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Review

Pathogenesis of bovine brucellosis

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ABSTRACT

Bovine brucellosis is one of the most important zoonotic diseases worldwide, and is of particular significance in developing countries. The disease, which results in serious economic losses due to late term abortion, stillborn and weakly calves, is caused by Gram negative coccobacilli bacteria of the genus *Brucella*. Lesions consist of necrotic placentitis and interstitial mastitis in pregnant cows, and fibrinous pleuritis with interstitial pneumonia in aborted fetuses and newborn calves. This article considers the pathogenesis of *Brucella abortus* and reviews the ability of the pathogen to invade phagocytic and non-phagocytic host cells, resist the acidified intraphagosomal environment, and inhibit phagosome–lysosome fusion. Significant aspects of innate and adaptive immunity against brucellosis are also discussed.

Introduction

Brucellosis is caused by Gram negative bacteria of the genus *Brucella*, which are facultative intracellular coccobacilli that belong to the α2-Proteobacteriacea family (Garrity, 2001). Although there is evidence supporting the notion that the genus *Brucella* should be re-classified as a monospecific genus with several biotypes (Verger et al., 1985), division of the genus into six classical *Brucella* species is still widely used for historical and clinical reasons. These species are *Brucella melitensis*, *Brucella abortus*, *Brucella suis*, *Brucella ovis*, *Brucella canis*, and *Brucella Neotomae* (Osterman and Moriyon, 2006).

Importantly, there is ~94% genetic similarity amongst the members of the genus (Verger et al., 1987; Delvecchio et al., 2002a), although specific genomic islands have been identified (Rajashekara et al., 2004). Whole genome sequencing of *Brucella* spp., including *B. melitensis* (Delvecchio et al., 2002b), *B. suis* (Paulsen et al., 2002), two strains of *B. abortus* (Halling et al., 2005; Chain et al., 2005) and *B. ovis* (R.M. Tsolis, et al., unpublished data) has demonstrated very low genetic variability. Conversely, other bacterial species such as *Salmonella enterica*, have much more genetic diversity amongst their serotypes (Tsolis, 2002). *Brucella* spp. are divided by their strong affiliation to specific natural hosts but can infect heterogeneous hosts (Boschiroli et al., 2001). With the exception of *B. ovis* and *B. neotomae*, all other species are capable of infecting man (Hartigan, 1997).

In addition to the classical *Brucella* spp., the genus has recently been expanded to include marine isolates, which have been divided into two species, *Brucella ceti* and *Brucella pinnipedialis*, based on their preferential hosts i.e. cetaceans and pinnipeds, respectively (Foster et al., 2007). Marine isolates have zoonotic potential, with one reported case of accidental laboratory-acquired infection (Brew et al., 1999) and three other cases of natural infection with a neurological manifestation (Sohn et al., 2003; McDonald et al., 2006). Interestingly, dolphins infected with *B. ceti* often develop a neurological disease (Hernández-Mora et al., 2008). Recently, a new *Brucella* species, named *B. microti*, has been isolated from the common vole *Microtus arvalis* (Scholz et al., 2008).

Brucellosis is one of the most important zoonotic diseases worldwide, particularly in developing countries (Trujillo et al., 1994), Mediterranean countries (Godfroid and Käsbohrer, 2002) and Central Asia (Pappas et al., 2006). In some of the endemic areas such as Africa, the Middle East, and Latin America, brucellosis results in significant human morbidity (Boschiroli et al., 2001). Clinical symptoms in human brucellosis include fever, anorexia, polyarthritis, meningitis, pneumonia, endocarditis, and other less common clinical manifestations (Sauret and Vilissova, 2002). In most cases, human infection is due to consumption of contaminated non-pasteurised milk and cheese or as an occupational exposure to infected animals or carcasses, uterine secretions or aborted fetuses. Less often accidental infection may occur due to manipulation of live vaccine strains or virulent *Brucella* in the laboratory (Young, 1983; Corbel, 1997). As human brucellosis is essentially a zoonotic disease, control and prevention of brucellosis in animals is essential for eradicating the disease in man.

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Bovine brucellosis

Although eight biovars of *B. abortus*, the causative agent of bovine brucellosis, have been identified, biovar 1 is most frequently isolated from cattle (Nicoletti, 1980) in countries where biovar prevalence has been studied, such as the USA (Bricker et al., 2003), Latin America (Lucero et al., 2008), Brazil (Poester et al., 2002) and India (Renukaradhya et al., 2002). *B. suis*, particularly biovars 1 and 3, is capable of infecting cattle, although infection is usually not associated with clinical signs (Ewalt et al., 1997). Importantly, *B. suis* and *B. melitensis* infections in cattle interfere with serological diagnosis of *B. abortus* infection (Rogers et al., 1989; Ewalt et al., 1997).

Outbreaks of bovine brucellosis are associated with abortion during the last trimester of gestation, and produces weak newborn calves, and infertility in cows and bulls (Enright et al., 1984; Poester et al., 2005; Xavier et al., in press). Therefore, due to its zoonotic potential, brucellosis must be differentiated from several other diseases that cause abortion in cattle. Importantly, the outcome of infection in cattle is dependent on age, reproductive and immunological status, natural resistance, route of infection, infectious challenge, and virulence of the infective strain (Nicoletti, 1980; Adams, 2002).

After the first episode of *Brucella*-induced abortion, the cow often has normal subsequent parturitions, although another abortion may occur (Nicoletti, 1980). Calves that acquire the infection vertically or by ingesting contaminated milk may remain serologically negative and not show any sign of the disease. However, heifers with latent asymptomatic infection may abort or give birth to infected calves, which are central in maintaining the disease in a herd (Wilesmith, 1978; Nicoletti, 1980). Other clinical signs of infected cows include reduced milk production, an increase in the number of somatic cells in the milk, and impaired reproductive efficiency (Emminger and Schalm, 1943; Meador et al., 1989).

