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The Development of New/Improved Brucellosis Vaccines: Report of WHO Meeting

with the participation of the Food and Agriculture Organization of the United Nations (FAO) and the Office International des Epizooties (OIE)

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Brucellosis is a major zoonotic disease, widely distributed in both humans and animals, especially in the developing world. The occurrence of the disease in humans is largely dependent on the animal reservoir and high rates of brucellosis infection in sheep and goats usually cause the greatest incidence of infection in humans.

The World Health Organization (WHO) has long been involved in brucellosis surveillance and control including research and development of vaccines to prevent animal brucellosis. Between 1987 and 1992, a number of meetings were held where the safety, antigenicity and protective capabilities of the *Brucella suis* strain 2 (S2) vaccine as a candidate vaccine for oral vaccination of small ruminants were evaluated and compared with the *Brucella melitensis* Rev. 1 vaccine.

More recently, a new vaccine, *Brucella abortus* rough strain RB51, has been developed and is in use in several countries, mainly in the Americas. Use of another vaccine, *B.melitensis* rough strain M111, has also been reported from China.

The participation in this meeting of the Food and Agriculture Organization of the United Nations, Rome (FAO) and the Office International des Épizooties, Paris (OIE) emphasizes the importance of this disease, as brucellosis has not only direct public health implications but also poses a potential barrier to international trade of animals and animal products. Such a barrier could seriously impair socio-economic development, especially for a vulnerable sector in many rural populations - the livestock owner.

The objectives of this meeting are to strengthen research on development of brucellosis vaccines, to obtain consensus on research and development approaches for new vaccines for animal and human brucellosis and to define a strategy for international collaboration. The link with industry has also been emphasized, whenever possible, as a partner in sharing resources and experiences.

Dr F.-X. Meslin welcomed the participants (Annex 3) on behalf of the Director-General of WHO. Dr G. Dubray was elected Chairperson, Dr G.G. Schurig as Vice-Chairperson and Dr M.J. Corbel was nominated as Rapporteur.

1. INTRODUCTION

Brucellae are gram-negative, facultative intracellular bacteria that can infect many species of animals and humans. Six species are recognized within the genus *Brucella*: *B.abortus, B.melitensis, B.suis, B.ovis, B.canis* and *B.neotomae.* This classification is mainly based on the difference in pathogenicity and in host preference (Corbel and Brinley-Morgan, 1976-1984). *Brucella* species and their different biotypes are currently distinguished by differential tests based on sero-typing, phage typing, dye sensitivity, CO2 requirement, H2S production, and metabolic properties. The main pathogenic species worldwide are *B.abortus*, responsible for bovine brucellosis; *B.melitensis*, the main etiological agent of ovine and caprine brucellosis; and *B.suis* responsible for swine brucellosis. These three *Brucella* species usually cause abortion in their natural hosts, resulting in huge economic losses. They also account for most cases of human brucellosis. *B.abortus, B.melitensis, B.neotomae* or *B.suis* strains may occur as either smooth or rough strains expressing smooth-lipopolysaccharide (S-LPS) or rough-

lipopolysaccharide (R-LPS) as major surface antigen, while *B.ovis* and *B.canis* are two naturally rough species, expressing R-LPS as major surface antigen; they are responsible for ram epididymitis and canine brucellosis respectively, and *B.canis* is occasionally involved in human disease. Other strains, hitherto unclassified, have been isolated from marine mammals.

The disease in humans is characterized by a multitude of somatic complaints, including fever, sweats, anorexia, fatigue, malaise, weight loss, and depression. Localized complications may involve the cardiovascular, gastrointestinal, genitourinary, hepatobiliary, osteoarticular, pulmonary and nervous systems. Without adequate and prompt antibiotic treatment, some patients develop a 'chronic' brucellosis syndrome with many features of the 'chronic fatigue' syndrome.

2. IMMUNOLOGICAL ASPECTS OF BRUCELLOSIS

Protective immunity against *Brucella* infection has been studied mainly in mouse models (principally BALB/c and CD-1 mice). The criterion used for measuring protection in immunized mice is the reduction, at a specified time, after a virulent challenge, of the number of colony-forming units (cfu) of *Brucella* recoverable from the spleen or liver or both. Protective immunity against *Brucella spp*. in the mouse has been shown, by passive and active immunization experiments, to be mediated by antibodies as well as by cell-mediated immune responses.

The protective activity of antibodies was first demonstrated by passive immunization with immune sera of broad specificity. In studies using a smooth *B.abortus* biovar 1 strain (A-dominant) as challenge, protection was demonstrated using sera prepared by infection with smooth *B.abortus* or immunization with whole cells or subcellular fractions mainly from the cell wall of *B.abortus*. Biochemical analysis of the protective sodium dodecyl sulphate-insoluble cell wall (SDS-I CW) fraction showed that it comprised mainly peptidoglycan and the major outer membrane proteins (Omps). This led to the assumption that the major Omps could play an important role in protection. However, the application of monoclonal antibody (MAb) and recombinant protein technology to characterization of protective fractions showed that such was not the case.

The first antigen to be clearly identified as protective against smooth *B.abortus* was the smooth-lipopolysaccharide (S-LPS), through passive immunization experiments with monoclonal antibodies (MAbs) directed against the O-chain or O-polysaccharide (O-PS) moiety of S-LPS. S-LPS and its O-chain are the most exposed antigenic structures on the surface of smooth brucellae whereas the Omps are less accessible to antibodies. MAbs to surface-exposed Omps show much lower binding to smooth *Brucella* strains than to their rough counterparts lacking the O-chain. The length of the latter and its density on the surface of smooth brucellae also affects the accessibility of Omp epitopes to MAbs. Probably for this reason, MAbs to Omps confer little or no protection against a smooth *B.abortus* challenge in mice. MAbs to rough-lipopolysaccharide (R-LPS) epitopes also bind poorly to smooth cells and give correspondingly little protection against smooth *B.abortus* infection in mice. The protective activity of S-LPS against smooth *Brucella* infection in mice has also been demonstrated by active immunization with various S-LPS preparations or purified O-PS conjugated either to bovine serum albumin (Jacques *et al.*, 1991) or to *B.abortus* porin protein (Winter *et al.*, 1988). The O-PS specificity i.e. A- or M-dominant, plays a relatively small role in determining protection against the

homologous serotype since all currently recognized A- and M-dominant *Brucella* biotypes, carry common O-PS epitopes , some of which have been clearly identified as protective (Cloeckaert *et al.*, 1992).

Attempts to identify protective proteins have been largely unsuccessful. In all protective subcellular fractions tested, mainly the SDS-I CW fraction and the *Brucella* Cell Surface Protein (BCSP) fraction, S-LPS or O-PS has always been present in fractions prepared from smooth *Brucella* strains thus rendering evaluation of protection by their protein content very difficult. Furthermore the SDS-I CW fraction has induced higher antibody levels to S-LPS than purified S-LPS. Moreover, SDS-I CW fractions isolated from rough *Brucella* strains have proved poor inducers of protection against smooth brucellae, indicating that the major Omps contained in this fraction probably make little contribution to this activity. The failure of pepsin digestion to influence the protection levels conferred by the SDS-I CW fraction also provided an indication of the minor role of the protein content of this fraction. Finally, the use of recombinant proteins completely devoid of *Brucella* S-LPS has confirmed that the major *Brucella* Omps contained in the SDS-I CW fraction and now called Omp25 (25 kDa Omp), Omp31 (31 kDa Omp), and Omp2b (36 kDa porin Omp) appear to be of little relevance in protection against smooth *B.abortus* and/or smooth *B.melitensis.* Other recombinant proteins contained in the BCSP fraction, including rBCSP20, rBCSP31, and rBCSP45, have also proved non-protective in mice. Thus, protection reportedly induced by the BCSP fraction is probably attributable to S-LPS contamination. In fact, some of the proteins of the BCSP fraction may even act as antagonists to immunity induced by S-LPS and have been suggested as virulence factors in *B.abortus* infections.

In summary, these data indicate that S-LPS and more precisely O-PS specific antibodies play a major role in protective immunity against brucellosis caused by smooth brucellae. Nevertheless, other antigens, probably proteins, must be involved in protection against smooth brucellae since active or antibody mediated passive immunization based on S-LPS, although it may considerably lower the infection level, does not eliminate all brucellae. In addition, vaccinations performed with rough strains devoid of O-PS have protected mice against challenges of smooth virulent *B.abortus, B.melitensis,* or *B.suis* strains. However, these proteins are not necessarily cell surface proteins and probably protect through their capacity to induce cellular immune responses.

Brucellae are facultative intracellular bacteria that survive and replicate in both phagocytic and non-phagocytic cells. Phagocytes play a key role in initiating T-cell responses by processing and presenting antigens. T-cells play a major role in the acquired specific resistance to intracellular bacteria determines the resolution of infection. Protective cell-mediated immunity to *B.abortus* infection in mice has been demonstrated by passive transfer assays with immune T-cell-enriched spleen cells. The combined transfer of immune serum and cells has given better protection than that provided by serum or cells alone given prior to the challenge. Passive transfer experiments after *in vivo* depletion with anti-CD4 or anti-CD8 MAbs or with purified CD4 and CD8 subsets have suggested that both CD4 and CD8 subsets are involved in cell-mediated protection. Consequently, acquired resistance to infection with *B.abortus* in mice is the result of independent, and probably interactive, effects of antibodies and effector T-cells of both CD4 and CD8 phenotypes (Araya *et al.*, 1989). Further studies using major histocompatibility complex (MHC) class I and class II gene knockout mice infected with *B.abortus* have demonstrated that CD8 T-cells (MHC class I restricted) are mainly involved. These cells have been shown to play an important role in clearance of brucellae

following the peak of infection, probably by lysing infected macrophages (Oliveira and Splitter, 1995).

The cytokines involved in cell-mediated immunity developed against *B.abortus* primary infection have been studied mainly in three mouse models (CBA, BALB/c, C57Bl/10 mouse strains). Proinflammatory cytokines such as tumour necrosis factor alpha (TNF-alpha), interleukin-1 (IL-1), IL-6, and granulocyte macrophage-colony stimulating factor (GM-CSF) as well as T-cell derived cytokines such as gamma interferon (IFN-gamma), IL-2 and IL-10 (but not IL-4) have been detected in supernatants of primed splenocytes restimulated with *Brucella* antigens. The role of these cytokines in the control of *Brucella* infection has been investigated by injection of recombinant cytokines or by inhibition of their activity using specific MAbs. Macrophage-derived cytokines such as IL-1, IL-12 and TNF-alpha contribute to the control of early *Brucella* infection; IL-12 by stimulating NK cells and T-cells to produce IFN-gamma and TNF-alpha via an IFN-gamma independent pathway, probably by recruiting phagocytes to the site of infection, activating macrophages and promoting granuloma formation.

