional and unknown defects (Schurig et al., 2002). Some studies suggest that other LPS genes, such the *wzt*, involved in the transportation of O-chain to the surface of bacteria are affected (Vemulapalli et al., 2000; Cloeckart et al., 2002).

RB51 is important as a booster in animals previously immunized during calfhood with S19. Used as such, it may provide immunological stimulation, without the inconvenience of antibodies specific to the lipopolysaccharide (LPS) (Moriyon et al., 2004).

Olsen and Stoffregen (2005), in a review on vaccines in brucellosis, conclude that S19 might be slightly more efficacious than RB51 under experimental conditions. The S19 might be the vaccine of choice for areas with a high prevalence of brucellosis. As many countries currently produce S19 vaccines, but do not produce a commercial RB51 product, use of S19 vaccine may also be less expensive. However, in areas where prevalence of brucellosis is low, serologic surveillance is high and regulatory programs are nearing their goal of brucellosis eradication, RB51 might be preferred to S19 owing to its lack of interference with surveillance activities and similar efficacy in protecting against brucellosis.

Many other vaccines have been developed, including nonliving vaccines, such as killed preparations or antigenic fractions with limited success, compared with the live attenuated vaccines. Novel adjuvants and delivery systems could be utilized to both

enhance and focus the immune response against subunit antigens (Cutler et al., 2005). DNA vaccines offer the possibility of inducing both cellular and humoral responses. Different antigens have been explored for their value as DNA vaccines against brucellosis providing various levels of protective efficacy in the mouse model (Al-Mariri et al., 2001, Leclercq et al., 2002, Velikovsky et al., 2002, Oñate et al., 2003). Disavantages of the DNA vaccination approach are the amount of DNA required to elicit response, and the often disappointing results obtained following assessment of the vaccines in the target animal (Babiuk et al., 2003). Investigation of enhanced delivery mechanisms may overcome these issues (Cutler et al., 2005).

3.5 Evaluation of anti-*Brucella abortus* vaccines

The most suitable way to control *Brucella* infections in cattle is by vaccination with the classical smooth *B. abortus* S19 or RB51 live vaccines (Nicoletti, 1990, Cheville et al., 1993, Schurig et al., 1991). The use of good quality commercial batches of these attenuated vaccines is an essential prerequisite for the success of vaccination campaigns.

While acknowledging that manufacturers are responsible for the quality of the vaccines they produce, the World Health Organization proposes a definition for "vaccines of assured quality" which depends on the existence of a component and fully functional regulatory authority as assessed by an external expert team using widely agreed indicators to regulate the product. "A vaccine of assured quality is defined as one that consistently meets appropriate levels of purity, potency, safety and efficacy as judged through an independent review system component to take an evidence-based decision on the product for a specified population in a specific context" (Milward, 1995) .

Although standard protocols basic methods for the production and control of brucellosis vaccines have been published, their production at a large scale requires additional expertise. At all levels, many factors must be taken into account to insure that the quality of the production output is homogeneous from batch to batch. Milward (1995) emphasizes that authorities must consider all these factors when implementing a brucellosis control program to guarantee that the vaccine they are using is of high quality, and reminds that although the cost of the vaccine is only one fraction of the total cost of a control program, its quality will directly and dramatically affect the outcome of the program.

Batch quality control must be considered as a series of methods that will detect gross defects of a production run and certain tests, such as immunogenicity in animals are cumbersome and costly to be applied on each batch (Milward, 1995). Quality control of anti-*Brucella* live vaccines is generally based on the exclusive *in vitro* criteria examination such as absence of contamination, identity (assessment of the typical colonial morphology), smoothness, enumeration of live bacteria, stability, pH and humidity (Brasil, 2004b; OIE, 2006).

3.5.1 Production and quality control of S19 vaccines

Technicians involved in the production of vaccines for brucellosis are at high risk of acquiring an infection as well as causing a dissemination of the agent. All technicians should be regularly tested for antibodies to *Brucella* antigens. Personnel must be well trained and reminded of the requirements for handling a class III pathogen (Milward, 1995).

According to Brasil (2004b), the initial strain used in the vaccine production must be obtained from an international reference centre recognized by MAPA. It must be accompanied by a certificate, adequately characterized, of uniform composition, with

proved safety and efficacy in female calf. It must be lyophilized and kept between 2°C and 8°C. Having obtained the suitable initial seed, the responsibility of the producing laboratory is to prepare and maintain a master seed lot and production seed lots in such a way that the characteristics of the initial seed are completely preserved. By performing two passages from the initial strain, enough seed lots can be produced with identical characteristics for a large number of vaccine batches. This procedure ensures that each seed lot is not more than three passages removed from the initial seed (Brasil, 2004b; Milward, 1995). Care must be taken at all steps of the production to ensure batch homogeneity (culture, harvest, ampoule filling, freeze-drying). Quality control of the production seed is critical. It must be identical to the master and initial seed. According to Milward (1995), using one ampoule of initial seed it is possible to produce 48 ampoules of master seed. And one ampoule of master seed originates other 48 ampoules of production seed lots.

The production and control of vaccine lots must be conducted according to a technical report of product registration. The regulation must be strictly followed and all steps of production must be registered in order to allow investigation of problems (Brasil, 2004). All final product lots are submitted to official control, which includes purity, dissociation, viable count, which must be between $6.0x10^{10}$ UFC/doses and $1.2x10^{10}$ UFC/doses when commercialized and not lower than $4.0\ x10^{10}$ UFC/doses at expiry, pH, humidity and pressure, which must be negative. Diluents are also tested. A S19 vaccine lot is approved when results in all tests are satisfactory (MAPA, 2004). Brazilian official control methods include physical-chemical controls and biological control *in vitro*. It does not include biological controls *in vivo*, which may be applied to each seed lot and the first batch of vaccine from a new seed lot according to OIE, WHO and FAO (Food and Agriculture Organization of the United Nations) (Milward, 1995; Nielsen and Ewalt, 2008)

Control methods should conform to international and local authority requirements. Stringent standards are necessary to insure that vaccines being used in long term control programs are of consistent quality.

Despite all the control methods applied by Brazilian official control, those *in vitro* requirements do not guarantee the immunological quality of vaccines, because vaccine batches with acceptable microbiological characteristics can show deficient immunological behavior (Bosseray, 1992, 1993). For an adequate quality control, those *in vitro* criteria should be complemented with the assessment of biological properties, which can be studied in laboratory models. Furthermore, the OIE (Nielsen and Ewalt, 2008) recommend checking representative seed lot batches of Rev 1 and S19 vaccines in those models. Briefly, these *in vivo* tests comprise two complementary studies based on spleen infection assays, i.e. residual virulence (Recovery Time 50%, RT_{50}) and immunogenicity or potency (OIE, 2004).

3.5.2 Laboratory animal models

Because of the high costs and long time span of the experiments in natural hosts, laboratory animals have been used as a preliminary step in the analyses of vaccines against brucellosis (Cloeckaert et al, 2004; Kurar and Splitter, 1997, Al-Mariri et al., 2001, Leclercq et al., 2002, Velikovsky et al., 2002, Oñate et al., 2003).

The ideal laboratory animal as an experimental model for brucellosis is the one that can develop lesions similar to those encountered in natural hosts, become infected through the same routes, have the same sensitivity, similar clinical signs and the same pathological changes. Findings in laboratory animals can sometimes, but not always, correlates to the findings in man, cattle, goats, etc. So, correlations can be established with some limitations (García-

Carrillo, 1990). The ideal method for vaccine testing should also be simple, quick and inexpensive, and should also give clear-cut results. In practice, guinea pigs and mice have been the laboratory animals used for this purpose. Only few studies use other laboratory models, which include rabbits, rats, hamsters, and gerbils.

3.5.2.1 Guinea pig

Guinea pigs (*Cavia porcellus*) are probably the animals that are most susceptible to *Brucella* spp. infection. As few as 11 cells of *Brucella* spp. are sufficient to cause infection (García-Carrilo and Casas-Olascoaga, 1977). They are very sensitive to infection by any route: subcutaneous, conjunctival, intraperitoneal, intranasal, intravenous, vaginal, oral or cutaneous scarification (García-Carrillo, 1990).

Female guinea pigs have generally been preferred for *Brucella* spp. studies, even though sensitivity to *Brucella* spp. infection is practically the same for both sexes (García-Carrillo, 1978).

Splenic lesions are very common. It is easy to observe macroscopic alterations in infected guinea pigs, which can be a discrete hypertrophy or a slight increase in spleen size relative to the expected normal size (García-Carrillo, 1990). Many researchers use alterations of splenic index (ratio of the spleen weight to the body weight of the animal) as indicators of infection, but García-Carrillo (1990) advertises that this ratio increases from birth to a body weight of 300 to 400g, with different curves for males and females. Thereafter, the ratio decreases for the rest of the animal's life. So, comparisons of indices are valid between groups only when they have equal body weights and are of the same sex.

Some authors consider that the guinea pig model is considerably more valuable than the mouse model for the evaluation of new vaccines. They defend that because, occasionally, results that were promising in mice failed to materialize in guinea pigs, whereas the results obtained using guinea pigs were usually correlated with those obtained in other animals (García-Carrillo, 1990).

3.5.2.2 Mouse

There are several hundred different strains of mouse (*Mus musculus*), including inbred, mutants and outbred strains. All have their specific characteristics and there is considerable variability in many of their physiologic parameters. So, their susceptibility to infections also varies according to their genetic constitution (García-Carrillo, 1990).

Many authors use mice for the initial testing of proposed vaccines for their immunogenic properties (Bosseray et al., 1984). Their main reason is that mice are less costly than other animals. Sex is important, according to García-Carrillo (1990), females are more resistant to brucellosis. Nielsen and Ewalt (2008) recommend the use of mice between 5 and 7 weeks of age. The same authors suggest that challenge should be done intraperitoneally. Duration of experiment with mouse model depends on the propose of the study, immunogenicity tests takes around 45 days, while residual virulence study takes 12 weeks of duration (Nielsen and Ewalt, 2008).

Bosseray et al. (1984) suggest mice as the animal model for titration of biological activity and quality control of anti-*Brucella* spp. vaccines. Immunity is evaluated relative to the number of *Brucella* spp. organisms recovered from the spleen.

Pugh Jr. et al. (1989) compared responses of five strains of mice (CBA/NJ, BALB/c, CD-1, C3H/HeN and C3H/HeJ) to *B. abortus* strain 2308 and conclude that mice can be used for comparative studies on the pathogenesis and immunogenesis of *B. abortus* infections and strains may vary in their responses to *Brucella* spp. infection. The CD-1 mice were studied in detail for

studies of brucellosis vaccines (Bosseray et al., 1984, Bosseray, 1993, Bosseray and Plommet, 1990).

Some authors use strain CD-1, which is the mouse strain recommended by OIE, to test new vaccines or to study or compare classical vaccines (Tibor et al., 1998; Plommet and Plommet, 1981; Bosseray and Plommet, 1990; Bosseray et al., 1984; Grilló et al., 2000; Nielsen and Ewalt, 2008). Nevertheless, the majority of the authors use BALB/c (Stevens et al., 1995; Kurar and Splitter, 1997; Cloeckaert et al., 2004; Oñate et al., 1999; Winter et al., 1996) or Swiss (Plommet et al., 1982).

3.5.3 Residual virulence

This parameter is useful in checking that there has been no change in the virulence of the attenuated vaccine strains *B. abortus* strain 19 or *B. melitensis* Rev. 1, but it can be used to test any attenuated vaccine. While virulent *Brucella* spp. cause long-lasting infections in mice, vaccine strains are usually eradicated in a short time. There are some different methods to test the virulence of *Brucella* spp. strains in laboratory animals.

The residual virulence can be expressed as the Recovery Time (RT_{50}) of the infection (Grilló et al, 2000; Bosseray, 1993), which means the time (in weeks) after inoculation at which half of the mice had recovered from infection in the spleen (Bosseray, 1991). In this test 32 female CD-1 mice, aged 5-6 weeks must be injected subcutaneously with 10^8 CFU/mouse of the test vaccine. In parallel, a similar inoculation is done in another 32 mice using the suspension containing the S19 reference strain, which has been shown satisfactory with respect to immunogenicity and/or residual virulence. The reference strain can be obtained from USDA (United States Department of Agriculture) or INRA (Institut National de la Recherche Agronomique – France). After 3, 6, 9 and 12 weeks mice are killed in groups of eight animals selected at random. Spleens are removed, homogenized in PBS and inoculated onto agar medium to detect any *Brucella* spp. colonies. An animal is considered infected when at least 1 CFU is isolated from the spleen. The number of cured mice (no colonies isolated in the spleen) is determined at each slaughtering point time (eight mice per point) and the percentage of cured accumulated mice over time is calculated by the Reed and Muench method (Bonet-Maury et. al., 1954). The function of distribution of this percentage describes a sigmoid curve, which must be linearised for calculating the RT_{50} values. If there is a parallelism (intercept and slope) between the distribution lines obtained for both tested and reference S19 strains, their RT_{50} values can be statistically compared. If parallelism does not exist, the residual virulence of the tested strain should be considered inadequate, and discarded for vaccine production (Nielsen and Ewalt, 2008). To be accepted for vaccine production, the RT_{50} obtained with the tested strain should not differ significantly from that obtained with the reference S19 strain (RT_{50} and confidence limits are usually around 7.0 ± 1.3 weeks). According to OIE (Nielsen and Ewalt, 2008), if the test has been done with good results on a representative seed lot or batch of the test vaccine, it does not have to be repeated routinely on other vaccine lots prepared from the same seed lot and using the same manufacturing process.

Pouillot et al. (2003) provide a description of the graphical method in which the currently recommended procedure for calculating the RT_{50} is based. According to them this graphical method, which is based on a parallel line assay and a classic probit method, has never been described in detail. Moreover, they describe Rev 2, a dedicated internet interface, as a simple statistical alternative for controlling the biological quality of both S19 and Rev 1 vaccines.

Alton et. al. (1988) suggest a method in which guinea pigs are injected,

intramuscularly, in the thigh with 5,000 cells of the culture under test, 12 weeks after inoculation animals are weighted and killed. Spleens are removed, weighted, lesions are observed and they are used to count *Brucella* spp. colonies. Some parameters are used to check if the strain has the characteristics of a vaccine strain or a virulent one: enlarged spleen and/or nodules, spleen weight, percentage spleens yielding *Brucella* spp., mean colonies/gram spleen, mean spleen/body weight ratio. The same authors consider it is possible to test virulence in mice by a similar protocol. The test uses twenty four female, outbred, Swiss mice, which are inoculated subcutaneously with 10^8 organisms of vaccine to be tested. Eight weeks later, mice are killed, spleens removed, ground in diluent and inoculated onto agar medium to detect any *Brucella* colonies. When the strain in test has a normal residual virulence, not more than 12.5% of the spleens will yield strain 19 and 25 to 50% will yield Rev.1, whereas if the organism inoculated has lost its normal residual virulence, practically 0% o spleens will yield *Brucella* spp.

3.5.4 Potency

Titration of immunogenic activity of anti-*Brucella* spp. vaccines must be done *in vivo*, by challenge of control and vaccinated laboratory animals. Also referred as immunogenicity, it is the ability to protect a laboratory model against a challenge with a virulent strain determined as the number of virulent bacteria in the spleen. The potency of vaccines against brucellosis can be routinely determined in guinea-pigs or mice.

Protective immunity against *Brucella* spp. infection has been studied mainly in mouse models, chiefly BALB/c, Swiss and CD-1 mice (Bosseray et al., 1984; Bosseray and Plommet, 1990; Cloeckaert et al., 2004; Grilló et al., 2000; Kurar and Splitter, 1997; Nielsen and Ewalt, 2008; Oñate et al., 1999; Plommet and Plommet, 1981; Stevens et al., 1995; Tibor et al., 1998; Winter et al., 1996). The criterion used for measuring protection in immunized mice is reduction, at a specific time, after a virulent challenge, of the number of colony-forming units (CFU) of *Brucella* spp. recoverable from the spleen or liver or both.

CD-1 mouse has been recommended by the OIE (Nielsen and Ewalt, 2008) as a reference laboratory animal for the control of S19 vaccines, although its use has not been generalized.

