ACELLULAR VACCINES FOR OVINE BRUCELLOSIS: A SAFER ALTERNATIVE AGAINST A WORLDWIDE DISEASE.

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Abstract

Ovine brucellosis is a very contagious zoonotic disease distributed geographically worldwide and constitutes a very important zoosanitary and economic problem. The control of the disease includes animal vaccination and slaughter of infected flocks. However, the commercially available vaccine in most countries is based on the attenuated strain *Brucella melitensis* Rev 1 which present important safety drawbacks. This review is focused on the most recent and promising acellular vaccine proposals.

Keywords

Brucellosis; acelular vaccine; adjuvant; mucosal vaccination; microparticle; nanoparticle.

1. BODY OF THE ARTICLE

Brucellosis is considered a reemerging zoonosis, producing low mortality but high morbidity in both animals and humans [1, 2].

Most *Brucella* species affect domestic ruminants (Table 1) and, consequently, affect severely on the economy of millions of people. Specifically, the costs associated with ovine brucellosis are related to losses in animal reproduction, infertility, abortion, delayed conception and reduced milk production. The causative agents of ovine brucellosis are *B. ovis* and *B. melitensis* [3]. *B. ovis,* is a not zoonotic rough strain, responsible for contagious ram epididymitis and also produce alteration on the blood circulation of sheep placenta, implying placentitis, abortion as well as neonatal death. This species infects sheep, as well as farmed red deer (*Odocoileus virginianus*) in New Zealand [3]. Experimental infections have been reported in goats and cattle, but there is no evidence that these species are infected in nature (Table1). On the other hand, *B. melitensis* is a major worldwide zoonosis with significant economic losses and importance to public health, is highly pathogenic for humans and responsible for classical testicular alterations in rams, reduced fertility and abortion. The infected females rarely clear the pathogen from their system and tend to shed through their next parturition. It may be venereally transmitted and shedding of the organism can be greater than four years in rams. Semen quality deteriorates rapidly and inflammatory cells are often present. Once fibrous atrophy of the testes occurs, they are permanent. Thus, the development of new vaccines against *B. melitensis* and *B. ovis* would be of great benefit worldwide.

Table 1

Different countries may require different strategies for its prevention and control in animals, depending on epidemiological and socioeconomic conditions [4]. The general strategies proposed in 1998 by the WHO to control animal brucellosis were [5]: (i) prevention of spread between animals and monitoring of brucellosis-free flocks/herds and zones; (ii) elimination of infected animals by test-and-slaughter program to obtain brucellosis free flocks/herds and regions, and (iii) vaccination to reduce the rate of infection. It is certain that vaccination is the most economic and effective measure to control infections. Accordingly to the established by WHO, an ideal vaccine should accomplish basically two goals, efficacy and innocuousness [6]: (i) innocuous for the vaccinated animals; (ii) not produce disease in the vaccinated animals; (iii) prevent infection, abortion and sterility; (iv) provide long-term protection against infection and abortion with a single vaccination (more than 90% of protection); (v) must minimize long-term production of antibodies, which may interfere with the serodiagnosis tests of field infections; (vi) non invasive administration (mucosal administration preferably); (vii) not be transmitted to other animals if the vaccine strain establishes a long-term latent infection; (vii) biologically stable and free of reversion to virulence *in vitro* and *in vivo*; (viii) stable at room temperature; (ix) non-pathogenic for humans; (x) not contaminate meat and milk products; (xi) culturable under large-scale conditions and (xii) should contain specific genetic or phenotypic markers that would make it easy to differentiate from field isolates.