IFN-gamma is one of the most important T-cell stimulated cytokines in resistance to *B.abortus* infection. It is a potent activator of macrophages and monocytes and up-regulates their metabolic activities to produce oxidative metabolites and other microbicidal molecules. Indeed, IFN-gamma has been reported to reduce *Brucella* growth in macrophages although activation with IFN-gamma alone did not result in total elimination of intracellular brucellae. Factors working together with IFN-gamma for the elimination of intracellular brucellae include iron and TNF-alpha. Nitric oxide production also plays a role in killing the bacyeria. IL-2 has also been shown to mediate the inhibition of growth of *B.abortus* in the macrophage although its effect was not augmented by combination with IFN-gamma. Other cytokines such as IL-1, IL-6, TNF-alpha, GM-CSF and IL-4 have shown no consistent effect on growth of *B.abortus* in the macrophage *in vitro* when tested individually. In contrast, IL-10 and IL-4 contribute negatively to resistance to *B.abortus* infection by down-regulating both macrophage effector function and the production of protective IFN-gamma. Strain differences seen in the resistance of mice to infection with *B.abortus* in mice may be attributable to differences in the balance of the T-helper 1 (Th1)-associated cytokines (IFN-gamma, IL-2) and T-helper 2 (Th2)-associated cytokines (IL-10) produced.

In summary, cytokine data indicate that for optimal resistance to *Brucella* infection, vaccines should preferentially induce a Th1 subset response, with production of the protective cytokines IFN-gamma and IL-2. The induction of Th2 subset responses with IL-10 production appears to be detrimental for the control of infection caused by smooth strains. Two particular points of note are: i) cytokines are likely to exert maximum effect early in infection and ii) a delicate balance exists between enhancing immunity and exacerbating disease (continuous versus discontinuous secretion of TNF-alpha, and balance between IFN-gamma and IL-10). In addition, a Th1 response with IFN-gamma production may promote secretion of antibody of further protective isotype; in mice for example, IgG2a and IgG3 against *Brucella* S-LPS.

Immunization of mice with killed *B.abortus* as opposed to live *B.abortus* resulted in the production of both Th1-associated cytokines (IFN-gamma) and Th2-associated cytokines (IL-10) but important macrophage-derived cytokines such as TNF-alpha was not induced by killed organisms. TNF-alpha was barely detectable in supernatants of cultured murine macrophages stimulated by killed organisms, whereas its production was

heightened by live brucellae. In contrast, live *Brucella* spp of U937-derived macrophages, a human macrophage cell line, . did not induced TNF-alpha excretion in contrast to killed brucellae, which promoted a significant excretion of TNF-alpha from these cells. The capacity of *Brucella* spp. to use pathways avoiding TNF-alpha production during infection may be an attribute of virulence. Evidence has been presented that secreted *Brucella* protease- and heat-sensitive factor(s) of high molecular weight, probably protein(s), specifically inhibit TNF-alpha expression and thus may be considered as virulence factors.

It has also been shown that immunization of mice with total soluble *B.abortus* proteins result in a high frequency of IL-4 producing CD4 T-cells and a very low frequency of IFN-gamma producing CD4 T-cells, indicating a Th2 cytokine oriented response. IL-4 producing CD4 T-cells from these mice fail to mediate resistance against challenge infection in recipient mice, whereas IFN-gamma producing CD4 T-cells from infected mice confer significant protection.

A number of studies have focused on the identification of antigens which induce a protective Th1 cell-mediated response. These antigens have been identified in several ways. By using cell-immunoblotting, protein antigens ranging in molecular mass from high (> 45 kDa) or medium (25 to 45 kDa), to low (< 25 kDa) and separated electrophoretically by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) have been shown to stimulate proliferation of bovine lymphocytes from cattle vaccinated with *B.abortus* S19. Two-dimensional cellular immunoblotting has allowed the resolution of 38 individual *Brucella* proteins that induce lymphocyte proliferation. Phenotypic analysis of the proliferating cell populations demonstrated the presence of CD4, CD8, and IgM producing lymphocytes. A 12 kDa protein identified within the area of greatest lymphocyte proliferation was shown by gene cloning to correspond to the L7/L12 ribosomal protein (Bachrach *et al.*, 1994a,b). The use of purified L7/L12 recombinant protein produced in *E.coli* confirmed its importance in inducing CD4 T-lymphocyte proliferation of *B.abortus*-primed bovine peripheral blood mononuclear cells. Furthermore, the purified L7/L12 recombinant protein stimulated CD4 T-cells, associated with a Th1 cytokine profile (IL-2 and IFN-gamma), from mice infected with *B.abortus* (Oliveira and Splitter, 1994). Finally, immunization of mice with recombinant L7/L12 ribosomal protein was shown to confer protection against *B.abortus* infection (Oliveira and Splitter, 1996). These results provide for the first time an explanation of why ribosomal vaccines may protect against intracellular infections. In fact, the immunogenic and protective activity of ribosomal fractions derived from *B.abortus* reported 20 years earlier but the active protein components were not identified (Corbel, 1976). Incidentally, the L7/L12 ribosomal protein is a major active component of brucellin INRA (Institute National de la Recherche Agronomique, 147, rue de l'Université, F-75007, Paris, France). However, brucellin INRA does not stimulate protection against *B.abortus* infection in mice, probably because of antagonism between L7/L12 and other components as in other *Brucella* fractions such as BCSP and SDS-I CW fractions. This suggests that individual proteins rather than crude fractions should be screened as potential protective T-cell antigens for inclusion in subunit vaccines.

Thus, the strategy developed to identify immunodominant T-cell antigens has proved useful for the identification of protective antigens. This emphasizes the importance of screening these antigens first on T-cells from target animals such as cattle, followed by assessment of immunogenicity and protective properties in mouse models. The Th1 response induced in mice correlates with the cytokine mRNA transcript response of primed bovine peripheral blood mononuclear cells.

Apart from the L7/L12 protein, no other proteins have yet been shown to stimulate protection against *Brucella*, although several, including the GroEL, GroES, Ssb and UvrA recombinant gene products, stimulate proliferation of lymphocytes from primed cattle or mice and induce a Th1 cytokine profile. The Cu-Zn superoxide dismutase failed to protect as a recombinant protein although a synthetic peptide derived from this gave some protection in mice. Other *Brucella* antigens identified could also possibly be involved in cellular immune responses, such as the stress response proteins DnaK and HtrA. These proteins are reported to play an important role in survival of brucellae in the macrophage and/or in mice.

In conclusion, current data indicate that both antibodies against the O-PS and a Th1 cellular response induced by proteins are required for optimal protection against smooth *Brucella*. Only the L7/L12 ribosomal protein has as yet been clearly identified as a protective antigen inducing such a Th1 cellular response. Further studies using the approaches described should be performed to identify additional protective antigens.

The immunological aspects of *B.ovis* infection are quite different from those relating to smooth brucellae. Passive transfer assays have revealed that protection in mice is mediated by both T lymphocytes and antibodies, but that the latter play a substantially greater role. Transfer of MAbs showed that R-LPS and the major outer membrane protein Omp31 were the most important protective antigens. Vaccination with a *B.ovis* hot saline (HS) extract, mainly composed of R-LPS, Omp25 and Omp31, conferred good protection against *B.ovis* infection in mice, as did recombinant Omp31. Protection provided by the HS extract resulted largely from immune responses to its protein moieties. In contrast to smooth brucellae, *B.ovis* lacks the surface O-PS and outer membrane proteins are readily accessible to antibodies. The HS extract has also been shown to provide protection against *B.ovis* infection in rams. Thus, a possible subunit vaccine against *B.ovis* infection could consist of recombinant Omp31 protein complexed with R-LPS, although the latter would interfere with current diagnostic tests.

3. REQUIREMENTS FOR AN IDEAL VACCINE FOR BRUCELLOSIS

Live vaccines have until now proved superior to inactivated products for the prevention of animal brucellosis (Nicoletti, 1990). They are effective, inexpensive, and immunity is more persistent. The ideal live vaccine should not produce disease in vaccinated animals; it should prevent infection in both sexes at any age, prevent abortion and sterility, provide long-term protection against infection and abortion with a single vaccination, not stimulate persistent antibodies interfering with accurate serodiagnosis of field infections, not be transmitted to other animals if the vaccine strain establishes a long-term latent infection, be biologically stable, free of reversion to virulence *in vitro* and *in vivo*, be non-pathogenic for humans, not contaminate meat and milk products, and, be readily culturable under large-scale fermentation conditions (Adams, 1990). The ideal live vaccine should also contain specific genetic or phenotypic markers that would make it easy to differentiate from field isolates.

For the prevention of human brucellosis, an ideal vaccine should be safe and effective (i.e. it should not produce disease, hypersensitivity or more than minimal local or systemic reactions), and should provide long-term protection, preferably for life, with a single dose.

4. AVAILABLE VACCINES

4.1. Animal brucellosis

Vaccination against *Brucella* infections in animals is usually performed by administration of the live attenuated smooth *Brucella* strains: *B.abortus* strain S19 and *B.melitensis* strain Rev.1. The non-smooth strain *B.abortus* RB51 has recently been introduced in some countries.

B.abortus S19 and *B.melitensis* Rev.1 are proven effective vaccines against *B.abortus* in cattle and against *B.melitensis* and *B.ovis* in sheep and goats, respectively (Elberg, 1996; Nicoletti, 1990). Both vaccines have the disadvantages of causing abortion in a proportion of pregnant animals, and of being pathogenic for humans. However, their main disadvantage is the induction of O-PS specific antibodies that interfere with the widely used serological tests which employ S-LPS as antigen.

In countries where *B.melitensis* infection in sheep and goats is widespread, cattle may also become infected with this species. *B.melitensis* Rev.1 has also been evaluated for the vaccination of cattle under these conditions. Experimental studies have shown that *B.melitensis* Rev.1 provided immunity to *B.melitensis* equal to or superior than the immunity induced by *B.abortus* S19 with a lower vaccine dose. Despite of these results, the use of *B.melitensis* Rev.1 in cattle has been very limited (Nicoletti, 1990).

The live attenuated smooth *B.suis* strain 2 (S2), produced in China by serial transfer of a virulent *B.suis* biovar 1 strain of swine origin, has been widely used in that country, particularly for immunization of sheep and goats (in drinking water). Its virulence is approximately the same as *B.abortus* S19 and is stable (Xie Xin, 1986). Although the S2 strain also gave a satisfactory protection rate in cattle (Nicoletti, 1990b), its efficacy against experimental *B.melitensis* infection in pregnant ewes (Verger *et al.*, 1995) or against *B.ovis* infection in rams (Blasco *et al.*, 1993) was inferior to that of *B.melitensis* Rev.1 vaccine.

Currently, *B.melitensis* Rev.1 appears to be the most generally effective vaccine strain available for prevention of animal brucellosis.

4.2. Human brucellosis

B.abortus S19 and *B.melitensis* Rev.1 can cause brucellosis in humans and are therefore unsuitable for human vaccination. A variety of live attenuated strains, such as *B.abortus* strain 19BA or *B.melitensis* 104M, have been used at some time in the former USSR and China, but tend to be reactogenic and of limited efficacy. Various *Brucella* fractions have been studied as potential human vaccines, but their efficacy remains uncertain. They include the phenol-insoluble SDS fraction of *B.abortus* or *B.melitensis*, composed mainly of peptidoglycan, proteins, and S-LPS (Lopez-Merino, *et al.*, 1976). This is protective against *Brucella* infection in the mouse model but induces substantial antibody responses against S-LPS which interfere with diagnostic tests. A polysaccharide fraction produced by mild acid hydrolysis developed in Russia seems to be protective with minimal reactogenicity in clinical trials but its current availability is uncertain

(Dranovskaya, 1991). An LPS-protein conjugate has given encouraging results in a mouse model and may warrant further study (Jacques, *et al.*, 1991).

