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REVIEW

Progress in *Brucella* vaccine development

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Abstract ***Brucella*** **spp. are zoonotic, facultative intracellular pathogens, which cause animal and human disease. Animal disease results in abortion of fetuses; in humans, it manifests flu-like symptoms with an undulant fever, with osteoarthritis as a common complication of infection. Antibiotic regimens for human brucellosis patients may last several months and are not always completely effective. While there are no vaccines for humans, several licensed live** ***Brucella*** **vaccines are available for use in livestock. The performance of these animal vaccines is dependent upon the host species, dose, and route of immunization. Newly engineered live vaccines, lacking well-defined virulence factors, retain low residual virulence, are highly protective, and may someday replace currently used animal vaccines. These also have possible human applications. Moreover, due to their enhanced safety and efficacy in animal models, subunit vaccines for brucellosis show great promise for their application in livestock and humans. This review summarizes the progress of brucellosis vaccine development and presents an overview of candidate vaccines.**

Keywords *Brucella*, brucellosis, zoonosis, livestock vaccines

Introduction

Brucellosis is a zoonotic disease caused by the bacteria of the genus *Brucella* (Bercovich, 2000). Brucellosis rarely results in human death, but it can pose a significant economic loss to owners of domesticated animals due to loss of progeny, reduced milk yield, and infertility. In animals, brucellosis causes epididymitis in males and abortion, placentitis, and infertility in pregnant livestock (Xavier et al., 2009; Silva et al., 2011b). In humans, brucellosis causes acute inflammation and manifests many symptoms of a flu-like infection, including undulating fever, sweats, headaches, back pains, and physical weakness. In some patients, symptoms of acute brucellosis can endure over one year and eventually result in chronic persistence (Castaño and Solera, 2009). Symptoms of chronic brucellosis include recurrent fevers, joint pain, fatigue, and complications of sacroiliitis, peripheral arthritis, spondylitis, osteomyelitis, and bursitis (Pourbagher et al., 2006).

The genus of *Brucella* is composed of at least ten species (spp.): *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis*, *B. neotomae, B. microti*, *B. inopinata*, *B. pinnipedialis*, and *B. ceti* (Haag et al., 2010). However, two additional species have been identified in nonhuman primates (Schlabritz-Loutsevitch et al., 2009) and foxes (Hofer et al., 2011), implicating further diversity of *Brucella*. The classification of *Brucella* spp. is based largely on the preferred hosts (origin of identification), as *Brucella* spp. show > 94% DNA sequence identity (Chain et al., 2005; Halling et al., 2005). The *B. abortus*, *B. melitensis,* and *B. suis* cause most of the animal and human disease (Corbel, 1997). *B. abortus* is the most widespread (Corbel, 1997), causing abortion in cattle (*Bos* spp.) and chronic infection in humans (Moriyón et al., 2004; Pappas et al., 2005). *B. melitensis* is predominantly a disease of goats (*Capra hircus*) and sheep (*Ovis aries*), but can infect cattle as well (Zowghi and Ebadi, 1985; Buyukcangaz and Sen, 2007), causing placentitis. It is considered the most pathogenic species to humans (Corbel, 1997; Contreras-Rodriguez et al., 2003; Eschenbrenner et al., 2006), at least by the median infective dose by aerosol exposure (Teske et al., 2011), although *B. abortus* appears to cause equally severe disease (Atluri et al., 2011). *B. suis* infects a broad range of animals, including swine (*Sus* spp.), reindeer (*Rangifer tarandus*), caribou (*Rangifer tarandus*), hares (*Lepus* spp.), various murine species (*Mus* spp.), and occasionally cattle and dogs (*Canis* spp.), and is the most diverse in its genome sequence (Moreno and Moriyón, 2001). Although each

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species has a relative host preference, cross-species infections occur frequently. *B. canis,* a species mostly limited to infecting dogs, is also infectious in cattle (Li, 1988), as *B. melitensis* is in swine (Borts et al., 1946).

Today, approximately half a million new human cases occur each year worldwide (Franco et al., 2007). However, the incidence of disease is believed to be under diagnosed, thus under reported (Abu Shaqra, 2000; Diju, 2009). In fact, for each case reported, as many as 26 cases are believed to be undiagnosed (Wise, 1980). The primary mode of exposure is the consumption of unpasteurized milk products, while occupational exposure is secondary (Atluri et al., 2011).

Brucellosis will continue to remain an important public health concern as long as natural reservoirs exist. Although live vaccines are available for livestock, their immunization programs must be maintained to limit human infection. Vaccination of uninfected animals and culling of the infected animals have been successfully used to eradicate brucellosis from most of the USA (Ebel et al., 2008; Treanor et al., 2010); however, this approach cannot be used in countries that are unable to provide restitution of the disposed livestock or removal of infected animals because of cultural beliefs. More problematic is the lack of brucellosis vaccines for humans. Hence, development of better vaccines for livestock and humans could circumvent some of the issues and possibly eventually limit the natural reservoir. Notwithstanding the complications in livestock, natural reservoirs for *Brucella* spp. also extend into wildlife (Galindo et al., 2010; Olsen, 2010). Because these *Brucella* reservoirs are unlikely to be eliminated from wildlife, the infection chain from wildlife to livestock and ultimately to humans will remain (Fig. 1). Thus, emphasis on the development of efficacious vaccines for livestock and humans is paramount. Moreover, conventional antibiotic regimens are still not a guarantee for elimination of brucellae from the host, and a relapse rate of 5%–10% is still observed in patients (Hall, 1990; Memish et al., 2000). Finally, *Brucella* was previously used as a bioweapon in World War II (Pappas et al., 2006a), and because of its ease of propagation, it can still today be readily disseminated as an aerosol, causing alarm for its illegitimate use (Valderas and Barrow, 2008; Audic et al., 2009). Given these problems associated with containing and treating this disease, significant efforts are warranted to develop better brucellosis vaccines and treatment regimens.

Brucella has devised multiple mechanisms to evade immune detection. Typically, Gram-negative bacteria lipopolysaccharide (LPS) induces strong proinflammatory responses via the interaction by host pattern recognition receptors, specifically, the toll-like receptor 4 (TLR-4) enabling host recognition (Bäckhed et al., 2003). Yet, *Brucella* LPS does not trigger such inflammatory responses (Haag et al., 2010), thus, avoiding host detection, which is attributed to their side-chain acylation of the LPS molecule that normally dictates the strength of the TLR4-mediated response (Dueñas et al., 2004). Furthermore, their LPS makes the brucellae highly resistant to cationic bactericidal peptides (Martínez de Tejada et al., 1995), thus, enhancing their survival. To further enhance their survival following infection, brucellae inactivate host defenses by impairing the macrophage response to IFN-γ (Barrionuevo et al., 2011), a

