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Brucellosis Vaccines: An Overview

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1. Introduction

Brucella is α -*Proteobacteria* causing an infectious disease in mammals that could be transmitted to humans. Ruminant and swine are prone to be infected by such microorganism all over the world, thus acting as a potential reservoir for domestic livestock and therefore, affecting humans. *Brucella* species differ in their hosts' preference, physiological abilities and cell surface structural characteristics. Those affecting domestic livestock are *B. melitensis* (sheep and goats), *B. abortus* (cattle), *B. suis* (swine), and *B. ovis* (sheep). Because domestic ruminants and swine are essential to the economy of millions of people, particularly in low income countries, brucellosis is a major cause of direct economical losses and a major impediment for trade. Moreover, human brucellosis is a severe and debilitating disease requiring a prolonged antibiotic treatment and often leaving permanent and disabling sequel. Thus, its control and if it possible its eradication are major goals of public health programs in affected countries (1,2).

2. The bacterium

Brucella belongs to the α -2 subdivision of the *proteobacteria*, along with *ochrobactrum*, *rhizobium*, *rhodobacter*, *agrobacterium*, *bartonella*, and *rickettsia*. The traditional classification of *Brucella* species is largely based on its preferred hosts. There are six classic pathogens, of which four are recognized human zoonoses. The presence of rough or smooth lipopolysaccharide is correlated to the virulence of the disease in humans. Two new *Brucella* species, provisionally called *Brucella pinnipediae* and *B. cetaceae*, have been isolated from marine hosts within the past few years (3,4).

Brucella is a monospecific genus that should be termed *B. melitensis*, and all other species are subtypes, with an interspecies homology above 87 percent. The phenotypic differences and host preferences can be attributed to various proteomes, as exemplified by specific outer-membrane protein markers. All *Brucella* species seem to have arisen from a common ancestor to which *B. suis* biotype 3 shares particular similarity. Although the scientific accuracy of this classification cannot be disputed, its practicality has been under scrutiny (5).

3. Nomenclature and classification

The Manchester report assumed the paper by Verger et al. on DNA hybridization studies and the proposition that all *Brucella* are just one species, with biovars; it was necessary to

reclassify *Brucella abortus* biovar 9 as *Brucella melitensis* biovar abortus 7, following deletion of biovars 7 and 8. It is remarkable that, according to the Manchester nomenclature, all 'biovars' *Melitensis* 1–3, *Abortus* 1–7 and *Suis* 1–5 were assigned to the same level of differentiation, irrespective of previous nomenclatures; this is undoubtedly correct and is based on genome studies, but misleading for brucellosis epidemiology considering the relatedness of nomenclatures with host animals and geographical spreading. Moreover, McGillivray et al. (1988) found that the restriction endonuclease profiles produced by *Bam*HI from DNA of five *Brucella abortus* isolates and the reference strain *B. abortus* biovar 2 were very similar. These results reinforced the existence of significant genetic homogeneity in *Brucella* genus. The report also emphasizes the relatedness of genus *Brucella* with the genera *Agrobacterium*, *Phyllobacterium* and *Rhizobium*. De Ley et al. identified *B. abortus* as a member in the α -2 subgroup of Proteobacteria on the basis of 16S rRNA gene sequence comparison and Moreno et al. suggested a close phylogenetic relation within the same group as a result of studying the composition of *Brucella* lipid A; later, Corbel published dendrogram considered it as serologically related (*Yersinia enterocolitica* O: 9, *Yersinia pseudotuberculosis*, *Salmonella typhimurium*, *Vibrio cholerae*, *Francisella tularensis*, *Escherichia coli* O: 157, *Pseudomonas putida*, *Rickettsia prowazekii*) (6,7).

4. Genetics of the *Brucella*

Classic genetic studies of *Brucella* was begun by spontaneous mutants in the early 20th century. The most widely studied spontaneous mutants are vaccine strains, such as *B. melitensis* Rev 1, *B. abortus* strain 19 and recently *B. abortus* strain RB51. The classic genetic studies are focused on phenotypic appearance, stability, metabolism and virulence of mutant colonies. Smoothness and roughness of the colonies usually attribute to high and low virulence of *B. abortus*, *B. suis*, and *B. melitensis*. Mutation causing changes in appearance of the colonies (smoothness \rightarrow roughness) usually decreases the virulence of these species and decreases or eliminates the stimulation of antibodies to the O antigen in animal hosts. *B. abortus* strain RB51 illustrates this well, it is a rough strain which is highly attenuated and does not induce anti-O antibodies. The *Brucella* genome has a GC content of approximately 58%. *B. melitensis*, *B. abortus*, *B. ovis*, *B. neotomae*, and *B. suis* biovar 1, each has two chromosomes of 2,100 kb and 1,150 kb. However, *B. suis* biovar 2 and 4 have two chromosomes of 1.85 Mb and 1.35 Mb, and *B. suis* biovar 3 has only one chromosome with a size of 3.1 Mb. These differences in size and number of chromosomes can be explained by rearrangements resulting from homologous recombination at chromosome regions containing the three *rrn* genes. The DNA sequences amongst different *Brucella* species share more than 90% homology. According to the present taxonomy and phylogeny based on 16S RNA, the classic 6 species belong to a single species. This fact has been used to propose that the genus *Brucella* contains only a single species *B. melitensis*, and that the remaining classic species be considered biovars. Insertion sequences (IS) are discrete segments of DNA that can transpose from one genomic site to another and promote genetic rearrangements. Insertion sequences are found on both chromosomes of *Brucella*. All *Brucella* species contain approximately 8–35 copies of an insertion sequence denominated IS711 (also known as IS6501). The position and copy number of this insertion sequence seems to vary in different species, a characteristic which can be used to differentiate them. For example, the *whoA* gene in *B. abortus* RB51 is disrupted by an IS711-like element. Based on this, a PCR assay has been developed to distinguish strain RB51 from other *Brucella* spp. and strains including its parent strain 2308. Many PCR assays based on gene differences

have been developed to detect or differentiate various *Brucella* strains. There are more than 50 *Brucella* genes with a variety of functions listed in GenBank. For example, GenBank includes genes that encode the chaperones such as dnaK, groEL, and groES. Both 16S RNA and 23S RNA DNA sequences of *Brucella* are found in GenBank. No resident plasmids have been found in *Brucella*. However, several plasmids have been shown to be able to replicate in the *Brucella* (8,9).