5. NEW VACCINATION APPROACHES

5.1. Animal brucellosis

Several approaches have been followed to overcome the main problem encountered in animal vaccination with live attenuated smooth *Brucella* strains, i.e. inability to distinguish vaccinated animals from infected animals by the current standard serological tests based on detection of antibody to S-LPS.

The first is to use live attenuated rough *Brucella* strains, in particular rough *B.abortus* strain RB51, a stable rough rifampin-resistant mutant of virulent *B.abortus* strain 2308 (Schurig, *et al.*, 1991). This is discussed in detail in Section 9. The efficacy of the *B.abortus* RB51 vaccine strain has yet to be fully determined in other animal species such as sheep, goats, and swine. In rams, *B.abortus* RB51 failed to protect against *B.ovis.* Possible alternative vaccines to *B.abortus* RB51 include the reported rough strains derived from *B.melitensis* or *B.suis* (Winter, *et al.*, 1996). These strains were produced by allelic exchange, in their smooth parental strains, of the rfbU gene encoding mannosyltransferase (an enzyme required for O-PS synthesis). The method used to construct such mutants offers the advantage of inserting in the *Brucella* genome stable genetic markers that can subsequently be used for identification purposes. The rough *B.melitensis* or *B.suis* mutants could potentially be more efficacious than *B.abortus* RB51 in protecting against virulent strains of the homologous species in the primary hosts.

Another approach consists in identifying diagnostic protein antigens and deleting the corresponding genes in the current live attenuated smooth *Brucella* strains. Such constructed mutants should not induce antibody responses against the target protein which could be further used in new diagnostic tests. The protein should be immunodominant in infection and allow the detection of a proportion of infected animals similar to that detected by the current serological tests. The introduction of stable genetic or phenotypic markers used to replace the genes of interest should make possible the differentiation of the newly constructed vaccine strain from field isolates. *B.abortus* S19 mutants deleted for Cu-Zn superoxide dismutase or a 31 kDa protein have been constructed and have provided protective immunity against *B.abortus* in cattle similar to that stimulated by the parental strain (Cheville, *et al.*, 1993). However, the proteins chosen were not immunodominant in infected cattle and therefore were unsuitable as diagnostic reagents. Other proteins investigated subsequently have included the cytoplasmic p15, p17 and p39 proteins. These have detected more than 80% of animals positive to conventional serological tests. However, when used as recombinant proteins expressed in *Escherichia coli,* they were unsuccessful in detecting non-pregnant cattle or sheep known to be infected with *Brucella.*

The Omp 28 or BP26 protein appears more promising. This periplasmic protein is immunodominant in cattle, sheep, goats and humans. It is particularly useful in sheep and has detected 90% of those positive to conventional serological tests. It also showed an 80% correlation with the intradermal test for delayed hypersensitivity in bacteriologically negative sheep and with conventional serological tests. Rams infected with *B.ovis* also showed significant antibody responses to BP26. Thus, BP26 appears to be a good diagnostic antigen candidate for deletion from the *B.melitensis* Rev.1 vaccine strain to

facilitate serological differentiation between vaccinated sheep and sheep infected with *B.melitensis* or *B.ovis*. A *B.abortus* S19 mutant lacking BP26 has already been constructed and shown to protect mice against *B.abortus* to an extent similar to the parental vaccine strain (Boschiroli, *et al.*, 1997).

Another approach to developing diagnostically non-compromising live attenuated *Brucella* vaccine strains could be the deletion of one or more specific O-PS epitopes that could subsequently be used as a diagnostic reagent. Competitive ELISA using specific MAbs and purified *Brucella* S-LPS or O-PS cannot be used for such a purpose since different *Brucella* O-PS epitopes are probably overlapping structures on the O-PS or S-LPS antigens. Suitable antigens are not currently available but potentially useful epitopes have been shown to be absent from *B.suis* biovar 2 LPS. The relevant epitopes could be produced by oligosaccharide synthesis or vaccine strains lacking these epitopes could be produced from *melitensis* Rev.1 or *B.abortus* B1.

As an alternative to live attenuated *Brucella* vaccine strains, subunit vaccines containing only protective antigens, other than those useful for differential diagnosis, could be developed. Such a potential subunit vaccine could consist of S-LPS or O-PS combined with or conjugated to the L7/L12 ribosomal protein for protection against smooth *Brucella spp*., together with Omp31 complexed with R-LPS for protection against *B.ovis* strains. Further work is needed to identify new protective antigens that could improve the protective capacity of such subunit vaccines. Complexing of ribosomes to LPS has been suggested as a potentiator or carrier for experimental subunit vaccines. Immune cellular or humoral immune responses of subunit vaccines can be further potentiated by addition of appropriate adjuvants or by encapsulating them in liposomes (Cox and Coulter, 1997). Enhanced cellular responses in mice have also been demonstrated by treatment with a *Brucella* antigen-liposome mixture. Monophosphoryl lipid A has induced immune enhancement of *B.abortus* LPS vaccines in mice (Tabatabai, *et al.*, 1992) and also has the advantage of modulating the cellular immune response towards a strong Th1 response.

DNA vaccination with plasmid DNA constructs containing bacterial genes is also reported to be particularly appropriate for preventing bacterial infections where cytotoxic T-cells confer protection, or where a Th1 type T-cell response mediates resistance (Strugnell *et al.*, 1997). This type of vaccination has indeed been found to induce protective immunity against complex intracellular pathogens such as *Mycobacteria* and may be an effective approach to *Brucella* vaccines, as indicated in recent studies with L7/L12 DNA vaccines.

5.2. Human brucellosis

The development of new vaccines against human brucellosis is discussed in Section 12. Subunit preparations of defined composition based on purified protective antigens free of toxic components are likely to be favored, but defined attenuated strains are also of potential interest.

6. ADVANTAGES AND DISADVANTAGES OF REV. 1 VACCINE FOR THE PROPHYLAXIS OF BRUCELLOSIS IN SMALL RUMINANTS

With few exceptions, sheep and goats are managed on extensive systems and brucellosis is usually prevalent at high rates, particularly in developing countries. Under these conditions, *B.melitensis* cannot be eradicated by testing and slaughtering alone, and a vaccination programme has to be applied to lessen the spread of disease. The live attenuated *B.melitensis* Rev.1 strain has until now been considered the best vaccine available for the prophylaxis of *B.melitensis* infection in small ruminants (Elberg, 1996). When applied to replacement animals (3-5 months old) by the standard method ($1x10^9$ cfu subcutaneously), the Rev.1 vaccine induces solid immunity against *B.melitensis*. However, infection in vaccinated animals, subcutaneous inoculation causes a generalised Rev.1 thus inducing an intense and long-lasting antibody response that interferes with subsequent serological screening. By contrast, when administered by the conjunctival route, the Rev.1 infection is mainly restricted to cranial lymph nodes. Thus, the immunity conferred is similar to that induced by the standard subcutaneous method but the serological response evoked is significantly reduced, making its use compatible with test and slaughter programmes. Accordingly, when eradication is the final objective of control programmes, conjunctival vaccination of replacement animals with Rev.1 is ideal for the prophylaxis of *B.melitensis* infection in small ruminants.

It has been hypothesised that after implementing an exclusive vaccination programme (i.e. a programme limited to replacements animals) for 5 to 6 years (the usually accepted productive life-span of sheep and goats), the whole population would have life-long immunity. This hypothesis, and the unproved assumption that Rev.1 vaccine induces life-long immunity, have contributed to the widely accepted opinion that vaccination limited to young replacements would be sufficient for adequate control of *B.melitensis* infection in small ruminants. However, this exclusive vaccination strategy is inapplicable in developing countries and has failed to control brucellosis even in developed countries. The main factors explaining this failure include: *i)* low vaccination coverage due to lack of concordance between replacement and vaccination times (owners make replacements continuously throughout the year); *ii)* use of vaccines lacking adequate quality control; and *iii)* a possible decrease with time of the level of immunity conferred (with special impact in some European countries, in which the productive life-span of small ruminants has increased significantly e.g. to 9 -10 years, as a direct consequence of compensation practices). Accordingly, whole-flock vaccination is the only feasible alternative for control of *B.melitensis* infection in small ruminants under the extensive management conditions characteristic of many developing countries (FAO/WHO/OIE, 1995).

The main problem arising from the use of Rev.1 in adult sheep and goats is that vaccination of pregnant animals with full standard doses administered subcutaneously is in most of these followed by abortion. The use of lower vaccine dose may resolve this safety problem in goats, at the cost of decreasing the level of immunity conferred by the standard doses (Alton, 1970). Accordingly, a reduced-dose vaccination strategy has been widely used and is reported as a safe and effective method of controlling small ruminant brucellosis, particularly when little or no monitoring is conducted after vaccination (Al-Khalaf, *et al.,* 1992). However, field and experimental results do not support the use of reduced doses of Rev.1, whether administered by subcutaneous or conjunctival routes, as an alternative to the full standard dose (Blasco, 1997).

When tested in a mouse model, differences in residual virulence and immunogenicity have been demonstrated among the different Rev.1 vaccines produced world-wide. These differences may account for the discordant safety results obtained in mass vaccination trials in different countries. The induction of abortions when vaccinating pregnant animals means that there is no entirely safe strategy for Rev.1 vaccination. Although conjunctival vaccination is safer than subcutaneous vaccination (see Table 1), it is not safe enough to be applied regardless of the pregnancy status of animals, and should be used only under restricted conditions (Blasco, 1997).

Table 1 Safety of conjunctival (conj) versus subcutaneous (sc) vaccination with Rev.1

Days of pregnancy at vaccination	55 days		120 days	
Vaccination route	sc	conj	sc	Conj
Rev.1 excretors	81.2%	41.2%	91%	22.2%
Aborted ewes	68.7%	11.7%	9.1%	0%

Comparative experiments in sheep with other live attenuated vaccine strains, such as the smooth *B.suis* S2 or the rough *B.abortus* RB51, have confirmed the outstanding immunity conferred by Rev.1 (Verger, *et al.*, 1995). Therefore, until a safe and effective alternative vaccine becomes available, *B.melitensis* Rev.1 has to be considered as the reference vaccine for the prophylaxis of brucellosis in small ruminants. Conjunctival vaccination with Rev.1 is the ideal tool for prophylaxis in replacement animals; some risk of induced abortions has to be accepted when used in whole-flock vaccination programmes. In this case, the conjunctival administration of standard doses of 10^8 cfu during the late lambing season or during lactation, periods of minimal risk for induced abortion, could be recommended as the optimal whole-flock vaccination strategy for control of *B.melitensis* in developing countries (Blasco, 1997).