Infected bulls may develop systemic signs of infection including fever, anorexia, and depression, although infection is often inapparent (Campero et al., 1990). The most significant lesion induced by *B. abortus* in bulls is orchitis (Lambert et al., 1963; Trichard et al., 1982), which is often associated with seminal vesiculitis and epididymitis (Rankin, 1965; McCaughey and Purcell, 1973). As a result of chronic orchitis and fibrosis of the testicular parenchyma, affected bulls may develop permanent infertility (Campero et al., 1990).

Aborted fetuses as well as fetal membranes and uterine secretions eliminated after abortion or parturition are the most important sources of infection (Samartino and Enright, 1993). The disease can also be transmitted to calves vertically (Ray et al., 1988) and through contaminated milk (Wilesmith, 1978; Nicoletti, 1980), but these routes of infection are much less important (Crawford et al., 1990). Venereal transmission is not a major route of infection under natural conditions, but artificial insemination with contaminated semen is a potential source of infection (Rankin, 1965).

Although infection may occur through the skin, conjunctiva or respiratory mucosa by inhalation (Crawford et al., 1990; Ko and Splitter, 2003), the most common route of infection in cattle is the gastrointestinal tract (Payne, 1959; Crawford et al., 1990), from where infection spreads to local lymph nodes where *Brucella* replicates intracellularly in phagocytes (Anderson et al., 1986a). Invasion of lymphatic vessels is followed by bacteraemia leading to systemic infection, favouring colonisation of the pregnant uterus, male genital organs, and mammary gland (Ko and Splitter, 2003).

B. abortus has a strong tropism to the uterus during the last trimester of gestation, which is thought to be due to high concentrations of erythritol and steroid hormones. Erythritol favours bacterial survival since it can be metabolised by *B. abortus* as a source of carbon and energy (Samartino and Enright, 1996). Erythrophagocytic trophoblastic cells located at the base of chorionic villi of ruminants (Santos et al., 1996) are considered the primary site of invasion of fetal placental tissues, from where *B. abortus* disseminates to intercotyledonary trophoblasts (Anderson et al., 1986a). *Brucella* multiplication induces infiltration of inflammatory cells, trophoblastic necrosis, vasculitis, and ulceration of the allantochorion. Consequently, fetal–maternal metabolic exchanges are compromised resulting in abortion (Anderson et al., 1986a).

We have recently performed a thorough histopathological, immunohistochemical and bacteriological analysis of experimental *B. abortus* infection in pregnant cows (Xavier et al., in press). In this study, pregnant cows were inoculated with *B. abortus* (strain 2308), resulting in abortion at the last trimester of gestation (Xavier et al., in press). Gross pathological changes in pregnant uteruses and fetuses are suggestive, but not specific for *B. abortus* infection and are insufficient for diagnosis. Usually pregnant cows develop a local lymphadenopathy at the site of infection that progresses to acute lymphadenitis (Schlafer and Miller, 2007). The infected uterus contains variable amounts of a fetid yellow to brownish exudate containing fibrin and necrotic debris (Xavier et al., in press). Importantly, placental lesions are randomly distributed amongst placentomes, with some normal placentomes and others with severe necrosis and haemorrhage (Fig. 1) (Payne, 1959; Xavier et al., in press). Less severe placental lesions may not result in abortion, but are often associated with weak newborn calves, resulting in high neonatal death rate (Schlafer and Miller, 2007; Xavier et al., in press).

Microscopic changes are also not pathognomonic, but they are more specific than gross changes (Xavier et al., in press). Histologically, trophoblastic cells, which are the primary target cells in the placenta, are swollen and filled with coccobacilli. There is usually a neutrophilic and histiocytic inflammatory infiltrate, associated with necrosis, oedema, fibrin deposition, and in some cases vasculitis (Fig. 2A) (Xavier et al., in press). The endometrium has a severe infiltration of neutrophils, lymphocytes, plasma cells, and a few eosinophils. The inflammatory reaction is associated with multifocal erosions or superficial ulcerations of the luminal epithelium (Payne, 1959; Meador et al., 1988). Necrotic debris in the caruncular crypts and endometrial surface contains large numbers of intra and extracellular *B. abortus* bacilli (Fig. 2B) (Xavier et al., in press). A few weeks after abortion, the uterine inflammatory infiltrate is

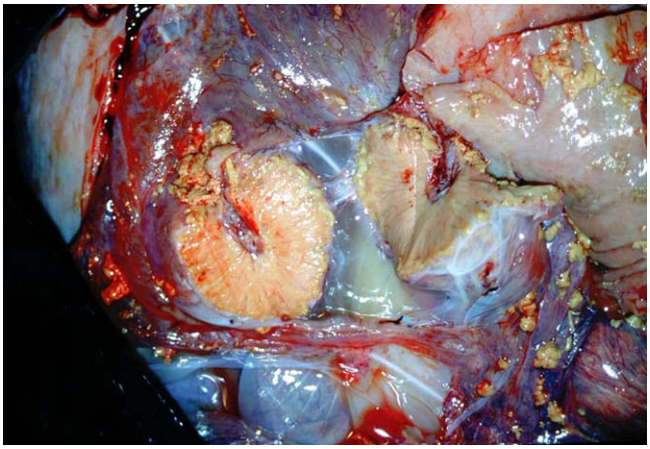

Fig. 1. Cow experimentally infected with *Brucella abortus*. Uterus containing multifocal fibrinous exudate on the caruncular surface. Cut surface of a placentome with fibrinous necrotic exudate and multifocal haemorrhage (necrotising placentitis).