5. Antigenic composition

A substantial number of antigenic components of *Brucella* have been characterized. However, the lipopolysaccharide constituents of the cell wall in *Brucella* species cause the antibody to response to such species. *Brucella* devoid of the o-polysaccharide (O-PS) are termed rough or “R” because their colonial surface contrasts with the glistening, smooth aspect of those carrying S-LPS. They can naturally be members of the R *Brucella* species (*B. canis* and *B. ovis*) or mutants derived from the S *Brucella* species (*B. melitensis*, *B. abortus* and *B. suis*)(18,19,20). Cultures of S *Brucella* spontaneously dissociate to generate mixtures of S and R colonies, the latter is formed by R mutants. Owing to their lack of antigenic O-PS, true R mutants neither induce anti O-PS antibodies nor react with antibodies of this specificity. They also show other outer membrane topology and physiological changes due to lack of O-PS. Manifestations of these changes are the uptake of crystal violet, the auto agglutination in acryflavin solutions and the sensitivity to *Brucella* phages specific for the R species. Since the S → R dissociation occurs spontaneously with a frequency that depends on the strain and growth conditions, repeated *in vivo* or *in vitro* passage has been used to obtain R- mutant strain for vaccines production. The *B. abortus* 45/20 and RB51 strains were developed in this way. Alternatively, R mutants can be generated by new molecular genetics techniques such as transposon mutagenesis or deletion of genes involved in S-LPS biosynthesis. It has been known for a long time that *Brucella* R mutants are attenuated spontaneously. This attenuation has been ascribed to the increase in both the antibody independent complement activation and the sensitivity to polycationic bactericidal peptides. In addition, R mutants display altered attachment to cells. Moreover, since the S *Brucella* are intracellular parasites able to alter constitutively intracellular trafficking (i.e. the one followed by inert particles or non-virulent *Brucella*), other factors related to the interplay of R mutant is the importance of the host cell, an aspect that has not been investigated so far. The outer membrane topology of rough mutants is altered LPS acylation patterns and could be relevant in this regard (4,21,22). The structure of S-LPS content of *Brucella* is known in part. According to nuclear magnetic resonance studies, the O-PS is a homopolymer of N-formyl-perosamine either exclusively in α -(1-2) linkages (for example in *B. abortus* biotype 1) or in α -(1-2) plus α -(1-3) in a $\geq 4:1$ proportion (4:1 in *B. melitensis* biotype 1). These O-PSs carry three basic types of overlapping epitopes: C (common to all chemical types of *Brucella* O-PS), M (present in those O-PS with α (1-3) linkages) and A (present in those O-PS with no α (1-3) linkages or with a proportion of α -(1-2) to α -(1-3) linkages higher than 4:1). Nuclear magnetic resonance studies also show that the S-LPS of *Yersinia enterocolitica* O:9 carries a homopolymer of N-formyl-perosamine in α -(1-2) linkages and, accordingly, it should be identical to O-PS such as those of the *B. abortus* biotype 1. It might be, however, that some aspect of these structures has escaped the nuclear magnetic resonance analyses because, whereas some monoclonal antibodies of O-PS specificity react equally with S *Brucella* and *Y. enterocolitica* O:9 (Cyb epitopes), others recognize epitopes common to all S *Brucella* but not

to *Y. enterocolitica* O:9 (Cb epitopes), strongly suggests subtle structural differences (4,23,24,25). The structure of the LPS core in *Brucella* is largely unknown and qualitative studies show 3-deoxy-D-manno-2-octulosonic acid, mannose, glucose, glucosamine and quinovosamine as the main sugars. The synthesis of LPS in *Brucella* is largely unknown but the genetic evidence available is fully consistent with a mechanism similar to that existing in some of the best studied gram-negative bacteria. First, lipid A is synthesized on the inner face of the cytoplasmic membrane. Second, through the sequential action of glycosyltransferases, sugars are added to lipid A until the core oligosaccharide is completed. These two pathways are intermingled since two 3-deoxy-D-manno-2-octulosonate residues are added before lipid A synthesis is finished. On the contrary, the O-PS is synthesized in an independent pathway and, once its biosynthesis is carried through, it is linked to the acceptor sugar of the completed lipid A-core (19,24,25). Depending on the particular O-PS, there are three known types of mechanisms of synthesis, and that of *Brucella* belongs to the so-called ABC transporter-dependent (or *wzy*-independent) type. In this pathway, a lipid carrier (undecaprenol pyrophosphate) on the cytoplasmic side of the membrane is first primed with an amino sugar by the *WecA* protein. Then, O-PS sugar units are inserted successively at the non-reducing end (i.e. the "tip" of the growing polysaccharide) by glycosyltransferases. Finally, the ABC proteins translocate the amino sugar-O-PS (possibly still linked to the undecaprenol) to the periplasmic side of the membrane where a ligase (*WaaL*) binds the amino sugar-O-PS to the completed lipid A-core. Thus, when the synthesis of the core is blocked, the O-PS is generally not incorporated to the LPS (25). In addition to the lipid A, core, and O-PS pathways, there are subsidiary pathways that provide the necessary nucleotide-sugar precursors. Some of them are exclusive to LPS biosynthesis whereas others are housekeeping pathways. The more recent nomenclature for the genes coding for the enzymes of LPS synthesis uses four letters: (i), *lpx** for those involved in the early steps of lipid A synthesis; (ii), *wa*** for those involved in the late steps of lipid A synthesis, in core synthesis and in the ligation of the amino sugar-O-PS to the lipid A-core (*waaL*); (iii), *wb*** for those involved in the O-PS synthesis; and (iv), *wz*** for those involved in O-PS processing (for example, *wzm/wzt* are the genes coding for the ABC transporters such as those acting on *Brucella* O-PS). The genes coding for the enzymes belonging to the precursor pathways follow a conventional nomenclature (for example *man** for mannose biosynthesis, *per* for perosamine synthetase, etc.) even though they functionally belong to LPS pathways. Sometimes there are two different genes for the same enzymatic function as there can be two pathways for the same sugar when it is present in both the core or the O-PS and, in this case, sub-indexes are used (for example, *manBcore* and *manBOAg* for the phosphomannomutases of core and O-PS [O Antigen] mannose synthesis). At least sixteen genes have been proven to be involved in *Brucella* LPS synthesis by analysis of the corresponding mutants and, as in many bacteria, most of the O-PS ones are clustered in a region (*wbk*) region. Although mutations in some genes outside *wbk* also bring about an R phenotype, their assignation to the core or O-PS pathways is less clear. As judged by the analyses derived from the complete sequence of the *B. melitensis* and *B. suis* genomes, genes encoding for LPS in *Brucella* are scattered in chromosomes with the exception of the *wbk* region (26). R strains of *B. melitensis*, *B. abortus* and *B. suis* should result from mutations in some *wa*** genes (including *WaaL*), in all *wb*** genes, in the *wzm/wzt* genes, and in genes of the pathways that lead to precursors of core and O-PS sugars (for example *manBcore* and *per*, respectively). But for the absence of an O-PS linked to the remaining LPS molecule, it can be predicted that not all these mutants are equivalent and they can be hypothetically grouped