Further research on the safety of Rev.1 needs to be carried out in lactating goats and billy goats. Moreover, although conjunctival vaccination has been reported to confer adequate protection in sheep for at least two subsequent pregnancies (Verger, *et al*, 1995), more information is needed about the duration of the immunity conferred by this method of vaccination.

7. THE ISRAELI EXPERIENCE OF FIELD APPLICATION OF S19 AND REV.1

The Israeli animal management system is divided between intensively kept dairy cattle and extensively reared beef cattle and small ruminants. Specifically, the small ruminant population includes sheep and goat in dairy farms that are free of brucellosis, and nomadic and semi-nomadic sheep and goat flocks in which infection with *B.melitensis* is endemic. The human population at risk of brucellosis infection includes farmers and their families who are exposed to the organisms, either by attending the infected animals or by consumption of their own unpasteurized dairy products, and field veterinarians, abattoir personnel and clinical laboratory personnel.

Human infection with *B.melitensis* has been notifiable in Israel since 1973. Initially the disease was rarely reported but the incidence of human brucellosis rose to a record level of 500 cases in 1988. Since then, the annual incidence has stabilized at about 300 cases. The limited availability of natural pasture land leads to the grazing of sheep and goats on agricultural fields after harvest. The intensively managed cattle are fed with fodder originating from these fields and possibly contaminated with brucellae. Moreover, dogs and wild canidae can carry the aborted placentas and fetuses into the corrals, exposing cows to *B.melitensis* infection. The dust raised during the movement of sheep and goats on dry pasture land is another potential source of *B.melitensis* infection for cattle. *B.abortus* was officially eradicated in 1985 and has not been reported since then. Nevertheless, the veterinary services have continued vaccination with subcutaneous, full dose S19 in female calves between the ages of 2 to 6 months. This programme has been successful, with only rare isolations of the strain from adult cows. Moreover, it has not hampered simultaneous surveillance.

Before 1988 *B.melitensis* infection was seen only sporadically in dairy farms; subsequently, it has frequently invaded intensively managed herds. The first outbreak was reported in 1989 when *B.melitensis* biotype 3 infected an Israeli-Holstein dairy herd, in a farm that also included several unvaccinated Simmental cows. The strain was isolated from placentas and fetuses obtained from both dairy and Simmental cows after slaughter. However, since, the slaughter of infected cows preceded the date of normal calving, the possible abortive capacity of *B.melitensis* in cows was not determined.

The threat to the human population together with that of possible transmission of *B.melitensis* to the national dairy cattle industry led to the implementation of a national elimination programme based on test-and-slaughter, and on the vaccination of ewe-lambs and kid-goats with *B.melitensis* Rev.1.

The programme was strengthened with the establishment of a National Reference Laboratory that established collaborations with public health laboratories, which referred all human isolate for biotyping.

From 1988 to 1993, *B.melitensis* biotype 1 was most often recorded biotype among human and animal isolates; *B.melitensis* biotype 3 was isolated less often. *B.melitensis* biotype 2 was rarely identified. The similarity of biotypes patterns isolated from animals and humans clearly demonstrate the zoonotic features of the disease. In 1987 a new atypical biotype 1 strain of *B.melitensis* was first isolated. The characteristics of the strain were peculiar in that, like the vaccine strain Rev.1, it was susceptible to penicillin and dyes, but differred in being susceptible to streptomycin. The isolation of a similar strain from a herd owner and his sheep clearly demonstrated its virulence and zoonotic potential. This raised the issue of its origin, from mutation of a field strain to an atypical biotype, or from acquisition of virulence by Rev.1.

Rev.1 infection of both sheep and humans was demonstrated in a single event. *Brucella,* identified as Rev.1, was isolated from the placenta of an aborting ewe from a Merino flock with quasi-complete vaccination coverage. Serological diagnosis identified more than 20% positive reactors among the ewes but attempts to isolate the strain from milk samples and lymph nodes from slaughtered animals all failed. Seven months later, the owner contracted brucellosis and his blood culture yielded a strain identified as *B.melitensis* Rev.1.

Subsequently, an atypical biotype 1 virulent strain was isolated from two fetuses recovered at slaughter from an unvaccinated imported Romanov ewe kept in a flock of 300 vaccinated locally bred Merino sheep which had yielded periodic serological reactors but no isolates. The strain had aberrant slow urease activity resembling that of the vaccine strain Rev.1 but with the biotyping characteristics of a normal field strain.

The occurrence of *Brucella* infection in an intensively managed sheep flock with a very high vaccination coverage raises the following questions:

i) Is Rev.1 vaccination sufficiently protective in the individual?
ii) Does it protect the flock adequately?
iii) How can serological responses due to vaccination and those due to infection be distinguished?
iv) How can serologically positive reactors that shed the vaccine strain be distinguished from those that shed a field strain?

The implementation, in 1993, of a national interim control programme provided indirect answers to these questions. *Brucella* was isolated only very rarely, proving the efficacy of vaccination in preventing *Brucella* secretion. In most cases the strain was Rev.1 like. In a very few cases, Rev.1 like organisms were isolated from unvaccinated ewes, demonstrating for the first time horizontal transfer of the vaccine strain (two isolates were in the rough form indicating possible dissociation of the strain in the host).

In semi-nomadic flocks the situation was even more complex. Almost no abortions were recorded in flocks with good vaccination coverage. This proved the value of vaccination for prevention of abortion thereby also protecting the environment from contamination with *Brucella* organisms. Positive reactors, however, were identified among vaccinated flocks. In a single case the vaccine strain was co-isolated with a field strain from an aborted placenta from a nulliparous ewe. In other cases the vaccine strain was isolated from the same flock in which a field strain was proven to be the cause of the infection. Mixed infection with two or three different biotypes was also demonstrated in intensively vaccinated flocks.

Given the possible changes that may have occurred in some of the commercial Rev. 1 seed strains, consideration was given to the use of the Rev.1 vaccine prepared from the original Elberg strain. This in combination with conjunctival vaccination might be expected to provide a more effective vaccination programme.

A *B.melitensis* biotype 1 infection was diagnosed in a dairy herd where, by the time of diagnosis, 60% of the milking cows reacted positively in the serological tests and about 20% of these secreted the strain in their milk. The infection source was not identified but sheep grazing in the vicinity were suspected. Since all the cows were vaccinated as calves with a full dose of subcutaneous S19 vaccine, the extremely high rate of infection demonstrated the failure of the vaccine to protect from *B.melitensis*. In fact, this case was similar to an early observation in sheep and goats where whole flocks contracted brucellosis even though the animals had been promptly vaccinated with Rev.1.

In a second case *B.melitensis* biotype 1 infected a dairy cattle herd; gestating cows in the third trimester had aborted and the strain was isolated from the foetal material. This demonstrated for the first time that *B.melitensis* can cause abortion not only in the natural

host but also in the heterologous host. It also confirmed the failure of S19 vaccine not only to protect against infection but also to prevent abortion.

In considering development or improvement of *Brucella* vaccines their design should be in the context of a feasible control programme and should recognize the need to confer protection against different *Brucella* species in different target animals. Prophylaxis could be achieved by vaccination of ewe lambs and kid goats, with the conjunctival Rev. 1 vaccine followed by annual booster vaccination with a protective subunit vaccine. The virulence of *B.melitensis* is far greater than that of other species and it easily infects cattle. The design of a new vaccine should take into consideration possible changes (genetic, bacteriological adaptation to new hosts) of local strains. Innocuity of the vaccine strain should be stringently controlled to maintain good safety and immunological characteristics. Finally, as indicated previously, new approaches should be developed to permit differentiation of serological responses resulting from vaccination and infection.

8. EXPERIENCE WITH *BRUCELLA* VACCINES IN CHINA

8.1 *Brucella suis* strain 2 vaccine

Brucellosis has a long history in China and formerly was a major cause both of economic loss in animal production and of morbidity in the human population. A national control programme began in 1949 and currently the disease is well controlled or has disappeared from most parts of the country. Vaccination with *B.suis* strain 2 (S2) played a major role in this although *B.melitensis* strain M5 was used for vaccination of sheep and goats in some areas. Control was conducted in a four-phased campaign.

The first phase lasted from 1964 to 1970. Immunization using S2 vaccine was implemented in most of the infected areas. During the second phase, from 1971 to 1980, the S2 vaccination was extended to other infected areas. The main control policy was intensive vaccination, and the vaccinated animals were not tested serologically. By 1977 vaccination had covered almost all of the brucellosis-infected areas, leading to a drastic decrease in the prevalence of animal brucellosis Approximately 40-50% of the sheep, goats and cattle were vaccinated every year in the infected areas.

During the third phase, from 1981 to 1990, S2 vaccine was used in sheep, goats, pigs, cattle and yaks in most of the infected areas of China, leading to a further decline in prevalence. In some brucellosis infected areas with low prevalence, vaccination was only applied to young livestock so that test-and-slaughter could be implemented. In other areas in which this was not feasible, an intermittent vaccination policy was applied. The animals were vaccinated continuously for 2 to 3 years, vaccination was stopped for 2 or 3 years, and then resumed again. After 10 years, with a coverage of >90%, the disease was well-controlled.

The current and fourth phase started in 1991. The National Brucellosis Control Programme was further strengthened, and by 1992 brucellosis was well under control in 76% of infected areas. The use of S2 vaccination has now been discontinued in most areas which rely on a test-and-slaughter and quarantine policy.

S2 vaccine has been successful in controlling brucellosis in some areas that formerly were very heavily infected. For example, in Inner Mongolia before 1970, up to

25% of cattle were sero-positive but by 1990 this had declined to 0.84%. In Shan Dong province the prevalence of infection in sheep and goats declined from 35% in 1960 to 0.09% in 1990. In Guang Dong province the sero-positive rate in pigs declined from 4.58% in 1986 before the introduction of S2 vaccination to 0.4% in 1991. The vaccine has also been effective in yaks, the prevalence of infection declining from 40% in 1983, before the introduction of the vaccine, to no detected cases by 1990.

8.2 Rough *Brucella melitensis* vaccine strain M111

A rough *B.melitensis* strain M111 vaccine was developed in China in the 1980s for use as a live vaccine. Strain M111 was obtained from a smooth *B.melitensis* strain by selection of non-agglutinating colonies. It has the cultural and biochemical properties of *B.melitensis* biotype 1 but is rough. Its virulence is slightly less than that of *B.suis* S2. It showed no change in properties after 50 sub-cultures or after 8 passages in guinea-pigs or 4 passages in pregnant sheep. Post mortem examinations showed that the strain did not produce lesions in sheep or goats, nor did it produce abortion in pregnant animals. Rabbits, sheep, goats and cattle vaccinated with strain M111 did not produce antibodies against smooth *Brucella* antigen detectable in the Rose Bengal, complement fixation, or standard agglutination tests. It did, however, stimulate antibodies to rough *Brucella* strains. When compared with S2, S19 and *B.abortus* 45/20 vaccines, M111 failed to stimulate antibodies to smooth *Brucella* after inoculation, for up to 330 days in sheep, and 138 days in goats.