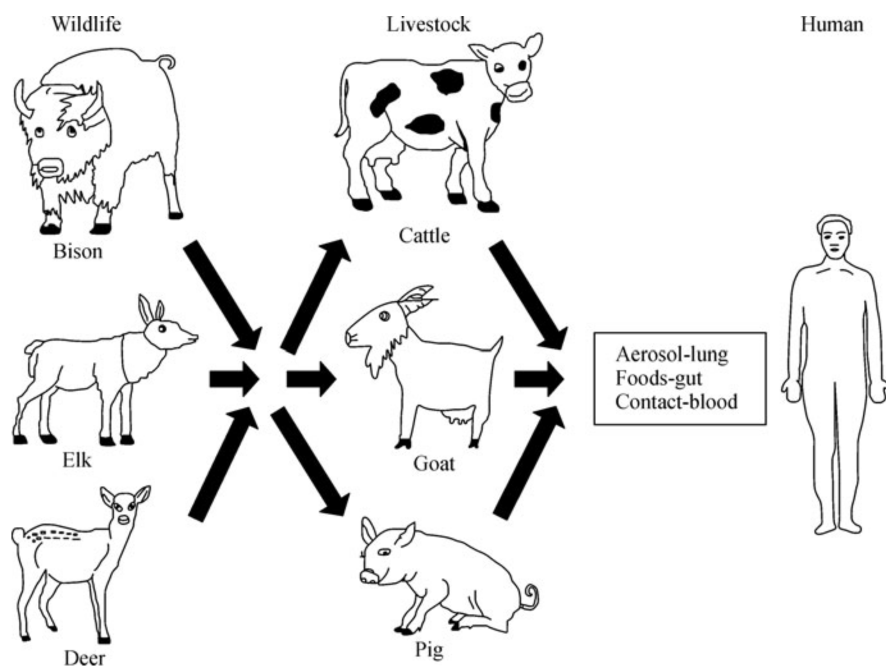

Figure 1 The transmission chain of *Brucella* spp. among wildlife, livestock, and human. Livestock acquires the *Brucella* diseases from the natural reservoirs, such as bison, elk, and deer. Subsequently, humans are infected by the sick livestock and/or its products, either via inhalation of contaminated aerosol, ingestion of foods such as unpasteurized milk and cheese, or direct contact with the sick livestock and its contaminated products.

key mediator of the innate and adaptive immunity produced during *Brucella* infection (Zhan and Cheers, 1993). To ensure their survival, intracellular brucellae do not induce cell death in macrophages (Barquero-Calvo et al., 2007), and they do not induce apoptotic death in respiratory epithelial cells (Ferrero et al., 2009). To enhance their defense, brucellae can modify their outer membrane by installing phosphatidylcholine to avoid fusion with lysosome (Conde-Alvarez et al., 2006). *Brucella* synthesizes cyclic β-1,2-glucans that interact with the lipid rafts contained in the host cell membrane to prevent fusion between phagosome and lysosome and to successfully survive and replicate intracellularly (Arellano-Reynoso et al., 2005). These studies show how efficient *Brucella* is at infecting the host and is able to survive phagocytosis. In fact, in some estimates, *B. melitensis* infection is highly efficient, the median infectious dose is 1840 colony forming units (CFUs) subcutaneously for the mouse; the median infectious dose by aerosol route is 1924 CFUs for macaques; and the median infectious dose by aerosol is 1885 CFUs for human (Teske et al., 2011).

The development of safe and effective vaccines against both animal and human disease will constitute an important step in curbing brucellosis. Much progress has been made in brucellosis vaccine research, especially during the past decade, expedited by molecular techniques successful sequencing of *Brucella* spp. genomes, and the utilization of the small-rodent animal model. As such, mice can be used as a tool to screen brucellosis vaccines. Experiments with the natural hosts are more appropriate, but often these are problematic due to longer periods of time to perform, special containment facilities, and high costs. Laboratory animal models, particularly the mouse, have the advantage to allow preliminary testing to determine the initial efficacy of vaccines against *Brucella* spp. (Smither et al., 2009). In addition, a number of different mouse strains can readily be infected by *B. abortus* (Silva et al., 2011a). Many symptoms of brucellosis mimic symptoms of large animals, including abortion (Kim et al., 2005), placental infection and lesion, and they also mimic symptoms of humans, including acute and chronic diseases. Mouse models are also able to mimic modes of infection that cause large animal or human *Brucella* infections, as evidenced by oral inhalational routes. However, there are several differences between brucellosis in mice and large animal or human disease: (1) Mice are often infected by the intraperitoneal route, while large animal and human are via the mucosal penetration; (2) brucellosis in mice does not cause fever, while human brucellosis is frequently associated with undulant fever (Silva et al., 2011a); (3) human brucellosis has a deleterious effect on the spleen, liver, heart, bones, and brain (Young, 1989), but brucellosis in mice is mostly limited to brucellae persistence in the spleen and liver (Dornand et al., 2004). Thus, these differences are needed to be considered when adapting results from rodent studies to large animals, and outcomes may differ in these large animals. Nonetheless, understanding basic mechanisms of pathogenesis and identification of a number of virulence factors have been revealed by such evaluations in mice. Moreover, mice can be more readily genetically manipulated to help understand correlates of immune protection and how the host responds to *Brucella* virulence factors. While perhaps only identifying fewer protective antigens in mice, progress can still be made in *Brucella* vaccine development. Thus, this review will summarize the significant achievements in *Brucella* vaccine research and development in both laboratory animals and the large animals.

Live vaccines

Live *B. abortus* vaccine strain 19

The live vaccine *B. abortus* strain 19 (S19) is a spontaneously attenuated mutant discovered in 1923 (Graves, 1943), and attributed to a deletion of 702 bp encompassing the erythritol catabolic genes, manifesting a phenotypic sensitivity to erythritol (Sangari and Agüero, 1994; Sangari et al., 1994). S19 had been used worldwide to prevent brucellosis in cattle for more than half a century until the introduction of the rough vaccine strain RB51 in the mid-1990s (Crasta et al., 2008). Although currently not used in the USA, S19 is still being employed in other countries, such as India (Mukherjee et al., 2005) and Argentina (Baldi et al., 2008). Summarized research results by the National Animal Disease Laboratory showed that 65%–75% S19 immunized cattle were completely protected against most kind of exposure. The remaining 25%–35% of the immunized cattle became infected, but many did not show symptoms, such as abortion (Manthei, 1959). Later, other studies showed that S19 provided 70%–91% protection against cattle abortion (Confer et al., 1985; Wyckoff et al., 2005), although the protective efficacy is challenge dose-dependent (Confer et al., 1985) (Table 1). S19 is also effective in protecting cattle against wild-type (wt) *B. abortus* infections (Cardena et al., 2009). A major drawback of S19 is that it maintains a smooth phenotype due to its intact extracellular O-antigen LPS layer. Since the smooth phenotype induces anti-LPS antibodies, it is difficult to distinguish between cattle naturally infected from those immunized. Since the card test bases positive reactivity on the presence of these anti-LPS antibodies, current vaccination programs in the USA and other countries rely on cattle being immunized with the rough RB51 vaccine because vaccinated animals lack these anti-LPS antibodies. Another problem with S19 is it can cause abortion if given to cows during pregnancy (Smith and Ficht, 1990), and it is fully virulent for humans (Spink et al., 1962). Subcutaneous immunization with S19 in pregnant cattle can result in a 3.2% abortion rate (Beckett and MacDiarmid, 1985), and intravenous immunization of pregnant cows can lead to 100% abortion (Mingle et al., 1941; Taylor and McDiarmid, 1949). To dampen its virulence, but retain its immunogenicity, some studies have