The method proposed by OIE to test immunogenicity of anti-*Brucella* spp. vaccines in mice is based on Bosseray (1992) and Bosseray (1993). The test uses three groups of six female CD-1 mice, aged 5-7 weeks. The vaccine suspensions, test vaccine and reference one must be prepared, adjusted spectrophometrically and injected subcutaneously at a dose containing 10^5 CFU in 0.1 mL/mouse. The third group serves as the unvaccinated group, which is inoculated subcutaneously with 0.1 mL of PBS. All mice are challenged 30 days after vaccination intraperitoneally with a suspension containing 2.0×10^5 CFU in 0.1 mL/mouse of *B. abortus* strain 544, immediately following 16 hours of starvation to make sure that challenge was really administered into the peritoneal cavity. The mice are killed by cervical dislocation 15 days later. Then, spleens are removed, weighted and homogenized in nine times its weight of PBS, to prepare three serial tenfold dilutions. Colonies of *Brucella* spp. are enumerated on tryptose plates. The numbers of *Brucella* spp. per spleen are first recorded as X and expressed as Y after transformation [Y=log(X/logX)]. Then, mean and standard deviation are calculated for each group. The OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Nielsen and Ewalt, 2008) considers the conditions of the experiment satisfactory when: Y value of unvaccinated mice is at least 4.5; Y value of vaccinated mice with the reference S19 vaccine is lower than 2.5 and standard deviation of each group is lower than 0.8. The test vaccine would be satisfactory if the

immunogenicity value of vaccinated mice is significantly lower than that obtained in the unvaccinated controls and does not differ significantly from that obtained in mice vaccinated with the reference vaccine.

Protective activity of living, killed or experimental vaccines against the three main species of *Brucella* can be controlled with this quantitative model (Bosseray and Plommet, 1990).

When testing live vaccines it is necessary to differentiate vaccine and challenge strains, since vaccines may survive until challenge and may be reactivated, induced to multiply again (Plommet and Plommet, 1988). Thus, it is necessary to differentiate them to avoid a biased estimation of protection (Bosseray and Plommet, 1980).

Bosseray and Plommet (1983) proposed the use of a reference vaccine, a lyophilized formalin-killed bacterial cell suspension of *B. melitensis* H38, in experiments with anti-*Brucella* spp. vaccines. According to the authors, from the dose response curve of the group vaccinated with this reference vaccine, the quantities giving reference values would be calculated and expressed in a unit system. It would make possible inter-laboratory comparisons of vaccinal activity, which could be expressed on unit basis, but it has not been widely used.

Although there is a method standardized for the quality control of the classical S live anti-*Brucella* spp. vaccines, which can be extrapolated to test new vaccines and is currently accepted by the OIE, there are variations in the protocols used by different authors (Cloeckaert et al., 2004; Bosseray e Plommet, 1990, Bosseray et al., 1984; Grilló et al. 2000; Guilloteau et al., 2006; Kurar and Splitter, 1997; Oñate et al., 1999; Phillips et al., 1989; Plommet and Plommet, 1981; Plommet et al., 1982; Pugh Jr. et al., 1989; Stevens et al., 1995; Tibor et al., 1998). They differ in breed of animals, route of inoculation, doses (vaccine and challenge), challenge strain and time intervals at which the number of *Brucella* spp. in spleens are determined.

Although the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Nielsen and Ewalt, 2008) indicates the use of *B. abortus* 544 as the challenge strain, it is not widely used. There is a preference by the European groups to use *B. abortus* 544 (Cloeckaert et al., 2004; Guilloteau et al., 2006; Tibor et al., 1998; Bosseray et al., 1984; Plommet and Plommet, 1981; Grilló et al. 2000; Bosseray e Plommet, 1990), while American groups tend to use *B. abortus* 2308 as the challenge strain (Phillips et al., 1989; Pugh Jr. et al., 1989; Stevens et al., 1995; Oñate et al., 1999; Kurar and Splitter, 1997).

Plommet and Bosseray (1984) defend that the protection given by living vaccines is poorly related to the dose, so a standard dose of 1x 10^5 colony-forming is reasonable for testing living vaccines in mice. The route of vaccination used by researchers is also variable. The most used route to test live vaccines is the subcutaneous (Garin-Bastuji, 1995; Grilló et al., 2000)

There are three methods described for assaying the potency of S19 vaccines by challenge tests in guinea-pigs: constant vaccine/graduated challenge, graduated vaccine/constant challenge and single level vaccine and challenge. Thornton and Muskett (1972) studied the response of guinea-pigs to graduated doses of vaccine and challenge (by the first two methods). By the first method, they conclude that the optimal vaccine dose to guinea-pigs is 1/15 cattle dose. By the second method, they conclude that vaccine dose was too high or challenge dose was too low to present a critical test. So, the authors suggest that the single level vaccine and challenge dose method would be satisfactory if used within the range where the protection is proportional to the dose. Le Garrec et al. (1976) studied the kinetics of *Brucella* spp. infection in guinea pigs after various doses of *B. abortus* 544. They suggest the use of a

challenge dose of at least 10^4 bacteria (instead of $5x10^3$ CFU).

3.5.5 Genotypic Profile of vaccine strains

In vivo assays are expensive, laborious and generate a real concern about the use of laboratory animals. *In vitro* tests should be preferred for routine assays when it is possible. If the objective of these i*n vivo* tests, such as immunogenicity and residual virulence, is to guarantee that the vaccine strains remain similar or identical to the reference strain, an *in vitro* test can be suggested for this comparison.

Tandem repeat sequences have been shown as interesting class of markers, because multiples alleles can be present at a single locus, and size differences are easily resolved by eletrophoresis (Lindstedt, 2005). Tandem repeat typing has proven to be highly appropriate for the typing of bacterial species with high genetic homogeneity (Le Flèche et al., 2001). Le Flèche et al. (2006) propose a selection of 15 markers to be used in a Multiple Locus Variable Number Tandem Repeats Analysis (MLVA) assay consisting of two complementary panels, panel 1 (8 markers) and panel 2 (7 markers). The fifteen markers are a combination of moderately variable (minisatellites, panel 1) and highly discriminant (microsatellites, panel 2) loci. Strains clustering together by MLVA frequently have identical origin (Le Flèche et al., 2006).

3.5.6 Efficacy

The real quality of a vaccine should be logically evaluated by studies conducted on the target species. Such studies are necessary when a new product is to be commercialized. They are long and expensive, and impracticable for the routine quality control of vaccines (Garin-Bastuji, 1995; Olsen et al., 1999; Poester et al., 2006). For these reasons this method cannot be done routinely. The efficacy of *B. abortus* S19 has been proven by extensive efficacy tests in cattle (McDiarmid, 1960; Redman et al., 1967). Immunogenicity tests on vaccine batches are, for economic reasons, carried out routinely in small laboratory animals. It is, however, very desirable that the results of such tests should be checked, from time to time, against the results of efficacy tests carried out in cattle (Cocks and Davies, 1973).

According to Moriyón et al. (2004), evaluations in the natural hosts can be performed in controlled experiments and in the field. Controlled experiments are based on clinical, bacteriological and serological findings in homogenous groups. This kind of experiment allows the strict control of the preparation, dosage and application of the vaccine. However, controlled experiments in cattle are very expensive, laborious, and require biosafety level 3 containment facilities, but provide solid data to make conclusions. Controls must include unvaccinated animals and vaccinated with a reference vaccine. Efficacy is determined after a time span that varies on the purpose of the experiments. The animals are usually challenged at 5-6 months of the first pregnancy. It is important to use a reference strain, *B. abortus* 2308 or 544, to prepare the challenge. However, perusal of the literature suggests that strain 544 is more virulent (Moriyón et al., 2004). The animals are usually challenged conjunctivally (that would represent the normal route of entry). Results are accessed by bacteriological procedures, which mean the search of challenge strain in the target organs, to obtain quantitative or semi-quantitative data for each animal.

According to Moriyón et al., 2004, a “real” test could be represented by observational studies, however the authors emphasizes some confounding factors, such as selection of animals (not only based on serological tests), breed, age, sex and reproductive status of the animals, the prevalence of the disease in the area of the study, the method for preparing and applying the vaccine, general management conditions, the use of control groups, the system of monitoring

disease and time span of the study, which should be long enough to establish conclusions. Thus, they consider a double blind system ideal for these studies (for farmers and researchers do not influence the management of the animals). The monitoring of the effect of the vaccine can be performed serologically and by bacteriological methods, but sensitivity and specificity must be taken into account. The rate of abortion is a useful clinical index. However, a reliable estimation of this index is difficult when breeding is extensive and reports from farmers must be taken with caution. Moreover, it is not a constant clinical sign, because 20% of infected cattle never abort and 80% of those that abort do so only once, some twice, but rarely more (Cunningham, 1977). So, a decrease in the rate of abortions does not necessarily mean that animals are not infected and non-aborting infected mothers act as shedders and transmitters of the disease (Plommet, et al., 1971).

3.6 Bovine Brucellosis Control

The justification for control measures in brucellosis are often given as economics and public health. The control measures could be classified into two general categories: hygiene and vaccination. Hygiene includes all procedures where the goal is to limit exposure to susceptible animals such as isolation, restriction in commerce, slaughter of positive animals, and disinfection (Lage et al., 2005a, Nicoletti, 2005).

Modern agricultural practices such as increasing herd size and greater livestock commerce have led to introduction of infected animals into previously healthy herds and an increased persistence of the disease (Nicoletti, 2005).

Temperature, humidity, and pH influence the ability of *Brucellae* to survive in the environment. The organisms are sensitive to sunlight, disinfection and pasteurization and under dry conditions survive only if embedded in protein (Nicoletti, 2005).

Numerous countries have brucellosis control measures in order to reduce the prevalence or eradicate the disease from domestic livestock in an effort to prevent transmission to humans. Another effect of brucellosis regulatory programs is to assist producers by preventing economic losses, reduced fertility and decreased milk production. Regulatory programs are usually influenced by the prevalence of the disease within livestock or human populations and economic considerations. Programs to eradicate or reduce *Brucella* infections include parts, or all of the following: test and removal programs, sanitation and/or vaccination (Olsen and Stoffregen, 2005).

In general, sanitization programs are more apt to be emphasized in high prevalence areas for educating producers on methods to reduce disease transmission by segregation, the prompt elimination of infectious materials, decontamination and other mechanisms for preventing exposure. Test and removal regulatory programs can either be tied to area surveillance activities or localized to individual herds. In countries with a high prevalence of bovine brucellosis, regulatory programs may be designed to reduce prevalence rather than eradicated disease. The use of extensive test and removal programs in areas of high prevalence may have unacceptable economic costs, and may be devastating to livestock production in that area. Successful test and removal programs require that the country has strong regulatory and diagnostic structures for livestock disease control. Other point is the importance of mandatory pasteurization of all milk, because nonpasteurized dairy products are the primary mechanism for food-borne transmission (Olsen and Stoffregen, 2005).

3.7 Brazilian National Program for Control and Eradication of Bovine Brucellosis (PNCEBT)

In Brazil, brucellosis is endemic throughout the territory (Poester et al., 2002). The data

from official reports indicate that the prevalence of seropositive animals remained between 4% and 5% in the period from 1988 to 1998, but it is not homogenous around the country. It was estimated for the State of Minas Gerais a prevalence of infected cattle of 6.3% in 1975 (Brazil, 1977) and 6.7% in 1980 (Castro 1982). Recently, the prevalence of animals with *Brucella* in Minas Gerais State was estimated to be around 1%. This significant reduction in the prevalence of the disease was caused by a compulsory vaccination program that began in the Triangle Mineiro in 1993 and covered the entire state in 1998. Santa Catarina has a very low prevalence, with only 0.02% of infected herds. In Paraná, the prevalence is very heterogenous, 0.34% in the south of the state and 14.72% in the north. In São Paulo the estimated prevalence is around 9.7% (Lage et al., 2005).

The technical regulation of PNCEBT (Brasil, 2004; 2006) for the control of bovine brucellosis is based on the interruption of the transmission chain, mainly by the early detection and investigation of positive cases in order to prevent the spread of the disease. The PNCEBT aims at lower the prevalence and incidence of new cases of brucellosis and to create a significant number of properties free of the disease, reducing the risk to public health, and offering good quality food for the population.

The mandatory action of the PNCEBT is vaccination of female calves against brucellosis, control the movement of animals for breeding and to entrance into livestock fairs/exhibitions, compulsory slaughter of cattle testing positive, in approved abattoirs and standardization of testing procedures through short courses for accredited veterinarians (Poester et al., 2002). The objective is reducing the prevalence and incidence of cases of the disease to a very low level at which an eradication program could be started. It is expected that within a decade it is possible to reduce the prevalence of affected herds to values close to 1% in States that implemented the program within the timeframe envisaged (Lage et al., 2008). It is important to emphasize that vaccination against brucellosis is a top priority in this program.

The strategic action of this program is clear: the voluntary accreditation of free herds and voluntary monitoring of beef herds. These are interesting strategies for producers and agro-industrial sectors because they can add some value to their products. Therefore, it is not only a program of federal and state government level, but a project that involves the productive sector and their communities, the industry and consumers, including veterinarians that work in the private sector. In other words, the public sector should act as a certifying agent within a process that directly involves the entire production chain (Lage et al., 2005).

Vaccination with S19 is mandatory and restricted to female calves, between 3 and 8 months of age. It is delivered under the supervision of an accredited veterinarian at full cost of the livestock owner. *B. abortus* strain 19 is manufactured at several private laboratories. All vaccine batches have to be approved by the federal reference laboratory. Brazil is capable of producing vaccine of international standards in sufficient quantities to supply its massive cattle industry (Poester et al., 2002).

Strategic vaccination of adult females with *B. abortus* RB51 is allowed and it is an additional tool for use under special circumstances, such as in adult animals that were not vaccinated with S19 as calves or in heavily infected herds (Brasil, 2004, Poester et al., 2006). The role of RB51 in the situations mentioned is to improve immunity of animals or herds without interfering with diagnostic tests. As with S19 vaccination, the RB51 must be done under the supervision of an accredited veterinarian.

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Chapter II - Growth of *B. abortus* reference strains S19, RB51, 544 and 2308 and some field strains on media containing different inhibitor agents

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1. Introduction

Brucellosis is a widespread zoonotic disease, transmitted mainly from ruminants to humans. This is a disease of major public health, animal welfare and economic significance worldwide. *Brucella* infections may result in significant economic losses due to abortion and slaughtering of infected animals. Humans are mainly infected through the consumption of contaminated dairy products or by direct contact with infected animals. *Brucella* species have also long been considered potential biological warfare agents and the organism remains in the list of Centers for Disease Control and Prevention category B potential biological warfare agents (Rotz et al., 2002).

Differentiation among vaccine, S19 and RB51, and field strains is required in areas where vaccination is performed due to the occasional isolation of vaccine strains from milk or other biological samples. Also, experiments evaluating vaccines should 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. The animals must be injected with the test vaccine and followed later with a challenge of a virulent *B. abortus* strain, such as 2308 or 544. Later, the animals are killed and the spleen counts of viable *B. abortus* organisms are determined, the protection index relative to the reference preparation is then calculated. When live vaccines are involved in these experiments, not only challenge strains, but vaccine strains can also be isolated and influence the protection index, so it is necessary to inhibit or estimate them to subtract from the total. In addition, a difficulty in differentiation between vaccine strains and field isolations of *B. abortus* is observed, thus a biochemical test to separate these strains would be very useful in laboratory routine of 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 strains on media containing different inhibitor agents.

2. Material and methods

2.1 Bacterial strains

B. abortus S19 original seed strain was obtained from United States Department of Agriculture (USDA), 2308 was provided by Dr. Samartino (Instituto Nacional de Tecnologia Agropecuaria -INTA- Argentina), 544 was provided by Dr. Mota (Laboratório Nacional Agropecuário - Lanagro-MAPA - Brazil) and RB51 strain was provided by Dr. Schurig (Virginia Polytechnic Institute and State University - VirginiaTech – USA). The other strains used are field isolates of *B. abortus,* isolated and identified in our laboratory by routine and molecular methods (Alton et al., 1988;

LeFleche et al., 2006) as *B. abortus* biovar 3 (strains A1, A4 and A6), biovar 1 (strains 13A and 13B) and biovar 6 (17A and 17B).