Attenuated vaccines

The classical live attenuated vaccines are made of attenuated pathogens in order to diminish their virulence, while retaining its immunogenicity. However, several risks are

associated with such vaccines including the residual virulence or hazardous re-infection, which occur when the inactivation of the microorganism is incomplete [7]. In this context, attenuated vaccines can regain their virulence in immunodeficient hosts causing the pathogen release and spread into the environment [8]. Thus, a careful and exhaustive evaluation of the potential impact of environmental release is required. This is the case of the currently best available immunoprophylaxis system against ovine brucellosis, the *B. melitensis* Rev 1 vaccine [9]. Rev 1 was developed in 1950 by Elberg and Herzberg derived from a virulent *B. melitensis* isolate which became streptomycin-dependent. Since its introduction in the marketplace in the 1960s, the benefit derived from its use is incalculable, protecting the ovine livestock and goats against *B. melitensis* and providing protection against *B. ovis* in ovine livestock. However, this vaccine is not free from virulence, present important disadvantages like causing infertility and abortion in pregnant animals [9] and of being pathogenic for humans [7]. Furthermore, is streptomycin resistant, the antibiotic that combined with doxycycline provides the most effective brucellosis therapy [10]. Moreover, this strain possesses a smooth phenotype, and, therefore, vaccinated animals produce long persistent antibodies against the LPS O-chain. As a consequence, the utilization of smooth vaccine strains makes the distinction between vaccinated and infected animals, including sheep, impossible [9, 11]. For all these reasons, the use of Rev 1 is prohibited in those countries where *B. melitensis* infection has been eradicated.

Due to the unacceptable levels of antibodies interfering with diagnostic tests, attempts were made to circumvent this problem. Blasco and co. found that conjunctival vaccination with Rev 1 did not induce so long-lasting serological rams response after vaccination. Moreover, rams conjunctival vaccination was safer than subcutaneous

vaccination, reducing the number of abortions but, however, there were still a significant proportion of vaccinated animals excreting Rev 1 bacteria [12, 13].

Another approach to avoid the interference of the O-chain with disease control surveillance is based on the use of rough vaccine strains [14]. The rifampicin-resistant mutant *B. abortus* strain 2308, denominated *B. abortus* RB51 strain [15], is a genetically stable, rough morphology mutant that lacks the polysaccharide O-side chains on the surface of the bacteria, responsible for the development of the diagnostic antibody responses of an animal to brucellosis infection. However, in sheep it has been clearly demonstrated that RB51 does not confer enough protection against *B. melitensis* [16] or *B. ovis* [17] infections. Moreover, even though the risks are low, human infections due to RB51 have also been described [18], and being resistant to rifampin is problematic, since this antibiotic combined with doxycycline, is widely used for treating brucellosis in humans [10].

B. melitensis B115 is a natural *Brucella* rough strain, according to the absence of agglutination with monospecific sera to A and M antigens of *Brucella* but it has a cytoplasmic O chain [19]. Adone et al [20, 21] have demonstrated to be highly protective against *B. melitensis* and *B. ovis* infections in mice, without inducing interfering antibodies, suggesting its potential usefulness to control brucellosis, but it must be tested in the natural host.

A further approach is the construction of recombinant strains deleted in relevant diagnostic proteins. Thus, a Rev 1 vaccine strain deleted in the gene coding for BP26 periplasmic protein resulted in the same protective efficacy as Rev 1 against *B. ovis* in sheep [22], but the performance of the BP26 based differential diagnostic test was only very limited [23].

The elucidation of the genome of *Brucella* is now providing new lights towards the development of safer attenuated vaccines against brucellosis [95]. Thus, Arenas-Gamboa et al. using a defective mutant *B. melitensis* Δ*mucR* against intraperitoneal and aerosol *B. melitensis* challenge obtained a significant protection in BALB/c mice. MucR is a transcriptional regulator that regulates exopolysaccharide biosynthesis, iron sequestration and storage, nitrogen metabolism, and stress response mechanisms [24].

Bacterins

The use of inactivated whole bacteria as a vaccine (bacterin) was introduced as a safer alternative. A commercial killed *B. ovis* vaccine is used in New Zealand, but its efficacy is limited and may display inactivation problems. Several new vaccination approaches have emerged with significant advantages over traditional former approaches. Magnani et al. [25] have proposed the use of inactivated *B. melitensis* by gamma-irradiation in order to inhibit its replication but retaining metabolic and transcriptional activity. These inactivated bacteria triggers danger signals that allow efficient processing and presentation of antigens, generate antigen-specific cytotoxic T cells, and, most important, protected mice against virulent bacterial challenge, without signs of residual virulence. This may be a promising strategy for a safely vaccination that should be tested in the natural host.