as follows: (i), R mutants have a complete lipid A-core plus a cytoplasmic O-PS, the incorporation of which to the LPS is blocked (at least the *wzm/wzt* and possibly the *WaaL* mutants); (ii), R mutants have a complete lipid A-core but no O-PS (mutants in *wb*** glycosyltransferases, in *wecA*, and in genes coding for enzymes necessary to synthesise some precursors, such as *manBOAg*, *gmd* and *per*) and (iii), R mutants have progressive deficiencies in the core and that may or may not accumulate cytoplasmic O-PS (mutants in some *wa*** genes and in some precursor genes such as *manBcore*). Mutants of each of these three groups have in fact been described, and the question then arises as to what extent they are equivalent in attenuation and immunizing abilities (27,28,29,30). Numerous outer and inner membranes, cytoplasmic, and periplasmic antigenic proteins have also been characterized. Some are recognized by the immune system during infection and are potentially useful in diagnostic tests. Hitherto, tests based on such antigens have suffered from low sensitivity as infected persons tend to develop a much less consistent response to individual protein antigens than to LPS. Thus, tests such as immuno-blotting against whole-cell extracts may have some advantages over more quantitative tests that employ purified individual antigens. Recently, ribosomal proteins have reemerged as immunologically important components. Interest in these, first arose more than 20 years ago when crude ribosomal preparations were demonstrated to stimulate both antibody and cell-mediated responses and to confer protection against challenge with *Brucella*. However, the individual components responsible for such activity were not identified until recently. It has been established that the L7/L12 ribosomal proteins are important in stimulating cell-mediated responses. They elicit delayed hypersensitivity responses as components of brucellins, and as fusion proteins, they have been shown to stimulate protective responses to *Brucella*. They appear to have potential as candidate vaccine components (31,32).

Bacterial pathogens that maintain long-term residence within host phagocytes probably express a variety of genes to help them adapt to the harsh environmental conditions of pH, nutrition deprivation, ROIs, and reactive nitrogen intermediates (RNIs) as well as lysosomal enzymes encountered within the phagosome. Prominent among these responses is the induction of heat shock proteins, suggesting that considerable protein misfolding and damage occurs within this compartment. However, the role of these proteins in *Brucella* pathogenesis was uncertain. *B. abortus* Lon transposon mutants were attenuated in BALB/c resident peritoneal macrophages but persistent in BALB/c mice except for a minor attenuation at 1 week postinfection, suggesting that Lon protease is important for *Brucella* survival during early infection. *B. suis* *dnaK* insertional mutants, defective in a member of the Hsp70 family, were attenuated in the human macrophagic cell line, U937. *B. abortus* *htrA* deletion mutants, deficient in a serine protease called high temperature-requirement A protein, have been considered to be attenuated in vitro and in vivo, but a recent report suggests that *htrA* mutants were in fact *htrA cycL* double deletion mutants. An additional report suggests that a *B. melitensis* authentic *htrA* deletion mutant was not attenuated in goats, suggesting that *HtrA* is not involved in *Brucella* pathogenesis. Also, the *B. suis* *clpA* deletion mutant was not attenuated in vitro or in vivo. Taken together, these reports suggest that not all heat shock proteins are critical to *Brucella* pathogenesis or that redundant function of heat shock proteins will compromise the functional deficiency caused by the loss of one heat shock protein (33,34,35,36).

6. Virulence

The basis for the virulence of *Brucella* can be attributed to the ability of these bacteria to escape the host defense mechanisms and to survive and replicate within the host cells.

Attempts to identify *Brucella* virulence factors have been made. The first studies reported that intracellular multiplication of *Brucella* was attributable to erythritol. Virulent *Brucellae* are capable of invading and residing in professional phagocytes, such as macrophages, as well as non-phagocytic cells. The mechanism of attachment and entry into these cells by *Brucella* has yet to be clearly elucidated. Virulence mechanisms identified so far is associated with the ability to reside within phagocytic and/or non-phagocytic cells are as follows: the ability to inhibit phagolysosome fusion, degranulation and activation of the myeloperoxidase-halide system, and the production of tumor necrosis factor (37,38).

In both phagocytic and non-phagocytic cells, *Brucella* has the ability to replicate within membrane-bound compartments. In non-phagocytic cells, such as *HeLa* cells, virulent *B. abortus* 2308 has been documented to replicate in the endoplasmic reticulum by utilizing the autophagic machinery of the *HeLa* cell. In professional phagocytes, the membrane-bound compartment within which virulent *Brucella* organisms can replicate is the phagosome. By some unknown mechanism, *Brucella* is able to block phagolysosome fusion. It is now thought that the production of adenine and guanine monophosphate can inhibit phagolysosome fusion. The ability to produce these compounds is therefore considered as virulence factor of *Brucella*. In contrast, attenuated strains of *Brucella* are unable to prevent such fusion and are thereby destroyed by the lysosomal contents. Research on intracellular survival and replication of *Brucella* within professional phagocytes has mainly focused on macrophages (39,40). Survival within macrophages is apparently associated with the production of many different proteins. These proteins tend to be stress induced proteins such as heat shock or acid-induced proteins. They include 17, 24, 28, 60, and 62 KD proteins. Two of these proteins, the 17 and 28 KD proteins, seem to be induced only during intracellular cohabitation of *Brucella* with macrophages (41,42). *HtrA*, another stress-induced protein, has been examined for its possible role in virulence and intracellular survival. Using deletion mutants, *HtrA* has been shown to be involved in inducing a granulomatous reaction and thus reduces the levels of infection during the early phase of infection (murine model). However, this does not result in reduced levels in the later phases of infection. In fact, overall, *htrA*-deficient mutants produce splenic bacterial loads comparable to their 11 wild-type counterparts. *RecA* mutants produce similar results as *htrA* mutants in early- and late-phase splenic load (43). Two other types of proteins that have been put forth as possible virulence factors are siderophores and Cu-Zn superoxide dismutase (Cu-Zn SOD). Iron-sequestration by siderophores may be an integral virulence factor in intracellular survival of *Brucella* species. Low levels of iron *in vivo* aid the host's ability to restrict microbial growth. *Brucella* species do carry iron-sequestering proteins and other siderophores, but their role in pathogenesis has not been clearly elucidated. Cu-Zn SOD may play a significant role in the early phase of intracellular infection, but contradictory results have been reported. Further studies are needed before the role of Cu-Zn SOD as a virulence factor of intracellular survival of *Brucella* can be accurately assessed (33,44). An auxotrophic mutation encoding for an essential enzyme (5'-phosphoribosyl-5-amino-4-imidazole carboxylase) necessary for the *de novo* synthesis of purines has been demonstrated to be essential for the intracellular