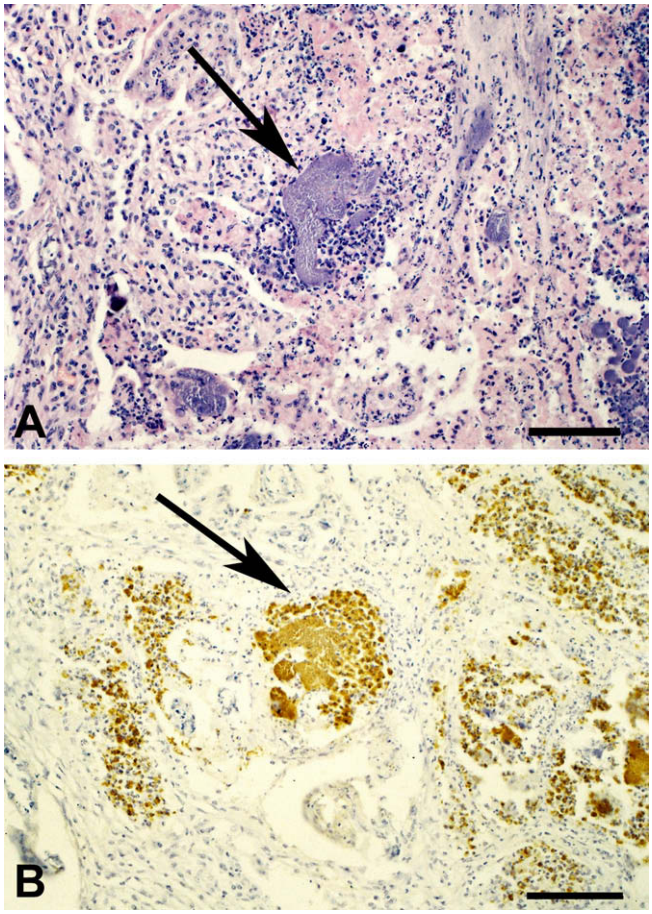

Fig. 2. (A) Cow placentome with caruncular crypts filled with necrotic debris, intense inflammatory infiltrate, and several bacterial colonies (arrow) (acute necrotising placentitis). Haematoxylin and eosin (50×), bar = 100 μm. (B) Cow placentome with immunolabelled colonies of *B. abortus* (arrow), streptavidin–biotin–peroxidase (50×), bar = 100 μm.

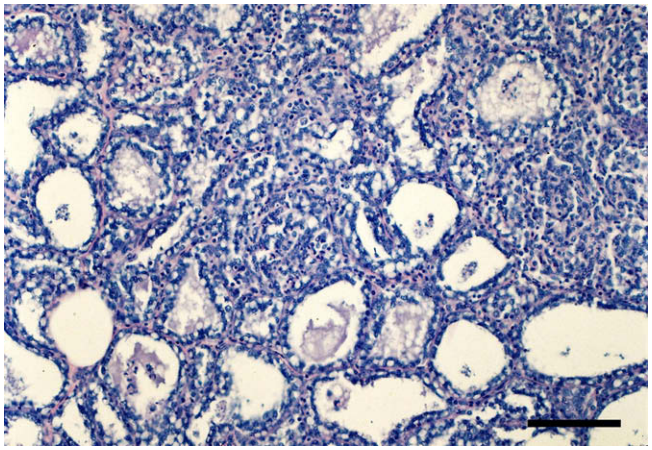

Fig. 3. Cow experimentally infected with *Brucella abortus*. Mammary gland with focal interstitial infiltration of lymphocytes, macrophages, and neutrophils in an acinar lumen. Haematoxylin and eosin (50×), bar = 100 μm.

restricted to a periglandular and perivascular distribution (Meador et al., 1988).

The mammary gland is another target organ that is important in transmitting the infection through contaminated milk. *B. abortus* induces a multifocal interstitial mastitis with interstitial accumulation of macrophages and intra-acinar infiltration of neutrophils (Fig. 3) (Emminger and Schalm, 1943; Payne, 1959; Meador et al., 1989; Xavier et al., in press), associated with moderate numbers of predominantly intracellular organisms (Xavier et al., in press). Histopathological changes also include lymphoid hyperplasia in lymph nodes that may be associated with a neutrophilic infiltrate that may progress to a granulomatous lymphadenitis (Payne, 1959; Meador et al., 1988, 1989).

Under field conditions, aborted fetuses are often not available or are severely autolysed so preventing any useful pathological examination. Nevertheless, experimental infections confirm that aborted fetuses may undergo autolysis within the uterus. Although not seen in all cases, pleuropneumonia is the most common fetal lesion (López et al., 1984; Xavier et al., in press). Grossly the pleura are thickened, oedematous, haemorrhagic, and recovered by fibrin, characterising a fibrinous pleuritis (Fig. 4A). The lung may have areas of pneumonia, which are firm and have thickened interlobular septa (Gorham et al., 1986; Xavier et al., in press). Fibrinous inflammation may also be seen in other body cavities so aborted fetuses may have, in decreasing order of frequency, fibrinous pleuritis, pericarditis and/or peritonitis (Xavier et al., in press). In addition, aborted fetuses may develop enlargement of internal iliac, bronchial, and hepatic lymph nodes (Enright et al., 1984; Gorham et al., 1986), enlargement of the adrenal glands, and hypotrophy of the thymus, which may be associated with petechial haemorrhages (Enright et al., 1984).