For the assays, frozen strains were thawed at room temperature, seeded on tryptose agar plates (Difco, USA) and incubated at 37°C, in a 5% CO_2 atmosphere, for 48h. Fresh bacterial suspensions were harvested in PBS (pH 7.2) and adjusted to MacFarland N° 3 standard, to have a suspension of approximately 10^9 CFU/mL. For the experiments, these fresh suspensions were diluted properly in PBS. From each suspension, six tenfold dilutions were prepared. Suspensions from RB51 were made in PBS with 0.5% Tween 80 (Sigma-Aldrich, USA).

2.2 Growth tests

Viable counts of each bacterial suspension of the reference strains (S19, RB51, 544 and 2308) were done in duplicate by the drop counting method (Miles and Misra, 1938) on tryptose agar (as control) and tryptose agar containing the following substances: i-erythritol (1.0 mg/mL) (Sigma Aldrich, Germany); basic fuchsin (20μg/mL and 80 μg/mL) (Merck, Germany); thionin (2.5 μg/mL and 10 μg/mL) (Merck, Germany); rifampicin (200μg/mL) (Merrell, United Kingdom); and safranin O (200 μg/mL) (Merck, Germany).

Plates were incubated at 37°C for up to 96h in 5% CO_2. Furthermore, each suspension was inoculated on two tryptose agar plates which were incubated at 37°C for 48h 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. All treatments were applied to the reference strains while field isolates were tested only on media containing erythritol (1.0 mg/mL), rifampicin (200μg/mL) and thionin (10 μg/mL), which were the agents really able to inhibit growth of some strains.

All tests were done in triplicate.

3. 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 (Figure1).

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, 544 and RB51 were completely inhibited (Figure1).

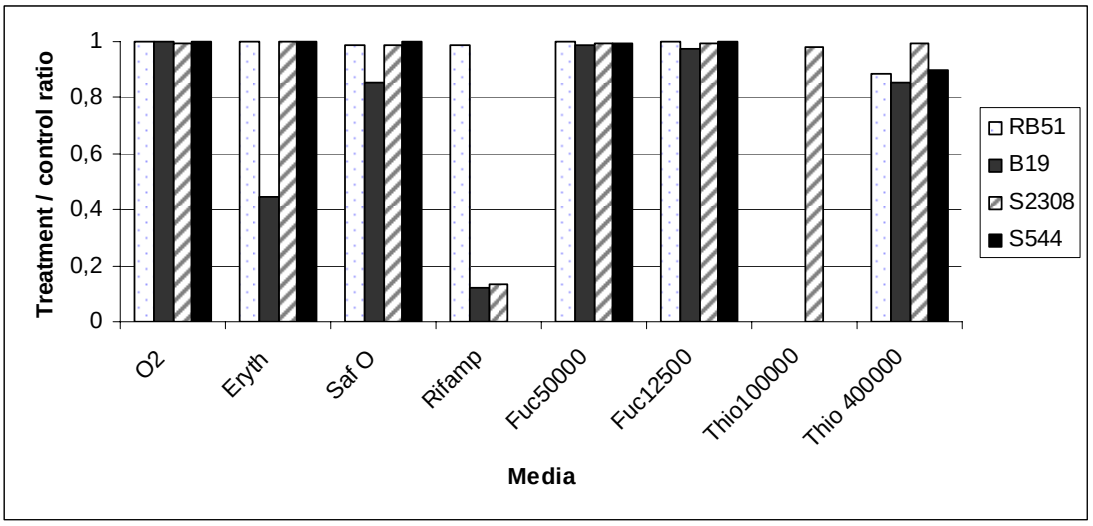

Figure1. Growth patterns of *B. abortus* strains RB51, S19, 544 and 2308 on different selective media or atmosphere condition.
O2= Suspensions inoculated onto tryptose agar plates and incubated in air
Eryth=Suspensions inoculated onto tryptose agar plates containing i-erythritol (1.0 mg/mL)
Saf O=Suspensions inoculated onto tryptose agar plates containing safranin O (200 μg/mL)
Rifamp=Suspensions inoculated onto tryptose agar plates containing rifampicin (200μg/mL)
Fuc 50000=Suspensions inoculated onto tryptose agar plates containing basic fuchsin (20μg/mL)
Fuc 12500=Suspensions inoculated onto tryptose agar plates containing basic fuchsin (80 μg/mL)
Thio 100000=Suspensions inoculated onto tryptose agar plates containing thionin (10 μg/mL)
Thio 400000=Suspensions inoculated onto tryptose agar plates containing thionin (2.5 μg/mL)

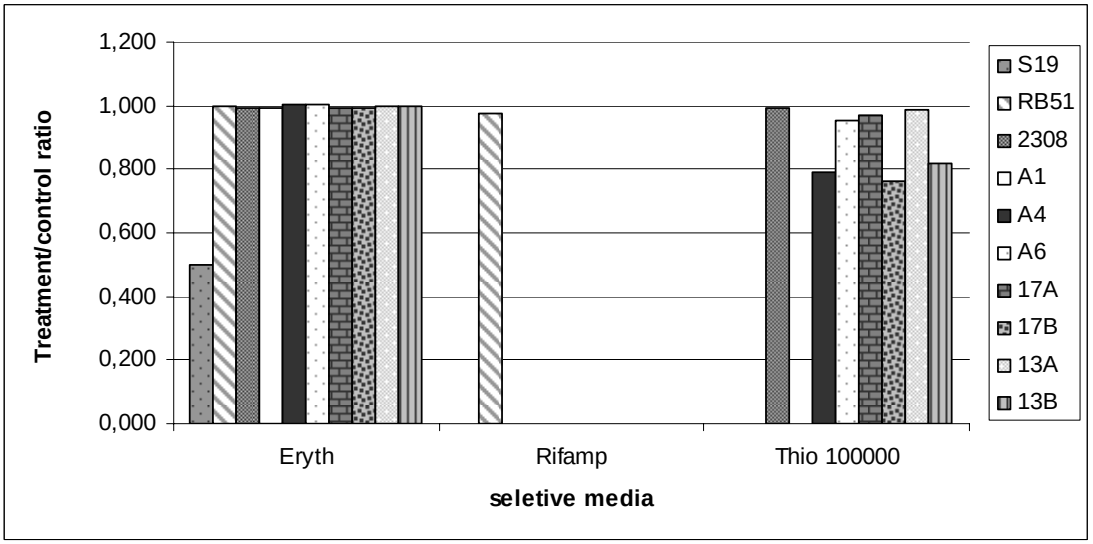

Figure2. Growth patterns of *B. abortus* reference strains RB51, B19, 544 and 2308 and field strains A1, A4, A6, 17A, 17B, 13A, 13B on different selective media.
Eryth=Suspensions inoculated onto tryptose agar plates containing i-erythritol (1.0 mg/mL)
Rifamp=Suspensions inoculated onto tryptose agar plates containing rifampicin (200μg/mL)
Thio 100000=Suspensions inoculated onto tryptose agar plates containing thionin (10 μg/mL)

All the field strains were able to grow normally on tryptose agar containing i-erythritol 1.0 mg/mL, just like *B. abortus* 2308 and RB51 (Figure2). All the field strains were totally inhibited on media containing rifampicin, where the only strain able to grow was RB51. On media containing thionin (10 μg/mL) the reference strain 544 and the field strain A1, *B. abortus* bivoar 3, were totally inhibited like the vaccine strains, S19 and RB51. However, the other *B. abortus* biovar 3, strains A4 and A6, were able to grow with only a small inhibition. All field strains, with exception of A1, could grow on agar tryptose with thionin (10 μg/mL); strains A4, 17B and 13B showed a treatment/control ratio of 0.75-0.80, which represents a small inhibition.

4. Discussion

There are several advanced molecular techniques that can be used as additional tools, such as PCR and MVLA (Bricker and Halling, 1995, Le Flèche et al., 2006) when the objective is the identification of the biovar of new isolates. However, most of laboratories are not equipped to perform these molecular tests and characterization and identification of *B. abortus* isolates remain based on phenotypic tests. Thus, the experiments with inhibitor agents for differentiation among *B. abortus* strains are very useful, specially to differentiate vaccine strains from field isolates.

The use of a special media that inhibits the growth of one strain, but not the other one is also very useful when evaluating potency (immunogenicity) of attenuated vaccines. In these cases, it is necessary to inhibit the vaccine strain, to count only challenge strains on target organs to predict protection.

According to Alton et al. (1988), for *B. melitensis, B. abortus* and *B. suis*, the identification at the 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. *B. abortus* biovar 1, 2, 3 and 4 requires CO_2, however, Alton et al. also present a note, stating that some *B. abortus* biovar 1 to 4 usually require CO_2 only on primary isolation. This feature was found among all strains of biovar 1 tested could grow in air (S19, RB51, 544 and 2308). Thus, this information of CO_2 dependence must be carefully used, because although CO_2 requirement is an important differential feature among *B. abortus* biovars, it is not always stable (Alton et al., 1988).

Some traits can distinguish live vaccine *B. abortus* strain 19 from typical *B. abortus* biovar 1, such as growth on media containing thionin blue (2 μg/mL) and i-erythritol (1 mg/mL), since typical *B. abortus* biovar 1 strains can grow on these media and S19 cannot (Alton et al., 1988). However, on primary isolation typical *B. abortus* biovar 1 strains may not grow on media containing 2 μg/mL of thionin blue. In addition, the mutation rate to tolerance to erythritol is fairly high and some suspected strain 19 isolates may grow on erythritol although resembling strain 19 in other tests (Corner and Alton, 1981).

In this study, S19 was inhibited on media containing erythritol, but not completely, there was a 5-log drop from initial inoculum (10^9 to 10^4 CFU/mL). The stability of this characteristic was confirmed by the results of three experiments. It demonstrates that this inhibitor agent can be very useful in the differentiation of S19 strain from challenge strains 544 or 2308 in immunogenicity studies of S19 and also in the differentiation of field isolates from S19 vaccine isolate.

According to Alton et al. (1988), 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. In fact, we did not test this concentration, but in a lower concentration (10 μg/mL), a *B. abortus* biovar 3, a field strain, was totally inhibited, what is

discordant from the information given by those authors. Other interesting finding was that the reference strain 544 was not able to grow on media containing thionin 10 μg/mL, while the reference strain 2308, which belongs to the same biovar of 544, could grow normally on this media. Thus, in this case, the use of media containing thionin 10 μg/mL would be useful to immunogenicity tests of S19 only if the challenge strain used was *B. abortus* 2308.

The present results showed that strain RB51 is really resistant to rifampicin, while all other strains tested were inhibited on media containing this antibiotic, which confirms rifampicin-resitance 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 not the growth of the challenge strain will be desirable. If the challenge strain chosen for this kind of experiment is 2308, it can be used media containing thionin 10 μg/mL, to inhibit the vaccine strain. The same is not possible when using challenge strain 544.

The Manual of Diagnostic Tests and Vaccines for Terrestrial Animals – OIE (OIE, 2008) suggests the use of *B. abortus* strain 544 CO_2 –dependent as challenge strain in studies of immunogenicity of S19 vaccine in mice. So, the plates for counting of challenge strain in target organs are 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, the characteristic of CO_2 requirement is not always stable, subculture provide 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. The *B. aboruts* strain 544 used in our laboratory is not CO_2 –dependent, and because of it we can not use this characteristic to differentiate challenge and vaccine strain in studies of immunogenicity of S19 vaccines in mouse model.

In summary, the overall results suggest that differentiation between S19 and 544 / 2308 can be done using i-erythritol (1 mg/mL). The differentiation between S19 / RB51 and 2308 can be done on media containing thionin (10 μg/mL). And differentiation between RB51 and challenge strains can be done using rifampicin (200μg/mL). In the case of having a *B. abortus* strain 544 CO_2 – dependent, differentiation can be done by incubating plates in air. When the inhibited strain is not the vaccine strain, it is necessary to calculate the difference of gowth in tryptose agar plates under 5% CO_2 atmosphere and the inhibition situation to estimate the growth of challenge strain.

5. Conclusion

These results show that tryptose agar with thionin (10μg/mL), i-erythritol (1.0 mg/mL) or rifampicin (200 μg/mL) could help in the differentiation among vaccine and challenge or field strains of *B. abortus.*

Growth characteristics of reference challenge strains and vaccine reference strains must be checked before executing experiments in which the differentiation using inhibitor agents is necessary, such as immunogenicity tests of S19 vaccines.

6. Acknowledgements

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Chapter III - Immunogenicity of S19 or RB51 in three strains of mice (CD-1, Swiss and BALB-c), challenged with *B. abortus* strain 544 or 2308

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1. Introduction

Brucellosis is a zoonotic disease of domestic and wild ungulates. *Brucella* spp. are facultative intracellular bacteria causing chronic disease that usually persists for life. The main clinical sign of *Brucella abortus* infections is abortion, which results in important economic losses to livestock around the world.

Because of the high costs and long time span of the experiments in natural hosts, laboratory animals have been used as a preliminary step in the analyses of vaccines against brucellosis (Bosseray et al., 1984, Cloeckaert et al, 2004; Kurar and Splitter, 1997, Al-Mariri et al., 2001, Leclerq et al., 2002, Velikovsky et al., 2002, Oñate et al., 2003). The ideal method for vaccine testing should also be simple, quick and inexpensive, and should also give clear-cut results. In practice, guinea pigs and mice have been the laboratory animals used for this purpose. Historically, the mouse has been used as a model in studies on the pathogenesis and immunology of brucellosis, specially because mice are less costly than other animals. Protection tests in laboratory animals are the usual method for measuring the potency of live *Brucella* spp. vaccines and also for evaluating experimental vaccines. Although the murine model is the most frequently used, there is no established standardized model. Due to large number of different strains of isogenic and heterogenic mice used, and with the lack of baseline data from the specific strains, extrapolation of results from one study to another has been difficult (Pugh et al., 1989).

Protective immunity against *Brucella* infection has been studied mainly, but not only, in BALB/c, CBA/NJ, C3H/HeN, C3H/HeJ, Swiss and CD-1 mice (Plommet and Plommet, 1981, Bosseray et al., 1984, Plommet and Bosseray, 1984, Pugh et al., 1989, Bosseray and Plommet, 1990, Bosseray, 1991, Kurar and Splitter, 1997, Grilló et al., 2000, Al-Mariri et al., 2001, Leclerq et al., 2002, Velikovsky et al., 2002, Oñate et al., 2003, Cloeckaert et al, 2004). The criterion used for measuring protection in immunized mice is reduction, at a specific time, after a virulent challenge, of the number of colony-forming units (CFU) of *Brucella* recoverable from the spleen or liver or both. CD-1 mouse has been recommended by the OIE (OIE, 2008), as a reference laboratory animal for the control of S19 vaccines, although its use has not been generalized. Other point is that the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2000 (OIE, 2003) allowed the use of other mice strain, BALB-c, what is not permitted anymore (OIE, 2008). This method proposed by OIE to test immunogenicity of anti-*Brucella* spp. vaccines in mice is based on Bosseray (1992) and Bosseray (1993). This group has extensively studied the CD-1 mouse model (Bosseray, 1991, 1992, 1993; Bosseray and Plommet, 1976, 1983, 1984, 1990; Grilló et al., 2000; Plommet and Bosseray, 1984; Plommet and Plommet, 1981, 1988). However, this strain of mice, which is a trade mark originated from Swiss mice, is just commercialized by one laboratory (Chales River Laboratories - France), what becomes difficult for all scientists to use it.

The aim of this study was to evaluate the employment of different strains of mice (CD-1, BALB/c and Swiss) and different challenge strains (*B. abortus* 544 and 2308) in the study of immunogenicity of anti-*Brucella* vaccines in the murine model. The choice of mice strains was based on three criteria: the most used (BALB-c), the recommended by OIE (CD-1), and a readily available strain, apparently very similar to CD-1 (Swiss).

2. Material and Methods

2.1 Animals

Thirty six healthy female mice from each of the three strains, CD-1 (USP-Brazil), BALB/c (UFMG-Brazil) and Swiss (Funed-Brazil) were used in the study. All mice used in experiments were between 5 and 6 weeks of age. They were randomly distributed in cages with a maximum of six mice/cage. Mice were maintained in an air-conditioned, air filtered facility that contained only mice in a biosafety level III building (Lanagro / MG, Pedro Leopoldo, Brazil). Mice were feed a balanced diet and water *ad libitum.* Humane practices were used in all animal manipulations throughout the study (NATIONAL…, 2004; AMERICAN…, 2007) and the experimental protocol was approved by the Ethics Committee in Animal Experimentation of the Universidade Federal de Minas Gerais (Cetea – UFMG).