survival of *B. melitensis*. Deletion of the gene, *purE*, encoding this enzyme in virulent *B. melitensis* drastically reduced its ability to survive within macrophages and demonstrated attenuated behavior in mice and goats. Recently, a two-component regulatory system has been discovered in *B. abortus*. The *Brucella* virulence related proteins (*Bvr*) system consists of a regulatory (*BvrR*) and a sensory protein (*BvrS*). This regulatory system, *BvrR-BvrS*, may play a critical role in the ability of *B. abortus* to invade and multiply within cells. *BvrR*-deficient mutants were obtained by transposon mutagenesis. Morphologically, these mutants produced smooth-type LPS (45). They were found to be increasingly sensitive to polycations surfactants and showed decreased *in vivo* replication and persistence in mouse spleens. This occurred even though no obvious 12 growth defects could be detected in the mutants *in vivo*. Complementation with the *bvrR* gene restores resistance to polycations and partially restored the ability of these mutants to multiply intracellularly. The results further suggest that restoration of full virulence requires both components of the regulatory system to be intact. Interestingly, LPS core and lipid A are known to be involved in polycationic resistance. Therefore, it is possible that these LPS features involved in polycationic resistance are under the *BvrR-BvrS* regulatory system. Analysis at the DNA level of *bvrR* and *bvrS* genes revealed that they are highly homologous to other regulatory systems being found within symbiotic plant pathogens such as *Rhizobium meliloti* (*ChvI-ExoS* system) and *Agrobacterium tumefaciens* (*ChvI-ChvG* system). It has been established that *B. abortus*, *R. meliloti*, and *A. tumefaciens* are phylogenetically related. Therefore, this suggests that the *BvrR-BvrS* system co-evolved with the other two systems listed above to aid in the ability of *Brucella* to survive intracellularly (22,46). Recently in *B. suis*, genes encoding a type IV secretion system homologous to the *Agrobacterium tumefaciens* *VirB* and *Bordatella pertussis* *Pt1* systems have been identified to be essential for the intracellular survival in *HeLa* cells and human macrophages. Further research is needed to clearly understand the actual role of this secretion system in the virulence of *Brucella* species (47). At present, there is no evidence to support a secretion system within *Brucella*. If *Brucella* is capable of secreting, it is probably in very small amounts. Non-protein components of *Brucella* may also contribute to its ability to survive within cells. One such cellular component, lipopolysaccharide (LPS) will be discussed in the section below (46,47).

The LPS of smooth strains of *Brucella* are comprised of a lipid A molecule, fatty acids, a core region, and a polysaccharide O-side chain. This O-side chain is made from a homopolymer of perosamine and is found on the surface of smooth strains, while rough organisms lack this chain on their LPS. Smooth *Brucellae* are able to survive intracellularly as compared to their rough counterparts. Therefore, smooth lipopolysaccharide (S-LPS) probably plays a significant role in pathogenesis. The simple explanation of rough versus smooth morphology and virulence, however, does not explain how naturally occurring rough species *B. ovis* and *B. canis* retain their virulence. Using *Tn5* transposon mutagenesis, several genes necessary for the synthesis of S-LPS have recently been identified. Both *in vitro* and *in vivo* studies with the rough mutants derived from the deletion of these genes clearly established that S-LPS is necessary for efficient intracellular survival and virulence of *B. melitensis*, *B. abortus*, and *B. suis*. *B. abortus* S-LPS is 100 times less potent than that of *E. coli* and *Salmonella* in inducing TNF from macrophages as well as oxidative metabolism and lysozyme release by human neutrophils. This feature of S-LPS has been proposed to contribute to the survival of *B. abortus* within phagocytic cells. In addition, *Brucella* S-LPS is

not susceptible to the actions of polycationic molecules, suggesting that smooth *Brucella* can resist the cationic bactericidal peptides of the phagocytes. S-LPS has also been found to confer anti-phagocytic properties to *Brucella* and does not activate the alternate pathway of the complement cascade (37,48,49).

7. *Brucella* vaccines

7.1 Live, attenuated vaccines

Both killed and live, attenuated vaccines have been examined for their potential role in the control and eradication of brucellosis in cattle, goats, and swine. Live, attenuated vaccines carry several advantages over their killed counterparts. First, immunity derived from their use tends to be cell-mediated and long lasting. Also, as they are administered live, the organism is allowed to replicate within the host, thus making them less expensive. However, some live, attenuated vaccines may cause abortion in pregnant females and therefore their use is often relegated to males and non-gravid females.

The two main live, attenuated vaccines used in the control of *B. abortus* infection in cattle are *B. abortus* strain 19 and *B. abortus* strain RB51. A brief discussion of each, plus the use of *B. melitensis* strain Rev. 1 in goats, follows (48,50).

7.2 Killed vaccines

Killed vaccines can offer protection to a disease while still retaining safety for those animals that are young, immuno-suppressed, or pregnant. Over the years, a variety of killed vaccines have been developed for protection against brucellosis. They have had limited success. None have approached the protection status afforded by the live, attenuated vaccines. Examples of vaccines in this category are *B. abortus* strain 45/20 and *B. melitensis* H38. In addition to the lack of sufficient protection in the face of challenge, killed vaccines such as 45/20 and H38 can induce persistent antibody titers that can interfere with common serological tests used (51,52,53).

7.3 *Brucella abortus* vaccines

7.3.1 *B. abortus* strain 19

Brucella abortus strain 19 (S19) is a smooth but attenuated strain. The molecular basis for the attenuation is not known. The strain S19 has been shown to contain a deletion in the erythritol catabolic genes rendering it sensitive to erythritol. However, such a deletion in virulent strains has been shown not to result in attenuation. Prior to the introduction of vaccine strain RB51 in 1996, *B. abortus* S19 was known to be the official vaccine strain. The strain S19 was quite effective in protecting cattle against subsequent infection with virulent strains of *B. abortus* (54). However, S19 did have several problems that restricted its use within the cattle. During protection studies, it was discovered that S19, when given to adult cattle (>1yr), often caused persistent titers which could not be distinguished from titers resulting from a natural infection using standard serological tests. The tests like a plate agglutination test, complement fixation (CFT), and tube agglutination tests can only detect the presence of antibodies against O-antigens. This directly undermined the brucellosis eradication program that was dependent on a test and slaughter strategy to reduce numbers

of infected cattle within the United States. Persistent antibodies could be detected for up to 10-11 months post vaccination when vaccinating adult cattle with the standard dose (3×10^{10} CFU). Although a rare finding, even some calves vaccinated S19 produced persistent antibodies (55,56). Use of S19 in pregnant cattle also resulted in abortions. Even when a reduced dose of S19 (1/20-1/100 of the standard dose) was used to vaccinate pregnant cattle, abortions in post-inoculation were still observed, although this reduced dose appeared to be less abortigenic (57,58). The use of the reduced dose vaccine did not eliminate the problem of persistent titers (51,59,60). In fact, these titers lasted about the same amount of time as the full dose (60). For this reason, Erasmus and Erasmus recommended that vaccination of adults with the reduced dose of S19 be relegated to herds heavily infected with *B. abortus*. As a result of the overwhelming experimental evidence, S19 was designated for use as a calf hood vaccine (4 to 12 month of age) (60). Calf hood vaccination with S19 is not completely without side effects. As with all other brucellosis vaccines, S19 cannot be administered to bulls or bull calves due to the resulting persistent orchitis (61). There have also been reports of an arthropathy (gonitis) linked to vaccination of female calves with S19. Immunological studies by Wyn-Jones and colleagues indicated the presence of *B. abortus* strain S19 antigenic material within the cells of the stifle, synovial membrane and the drainage lymph nodes (54). With the discovery of *B. abortus* strain RB51, the benefits of S19 vaccination diminished and RB51 replaced S19 as the official vaccine for the brucellosis eradication program. The use of S19 has also raised concerns about human exposure to brucellosis vaccines.