Strain M111 was able to produce effective immunity against challenge with virulent *B.melitensis* strain M28 in guinea-pigs, sheep and goats. The vaccine could be applied by parenteral injection or orally. The protective efficacy was 84% in sheep and 78% in goats. In field trials, the vaccine proved to be safe for sheep and goats and was effective in stimulating immunity against wild type *B.melitensis.* In Northern China, it was effective in reducing abortion rates from about 50% to zero within 2 years. Since it did not interfere with conventional serology, strain M111 could be used in conjunction with test-and-slaughter for brucellosis control.

9. NOVEL ROUGH BRUCELLA VACCINES: *B.ABORTUS* VACCINE STRAIN RB51 AND ANALOGOUS ROUGH VACCINE STRAIN FOR *B.MELITENIS*

9.1 *B.abortus* strain RB51

When cultured on solid medium, *Brucella* can present a smooth or rough colonial morphology, some strains showing a mucoid phenotype. It is possible for smooth colonies to become rough spontaneously and for some rough *Brucella* strains to revert to the smooth form. The composition of the lipopolysaccharide (LPS) molecules of the *Brucella* outer membrane is coupled to the rough vs. smooth morphology. Smooth organisms have LPS molecules containing a polysaccharide O-chain composed of a homopolymer of N-formylated perosamine (N-formyl-4-amino-4,6-dideoxy mannose) while rough organisms have an LPS molecule which lacks an O-chain. The N-formylated perosamine O-chain plays a central role in the serological diagnosis of brucellosis, since it is an immunodominant antigen that induces antibody responses in most animals exposed to smooth *Brucella* organisms. Furthermore, virtually all diagnostic serological tests in common use are based on the detection of antibodies to the O-chain.

Prevention of infection, and hence disease, can be achieved with vaccines. As a general rule, induction of an effective, long lasting protective immune response to facultative intracellular parasites requires the use of live vaccines or in some cases the use of multiple injections of appropriate protective antigens in the presence of adjuvants which favour cell mediated immune (CMI) mechanisms. The latter are a crucial component of the protective immune response to infection with smooth *Brucella spp*., although antibodies against the O-chain play a more or less important role in protection, depending on the animal species involved.

B.abortus S19 and *B.melitensis* Rev.1 are live vaccines based on attenuated smooth strains. Both can induce varying degrees of protective responses to *Brucella* and both also stimulate antibodies to smooth O-chain.

B.abortus strain 45/20, a rough organism with little or no ability to induce anti O-chain antibodies, was originally developed as a live vaccine but abandoned in this form because of a tendency to revert to virulence. It was subsequently used as a killed vaccine in oily adjuvant with some success. Its ability to induce significant protection against infection with *B.abortus* indicates that rough organisms can be used to induce a protective immune response, thus avoiding the diagnostic problems associated with smooth strains.

B.abortus strain RB51 was selected by growth of *B.abortus* strain 2308 in the presence of rifampicin. Strain RB51 is essentially devoid of the O-chain, its roughness being very stable after multiple passages *in vitro* and *in vivo* through various species of animals. Since it lacks an O-chain, it does not induce anti-O-chain antibodies measurable by conventional serological tests or ELISA, regardless of age, dose or frequency of injections. Strain RB51 is attenuated, as indicated by studies in mice, guinea pigs, goats and cattle from which it is cleared in a relatively short time with little or no abortifacient activity. When used in single vaccination protocols, its protective effect in cattle is similar to that of S19. Recent field experiments carried out in areas of high and low brucellosis prevalence indicate that strain RB51-induced immunity (at least one year after vaccination) in cattle is similar or better than the immunity induced by S19. The mouse model indicates that the protective immunity induced by strain RB51 is solely T cell-mediated, since passive transfer of RB51-induced antibodies does not protect, while adoptive T cell transfer does. Recent unpublished work (presented at the 50th Brucellosis Research Conference in Chicago, November 1997) suggests that specific cytotoxic T cells able to kill *Brucella* infected macrophages are induced by vaccination with strain RB51. Strain RB51 has been approved for use as the official vaccine in the USA, replacing S19, and its use has been implemented in Mexico and Chile. It can be differentiated from field isolates of *B.abortus* by molecular methods such as pulsed field gel electrophoresis and PCR.

Studies in mice indicate that strain RB51 can protect against infections with *B.abortus, B.melitensis, B.suis* and *B.ovis*. However, the level of protection afforded against the smooth virulent strains is lower than that conferred by the current live attenuated smooth strains, probably because of the absence of anti-O antibodies which are important in the mouse model. Ongoing work with various animal species confirms excellent protective activity in swine against infection with *B.suis* under field conditions (Lord, *et al.,* 1998) and has shown that RB51 can protect up to 93% of RB51 vaccinated goats against *B.melitensis* infection. Preliminary results in sheep suggest that RB51 does not induce protective immunity in this species if used as a single vaccine; further information is required.

After RB51 vaccination, all species tested have remained serologically negative in all conventional serological tests for brucellosis. At present, over 5 million calves have been vaccinated subcutaneously with the recommended dose of 1 -3.4 x 10^{10} organisms without deleterious effects. Unpublished observations regarding protective efficacy suggest that immunization should start with animals not younger than 4 months. Pregnant cattle can be safely vaccinated subcutaneously with 10^9 RB51 organisms without the induction of abortion or placentitis. Intravenous inoculation of pregnant cattle with 10^{10} organisms led to placental and fetal infection but not to abortion, suggesting that vaccination of non-pregnant, adult cattle with a full dose should be safe. However, controlled studies employing the recommended subcutaneous route and a larger number of cattle are needed. Oral administration of strain RB51 in mice and cattle has also induced protective immunity, opening a practical approach to wildlife immunization. Finally, the investigation of several incidents of accidental inoculation with strain RB51 suggests that this strain is non-pathogenic for healthy humans (E.J. Young, personal communication; MMWR, 1998) and that it may have potential as a live vaccine for humans.

9.2 Analogue mutants of other *Brucella* species

The *rfbU* gene in *Brucella* which codes for mannosyl transferase, an enzyme necessary for the assembly of the *Brucella* O-chain, has been identified. The rough strains *B.abortus* VTRA1, *B.melitensis* VTRM1 and *B.suis* VTRS1 have been constructed using the Brucella *rfbU* gene sequence to produce disruption mutants of *B.abortus* 2308, *B.melitensis* 16M and *B.suis* 40 (biotype 4). This strategy can be utilized to generate rough mutants from any smooth *Brucella* spp. Recently the *rfbE*, *rfbK* and *rfbD* genes have been described and an *rfbM* gene reported. Deletion or interruption of these genes can also lead to expression of rough morphology. Double mutants could therefore be constructed to further decrease the possibility of rough to smooth reversion.

Strains VTRM1 and VTRS1 were tested in mice for their virulence (spleen clearance after intraperitoneal injection) and their ability to protect mice against infection with heterologous and homologous strains of *Brucella*. The virulence of VTRM1 and VTRS1 was lower than that of the parent strains but the attenuation achieved was not as great as with RB51. This indicates that *rfbU* is involved in virulence but also suggests that other genes, most probably not involved in the synthesis of LPS components, play an important role in attenuation. Various groups are working in the area of *Brucella* attenuation by interrupting or deleting a variety of potential virulence genes. For example, *B.melitensis* 16M with interrupted *purE* appears to be attenuated in goats and has good protective characteristics.

Strains VTRM1 and VTRS1 induced immunity in the mouse model against infection with *B.abortus*, *B.melitensis*, *B.suis* and *B.ovis* strains isolated from a variety of animal species. In several instances, the protection induced by VTRM1 and VTRS1 was superior to that afforded by strain RB51, probably because these strains replicate more vigorously and remain longer in the host than strain RB51. This characteristic may lead to a stronger CMI response. Neither strain induced antibodies to the O-chain. Studies using VTRM1 in goats and VTRS1 in swine are in progress.

It is clear that rough strains can produce very good protection, at least in selected animal species. However, the optimal conditions for these need to be defined.

10. PURIFIED SUB-UNIT AND LIVE ROUGH O-SIDE CHAIN DEFICIENT *B.ABORTUS* VACCINES FOR PROTECTION AGAINST ABORTION AND INFECTION IN CATTLE AND GOATS

Modern animal management systems demand continuous improvement in vaccines against zoonotic diseases, particularly brucellosis, occurring in livestock species utilized for food production. An improved brucellosis vaccine has been developed using transposon mutagenesis of *B.abortus* to create a panel of more than 1000 mutants. From *E.coli*, a TN5 transposon expressing kanamycin resistance was amplified, purified and electroporated into *B.abortus* strain 2308 expressing resistance to nalixidic acid. Mutants were selected by growth on media containing both kanamycin and nalidixic acid. All mutants were further selected for reduced survival in the macrophage-like cell-line, J774-A1 and subsequently reconfirmed for reduced survival in monocyte-derived macrophages from cattle phenotyped and genotyped for susceptibility to *B.abortus*.

Of more than 1,000 mutants produced in this way, over 60 were further selected for either deficient or undetectable expression of the O-chain of *B.abortus* LPS, but confirmed expression of Lipid-A, using MAbs specific for these moieties of *B.abortus*. Of these, two mutants expressed no O-side chain and were subsequently determined to have greatly reduced *in vivo* survival in BALB/C mice as compared with strain S2308 WT, and were in fact cleared from the spleens by four weeks post-challenge. Neither of these two mutants colonized the pregnant uteri of unvaccinated susceptible goats. The sequences of DNA flanking the TN5 transposon in these mutants were determined, and one mutant was shown to contain the homologue of the phosphomannomutase (rfbK) gene of *Salmonella typhimurium*, while the other was determined to contain the homologue of the TDP-rhamnose synthetase (rfbD) gene of *Vibrio cholerae*. The rfbK mutant of *B.abortus* was selected to produce killed and live vaccine preparations for protective immunity studies in cattle and goats.

Studies to assess protective immunity in cattle were conducted as follows: killed vaccines were injected intramuscularly on day 0 and day 60 into 12-14 month heifers (Table 2). These were oestrus-synchronized and bred naturally between day 56 and day 116. Only pregnant heifers were challenged intraconjunctivally, on day 245 with 10^7 cfu *B.abortus* strain 2308. Serology (card test, rivanol, CFT, competitive ELISA, rough indirect ELISA and smooth indirect ELISA) was done every other week from day -20 through day +392. Samples of 11 tissues from fetuses and 54 tissues from cows were collected and cultured for *B.abortus* at parturition to determine protection against abortion and infection by the challenge strain.

Table 2. Dosage and types of *B.abortus* sub-unit vaccines evaluated in pregnant first calf heifers

Vaccine	Dose/Route	Adjuvant	Injections	Interval (days)
S19	5×10^8 cfu/sq	No	1X	None
Adjuvant*	2ml/im	Yes	2X	60
Fusion Proteins of 7KDa Omp I,II, III	30mg.ea./im	Yes	2X	60

S2308 LPS	30 mg/im	Yes	2X	60
OM-PG rough	400 mg/im	Yes	2X	60
Purified S2308 7KDa, 8KDa	30 mg.ea./im	Yes	2X	60
Purified S2308 Omp I,II and III	30 mg.ea./im	Yes	2X	60

*0.25mg monophosphoryl lipid A, 0.25 mg. trehalose dimycolate, 0.25 mg muranyl dipeptide, 0.02 ml squalene and 0.002 ml Tween 80.