2.2 Bacterial strains

B. abortus S19 original seed strain was obtained in lyophilized ampoules from United State Department of Agriculture (USDA), 2308 was provided by Dr. Samartino (INTA), 544 by Dr. Mota (Lanagro-MG/MAPA) and RB51 strain was provided by Dr. Schurig (Virginia Tech – USA).

Before the assays, frozen strains were thawed at room temperature, seeded on tryptose agar plates (Difco, USA) and incubated at 37ºC, in a 5% CO_2 atmosphere, for 48h. Fresh bacterial suspensions were harvested in PBS (pH 7.2), and then spectrophotometrically adjusted to 10^9 CFU/mL. For *in vivo* studies, these fresh suspensions were diluted properly in PBS. Exact doses inoculated into mice were assessed retrospectively (Miles and Misra, 1938). Challenge strains (2308 and 544) were reactivated by passing twice in guinea pigs.

2.3 Experimental design

A factorial experimental design using three strains of mice – CD-1, BALB/c and Swiss – , two challenging strains – 2308 and 544 – ,and three treatments – S19 and RB51 *B. abortus* vaccine strains plus PBS as control – was established. The 18 resulting experimental groups are shown in Table 1.

Table 1. Experimental groups, composed by six animals, according to mice strains – CD-1, BALB/c and Swiss –, challenging strains – 2308 and 544 –, and treatments – S19 and RB51 *B. abortus* vaccine strains plus PBS as control.

Challenge strain	Treatment	Mice Strain		
		BALB/c	Swiss	CD-1
2308	B19	Group 1	Group 2	Group 3
	RB51	Group 4	Group 5	Group 6
	PBS	Group 7	Group 8	Group 9
544	B19	Group 10	Group 11	Group 12
	RB51	Group 13	Group 14	Group 15
	PBS	Group 16	Group 17	Group 18

2.4 Vaccination

Groups of six mice were vaccinated subcutaneously with approximately 1.0 x 10^5 CFU/100 µL/mouse of each S19 or SRB51 vaccine suspension or with 100 µL/mouse of PBS (pH 7.2) (unvaccinated control mice). The precise numbers of CFU were determined retrospectively by viable plate counts (Miles and Misra, 1938).

2.5 Challenge exposure

All mice were challenge exposed with a virulent 40 - 48 h culture of *B. abortus* strains 2308 or 544, four weeks after treatment (S19, RB51 or PBS), according to the experimental group (Table 1). Mice were inoculated intraperitoneally with 2.0 x 10^5 CFU in 100 µL of the PBS / mouse.

2.6 Culture examination of spleen

Mice were killed by cervical dislocation (AMERICAN…, 2007) two weeks after challenge and total splenic counts were determined as follows: each spleen was removed, weighted and homogenized with diluent (PBS, pH 7.2). Its weight in grams was multiplied by 9 to determine the amount of diluent needed to make a $1x10^{-1}$ solution of spleen. Then, serial tenfold dilutions were made. Three plates of tryptose agar (Difco, USA) containing 1% of erythritol (Sigma-Aldrich, Germany) were used for each dilution of spleen. The inoculated plates were incubated at 37°C for 96 hours and the colonies were counted. The total colonies on the three plates representing that dilution of spleen were counted and the average CFU were determined. Erythritol was used to inhibit S19 vaccine strain and rifampicin to inhibit RB51 vaccine strain, since vaccines may survive until challenge and be reactivated (induced to multiply again) (Plommet and Plommet, 1988). So, enumeration in target organs that would not differentiate the vaccine from challenge strain might give a biased estimation of protection. After incubation, bacterial counts per organ (X) were transformed as Y = log(X/log X); to normalize the distribution of individual counts (Bosseray and Plommet, 1976). Mean and standard deviation of transformed values per group were then computed.

2.7 Analysis of data

Statistical analysis of this experiment of factorial design was performed accordingly by complete variance analyses of effects and interactions by use of SNK test, using an α error of 0.05 (Sampaio, 2002).

3. Results

The immunogenicity of mice, measured by the logarithmic transformation of CFU/spleen, was compared two weeks after challenge exposure to virulent *B. abortus* by analyses of variance. Results are shown on Table 2 and figure 1, according to the strain of mice (CD-1, BALB/c or Swiss), the treatment received (S19, RB51 or PBS), and the challenge strain (2308 or 544).

No significant difference was found between the use of *B. abortus* 2308 or *B. abortus* 544 challenging strains in the mouse model of *Brucella* spp vaccine potency assay (Fig.1). Comparisons are shown on figure 1.

Table 2. Results of mean CFU from spleen after transformation [y = log(x/log x)], in three strains of mice according to vaccination and challenge.

Challenge strain	Vaccinated or control	Immunogenicity: log (X/log X) ± sd					
		BALB/c		Swiss		CD-1	
2308	S19	2.974 ±	0.29	2.163 ±	0.56	2.226 ±	0.37
	RB51	3.087 ±	1.34	3.001 ±	0.64	3.480 ±	0.85
	Control	4.033 ±	0.42	5.348 ±	0.18	4.898 ±	0.45
544	S19	2.644 ±	0.28	2.229 ±	0.07	2.333 ±	0.65
	RB51	2.535 ±	0.37	2.796 ±	0.80	2.523 ±	0.52
	Control	4.297 ±	0.46	4.630 ±	0.35	4.527 ±	0.62

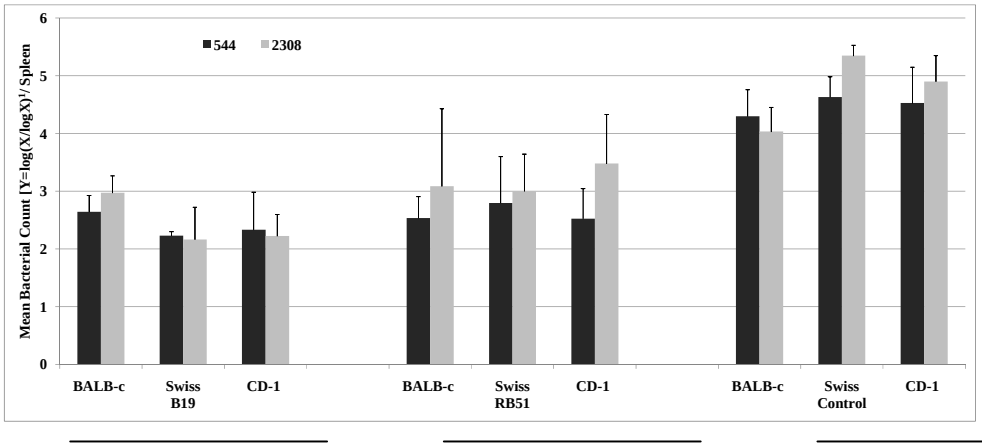

Fig.1. Mean bacterial count per spleen after transformation [Y = log (X/log X)] for the three strains of mice, the two challenging strains and the three treatments. Bars indicate the standard deviation of the mean.

Results of variance analysis show an interaction between treatment and strain of mice in this kind of study, thus these parameters can not be compared separately. Protective differences were observed between the treatments within the same strain of mice (Table 3). When CD-1 groups were compared, those vaccinated showed significantly lower counts than nonvaccinated ($P \leq 0.05$), independently of the vaccine received (S19 or RB51), and the same was observed in BALB/c mice. However, in Swiss mice, S19 was more protective than RB51 ($P \leq 0.05$), which was protective when compared to the nonvaccinated groups ($P \leq 0.05$).

Table 3. Comparison of mean bacterial count per spleen after transformation [Y = log(X/log X)], according to the strain of mice and treatment.

Treatment	Mouse strains		
	CD-1	BALB/c	Swiss
S19	2.5^{Ab}	2.8^{Ab}	2.3^{Ac}
RB51	2.9^{Ab}	2.8^{Ab}	2.8^{Ab}
Control	4.7^{Aa}	4.2^{Ba}	5.05^{Aa}

Different uppercase letters indicate differences within line; different lowercase letters indicate differences within column. Statistical analyses were performed by ANOVA for a factorial experimental design and SNK test ($P \leq 0.05$).

4. Discussion

The overall results indicated that different strains of mice can be used for comparative studies on the immunogenicity of vaccines against brucellosis. All three strains of mice can be used to this propose. It is possible because all strains of mice tested were susceptible to *Brucella* infection and could be protected with vaccines currently used in cattle.

Results showed that BALB/c and Swiss can also be used in *in vivo* tests to evaluate S19 vaccines, the same way that CD-1, which is the strain indicated by the actual OIE Manual (OIE, 2008) for the mouse potency assay of vaccines. The use of other alternative strains of mice would facilitate the implantation of *in vivo* tests in the routine of Brazilian and other official laboratories, because CD-1 is a trade mark of mice, commercialized only by one laboratory (Charles River Laboratories), localized in France and United States.

According to OIE (2003), a potency test on *B. abortus* strain RB51 is not routinely carried out and the usefulness of this test to predict protection in cattle is questionable. However, if desired, it could be carried out in BALB/c female mice, using *B. abortus* strain 2308 as the challenge strain (OIE, 2003). The Manual of Diagnostic Tests and Vaccines for Terrestrial Animals – OIE (2008) says nothing about potency tests on RB51. The results found in this study show that the three strains of mice could be used to evaluate potency of strain RB51, because it was possible to observe a statistical difference between the groups vaccinated with this strain and the control groups, which received only PBS in all strains of mice. However, we can not conclude if this test could predict protection in cattle. Anyway, independent of the strain of mice used in the potency test of RB51, it is not possible to apply the same values stated by OIE for S19 vaccine (response of vaccinated mice lower than 2.5) (OIE, 2008), because all results of immungenicity in mice vaccinated with RB51 were greater than 2.5, achieving 3.5, but always significantly lower than the control (nonvaccinated group), what valid this model.

In a similar comparison, when using BALB-c in evaluation of potency of S19 vaccines, the values stated by OIE for protected and non-protected animals can not be applied (response of mice nonvaccinated at least 4.5 and vaccinated lower than 2.5) (OIE, 2008). The difference of results obtained in vaccinated and nonvaccinated BALB/c is lower than in the other strains of mice tested, but still statistically different. It would be necessary more studies to standardize great limit values of response of BALB/c mice vaccinated with a reference S19 vaccine and nonvaccinated. More studies are necessary to establish the cut-off points to protected and non-protected animals in immunogenicity studies using the strain of mice BALB/c or in immunogenicity tests of RB51, because the parameters established by OIE for immunogenicity test f S19 in CD-1 mice could not be used in these two cases, what can be visualized in table 2.

According to the results, the OIE pattern of responses expected in potency tests with CD-1 could be applied to Swiss mice. Thus, Swiss mice can be alternatively used in immunogenicity tests of S19, applying the same parameters established by OIE to CD-1 mice. It is very advantageous, because Swiss mice are readily available for most laboratories, different from CD-1 mice, which is a trade mark of difficult access for most scientists. The possibility of using Swiss mice in place of CD-1 mice is a very important finding of the present study. It was an expected result, because it reflects the same origin of these strains.

OIE indicates the use of *B. abortus* strain 544 (OIE, 2008), because that Manual is based on extensive studies realized on CD-1 mice using *B. abortus* 544 as challenge strain. However, our results on comparison of challenge strains, *B. abortus* 2308 and 544, indicate that both strains are useful in studies of immunogenicity of *Brucella* vaccines, with no significant difference. In addition, our data demonstrate that both of them can be used in immunogenicity studies of either S19 or RB51 using the three strains of mice, because there was no interaction between the challenge strain used and the other parameter studied (vaccine and strain of mice). Although both challenge strains can be used, it is evident that European groups do prefer using the challenge strain 544 and American groups do prefer using strain 2308.

These results do represent an up-to-date documentation of a comparative study in mice strains currently used in most research laboratories, as well as the two reference virulent strains of *B. abortus* most used. This data, therefore, will be useful for future reference for those interested in the evaluation and the development of new *Brucella* spp. vaccines.

5. Conclusion

The strains of mice CD-1, BALB/c and Swiss, as well as both challenge strains *B. abortus* strains 544 and 2308 can be used in immunogenicity tests of S19 and RB51 vaccines.

6. Acklowledgements

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Chapter IV - Evaluation of *Brucella abortus* S19 vaccines commercialized in Brazil: immunogenicity, residual virulence and genotyping

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1. Introduction

Brucellosis, a zoonotic disease, is caused by members of the genus *Brucella*. *B.* abortus is a Gram-negative, aerobic, facultative intracellular bacterium and one of the species in the genus. The natural host reservoir is cattle, and infection of cows induces spontaneous abortion. Also, *B. abortus* infection of humans causes persistent undulant fever, endocarditis, arthritis and meningitis. A direct relationship has been established between animal and human brucellosis, ruminants being the main source of human infection (Corbel, 1997). Ruminant brucellosis may be controlled and eradicated by means of adequate testing and slaughtering program, but in areas with high prevalence, vaccination is the best way to start controlling the disease, reducing its prevalence.

The current vaccine for cattle used in most of the world against this disease is an attenuated strain, *Brucella abortus* S19. According to OIE (World Organization for Animal Health), this vaccine must be prepared from USDA-derived seed and each batch must be checked (OIE, 2008). Regular control of the biological quality of live *B. abortus* S19 vaccine is essential for the successful management of bovine brucellosis. The reference procedures recommended by the OIE include all *in vitro* (purity, number of viable bacteria, smoothness, and diluent's analysis) tests and also some *in vivo* tests, the determination of residual virulence and immunogenicity (OIE, 2008).

Since 2001, Brazil has the National Program for Control and Eradication of Brucellosis, which aims at lower the prevalence and incidence of new cases of brucellosis and to create a significant number of properties free of the disease, reducing the risk to public health, and offering good quality food for the population. The mandatory actions of the PNCEBT are vaccination of female calves against brucellosis with S19, control the movement of animals for breeding and to entrance into livestock fairs/exhibitions, compulsory slaughter of cattle testing positive, in approved abattoirs and standardization of testing procedures through short courses for accredited veterinarians (Poester et al., 2002). The use of good quality commercial batches of this attenuated vaccine is an essential prerequisite for the success of vaccination campaigns.

Official Brazilian control of S19 vaccine includes *in vitro* criteria (purity, number of viable bacteria, smoothness, thermal stability and diluent's analysis) (BRASIL, 2004), but not biological properties of the vaccine, such as potency or residual virulence. It must be emphasized that the cost of a vaccination program in a given region is only in a small part affected by the cost of the vaccine itself. The entire success of the program rests on the quality of the vaccine used. Accordingly, it must be recommended that the upmost care should be taken in quality of the S19 vaccine that is used. According to Milward (1995), a vaccine is not just a strain, grown on a

defined culture media, and tested once by challenging animals. A vaccine is a product that, put into the hands of the final user, will reliably and reproducibly provide the expected results. Indeed, a vaccine truly exists only when its production has been scaled up and shown to be reproducible and that is the purpose of any industrial process for the production of vaccine.

The assessment of genetic stability is one of the essential elements to guarantee the biological quality of live anti-bacteria vaccines (García-Youldi et al., 2007). LeFléche et al. (2006) evaluated and selected a combination of 15 genetic markers to be used in a *Brucella* multiple locus variable number tandem repeats analysis (MLVA), which has been used to estimate the genetic stability of *B. melitensis* Rev1 vaccine strains (García-Youldi et al., 2007) and could also be used in S19 vaccines control.

The objective of this work was to compare the biological properties (immunogenicity and residual virulence) and genotypic profile (MLVA) of the eight S19 vaccines commercialized in Brazil to the reference strain obtained from USDA.

2. Material and Methods

2.1 Mice

Swiss female (Fundação Ezequiel Dias) mice were housed in a biosafety level III building (Lanagro, Pedro Leopoldo, Brazil) and were randomly allocated to experimental groups one week before being inoculated or vaccinated, at the age of 5-6 weeks. Human practices were used in all animal manipulations throughout the study (NATIONAL…, 2004; AMERICAN…, 2007) and the experimental protocol was approved by the Ethics Committee in Animal Experimentation of the Universidade Federal de Minas Gerais (Cetea – UFMG).