Table 1 Live vaccine immunization regimens and protective efficacies

Live vaccine strains	Animals	Immunization dose/ route[1]	Challenge strain[2]/ dose/route[1]	Efficacy[3] (protection against abortion)	References
B. abortus					
S19	heifers	10^9–10^{10}/s.c.	2308/9×10^6/i.c.	70%–91%	(Confer et al., 1985)
	heifers	10^9–10^{10}/s.c.	2308/5×10^7/i.c.	0%–20%	(Confer et al., 1985)
	heifers	10^7 (×2)/s.c.	2308/9×10^5/i.c.	86%	(Wyckoff et al., 2005)
	heifers	1.0×10^{10}–1.4×10^{10}/s.c.	2308/1×10^7/ i.c.	100%	(Cheville et al., 1996b)
	bison	5×10^8/s.c.	2308/1×10^7/i.c.	67%	(Davis et al., 1991)
RB51	heifers	1.0×10^{10}–1.4×10^{10}/s.c.	2308/1×10^7/ i.c.	100%	(Cheville et al., 1996b)
	heifers	1.0×10^{10}–1.4×10^{10}/s.c.	2308/1×10^7/i.c.	100%	(Cheville et al., 1993)
	cattle	5×10^9/s.c.	field spp.	100%	(Lord et al., 1998)
	cows	1.5×10^{10}–1.6×10^{10}/s.c.	2308/3×10^7/i.c.	75%	(Poester et al., 2006)
	elk	10^8–10^9/i.m.,bb	2308/1×10^7/i.c.	12%–25%	(Cook et al., 2002)
	elk	10^{10} /i.m.	2308/1×10^7/i.c.	0%	(Kreeger et al., 2002)
	elk	10^{10} (×2)/i.m.	2308/1×10^7/i.c.	7%	(Kreeger et al., 2002)
				($\log_{10}$ units)	
Δ*pgk*	BALB/c mice	10^5/i.p.	2308/1×10^6/i.p.	0.96	(Trant et al., 2010)
	C57BL/6 mice	10^5/i.p.	2308/1×10^6/i.p.	1.36	(Trant et al., 2010)
	129/Sv mice	10^5/i.p.	2308/1×10^6/i.p.	3.28	(Trant et al., 2010)
Δ*pgm*	BALB/c mice	10^7/i.p.	2308/5×10^5/i.p.	1.9–2.3	(Ugalde et al., 2003)
Δ*znuA* 2308	BALB/c mice	10^8/i.p.	2308/5×10^4/i.p.	1.88	(Yang et al., 2006)
				(protection against abortion)	
B. melitensis					
Rev-1	goats	1.5×10^9/(uk)	640/4×10^{10}/i.m.	100%	(Alton, 1966)
	sheep	2×10^9/s.c.	Isfahan/6×10^9/s.c.	100%	(Entessar et al., 1967)
	goats	1.5×10^9/(uk)	field spp.	100%	(Alton, 1968)
				($\log_{10}$ units)	
RBM17	BALB/c mice	10^8/i.p.	16M/2×10^5/i.p.	2.83	(Adone et al., 2005)
Bm16MR*wa*	BALB/c mice	10^8/i.p.	H38/1×10^4/i.p.	3.9–4.4	(González et al., 2008)
Bm16MR*wzm*	BALB/c mice	10^8/s.c.	H38/1×10^4/i.p.	4.1	(González et al., 2008)
Δ*znuA* 16M	BALB/c mice	3×10^{11}/oral	16M/2×10^4/i.n.	3.0–4	(Clapp et al., 2011a)
	IFN-$\gamma^{-/-}$ mice	3×10^{11}/oral	16M/2×10^4/i.n.	1.85	(Clapp et al., 2011a)
RWP5	goats	10^9/s.c.	16M/1×10^7/i.c.	100%	(Phillips et al., 1997)
Δ*omp25* 16M	goats	10^9/s.c.	16M/1×10^7/i.c.	100%	(Edmonds et al., 2002b)
16MΔmucR	BALB/c mice	10^6/i.p.	16M/6×10^5/i.p.	2.79	(Arenas-Gamboa et al., 2011)
16MΔvjbR	BALB/c mice	10^6/i.p.	16M/1×10^5/i.p.	~2.4	(Wang et al., 2011)
vjbR::Tn5 16M	BALB/c mice	10^5/i.p.	16M/1×10^5/i.p.	4.98	(Arenas-Gamboa et al., 2008)
				(protection against abortion)	
B. suis					
S2	sheep	0.2×10^{10}–5×10^{10}/oral	*B. melitensis* 28/10^7/i.c. or 10^9/oral	82.7%	(Xin, 1986)
	goats	0.25×10^{10}–5×10^{10}/oral	*B. melitensis* 28/10^7/i.c. or 10^9/oral	82.1%	(Xin, 1986)
	sows	2×10^{10}×10^{10} (×2)/oral	*B.suis* 12/6×10^7/oral	75.0%	(Xin, 1986)
	cows	0.25×10^{10}–5×10^{10}/oral	*B.abortus* 387/10^6–10^7/ i.c.	71.4%	(Xin, 1986)

1 Animals dosed once unless indicated with "(×2)"; s.c.: subcutaneous; i.m.: intramuscular; bb: biobullet; i.p.: intraperitoneal; i.n.:intranasal; i.c.: intraconjunctival; uk: route of immunization is unknown.
2 Challenge strains: *B. abortus* 2308; *B. melitensis* 16M; or otherwise stated.
3 Efficacy in livestock is depicted as "protection against abortion" and in mice as "$\log_{10}$ unit" reduction in splenic colonization.

focused on modifying S19 (Robertson et al., 1996; Arenas-Gamboa et al., 2009c; Briones et al., 2001). One promising candidate shows a *cgs* (encoding cyclic – 1,2-glucan synthetase) mutant nullifies the host immune response against LPS, while successfully retaining protective efficacy against the wt strain *B. abortus* 2308 (Briones et al., 2001). However, studies with these vaccines await further testing in large animals.

B. abortus can also infect American bison (*Bison bison*) and elk (*Cervus elaphus*) (Scurlock and Edwards, 2010), which serve as wildlife reservoirs for the disease in the Greater Yellowstone Area, USA (Thorne, 1997; Cheville et al., 1998). Subcutaneous or intramuscular vaccination with S19 results in 58% abortion rate in pregnant bison (Davis et al., 1991). S19 is poorly immunogenic in elk (Olsen et al., 2006), but partially protective in red deer (*Cervus elaphus*), a related species of elk, when S19 is microencapsulated (Arenas-Gamboa et al., 2009a). Of note is the microencapsulated S19 is only protective in the spleen by reducing the splenic bacterial load but not the liver and the lung since both organs show no difference from the unvaccinated control in bacterial CFUs. This difference in splenic efficacy between elk and red deer might be due to differences in immunization regimen, since the red deer were orally vaccinated with microencapsulated S19, while the elk were immunized parenterally with S19. Alginate (a naturally occurring polymer combined with a protein of *Fasciola hepatica* vitelline protein B), the main component of the capsule, may display an adjuvant role in enhancing the S19 immunity to red deer. While not eliminating the possibility of differences attributed to species, future studies will be needed to discern whether microencapsulated S19 is effective in protecting elk.

Live *B. abortus* vaccine strain RB51

In 1996, to circumvent the problem incurred by the S19 O-antigen, *B. abortus* RB51 subsequently replaced S19 for vaccinating cattle in the USA against brucellosis (CDC, 1998). This rough mutant strain was derived from smooth wt *B. abortus* 2308. Unlike S19, RB51 does not induce anti-LPS antibody responses, enabling conventional serologic tests for diagnosing brucellosis in cattle, including card, complement fixation, particle concentration fluorescence immunoassay, and tube agglutination tests (Stevens et al., 1994; Stevens and Olsen, 1996). Compared to S19, RB51 is relatively safe as intravenous administration to cattle only causes 25% of heifers to deliver prematurely (Palmer et al., 1996a). Cattle vaccinated with S19 or RB51 display similar lymph node immune responses to *B. abortus* 2308 (Stevens et al., 1995a), and RB51 confers similar protection as S19 to abortion and infection (Cheville et al., 1996b). RB51 can confer full protection in cattle, as none of the RB51-immunized cattle aborted, while 83.3% of the cattle aborted or delivered small, weak calves in the unimmunized control group (Cheville et al., 1993). RB51 induced 100% protection against abortion in cattle when a field strain of *B. abortus* was used, and found to be more effective than S19 (Lord et al., 1998). However, vaccination with RB51 does not protect cattle against *B. suis* infection, as evidenced by infection rates in maternal or fetal tissues (Olsen and Hennager, 2010).