Subunit vaccines

The greatest challenge for vaccine development against bacterial diseases is the development of vaccines against intracellular pathogens, such as *Brucella spp.*. The ideal brucellosis vaccine, that provides effective and safe protection against all species of *Brucella* in all animals, has not been developed yet.

To face the limitations of the rough vaccine strains, residual virulence and diagnosis interference, interesting approaches to develop a new generation of "ideal vaccines" are being investigated. It is probable that some of these vaccines will become serious candidates to replace the available classical vaccines in the near future.

Concerning the quantitative costs and benefits of a vaccine, we should project not only the economic costs derived from investigation, development and production, but also the costs derived from the negative effects like the residual virulence in the host. In the case of brucellosis, the cost/benefit ratio is not favourable to live attenuated vaccines, like Rev 1. In fact, acellular extracts could be effective to face infections caused by smooth and rough species, with the advantage that it avoids the problems of obtaining seropositive reactions against the S-LPS, making possible the differentiation between the infected animals and the vaccinated with Rev 1, as well as it would avoid the risk of infecting humans, specially veterinarians or whom manipulate existing vaccines.

One of the alternative approaches is the development of acellular vaccines, specifically subcellular fractions able to stimulate an adequate *Brucella spp.* immune response. To reach the purpose is required the use of highly conserve immunogenic antigens. The success of a subunit vaccine is strongly associated with its composition. Under this approach, it is required the use of highly conserved immunogenic antigens [26]. Various authors have challenged either rams or murine model, acellular extracts of *Brucella spp.* derived, among others, from smooth or rough strains as potential protective antigens [27-30], recombinant proteins [31, 32], synthetic peptides [33], DNA vaccines [34, 35] or anti-idiotypic antibodies that mimic the O-chain [36]. Thus, the outer membrane of *Brucella* contains the main immunodominat antigens involved in the host specific immune response. As other gram-negative bacteria, the outer membrane of rough *Brucella spp.* is composed of phospholipids, outer membrane

proteins (OMPs) and rough lipopolysaccharide (R-LPS), which are the immunodominant antigens. *B. ovis* is a stable rough form which lacks the OPS chains characteristic of the smooth strains, but contains an OMP profile similar to other members of the genus.

In the case of the rough strains the mostly immunogenic antigens are the OMPs, mostly because these are the most exposed antigens on the cellular surface (due the O-chain lack) [37]. OMP31 appear as the immunodominant antigen during the course of infection of rams [38], and the OMP25 has been shown to be involved in the virulence of *B. melitensis*, *B. abortus* and *B. ovis* [39]. In previous studies it was demonstrated that the OMPs pattern obtained for *B. melitensis* and *B. ovis* strains from different geographical origin were very similar [37]. Considering that 97% of their amino acids are conserved, and along with their strong immunogenicity, an acellular vaccine containing an outer membrane extract from *B. ovis* might be effective and protecting against infections caused by both rough *B. ovis* and smooth *B. melitensis* species. In fact, as it was previously mentioned, the smooth vaccine strain *B. melitensis* Rev 1 protects against the rough type *B. ovis*.

The enzyme lumazine synthase from *Brucella spp*. (BLS), a highly immunogenic decameric protein and activates dendritic cells via TLR4 [40,41], with adjuvant properties when a foreign antigen is attached covalently. Given the fact that Omp31 and BLS have been implicated in the generation of protective cellular and humoral immune responses, authors have generated chimera vaccines of the recombinant protein or DNA [42-44]. The coadministration with Incomplete Freund Adjuvant (IFA), has been shown to confer some protection degree against *B. abortus* in mice [42,43] and *B. ovis* in rams [45], and similar protection to *B. melitensis* Rev. 1 against *B. ovis* infection in BALB/c mice [44].

Immunoadjuvants

The main limitation of subunit vaccines is the low immunogenicity usually achieved, thus requiring booster doses in order to induce protection [46]. Moreover, immunity against *Brucella* requires cell-mediated mechanisms, in particular Th1 immune responses, characterized by INF-γ production [29]. Therefore, it is necessary to associate the subcellular components to suitable adjuvants, in order to reach the appropriate immunity. The selection of the correct adjuvant to tailor the adequate immune mechanisms requires a good knowledge of its potential and characteristics, the understanding of the pathogenesis and the molecular basis of the disease, as well as the role of the immune system [47].