Purified fusion proteins, LPS, outer membrane peptidoglycan (OM-PG), P7 + P8, and Omp I, II and III gave 14%, 50%, 60%, 50% and 19% protection respectively against abortion in pregnant heifers. In comparison, adjuvant alone and S19 conferred 46% and 86% protection respectively. The corresponding figures for protection against infection were 14%, 21%, 47%, 25%, and 19% respectively for the sub-unit preparations, and 79% and 8% respectively for the S19 and adjuvant. In contrast, the OM-PG derived from *rfbK* TN5 mutant of *B.abortus* 2308 was more effective than S19 in protecting against abortion (88% and 80% respectively vs 45% for adjuvant control). For protection against infection, it was rather less effective than S19 (49% against 67%, compared with 0% for adjuvant alone).

The live mutant strains were actually more effective than S19 in protecting against both abortion and infection (Tables 3 and 4). They were also effective in protecting goats against *B.melitensis* infection (Table 5).

Table 3. Efficacy of live rough *rfbK* mutant of *B.abortus* S2308 in protecting pregnant Heifers against abortion

Vaccine	Dose	Calf		% Protected	vs. Saline	vs. S19
		Live	Dead			
Saline	-	11	13	45	-	0.030*
S19	$5x10^8$ cfu	16	4	75	0.030	-
rfbK mutant	$1x10^{10}$ cfu	17	4	77	0.028	0.938

** Fisher's Exact Test

Table 4. Efficacy of live rough *rfbK* mutant of *B.abortus* S2308 in protecting Pregnant heifers against *Brucella* infection

Vaccine	Dose	Calves *B.abortus*		% Protected	vs. Saline	vs. S19
		Positive	Negative			
Saline	-	25	0	0	-	0.00002*
S19	5×10^8 cfu	8	16	67	0.00002	-
rfbK mutant	1×10^{10} cfu	7	17	71	0.000007	968

* Fisher's Exact Test

All animals vaccinated with rough OM-PG sub-units or live rough *rfbK* mutant vaccines were negative to all standardized serological tests but, on the other hand, all developed antibodies to the rough-specific indirect ELISA post-vaccination.

Table 5. Efficacy of live rough *rfbK* mutant of *B.abortus* S2308 in protecting goats against infection with *B.melitensis* 133M

Vaccine	Dose	Culture *B.melitenis*		% Protected	vs. Saline	vs. *RfbK*
		Positive	Negative			
Saline	-	6	4	40	-	0.028**
rfbK mutant	$4x10^{10}$ cfu	2	13	87	28	-
RB51	$1x10^{9}$ cfu	1	14	93	6	1.000

*Goats challenged intraconjunctivally with $9x10^{5}$ cfu Mexican field isolate of *B.melitensis* 133M at 116 days post-vaccination.
**Fisher's Exact Test

In conclusion, cattle were protected against abortion (88%) and against infection (49%) by the (OM-PG) complexes harvested from a *rfbK* mutant of *B.abortus* S2308 and emulsified in a monophosphoryl Lipid A, muramyl dipeptide, and trehalose dimycolate in squalene based adjuvant when challenged at 245 days after vaccination with 1×10^{7} cfu of S2308. No cross reactive anti-LPS was detected in the rough OM-PG vaccinates for 245 days post-vaccination. Thus, rough OM-PG *Brucella* vaccines induce good protection against abortion and adequate protection against infection without stimulating cross-reactive antibody production against S-LPS .

Cattle were better protected against abortion (77%) and against infection (71%) by the genetically-defined live *rfbK* O-side chain-depleted mutant of *B abortus* S2308 than by S19, again without inducing anti-S-LPS antibodies. Thus, the *rfbK* mutant appears ideal for protecting cattle against *B.abortus* field strains. Likewise, goats were also protected against infection (87%) when challenged with a virulent *B.melitensis* Mexican field strain, and once again anti-S-LPS antibodies were not induced.

These data confirm that the *rfbK* rough mutant *B.abortus* vaccine, whether sub-unit or live, affords protective immunity against wild type *B.abortus* without inducing anti-S-LPS antibodies that confuse standard diagnostic tests for brucellosis.

11. NUCLEIC ACID VACCINES FOR BRUCELLOSIS

Although some developed countries have succeeded in eradicating brucellosis, the problem in these countries has usually been confined to cattle infected with *B.abortus* alone. The success rate in eliminating *B.melitensis* infection from small ruminants has

been very poor, and in several countries the situation is currently deteriorating still further. It is infection with this latter organism that poses the greatest threat to the public health.

Thus the threat to human health has generally not diminished in many regions where cultural practices hinder control and heighten public health risks. In addition, the prospect of biological warfare has reinforced the need for a vaccine suitable for the protection of human beings. Many countries accept that *Brucella* is one of the agents that could be used by an aggressor to target both troops and civilian populations (Franz *et al.*, 1997). There is no currently feasible means of protecting whole populations.

Although the classical vaccines, S19 and Rev.1, have been successful in controlling brucellosis in many countries over several decades, their use is associated with the type of problems already described. The features of ideal vaccines for the control of brucellosis in animals and for the protection of humans have been summarized in Section 3. In essence they should be cheap, non-pathogenic, provide long-term protection and not confuse diagnosis. The advent of nucleic acid or genomic vaccines offers a new approach to this problem.

This type of vaccine is based on the construction of plasmids containing cloned gene sequences encoding proteins capable of eliciting protective immune responses. These constructs usually also contain regulatory sequences designed to ensure translation and expression of the protective antigen in eukaryotic cells. Although either DNA or RNA may be used as the basis for such vaccines, in practice nearly all studies have used the former. Thus, Wolff *et al.* (1990) observed that plasmid DNA encoding marker genes could be expressed in mice following intramuscular injection and initiated an immune response related to *in vivo* gene transfer. The transfection rate was low, but internalised DNA could persist and be expressed for long periods, apparently without chromosomal integration of the transgene. Since then, rapid progress has been made with successful immunization against viral, bacterial and parasitic models. These include:

- Influenza HA and NP in mice, chickens and ferrets.
- *Plasmodium yoelii* CS AG in mice.
- *Leishmania major* gp63 in mice.
- Hepatitis B surface Ag in rabbits.
- Bovine herpes virus in cattle.
- *Mycobacterium tuberculosis* HSP65 and 85A Ag in mice.
- HIV-1, gp160 in mice and primates.
- *Brucella* L7/L12 gene in mice.

DNA vaccines can be delivered by a variety of routes including intradermal, intramuscular, or mucosal administration. Following intramuscular injection, the antigen expression occurs in skeletal muscle. However, it is not entirely clear whether the muscle cells or bone marrow derived cells within the muscle act as antigen presenting cells (Robinson, 1997). A number of hypotheses have been suggested to explain the effective immune responses seen.

Thus, transfected muscle cells could produce antigens which are subsequently taken up by lymphoid cells, or the lymphoid cells might themselves be transfected. In any case, these cells may travel to the lymph nodes where antigen presentation would occur with considerable efficiency, generating both a humoral and cell-mediated immune

response. However, there is some conflicting evidence that suggests that expression and presentation may be mediated solely by muscle cells *in situ.*

The route of delivery and subsequent processing has a profound effect on the balance of the immune response. For example, intramuscular administration of DNA in saline seems to promote a Th-1 response mediated by IL-2 and IFN gamma, whilst administration via a gene-gun appears to favour a Th-2 type response mediated by IL-4, 5, 6 and 10. A number of other delivery methods have been described, including incorporation in liposomes, cationic lipids and biodegradable microbeads.

Parenteral administration of DNA results in a strong humoral- and cell-mediated response, but does not provoke a mucosal response. Administration to mucosal surfaces has been shown to stimulate mucosal immunity, but only if the DNA is protected from extra-cellular enzymes by liposomes or cationic lipids or polymeric microbeads. This novel vaccine technology has potential advantages compared with conventional vaccines in terms of the following:

i) Expression of antigen in their native form resulting in improved processing and presentation.
ii) Ability to combine vaccines easily.
iii) Ease of industrial manufacture.
iv) Possibility of reducing the number of doses necessary for prolonged protection.

However, before such vaccines could achieve clinical reality certain hurdles would have to be overcome:

i) Post-mitotic muscle fibres alone should be transfected.
ii) The injected plasmid DNA should not be incorporated into the genome.
iii) There should be no deleterious effects of prolonged expression of the foreign antigen.
iv) Anti-DNA antibodies should not be elicited.

One limitation recognized is the feasibility of producing sufficient quantities of DNA for a successful vaccine programme. However, this may not be an insurmountable problem. Whilst current immunization protocols require DNA in relatively large quantities, improvements in vector design and delivery systems are likely both to improve expression levels and to reduce the amount of DNA required to elicit successful protection.

DNA vaccination potentially offers a number of advantages over not only the existing classical live vaccines and also over conventional vaccines of the future. The technology offers the potential for the protection of both animals and humans, using a vaccine safe to produce and administer.

Although humoral immune response plays an important role in immunity to *Brucella,* it is the cell-mediated response that is most important in providing protection. Abundant data suggest that the Th-1 type response is most effective and that the Th-2 type response may even be deleterious. Thus, optimal resistance to *Brucella* would be provided by vaccines that elicit a predominantly Th-1 response with the production of the protective cytokines, IFN-gamma and IL-2, which can be evoked by DNA immunization (Robinson,

1997). The stimulation of additional protection at the mucosal surface would potentiate resistance by limiting entry of infecting organisms.

The Brucella Research Group at the Central Veterinary Laboratory, Weybridge, UK (CVL) has a programme for the development of a DNA vaccine for brucellosis. The group works in collaboration with another that has successfully used this approach to immunize with a construct bearing the HSP-65 gene of *Mycobacterium*. This construct has given protection in mice against challenge with virulent mycobacteria. The Brucella Research Group have used PCR to clone the gene encoding the homologous *Brucella* HSP-62 gene into pcDNA3.1. This vector makes use of the strong CMV promoter for high-level expression in eukaryotic cells. Modifications to enhance expression (e.g. Kozak site) have been introduced during the PCR amplification process.

Prior to animal work it is important to confirm eukaryotic expression *in vitro*. This is done by the transfection of COS cells with the DNA vaccine construct, followed by immunofluoresent staining for expressed antigen. The DNA vaccine construct will initially be evaluated in a mouse model using the intramuscular route of immunization, known to induce a Th-1 response. In addition, alternative delivery methods will be evaluated, such as those involving microencapsulation in polymeric microparticles. The poly(lactide-co-glycolide) beads can induce a good mucosal immune response, as well as a good systemic response.

Should mouse studies prove successful, DNA immunization will be evaluated in large agriculturally important livestock. Whilst initial studies are concerned with protection against *B.suis*, the gene encoding the *Brucella* HSP-62 is highly conserved amongst all *Brucella* spp. This offers the potential to vaccinate against brucellosis in a range of animal species.