RB51 has been tested in other animals, including water buffalo (*Bubalus bubalis*), sheep, goats, bison, elk, and red deer. RB51 can be safely used in water buffalo (Diptee et al., 2006), but it is not protective against natural *Brucella* infections, as evidenced by the percentage of vaccinated animals seroconverting was greater than the control group (43% versus 15%), and all isolates were confirmed to be *B. abortus* biovar 1 and not RB51 (Fosgate et al., 2003). RB51 is safe in small ruminants (Roop et al., 1991), but it does not protect sheep against abortion induced by wt *B. melitensis* (el Idrissi et al., 2001), nor does RB51 confer protection against *B. ovis* in goats (Jiménez de Bagüés et al., 1995; Herrera et al., 2011). The safety data for RB51 in bison are ambiguous. One study found RB51 causes both abortion (25%) and tissue infection, with the latter leading to placentitis and endometritis (Palmer et al., 1996b). However, another study found RB51 is safe in bison since subcutaneous vaccination did not cause prolonged bacterial colonization of tissues in both calves and adults nor did it induce abortions following vaccination of pregnant bison (Elzer et al., 1998). Efficacy data are also conflicting. One study has found the incidence of abortion due to *B. abortus* challenge is significantly reduced in bison vaccinated with RB51 (1.2×10^{10}–6.1×10^{10} CFUs) when compared to unvaccinated controls (15% versus 62%) following conjunctival challenge with 3×10^{7} CFUs 2308 (Olsen et al., 2003). Furthermore, revaccination of bison with RB51 did not cause abortion, but RB51 was recovered from tissue samples in 16.7% pregnant bison (Olsen and Holland, 2003). In a separate study, RB51 showed little efficacy in adult and calf bison despite repeated subcutaneous vaccinations with 1×10^{7}–1×10^{9} CFUs given as a primary immunization and boosted with 1×10^{9} CFUs followed by a conjunctival challenge with 1×10^{7} CFUs 2308 (Davis and Elzer, 2002). Surprisingly, RB51 was found to be pathogenic to elk since this vaccine induced a 71% abortion rate (Kreeger et al., 2000). Elk that did not abort and were challenged with wt *B. abortus* 2308 produced fetuses that possessed both RB51 and 2308 (Kreeger et al., 2000, 2002). Testing alternative methods to vaccinate, the closely related red deer (*Cervus elaphus elaphus*) has been used as a model for the Rocky Mountain elk (*Cervus elaphus nelsoni*). Red deer vaccinated with microencapsulated RB51 by either the oral or subcutaneous route, showed a significant reduction in splenic colonization (Arenas-Gamboa et al., 2009b).

Two major hindrances are associated with RB51. One is RB51 that is infectious to humans and can cause human brucellosis (Ashford et al., 2004); the other is it is resistant to rifampin (Schurig et al., 1991; Adone et al., 2005), one of the most potent and effective antibiotics for treating brucellosis (Eker et al., 2011; Gulsun et al., 2011).

Attenuated *B. abortus* mutants as vaccine candidates

To develop better performing vaccines, significant efforts have focused on defining the relevant *Brucella* virulence genes. Mutants of the purine biosynthesis pathway genes *purL*, *purD*, and *purE* remained viable in mice 12 weeks after infection (Alcantara et al., 2004), suggesting these were insufficient to attenuate *Brucella*. In contrast, the lipid A fatty acid-transporting gene *bacA* mutant (Walker et al., 2000; Ferguson et al., 2004), the ferrochelatase *hemH* mutant (Almirón et al., 2001), and the type IV secretion *virB* mutant (den Hartigh et al., 2004) were highly attenuated phenotypes, but their protective efficacies remain to be analyzed. Deletion of the phosphoglycerate kinase encoding gene (*pgk*) from wt strain *B. abortus* 2308 resulted in dramatic attenuation. Although Δ*pgk B. abortus* induced protection as S19 in interferon regulatory factor-1-deficient mice, which have defects in Th1-type $CD8^+$, NK, and γδ T cell development, and are highly susceptible to brucellosis (Rajashekara et al., 2005). In BALB/c and C57BL/6 mice, Δ*pgk B. abortus* was as protective as S19 and RB51 vaccines (Trant et al., 2010). Mutants in the phosphoglucomutase (*pgm*) gene (responsible for isomerizing glucose-6-phosphate to glucose-1-phosphate) or high-affinity zinc uptake system (*znuA)* gene from wt 2308, conferred equivalent protection to S19 (Ugalde et al., 2003; Yang et al., 2006). Δ*pgm B. abortus* did not induce a detectable antibody response against O-antigen (Ugalde et al., 2003); therefore, similar to RB51, Δ*pgm B. abortus*-infected animals can be distinguished from animals infected with wt brucellae.

Live *B. melitensis* vaccine strain Rev-1

The live vaccine strain Rev-1 was originally derived from wt *B. melitensis* 6056 in 1957 by sequential passage on streptomycin-containing media until streptomycin-resistant clones developed (Elberg and Faunce, 1957). Rev-1 vaccine is innocuous when administered to rams (Muñoz et al., 2008). Rev-1 immunization of goats induces strong immunity that can be maintained for over 2 years (Alton, 1966; Entessar et al., 1967) without side effects, such as prolonged interference with serological tests or shedding (Alton, 1966). Rev-1 has also been found to be protective against natural infections in goats for at least 4.5 years after vaccination, including full protection against abortion versus 45% abortion rate in unvaccinated controls, and 92% versus 16% protection against tissue infection (positive mammary gland, uterus or both) for vaccinated ewes relative to controls (Alton, 1968). On a large scale, Rev-1 was found to be fairly effective in preventing goat and sheep from natural *Brucella* infections. In 2004, Tajikistan adopted a Rev-1 immunization program in small ruminants with 13006 animals. Five years later, the seroprevalence dropped by 80%, but for those districts where no vaccinations were carried out, no change in seroprevalence was observed (Ward et al., 2011). After sheep and goat brucellosis eradication program was implemented in 1999 in Portugal involving 2.4 million animals, the serological positive rate dropped from 2.8% to 0.3% and the serologically positive herds dropped from 8.4% to 1.2% (SCOFCAH, 2011).

However, Rev-1 was not found to be safe for pregnant goats. Rev-1 could be isolated in vaginal discharge from ewes, regardless of subcutaneous or conjunctival route of immunization (Jiménez de Bagüés et al., 1989). Rev-1 vaccination of lactating goats demonstrated occasional excretion of brucellae into the milk (Banai, 2002). Further, Rev-1 is virulent to humans (Blasco and Díaz, 1993), and in fact, is more virulent than S19 (Spink et al., 1962). Another drawback rooted in Rev-1 is that immunization can stimulate strong anti-O antigen antibody titers interfering with the serological diagnosis to distinguish between vaccinated animals and natural infection by both *B. melitensis* and *B. ovis* (Fensterbank et al., 1982; Marín et al., 1999). Rev-1 is also subject to varying its morphological and immunological properties (Bosseray, 1991), which can impact its efficacy.