For standard prophylactic immunization, only adjuvant that induces minimal adverse toxic effect will be accepted. In addition, practical issues that are considered important for adjuvant development include biodegradability, stability, easy manufacture, cost and applicability. Despite the recognition of many different types of adjuvant, the events triggered by them are poorly understood. However, two main mechanisms, which are not independent, were suggested and deeply reviewed [48]. The first based on the depot effect induced by the adjuvant, and the second related to the role on the antigen presenting cells (APCs). The adjuvant may be able to present the antigen directly to the competent cells (i.e. macrophages, dendritic cells). They are categorized as "Vacine Delivery Systems", and are generally emulsions, nano or microparticles, ISCOMS and liposomes. APCs adjuvant-induced enhancement of an immune response may improve the delivery of vaccine antigen into the draining lymph nodes. This can be achieved by facilitating antigen delivery to APCs, in particular to dendritic cells, which are known to be the most prominent T-cell activators [49]. The results increase the

provision of antigen-loaded APCs for cognate naive T cells, promoting upregulating of costimulatory signals or MHC expression, inducing cytokine release and thus enhancement the magnitude and duration of the immune response [48, 50].

Adjuvants can be used also used to promote the induction of mucosal immune responses, critical against *Brucellosis* as being pathogens that initiate infection and colonization at mucosal surfaces. This can be achieved by the mucosal delivery of the vaccine, since administration by injection generally stimulates poor mucosal immune responses. In this context, the classical vaccine adjuvants (aluminium hydroxide, aluminium phosphate or calcium phosphate and MF59 emulsion) do not elicit an effective mucosal immune response, as demonstrated when administered by the oral or nasal route [51]. In fact, the development of adjuvants for mucosal immunization is an important current area of vaccine development [52]. New mucosal adjuvants need to consider the ability to stimulate the APCs throughout the various mucosal routes. In this context, several adjuvants has been described [53, 54] including monophosphoryl lipid A (MPL®), CpG oligonucleotides, cholera toxin and *Escherichia coli* heat-labile enterotoxin.

Omp19 is a lipoprotein broadly expressed within the *Brucella* genus that when administered with the mucosal adjuvant cholera toxin (CT) confers protection against an oral challenge with virulent *Brucella* [55]. Recently [56], in order to avoid the toxicity due to the CT adjuvant, Omp19 was expressed in *Nicotiana benthamina* and the transgenic leaf material was used as edible plant-made vaccine against brucellosis, demonstrating its protective capacity when orally administered without the need of additional adjuvants.

Nowadays, new adjuvants under investigation are the particulate polymeric systems [57-63]. These particulate systems can act as vaccine adjuvants or/and as

immunomodulators, increasing the antigen response with which it is administered jointly. Biodegradable and biocompatible polymeric particles are highly useful and many antigens, regardless of their structure and water solubility, can be loaded into these systems by the use of different manufacturing techniques. Microparticles are defined as relatively solid spherical particles, with diameter between 1 and 1000 micrometers that form a continuous network or matrix system composed by one or more polymeric substances, in which the antigen is dispersed in the molecular form or solid dispersion. According to their structure, microparticles can be classified in microcapsules and microspheres. Thus, microcapsules are vesicular systems in which the active substance is confined to a cavity and is surrounded by a unique polymeric membrane; and microspheres are matricial systems in which the active substance is dispersed. The many advantages that these systems offer in biomedicine applications include: (i) increase the stability of the antigens incorporated; (ii) protection against chemical and enzymatic inactivation in the environmental conditions of the organism; (iii) improve the antigen transport to areas of the body in which produce its beneficial action, including the ability to interact with systemic immune cells (phagocytic cells, dendritic cells and macrophages that efficiently presenting carried-antigens to T and B cells [64, 65]; and (iv) prolong time of residence of the drug in the organism.