Histologically, fibrinous pleuritis may be associated with bronchointerstitial pneumonia (Figs. 4B and C), characterised by the accumulation of macrophages and neutrophils, variable amounts of fibrin, and haemorrhage, and the presence of the bacteria associated with the lesions (Figs. 4D and E). In some cases, necrotising vasculitis is observed in the pulmonary parenchyma (Enright et al., 1984; López et al., 1984; Meador et al., 1988; Xavier et al., in press). Fibrinous pericarditis may also occur in fetuses (Fig. 4F) (Xavier et al., in press). There is lymphoid hyperplasia in lymph nodes and spleen, whereas lymphoid depletion occurs in the thymus (Enright et al., 1984). A granulomatous inflammatory process may be eventually seen in the liver, spleen, and kidney (Meador et al., 1988; Hong et al., 1991), and rarely the central nervous system may be affected with multifocal or diffuse histiocytic meningitis (Hong et al., 1991).

A definitive diagnosis must be supported by laboratory tests, including serological assays or direct diagnostic tests, i.e. isolation and biochemical characterisation of the organism (Nielsen and Ewalt, 2004). An alternative to isolation is the use of PCR-based methods for detecting *Brucella* genomic DNA (Leal-Klevezas et al., 1995; Bricker, 2002). Immunohistochemistry is another approach for direct diagnosis based on detection *Brucella* antigens in tissue sections. Although not widely used for diagnostic purposes, this method is a very useful tool for studying pathogenesis of the disease (López et al., 1984; Meador et al., 1986; Santos et al., 1998; Xavier et al., in press).

Serological assays are based on the fact that *B. abortus* as well as other smooth *Brucella* have the O polysaccharide that induces a humoral response with an initial production of IgM followed by IgG1, and IgG2/IgA (Beh, 1974; Allan et al., 1976; Nielsen et al., 1984). Considering that IgM often cross-reacts with other agents, and IgG2 or IgA are produced in low concentrations and later during the course of infection, IgG1 is recognised as the major target in serological assays (Allan et al., 1976; Lamb et al., 1979; Nielsen et al., 1984; Butler et al., 1986). Screening tests include the buffered acidified plate antigen test and the milk ring test, both of which having high sensitivity. These assays are complemented by confirmatory tests such as reduction by 2-mercaptoethanol or complement fixation. Indirect or competitive ELISA and fluorescent polarisation assay are also employed as confirmatory tests (Nielsen, 2002). Serological diagnosis may be compromised by use of

