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Confronting the barriers to develop novel vaccines against brucellosis

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Brucellosis is an important zoonotic disease of nearly worldwide distribution. This pathogen causes abortion in domestic animals and undulant fever, arthritis, endocarditis and meningitis in humans. Currently, there is no vaccine licensed for brucellosis in humans. Furthermore, control of brucellosis in the human population relies on the control of animal disease. Available animal vaccines may cause disease and in some cases have limited efficacy. This article discusses recent studies in the development of recombinant protein, DNA and live-attenuated vaccines against brucellosis. Furthermore, we call the attention of the scientific community, government and industry professionals to the fact that for these novel vaccine initiatives to become licensed products they need to be effective in natural hosts and bypass the regulatory barriers present in several countries.

Keywords: *Brucella abortus* • brucellosis • DNA vaccine • live-attenuated vaccine • recombinant protein vaccine • vaccine

Brucellosis is a zoonotic infection of domestic and wild animals. *Brucella* causes third trimester abortions in pregnant females and orchitis and epididymitis in males [1]. Among *Brucella* species are *B. melitensis*, *B. abortus*, *B. suis*, *B. canis*, *B. neotomae*, *B. ovis*, *B. ceti*, *B. penipedialis*, *B. microti* and *B. inopinata*. The first four species are pathogenic to humans in decreasing order of severity making brucellosis a zoonotic disease with more than 500,000 new cases reported annually [2]. Human brucellosis causes a chronic disease with symptoms such as intermittent fever, endocarditis, arthritis and osteomyelitis [2]. Since this is a zoonosis of great importance for human and animal health, effective regulatory programs are required to control animal brucellosis, which is the main reservoir for human infection [3].

Currently, three *Brucella* strains have been used in vaccines for brucellosis prevention, S19 and RB51 for cattle and Rev1 for small ruminants [3]. Although these vaccines have been successfully used worldwide and helped disease eradication in developed countries, they are still far from ideal. Although the smooth strains S19 from *B. abortus* and Rev1 from *B. melitensis* are able to induce effective levels of protection in cattle, goat and sheep, respectively, they have some drawbacks. These vaccine strains can sometimes cause abortion in pregnant adult cows [4]. Both strains are pathogenic to humans and interfere with the diagnosis because they possess the lipopolysaccharide (LPS) bearing the intact O-chain. Antibody production against the O-chain complicates the differentiation between vaccinated from infected animals [4]. Additionally, S19 and Rev1 vaccines do not induce full protection against infection with virulent strains [4]. On the other hand, the mutant strain RB51 derived from *B. abortus* does not possess an intact O-chain, which avoids interference in serological diagnosis. RB51 is stable and is less virulent than the smooth strains [5]. However, this strain is resistant to rifampicin, which is the first antibiotic of choice for human brucellosis treatment [6]. Moreover, immunization effectiveness of RB51 when compared with S19 with the same vaccine dose is controversial in cattle [7]. Therefore, the development of effective vaccines that completely prevent the infection and protect the different hosts from *Brucella* infection, are required for elimination of this illness. Together with a better vaccine, better animal management conditions (e.g., extensive breeding, coexistence of several livestock species, and so on) and structural weaknesses are also important barriers to bypass in order to reach control and eradication of brucellosis in endemic countries.

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Rationale for developing an anti-brucellosis vaccine

The development of an effective vaccine against brucellosis has been a challenge to scientists around the world. According to Adams [8], an ideal vaccine against *Brucella* should have the following properties: prevents bacterium infection in both genders; does not provoke disease in vaccinated animals; prevents abortion; promotes a long period of protection with only one dose; does not interfere with serological diagnosis; is biologically stable and does not present risk of virulence reversion; is not shed to humans and is not shed in milk; and can be produced in large scale and at low cost.

Brucella spp. are facultative intracellular pathogens which resist killing by neutrophils, replicate inside macrophages and in nonprofessional phagocytes, and maintain a long lasting interaction with the host cells [9]. As intracellular organisms, protection against *Brucella* infection requires cell-mediated immunity, which includes $CD4^+$ and $CD8^+$ T lymphocytes, Th1-type cytokines such as IFN-γ and TNF-α, and activated macrophages and dendritic cells (DCs) [10]. Therefore, host control of infection requires a set of cells and components of the immune system which together promote a complex response against *Brucella*.

The first encounter of a pathogen by innate immunity is triggered by pattern recognition receptors (PRRs) that are capable of sensing pathogen-associated molecular patterns [11]. Several PRRs have identified, among them Toll-like receptors (TLRs), nucleotide binding and oligomerization domain-like receptors and retinoic acid-inducible gene 1-like receptors. To date, TLRs are the best described PRRs [12]. The involvement of TLRs in the host resistance to infection by *B. abortus* has been investigated by different groups using TLR-2-, TLR-4-, TLR-9- and MyD88-deficient mice. TLR-2 clearly does not play any role in controlling *B. abortus* infection *in vivo* [13,14], whereas TLR-9 has been shown to be required for clearance of this bacterium in infected mice [15]. The role of TLR-4 is a matter of controversy in the literature [14]. These receptors signal through the adaptor molecule MyD88. Furthermore, MyD88-dependent signaling was shown to be essential for the activation of IFN-γ-producing cells and DCs during *Brucella* infection [15,16]. Copin *et al.* [16] also demonstrated that MyD88 deficiency strongly reduces the frequency of IFN-γ cells during the *B. melitensis* infection. In addition, they reported that TRIF play no role *in vivo* control of *B. melitensis* infection [16]. In a study carried out by our research group, we demonstrated that *B. abortus*-mediated DC maturation is dependent on the adaptor molecule MyD88 [15]. Additionally, we have reported that MyD88 knockout mice are highly susceptible to *B. abortus* infection *in vivo* and this result was also confirmed by Copin *et al.* using *B. melitensis* [16]. Therefore, the use of TLR-9 agonists as adjuvants would be a critical strategy to develop an anti-brucellosis vaccine.

Regarding $CD4^+$ and $CD8^+$ T lymphocytes, both subsets are involved in host resistance against *Brucella*. Experiments transfering $CD4^+$ or $CD8^+$ T cells from immunized mice to naive recipients prior to infection demonstrated that both T-cell subpopulations are required to induce resistance to *Brucella* [17]. These studies were also confirmed by gene knockout mice [18]. Protective immunity can be achieved by production of type 1 cytokines, mainly IFN-γ, and lysis of *Brucella*-infected macrophages [19]. The importance of IFN-γ is supported by studies in which IFN-$\gamma^{-/-}$ mice infected with *Brucella* organisms died within 6 weeks of infection [20]. Activated macrophages show increased anti-brucellae mechanisms and are able to destruct the pathogen, inhibiting *Brucella* spread [21]. Moreover, the type 1 cytokines produced by $CD8^+$ T cells induce downregulation of Th2 cytokines and IL-10 [18,19].