To assess its ability to be cross-protective, Rev-1 was further evaluated for its efficacy against wt *B. abortus*. The results showed Rev-1 being more protective in cows than S19 at protecting against abortion using a challenge dose of 8×10^6 CFUs with wt *B. abortus* 2308 (90% versus 80% in normal parturition) or a high challenge dose of 1×10^9 CFUs with 2308 (40% versus 0% in normal parturition) (García-Carrillo, 1980). However, a recent study isolated Rev-1 from the aborted cattle fetuses subsequent exposure of cattle to Rev-1-immunized ewes, implicating Rev-1's virulence to cows (Pishva and Salehi, 2008). Later, it was found that Rev-1 is able to enter the sheep blood at 1 day after immunization, and persist in blood over 60 days. The persistent bacteremia may account for its transmission capability among animals (Kojouri and Gholami, 2009). Rev-1 also seems to be safe in camels since immunized animals did not abort, and no Rev-1 was recovered from udder secretion samples collected from all vaccinated lactating camels (Radwan et al., 1995).

Live *B. melitensis* mutant vaccine candidates

To develop live vaccines against *B. melitensis* with superior performance to Rev-1, efforts have focused on the deletion of various virulence genes. However, these studies have achieved varied success. Since Rev-1 carries a smooth LPS with an O-antigen that evokes antibodies interfering in serodiagnosis in eradication campaigns, rough *Brucella* mutants without O-antigen have been proposed as vaccines. In this respect, in an attempt to block the expression of LPS from *B. melitensis*, three mutants, defective in core and O-polysaccharide synthesis, were derived: *wbkF* mutant, *per* mutant, and *wa* mutant, each found to be less effective than Rev-1 in sheep. The *wbkF* mutant, the best candidate among the three mutants, still exhibited 38% abortion rate in vaccinated ewes, while Rev-1 vaccinated ewes did not abort

after challenge at mid-gestation with virulent wt *B. melitensis* H38. Forty-six percent of *wbkF* mutant-immunized ewes secreted wt *Brucella* in their milk, vaginal swabs, and/or aborted or produced non-viable lambs, while none of the Rev-1 immunized sheep secreted brucellae (Barrio et al., 2009). In a separate study, six other rough *B. melitensis* mutants were developed with different *rpoB* genotypes, and these were able to confer variable levels of protection against wt *B. melitensis* infection in mice via intraperitoneal challenge. Two of these vaccines provided similar efficacy as Rev-1 and were superior to RB51, without inducing O-antigen antibodies (Adone et al., 2005). Another group investigated 14 *B. melitensis* LPS mutants in mice, from which two were able to confer protection equivalent to that obtained with Rev-1 via intraperitoneal challenge, but required a vaccine dose ~1000-fold greater than Rev-1 (González et al., 2008). Recently, our group showed that oral immunization with a Δ*znuA B. melitensis* 16M mutant conferred better protection than RB51 against intranasal challenge with wt *B. melitensis* 16M, and this observed protection was IFN-γ-dependent (Clapp et al., 2011a). Interestingly, this vaccine is cleared relatively rapidly within 1–2 weeks following oral immunization, suggesting, possibly, oral delivery represents another mode of *Brucella* attenuation. Until tested in livestock, these studies suggest LPS may be an essential protective antigen against *B. melitensis* infections.

In addition to LPS, interruption of other virulence genes in *B. melitensis* has been examined. Immunization of goats with *B. melitensis* high-temperature-requirement A (*htrA*) deletion mutant did not cause abortion, but the vaccine did colonize both the fetus and the nanny (Phillips et al., 1997; Roop et al., 2001). A *purE B. melitensis* mutant appeared to be safe in goats, but the protective efficacy remains to be determined (Cheville et al., 1996a). Interruption of *B. melitensis*' major outer membrane protein Omp25 resulted in the attenuation in mice, and the resulting *omp25* mutant provided measurable protection against wt *B. melitensis* challenge (2.29 versus 2.45 $\log_{10}$ units for *omp25* mutant compared to Rev-1); however, this mutant retained residual virulence as mouse spleens contained $> 10^3$ CFU by week 8 post-immunization (Edmonds et al., 2002a). Later, *omp25* mutant was evaluated in goats, and upon immunization of pregnant goats at late gestation, it did not cause abortion (0%), unlike the parental strain *B. melitensis* 16M that induced 100% of the dams to abort (Edmonds et al., 2002b). Prior to breeding, vaccination with *omp25* mutant conferred full protection against abortion following challenge in late gestation with wt strain 16M, a protective efficacy equivalent to Rev-1 (Edmonds et al., 2002b). While the mutant retained virulence for mice, it performed well in a small ruminant model, indicating the murine models may not always predict protection in ruminants.

A *B. melitensis mucR* mutant strain has been found to be highly protective against both intraperitoneal and aerosol challenges with wt *B. melitensis* 16M in BALB/c mice (Arenas-Gamboa et al., 2011). The *mucR* gene is believed to encode for a transcriptional factor involved in expolysaccharide production for biofilm formation. However, this mutant can persist in mice for as much as 12 weeks post-infection. In contrast, the *vjbR* (encoding quorum sensing regulator) mutation of strain 16M is more readily cleared from mice since a dramatic reduction in splenic CFU burden was observed by 28 days post-infection ($10^{0.5}$ CFUs), and it conferred protection similar to Rev-1 vaccine (Wang et al., 2011). Microencapsulation of a related mutant, *B. melitensis vjbR*::Tn5, in alginate microspheres, resulted in protective efficacy being enhanced by 1.84 $\log_{10}$ units compared to the unencapsulated strain (Arenas-Gamboa et al., 2008). A deletion mutation of *galE* (involved in galactose metabolism) from *B. melitensis* provided similar protective efficacy to Rev-1 in mice, yet its virulence was similar to the parental wt *B. melitensis* 16M strain (Petrovska et al., 1999), making this strain less desirable for application to small ruminants.

Live *B. suis* vaccine strain 2

B. suis strain 2 (S2) is a laboratory adapted strain isolated in 1953 from an aborted sow, which was attenuated by serial passage (Bosseray and Plommet, 1990). Although S2 possesses a smooth phenotype, its virulence is significantly less than wt *B. suis* (Xin, 1986). Since 1958 in China, S2 has been used for oral immunization of livestock by adding the vaccine to the animals' drinking water. S2 has been found to be efficacious by preventing infections in 82.7% of sheep, 82.1% of goats, 75.0% of sows, and 71.4% of cows. As swine are the preferred host for *B. suis*, oral immunization of pigs with S2 once a year for two years lowered the serologic reactivity on farms from 62.8%–75% to 0%–2.4% (Xin, 1986). When evaluated in mice relative to Rev-1 and S19 vaccines, S2 was found to be less virulent than these other vaccines. For short-term immunity, no differences were observed among the three vaccines, but for long-term immunity, immunity induced by S2 declined relative to those vaccinated with Rev-1 and S19 vaccines (Bosseray and Plommet, 1990). S2 was also evaluated for its ability to be delivered by the conjunctival route in rams and ewes for protection against wt *B. ovis*. The results showed that S2 was less protective than Rev-1 in rams (43.7% versus 78.6%, respectively) (Blasco et al., 1993), and in ewe abortion (71%–81% versus 20%–38% for S2 and Rev-1 aborted ewes, respectively) (Verger et al., 1995). This may imply that S2 is not appropriate for usage via conjunctival immunization route, or alternatively, S2 is not effective in protecting infections caused by the species *B. ovis* or *B. melitensis*. Another drawback associated with S2 is that its immunization via drinking water cannot ensure reproducible vaccine doses.