Microparticles can be prepared with natural (albumin, collagen, gelatine, polysaccharides) or synthetic materials (hyaluronic acid, alginic acid, chitosan, polyesters, polyorthoesters, polyalkylcarbonates, polyaminoacids, polyanhydrides, polyacrylamides and polyalkylcyanoacrylates). Poly(ε-caprolactone) (PEC), a biocompatible and biodegradable polyester polymer that degrades slowly and does not generate an acid environment, unlike the PLGA copolymers. Other advantages of PEC include its hydrophobicity, stability and low cost. Several experiments have

demonstrated that the outer membrane complex of *B. ovis* (HS complex) incorporated into PEC-microparticles (HS-PEC) induces adequate immune response and protection against experimental brucellosis in mice and rams [29, 32, 66].

The mucosal route is one of the preferred ways for antigen delivery, not only because it represents one of the most safer and comfortable routes of administration, but also, in the case of *Brucella spp.*, mucosal vaccination would imitate the infection pathway. The delivery of antigens of *Brucella* to mucosal surfaces is of remarkable interest, as it has been shown by the ocular administration of Rev 1 vaccine [9]. Arenas-Gamboa et al. [67] investigated the possibility of delivering the currently licensed vaccine against bovine brucellosis, *B. abortus* S19 strain, in a controlled microencapsulated format consisting of alginate microspheres. In a challenge study using red deer (*Cervus elaphus elaphus*) the encapsulated strain vaccine delivered by oral route conferred protection against an experimental challenge.

Finally, the enzyme lumazine synthase from *Brucella* spp. has been shown to confer protection against *B. abortus* and it has been described to be an antigen delivery system to effectively induce oral immunity. Thus, virus-like particles comprising repeating units of lumazine synthase decorated with OMP31 provided protection against *B. ovis* in mice similar than the control vaccine Rev1 [68, 69].

2. EXPERT COMMENTARY

Brucellosis is considered as one of the major extended bacteriological diseases worldwide, and constitutes an important socioeconomic and sanitary problem. Nowadays, the importance of vaccination in the control of animal brucellosis diseases is unquestionable and however, there are many concerns on this practice. Live attenuated vaccines are still by far the most utilized for their efficiency with respect to

inactivated and subunits ones [70]. However, the level of attenuation requires so fine-tuning to provide a protective immune response while maintaining safety that makes the benefit/risk ratio in favor of the subunit vaccines. Different approaches are being investigated, but however, in general, subunit vaccines induced inadequate immune responses without the use of adjuvants. Adjuvants can be used also used to promote the induction of mucosal immune responses, critical against pathogens like *Brucella* that use mucosae as main portal of entry. In fact, the development of adjuvants for mucosal immunization is an important current area of vaccine development [52]. Activation of mucosal immunity induce both mucosal and systemic immune responses. In contrast, parenteral immunization usually fails to stimulate mucosal lymphatic tissues to generate protective immunity at these sites. The need of adequate adjuvants capable of increasing the mucosal immune response is leading to suggest the use of particulate delivery systems, such as nanoparticles [29]. Particulate delivery systems belong to the category of adjuvants that facilitate the antigen uptake by APCs or by increasing the influx of professional APCs into the injection site. Among them, polymer nanoparticles are a group of delivery systems with interesting abilities as adjuvants for both conventional and mucosal vaccination. In particular, poly(anhydride) nanoparticulate systems made by the copolymer of methyl vinyl ether and maleic anhydride have demonstrated their efficacy as adjuvants to induce Th1 immune responses [57].

In summary, the multiplicity of delivery systems and adjuvants tested experimentally has not yielded many effective candidates for mucosal vaccination. Thus, the need of potent and safer mucosal adjuvants is still required

FIVE-YEAR VIEW

Vaccinology is a fast moving science that requires the interdisciplinary collaboration of other sciences like the pharmaceutical technology. The new technologies to produce purified antigens require the intervention of pharmaceutical technology to produce new adjuvants, more convenient to use, safer and easy to administer, even through mucosal routes. In such a way that in the near future, the mucosal administration of vaccines, such as oral, intranasal, or conjunctival routes are the method of choice for vaccine delivery [71].