Overall, there are two mechanisms of the adaptive immune response during *Brucella* infection that appear to be important: first, IFN-γ produced by $CD4^+$, $CD8^+$ and γδ T cells activates the bactericidal action of macrophages to control the intracellular *Brucella* infection; second, lysis of *Brucella*-infected target cells by $CD8^+$ and γδ T cells. Therefore, to develop an effective vaccine against brucellosis these arms of the host immune system should be activated.

Brucella subvert the host immune system

There is a considerable amount of evidence that indicates the ability of *Brucella* spp. to avoid or interfere with components of the host innate and acquired immune responses, which plays a critical role in their virulence. *Brucella* have developed ways to subvert the host innate immune system via reduction, modification and hiding of pathogen-associated molecular patterns such as LPS and flagellum. This strategy allows the bacteria to enter the *Brucella*-containing vacuole (BCV), avoiding activation of adaptive immune responses [22].

Brucella abortus lacks some surface structures commonly recognized by innate immunity, such as capsules, fimbriae and pili, but has a flagellum expressed on its surface, even though it is a nonmotile bacteria. It was proposed that this flagellum could be used somehow to subvert the specific immune response against *Brucella* [23]. Indeed, the flagella of *Brucella* displays an amino acid sequence that is not recognized by its cognate receptor, TLR-5 [24], thus being a poor inducer of TLR-5-mediated inflammatory responses [25].

Another feature of *Brucella* is its so called noncanonical LPS structure [26]. When compared with other Gram-negative bacteria, the lipid A of *Brucella* possesses a diaminoglucose backbone rather than glucosamine and longer acyl groups (C18–C19 and C28 rather than C12 and C14), which are only linked to the core by amide bonds rather than ester and amide bonds [27]. The lipid A moiety of the LPS of *Brucella* elicits a reduced and delayed inflammatory response in the infected hosts compared with the endotoxins from other Gram-negative bacteria [28]. The O-antigen, the most distal portion of the LPS of *Brucella*, plays a crucial role in intracellular niche establishment by dictating the interactions between *Brucella* and specific cell surface receptors, minimizing macrophage activation and enhancing *Brucella* survival and/or persistence [29]. In addition, the O-antigen portion can interact with MHC class II molecules and form complexes, interfering with the ability of *Brucella*-infected macrophages to present exogenous proteic antigens acting as downmodulator of T-cell activation [30]. Furthermore, recent studies have demonstrated that heat-killed *B. abortus* or *Brucella* lipoproteins

downmodulate MHC class II expression on antigen-presenting cells and this phenomenon was dependent on TLR-2 and mediated by IL-6 [31].

In addition to the LPS of *Brucella*, other factors also contribute to bacteria entrance and replication inside phagocytic cells without efficiently activating antimicrobial mechanisms. *Brucella* enters macrophages through lipid rafts [32] and once inside the host cell the bacteria are found within a compartment termed the *Brucella*-containing vacuole (BCV). BCV interacts transiently with early endosomes, escapes lysosome fusion and further fuses with the membrane of the endoplasmic reticulum (ER), thereby establishing a replicative organelle [33]. This intracellular process is dependent on the *Brucella* type IV secretion system VirB [34] and it is hypothesized that effector molecules secreted by the VirB system into the host cell, actively redirect the intracellular trafficking and target the bacteria to their replicative niche [35]. *Brucella* spp. also produce a periplasmic cyclic β-1,2-glucan, which was hypothesized to interact with cholesterol and reorganize lipid rafts in macrophages, interfering with host cell functions and contributing to perturbation of intracellular trafficking to the advantage of the pathogen [36].

Furthermore, several studies have shown the presence of a *Brucella* protein called Btp1 (also known as TcpB), which bears significant homology with the TIR domain present in TLRs and adapter molecules such as MyD88, MAL/TIRAP, TRIF and TRAM [37–40]. Btp1/TcpB has been shown to interfere with signaling via TLR-2 which downmodulates maturation of infected DCs [39]. The interference with signaling via TLR-2 may occur through interactions between Btp1/TcpB and MAL/TIRAP, an adapter molecule that recruits MyD88 to trigger TLR signaling [38]. The presence of Btp1/TcpB leads to enhanced polyubiquitination of MAL, which is likely responsible for its accelerated degradation. Therefore, Btp1/TcpB can also inhibit signaling by TLR-4 and TLR-6 [40]. Therefore, *Brucella* could subvert TLR signaling pathways to suppress host immune responses to benefit their survival and persistence. Therefore, to develop an efficient vaccine against brucellosis it is necessary to fully understand the mechanisms by which the bacteria manipulate the host immune response.

Recombinant protein vaccines

Subunit vaccines, such as recombinant proteins, are promising vaccine candidates, because they can be produced at high yield, purity and can be manipulated to maximize desirable activities and minimize undesirable ones. In this particular case, selecting an antigen for vaccination different to the one used in diagnosis tests will allow the ability to differentiate vaccinated from *Brucella*-infected animals. Moreover, they are safer for manipulators, well defined, not infectious and can not revert to a virulent strain. However, despite these advantages, recombinant proteins tend to be poorly immunogenic *in vivo*, and require the coadministration of adjuvants that indirectly enhance the immune response against recombinant proteins. Therefore, recombinant vaccine success is usually dependent on the use of these substances with immunomodulatory properties, which instruct and control the selective induction of diverse antigen-specific immune responses [41–43].

Numerous cell surface and intracellular components have been assessed as protective antigens (Ags) against *Brucella* infection (Table 1). The L7/L12 ribosomal protein, apart from being one of the first recombinant purified proteins tested against *Brucella*, is a good example of the importance of trying different Ag formulations when developing a subunit vaccine. L7/L12 administered with adjuvant induced significant protection against *B. abortus* infection, yet the degree of protection was less than that elicited by the control attenuated vaccine S19 [44]. In an attempt to improve the immunogenicity of L7L12, Mallick and colleagues [45] demonstrated that the egg phosphatidyl-choline/cholesterol liposome encapsulated recombinant L7/L12 protein or *Escherichia coli* lipid liposome (escheriosome)-mediated cytosolic delivery of rL7/L12 protein [46] induced strong Th1 immune responses and these strategies elicited protection levels against *B. abortus* 544 comparable to S19 vaccine at 30 days postimmunization.

More recently, Yang *et al.* [47] have identified by liquid chromatography tandem mass spectrometry one immunodominant protein from *Brucella S*-adenosyl-homocysteine hydrolase (AdoHcyase). Recombinant AdoHcyase induced a strong Th1 type response and induced similar level of protection to Rev1 vaccine against *B. melitensis* 16M challenge at 4 weeks postchallenge.