There have been several reports of illness following accidental self-inoculation with the S19 vaccine (54,62). This stresses the importance of safe-handling practices when vaccinating herds for brucellosis using the S19 vaccine. It also led investigators to try and develop a more efficacious cattle vaccine that would also be safer in terms of potential human exposure (62,63,64).

7.3.2 *B. abortus* strain RB51

The use of *B. abortus* strain RB51 was approved to be the official strain employed to manufacture calf hood vaccine for protection against brucellosis in 1996. Vaccine strain RB51 is a stable, rifampin-resistant, and derived from rough mutant of *B. abortus* 2308. It was derived by serial passage of parental strain 2308 on trypticase soy agar supplemented with varying concentrations of rifampin and penicillin. Colonies of RB51 are rough in morphology as indicated by their ability to absorb crystal violet as well as auto-agglutinate when in suspension. The LPS of RB51 is deficient in O-side chain, unlike its parental strain 2308. Metabolically, RB51 shares the ability to use erythritol with strain 2308 and RB51 has proven to be an extremely stable rough mutant of *B. abortus*. Its stability and efficacy have been shown in vitro and in vivo (65,66). Like the strain S19 vaccine, calves must be vaccinated with strain RB51 between the ages of 4-12 months of age with the calf dose ($1.0-3.4 \times 10^{10}$ CFU) and in high risk area animals receive the vaccine after 12 month of age (67). Advantages of RB51 over other vaccines for protection against bovine brucellosis are numerous. It does not produce any clinical signs post-vaccination, nor does it produce a local reaction at the site of injection (65). It is rapidly cleared from the bloodstream, as early as 2 weeks after post-inoculation. It is not shed in the nasal secretions, saliva, or urine. Therefore, the organism appears to be unable to spread from vaccinated to non-vaccinated animals through these routes. In immuno-suppressed animals, no recrudescence of infection has been documented. In addition, vaccination with RB51 affords a high level of protection,

characterized by good cell mediated immunity. In one study, vaccination of cattle at least one year prior to mating induced 100% protection against abortion caused by exposure to field conditions of high and low brucellosis levels. The use of RB51 has also helped clear up the issue of *Brucella*-positive/"reactor" animals. Since RB51 lacks O-side chain, vaccination with the strain (unlike strain 19) produces no antibodies to O-side chain. This is particularly advantageous because all of the diagnostic tests used to screen for brucellosis in herds are directed toward the detection of O-antibodies in the serum or milk. Cattle vaccinated with RB51 are negative on all subsequent serological tests, including agar gel diffusion test. This lack of interfering antibodies is even true in the face of calf hood vaccination with strain 19 and subsequent adult vaccination with RB51 (68).

Although, sera from RB51 vaccinated cattle do not respond to standard diagnostic tests, they do contain antibodies that react to a dot-blot ELISA containing RB51 antigen (65). As these antigens are common to both RB51 and 2308, the dot-blot ELISA test cannot differentiate between vaccinated and infected animals; it is, therefore, relegated to assessing the humoral, non-protective immune response of cattle post-inoculation (69). In addition, there are two molecular methodologies that may be used to differentiate RB51 from other isolates: pulse field gel electrophoresis (PFGE) and polymerase chain reaction (PCR). RB51 possesses a unique fingerprint using the pulsed-field gel electrophoresis patterns of genomic DNA digested with restrictive endonuclease *Xba* I. The fingerprint of RB51 contains a unique band at 104 kb, as opposed to a 109-kb fragment within genomic DNA samples of *B. abortus* isolates from naturally infected cattle, bison, and elk (70). In addition, there is a specific PCR test that can differentiate RB51 isolates from all other *Brucella* isolates tested. This PCR method is based on the interruption of the *whoA* gene by an insertion element (IS711), a unique mutation present only in RB51 (71). In a murine model, *B. abortus* strain RB51 has been shown to confer protection against challenge with *B. melitensis*, *B. suis*, and *B. ovis*. However, in rams this vaccine did not induce protection against *B. ovis* challenge. Field trials indicate that *B. abortus* strain RB51 is also protective against swine brucellosis.

In addition to domesticated species, *B. abortus* strain RB51 has also been used to vaccinate wild animals such as bison and elk. Oral vaccination of mice and cattle with RB51 has been shown to be effective in inducing protective immune responses. These results are encouraging and highlight the feasibility of oral vaccination of wild life on a large scale. RB51 may appear to be a safe vaccine with respect to human exposure (72,73).

7.4 *Brucella melitensis* vaccines

7.4.1 *B. melitensis* Rev. 1.

B. melitensis Rev. 1 (Rev1) is currently the only approved vaccine available for protection against *B. melitensis* infection. In 1957, a smooth attenuated strain of *B. melitensis* was isolated from a streptomycin-dependent population that had been grown in a streptomycin deficient medium. In experimental challenge trials in goats, this strain was found to induce significant protection against the virulent challenge strain without shedding the organism. The organism was later designated *B. melitensis* Rev. 1. Use of the Rev1 vaccine has both advantages and disadvantages. Vaccination with Rev1 induces significant protection in sheep and goats. Rev1 has been found to be much more protective in goats and sheep challenged with virulent *B. melitensis* than those animals vaccinated with S19. The Rev1

vaccine does have some disadvantages. It can cause abortions if used in pregnant animals. Vaccination with Rev1 can result in persisting agglutinins that can interfere with various serological diagnostic tests. Rev1 is pathogenic to humans via aerosol exposure or self-inoculation causing generalized brucellosis in affected individuals. Like all other *Brucella* vaccines, Rev1 can cause local hypersensitivity reactions in cases of accidental inoculation (12,74,75,76,77,78).

8. *Brucella* R mutants for vaccine studies

8.1 *B. abortus* 45/20

This R vaccine was obtained after twenty passages in guinea pigs of a field isolate (*B. abortus* strain 45) in 1938. However, the original 45/20 strain was reported to revert to S pathogenic forms when injected into cattle. Alton reports of experiments with several 45/20 stocks which, after repeated passages in guinea pigs, showed either S-intermediate and R colonies or only R forms depending on the origin of the stocks. This suggests that the original strain contained in fact several different clones so that the S-intermediate ones were selected when injected into cattle. Also, it seems likely that different laboratory variants of this strain have coexisted for years. The genetic defects in this strain are unknown and the vaccine is not presently marketed (65,79).