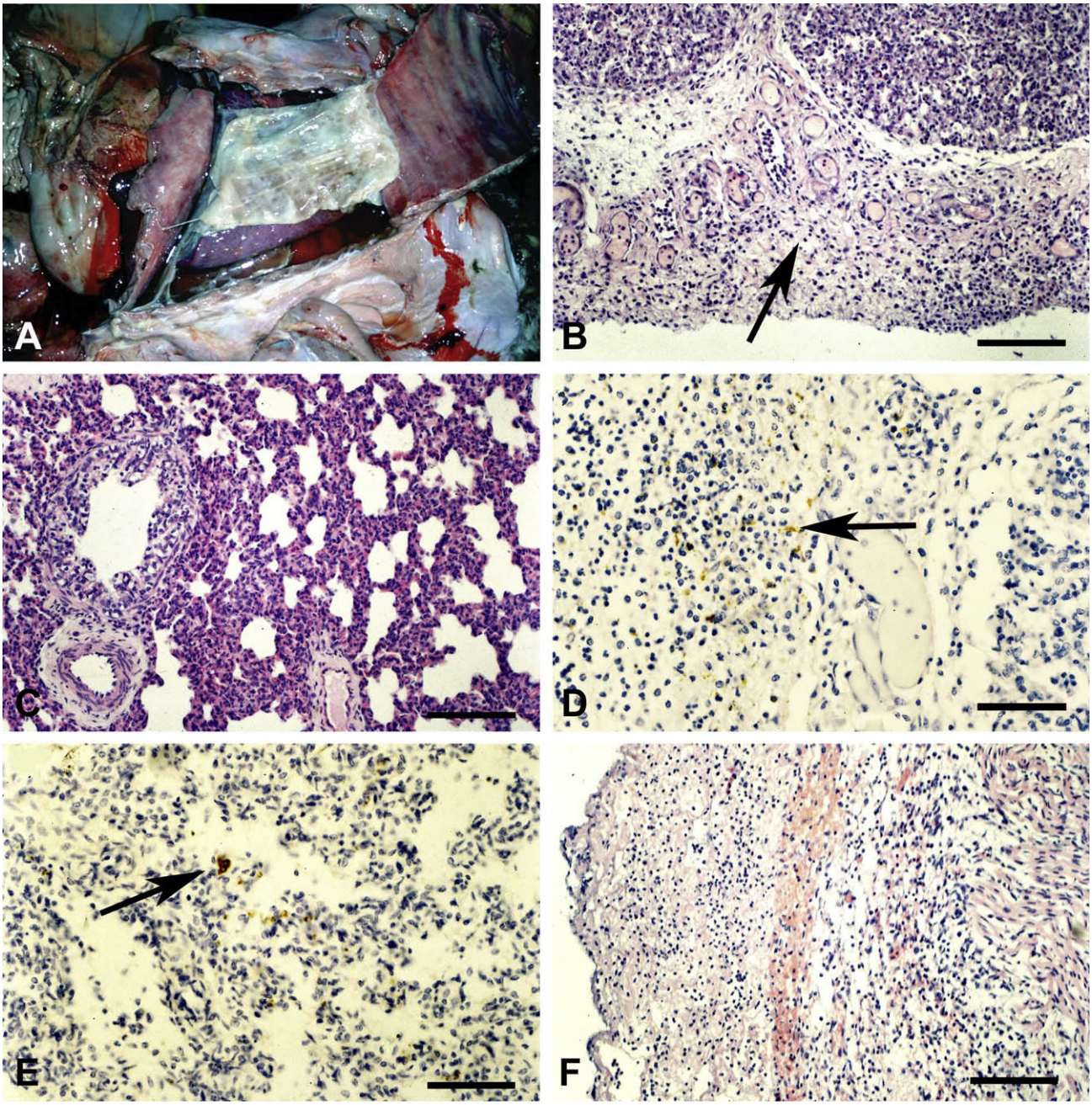

Fig. 4. Aborted fetuses and weak premature calves experimentally infected with *B. abortus*. (A) Aborted fetus with a large amount of fibrinous exudate on the pleural surface of the lung and a moderate amount of fluid in the thoracic cavity (fibrinous pleuritis). (B) Aborted fetus with severely thickened visceral pleura (arrow), fibrin accumulation and diffuse inflammatory infiltrate (fibrinous pleuritis). Haematoxylin and eosin (50×), bar = 100 μm. (C) Weak premature calf showing lung with diffusely thickened alveolar walls and interstitial inflammatory infiltrate (interstitial pneumonia). Haematoxylin and eosin (50×), bar = 100 μm. (D) Aborted fetus; visceral pleura with immunolabelled colonies of *B. abortus* (arrow), streptavidin–biotin–peroxidase (100×), bar = 50 μm. (E) Weak premature calf showing lung with immunolabelled colonies of *B. abortus* (arrow), streptavidin–biotin–peroxidase (100×), bar = 50 μm. (F) Aborted fetus with severely thickened pericardium, fibrin accumulation and diffuse inflammatory infiltrate (fibrinous pericarditis). Haematoxylin and eosin (50×), bar = 100 μm.

the Strain 19 vaccine since this is a smooth strain. This limitation has been overcome by the use of the RB51 vaccine, which is a rough strain that does not interfere with serological tests (Schurig et al., 1991; Cheville et al., 1993; Poester et al., 2006).

Invasion of host cells by *B. abortus*

A key step in the pathogenesis of *B. abortus* is its ability to invade phagocytic and non-phagocytic host cells. *B. abortus* is capable of invading the intestinal mucosa preferentially through the M cells. Intra-epithelial phagocytes may also favour transepithelial migration and transport of *B. abortus* to the lamina propria and submucosa (Ackermann et al., 1988). Opsonised *B. abortus* is internalised in phagocytes through complement or Fc receptors, whereas non-opsonised organisms apparently invade by interacting with lectin and fibronectin receptors (Campbell et al., 1994).

Opsonised bacteria phagocytosed by activated macrophages are usually killed within the phagolysosome before they reach the sites of intracellular replication. Conversely, non-opsonised organisms are more likely to survive and replicate intracellularly (Gorvel and Moreno, 2002). Attenuated strains adhere and invade host cells, but they do not survive intracellularly (Pizarro-Cerdá et al.,

2000; Gorvel and Moreno, 2002). Although *B. abortus* is capable of invading epithelial cell lines (Detilleux et al. 1990a,b; Guzmán-Verri et al., 2001) and bovine trophoblastic cells (Carvalho Neta et al., 2008), it is much less invasive than other facultative intracellular bacterial pathogens such as *Salmonella enterica* (Santos and Baumler, 2004).

No specific receptors for *B. abortus* have been identified in non-phagocytic cells, but there is evidence of specific bacterial molecules involved in adhesion (Gorvel and Moreno, 2002). A surface protein 41Kd of *Brucella* (SP41) is associated with adherence and invasion in host cells (Castañeda-Roldán et al., 2006). In vitro studies have demonstrated that invasion of epithelial cells by *B. abortus* is associated with recruitment of actin filaments to the site of interaction of the bacterium with the host cell membrane (Pizarro-Cerdá et al., 1999; Guzmán-Verri et al., 2001).

The internalisation of *B. abortus* by non-phagocytic cells involves activation of small GTPases of Rho subfamily such as Rho, Rac and Cdc42 (Guzmán-Verri et al., 2001), which are known to be regulators of cytoskeleton. Apparently activation of Cdc42 is directly due to the contact of *B. abortus* with the host cell, whereas activation of Rac ad Rho appears to be indirect (Waterman-Storer et al., 1999). In addition, molecular mediators of signalling pathways, including cyclic GMP, PIP3-kinase, tyrosine kinase, and MAP kinases are also involved in the internalisation process of *B. abortus* as second messengers of signal from GTPases, since the inhibition of these molecules impairs bacteria invasion (Guzmán-Verri et al., 2001; Gorvel and Moreno, 2002).

Intracellular survival of *B. abortus*

B. abortus intracellular survival is dependent upon its ability to resist the acidified intraphagosomal environment and to inhibit phagosome–lysosome fusion (Pizarro-Cerdá et al., 1998a; Porte et al., 1999; Wang et al., 2001). These processes are triggered by the internalisation of *B. abortus* that redirects intracellular trafficking, changing the normal maturation process of the phagosome and blocking fusion of *Brucella*-containing phagosomes with lysosomes (Gorvel and Moreno, 2002). Intracellular trafficking of *B. abortus* and its capability of surviving intracellularly is not identical in all cell types. For instance, neutrophils are not permissive to intracellular growth of *B. abortus*, whereas the organism survives well in other phagocytes such as macrophages in which the intracellular trafficking of the pathogen is similar to non-phagocytic cells (Jiang and Baldwin, 1993; Gorvel and Moreno, 2002).

Soon after internalisation of *B. abortus,* the early phagosomal complex is formed, which expresses the small GTP-binding protein Rab 5 and early endosomal antigen 1 (EEA1) (Pizarro-Cerdá et al., 1998b; Chaves-Olarte et al., 2002). GTPases are also activated during intracellular trafficking of *B. abortus* but they are different from those activated during the invasion process. Thus, invasion and intracellular trafficking appears to be independent (Pizarro-Cerdá et al., 1998a,b; Guzmán-Verri et al., 2001; Gorvel and Moreno, 2002). Acidification of the *Brucella*-containing vacuole during early steps of infection is also required for intracellular survival. This acidified environment induces changes in the profile of bacterial gene expression favouring intracellular survival (Porte et al., 1999).