The enzyme lumazine synthase from *Brucella* spp. (BLS) is highly immunogenic [48], presumably due to its decameric arrangement and remarkable stability as shown in biophysical studies [49]. It is also a potent activator of bone marrow DCs [50]. BLS has been shown to confer partial protection against *B. abortus* independent of the adjuvant formulation used (incomplete Freund's adjuvant [IFA], monophosphoryl lipid A or aluminum hydroxide) [48], however, it did not confer protection without adjuvant [48,51]. These results indicate that the need for an adjuvant is critical but the choice is less important in this case. In our opinion and because of the particular immunostimulatory properties of some Ags, the final choice of the adjuvant must be made only after experimentally testing the formulation (Ag + adjuvant).

Similar results were obtained with an outer membrane protein of 31 kDa (Omp31). In BALB/c mice, recombinant Omp31 in aluminum hydroxide induced similar levels of protection against *B. melitensis* compared with Omp31 in IFA [52]. Generally, water in oil emulsions (e.g., IFA) are recommended for bovine animals, small ruminants, poultry and fish when long-term immunity is required [53]. However, currently only aluminum-based adjuvants are approved by the US FDA to be used in humans [53]. Therefore, BLS and Omp31 could be used as vaccine candidates in larger animals and humans.

The main functions of vaccine adjuvants are formation of antigen–adjuvant depots and induction of an inflammatory response [54]. Research programs that are based on the identification of novel adjuvants is critical to the development of more efficient vaccines. Recently, Denisov *et al.* [55] tested several adjuvants such as larifan, polyoxidonium, natrium thiosulfate, TNF-β and Ribi for their ability to enhance immune responses to live *B. abortus* strain 82-PS vaccine. Combination of adjuvants with live vaccine enhanced antibody and cell-mediated responses to strain 82-PS,

Table 1. Recombinant proteins and synthetic peptides from *Brucella* spp. tested as vaccines against brucellosis.

Antigen	Vaccination (adjuvant, route)	Challenge (*Brucella* species, interval vaccination/challenge)	Protection†	Ref.
L7/L12 (ribosomal protein)	None, sc. or ip.	*B. abortus*, 1 and 4 weeks postvaccination	None	[45, 46, 82]
	IFA, sc.	*B. abortus*, 1 week postvaccination	0.6	[45, 46]
	Liposomes, sc.	*B. abortus*, 1 week postvaccination	1.65	[45]
	Escheriosomes, sc.	*B. abortus*, 1 week postvaccination	1.65	[46]
MBP-L7L12 (fusion protein between maltose-binding protein from *Escherichia coli* and L7/L12)	Immune plus commercial adjuvant, ip.	*B. abortus*, 4 weeks postvaccination	1.21	[44]
GroEL (heat shock protein)	IFA, im.	*B. abortus*, 2 weeks postvaccination	None	[59]
	Ribi	*B. abortus*, 5 weeks postvaccination	None	[60]
GroES (heat shock protein)	Ribi	*B. abortus*, 5 weeks postvaccination	None	[60]
HtrA (heat shock protein)	Ribi	*B. abortus*, 5 weeks postvaccination	None	[60]
GroEL + GroES + HtrA	Ribi	*B. abortus*, 5 weeks postvaccination	None	[60]
Cu-Zn SOD	None or MPA, ip.	*B. abortus*, 4 weeks postvaccination	None	[62]
GGDNYSDKPEPLGG (peptide derived from SOD)	None or MPA, ip.	*B. abortus*, 4 weeks postvaccination	None	[62]
LAEIKQRSLMVHGG (peptide derived from SOD)	None or MPA, ip.	*B. abortus*, 4 weeks postvaccination	None	[62]
GGAPGEKDGKIVPAG (peptide derived from SOD)	None, ip.	*B. abortus*, 4 weeks postvaccination	0.84	[62]
	MPA, ip.	*B. abortus*, 4 weeks postvaccination	2.19	
CP24 (ribosome recycling factor-like protein of 24 kDa)	CFA/IFA, ip.	*B. melitensis*, 4 weeks postvaccination	None	[58]
BLS (*Brucella* lumazine synthase)	None, ip.	*B. abortus*, 4 weeks postvaccination	None	[48]
	IFA, ip.	*B. abortus*, 4 weeks postvaccination	1.26	
	MPA, ip.	*B. abortus*, 4 weeks postvaccination	1.31	
	$Al(OH)_3$, ip.	*B. abortus*, 4 weeks postvaccination	1.40	
Omp31	IFA, ip.	*B. melitensis*, 4 weeks postvaccination	1.25	[52]
Omp3148-74 (27 amino acid peptide derived from Omp31)	IFA, ip.	*B. melitensis*, 4 weeks postvaccination	1.15	[52]

Mice were used as a host in all vaccine trials.
† Units of protection are the average of $\log_{10}$ colony-forming units (CFU)/spleen in control mice minus average of $\log_{10}$ CFU/spleen in vaccinated mice.
CFA: Complete Freund's adjuvant; IFA: Incomplete Freund's adjuvant; im.: Intramuscular; ip.: Intraperitoneal; MPA: Monophosphoryl lipid A; Omp: Outer membrane protein; sc.: Subcutaneous; SOD: Superoxide dismutase.

Table 1. Recombinant proteins and synthetic peptides from *Brucella* spp. tested as vaccines against brucellosis (cont.).

Antigen	Vaccination (adjuvant, route)	Challenge (*Brucella* species, interval vaccination/challenge)	Protection†	Ref.
BLSOmp31 (chimera between BLS and Omp3148-74)	IFA, ip.	*B. melitensis*, 4 weeks postvaccination	1.34	[65]
p39 (periplasmic-binding protein)	None, im.	*B. abortus*, 3 weeks postvaccination	None	[56]
	CpG, im.	*B. abortus*, 3 weeks postvaccination	2.48	
Bacterioferritin	None, im.	*B. abortus*, 3 weeks postvaccination	None	[56]
	CpG, im.	*B. abortus*, 3 weeks postvaccination	None	
DnaK (chaperone of the HSP70 family)	None, ip.	*B. abortus*, 4 weeks postvaccination	None	[64]
	CFA/IFA, ip.	*B. abortus*, 4 weeks postvaccination	0.95	
SurA (periplasmic peptidyl prolil *cis–trans* isomerase)	CFA/IFA, ip.	*B. abortus*, 4 weeks postvaccination	1.45	[64]
bp26 + TF (diagnostic antigen + trigger factor)	Choleric toxin, nasal	*B. melitensis* (ip.), 2 weeks postvaccination	0.3	[71]
CGH (bile salt hydrolase)	Choleric toxin, oral	*B. abortus* (oral), 4 weeks postvaccination	0.98	[69]
U-Omp16 (unlipidated Omp16)	None, ip.	*B. abortus*, 4 weeks postvaccination	1.33	[73]
	None, oral	*B. abortus* (oral), 4 weeks postvaccination	1.26	[73]
	IFA, ip.	*B. abortus*, 4 weeks postvaccination	1.61	[70]
	$Al(OH)_3$, ip.	*B. abortus*, 4 weeks postvaccination	1.48	
	Choleric toxin, oral	*B. abortus* (oral), 4 weeks postvaccination	1.15	
U-Omp19 (unlipidated Omp19)	None, ip.	*B. abortus*, 4 weeks postvaccination	1.84	[74]
	None, oral	*B. abortus* (oral), 4 weeks postvaccination	1.26	[74]
	IFA, ip.	*B. abortus*, 4 weeks postvaccination	1.70	[70,74]
	$Al(OH)_3$, ip.	*B. abortus*, 4 weeks postvaccination	1.27	
	Choleric toxin, oral	*B. abortus* (oral), 4 weeks postvaccination	1.26	
Omp28	None, im.	*B. abortus*, 3 weeks postvaccination	None	[57]
	CpG, im.	*B. abortus*, 4 weeks postvaccination	0.96	
S-adenosyl-L-homocysteine hydrolase	CFA/IFA, ip.	*B. melitensis*, 3 weeks postvaccination	2.13	[47]