2.2 Bacterial strains and S19 vaccines

B. abortus S19 strain was obtained in lyophilized form from United State Department of Agriculture (USDA- USA) and *B. abortus* biovar 1 544 (ATCC 23448^T) reference strain was provided by Lanagro-MG (Brazil). One vial of each of two batches of each commercial S19 vaccine manufactured in Brazil in the period 2005 to 2007 were obtained from Lanagro – MAPA. These vaccines were produced by the eight Brazilian S19 vaccine manufacturing laboratories, named in this study from A to H. Only one vial of one batch was collected from laboratory H, because at the time we obtained the first batch of each laboratory, this one was not producing S19 vaccine. All the commercial vaccines used in this study had been approved in the mandatory *in vitro* controls tests perfomed by the Ministério da Agricultura, Pecuária e Abastecimento (MAPA) (Brazilian Ministry of Agriculture, Livestock and Food Suply).

The lyophilized reference and commercial vaccines were rehydrated in sterile distilled water, according to the instructions of manufacturer. Once reconstituted, all strains were grown in tryptose agar, harvested in tryptose broth with glycerol 5% and stored at -70^{o}C for use in subsequent assays.

For each assays, the strains were thawed at room temperature, seeded on tryptose agar plates (Difco, USA) and incubated at 37^{o}C, in a 5% CO_2 atmosphere, for 48h. Logarithmic phase bacterial suspensions were harvested in PBS (pH 7.2) and spectrophotometrically adjusted to 10^9 CFU/mL. For *in vivo* studies, these suspensions were diluted properly in PBS. Exact doses inoculated into mice were assessed retrospectively (Mile and Misra, 1938).

2.3 Immunogenicity tests

For immunogenicity tests, groups of six mice were vaccinated subcutaneously with approximately 1.0 x 10^5 CFU/mouse of each S19 vaccine in 100 μL suspension or with

100 µL/mouse of PBS (unvaccinated control mice). Vaccinated and nonvaccinated control mice were challenged 30 days after vaccination, intraperitoneally with *Brucella abortus* strain 544 (2.0 x 10^5 CFU in 100 µL of PBS, exactly 1.9 x 10^5 CFU, checked retrospectively). The precise numbers of challenged bacteria were determined retrospectively by viable plate counts (Miles, Misra, 1938). Mice were killed by cervical dislocation 15 days after challenge. Spleens were then removed aseptically, weighted and homogenized in nine times its weight of PBS, pH 6.8 and serial tenfold dilutions of each homogenate were made in the same diluent. Number of *B. abortus* in each lysate was determined by plating dilutions onto tryptose agar containing 1% of erythritol (Miles and Misra, 1938; Alton et al., 1988). Plates were incubated at 37°C, in a 5% CO_2 atmosphere, for 5 days. Erithritol was used to inhibit S19 vaccine, since vaccine strain may survive until challenge and be reactivated (induced to multiply again) (Plommet and Plommet, 1988) and introduces a bias in the estimation of protection. Thus, enumeration of *B. abortus* in target organs must differentiate the vaccine from challenge strain. After incubation, bacterial counts per organ were determined (named X) and a logarithmic transformation of the bacterial counts [y = log(x/log x)] was used to normalize the individual count distributions (Bosseray and Plommet, 1976). Mean and standard deviation of transformed values per group were then computed. The statistical analysis were performed with variance analysis and compared by Tukey Test ($P<0.05$).

2.4 Residual Virulence

For residual virulence, lots of 32 female Swiss mice were injected subcutaneously with 100 µL of PBS containing 10^8 CFU of each vaccine strain to be tested. In parallel, a similar inoculum was done in another 32 mice using the S19 USDA original seed reference strain suspension. Mice were killed by cervical dislocation, in groups of eight selected at random 3, 6, 9, and 12 weeks after inoculation. Then, spleens were removed, individually and aseptically homogenized in 1.0 mL of PBS, and plated onto tryptose agar plates, which were incubated at 37°C, in 5% CO_2, for 5 to 7 days. Persistence of the vaccine in spleen was first expressed by the number of animals found infected at each time point. An animal was considered infected if at least one *B. abortus* colony was isolated from the spleen. After completion of experiment, the times at which 50% of the mice had recovered from the vaccine infection were calculated using an internet interface developed to perform RT_{50} calculations (Pouillot et al., 2003), available on the web (www.afssa.fr/interne/rev2.html), which is a modified procedure based on graphical method of Bonet-Maury et al. (1954). Recovery time (RT_{50}) of each vaccine batch was compared to the recovery time of the reference S19 USDA original seed vaccine strain.

2.5 Multiple Locus Variable Number Tandem Repeats Analyses (MLVA)

DNA from all *B. abortus* (each vaccine batch, the S19 USDA original seed strain and strain 544) were extracted according to Pitcher et al. (1989). MLVA was performed using 15 *loci* as previously described (Le Flèche et al., 2006). Gel images were managed using Bionumerics software package (Version 5.1, Applied-Maths, Belgium). Band size estimates were converted to a number of repeat units within a character database using Bionumerics software. Results obtained were compared to a data bank available on the web (http://bacterial-genotyping.igmors.v-psud.fr).

3. Results

The exact count of CFU/mL of S19 *B. abortus* per vaccine dose used in the potency (immunogenicity) tests is shown in Table 1.
In the potency tests, *B. abortus* counts per spleen (X) were expressed as Y ± standard error of the mean (Table 2, Fig. 1), after the

transformation Y = log (X/log X). There was no statistical difference among any vaccine batch tested and the S19 USDA original seed strain in potency (immunogenicity) tests, after analysis of variance and Tukey test.

Table 1. Exact S19 *B. abortus* count per vaccine dose used in each group.

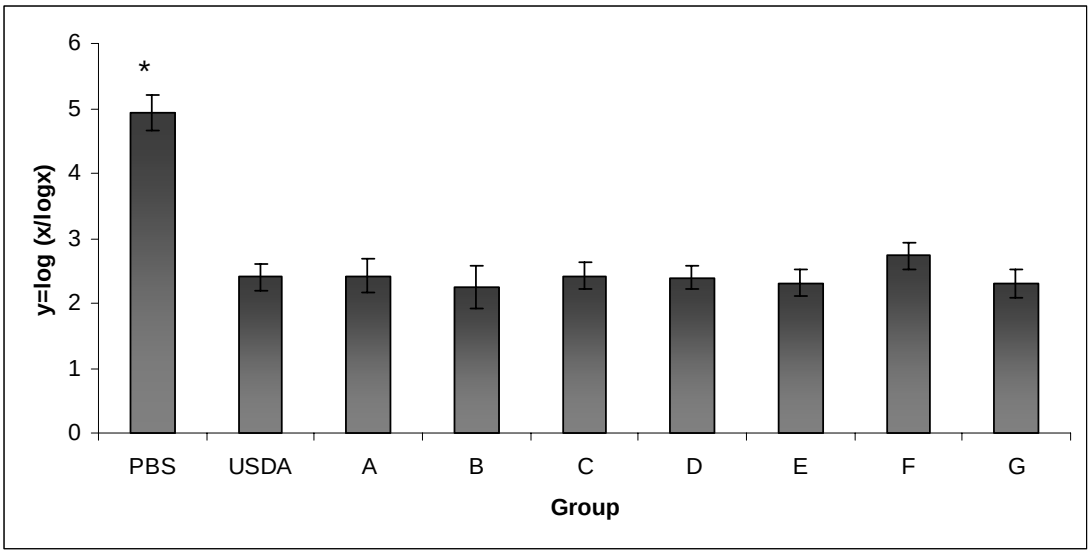
Group	Vaccine inoculum (UFC/100 µL)	
	Batch I	Batch II
A	1.03×10^5	1.10×10^5
B	1.25×10^5	1.10×10^5
C	1.15×10^5	1.25×10^5
D	1.00×10^5	1.30×10^5
E	1.02×10^5	1.20×10^5
F	1.28×10^5	1.32×10^5
G	1.10×10^5	9.9×10^4
H	-	1.02×10^5
USDA	1.025×10^5	1.025×10^5

Table 2. Immunogenicity values of eight commercial S19 vaccines and the reference S19 USDA original seed strain.

Experimental Group	Immunogenicity log (X/log X) ± sd	
	Batch I	Batch II
A	2.424 ± 0267 [a]	2.314 ± 0.178 [a]
B	2.246 ± 0326 [a]	2.213 ± 0.218 [a]
C	2.414 ± 0.206 [a]	2.285 ± 0.289 [a]
D	2.393 ± 0181 [a]	2.317 ± 0.264 [a]
E	2.313 ± 0204 [a]	2.291 ± 0.191 [a]
F	2.732 ± 0204 [a]	2.313 ± 0.268 [a]
G	2.296 ± 0220 [a]	2.303 ± 0.121 [a]
H	-	2.294 ± 0.207 [a]
USDA	2.398 ± 0202 [a]	2.295 ± 0.294 [a]
Control	4.935 ± 0.276 [b]	4.612 ± 0.202 [b]

Tukey Test ($P<0.05$); SMD batch I =0.4436; SMD batch II =0.4394; CV batch I = 8.9; CV batch II = 9.1. sd = standard deviation.

A



B

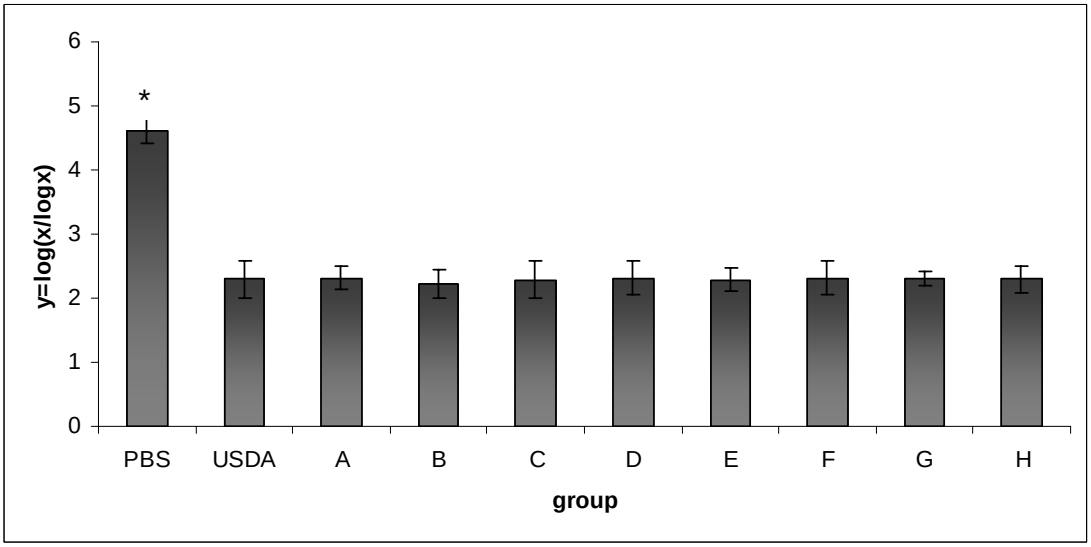


Fig.1. Immunogenity of S19 commercial vaccines compared to USDA and to the PBS control. **A.** Batch I. **B.** Batch II. *Statistically significant (P<0,05).

The real vaccine inocula used for the estimation of the recovery time is shown on Table 3.

RT_{50} values of tested vaccines and the S19 USDA original seed reference strain were obtained and compared using the program Rev2, the statistical procedure described by Pouillot et al. (2003). The program estimates a RT_{50} for the reference strain and a RT_{50} for the tested vaccine, as well as the confidence intervals (Fig.2, Table 4). Moreover, it answers if the model could be valid, analyzing the existence of parallelism between the curves, and finally if there is statistical difference between the reference

strain and the tested vaccine (Pouillot et al., 2003). RT_{50} values obtained for both tested and reference S19 USDA original seed strains, about 7.5 ± 3.3 weeks, were statistically similar. A time-series plot of the number of infected mice per time point for the eight laboratory producing S19 vaccine in Brazil is shown on figure 3. The results of RT_{50} can be visualized with the results if immunogenicity at figure 4.

The results obtained show that the model was valid for all tested vaccines, which means that the comparison could be applied, because the parallelism between the curves (tested and reference strains) was observed. Moreover, it was shown that none of the vaccine strains from any batch were statistically different from the S19 USDA original seed reference strain.

Table 3. Vaccine inoculums per mouse in Residual Virulence test, according to the experimental group, for batches I and II.

Laboratory	Dosage/animal	
	Batch I	Batch II
A	2.5×10^8	2.5×10^8
B	2.3×10^8	2.4×10^8
C	2.8×10^8	3.05×10^8
D	2.0×10^8	2.3×10^8
E	2.35×10^8	2.45×10^8
F	2.95×10^8	2.65×10^8
G	2.15×10^8	2.35×10^8
H	-	2.95×10^8
USDA	3.05×10^8	2.85×10^8

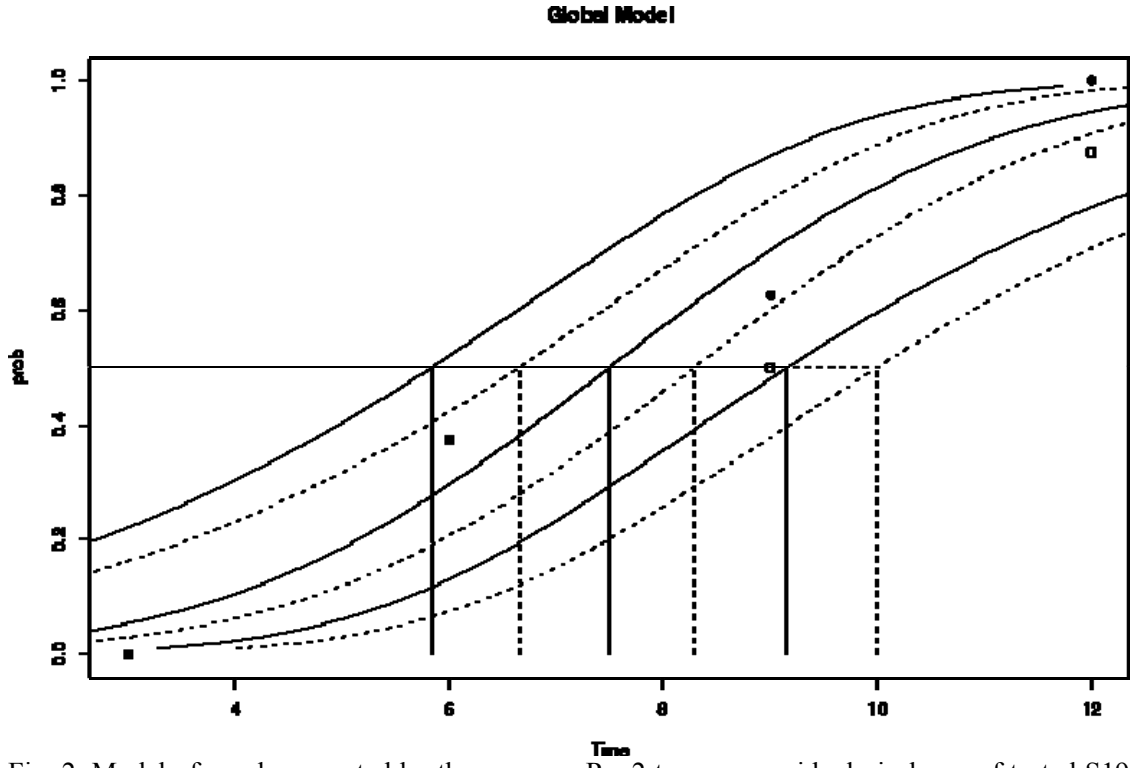
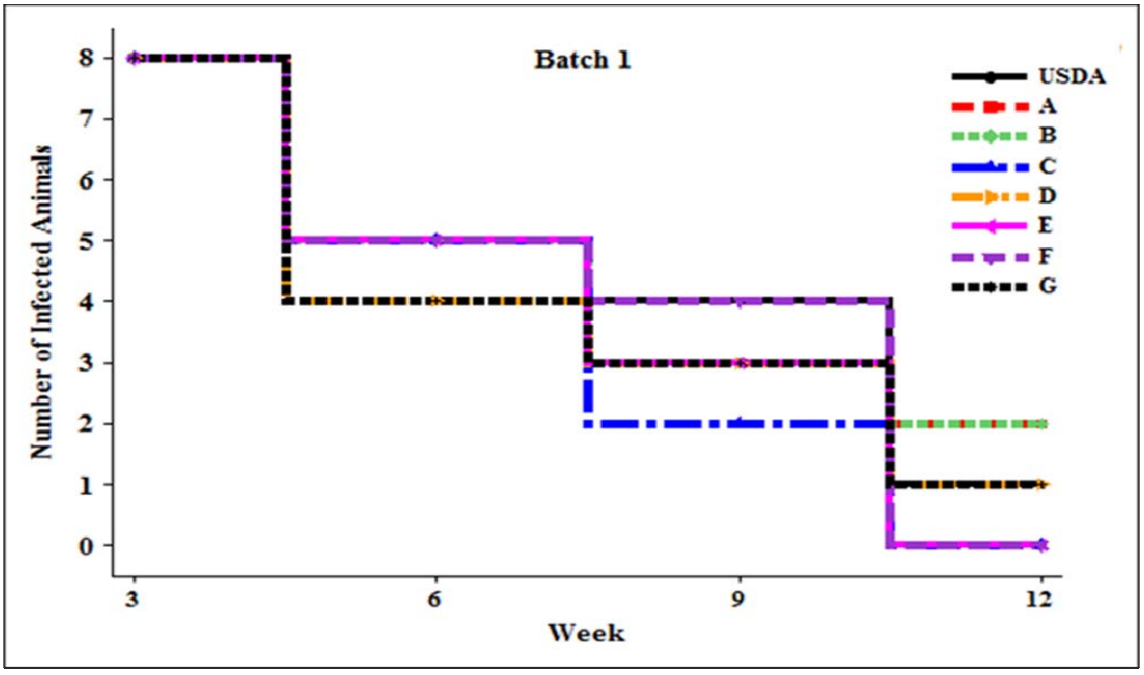


Fig. 2. Model of graph generated by the program Rev2 to assess residual virulence of tested S19 vaccines. Tested laboratory, represented by discontinuous line, compared to the reference strain, represented by continuous line.