Live *B. suis* mutant vaccine candidates

Mutations of *B. suis* in *aroC* (aromatic amino acid

biosynthetic pathway) (Foulongne et al., 2001), *bvfA* (*Brucella* virulence factor A) (Lavigne et al., 2005) or *norD* (nitric oxide reductase) (Loisel-Meyer et al., 2006) resulted in the loss of virulence in mice. However, no efficacy data were provided to compare how well these mutants behaved. Interruption of *eryC* (erythritol metabolism) or *ntrC* (two-component regulatory system) in *B. suis* did not drastically reduce virulence in mice (dropping by only 1.49 $\log_{10}$ units in spleen) (Burkhardt et al., 2005), implying that these mutants are not sufficiently attenuated for further investigation in pigs.

Subunit *Brucella* vaccines

The advantage of subunit vaccines is they eliminate safety concerns associated with live vaccines, although replicating the immunogenicity of live vaccines is more problematic. Since subunit vaccines avoid utilizing living brucellae, they do not result in animal infection or abortion, and thus are safe for both animals and humans. A number of protective antigens for brucellosis have been identified during the past two decades. These subunit vaccines are formulated either as DNA or purified proteins to stimulate immune responses in animals. As protection to *Brucella* in murine models is typically measured as a reduction in splenic colonization in CFUs ($\log_{10}$ units) relevant to non-vaccinated but challenged controls, various subunit vaccines have been tested. These include: lumazine synthase (Velikovsky et al., 2002), Bp26 and trigger factor together (Yang et al., 2005), InfC (Cespedes et al., 2000), L7/L12 (Kurar and Splitter, 1997), Omp16 and Omp19 (Pasquevich et al., 2009), Omp25 (Commander et al., 2007), Omp28 (Kaushik et al., 2010), Omp31 (Cassataro et al., 2005), P39 (Al-Mariri et al., 2001), S-adenosyl-l-homocysteine hydrolase (Yang et al., 2011), DnaK and SurA (Delpino et al., 2007), and SodC (Muñoz-Montesino et al., 2004) (The immunization dose and route and challenge dose and route are detailed in Table 2). Subunit vaccines have the advantage of being effective for multiple *Brucella* species because of the $>94\%$ gene homology among *Brucella* species (Whatmore, 2009). Although significant protection can be achieved by these subunit vaccines, these too have limitations. Each of these vaccines has a limited scope of efficacy of generally <2 $\log_{10}$ units of protection and usually requires several boosts to achieve immunity. This is generally less effective than the control live vaccines with merely one dose, such as RB51, S19, or Rev-1. Thus, their lack of potency remains problematic compared with live vaccines, and their manufacture can be cost prohibitive. Additionally, a majority of these studies were only performed in mice, which is not always an adequate predictor for protection in livestock or humans.

A few pilot studies were initiated to assess whether subunit vaccines are capable of protecting livestock. The use of *B. ovis* outer membrane proteins entrapped in poly(ε-caprolactone) microparticles resulted in nearly equivalent

Table 2 Subunit vaccine immunization regimens and protective efficacies.

Subunit vaccines	Mice[1]	Immunization/ formula/dose/route[2]	Challenge strain[3] /dose/route	Efficacy[4] ($\log_{10}$)	References
B. abortus					
BLS	BALB/c	DNA/100 µg (×4)/i.m.	544/10^5/i.p.	1.25–1.65	(Velikovsky et al., 2002)
InfC	BALB/c	protein/5 µg (×1)/i.p.	2308/1×10^4/i.p.	1.92	(Cespedes et al., 2000)
L7/L12	BALB/cByJ	DNA/100 µg (×1)/i.m.	2308/1×10^6/i.p.	0.47–1.26	(Kurar and Splitter, 1997)
Omp16	BALB/c	protein/10 µg (×2)/i.p.	544/4×10^4/i.p.	1.97	(Pasquevich et al., 2009)
Omp19	BALB/c	protein/10 µg (×2)/i.p.	544/4×10^4/i.p.	1.85	(Pasquevich et al., 2009)
Omp28	Swiss Albino	protein/30 µg (×2)/i.m.	544/1×10^5/i.p.	0.97	(Kaushik et al., 2010)
P39	BALB/c	protein/20 µg (×2)/i.m.	544/5×10^4/i.p.	1.21–2.48	(Al-Mariri et al., 2001)
DnaK	BALB/c	protein/30 µg (×2)/i.p.	2308/1×10^4/i.p.	0.90–0.95	(Delpino et al., 2007)
SurA	BALB/c	protein/30 µg (×2)/i.p.	2308/1×10^4/i.p.	0.97–1.45	(Delpino et al., 2007)
SodC	BALB/c	DNA/10 µg (×1)/i.s.	2308/1×10^4/i.p.	1.52	(Munoz-Montesino et al., 2004)
SodC	BALB/c	DNA/10 µg (×1)/i.m.	2308/1×10^4/i.p.	1.15	(Munoz-Montesino et al., 2004)
B. melitensis					
Bp26	BALB/c	DNA/300 µg (×4)/i.m.	16M/2.4×10^4/i.p.	1.16	(Yang et al., 2005)
Omp25	BALB/c	DNA/400 µg (×4)/i.m.	16M/1×10^4/i.p.	2.54	(Commander et al., 2007)
IalB	BALB/c	DNA/400 µg (×4)/i.m.	16M/1×10^4/i.p.	2.70	(Commander et al., 2007)
Omp31	BALB/c	DNA/100 µg (×4)/i.m.	H38S/1×10^4/i.v.	1.30–1.45	(Cassataro et al., 2005)
AdoHcyase	BALB/c	protein/100 µg (×2)/i.p.	16M/5×10^5/i.p.	1.13–2.13	(Yang et al., 2011)

1 Strain of mice used.
2 Vaccine composition being DNA or protein; number of doses given; and route of immunization: i.s.: intraspleen.
3 Challenge strains: *B. abortus* 2308 or 544; *B. melitensis* 16M or H38S.
4 Efficacy is defined as reduction of "$\log_{10}$ units" in splenic colonization.

protection in rams (46.2% uninfected) as Rev-1 (42.9% uninfected) (Da Costa Martins et al., 2010). Using *B. abortus* soluble antigens combined with the adjuvant of dimethyl-dioctadecyl ammonium bromide to immunize steers, both humoral and cell-mediated immune responses were elicited (Dzata et al., 1991). Bison immunized intramuscularly with Bp26 and TF DNA vaccines produced enhanced antibody, proliferative T cell, and IFN-γ responses, indicating elicited cellular immune responses (Clapp et al., 2011b). Future studies are needed to assess the efficacy of this approach in livestock.