Mice were used as a host in all vaccine trials.
† Units of protection are the average of $\log_{10}$ colony-forming units (CFU)/spleen in control mice minus average of $\log_{10}$ CFU/spleen in vaccinated mice.
CFA: Complete Freund's adjuvant; IFA: Incomplete Freund's adjuvant; im.: Intramuscular; ip.: Intraperitoneal; MPA: Monophosphoryl lipid A; Omp: Outer membrane protein; sc.: Subcutaneous; SOD: Superoxide dismutase.

and phagocytosis by macrophages. Furthermore, the highest protection was demonstrated by combining TNF-β or polyoxidonium with *B. abortus* strain 82-PS. This study demonstrated that adjuvants may be successfully used for stimulation of the appropriate immune response and protection when designing a brucellosis vaccine.

The need for an adjuvant was also important in the case of a putative periplasmic-binding protein of *Brucella* (P39), which only induced Th1 and protective responses against *B. abortus* infection when combined with CpG oligonucleotides [56]. Recently, it has been described that vaccination with rOmp28 adjuvanted with CpG conferred moderate levels of protection against *B. abortus* infection [57]. Of note, the protection afforded by the formulation P39-CpG at 4 weeks postchallenge was similar to the live-attenuated S19 strain and the mice were still significantly protected at 8 weeks postchallenge, although to a lesser extent than the S19-vaccinated group [56]. These data were important examples that indicated in mice that a recombinant protein vaccine would be as successful as live-attenuated vaccines against brucellosis.

By contrast, vaccination with CP24 [58], GroEL, GroES and Htra in adjuvant [59,60] or bacterioferritin with CpG [56] did not induce protection against *Brucella* challenge (Table 1), highlighting that the selection of the correct Ag will be critical when developing a vaccine to brucellosis.

The production of a subunit vaccine that is antigenically defined and pure is of interest in terms of safety and production. Furthermore, a vaccine based on peptides does not require storage at cold temperatures. This issue is critical when considering mass vaccination in rural areas of developing countries [61]. Tabatabai *et al.* [62] tested the protective immunity induced by three different synthetic peptides from Cu-Zn superoxide dismutase (SOD) from *B. abortus*. From three peptides tested, only one engendered significant levels of protection [62]. In a similar approach, a known exposed region of Omp31 situated between amino acids 48–74 was chosen as immunodominant [63]. This peptide was tested in BALB/c mice and induced IFN-γ production in $CD4^+$ T and $CD8^+$ T cells. These results demonstrated that this peptide possesses a Th1 and Tc1 epitope [52]. Immunization with $Omp31_{48–74}$ in IFA conferred significant levels of protection against *B. melitensis*, similar to that obtained with rOmp31 [52]. Of note, immunization with this peptide plus BLS in IFA induced similar protection against *B. melitensis* infection compared with Rev1 vaccination.

The recombinant proteins SurA (a periplasmic peptidyl prolyl *cis-trans* isomerase) and DnaK (a chaperone from the HSP70 family) were also evaluated in mice. Both proteins induce protection against *B. abortus* infection (moderate levels lower than control vaccine). Vaccination with rDnaK or rSurA engendered similar levels of protection despite inducing different patterns of immune responses [64]. rDnaK induced a cytotoxic T lymphocyte (CTL) and Th1 type immune response while rSurA elicited a Th1/Th2 profile with no CTL activation.

The general idea of combining several antigens in a vaccine formulation not always induces higher levels of protection than using single antigens. For instance, immunization with rDnaK and rSurA had no synergistic effect compared with vaccination with rDnaK or rSurA separately [64]. Similarly, we tested protective efficacy of *Brucella* Omp31 and BLS with no additive effect of these antigens observed [65]. However, in a chimeric formulation Omp31–BLS augmented the protection achieved by single antigen vaccination [65]. These results suggest that the lack of synergy may be caused by an epitope interference *in vivo,* as suggested for other antigens [66,67]. Therefore, the choice of adequate antigens to compose a subunit vaccine that engenders protection in natural hosts together with the correct Ag delivery system is a critical step towards the development of effective anti-brucellosis vaccine. Furthermore, a study performed by Laplagne *et al.* [68] demonstrated that is possible to insert foreign peptides at the N-terminal domain of BLS without disturbing the proper folding of this protein. Based on this study, we genetared a chimera composed of BLS scaffold associated with an Omp31 protective epitope. Mice immunization with BLS–Omp31 chimera engendered higher protection levels compared with single antigen vaccination; however, protective immunity was lower than afforded by vaccine strain Rev1 [65]. This vaccine formulation induced a specific Th1 and CTL response in immunized mice.

Infection by the oral route is one of the main entry sites for *Brucella*. Domestic animals usually acquire brucellosis by eating food contaminated with tissue remaining from abortions [69]. As a zoonosis, brucellosis transmission from animals to man occurs via ingestion of contaminated food products, contact with infected animals or inhalation of *Brucella* particles [2,69,70]. Thus, another key point in brucellosis is the induction of immune responses on the mucosal surfaces.

Three recombinant proteins: CGH, Omp16 and Omp19 induced protection against oral *Brucella* challenge when delivered by the oral route with choleric toxin (CT) as mucosal adjuvant [69,70]. Nasal immunization with trigger factor plus BP26 plus CT has also been conducted and induced local immune responses and a low level of protection against systemic infection. Unfortunately, the authors did not evaluate protection against nasal challenge [71].