8.2 *B. abortus* RB51

Strain RB51 is a spontaneous R mutant selected after repeated in vitro passages of *B. abortus* 2308 on media containing rifampin and penicillin. Selection was performed using crystal violet and acryflavin tests. RB51 carries an IS711 spontaneously inserted into *wboA* (putatively coding for a glycosyltransferase). However, a *wboA* transposon mutant obtained from strain 2308 is not as attenuated as RB51 and the protection afforded by *wboA* mutant vaccines in mice is better than that provided by RB51, which shows that RB51 carries additional and unknown defects. In the complete sequence of *B. melitensis*, *B. suis* and *B. abortus* genomes (an annotated *B. abortus* complete sequence is not available) *wboA* maps outside of the main *wbk** O-PS genetic region. RB51 accumulates small amounts of O-PS. This is noteworthy because, accepting that the current model of the *B. abortus* LPS structure is correct, mutation in a *wb*** gene should prevent O-PS synthesis. Complementation of RB51 with *wboA* increases O-chain expression but does not restore the S phenotype suggesting that other LPS genes are affected. In addition, although sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) migration patterns have been interpreted to mean that RB51 carries a LPS with a complete core, chemical analyses showed that this R-LPS contains 2.5 times less mannose than the *B. abortus* RA1 *wboA* mutant. The presence of additional mutations in genes of strain RB51 are not involved in LPS synthesis and cannot be excluded either (80,81). On the contrary to 45/20, RB51 is stable and it is currently being used in some countries instead of S19. Although it should show very low or no virulence in humans, there is little information on this point and there has been at least one case of RB51 infection in a veterinarian demonstrated by bacteriological isolation and typing of the strain. It has to be stressed that RB51 is resistant to rifampin, an antibiotic currently used in the groups of brucellosis patients that cannot be treated with streptomycin (pregnant women, children of young age, and endocarditis and neurobrucellosis cases) (34).

RB51 has been used as the starting strain in two genetic manipulations. First, the *wboA* defect has been complemented with a functional *wboA* gene to generate strain *RB51WboA*. This strain keeps the R phenotype manifested in the crystal violet and acryflavin tests, but expresses increased amounts of O-PS which by immuno-electron microscopy seems to be accumulated in the cytoplasm. However, at least part of this O-PS may be linked to a lipid to give an immunogenic form because it migrates in SDS-PAGE and vaccination of mice with *RB51WboA* elicits IgG antibodies of at least C specificity. Second, the *Brucella* Cu/Zn superoxide dismutase gene has been introduced in RB51 to obtain strain *RB51SOD* which over expresses (tenfold) this protein. The aim of this manipulation is to increase the expression of a *Brucella* antigen and a possible virulence factor on the RB51 background (33,82,83).

8.3 *wboA* mutants other than RB51

Mutants in this putative glycosyltransferase gene have been obtained from *B. melitensis* 16M and *B. suis* 2579 by allelic exchange to generate the strains VTRM1 and VTRS1, respectively. Both are kanamycin resistant since they carry a *Tn5* element. Although, it was originally named *rfbU*, blast analysis of the *Salmonella typhi* RfbU prototype against the *B. melitensis* genome shows the highest similarity (E value $1e-16$) with *WbkA*, and more recently the gene has been renamed *wboA* (E value for *RfbU* versus *WboA* is only $2e-04$). The VTRM1 and VTRS1 mutants are stable in mice, lack reactivity with monoclonal antibodies of C specificity and have an R phenotype but they have not been tested for the absence of core defects or expression of cytoplasmic O-PS (84,85,86).

8.4 Mutants in the *wbk* region

Several mutants in this cluster of O polysaccharide genes have been describe, and two have been analyzed as vaccines. *B. abortus* 2.17 and *B. abortus* 9.49 have been obtained from *B. abortus* 2308 by transposon mutagenesis and selection for polymyxin B sensitivity, and they carry the *Tn5* insert (they are kanamycin resistant) in *wbkA* and *per*, respectively. Both are resistant to the S *Brucella* specific phages, sensitive to the R *Brucella* specific R/C phage and positive in the crystal violet and acryflavin tests and do not express O-PS. As judged by the electrophoretic mobility and the reactivity with monoclonal antibodies specific for the inner and outer core epitopes, the R-LPS of both mutants keeps an intact core oligosaccharide, which is consistent with the position of *wbkA* in the major O-PS genetic region and the putative role of *Per*. 4.6. Mutants in genes affecting the LPS core structure (27,28,29).

8.5 *B. abortus* B2211 *pgm*

This mutant carries a gentamicin-resistance non polar cassette in the *pgm* (phosphoglucomutase) gene of *B. abortus* 2308. It is resistant to the S-*Brucella* specific Tb phage and carries R-LPS as judged by SDS-PAGE analysis. The central role of this enzyme in the synthesis of hexoses derived from glucose makes pleiotropic effects on the synthesis of oligo- and polysaccharides likely and, at least, the *pgm* mutant is also blocked in the synthesis of the periplasmic $\beta(1,2)$ cyclic glucans. Mutation in the homologous gene of *B. melitensis* causes a core defect as judged by the electrophoretic mobility in SDS-PAGE of its R-LPS (30,87,88).

8.6 *B. abortus* mutant 80.16 wa**

This is a mutant in a putative glycosyltransferase gene involved in core synthesis (hence its provisional denomination as wa**) as shown by SDS-PAGE and Western blots with monoclonal antibodies to core epitopes. Like *B. abortus* 9.49, it was obtained by Tn5 transposon mutagenesis of *B. abortus* 2308 and selection for polymyxin B sensitivity. This mutant is resistant to kanamycin, to the *S. brucella* phages, sensitive to phage R/C and does not express O-PS. A remarkable feature of the R-LPS of this mutant is that while keeping a fully reactive outer core epitope, it shows a reduced reactivity with monoclonal antibodies to the inner core epitope suggestive of a branch in the inner core in which the missing sugar(s) would be placed (29).

8.7 *B. abortus* manBcore mutants

Two different mutants in this gene, both from *B. abortus* 2308, have been described: the *rfbK* mutant and mutant 55.30.

The mutated gene (formerly *rfbK* but *manB* according to recent nomenclature) putatively codes for a phosphomannomutase and is thus predicted to be involved in the synthesis of mannose-1-P. Although mannose-1-P is a precursor of perosamine (the O-PS sugar), the mutated gene is not homologous to the *manB* of the *wbk* region (manBOAg) and its location, the lack of reactivity with monoclonal antibodies of outer core specificity and the SDS-PAGE profile of the corresponding R-LPS show that it acts as a manBcore. This is consistent with the presence of mannose in the core of *B. abortus* (12,29,89).

9. Can proteomics help in developing vaccines to protect animals or humans against brucellosis?

The identification of immunogenic proteins will also be a further step towards the understanding of the humoral immune response during *Brucella* infections. Most studies on the antigenicity of *Brucella* proteins are either hampered by the limited number of proteins investigated or the complexity of the protein mixtures used. Differences in the seroreactivity of various protein classes are well known, e.g. cytoplasmic proteins induce a higher antibody response than outer membrane proteins. Additionally, the production of antibodies directed against proteins may be host specific, e.g. anti-OMP28 antibodies were detected in *Brucella* infected humans and goats, but not in pigs and cattle (90).