Heterologously expressed *Brucella* vaccines

An alternative approach for delivering subunit vaccines is to use live vaccine vectors heterologously expressing *Brucella* protective antigens. This is an effective approach in delivering various antigens (Ascón et al., 2005; Yang et al., 2007). Many of these attenuated vaccine vectors are licensed for use in humans (Osorio et al., 2009; Keller et al., 2010) and have the advantage of being safe. As an example, the attenuated *Salmonella enterica* serovar Typhimurium was used to express *Brucella* fusion protein between L7/L12 and lumazine synthase. It was found that the recombinant fusion vaccine was more protective than the individual recombinant proteins against wt *B. abortus* challenge (1.32–1.58 versus 0.95–1.11 $\log_{10}$ units) (Zhao et al., 2009). However, when compared with *Brucella* live vaccine 104M (1.91 $\log_{10}$ units), the *Salmonella*-based vaccine was still less protective. *B. abortus* protective antigen, BCSP31 (Pugh et al., 1990), was expressed in attenuated *S. choleraesuis* strain χ3781. Oral immunization of this live vaccine elicited immune responses in both mice and swine (Stabel et al., 1990, 1991; Stabel et al., 1993); however, its protective efficacy was not evaluated. *B. abortus* L7/L12 expressed in *Lactococcus lactis* induced a strong copro-IgA titer, but yielded only 0.5 $\log_{10}$ units of protection in mice and was less effective than S19 (1.3 $\log_{10}$ units) (Pontes et al., 2003).

In some recent studies, RB51 has been adapted as a live vaccine vector for delivering homologous antigens to enhance its anti-*Brucella* immunity. RB51 expressing *sodC* conferred 1.9 $\log_{10}$ units protection relative to the 1.0 $\log_{10}$ unit conferred by RB51 (Vemulapalli et al., 2004). By co-overexpressing *sodC* and *wboA* in RB51, it produced a protective vaccine conferring 3.5 $\log_{10}$ units of protection against wt *B. suis* 1330 challenge (Rajasekaran et al., 2011). *wboA* was expressed in O-antigen deficient *B. melitensis* strain WRRP1, and the recombinant strain conferred robust protection against *B. melitensis* 16M challenge (3.2–4.2 $\log_{10}$ units) in mice (Bandara et al., 2009).

Viral vectors have also been tested for expressing *Brucella* antigens. L7/L12 was expressed in vaccinia virus, but failed to evoke protection against wt *B. abortus* challenge, despite inducing anti-L7/L12 immune responses (Baloglu et al., 2005). *B. abortus* translation initiation factor 3 (IF3) and *sodC* were expressed in replication-deficient Semliki Forest virus, and significant resistance against challenge of wt *B. abortus* 2308 was achieved (1.1 and 1.5 $\log_{10}$ units, respectively); however, this level of protection was less than that of RB51 (1.9 $\log_{10}$ units) (Cabrera et al., 2009; Oñate et al., 2005).

Future directions of *Brucella* vaccine development

Alternative marker genes for developing next generation live *Brucella* vaccines

Live *Brucella* vaccines will still need to be researched and improved regarding both safety and protective efficacy despite the availability of current commercial animal vaccines. *Brucella* LPS is very immunogenic, and it is used to differentiate immunized cattle from those naturally infected. While the RB51 vaccine is convenient, it has still proven to be less than ideal, given the susceptibility of vaccinated cattle to brucellosis in the Greater Yellowstone Area in the USA (Olsen et al., 2009; Van Campen and Rhyan, 2010). This reduced efficacy may be in part attributed to the lack of LPS by RB51, and LPS may in fact be important for protection. *B. melitensis* LPS is a protective antigen in mice, conferring 2.0 $\log_{10}$ units (Bhattacharjee et al., 2006). *B. abortus* LPS also is an essential component of an effective subcellular vaccine, conferring protective immunity in mice (Winter et al., 1988). Comparison of S19 and RB51 efficacy in mice reveals that S19 is more efficacious against wt 2308 challenge than RB51 despite a 100-fold greater RB51 was used than S19 (Stevens et al., 1995b). A recent study shows that a single S19 immunization dose conferred sterile protection against wt *B. abortus* 544 challenge in water buffalo in all the tissues examined, unlike RB51 provided only 80% protection in the retropharyngealymph nodes, despite animals being immunized twice (Caporale et al., 2010). Without LPS, in many circumstances, the rough *Brucella* mutants are not protective. Therefore, it may be time to re-examine the notion that a live *Brucella* vaccine should include LPS and use other brucellae markers or genes to differentiate infected from vaccinated animals (DIVA). Currently, multiple candidates can be used as marker (DIVA) genes, including bp26, P39, and green/red fluorescent protein (GFP/RFP). Bp26 is a potent immunogen (Cloeckaert et al., 1996); however, its deletion does not lower the protective capacity of S19 in either mice (Boschiroli et al., 1997) or pregnant heifers (Fiorentino et al., 2008) despite the abatement of anti-Bp26 titers. Similarly, the immunodominant protein P39, whose deletion from S19 did not affect the protective efficacy in mice (Tibor et al., 1998), may also serve as a marker gene. Heterologous proteins expressed in

Brucella vaccines, e.g. GFP or RFP, can also be used as marker genes to distinguish vaccinated mice from those naturally infected with wt *Brucella* (Chacón-Díaz et al., 2011). By selecting alternative marker genes, either by deleting immunogenic proteins or expression of heterologous antigens, the protective antigen LPS can be maintained, imparting the live vaccines with the ability to stimulate robust protection.

Construction of Th1 cell-biased live *Brucella* vaccine

Two indices are important for evaluating a live *Brucella* vaccine in the laboratory murine model: residual virulence and protection against both infection and abortion subsequent to virulent *Brucella* challenge. Any residual virulence requires examining the vaccine's ability to infect and how long it can persist in the host's tissues and organs. Previous work with *Salmonella*-based vaccines suggests some level of persistence is required for protection (O'Callaghan et al., 1988). Obviously this residual virulence cannot be too great to cause disease; yet this residual virulence cannot be too low being unable to stimulate protective immunity. An appropriate residual virulence carried by the live vaccine would allow it to elicit potent protective immunity while not causing disease or abortion. This has been demonstrated in our laboratory when two virulence factors were deleted from *B. abortus* 2308, creating the double mutant, Δ*znuA*Δ*purE B. abortus* strain, which was found to be highly attenuated in mice, and no brucellae could be detected in spleens after 8 weeks post-infection (Yang et al., 2010). In contrast, its parent strain Δ*purE* or Δ*znuA B. abortus* could be detected at 8 and 12 weeks post-infection, respectively (Alcantara et al., 2004; Yang et al., 2006). This suggests Δ*znuA*Δ*purE B. abortus* is safer than either Δ*znuA* or Δ*purE B. abortus* strains. The Δ*znuA*Δ*purE B. abortus* strain required two doses for immunization to elicit optimal protection efficacy (0.79 $\log_{10}$ units), while such an efficacy could be achieved by a single dose of the Δ*znuA B. abortus* strain (Yang et al., 2010). This indicates that the more attenuated the mutant is, the less protective it will be. Thus, this shows the importance of generating a mutant strain with minimal residual virulence that retains maximal immunogenicity.