Table 4. Model of RT_{50} results obtained with program Rev2.

RT_{50}	Lower limit	Estimate	Upper limit
Tested strain	6.4	8.3	10.0
Reference strain	5.8	7.5	9.2

Validity of the model: The model could be valid.
Comparison of strains: Strains are not different.



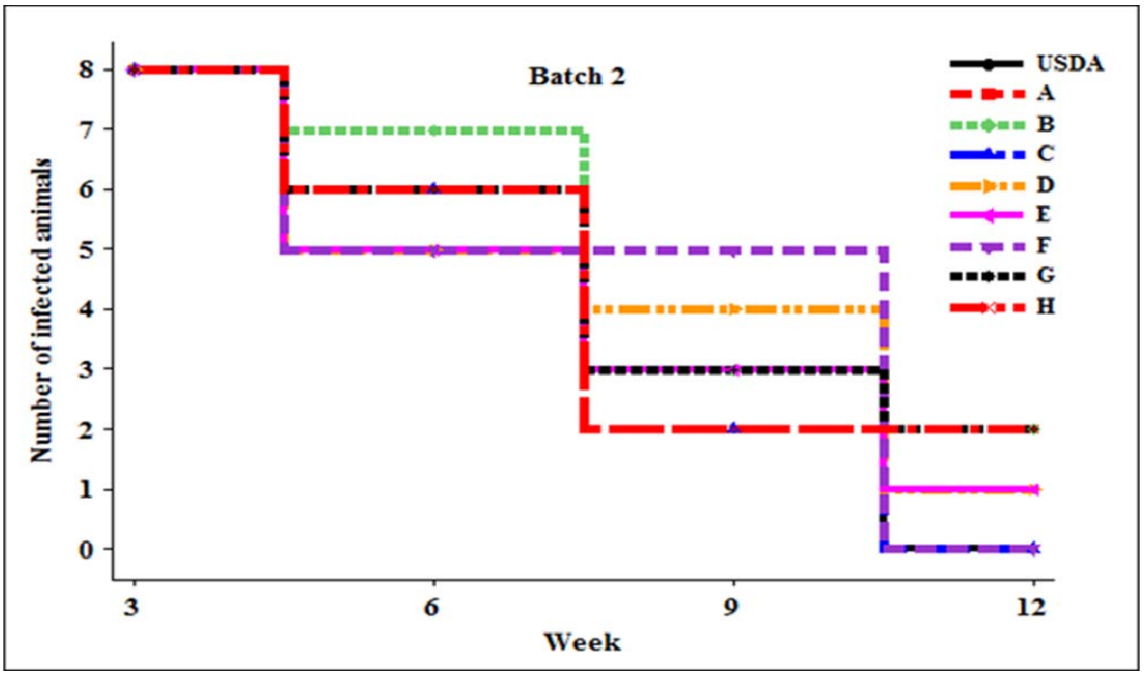


Fig. 3. Residual Virulence of S19 vaccines produced in Brazil. Time-series plot of the number of infected mice per time point for the eight laboratory producing S19 vaccine in Brazil. Data are showed for the two batches tested

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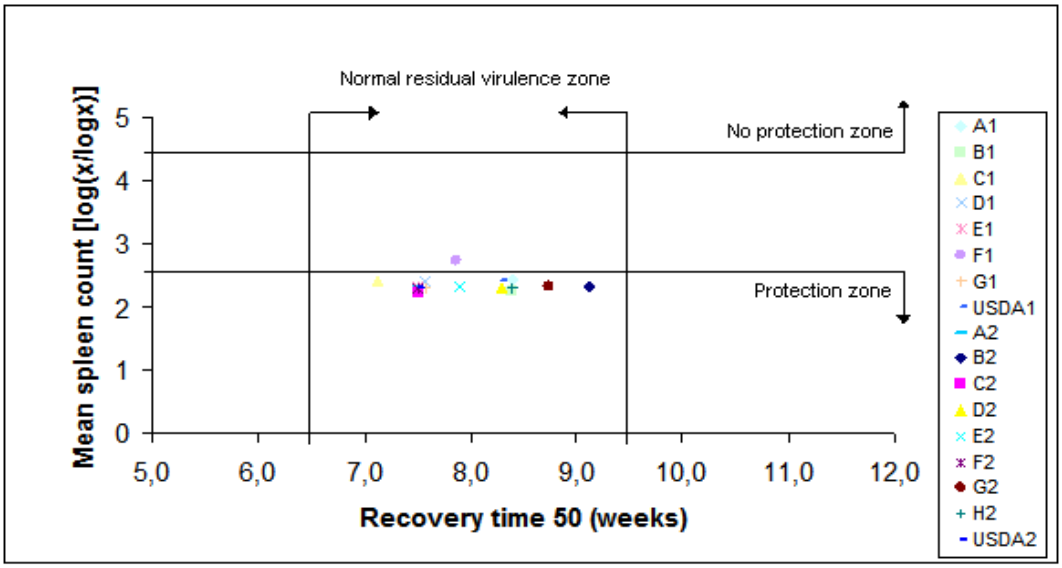


Fig. 4. Evaluation of biological activity (residual virulence and immunogenicity) of *B. abortus* S19 vaccines (Bosseray, 1991).

The figures 5 and 6 show the MLVA results of the vaccine strains tested, for panel 1 and B (Le Flèche et al., 2006). The figure 7 shows a graphical representation of the number of repeat units per *loci* of *Brucella* MLVA typing system of the eight commercial vaccine strains, the reference S19 strain (USDA) and the virulent strain *B. abortus* 544.

According to the results, the vaccine strain from the laboratory G presents a different genotypic profile when compared to the reference strain from USDA. This difference occurs in *locus* Bruce07, of panel 2, where all the tested strains, including the S19 USDA original seed, showed five repeat units, but both batch from laboratory G showed six repeats (Fig. 6A). The reproducibility of the MLVA 15 (Le Flèche et la., 2006) was shown to be very high as both batches of each vaccine showed the same profile.

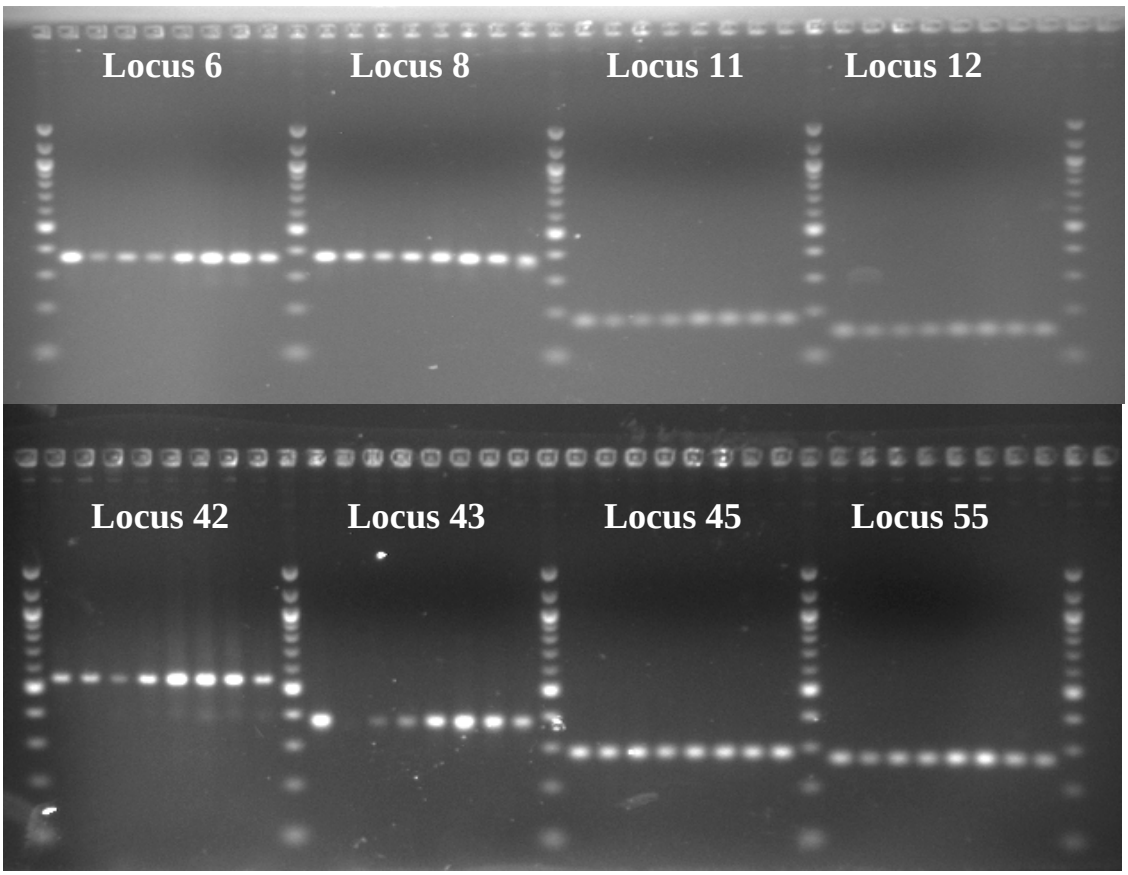
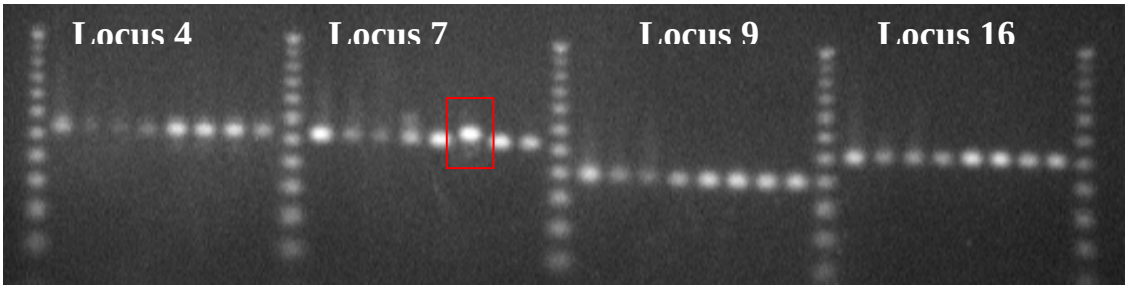


Fig. 5. Amplification patterns of *loci* Bruce06, Bruce08, Bruce11, Bruce12, Bruce42, Bruce43, Bruce45 and Bruce55 of MLVA panel 1 on the eight commercial S19 vaccine strains and the reference strain (USDA).

A



B

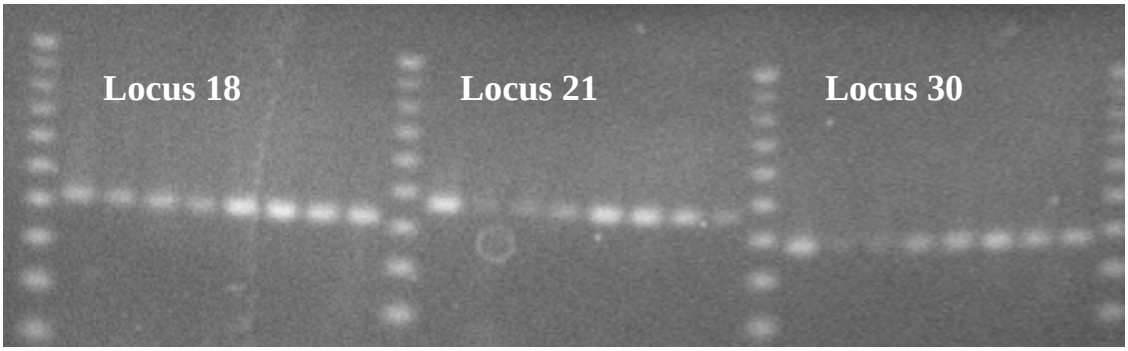


Fig. 6. Amplification patterns of *loci* Bruce04, Bruce07, Bruce09 and Bruce16 (A), and Bruce18, Bruce21 and Bruce30 (B) of MLVA panel 2 on the eight commercial S19 vaccine strains and the reference strain (USDA). The highlighted strain on locus 7 is from laboratory G.

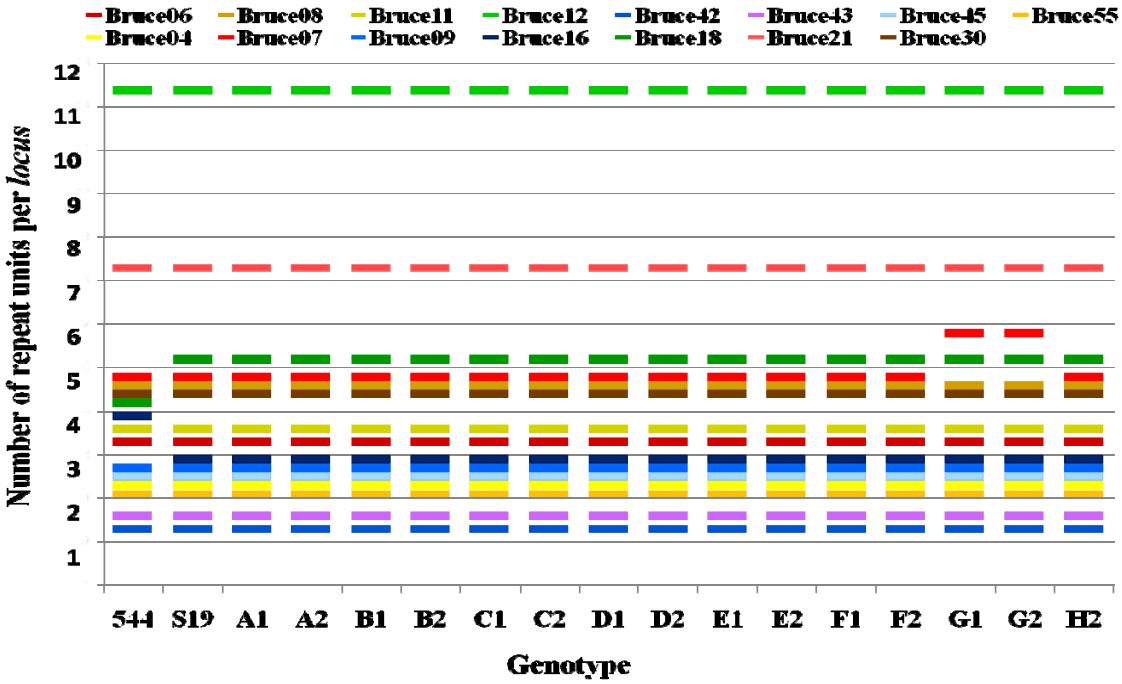


Fig.7. MLVA 15 Genotypes of commercial *B. abortus* S19 vaccines and reference strains (Le Flèche et al., 2006). Graphical representation of the number of repeat units per *loci* of *Brucella* MLVA typing system. 544 – *B. abortus* biovar 1 strain 544 (ATCC 23448^{T}); S19 – *B. abortus* S19 USDA original seed strain; A1 to H2 - S19 commercial vaccines from laboratory A to H and from batch 1 or 2.