In cultured HeLa cells, at 2 h post inoculation, *B. abortus* is located in multi-membranous intracellular compartments that co-localise with the lysosome-associated membrane glycoprotein 1 (LAMP1), a marker for autophagosomes (Pizarro-Cerdá et al., 1998b, 2000). The *Brucella*-containing vacuole then acquires markers of the rough endoplasmic reticulum (RER), such as calreticulin and sec61β. These findings demonstrate that *B. abortus* traffics from a phagosome compartment (Pizarro-Cerdá et al., 1998b) towards the RER of the host cell, where the organism has an optimal environment for replication (Anderson et al., 1986b; Pizarro-Cerdá et al., 2000). Early ultrastructural analysis of *Brucella*-infected cells has also identified the RER as the site of replication (Anderson et al., 1986b; Meador and Deyoe, 1989; Detilleux et al., 1990a). *Brucella* has limited replication in early compartments, and the RER is the only compartment that sustains optimal bacterial replication (Comerci et al., 2001; Chaves-Olarte et al., 2002; Gorvel and Moreno, 2002).

Intracellular replication of *B. abortus* in trophoblastic cells is strongly influenced by the stage of gestation, with higher replication rates within trophoblasts in late gestation when the cells actively secrete steroid hormones (Samartino et al., 1994). In trophoblasts, *B. abortus* induces steroid synthesis and modulates the metabolism of prostaglandin precursors, favouring bacterial growth (Anderson et al., 1986b). In addition, hormonal changes take place in infected placentas, with increase in the levels prostaglandin $F_{2\alpha}$, a decrease in progesterone, and an increase in oestrogen and cortisol. These changes mimic to some extent what happens during parturition (Gorvel and Moreno, 2002), and are likely to contribute to the abortion.

Molecular mechanisms of *B. abortus* pathogenesis

Brucella does not have classical virulence factors such as exotoxins, cytolysins, capsule, fimbria, flagellum, plasmids, lysogenic phages, antigenic variation, endotoxic lipopolysaccharide (LPS), and inducers of host cell apoptosis (Moreno and Moriyón, 2001). The mechanisms of *Brucella* spp. virulence are factors that are required for invasion (Guzmán-Verri et al., 2001) and intracellular survival (Moreno and Moriyón, 2001), which allow the organism to reach its intracellular replication site (Detilleux et al., 1990a,b; Pizarro-Cerdá et al., 1998b, 1999).

Smooth strains of *Brucella* generally invade host cells more efficiently than rough strains, suggesting that the LPS O chain plays a role in virulence, although some rough strains are naturally virulent (Sola-Landa et al., 1998; Ko and Splitter, 2003). *Brucella* LPS was originally recognised as a virulence factor due to its relatively low immunogenicity, which prevented activation of the alternative complement pathway (Sangari and Aguero, 1996). The role of LPS was confirmed by mutagenesis of the O chain that rendered *Brucella* more susceptible to complement-mediated bacterial lysis (Allen et al., 1998) and to bactericidal cationic peptides such as defensins and lactoferrins (Lapaque et al., 2005). Furthermore, *Brucella* LPS has long been recognised to be a weaker inducer of the immune response compared to enterobacterial endotoxins (Keleti et al., 1974). The LPS O chain inhibits cellular apoptosis avoiding immune response activation (Jimenez de Bagues et al., 2004; Pei and Ficht, 2004; Pei et al., 2006). It is noteworthy that *Brucella* LPS plays a more relevant role in virulence whilst the organism is in the extracellular environment prior to invading host cells (Ko and Splitter, 2003). Nevertheless, *B. abortus* rough mutant strains have a lower ability to survive intracellularly than smooth strains since the LPS O chain is essential for entry and early intracellular phase of *Brucella* in macrophages (Porte et al., 2003; Lapaque et al., 2005).

During internalisation, *B. abortus* relies on a two-component regulatory system named BvrR/BvrS, which is required for recruitment of GTPases as described above, and maintenance of the outer membrane. Thus, *bvrS–bvrR* mutants are impaired for invasion of non-phagocytic cells and intracellular survival (López-Goñi et al., 2002). The two components of this system are BvrS, a sensor protein member of the histidine-kinase superfamily, and BvrR, which is a regulator protein. This system regulates expression of outer membrane proteins (Omp) involved in invasion of host cells (López-Goñi et al., 2002; Guzmán-Verri et al., 2002). *B. abortus*

strains with mutated *bvrR* and especially *bvrS* lack the ability to recruit GTPases of the Rho subfamily, particularly Cdc42, which is required for actin polymerisation and invasion of host cells. Additionally, when invasion of the host cells by these mutants is stimulated artificially by enzymatic treatments, the mutants are more susceptible to host cell killing mechanisms. Attenuation in intracellular survival in this case is caused by the incapacity of the mutants to prevent phagosome–lysosome fusion (Sola-Landa et al., 1998; López-Goñi et al., 2002).

Depletion of *Brucella* cyclic β-1,2-glucans synthetase results in lack of cyclic β-1,2-glucans, which are constituents of outer membrane that are required to the survival of *B. abortus* in mice and intracellular replication in HeLa cells (Briones et al., 2001). Cyclic β-1,2-glucans are essential during early intracellular phase of *Brucella* infection since they prevent phagosome–lysosome fusion although they are not important for the trafficking of *Brucella* to the RER.