Immunoproteomics is the approach to identify specific immunogenic proteins in high resolution in the wide range of proteins expressed by *Brucella*. Previous studies of the *Brucella* proteome mainly focused on *B. melitensis* and the protein map of *B. melitensis* 16M (<http://www.proteome.scranton.edu>) may be used as a reference map for other *Brucella* spp.. However, crucial phenotypic differences responsible for host specificity, virulence, and immunogenicity may exist despite the close genetic relatedness within the genus *Brucella*. Five hundred fifty-seven protein spots representing 232 discrete ORFs were identified in *B. melitensis* using 2-D and MALDI-MS. Protein expression profiles of *B. melitensis* under various growth conditions, in wild type and attenuated vaccine strains have also been investigated. *B. abortus* proteomic studies have primarily been directed at the identification of virulence factors (Sowa et al., 1992; Lin and Ficht, 1995; Rafie-Kolpin et al., 1996). However, only one immunoproteomic study of *Brucella* has been published so far. In this, Teixeira- Gomes et al. (1997a) identified immunogenic proteins of *B. ovis* (91,92).

A lot of experiences have been involved in a comprehensive analysis of the *B. melitensis* 16M proteome, and initial results have been published recently. Previous proteomics studies using *B. melitensis* cells grown under different conditions have been reported, and initial work on the *B. abortus* proteome has been described. A comparative study was conducted with *B. abortus* vaccine strains S19 and RB51 and virulent strain 2308. recently, Eschenbrenner *et al.* compared the proteome of laboratory-grown strain Rev 1 to that of strain 16M by using two-dimensional (2-D) gel electrophoresis and matrix-assisted laser desorption/ionization (MALDI)-mass spectrometry (MS) to elucidate differences between the protein expression patterns of the two strains. Differentially expressed proteins were identified and grouped into three major classes: (i) protein spots unique to either 16M or Rev 1, (ii) proteins overexpressed in Rev 1, and (iii) proteins underexpressed in Rev 1 (93,94).

Comparative proteome analysis of vaccine strain Rev 1 and virulent strain 16M of *B. melitensis* indicates that the two strains have significant metabolic differences. Differentially expressed proteins involved in iron metabolism, sugar transport, lipid metabolism, and protein synthesis were identified. The expression of proteins essential for both low and high iron availability suggests a misregulated system for iron metabolism and capture, leading to possible unnecessary expenditure of energy. This may be a consequence of successive in vitro passages of *B. melitensis* in the presence of streptomycin. It is difficult to state what changes were directly or indirectly effected by this stressful growth condition. However, one plausible theory is that to compensate for these changes in gene expression, Rev 1 may have up-regulated other pathways, such as those involved in the α -oxidation of fatty acids and protein synthesis, to generate more reducing equivalents, ultimately for use in the production of ATP. These alterations would compensate for the energy loss due to misregulation of iron metabolism (72,95).

Briefly, The proteomes of selected *Brucella* spp. have been extensively analyzed by utilizing current proteomic technology involving 2-DE and MALDI-MS. In *B. melitensis*, more than 500 proteins were identified. The rapid and large-scale identification of proteins in this organism was accomplished by using the annotated *B. melitensis* genome which is now available in the GenBank. Coupled with new and powerful tools for data analysis, differentially expressed proteins were identified and categorized into several classes. A global overview of protein expression patterns emerged, thereby facilitating the simultaneous analysis of different metabolic pathways in *B. melitensis*. Such a global characterization would not have been possible by using time consuming and traditional biochemical approaches. The era of post-genomic technology offers new and exciting opportunities to understand the complete biology of different *Brucella* species (93,95,96).

Comprehensive proteome maps of all six *Brucella* species will be generated in order to obtain vital information for vaccine development, identification of pathogenicity islands, and establishment of host specificity and evolutionary relatedness.

10. *Brucella* subunit vaccine

Different studies have evaluated surface structures and antigens of *Brucella* as immunopotent components to design an efficient brucellosis subunit vaccine.

Currently subunit vaccines are being considered to develop effective vaccines for human which has been evidenced by vaccines currently available against the infections such as

meningococcal diseases and influenza. In parallel, subunit vaccines are hot topics in the development and design of human brucellosis vaccine. Jacques et al., showed the efficacy of *Brucella* O-polysaccharide-BSA conjugate in protection against *Brucella melitensis* H38 (97). Other studies have been carried out to design subunit vaccines using other components and conjugated compounds such as porins and smooth lipopolysaccharide, recombinant ribosomal proteins and anti-OPS specific monoclonal antibodies (98, 99, 100, 101). *Brucella* antigens have been applied along with different adjuvants to augment immune responses against this organism.

The latest studies in the field of brucellosis subunit vaccines have been carried out by Bhattacharjee et al (102) and Sharifat et al. (103) have evaluated the (Group B Outer Membrane Proteins) GBOMP – *B. melitensis* strain 16M LPS non-covalent complex to elicit the immunity against brucellosis in mice. In order to explore the efficacy of *Brucella abortus* LPS combined with different adjuvants and proteins (as a vaccine candidate) in the induction of response as an effective and long-lasting immunity against *Brucella*, Sharifat et al., evaluated and reported the outer membrane vesicles of *Neisseria meningitidis* serogroup B (GBMOMV) as a potent subcutaneous adjuvant and a part of a brucellosis candidate vaccine to induce high titres of specific anti-*Brucella abortus* S99 LPS in animal model.

The other candidate antigens are *Brucella* proteins with different cellular locations (Table 1). Four proteins are outer membrane proteins. The other nine proteins are located in cytoplasm (5 proteins), periplasm (4 proteins), and cytoplasmic membrane (1 protein) (104).

Symbol	Protein Description	Location
BLS	Brucella lumazine synthase	Cytoplasm
L7/L12	Ribosomal protein L7/L12	Cytoplasm
P39	sugar-binding 39-kDa protein	Periplasm
Bfr	Ferritin:Bacterioferritin	Cytoplasm
Bp26	Periplasmic immunogenic protein	Periplasm
DnaK	Molecular chaperone DnaK	Cytoplasm
IalB	Invasion protein B	Cytoplasmic membrane
Omp16	Outer membrane protein MotY	Outer membrane
Omp19	Lipoprotein Omp19	Outer membrane
Omp25	25 kDa outer-membrane immunogenic protein precursor	Outer membrane
Omp31	OmpA-like transmembrane domain	Outer membrane
SodC	Cu/ Zn superoxide dismutase	Periplasm
SurA	Peptidyl-prolyl cis-trans isomerase	Periplasm
Tig	Trigger factor	Cytoplasm

Table 1. Brucella proteins studied as sub-unit vaccines

11. Bioinformatic application and reverse vaccinology

Reverse vaccinology is an emerging vaccine development approach that starts with the prediction of vaccine targets using bioinformatics screening of an entire genome of a pathogenic organism (105). Vaxign is the first web-based vaccine design program that predicts vaccine targets based on reverse vaccinology. The Vaxign computational pipeline includes the following features: subcellular localization, topology (transmembrane helices

and beta barrel structure), adhesin probability, similarity to other pathogen sequences, similarity to host genome sequences (e.g., human or mouse), and MHC class I and II epitope predictions. Vaxign has been used to predict *Brucella* outer membrane proteins (OMP) as potential vaccine targets using *B. abortus* strain 2308 genome as the seed genome (106). Vaxign has identified 46 *Brucella* periplasmic proteins that are conserved in all *B. abortus*, *B. melitensis*, and *B. suis* genomes and lack sequence similarity with proteins in human or mouse genomes. The values of these proteins for vaccine development also deserve further analysis.