Mucosal adjuvants derived from bacterial toxins are associated with toxicity or side effects [72]. In this regard, the protein portion of lipoproteins Omp16 and Omp19 (U-Omp16 and U-Omp19) are endowed with self-adjuvanting properties [73,74]. They induced significant protection against oral or systemic *B. abortus* challenge when delivered by parenteral or oral routes without adjuvants, yet by different immune mechanisms. U-Omp16 required TLR-4 while U-Omp19 did not. The protective capacity is neither improved by the lipidation of the proteins nor by the addition of external adjuvants (CT when orally administered or IFA when parenterally delivered). This unique quality represents an exceptional benefit because external adjuvants might sometimes present a risk, inducing adverse reactions including local inflammation at the injection site with the induction of granuloma or sterile abscess formation [75].

Among oral delivery systems, plant-based vaccines have all the attractive features of mucosal vaccines along with other distinct features unique to plant expression systems, such as the lack of requirement for fermentation and protein purification processes, a cost-effective production process owing to the low energy input,

a low cost of supplies and ease of vaccine shipping, conservation and delivery [76]. Moreover, plant-made vaccines could be particularly suited for farm animals destined for meat markets, as repeated administrations can deteriorate the quality of meat [77]. Ags expressed in plants would be protected by bioencapsulation enhancing antigen delivery to the gut-associated lymphoid tissue. Moreover, it has been described that rumination could be exploited for exposure of nasopharyngeal tissues during cudding if vaccine antigen is expressed by a fibrous feed such as alfalfa [77].

Our results indicate that plant expressed U-Omp16 or U-Omp19 are able to induce significant protective immune responses when administered to mice by the oral route as purified proteins as well as within the crude leaf material of transgenic tobacco plants [73,74]. In both cases, the protection levels achieved were equivalent to those elicited by the purified *Escherichia coli*-derived Ags and statistically similar to protection levels elicited by oral delivery of live RB51 or S19 [73,74]. These results are encouraging and other plant expression systems (such as alfalfa or barley) might be optimized to develop an edible vaccine against brucellosis for cattle.

In summary, until now, significant protective activity has been identified against *Brucella* using the following purified recombinant Ags: L7/L12, 22.9 kDa, BLS, Omp31, p39, DNAk, SurA, Omp28, CGH, Omp16, Omp19 and *S*-adenosyl-L-homocystein hydrolase (Table 1). An efficient subunit vaccine must be protective to any host and induce protection to any *Brucella* species. This ideal vaccine will probably be composed of more than one *Brucella* antigen.

DNA vaccines

Vaccination with a plasmid expressing a gene coding for a specific antigen has become an important strategy to develop new generation vaccines. DNA immunization induces preferentially robust Th1 and CTL responses [78–80], as well as protection against a wide range of microbial pathogens [81]. Although the technology was initially developed in the 1990s for gene therapy application; published literature has increased dramatically ever since due to the promise of such vaccines replacing expensive subunit vaccines based on recombinant purified proteins.

Since the work of Kurar and Splitter [82], many antigens have been explored as DNA vaccines against *Brucella* infection. The majority of effort has been made in the murine model of infection. In the beginning, these studies resulted in the induction of a diverse immune response, which led to different levels of protection that, in many cases, were not as high when compared with live-attenuated *Brucella* vaccines [51,59,83–89]. This raised the question of whether DNA vaccines expressing a single *Brucella* gene encoding for a putative protective antigen would be able to induce high levels of protective immunity [90]. Yet, in the past 5 years, the use of combined DNA vaccines expressing several antigens or DNA plasmids encoding a chimerical *Brucella* antigen rendered significantly higher levels of protection compared with commercial live-attenuated *Brucella* vaccines [88,91–94] (Table 2). Although is tempting to speculate that the reason for the higher protection achieved with these vaccines was due to the fact that more than one immune target was attacked, it appears that the reason why these preparations achieved higher protection is because they induced a vigorous coordinated immune response that includes the three main mechanisms of *Brucella* immunity [95]: IFN-γ production; specific Th1-type antibodies; and, what seems to be mainly attributed as a correlate of protection [88,89,93], $CD8^+$ CTL activity. Moreover, vis-à-vis results obtained for monovalent DNA vaccines [51,82,83,86,89] and multivalent DNA vaccines [88,91–94] suggest that a robust and long-lasting $CD8^+$ response may be critical for protection against *B. abortus* infection.

Since eliciting a coordinate appropriate immune response seems to be a key factor when designing an ideal vaccine for brucellosis, strategies to enhance the efficacy of DNA vaccines are constantly emerging in order to maximize appropriate immune responses [96]. This is particularly important to support the transition of these vaccines into larger animal models and even humans [95]. In the past 5 years, the most tested strategy to improve brucellosis DNA vaccines in the mouse model was to modulate the immune response by the co-expression of cytokines as immune adjuvants with the goal of enhance CTL responses or prolong the duration of protection. Hitherto, results indicated that when a cytokine was fused to the antigen within single gene DNA vaccines, enhancement of vaccine efficacy was not achieved. When IL-2 [97] or IL-18 [98] gene fusions to SOD were expressed from a single DNA vaccine, the inclusion of the cytokine failed to increase protective efficacy over the SOD-expressing DNA vaccines against *B. abortus* infection. By contrast, when IL-15 [94] or IL-12 [92] were coadministered on a separate plasmid to the multigenic vaccine containing the *BCSP31*, *SOD* and *L7/L12* genes, efficacy against *B. abortus* infection was significantly improved and reached higher levels than the live-attenuated S19 control vaccine. Enhancement in protection levels strongly correlated with an increase in $CD8^+$ CTL activity in both cases. Moreover, the coadministration of IL-12 rendered a long-term protection that was evident at 6 weeks after challenge [92]. Furthermore, IL-12 associated with *Brucella* glyceraldehyde-3-phosphate dehydrogenase (*gap*) gene induced partial protection against infection that was not achieved with the *gap* gene alone (Table 2) [99].

Commander *et al.* [100] tested Omp25 and ialB DNA vaccines against *B. melitensis* challenge. Omp25- and ialB-expressing plasmids when administered separately induced 2.54 and 2.70 log units of protection, respectively.

Other strategies have been also tested to improve the immunogenicity and protective efficacy of DNA vaccines against *Brucella* infections. Several laboratories have attempted to manipulate the DNA construct in which the antigen of interest is expressed to achieve better expression of the antigen (by changing the promoter) [82] or the cellular location of the expressed protein is altered to obtained a secreted antigen (by adding secretion signal sequences) [59,97]. However, these have failed to improve both the elicited immune response and the protective efficacy, at least to levels comparable to that of live-attenuated vaccines. Another promising strategy to improve protection induced by DNA vaccines is first to vaccinate with DNA and boost with recombinant protein [101]. Several studies have validated this approach [101,102].

Table 2. Protective DNA vaccines in mouse models of brucellosis.