From our point of view, the appropriate design of micro or nanoparticles is an important task in vaccinology against brucellosis, not only to obtain successful mucosal delivery of antigens, but also to modulate the immune response and achieve adequate protection against *Brucella* infections. Nanoparticles have been proposed as mucosal adjuvants since they: (i) protect the loaded antigen from enzymatic degradation, (ii) prolong immune response, (iii) enhance the antigen delivery to the mucosal associated lymphoid tissue and (iv) exhibit a strong bioadhesive performance [72-75]. The direct delivery to the mucosal associated lymphoid tissues seems to be the determinant step in mucosal immunization design. For that reason, the using of specific ligands associated to the nanoparticles has been considered an important strategy for mucosal antigen delivery [62, 76, 77]. In order to promote specific bioahesion a large number of cytoadhesive ligands can be associated to conventional nanoparticles, such as lectins, bacterial adhesive factors (i.e., pilli, flagellin), antibodies, sugars or vitamin B_{12} which are being deeply reviewed [78-80].

Salman *et al.* described that poly(anhydride) nanoparticles surface enriched in mannose residues promoted a better interaction with gastrointestinal mucosal tissues [80]. In vaccinology, this targeting may be related to the high binding affinity of mannose

residues to the mannose-binding lectins and mannose-receptors highly expressed in cells of the immune system [83, 84]. In fact, several authors have been shown that both antigen loading in nanoparticles and mannosylation of the systems as effective approaches to potentiate immunogenicity after mucosal delivery [81, 83].

On the other hand, using specific ligands, which enhance the targeting to receptors on APCs (i.e. TLRs), it is possible to effectively enhance the antigen presentation with a successful systemic immune response [57, 85]. Thus, the specific recognition of the corresponding pathogen-associated molecular patterns (PAMPs) will direct a differential innate and, further, adaptive immune responses. For example, TLR3 (that recognizes double-stranded RNA from virus), TLR4 (bacterial lipopolysaccharide), TLR5 (bacterial flagellin); TLR7 (single-stranded RNA from virus) and TLR9 (bacterial CpG-containing DNA) are related with a preferential Th1 response. In contrast, TLR2 (recognizes bacterial peptidoglycane and lipopeptides) elicits a Th2 response, although in combination with TLR6 elicit a regulatory response (Th3/Treg) [48]. Therefore, modifying the vaccines with immunological adjuvants that carry PAMPs or other PRR-agonists may be a way out to face the natural mucosal tolerogenic tendency. Thus, nanoparticles have revealed as good oral adjuvants, being able to induce simultaneously a peripheral and a mucosal immunity. Through the use of nanoparticulated delivery systems it is possible to abrogate tolerance and eliciting the desired balance Th1/Th2/Th17 type responses in both mucosal and systemic sites.

Recently, it has been shown that poly(anhydride)-nanoparticles induced innate immune responses mediated by a TLR-2 and TLR-4 dependent manner [57, 86]. These nanoparticles induced maduration of DC with a significant up-regulation of CD40, CD80, and CD86 and a biased Th1 present response in animal models. This is an important finding since it has been recently shown that the use of multiple TLR-

agonists carried by nanoparticles influence the induction of long-term memory cells, being the ultimate goal for any vaccine the stimulation of long-lasting protective immunological memory [87].

Interestingly, although TLR are usually linked with DC antigen recognition, intestinal mucosa also recognizes the antigenic challenge by TLR, and this fact is of vital importance for the host. Actually, the expression of TLRs in the intestinal epithelium is greater than that of other major organs, like the liver. Intracellular PRRs, such as NOD-like receptors (NLR), also play an important role in the intestinal epithelium. Both TLR and NLR are expressed differentially along the intestine. This is again a critical evolving consequence to cover the expected pathogen signals along the epithelial intestinal surfaces, and has a direct effect in vaccinology since each intestinal section may responds differently to the same antigenic stimulus.

Nanotechnology, PRR such as Toll-like receptors-agonist polymers and the bioadhesion concept are opening new dimensions in the adjuvant field for mucosal vaccination.