The DNA immunization strategy allows for the evaluation of the protective efficacy of any *Brucella* protein antigen for which the DNA sequence is available. This circumvents the need to prepare large quantities of recombinant antigen and overcomes the associated problems.

12. APPROACHES TO THE DEVELOPMENT OF A NEW HUMAN BRUCELLOSIS VACCINE

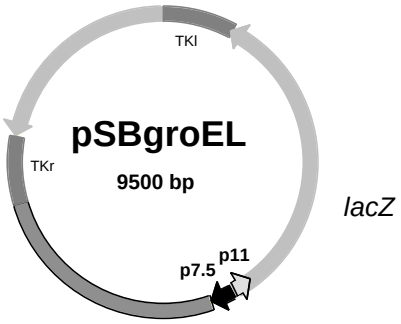
Brucellosis caused by *B.melitensis, B.suis, B.abortus* and *B. canis*, in decreasing order of pathogenicity, affects millions of people worldwide. The disease is endemic in many Mediterranean and Middle Eastern countries, Asia, Mexico, Central America and parts of South America. If untreated, the clinical phase can last from a few weeks to several years. Serious complications leading to disability or death can occur. While treatment with appropriate antibiotics is usually effective, prevention would be a better option. Currently available vaccines cannot be used in humans because of their side effects or lack of efficacy.

While antibodies may play an important role in protection in some animal species, as demonstrated in the mouse model, immunity to *Brucella* infection requires a strong CMI component. Specific antigens which can lead to CMI induction and protection include the L7/L12 protein. T cells, particularly those responsible for the production of INF-gamma (T helper1-CD4+ response), play a major role in protection. Cytotoxic T

cells (Tc, CD8+) are also important, since protective immunity can be demonstrated by passive transfer of either Th or Tc cells. It should be stressed that, although there is a variety of assays to measure induction of CMI, no assay has been demonstrated to be an unequivocal indicator of protective immunity in brucellosis.

Protective antigens may be cloned into expression systems that can present them appropriately to the immune system. One such approach currently under development at the Virginia Tech Center of Molecular Medicine and Infectious Diseases, in collaboration with the US Army, is to deliver *Brucella* antigens using vaccinia virus, by constructing vaccinia/*Brucella* antigen recombinants. In an ongoing study, various *Brucella* antigens are being expressed and the recombinants are being tested for their ability to induce immune responses in mice and to induce protection against challenge with a virulent *Brucella* strain. A *Brucella* GroEL vaccinia recombinant which expresses the whole GroEl protein has been constructed from the Western Reserve vaccinia strain using an early/late vaccinia promoter p7.5. *Brucella* antigens are expressed by this system and antibody and CMI responses can be induced but, protection has not yet been achieved.

Figure 1 Figure 2



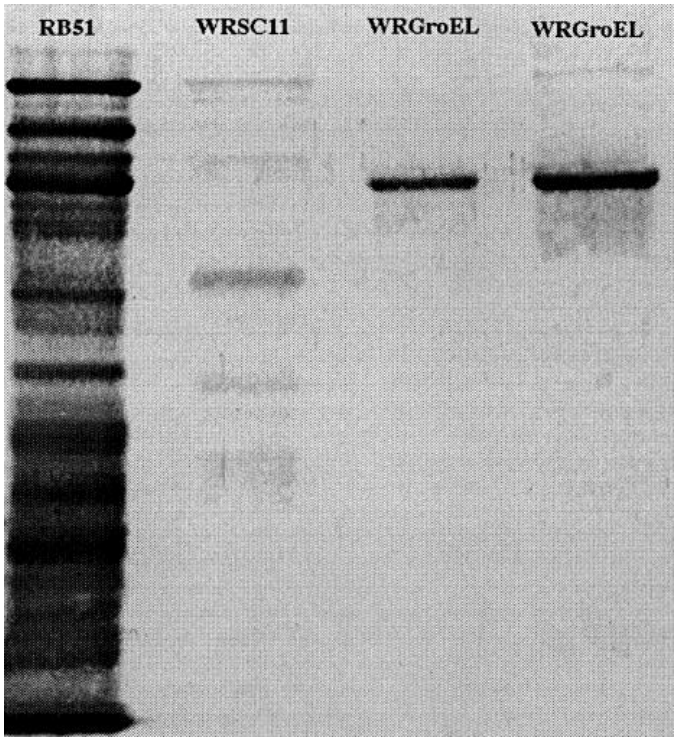


Figure 1. Diagram of recombinant plasmid pSBGroEL. A 1.7 kb fragment containing the *B.arortus groEL* gene (cross hatched line) was cloned into the shuttle vector pSC11 (light grey regions). The early/late vaccinia virus promoter p7.5 (solid arrow) regulates expression of the *groEL gene* and a late vaccinia virus promoter p11 (dotter arrow) regulates the expression of the *lacZ* gene. The vaccinia virus thymidine kinase sequences (TK1, Tkr) flank the expression cassette controlled by the p7.5 and p11 promoters.

Figure 2. Western blot analysis of vaccinia virus recombinant WRGroEL (2 different stocks) and plasmid control (WRSC11) and *B.abortus* strain RB51 antigens with goat anti-RB51 serum.

It is believed that this lack of protection is linked to the fact that the p7.5 promoter does not give the appropriate *in vivo* expression levels of GroEL needed to induce an adequate CMI response. For this reason, new vaccinia recombinant constructs are being prepared using a synthetic early/late promoter. Recently, shuttle vectors (pMCO2 and

pSC65) with a synthetic early/late promoter have been developed. Expression of the cloned genes by the recombinant vaccinia viruses prepared with these new vectors should be 1000 times greater than with natural early or late vaccinia promoters. Higher levels of *Brucella* protein expression by the recombinant vaccinia viruses which will also express IL-12 should enhance the specific and hopefully appropriate CMI responses in the vaccinated mice, leading to a better chance of protection against a virulent *Brucella* challenge.

Based on this rationale, the 18kDa gene was cloned in shuttle vector pMCO2 and recombinant vaccinia virus was constructed using the WR strain (vWRMC18). Mice were vaccinated intraperitoneally with vWRMC18 at 10^7 pfu/mouse and bled at several intervals post vaccination. Strong antibody responses were observed against the 18kDa antigen and the vaccinia virus antigens, confirming replication of the virus as well as *Brucella* antigen recognition (Fig.3). Lymphocytes obtained from the spleens of these mice proliferated *in vitro* upon stimulation with purified MBP-18 kDa fusion protein and produced IFN-gamma. The *in vitro* CMI results combined with the strong antibody responses to the 18 kDa protein, encourages the use of the synthetic promoter in future constructs with GroEL or other potentially protective antigens (Fig.4).

Figure 3

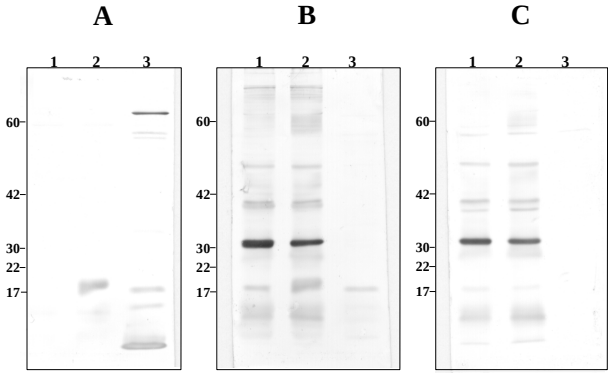


Figure 4

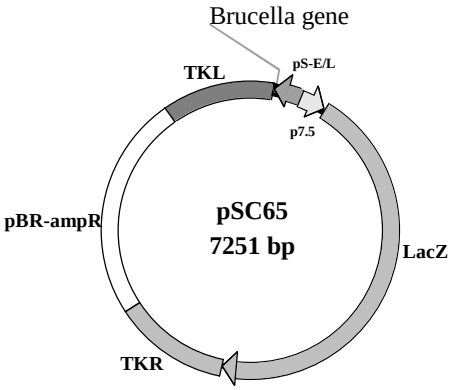


Figure 3. Extracts from cells infected with the recombinant vaccinia virus containing the 18 kDa protein gene either in the wrong orientation (lane 1) or in the right orientation (lane 2), and *B.abortus* strain RB51 (lane 3) were reacted with sera from mice vaccinated with *B.abortus* strain RB51 (panel A), the recombinant vaccinia virus expressing the 18 kDa protein (panel B), and the recombinant vaccinia virus not expressing the 18 kDa protein (panel C).

Figure 4. Schematic representation of the vaccinia shuttle vector pSC65 where *Brucella* genes can be cloned under a synthetic early/late promoter.

The US Army is developing human vaccines to protect soldiers against *Brucella* delivered via aerosol or other mucosal routes. The noncovalent complex of *Brucella* LPS and *N.meningitidis* group B outer membrane protein is to be tested in a primate model, since this complex gives good protection in the intranasal mouse challenge model. The *B.melitensis purE* deletion mutant shows evidence of attenuation in human monocyte-

derived macrophages and in the mouse model and will be tested in primates. However, this mutant has shown evidence of residual virulence in other species and may not be suitable for use as a human vaccine. Additional deletions could produce further attenuation and a greater margin of safety. Recent observations that vaccine strain RB51 appears to be avirulent for humans indicates that use of "debilitated" rough strains for human immunization purposes is a possibility. The relevance of mice and/or ruminants as models for human disease is unclear and this has forced the use of non-human primate models for vaccine testing.

An important aspect of development of human brucellosis vaccines is the extension from research results to commercial application. With few exceptions, vaccines against bacterial zoonoses have not attracted much interest from industry in the recent past. This is largely because of financial factors. Understandably, manufacturers are reluctant to commit resources to products for which the market is perceived to be limited and which may yield a low return on investment. This situation is likely to apply to vaccines against many zoonoses, most of which are prevalent in developing countries with little means to finance vaccine development or purchase. Manufacturers will need to be encouraged to undertake promotion of such products and this will require a commitment from international development agencies.

13. GENETIC RESISTANCE TO BRUCELLOSIS

The animal genome always influences and sometimes determines susceptibility to bacterial diseases, yet because of the huge variety of pathogens and the multitude of complex host defence mechanisms involved, a simple understanding of resistance rarely emerges. Natural disease resistance refers to the inherent capacity of an animal to resist disease when exposed to pathogens, without prior exposure or immunization. Although some of the observed variation in natural resistance is related to environmental factors, a significant component of variation in natural disease resistance is heritable and, therefore, passed consistently from parent to offspring. Given this series of complex host-pathogen interactions, it is obvious that control of natural bacterial infection and resulting disease would rarely be controlled by a single gene, although expression of an allele at one locus can significantly affect disease pathogenesis in individuals; at the herd and population levels, however, many genes would be operational in controlling the spectrum of disease expression.

Why then is genetic resistance not being used more in modern livestock industries, since there is no lack of evidence of genetic control for disease resistance? Perhaps it is because regulatory officials, owners, producers and other industry managers do not recognize the potential for genetic resistance, which would not necessarily be the tool to replace traditional methods of disease control, but would add another approach to reduce the impact of bacterial pathogens on animal health and to play a role in system-based approaches such as the pre-harvest pathogen reduction programme. Newer strategies to increase the overall level of resistance at herd and population levels by using selective breeding programmes to enhance natural resistance would be expected to contribute significantly in this regard.