Antigen	Vaccination (plasmid, route)	Challenge (*Brucella* species, interval vaccination/challenge)	Protection (log units)	Ref.
L7/L12 (ribosomal protein)	pcDNA3.1-L7/L12, im.	*B. abortus*, 4 weeks postvaccination	1.26	[82]
L7/L12 (ribosomal protein)	pcDNA3.1-L7/L12, im.	*B. abortus*, 2 weeks postvaccination	1.59	[104]
P39 (periplasmic-binding protein)	pcI-p39, im.	*B. abortus*, 8 weeks postvaccination	0.73	[83]
P39-L7/L12 (fusion protein)	pcDNA3.1-p39-L7/L12, im.	*B. abortus*, 5 weeks postvaccination	2.03	[91]
GroEL (heat shock protein)	pCMV-link/GroEL, im.	*B. abortus*, 2 weeks postvaccination	None	[59]
Cu-Zn SOD	pcDNA3-SOD, im.	*B. abortus*, 5 weeks postvaccination	2.25	[86, 87]
CP24 (ribosome recycling factor-like protein)	pcDNA3-CP24, im.	*B. abortus*, 4 weeks postvaccination	0.32	[58]
Omp16-L7/L12 (fusion protein)	pcDNA3.1-Omp16-L7/L12, im.	*B. abortus*, 2 weeks postvaccination	2.05	[104]
BCSP31 (31 kDa cytoplasmic protein) + SOD + L7/L12	pJW403-BCSP31 + pJW403-SOD + pJW403-L7/L12 + pCI-IL12 (adjuvant), im.	*B. abortus*, 6 weeks postvaccination	2.55 (DNA vaccine) 4.37 (DNA vaccine + IL-12)	[92, 93]
Omp31 (hemin-binding protein)	pCI-Omp31, im.	*B. melitensis*, 4 weeks postvaccination	1.30	[89]
BLS (*Brucella* lumazine synthase)	pcDNA3-BLS, im.	*B. abortus*, 4 weeks postvaccination	1.65	[51]
BLSOmp31 (fusion protein)	pC-IBLSOmp31, im.	*B. melitensis*, 4 weeks postvaccination	1.77	[88]
GADPH (glyceraldehyde-3-phosphate-dehydrogenase)	pCMV-Gap + pCI-IL12 (adjuvant), im.	*B. abortus*, 2 weeks postvaccination	0.68	[99]
Omp25 (25 kDa Omp)	pcDNA3.1-Omp25, im.	*B. melitensis*, 4 weeks postvaccination	2.54	[100]
IalB	pcDNA3-ialB, im.	*B. melitensis*, 4 weeks postvaccination	2.70	[100]

im.: Intramuscular; Omp: Outer membrane protein; SOD: Superoxide dismutase.

In our laboratory, boosting with recombinant Omp31 led to a moderate improvement in protection of an Omp31-expressing DNA vaccine against a challenge with *B. melitensis* [103]. Of note, although the prime–boost regimen induced specific cytotoxic responses, these responses could not reach those achieved by DNA immunization.

Drawing on the aforementioned publications in the field, it can be tentatively assumed that so far the most successful strategy to improve the efficacy of DNA vaccines and reach levels of protection better than live-attenuated commercial vaccines, at least in the murine model of infection, implies the use of DNA vaccines expressing several antigens (combined or expressed as a chimera) in combination with cytokine-expressing plasmids, which elicit a cytotoxic response, and better correlates with protection. Luo *et al.* [104] tested a DNA vaccine chimera containing *L7/L12* and *Omp16* genes (*L7/L12–Omp16*). This divalent DNA vaccine induced protection against challenge with virulent strain *B. abortus* 544 significantly higher than the univalent DNA vaccine administered separately. However, as suggested by Perkins *et al.* [95], most of these DNA vaccines rely on intramuscular immunizations that require large amounts of DNA, causing problems with scale-up when testing these vaccines in larger animal models. The use of gene gun-mediated vaccination, which requires significantly less plasmid DNA than intramuscular immunization, could solve this issue, although convention suggests that gene gun-based DNA vaccination generally elicits Th2-type immunity [81,105]. Alternatively, *in vivo* electroporation and the use of nanoparticles, which enhance cellular DNA uptake or the half-life of DNA, respectively, would ease that problem [106].

One of the main concerns that has arisen in the past in the field of brucellosis DNA vaccines was whether achieving sufficient protection in the mouse model would warrant the translation of this efficacy to larger farm animals [90]. Two promising studies have come to answer this uncertainty. Oñate *et al.* [86] have demonstrated that the DNA vaccine expressing Cu-Zn SOD was able to elicit a good protective immune response in mice and Saez *et al.* [107] also reported that this same vaccine induced both antibody and cell-mediated immune responses in cattle.

Finally, getting a small step closer to the Holy Grail of DNA vaccines, four DNA vaccine products have recently been approved, all in the area of veterinary medicine [108]. These licensures are an important validation of the DNA vaccine platform because they illustrate its commercial potential. Moreover, the success of these products shows that DNA vaccines can be manufactured to scale and at low cost complying to regulatory issues. Thus, current studies in large animals in the field of *Brucella* DNA vaccine should be encouraged to provide more vaccine candidates to the pipeline of a better, safe and efficacious subunit vaccine against this important zoonosis.

Live-attenuated *Brucella* vaccine

To overcome the drawbacks presented by the currently available vaccines, several efforts have been made to improve these immunogens. Rough vaccines are usually less effective than smooth *Brucella* vaccines [109]. Barrio *et al.* [109] tested the efficacy of three *B. melitensis* mutants *wbkF* (bactoprenol priming for O-PS polymerization), *per* (perosamine synthesis) and *wa*** (core glycosyltransferase) (Table 3). Even though good protection level in mice was achieved, 54% was the highest protection engendered by the vaccines in sheep compared with the Rev1 strain (100%). Furthermore, González *et al.* [110] also tested rough *wa*** and *wzm* mutants and showed them to be protective in mice; however, higher doses generated abscesses and other untoward effects. However, Ugalde *et al.* [111] have used the rough strain mutant for the phophoglucomutase (*pgm*) enzyme in a vaccine, with relative success. These authors showed that the Δ*pgm* strain produces O-antigen of approximately 45 kDa in size but it is still rough. This phenomenon occurs because this mutant strain cannot assemble the O-side chain in the LPS architecture. Immunization with Δ*pgm* engendered protection similar to that observed for S19. As an important diagnostic tool, they were unable to detect a specific anti-O-antigen antibody response in animals immunized with the Δ*pgm* strain using the fluorescence polarization assay. This mutant strain is now undergoing clinical trials in cattle in Argentina. Our group has also recently tested the efficacy of the *Brucella* formyltransferase enzyme mutant strain (Δ*wbkC*) involved in LPS biosynthesis. We observed that *B. abortus* Δ*wbkC* mutant was attenuated in macrophages and elicited higher levels of proinflammatory cytokines when compared with the wild-type strain 2308 [112]. Furthermore, Δ*wbkC* showed reduced virulence in C57BL/6 and IRF-1 knockout mice. Nevertheless, these rough mutants engendered lower protection compared with the S19 vaccine strain.