Brucella type VI secretion system is encoded by the *virB* operon (Comerci et al., 2001; Delrue et al., 2001), which is composed of 12 genes, namely *virB1* through *virB12*. An orthologue T4SS was originally identified in the plant pathogen *Agrobacterium tumefaciens*, and later was recognised as an essential virulence mechanism of *B. abortus* that is required for intracellular multiplication of the organism (O'Callaghan et al., 1999; Hong et al., 2000; Sieira et al., 2000). The *virB* encoded T4SS is required for intracellular growth of *B. abortus* in both phagocytic and non-phagocytic cells such as HeLa cells (O'Callaghan et al., 1999; Sieira et al., 2000; Comerci et al., 2001; Delrue et al., 2001).

Although the molecular mechanisms by which the T4SS system influences intracellular trafficking of *B. abortus* is not clear, apparently secreted effectors play a role in the biogenesis and maturation of the *B. abortus*-containing vacuole, and transport of *B. abortus* to its intracellular site of replication (Delrue et al., 2001; Comerci et al., 2001; Boschiroli et al., 2002), with evidence that the system is required for fusion of the autophagosome-like vacuole with the RER (Arellano-Reynoso et al., 2005). Experimental infection of mice and cultured cells with strains of *B. abortus* with a defective T4SS results in intracellular killing since the mutant strains fail to reach the RER (O'Callaghan et al., 1999; Hong et al., 2000; Sieira et al., 2000; Comerci et al., 2001; Delrue et al., 2001; Sun et al., 2002; Watarai et al., 2002; Den Hartigh et al., 2004, 2008; Kim et al., 2004; Celli, 2006). Although the T4SS is absolutely required for intracellular survival and replication of *B. abortus* (Hong et al., 2000; Boschiroli et al., 2002; Celli, 2006), apparently the system does not play any role during invasion and the initial steps of infection of host cells (Celli, 2006).

Brucella T4SS is also required for persistent infection in mice and to induce the host immune response (Rolán and Tsolis, 2007, 2008;Roux et al., 2007). It is also essential to elicit inflammatory and immune responses during *Brucella* infection in mice. *B. abortus* without functional T4SS is unable to stimulate the expression of pro-inflammatory genes and types I and II interferon response as induced by the wild type strain in the spleen (Roux et al., 2007). In addition, a *virB* mutant strain persists longer in B- and T-cells knockout mice than in control mice (Rolán and Tsolis, 2007), whereas the T4SS is required for early response of cytokines such as interleukin (IL)-12 and interferon (IFN)γ that contributes to T helper cell type 1 (Th1) polarisation of the immune response (Rolán and Tsolis, 2008).

Immune host response against *B. abortus*

Innate immunity plays an important role during *B. abortus* infection since it decreases the initial number of bacteria and may influence the development of a protective adaptive immunity. The initial recognition of *Brucella* by neutrophils, macrophages and dendritic cells (DCs) involves Toll like receptors (TLRs) (Campos et al., 2004; Dueñas et al., 2004; Weiss et al., 2005). Mammalian TLRs, similar to Toll from *Drosophila melanogaster* that has an antimicrobial function (Rock et al., 1998; Kopp and Medzhitov, 1999), are membrane receptors located either in the plasma membrane or in the phagolysosome membrane. TLRs are stimulated by conserved components of microorganisms known as pathogen-associated molecular patterns (PAMPs). PAMPs from bacteria such as lipoproteins, LPS, flagellin, and DNA are recognised by TLR2, TLR4, TLR5, and TLR9, respectively (Takeda and Akira, 2005).

B. abortus LPS is recognised by CD14 that connects to molecules with transmembrane domains essential for signalling, particularly the TLR4. Although *Brucella* LPS stimulates TLR4, it has lower immunostimulatory activity compared to other Gram negative bacteria such as *Salmonella enterica* serotype Typhimurium (Ritting et al., 2003; Dueñas et al., 2004), which has the ability to induce an intense and acute inflammatory response (Santos et al., 2003). Nevertheless, experiments with TLR4$^{-/-}$ mice have demonstrated the role of this molecule in inducing an immune response and resistance to *B. abortus* (Campos et al., 2004). TLR2 and TLR9 also have been shown to recognise components of *B. abortus* and to stimulate an immune response (Huang et al., 2003, 2005; Macedo et al., 2008). A downstream adaptor molecule named myeloid differentiation factor 88 (MyD88) also participates in macrophage activation upon TLR-mediated recognition of PAMPs. MyD88 is required for TLR-mediated induction of pro-inflammatory cytokines via NF-κβ, and has a critical function in mounting an immune response against *B. abortus* (Weiss et al., 2005).

DC activation during *B. abortus* infection results in an important regulatory pathway by inducing IFNγ production by T-cells. DC activation and maturation requires MyD88, whose signalling appears to be required for development of IFNγ production by the T-cell, and control *B. abortus* infection in mice (Copin et al., 2007; Macedo et al., 2008). Importantly, smooth *Brucella* LPS stimulates DCs to produce IL-12, so stimulating CD4+ T-cells (Billard et al., 2007).

Oxidative bursts and production of nitric oxide are efficient mechanisms by which macrophages kill bacteria and are stimulated by cytokines of innate immunity such as IFNγ and tumour necrosis factor (TNF)α (Jones and Winter, 1992; Jiang et al., 1993). An in vitro study demonstrated that reactive oxygen intermediates are required for the intracellular control of *B. abortus* during early phases of infection in macrophages (Jiang et al., 1993; Ko et al., 2002).

Natural resistance against brucellosis in cattle is another important element of innate immunity (Adams and Templeton, 1998). The resistant phenotype is associated with the ability of bovine macrophages to inhibit the intracellular growth of *B. abortus* (Campbell and Adams, 1992; Campbell et al., 1994; Qureshi et al., 1996). Studies correlate this resistant phenotype with polymorphisms of gene *NRAMP1* (Natural resistance associated macrophage protein 1) (Adams and Templeton, 1998; Barthel et al., 2001). Polymorphisms at 3′untraslated region (3′UTR) of bovine *NRAMP1* were thought to be associated with the ability of macrophages to control intracellular multiplication of *B. abortus* (Barthel et al., 2001). However, we have recently performed a thorough study that demonstrated no correlation between polymorphisms at the *NRAMP1* 3′UTR and natural resistance against bovine brucellosis (Paixão et al., 2006, 2007). Additional studies are underway to verify the possible association of recently identified polymorphisms of the bovine *NRAMP1* (Martínez et al., 2008) with natural resistance.