4. Discussion

All the commercial batches of S19 vaccines tested in this experiment were shown to be in accordance to *in vitro* and *in vivo* parameters established by Brazilian and international authorities (Brasil, 2004; OIE, 2008). The results obtained with this study show that all S19 vaccines commercialized in Brazil would be approved according to OIE conditions in immunogenicty and residual virulence tests. This is a very important finding, as it is the first time *in vivo* tests were performed on Brazilian S19 commercial vaccines, because Brazilian regulations does not require *in vivo* tests for the approval of a S19 vaccine batch. *In vitro* Official Brazilian control of S19 vaccine includes purity, viability, smoothness, thermal stability, humidity, ph and vacuum. All the strains included in this study were previously tested and approved by Brazilian Official Control, however these requirements do not guarantee the immunological quality of vaccines (Grilló et al., 2000). The results obtained about biological activity of Brazilian commercial S19 vaccines give a guarantee of the immunological quality of these vaccines.

Bosseray (1991) and Bosseray et al. (1984) reported that some anti-*Brucella* vaccines having adequate *in vitro* markers showed a deficient biological activity in mice, and Blasco (1997) evidenced the same fact studying Rev1 vaccines in sheep. Inadequate manipulation of Rev1 and S19 strains during manufacture, can result in a loss of biological activity for both vaccines. Thus, the assessment of the biological properties of seed lots and representative final lots is an essential element to guarantee the biological quality of the live anti-*Brucella* vaccines that have fulfilled the *in vitro* controls.

Some *in vitro* markers, such as dissociation of colonial phase, have been demonstrated to be an important element affecting the biological activity of vaccines (Bosseray, 1991), because rough mutants are rapidly cleared from spleens and induce low immunogenicity. Moreover, rough mutants may revert to apparently normal smooth colonies that show significantly reduced residual virulence (Bosseray, 1991). Therefore this *in vitro* marker is a good parameter, but it does not imply always an adequate biological quality. Grilló et al. (2000) showed a commercial Rev1 vaccine that was adequate according to the standard criteria for dissociation (less than 5% of rough colonies), but was deficient in biological activity. All the vaccines used in the experiments were previoulsly tested and approved by Brazilian Official Control for absence of contamination, adequate viable counts, assessment of the typical colonial morphology, sterility of diluents, pH, humidity and negative pressure.

In 2001, the Ministério da Agricultura, Pecuária e Abastecimento (MAPA) (Brazilian Ministry of Agriculture, Livestock and Food Supply implemented the Programa Nacional de Controle e Erradicação de Brucelose e Tuberculose (PNCEBT) (National Program on the Control and Eradication of Brucellosis and Tuberculosis). The PNCEBT is based mainly on testing-slaughtering of positive animals and mandatory vaccination of female calves aged between three and eight months with S19 vaccine (Brasil, 2006b). Although PNCEBT also previews voluntary accreditation of free and monitoring herds (Brasil, 2001; Poester et al., 2002), the main current goal of the program is to implement an S19 vaccination program to reduce the high cattle and herd prevalence found in the country (Brasil, 2006a; Lage et al., 2008). It is known that the success of the control program of brucellosis rests on the quality of the vaccine used, specially when this vaccine is used once in the animal life, as it occurs with S19. Thus, these results give us the certainty that this tool, mandatory vaccination with S19, can contribute to achievement of the goal established by the PNCEBT of reducing brucellosis prevalence to less than 1% within a decade.

Anti-*Brucella* live vaccines with increased residual virulence could be involved in the induction of undesirable side effects such as abortions or long lasting persistence of infection (Blasco, 1997) and should be discarded. Live attenuated vaccines show decreased virulence when compared to wild pathogenic strains. However, to induce immunity they have to survive enough time in immunoreactive tissues of the reticuloendotelial system to guarantee an adequate and durable immunity (Plommet and Plommet, 1987), but not so long that it could interfere to serological or bacteriological tests. The adequate viable count of vaccine is one of the essential parameters to guarantee that vaccine strain induce adequate immunity in animals. Thus, the Official Control must check and guarantee that viable counts persist up to expiration time of the product. This *in vitro* criteria has been recently evaluated by Caldeira (2008). About the *in vivo* parameters, a direct relationship between immunogenicity and residual virulence should be expected in vaccines, but it has not been always found (Bosseray and Plommet, 1990, Grilló et al., 2000). Thus, because of this lack of relationship between RT_{50} and immunogenicity, the model requires the combined determination of both parameters for a proper evaluation of the biological quality of vaccines.

The statistical method used for determining the RT_{50} of S19 vaccines, the program Rev2 (Pouillot et al., 2003), was easy to use and currently available on the internet (www.afssa.fr/interne/rev2.html), and is based on the same biological assumptions used by Bonet-Maury et al. (1954), which was the method indicated by World Organization for Animal Health in (OIE, 2004). In 2008, the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2008 alternatively gives the option of using the program Rev2 to calculate RT_{50}, what we think to facilitate the appliance of this test.

The efficient DNA-based method, MLVA, was performed in all vaccine strains tested to give an additional tool to analyze and compare strains in the case they present different biological activity, which did not occur in the present study. Considering MLVA results on the S19 strains we conclude that the group of commercial and reference vaccines is genetically very homogenous. The genetic stability has been demonstrated from one batch to another. The comparison of all genotypes showed variation in the number of repeats only in one locus, in one strain (from laboratory G). It was observed in *locus* Bruce07, on panel 2. The fifteen markers used in the *Brucela* MLVA are a combination of moderately variable (minisatellites, panel 1) and highly discriminant (microsatellites, panel 2) loci (Le Flèche et al., 2006). The difference found in our study corresponds exactly to a panel 2 locus, where we can find more variability. In addition, it was a little difference; the change corresponds to a gain of a single repeat unit. This strain from laboratory G had a consistent genotypic profile; because both batches tested showed the same results and it did not interfere with the ability of this strain to induce immunity in mice, as it was shown in immunogenicity and residual virulence studies that were identical to the reference strain.

After obtaining the initial seed from a reference bank, the industry has the responsibility to produce vaccine preserving the initial characteristics. Reduced passages of reference vaccine strains are essential to avoid changes in it. By performing two passages from the initial strain, enough seed lots can be produced with identical characteristics for a large number of seed batches (initial seed, master seed, production seed lots and finally production batches). The Brazilian legislation on S19 vaccine production obligates the producing laboratories to adopt this system of reduced passages (Brasil, 2004) and the MLVA results show that this normative has been efficacious to preserve the characteristics of vaccine strains.

Bricker et al., 2003, in a study including some of the markers used in the MLVA-15 (LeFleche et al., 2006), tested some field, reference and vaccine strains. They tested four-non consecutive production lots of commercially prepared RB51 vaccine and demonstrated that limited *in vitro* passage of RB51 did not induce any allele mutation.

García-Youldi et al. (2007) investigated the stability of *B. melitensis* Rev1 vaccine strains by MLVA. They studied 36 vaccines, and found 7 different genotypes. The differences found in that case occurred at markers Bruce07, 09, 16 or18, which belong to the highly discriminatory panel 2. As it seems, Rev1 is more susceptible to mutations than S19, however, just like in our study, must of differences corresponded to gains or losses of a single repeat unit in a locus of panel 2. Thus, they concluded that the Rev1 strains studied were genetically homogenous. Unfortunately, in that case they did not compare the genotypic profile to the immunological activity of those strains, but they also believe that MLVA methodology could be an essential assay to test quality and stability of live anti-bacterial vaccines produced worldwide and that this technique could be included as *in vitro* control.

The overall results, added to the literature data of *Brucella* MLVA make us believe that this methodology could belong to the list of *in vitro* tests applied to the commercial S19 vaccines tested by the Official Control, because it would rapidly identify expressive changes in the strains' characteristics.

5. Conclusion

All the S19 vaccines commercialized in Brazil present adequate biological properties, which were assessed through the study of residual virulence and immunogenicity in mice.

Results suggest that Official Control of S19 vaccines does not need to apply the i*n vivo* tests routinely, thus it is not necessary to include these tests in Brazilian legislation of S19 vaccine control. However, these *in vivo* tests can be done sporadically just to check the biological activity of commercial vaccines.

Based on the results we suggest that the MLVA-15 typing, previously described by LeFleche et al. (2006), could be included in the control tests of S19 as an efficient assay to guarantee the quality and stability of the vaccine strains.

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Chapter V - Persistence of *Brucella abortus* RB51 in the milk of vaccinated adult cattle

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1. Introduction

Brucellosis caused by *Brucella abortus* is a chronic disease of cattle of worldwide economic and public health importance resulting in abortion and infertility. Brucellosis in cattle in Brazil is widespread in the country, although it has an uneven distribution with areas with very low and others with high prevalences of cattle and herd infections (Poester et al., 2002; Brasil, 2006a). The Programa Nacional de Controle e Erradicação de Brucelose e Tuberculose (PNCEBT) (the Brazilian national program on control and eradication of brucellosis and tuberculosis) is based on compulsory vaccination of heifers aged 3–8 months with strain 19; voluntary accreditation of free herds, in accordance with international standards; voluntary vaccination of adult animals with RB51; voluntary monitoring of beef herds based on a periodic sampling scheme; and compulsory slaughter of cattle testing positive, in approved abattoirs (Poester et al., 2002; Brasil, 2006b, Brasil, 2007).

B. abortus RB51 is a lipopolysaccharide O-antigen deficient mutant derived from the virulent strain *B. abortus* 2308 used as an alternative vaccine to the S19 strain, with the advantage of not inducing an antibody specific anti-LPS or anti-O-side chain response that could be detected by routine serological tests (Schurig et al., 1991). This feature allows RB51 vaccination to be performed at any age, while vaccination with S19 is normally restricted to calves between 3 and 8 months of age (Manthei, 1959, Cocks and Davies, 1973). In Brazil, S19 vaccination is compulsory in young female calves and RB51 is approved as additional tool for controlling bovine brucellosis, for use in female cattle older than 8 month (Brasil, 2006b; Poester et al., 2006, Brasil, 2007). As most of adult cattle population in Brazil was never vaccinated, the vaccination of lactating cows with RB51 is prone to become frequent, raising concerns on the public health safety of those measures as there is only scanty data on the shedding of RB51 in the milk (Samartino et al., 1999, Uzal et al., 2000).

Humans can be directly infected through contact with post-parturition or post-abortion vaginal secretions, placenta or fetuses from *Brucella*-infected animals. Consumption of unpasteurized dairy products infected with *Brucella* spp. is also a major route for human infection (Corbel et al, 2006). Brucellosis in humans is a systemic disease that has an acute or insidious onset. Signs and symptoms of the disease include continued, intermittent, or irregular fever of variable duration; headache; weakness; profuse sweaty chills; arthralgia; endocarditis; depression; weight loss; and generalized aches (Young, 1995; Corbel et al., 2006). The disease can persist for periods ranging from days to years if not treated properly. It is a debilitating disease with impact on public health. *B. abortus* RB51 infection in humans is possible but has been rarely documented (Villarroel et al, 2000, Ashford et al., 2004).

Detection of possible human infection with RB51 vaccine strain and development of recommendations for chemoprophylaxis are complicated by two characteristics of this strain. First, humoral response to the RB51 strain is not detected by routine available serologic tests for *Brucella* spp. due to the rough aspect of the RB51 strain (Schurig et al., 1991). Experimental dot-blot assay used for serologic RB51 titration has been evaluated under experimental and field conditions in cattle, but has not been used in human sera (Olsen et al., 1997). Second, due to its selection in rifampicin-enriched media, RB51 is resistant to rifampicin, which is one of the therapeutic choices for treating human cases of brucellosis (Agalar et al., 1999, Solera et al., 1997). Thus, if the RB51 poses a risk for human infection, chemoprophylaxis recommendations require modification.

The colonization of mammary gland and associated lymph nodes with *B. abortus* have been demonstrated, and organisms may be excreted in the milk (Xavier et al., 2009). In a study of efficacy of RB51, with a group of heifers vaccinated with full calf dose at 60 days of gestation and challenged between 6 and 7 months of pregnancy with virulent strain, *B. abortus* was isolated from milk samples of some animals; however *B. abortus* RB51 was not isolated from any sample (Poester et al., 2006). The shedding of RB51 in milk was not also observed in a study of biosafety of RB51, in which 210 heifers were vaccinated with full dose between the first and seventh month of pregnancy (Samartino et al., 1999). However, RB51 was isolated from milk samples of some cows revaccinated with reduced dose of RB51 during pregnancy (Leal et al., 1999, Uzal et al., 2000).

The aim of this study was to evaluate by culture and PCR the shedding of *B. abortus* in the milk of cows vaccinated with full dose of RB51 during lactation.

2. Material and Methods

2.1 Animals and local

This experimental protocol was approved by the Ethics Committee in Animal Experimentation of the Universidade Federal de Minas Gerais (CETEA—UFMG)

Eighteen crossbred cows, aging 25 to 60 months, from brucellosis-free herds were used in the present study. They were divided in two groups. Group 1 were composed of nine cows selected from the herd of Escola de Veterinária da UFMG, at Pedro Leopoldo, Minas Gerais State, Brazil. All animals were vaccinated with S19 as calves (between three and eight months of age) (Brasil, 2004b) and chosen between those animals that have delivered 30 to 60 days earlier. The nine cows from group 2 were selected from herds at Lages, Santa Catarina State, Brazil, where vaccination with S19 is prohibited (Brasil, 2004b). These animals were also between 30 and 60 days after parturition when they entered the experiment.

All cows were raised semi-intensively and fed a balanced diet of corn silage, concentrate and a mineral salt mixture.

2.2 Vaccination

In day 0 of the experiment, when all animals have 30 to 60 days from delivery, the eighteen cows were subcutaneously vaccinated with 1.3×10^{10} CFU of viable *B. abortus* strain RB51, prepared according to Nielsen and Ewalt (2008).

2.3 Milk sampling

Milk samples were collected in sterile polypropylene tubes on days 1, 2, 3, 4, 7, 14, 21, 28, 35, 42, 49, 56 and 63 after vaccination. Teats were disinfected using alcohol 70º. The first milk streams of each teat were discarded, and then 50 mL of milk samples were manually collected from all quarters and stored at -20ºC.

2.4 Bacteriology of the milk

Samples were thawed and centrifuged at 2500 x G, for 15 minutes. The intermediate phase was discarded and the supernatant was mixed with the pellet. The mixtures were immediately inoculated in plates of tryptose agar (Difco, USA) with antibiotics (Farrell's supplement) (Oxoid, UK) in duplicates (Alton et al., 1988). Moreover, 1.0 mL of each mixture was diluted in 9.0 mL of enrichment media (tryptose broth with Farrell's sellective supplement) and incubated into 5% CO2 at 37ºC for 5 days, and then inoculated in tryptose agar with antibiotics (Minharro, 2009). All plates were incubated into 5% CO2 at 37ºC for 9 days. Another aliquot was separated for PCR assay.