In an attempt to increase the protection provided by the rough vaccine strains, Grilló and colleagues [113] demonstrated that the coadministration of rough and smooth *Brucella* mutants can confer protection against murine brucellosis. In this study, the investigators produced mutants to the O-chain, specifically to the *wbkA* gene, and to the two component regulatory system *bvrS/bvrR*. The vaccination combining the two mutants conferred better levels of protection when compared with that conferred with S19.

Regarding smooth strains, Izadjoo and coworkers [114] evaluated an orally administered live-attenuated purine auxotrophic (*purE*) *B. melitensis* mutant strain, WR201. The ability of this mutant strain to elicit cellular and humoral immune responses and to protect mice against intranasal challenge with *B. melitensis* 16M was evaluated. In this report, WR201 was able to induce cellular, humoral and mucosal immune responses. Moreover, oral immunization induced protection against systemic bacterial spread and enhanced clearance of bacteria from the lungs after intranasal challenge. These results suggest that purine auxotrophy is an attractive attenuating strategy for further vaccine development. However, in another study, these researchers showed that the WR201 mutant retains its infectivity for reproductive tissues [115]. This tropism may lead to signs and symptoms of disease in man.

Tibor *et al.* [116] have also tested the *Brucella* mutant of the periplasmic protein P39, which protected mice against challenge with lower levels when compared with strain S19 at 8 weeks postchallenge (Table 3). Furthermore, Kahl-McDonagh and Ficht [117] screened signature-tagged mutant banks to identify mutants attenuated for survival. They constructed unmarked deletion mutants of three gene candidates, *manBA*, *virB2* and *asp24*, in both *B. abortus* and *B. melitensis*. At 13 weeks postvaccination, mice were protected by Δ*asp24* at a higher degree than S19-vaccinated mice, both significantly greater than naive controls. Mutants that are cleared more quickly from the host, Δ*virB2* and Δ*manBA*, protected mice to a lesser degree.

Using bioluminescence imaging to gain greater insight into *B. melitensis* pathogenesis, Rajashekara *et al.* [118] described the reduced virulence of *B. melitensis* GR019 (*virB4*), GR024 (*galE*) and GR026 (BMEI1090-1091) mutant strains. Among the mutants studied, *Brucella galE* induced protection against infection in IRF-1 knockout and C57BL/6 mice and no pathology was detected in the liver and spleen of infected animals. Therefore, *galE* mutant strains have great potential as vaccine candidate to control brucellosis.

Arenas-Gamboa *et al.* [119], using the vaccine strain S19, developed a mutant to the *vjbR* gene. *Brucella* VjbR is a quorum sensing-related transcriptional regulator. A *Brucella vjbR* mutant has shown a downregulated expression of both *virB* operon and flagellar genes during intracellular infection. This potential vaccine was delivered in microcapsules as an improved delivery system. They reported higher level of protection induced by this vaccination strategy when compared with the S19 strain. Inflammation and persistence was also decreased.

More recently, our group has generated a smooth *Brucella* strain mutant deficient in the phosphoglycerate kinase enzyme. The *B. abortus* Δ*pgk* mutant showed reduced virulence in C57BL/6, 129/Sv, BALB/c and IRF-1 knockout mice [120]. Intracellularly, Δ*pgk* was found in BCVs rich in LAMP1 but not containing ER markers. Localization of *Brucella* in BCVs containing ER markers

Table 3. Genetically engineered live-attenuated vaccines against brucellosis.

Gene deleted	Host	Vaccination (dose, route)	Challenge (*Brucella* species, interval vaccination/challenge)	Protection	Ref.
wbkF (bactoprenol priming for O-PS polymerization)	Sheep	1×10^{10} CFU, sc.	*B. melitensis,* 26 weeks postvaccination	54%†	[109]
per (perosamine synthesis)	Sheep	1×10^{10} CFU, sc.	*B. melitensis*, 26 weeks postvaccination	36%	[109]
*wa*** (glycosyltransferase)	Sheep	1×10^{10} CFU, sc.	*B. melitensis*, 26 weeks postvaccination	31%	[109]
wzm (ATP-binding cassette)	Mice	1×10^{8} CFU, sc.	*B. melitensis,* 8 weeks postvaccination	4.07 log	[110]
pgm (phosphoglucomutase)	Mice	1×10^{7} CFU, ip.	*B. abortus*, 8 weeks postvaccination	2.25 log	[111]
wbkC (formyltransferase)	Mice	1×10^{8} CFU, ip.	*B. abortus,* 6 weeks postvaccination	0.58 log	[112]
wbkA (mannosyltransferases) and *bvrS/bvrR* (two-component regulatory system)	Mice	0.5×10^{8} CFU, ip.	*B. abortus*, 4 weeks postvaccination	4.46 log	[113]
purE (purine)	Mice	1×10^{11} CFU, orally	*B. melitensis,* 8 weeks postvaccination	2.46 log	[114, 115]
P39 (periplasmic-binding protein)	Mice	1×10^{5} CFU, sc.	*B. abortus*, 4 weeks postvaccination	0.80 log	[116]
manBA (enzyme related to mannose)	Mice	1×10^{6} CFU, ip.	*B. abortus,* 12 weeks postvaccination	0.90 log	[117]
virB2 (type IV secretion system)	Mice	1×10^{6} CFU, ip.	*B. abortus*, 12 weeks postvaccination	1.50 log	[117]
asp24 (protein induced by acid shock)	Mice	1×10^{6} CFU, ip.	*B. abortus,* 12 weeks postvaccination	4.7 log	[117]
galE (UDP-glucose 4′-epimerase)	Mice	1×10^{7} CFU, ip.	*B. melitensis,* 8 weeks postvaccination	3.0 log	[118]
vjbR (transcriptional regulator)	Mice	1×10^{5} CFU, ip.	*B. abortus*, 20 weeks postvaccination	3.06 log	[119]
pgk (phosphoglycerate kinase)	Mice	1×10^{5} CFU, ip.	*B. abortus*, 12 weeks postvaccination	3.28 log	[120]
31 kDa (protein)	Cattle	1×10^{10} CFU, sc.	*B. abortus*, 44 weeks postvaccination	100%	[121]
SOD (superoxide dismutase)	Cattle	1×10^{10} CFU, sc.	*B. abortus*, 44 weeks postvaccination	100%	[121]
bp26/Omp19 (periplasmic protein/outer membrane protein 19)	Cattle	1×10^{10} CFU, sc.	*B. abortus*, 68 weeks postvaccination	45.5%	[122]
bp26 (periplasmic protein)	Cattle	1×10^{10} CFU, sc.	*B. abortus*, 68 weeks postvaccination	81.8%	[122]

†Animals were considered to be protected when no abortion, no excretion of the challenge strain and no infection at slaughter occurred.
ip.: Intraperitoneal; sc.: Subcutaneous.

is a hallmark of virulent strains. Finally, the Δ*pgk* mutant induced superior protection compared with the S19 vaccine strain in immunocompetent and immunocompromised mice. This strain has great potential to enter clinical trials in cattle in the near future.