Since the majority of the *Brucella* live vaccine selection is primarily based upon mouse models, and the results achieved from mice do not always reflect results obtained in livestock, indices other than the virulence and protection could be useful. A cytokine profile may be such an index since previous studies have shown that Th1 cell-dominant responses are required for protection against *Brucella* infection (Arenas-Gamboa et al., 2008; Rafiei et al., 2006). The Δ*pgm B. abortus* yielded a cytokine profile typical of a Th1-type response with a high-level induction of IFN-γ and the absence of IL-4 secretion (Ugalde et al., 2003). As anticipated, it induced protection (~2.0 $\log_{10}$ units) against wt *B. abortus* 2308 in mice, compatible to that conferred by S19. Oral immunization with the Δ*znuA B. melitensis* resulted in undetectable CFUs in spleens, Peyer's patches (PPs), and mesenteric lymph nodes (MLNs) by two weeks following immunization, unlike mice orally vaccinated with a similar dose of RB51 or S19 that contained elevated CFUs in these same lymphoid tissues (Clapp et al., 2011a). This suggests that Δ*znuA B. melitensis* is considerably less virulent than the livestock vaccines of RB51 and S19, indicating the Δ*znuA B. melitensis* is potentially safe. Although the Δ*znuA B. melitensis* vaccine was cleared relatively rapidly from the host, it stimulated potent immunity in which 83% of the mice nasally challenged with wt *B. melitensis* showed no brucellae in their spleens (Clapp et al., 2011a). This protective effect was considerably subdued in IFN-$\gamma^{-/-}$ mice, although significant protection (1.85 $\log_{10}$ units) was still observed. To evaluate the cytokines induced by Δ*znuA B. melitensis*, lymphocytes were restimulated with heat-killed RB51 and found to produce elevated levels of IFN-γ, as did RB51-vaccinated mice prior to and after pulmonary challenge. Thus, the Δ*znuA B. melitensis* data suggest short-term infection is sufficient to stimulate enduring IFN-γ responses, which could be attributed to the expressed LPS, and RB51-vaccinated mice's duration of infection may be important since RB51 lacks LPS. Alternatively, RB51 stimulates different attributes of the immune system. Nonetheless, short-term infection with Δ*znuA B. melitensis* achieved robust protection against wt *B. melitensis* challenge, as opposed to RB51-vaccinated mice that showed only 42% with sterile immunity and variable levels of protection or no protection (Clapp et al., 2011a). The differences in protection observed between Δ*znuA B. melitensis* and RB51 may, in part, be due to the co-stimulation of IL-17. Although neutralization of IL-17 in unvaccinated mice did not show enhanced *B. melitensis* colonization, IFN-$\gamma^{-/-}$ mice required IL-17 for protection. Moreover, while Δ*znuA B. melitensis*-vaccinated wt mice showed no dependence upon IL-17 for protection, IL-17 was required for Δ*znuA B. melitensis*-mediated protection in IFN-$\gamma^{-/-}$ mice. IFN-γ competent and deficient mice vaccinated with RB51 displayed higher bacterial burdens when IL-17 was neutralized (Clapp et al., 2011a). Thus, this evidence suggests some vaccines may have a co-reliance upon alternative cell-mediated immune mechanisms.

In a similar vein, subunit vaccines that promote cell-mediated immunity generally show enhanced protective capacities. BCSP31, SodC, and L7/L12 (Yu et al., 2007), Omp16 or Omp19 (Pasquevich et al., 2009), and Omp25 (Commander et al., 2007) have been shown to stimulate antigen-specific IgG responses with IgG2a > IgG1 titers, indicating a Th1-type biased immune response. Due to the effectiveness of Th1-type immunity in predicting the vaccine's performance in the murine model, Th1 cell immunity can be used as a parameter to predict potential *Brucella* vaccine's efficacy in the preliminary screenings of candidate vaccines.

Human and other large animal brucellosis vaccines

The lack of a human brucellosis vaccine remains problematic due to the risk of *Brucella* as a possible bio-terrorist agent, and because brucellosis remains a global disease affecting more than 500000 people annually (Franco et al., 2007). Since *B. melitensis* is the most frequently acquired among all the *Brucella* spp., a live human *Brucella* vaccine based upon *B. melitensis* should be considered. Due to its low virulence and the excellent protective efficacy, Δ*znuA B. melitensis* makes an excellent candidate (Clapp et al., 2011a). Likewise, another promising candidate for *B. melitensis* is one deleted of *pgm*, and Δ*pgm B. abortus* may be a promising candidate for cattle due to its capability of not eliciting anti-LPS antibody while conferring substantial protection in mice. These vaccines will require further evaluation to learn whether these strains are sufficiently attenuated in primates. If not, *pgm* could be deleted from Δ*znuA B. melitensis* to generate a double mutant strain, or an additional virulence factor will be deleted from either Δ*znuA B. melitensis* or Δ*pgm B. melitensis* to further strengthen their safety.

Subunit and recombinant human vaccines against *Brucella*, due to their enhanced safety and cross-species protection traits, should also be considered. For subunit vaccines, efforts should be focused on how to optimize the formulation, e.g., selecting the optimal adjuvant. For the live recombinant *Brucella* vaccines, using the licensed human vaccines as vaccine vectors, such as *S. typhi* Ty21a (Levine et al., 1999) or gut commensal bacteria such as *L. lactis* (Shi et al., 2006), may further human brucellosis vaccine development.

Until human vaccines become available, to control human brucellosis, animal vaccination campaigns need to be maintained because human health can benefit tremendously from livestock vaccination campaign (Roth et al., 2003; Jelastopulu et al., 2008). Brucellosis is the most commonly acquired zoonotic disease, and humans become infected upon exposure to infected animals and their products, so preventing infection in animals is of paramount importance to eradicate human brucellosis. Control of brucellosis via mass immunization campaigns of livestock has proven effective in the USA (Pappas et al., 2006b), Mongolia (Kolar, 1977), China (Xin, 1986), and Greece (Minas et al., 2004). In the USA, because of the massive eradication campaign, human brucellosis cases dropped from 6321 cases in 1947, to 2215 cases in 1973–1982, to 1201 cases in 1983–1992, to 1056 cases in 1993–2002 (Pappas et al., 2006b). From 1971 to 1981, in the Inner Mongolia Autonomous Region of China, sheep and goats were immunized with S2 vaccine. Due to the cessation of *Brucella*-induced abortion in small ruminants, the incidence of brucellosis in humans also significantly dropped, with no new cases occurring since 1983 (Xin, 1986). In contrast, in Mongolia, the country-wide mass-vaccination program of livestock successfully reduced human brucellosis to less than one case per 10000 people per year (Kolar, 1977), but this program was interrupted in the early 1980s due to the termination of WHO and assistance from the former Soviet Union in 1990, and human brucellosis re-emerged (Zinsstag et al., 2005). Vaccination of sheep and goats with Rev-1 for 15 years in Greece decreased their abortions and the incidence of brucellosis in humans. After the vaccination program was stopped in 1994, the prevalence of brucellosis in animals and humans quickly increased in Greece (Minas et al., 2004).

Concluding remarks

Brucellosis remains a global health problem affecting at least a half million people annually (Franco et al., 2007) and potentially as many as 13 million because of misdiagnosis (Wise, 1980). Although brucellosis can be treated with antibiotics, the extended time for treatment impacts compliance, and the lack of vaccines for humans will continue to make this disease a global health threat. This is further complicated by the prevalence of brucellosis in livestock, particularly, in countries lacking the resources to have an effective eradication program. While vaccination of livestock with conventional vaccines could certainly dampen the impact of brucellosis, better livestock vaccines are needed, especially for *B. abortus,* since infections can still occur in vaccinated animals. Thus, continued efforts will be needed to develop more efficacious livestock vaccines and to generate human vaccines. To date, live *Brucella* vaccine candidates show the best promise for livestock, and further testing, including oral formulations (Clapp et al., 2011a) in relevant livestock and wildlife will be needed. While surely live *Brucella* vaccines offer one possibility, such live vaccines would have to be sufficiently mutated, minimizing multiple virulence factors to ensure these are safe and not overly attenuated where it is rapidly cleared. Alternatively, subunit *Brucella* vaccines that can recapitulate aspects of protective immunity would certainly be desirable and may require multiple epitopes (vaccine targets) for optimal efficacy. Thus, much work is needed at multiple fronts to minimize contamination of food products, improve livestock health status, and provide intervention strategies to prevent and treat brucellosis.

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