KEY ISSUES

- Brucellosis has a worldwide distribution with important socio-economic implications.
- The ideal brucellosis vaccine, that provides effective and safe protection against all species of *Brucella* in all animals, has not been developed yet. Currently licensed vaccines are live-attenuated with undesirable side effects. *B. melitensis* Rev1 strain is the vaccine of choice for small ruminants, but it is not allowed in areas in which *B. melitensis* has been eradicated because.
- It is relevant in a discussion on vaccination to consider the virulence

mechanisms that the pathogen uses to face the host immune system. Available genetic techniques allowed the identification of some *Brucella spp.* virulence factors allowing them to invade, resist intracellular killing, and reach their reproductive niche in professional and non-professional phagocytes like: an special outer membrane, cyclic ß-1,2 glucans, BacA, BvrR/BvrS, MucR, or heat shock proteins [88-91].

- Various authors have used acellular extracts, derived from both smooth and rough strains, as potential protective antigens, as well as recombinant proteins and idiotypic antibodies mimicking the O-chain. Considering all, a subcellular vaccine containing outer membrane extracts with OMPs and R-LPS from *B. ovis* could be effective to face infections caused by smooth and rough species, with the advantage that it avoids the problems of obtaining seropositive reactions against the S-LPS, making possible the differentiation between the infected animals and the vaccinated with Rev 1, as well as it would avoid the risk of infecting humans, specially veterinarians or whom manipulate existing vaccines.
- The need of potent and safer mucosal adjuvants is still required. Nowadays, promising adjuvants under investigation are the particulate polymeric systems [54,57-59,61,62,66,92-94]. These particulate systems can act as vaccine adjuvants or/and as immunomodulators.
- Concerning the quantitative costs and benefits of a vaccine, we should project not only the economic costs derived from investigation, development and production, but also the costs derived from the negative effects like the residual virulence in the host. In the case of brucellosis, the cost/benefit ratio is not favourable to live attenuated vaccines, like Rev 1.

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subcutaneous vaccination but is not safe enough to be applied regardless of the pregnancy status of the animals, and should be used only under restricted conditions. For sheep, conjunctival administration of standard doses of Rev 1 during the late lambing season or during lactation is recommended as a whole-flock vaccination strategy.

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Table 1. *Brucella* species and biovars characteristics (natural reservoirs, LPS type, pathogenicity in humans and humans cases).

Species	Biovar	LPS type	Natural reservoir(s)	Pathogenicity in humans	Human cases
B. melitensis	1-3	smooth	goat, sheep, ram, cat, camel, cattle	high	70% of cases
	1, 3	smooth	swine	high	
B.suis	2	smooth	Hare, wild boar	low	5% of cases
	4	smooth	reindeer, caribou	high	
	5	smooth	rodent	no	-
B. abortus	1- 6, 9	smooth	cows, cattle, buffalo, yaks, bison, horse	high	25% of cases
B. canis	-	rough	dogs, other canids	moderate	few
B. ovis	-	rough	sheep, ram	no	-
B. neotomae	-	smooth	desert wood rat	moderate	few
B. pinnipedialis	-	smooth	seal	?	?
B. ceti	-	smooth	cetacean	?	?
B. microti	-	smooth	soil, vole, fox	?	?
B. inopinata	-	smooth	human	?	?

Members of the genus *Brucella* are Gram-negative aerobic coccobacilli, nonmotile and no esporulated bacteria, which Proteomics situates in the α2 subdivision of the Proteobacteria. The genomes of the members of the genus *Brucella* are very similar in size (of about 3.29 Mb) and gene makeup. At present, the genus contains nine species with multiple biotypes that vary in terms of biochemical reactions, host specificity and pathogenicity in humans. *B. abortus*, *B. melitensis*, *B. suis*, *B. neotomae* and *B. microti*

(smooth species), *B. ovis* and *B. canis* (rough species), affect terrestrial animals. Recently, have been proposed two new species that affect marine mammals, *B. ceti* and *B. pinnipedialis* (both smooth species isolated from aquatic mammals), changing the concept of a land-based distribution and associated control measures. The smooth or rough phenotypes depend on, respectively, the presence or absence in the LPS of the O polysaccharide chain.

This table was adapted from the references [1,2].