Regarding trials in cattle, Cheville *et al.* [121] tested *B. abortus* mutants for 31 kDa protein or SOD. All vaccinated heifers were protected against intraconjunctival challenge with virulent *B. abortus* strain 2308, none aborted and none had *Brucella* isolated from their tissues. Additionally Fiorentino *et al.* [122] constructed two *Brucella* mutants *M1-luc* and *I2*. *B. abortus M1-luc* is a mutant strain in which most of bp26 has been replaced by the luciferase-coding gene. I2 is a double mutant strain derived from M1-luc in which most of omp19 has been deleted. These two mutant strains were tested in cattle. Four groups of 15 animals of 6 months of age were vaccinated with M1-luc, I2 or S19 as a positive control or were left unvaccinated as a negative control. After challenge infection, protection was measured as percentage of animals that aborted. The S19 vaccine strain protected 78.6% of the cattle, M1-luc protected 81.8% and I2 protected 45.5%. The negative control unvaccinated animals presented 25% of heifers protected against abortion. Similarly, Edmonds *et al.* [123] have constructed a *htrA-cycL* double gene deletion mutant, PHE1. These authors tested this strain for attenuation and immunogenicity in cattle. PHE1 did not cause any abortion while S2308 induced abortion or weak calves in four out of four animals. This study revealed that PHE1 is attenuated in cattle and it is killed more rapidly by bovine neutrophils and macrophages than the wild-type strain S2308.

To date, live-attenuated vaccines provide the best protection against *Brucella* challenge in larger animals. However, we should bear in mind that mutations may attenuate the organism too much, so that the level of protection induced is insufficient.

Regulatory issues

Brazil and Argentina are important endemic countries for brucellosis. Currently, Brazil has the biggest herd for commercial purposes. As for Argentina, it ranks at the fifth position worldwide. Bovine brucellosis causes economic losses of approximately US$32 million annually in Brazil [124] and US$60 million in Argentina [125].

Efforts to control bovine brucellosis in Brazil date back from 1940–1950. Control measures were defined as serological survey of animals with isolation of positive reactors and immunization

with S19 vaccine. In 2001, the Brazilian Ministry of Agriculture initiated a new National Program for the Control and Eradication of Brucellosis and Tuberculosis. The main control measures were: vaccination with S19 of females aged 3–8 months; accreditation of herds free of brucellosis; periodic serological survey of beef herds; requirement of serological testing for interstate movement and for entry into livestock fairs; compulsory slaughter of animals that tested positive; and permanent training for accredited veterinarians. So far, *B. abortus* S19 is the only approved vaccine and is produced by different pharmaceutical companies. Vaccination of adult females with rough strain RB51 can occur strictly in an endemic situation.

In Argentina, measures to control bovine brucellosis date back from 1932. Furthermore, in 1947 voluntary vaccination with S19 in cattle was implemented. In 1999, a National Control and Eradication Program was launched by the Agriculture Department in Argentina. The main resolutions of the program were: registration of accredited veterinarians; registration of private laboratories to standardize the certification of brucellosis-free herds; integration of accredited laboratories for brucellosis diagnostics; vaccination of females 3–8 months of age with S19; categorization of herds according to their brucellosis status.

In summary, in both endemic countries, to date S19 is the vaccine of choice approved to control bovine brucellosis.

Expert commentary

Health and sanitary regulations predicated on fear of spreading virulent domestic animal diseases, such as brucellosis, have limited the marketing opportunities of cattle products among countries. Despite great control efforts worldwide, pathogenic *Brucella* spp. can persist in domestic livestock or free-ranging wildlife. Vaccination is a critical measure of control programs and although S19 and Rev1 vaccines have been successfully used worldwide, they have drawbacks; therefore, the ideal brucellosis vaccine is still very much awaited. Thus, the development of effective vaccines that completely prevent the infection at the different entry sites and protect the different hosts of *Brucella*, together with control measures are required for elimination of this illness. An effective *Brucella* vaccine has to activate $CD4^+$ and $CD8^+$ T cells and induce IFN-γ production. Recently, a role for IL-17 in adaptive mucosal immunity against *Brucella* has been described for a particular recombinant vaccine but this needs further studies to be broadly applied.

We believe that: newly genetically engineered live-attenuated vaccines, currently used attenuated vaccines together with a boost of subunit vaccines or subunit vaccines alone (composed of different Ags), will be suitable for larger animals. When developing a human vaccine against brucellosis, a subunit vaccine will be the best option. We visualize that this vaccine would need to be made from different *Brucella* proteins to ensure that the microorganism is adequately confronted by the immune effectors during infection.

Five-year view

We anticipate that within the next 5 years there will be several laboratories worldwide testing different types of vaccines against brucellosis. A major issue related to this is the fact that at present government regulatory agencies in endemic countries are resistant to work with other *B. abortus* strains or subunit vaccines than strain S19. If this never changes, it may discourage work by many researchers and the investment of the industry to support the development and clinical trials of new vaccine initiatives. Therefore, if unchanged, no major advance in the development and licensing of these products will be achieved. However, we believe that if we can pass the regulatory requirements for the pharmaceutical and preclinical safety assessment of the products, showing better protection results without the drawbacks of attenuated vaccines in use, our national regulatory agencies must at last change their minds. Finally, all the work already made and to be done to ensure a continued influx of innovation into the development of a better vaccine against brucellosis is of great value.

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Key issues

- *Brucella* is a CDC category B select agent.
- Brucellosis is a zoonosis worldwide that causes annual economic losses of US$32 and US$60 million to Brazil and Argentina, respectively.
- Approximately 500,000 new cases of human brucellosis are reported annually.
- *Brucella* infection control requires IFN-γ production by host T cells and activation of cytolytic $CD8^+$ T lymphocytes.
- Although S19 and Rev1 vaccines have been successfully used worldwide and have helped disease eradication in developed countries, they are still far from ideal.
- There is no available human vaccine against brucellosis.
- Regulatory agencies should work closely with scientists and industry to encourage them to invest in the development of new vaccines against *Brucella*, suitable for use in animals and humans.

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