Cytotoxic activity of natural killer cells (NK) is also part of innate immunity against *B. abortus* (Salmeron et al., 1992). Bovine NK cells may act directly through the secretion of IFNγ (Golding

et al., 2001) which is a cytokine that stimulates bactericidal activity of macrophages (Oliveira et al., 2002; Wyckoff, 2002). Proteins of the complement system contribute to the innate immune response primarily through their role in opsonisation and immediate elimination of extracellular *Brucella* or by interacting with bacteria neutralised by antibody (Corbeil et al., 1988).

In contrast to the abundant studies on the interaction of *Brucella* and macrophages, DCs, and non-phagocytic cells, there is very little information about its interaction with trophoblastic cells, which are key target cells in bovine brucellosis. This lack of information is due to the absence of suitable bovine trophoblastic cell lines. Experimental in vivo and in vitro studies of bovine placenta have demonstrated the ability of *B. abortus* to infect trophoblastic cells (Anderson et al., 1986a; Samartino et al., 1994; Samartino and Enright, 1996). We have recently studied the profile of bovine trophoblast gene expression during the early stages of infection with *B. abortus* using a chorioallantoic membrane explant model (Carvalho Neta et al., 2008). Interestingly, *B. abortus* suppressed pro-inflammatory genes during the early stages of infection, which was followed by a delayed and mild expression of pro-inflammatory chemokines, particularly CXCL6 (GCP-2) and CXCL8 (IL-8) in trophoblastic cells in vitro. These in vitro results correlated well with chemokine expression in the placenta in vivo (Carvalho Neta et al., 2008).

It has been recently demonstrated that *B. abortus* does not induce significant pro-inflammatory responses during infection in mice, and has low immunostimulatory activity and toxicity for host cells (Barquero-Calvo et al., 2007). Apparently, this ability to inhibit, avoid or delay the host immune response may be a strategy of the organism to cause persistent infection in the host. This notion was further supported by the recent identification of a *Brucella* TIR (Toll/Interleukin-1 receptor) domain containing protein, which interferes with TLR signalling, inhibiting DC maturation, compromising DC cytokine secretion and antigen presentation (Salcedo et al., 2008; Cirl et al., 2008).

An effective adaptive immune response against *B. abortus* requires a cell-mediated immunity promoted by specific T-cell activation (Oliveira et al., 1998). T-cells recognise *B. abortus* through α/β receptors associated with co-receptor molecules CD4+ in T helper cells or CD8+ in T cytotoxic cells, after which bacterial antigens can be processed and presented to antigen-specific MHC class II or I (Janeway, 1992; Wyckoff, 2002). A T-cell subset population with γ/δ receptors is probably important in infected calves since a high number of γ/δ T-cells was observed in young calves (Ko and Splitter, 2003). This T-cell subset population did not express CD4+ nor CD8+, so its antigen presentation pathway is not well defined (Janeway, 1992). However, γ/δ T-cells have lytic activity in vitro, produce cytokines such as TNFα and IFNγ so they may activate macrophages and modulate α/β T-cell activation (Ottones et al., 2000).

CD4+ T-cell activation is stimulated by IL-12 secreted by infected macrophages (Jones and Winter, 1992; Jiang and Baldwin, 1993). IL-12 promotes T helper cell (Th0) differentiation into Th1 that is the most significant cell type in host immune response against *B. abortus*. IL-12 is also involved in adaptive immunity to direct activation of CD8+ T-cells (Zhan et al., 1993). Other cytokines secreted by active phagocytes are TNFα and IL-1. Although TNFα does not seem to be essential in controlling intracellular replication of *B. abortus*, it is required for activation of proinflammatory effector cells that limit bacterial multiplication (Zhan et al., 1996). IL-1 may stimulate and/or increase the number of neutrophils and macrophages in the spleen during *Brucella* infection (Zhan et al., 1991).

The chief cytokine produced by Th1 cells is IFNγ, which is responsible for macrophage activation and restriction of *Brucella* infection in vitro and in vivo (Jones and Winter, 1992; Jiang and Baldwin, 1993; Stevens et al., 1992; Golding et al., 2001). Th1 cells also secrete IL-2 that promotes T-cell clonal expansion and contributes to control of *B. abortus* multiplication in macrophages (Jiang and Baldwin, 1993). Conversely, cytokines associated with a Th2 response such as IL-10 may act during *B. abortus* infection limiting the inflammatory response and favouring the establishment of persistent infection in mice (Golding et al., 2001).

CD8+ T-cells also play an important role in protection against *B. abortus* (Oliveira et al., 1998; Wyckoff, 2002). Indeed, CD8+ knockout mice are more susceptible to *Brucella* infection (Oliveira et al., 1998). In addition, CD8+ T-cells secrete IFNγ and increase the cytotoxic ability of *B. abortus* infected macrophages (Oliveira and Splitter, 1995; Oliveira et al., 2002).

Although the protective effect of the humoral immune response in brucellosis is mild or questionable, it may have some significance in controlling *B. abortus* infection, since passive transfer of specific IgG2a decreases bacterial load in mice (Phillips et al., 1989). However, the role of IgG during active *B. abortus* infection in cattle remains controversial (Hoffmann and Houle, 1995). Importantly, humoral immune response is the basis for serological diagnosis of bovine brucellosis (Nielsen, 2002).

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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