2.5 PCR assay

DNA was extracted according to Pitcher et al. (1989). PCR assay for detection of *Brucella* spp. is genera specific and was carried out as described by Baily et al. (1992) with some modifications. The expected size of amplicon was 223 bp from the 31 kDa outer membrane protein gene. The PCR assay employed the primers B4 (5' – TGGCTCGGTTGCCAATATCAA – 3') and B5 (5' – GGCGGCGCGTCTTAAACATG – 3'). The PCR assay was carried out in a final volume of 25 µL with 2.5 µL of reaction buffer 10X (500 mM KCl, 100 mM Tris-HCl, pH 9.0), 2.5 µL of dNTP mix (2 mM), 0.75 µL of $MgCl_2$ (50 mM), 2.5 µL of each primer at 25 µM, 0.25 µL of Taq DNA polymerase (5 U/ µL) and 2 µL of extracted DNA. The amplification conditions employed in PCR assay were: initial denaturation at 94°C for 5 minutes, DNA denaturation at 94°C for 30 seconds, primers annealing at 60°C for 30 seconds and DNA extension at 72°C for 30 seconds, in a total of 30 cycles and final extension at 72 °C for 10 minutes.

The PCR products were visualized after eletrophoresis in 1% agarose gels and stained by ethidium bromide (Sambrook et al., 1989). Any positive results were tested by the enhanced AMOS PCR (Bricker and Halling, 1995), to confirm the presence of RB51 strain.

3. Results

No *Brucella abortus* was isolated from any sample tested by bacteriology. By the genera specific PCR, only a very faint band was amplified (Fig. 1, lane 4) from all milk samples. This sample was collected in the first day after RB51 vaccination from an animal of group 1 (vaccinated with S19 as calf). No other animals showed positive results in milk by the PCR technique.

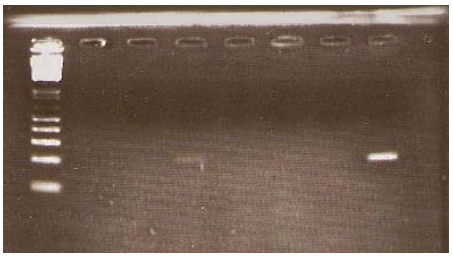

Fig.1. *Brucella* spp. specific PCR from milk samples of RB51 vaccinated cows. The PCR amplification performed in milk samples from cows of group 1 on day 1 after RB51 vaccination are shown for selected animals in lanes 2 to 7. Lane 1 - 1Kb plus marker (Invitrogen, USA). Positive (RB51 in milk) and negative control were shown in lanes 8 and 9, respectively. Gel was stained with ethidium bromide 1%.

The strain amplified by the genera specific PCR assay was further tested by the enhanced AMOS PCR, being confirmed as RB51 strain.

4. Discussion

In 2001, the Ministério da Agricultura, Pecuária e Abastecimento (MAPA) (Brazilian Ministry of Agriculture, Livestock and Food Supply) launched the PNCEBT, in which the strategy of compulsory vaccination of heifers from 3 to 8 months of age with live S19 plays an important role. In addition, the program also envisages the strategic use of the non-agglutinogenic vaccine, RB51, in adult animals that were not vaccinated with S19 as calves or that are negatives in brucellosis infected herds (Brasil, 2004, Poester et al., 2006; Brasil, 2007).

Xavier et al. (2009) demonstrated in a study of pathology, immunohistochemistry, and bacteriology of tissues and milk of experimentally infected cows, that *B. abortus* was detected in milk and mammary tissues of many animals. *B.* abortus-induced mastitis has been described (Emminger and Schalm, 1943, Xavier et al., 2009) and it has been demonstrated that an infected cow that develops mastitis has a high risk of shedding the pathogen in the milk, which might be destined to human consumption (Xavier et al., 2009). The foodborne transmission of *Brucella* spp. is well known and is especially common through the consumption of contaminated raw milk and cheese (Altecruse et al., 1998). Being a live attenuated strain of *B. abortus*, RB51 is approved in Brazil for using in adult animals (Brasil, 2007), which may include lactating cows, creating a real concern of local authorities about its possible danger to public health.

Some studies show that RB51 can be recovered from lymph nodes at various times after inoculation. Studies in cattle show that the vaccine strain was cleared from superficial cervical lymph nodes between 6 and 14 weeks after vaccination (Olsen et al, 1999; Cheville et al., 1996; Cheville et al., 1992). Diptee et al. (2006) found positive bacterial culture of prescapular lymph nodes of water buffalos 6 weeks after vaccination and RB51 was isolated from superficial lymph nodes of bison up to 24 weeks after inoculation (Olsen et al., 1998). However, it does not mean that RB51 can be found for the same period of time in supra mammary lymph node of cows vaccinated with RB51, neither should the cows shed the strain in milk for all this period. Persistence and intermittently shedding of *B. abortus* strain 19 from udder was also demonstrated in adult cows for up to three years (Meyer and Nelson, 1967, Nicoletti, 1981).

Our results show that no *B. abortus* was isolated from any sample tested. It is known that the use of non-selective media (most trivial microorganisms overgrow *Brucella abortus*) or Farrell's selective media (that could considerably reduce the numbers of *Brucella* CFU) results in a further reduction in sensitivity (Marin et al., 1996). In order to improve sensitivity of the isolations and to reduce false negative results, tryptose broth supplemented with antimicrobials (Farrell´s supplement) was used before platting the milk samples. Minharro (2009) showed that this methodology provided an increasing of more than 50% in the rate of isolation of *B. abortus* from infected samples, demonstrating its effectiveness for the isolation of *B. abortus*. To further increase the sensitivity of the diagnosis of *B. abortus* infection, samples were also submitted in parallel to a more sensitive technique, the PCR described by Baily et al. (1992). The analytical sensitivity of this PCR technique was checked by Richtzehain et al. (2002) using two different extraction protocols, and was confirmed between 2 and 20 CFU/mL. This detection threshold was confirmed in our pre-experiments using the DNA extraction protocol described by Pitcher et al. (1989) with milk samples, in which we could detect 10 CFU/mL (data not shown).

With the highly sensitive diagnostic strategy used in the present study, employing two analytical sensitive techniques in parallel,

broth enrichment culture and PCR, just one milk sample from postpartum RB51-vaccinated cows was found positive to *B. abortus* by PCR. This milk sample was from a cow that were previously vaccinated with S19 as calf and the faint band detected by the *Brucella* spp. generic PCR (Baily et al., 1992) (Fig. 1) was confirmed to be from RB51 by the enhanced AMOS PCR (Bricker and Halling, 1995). That result could be due to sample contamination in the laboratory, but this was unlikely because all experiments were performed adhering strictly the measures suggested by Kwok e Higuchi (1989) to avoid cross-contamination and none of the negative controls that were included in all PCR runs showed any sign of contamination. As no *B. abortus* was isolated from this sample or any other following milk sample from the same cow, it could be suggested that or the number of bacteria present in that sample was too small to be detected by culture or the RB51 DNA found in that sample was from a non-viable bacteria. The use of broth enrichment culture (Minharro, 2009) for *B. abortus* isolation probably prevented false-negative results from culture as it has been shown to be very feasible to be used in clinical samples with low number or damaged bacteria. Thus, it is more likely that the positive PCR resulted from DNA of a non-viable *B. abortus* RB51 present in the sample. The public health risk of RB51 infection by ingestion of infected milk from adult RB51-vaccinated cows seemed to be very low, because of the low frequency of infected samples, just in the first day after vaccination, and that this samples has very few or non-viable bacteria. However, pasteurization of all milk should be strictly followed to further reduce the risk of human infection.

The results found in this study differed from those of Uzal et al. (2000), where strain RB51 was isolated from the milk of vaccinated cows until 60 days after vaccination. The isolation technique used by those authors was the inoculation of milk samples subcutaneously into guinea-pigs, which are very susceptible to *B. abortus* infection (Garcia Carrillo, 1990), and later the lymph nodes, spleen and liver from those animals were processed and inoculated onto suitable media. Although the inoculation of susceptible animal is a very sensitive technique, its precise analytical sensitivity is not described. However, according to Alton et al. (1988), this technique is indicated when cultures are overgrown by contaminants, otherwise culture on media is at least as effective as animal inoculation. One major point that must be stressed in Uzal et al. (2000) study is that the period of RB51 shedding in the milk coincides with the time of peripartum immunosuppression of the cows. This immunosuppression occurs in the transition period (from 3 – 4 weeks before up to 3 – 4 weeks after parturition) (Detilleux et al., 1995; Bonizzi et al., 2003; Karcher et al., 2008), and could facilitate the colonization of the attenuated RB51 vaccine strain and consequently its shedding in milk for a long period.

In the present study, the full calf dose of RB51 ($1.0 – 3.4 \times 10^{11}$ CFU) was used to vaccinate postpartum cows. Samartino et al. (1999) also vaccinated cows with a full dose of RB51, between the first and third month of pregnancy and milk samples were cultured up to 30 days after vaccination. Isolation was attempted by direct culture on selective media and also by guinea pig inoculation. No *B. abortus* was isolated from any milk sample post-vaccination. Hence, those results together emphasizes that the RB51 shedding in milk found by Uzal et al. (2000) is probably the result of vaccinating animals during the periparturient immunosuppression period. It has already been demonstrated that neutrophil function, complement activity, immunoglobulin concentration, expression of IFN-γ, TNF-α and IL-12, as well as Th1 cells were reduced during the peripartum (Detilleux et al., 1995; Bonizzi et al., 2003; Karcher et al., 2008). This could explain the long shedding of RB51 in milk of cows vaccinated at 8-9 month of gestation, even using the lower calf dose ($1,0 \times 10^9$ CFU) (Uzal et al., 2000), because most of the protective mechanisms

against *Brucella* spp. infection (Wyckoff III, 2002; Baldwin and Goenka, 2006) is impaired in the peripartum period. Moreover, some authors have demonstrated that inoculation of pregnant cattle with RB51 lead to placental and fetal infection (Palmer et al., 1996) and it may cause fetal losses (Van Metre et al., 1999), which seems to be associated with mid- to late gestation (Van Metre et al., 1999; Poester et al., 2006). Thus, the vaccination of pregnant cows with RB51 could cause economic losses and constitute a real public health hazard, which should be avoided.

5. Conclusion

Only one of the eighteen animals shed the RB51 in milk, in a very low concentration, and on the first day post vaccination. Because of this, it should not be considered a public health problem. However, it is highly recommended to pasteurize the milk of cows after vaccination with RB51.

The public health hazard of milk consumption from RB51-vaccinated cows is very low, since no cow is vaccinated in the peripartum and the recommendation of pasteurization of all milk should be strictly followed to further reduce the risk of human infection.

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Chapter VI - General Discussion

Due to the high prevalences of brucellosis found throughout the country, one of the most important current strategies in PNCEBT is mass vaccination of 3 to 8 month-old female calves. First, the aim of the PNCEBT is to reduce the prevalence of the disease, at least to 1%. The control of the disease will be in fact achieved if mass vaccination is really applied. According to the Brazilian program, there is a mandatory vaccination of female calves, between three and eight months of age, with S19. This program also allows the use of RB51, which represents an additional tool that can be used in adult cows. The major aim of this study was to provide information on specific topics concerning the vaccines against brucellosis used in Brazil, thus supporting the activities of PNCEBT.

The official control held in S19 vaccines nowadays in the country includes all *in vitro* tests, such as viable counts, purity, dissociation, pH, humidity, and vacuum test. These tests are conducted by MAPA on all batches of S19 produced by authorized laboratories.

In fact, it is expected that application of the *in vitro* tests, added to the legislation that regulates the production of brucellosis vaccine ensure that the product that is commercialized has good quality. However, the OIE also recommends that each new seed lot should be tested for residual virulence and immunogenicity to ensure that the vaccine produced by a new seed, has a suitable efficacy when used in calves.

The aim of Chapter II was to establish some data to guide the procedure of differentiation of challenge strain and vaccine strain on studies of immunogenicity of S19 vaccines described on chapter IV. The results guided us to use erythritol and rifampicin for this propose. Despite the results of chapter show that media containing erythritol (1.0 mg/mL) could not inhibit completely the growth of *B. abortus* S19, it was sufficient to inhibit completely the vaccine strain from spleen of animals, because the concentration of S19 in this case was much lower.

Chapter III establishes data for comparison the mouse model for testing vaccines against brucellosis, and the use of different challenge strains, aiming to facilitate the deployment of these *in vivo* tests. According to this study it was possible to conclude that other strains of mice, other than recommended by the OIE can be used. The mice strain recommended by the OIE for such studies is CD-1, in which criterious studies for standardization of techniques were conducted. However this strain is commercialized by only one laboratory, with units in France and the United States, making it difficult and expensive to be use this mouse strain in routine. With the results obtained, it is clear that other strains of mice can be possibly used. These other strains are easily found, and with a lower cost, such as BALB/c and Swiss. For the use of BALB-c in potency tests, studies must be developed to establish mean values of immunogenicity of reference vaccine, as was done with CD-1 mice. With the comparison of the strains of mice, we could conclude that CD-1 mice vaccinated with S19 and challenged with virulent *B. abortus* comported the same way of Swiss mice and the same was observed in control groups of both strains. In addition, we could compare the two challenge strains most used by researchers, which showed no significant difference on their use in the mouse model assay of evaluating *Brucella* vaccines.

The results obtained in Chapter IV showed that all the batches of S19 vaccine from the eight laboratories tested would also be approved by the *in vivo* tests. The confirmation of the quality of S19 vaccines used in the country is critically important, since this vaccine is the major tool of PNCEBT in the initial phase, as the use of vaccines of questionable quality would have made difficult the achievement of the goals stated by the program.

In the same chapter, a difference in the genotypic profile of one of the laboratories tested was observed. This genotypic difference did not result in difference of immunogenicity and residual virulence, at least in the mouse model. This difference may be found due to different origin of the seed, since it is allowed to be acquired from several different banks. The fact that neither the producer laboratories nor the official laboratory tests potency or efficacy of the new vaccine batches that are produced, it would be important to implement the MLVA methodogy in the routine of Official control, because it would guarantee the quality and stability of S19 vaccine strains. Anyway, all the tested vaccines showed identical biological properties to the reference vaccine, with the same level of protection and presenting the same pattern of residual virulence in mice.

The *in vivo* tests are very laborious and expensive. They require well trained technicians, and a detailed standardization of techniques. To implant these tests in the routine of the official laboratory it would require a very careful planning, because it requires a very large functional structure, which may not be suitable to the existing one. The residual virulence test uses a large number of animals per batch of vaccine tested, 32 mice in each group. Beyond this, every experiment must also have a group of more 32 animals vaccinated with a reference S19. In addition, each experiment lasts for at least three months. The immunogenicity test, which evaluates potency of vaccines, uses a smaller number of animals per group, a total of six; however it has the disadvantage of requiring the manipulation of virulent challenge strain. So, with the volume of S19 vaccines produced today in the country, the deployment of these tests requires a considerable investment in staff and structure. It should be thoroughly evaluated, since the S19 vaccine has been produced and controlled in Brazil for decades, and the results of the *in vivo* tests showed adequate and statistically identical results to the obtained with the reference S19. Thus, the investment required for implementation of these tests in the routine of the official laboratory does not justify it. These *in vivo* tests could be done just sporadically.

Regarding the RB51 vaccine, Chapter V has shown its safety for use in adult dairy cows. However, it is possible to occur the shedding of vaccine strain in the milk of some newly-vaccinated animals, but when it occurs, its shedding is in a very low concentration. Anyway, the milk of cows newly vaccinated with RB51 should be submitted to pasteurization. The completion of this study was of great importance, because RB51 is approved in Brazil for use in adult cows, different from other countries, such as the United States, where the vaccine is used only in calves.

1. Conclusions

1. Culture media containing rifampicin, erythritol and thionin are useful in the differentiation between vaccine strains and challenge strains in studies of immunogenicity of live attenuated vaccines against *Brucella abortus*.

2. The strains of mice CD-1, BALB-c and Swiss, as well as the challenge strains *B. abortus* 544 and 2308 can be used in immunogenicity tests of either S19 or RB51 vaccines.

3. All Brazilian commercial S19 vaccines shows adequate immunogenicity and residual virulence, statistically similar to the reference vaccine strain (USDA).

4. MLVA can be included in the list of *in vitro* tests performed by Official Brazilian Control in order to assess genetic stability of S19 vaccine strains.

5. The public health hazard of milk consumption from RB51-vaccinated cows is very low.