Using the same criteria (sequence conservation and dissimilarity from human or mouse proteins), Vaxign has detected approximately 1,000 cytoplasmic proteins. It is impractical to individually test this high number of proteins for vaccine development. Considering only five cytoplasmic proteins have been experimentally confirmed to be protective antigens out of 1,000 conserved cytoplasmic proteins, it is much less likely that cytoplasmic proteins serve as protective antigens compared to outer membrane and periplasmic proteins. Vaxign also contains an epitope prediction component that can predict MHC class I and II binding epitopes (107). The addition of epitope prediction allows further analysis for the existence of potential *Brucella* vaccine targets.

12. *Brucella* DNA vaccines

DNA vaccination is a novel and powerful method of immunization that induces both humoral and cellular (Th1 and CTL) immune responses and protection against a variety of pathogens (104). Based on the results obtained with DNA vaccines against other pathogenic intracellular bacteria, many studies of brucellosis have been conducted (108, 109, 110, 111, 112, 113, 114). These vaccines induced strong Th1 responses, and some of them conferred protection against challenge with *B. abortus* (108, 110, 112, 113, 114).

Immunization of BALB/c mice with *B. melitensis* Omp31 gene cloned in the pCI plasmid (pCIOmp31) conferred protection against *B. ovis* and *B. melitensis* infection. Mice vaccinated with pCIOmp31 developed a very weak humoral response, and in vitro stimulation of their splenocytes with recombinant Omp31 did not induced the secretion of gamma interferon. Splenocytes from Omp31-vaccinated animals induced a specific cytotoxic-T-lymphocyte activity, which leads to the in vitro lysis of *Brucella*-infected macrophages. pCIOmp31 immunization elicited mainly CD8₊ T cells, which mediate cytotoxicity via perforins, but also CD4₊ T cells, which mediate lysis via the Fas-FasL pathway. In vivo depletion of T-cell subsets showed that the pCIOmp31-induced protection against *Brucella* infection is mediated predominantly by CD8₊ T cells, although CD4₊ T cells also contribute. Our results demonstrate that the Omp31 DNA vaccine induces cytotoxic responses that have the potential to contribute to protection against *Brucella* infection. The protective response could be related to the induction of CD8₊ T cells that eliminate *Brucella*-infected cells via the perforin pathway (115).

Kurar and Splitter (110) showed that DNA vaccination with the *B. abortus* ribosomal L7/L12 gene elicits humoral and cellular immune responses and partial protection. Thus, plasmid DNA vaccination may be a successful alternative method for conferring protection against *Brucella*. In addition, a genetic vaccine, by inducing an immune response to a single protein, would make possible the development of diagnostic tests that could differentiate vaccinated animals from infected animal.

Velikovsky *et al.*, (113) showed that injection of plasmid DNA carrying the *Brucella abortus* lumazine synthase (BLS) gene (pcDNA-BLS) into BALB/c mice elicits both humoral and cellular immune responses. Antibodies to the encoded BLS included immunoglobulin G1 (IgG1) IgG2a, IgG2b, IgG3, and IgM isotypes. Animals injected with pcDNA-BLS exhibited a dominance of IgG2a over IgG1. pcDNA-BLS is a good immunogen for the production of humoral and cell-mediated responses in mice and is a candidate for use in future studies of vaccination against brucellosis.

Gonzalez-Smith et al (116) showed that Injection of mice with a plasmid DNA carrying the gene for superoxide dismutase (pSecTag-SOD) leads to the development of significant protection against *B. abortus* challenge. They also evaluated the effect of delivering IL-2 on the efficacy of SOD DNA vaccine by generating a plasmid (pSecTag-SOD-IL2) that codes for a secretory fusion protein of SOD and IL-2. Although mice immunized with pSecTag-SOD-IL2 showed increased resistance to challenge with *B. abortus* virulent strain 2308, this increase was not statistically significant from that of pSecTag-SOD vaccinated mice. These results suggest that a SOD DNA vaccine fused to IL2 did not improve protection efficacy (116).

DNA vaccination approaches offer the possibility of inducing both cellular and humoral responses. Approaches have varied from use of a whole library from *B. abortus* (117), overcoming the need for prior knowledge and selection of specific antigens to selection of specific candidates and their subsequent evaluation as DNA vaccines against brucellosis. Various candidates have been explored for their value as DNA vaccines against brucellosis providing various levels of protective efficacy in the mouse model (108, 111, 114, 113, 112). Disadvantages of the DNA vaccination approach are the amount of DNA required to elicit the required response, and the often disappointing results obtained following assessment of the vaccines in the target animal (118). Investigation of enhanced delivery mechanisms may overcome these issues.

The availability of the genome sequences and the application of postgenomic approaches to identify potential vaccine candidate antigens, together with the improving knowledge of the protective immune response would provide an efficient nonliving vaccine.

13. Conclusion

Brucellosis is a disease which causes economic disadvantages in developed as well as developing/ underdeveloped countries. However to overcome such economic disaster, it is essential by employing various techniques ranging from conventional techniques to advanced ones such as genetic , proteomics, metabolic engineering. However by employing such techniques it will be possible to develop a vaccine against Brucellosis either for animals or humans.

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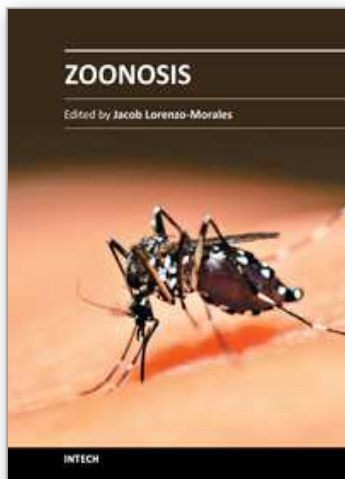
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Zoonotic diseases are mainly caused by bacterial, viral or parasitic agents although "unconventional agents" such as prions could also be involved in causing zoonotic diseases. Many of the zoonotic diseases are a public health concern but also affect the production of food of animal origin thus they could cause problems in international trade of animal-origin goods. A major factor contributing to the emergence of new zoonotic pathogens in human populations is increased contact between humans and animals. This book provides an insight on zoonosis and both authors and the editor hope that the work compiled in it would help to raise awareness and interest in this field. It should also help researchers, clinicians and other readers in their research and clinical usage.

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