In time, any gene involved in resistance to brucellosis should be genetically linked to a useful marker gene. Further advances in genetic technology should provide practical methods for using knowledge of disease resistance genes to improve the overall health of

livestock. Together with current approaches to improved vaccines and health management, genetic manipulation to increase natural disease resistance to brucellosis is predicted to improve significantly the health and productivity of domestic animals.

13.1 Host genes involved in resistance to *Brucella*

The best defined host-pathogen systems from the perspectives of effector mechanism and genetics are those involving facultative or obligate intracellular bacterial pathogens.

Nramp1 [*Bcg/Ity/Lsh*]. Variations in the functional capacity of phagocytes affect disease susceptibility, an example of which is the expression of the *Nramp1* gene. Studies over the last two decades have led to the identification of a gene, *Bcg/Ity/Lsh*, on chromosome 1 in mice which alters the early stages of resistance to diverse and antigenically unrelated facultative and obligate intracellular pathogens, including *Mycobacterium bovis* (Bacille Calmette-Guérin) (*Bcg*), *M.intracellulare, Salmonella typhimurium* (*Ity*), and *Leishmania donovani* (*Lsh*). In domestic animals, homologues for *Nramp1* have been identified, sequenced and/or mapped in cattle, chicken, swine, and sheep, but thus far associations with disease resistance have only been documented in cattle (Tables 6 and 7).

MHC (major histocompatibility complex). The MHC is an important genetic complex controlling immune responses). The ability of an individual to respond to certain antigens is under genetic control through the MHC.

Bovine brucellosis. Studies to determine if resistance to *B.abortus* is heritable and to identify genes controlling resistance to brucellosis in cattle began in the late 1970s. Unvaccinated and previously unexposed sexually mature bulls or heifers at mid-term gestation were challenged with a standardized discriminating challenge inoculum of *B.abortus* strain 2308, scored for the outcome of parturition, and quantitative cultures were collected from tissues and secretions 3-5 months later. Immune correlates with respect to macrophage function, *BoLA* alleles, and immunoglobulin allotypes associated with natural resistance to *Brucella* were determined retrospectively (as summarized in Table 6) and breeding experiments were begun to identify genes controlling differential immune responses.

Table 6. Characteristics of macrophages, T cells, and antibody responses in cattle naturally resistant or susceptible to *B.abortus*

Biological Activity	Resistant	Susceptible
Intracellular growth of *B.abortus, M.bovis, S.dublin*	Restrictive	Permissive
Phago-lysosomal fusion	Increased	Reduced
Production of Reactive Oxygen Intermediates	High	Low
LPS + IFNgamma induced Nitric Oxide production	High	Low

B.abortus binding to macrophages	RGDS tetrapeptide, *B.abortus* LPS and MAb anti-LFA-1 inhibit 90% binding	Minimal inhibition
Antibody response to *B.abortus* LPS	Minimal, short duration	Massive, long duration
IgG_{2a} A1 and A2 allotypic response to *B.abortus* LPS at 6 wks.	Å50:50	95% A1
Oligoclonal T cell response	Only *B.abortus* stimulates	*B.abortus, B.suis, B.melitensis, and B.canis* stimulates

The differential response between macrophages from resistant and susceptible cattle were similar to those observed in mice strains resistant and susceptible to *M.bovis* BCG, *L.donovani, M.paratuberculosis*, and *S.typhimurium*. Furthermore, macrophages from cattle which were selected for *in vivo* resistance to *B.abortus* restricted the intracellular replication of *B.abortus*, *M.bovis* BCG, and *S.dublin* significantly better than macrophages from cattle are susceptible to *B.abortus* challenge. Consequently, the bovine homologue to the murine *Nramp1* gene-designated bovine *NRAMP1*, was cloned and the cDNA sequenced. Thus, the hypothesis was further supported by the conservation of the *Bcg/Lsh/Ity*-bovine *NRAMP1* aminoacid sequences and the conservation of the linkage group on mouse chromosome 1 and bovine chromosome 2. Northern blotting confirmed that bovine *NRAMP1* was primarily expressed in macrophages of the RES. Single-stranded conformational analysis (SSCA) disclosed a highly significant association of a single stranded conformation polymorphism located in the 3' untranslated region of bovine *NRAMP1* with cattle naturally resistant to brucellosis (See Table 7).

Table 7. Association of bovine *NRAMP1* SSCA polymorphism with bovine brucellosis resistant and susceptible phenotypes

NRAMP1 SSCA Type	*In vivo* Resistant Phenotype	*In vivo* Susceptible Phenotype
Resistant SSCA	9*	2
Susceptible SSCA	2	9
Total	11	11

*Significant association - p = 0.0089 (Fisher's Exact Test)

In classical breeding studies, natural resistance to *Brucella* was demonstrated to be dramatically increased from 20% to 60% by simple mass selection in one generation of selective breeding. The genetic analysis of these mating results was consistent with the existence of two or more genes controlling the resistant phenotypes. This study also illustrates that individual genes, or gene effects, that have an effect on natural resistance can be identified, despite of complex patterns of inheritance, and that the frequency of these genes could realistically be increased through selection or gene manipulation procedures.

Porcine brucellosis: Studies of genetic control of resistance to *B.suis* demonstrated that 73% (24 of 33) of the progeny from the resistant swine were resistant to the challenge infection as compared to only 9% resistant (3 of 24) progeny in the control. It is remarkable that in one generation of mass selection, resistance to *B.suis* could be increased by approximately 64% over unselected controls. Obviously, few genes are involved in controlling resistance to *B.suis* in swine.

13.2 Future strategies for genetic resistance to brucellosis

Clearly any form of resistance to any infectious disease is relative rather than absolute. This is true for vaccine-induced resistance as well as for natural resistance. Thus, breeding animals to increase their level of natural resistance will not completely prevent infectious diseases. In combination with a vaccination programme, genetic resistance to brucellosis could provide even better protection than using either measure alone. Indeed, a strategy could include selection for appropriate immune responses to vaccination. Several strategies for improving genetic disease resistance have been previously proposed. Current techniques of animal breeding, using direct marker-assisted genetic selection for measurable traits, such as for bovine brucellosis, can be applied to the problem of increasing natural disease resistance. With the progress in the development of genetic and physical maps of domestic animal genomes having informative markers throughout the genome, identity-by-descent and identity-by-state analyses of affected sib pairs becomes a very powerful tool to identify major disease resistance loci. In this way, the linked markers or the gene can be used to determine the relative risk of clinical disease in exposed individuals.

It is becoming increasingly possible to transfer innate and adaptive disease resistance genes into embryos taken from animals that have superior production traits by micro injection of pronuclei, retroviral insertion and stem cell insertion.

Without doubt, vaccination programmes and other modalities have contributed significantly to reducing losses from brucellosis. However, with the exception of a few diseases in specific geographical areas, bacterial diseases such as brucellosis have not been eradicated and remain a major cause of animal and economic loss. The aim of applying genetic selection to the problem of controlling infectious diseases in domestic animals is not so far into the future. Use of such basic information to enhance herd health would be a significant adjunct to current and future bacterial disease control modalities for diseases of domestic animals, including brucellosis, within the next decade.

14. REGULATORY ASPECTS OF NEW BRUCELLOSIS VACCINES: POINTS TO CONSIDER

Before a new vaccine against brucellosis can reach the implementation stage, it will have to undergo a complex process to satisfy regulatory requirements and to achieve marketing authorization. This will require provision by the manufacturer of information satisfying the minimum requirements for assurance of safety, efficacy, quality and consistency. Usually this information is obtained in several stages, some of which may run concurrently. A brief outline of these requirements is as follows.

14.1 Pre-clinical evaluation

This will be a laboratory-based assessment in which the emphasis will be placed on testing in animals to identify potential safety problems. The principal aims will be to:

i) Identify inherent toxicity or other adverse effects.
ii) Identify potential target organs for adverse effects.
iii) Identify factors promoting risk of adverse effects.
iv) Estimate safe starting dose.
v) Identify appropriate parameters to monitor in clinical trials.

Studies to identify potential adverse effects need to address the following issues:

i) Acute toxicity.
ii) Local injection site reactogenicity.
iii) Induction of hypersensitivity.
iv) Chronic toxicity.
v) Carcinogenicity.
vi) Teratogenicity.
vii) Genotoxicity.

For traditional vaccines based on killed whole-cells or live attenuated strains, the points listed under (iv), (v), (vi) and (vii) are not usually considered applicable. However, for new types of vaccine based on recombinant strains or nucleic acids, these issues must be addressed.

Vaccines differ from therapeutic drugs in a number of important aspects, and these should be taken into account when designing pre-clinical testing protocols. These factors include:

i) Few doses are usually needed per lifetime.
ii) The dosage is usually low - micrograms compared with milligrams or grams for therapeutic drugs.
iii) Overdosage is unlikely to be achieved, although the possibility of immunological impedance, e.g. through blocking of pre-existing antibodies, should not be disregarded.
iv) Daily or more frequent dosage is not used.
v) The aim is to stimulate a response to foreign (non-self) antigens.
vi) Hyper-stimulation is unnecessary and may be undesirable.
vii) The vaccine is intended to target the immune system; involvement of other systems should be minimal.

It is usually desirable to test the product in at least two animal species, one of which may be a rodent. Primate testing is not usually required, unless the product is completely novel and raises safety issues which might not be assured by testing in non-primates. The dosage and schedule used should relate to those intended for final use. In practice, overdosage is almost invariably used in animal testing.

14.2 Clinical evaluation

In some countries this will require formal authorization (e.g. Investigational New Drug in USA and Clinical Trial Certificate Exemption in UK). To obtain this, information must be provided on the product, manufacturing process and pre-clinical testing.

The clinical studies will normally be performed on a phased basis.

Phase 1 uses adult volunteers and is intended to identify problems of toxicity and reactogenicity. Immunogenicity may also be examined at this stage.

Phase 2 is intended to assess reactogenicity and immunogenicity in the target age-groups. Unlike phase 1 which normally only requires a small number of subjects, usually 10-50, phase 2 will usually involve groups of 50-100 or more subjects.

Phase 3 is intended to assess efficacy under field conditions. It will usually involve thousands of subjects. Ideally, assessment is made under randomised double-blinded conditions, comparing the test vaccine with a placebo. In practice, constraints are often imposed by ethical factors or by the low incidence of disease in the target population. Various compromise systems may then be used. These will provide information of lesser precision and with a greater degree of statistical uncertainty.

Phase 4 is intended to assess the safety and efficacy at the population level. The number of subjects may amount to hundreds of thousands or millions. Usually effects have to be assessed by a case-control method. This phase is not usually an obligatory requirement for marketing authorization.

14.3 Implementation at the population level

This will normally only be feasible once the vaccine has produced acceptable results in pre-clinical studies and phases 1, 2 and 3 of clinical trials. However, marketing authorization may be permitted without phase 3 data when circumstances make the acquisition of such data impracticable. Full details must be provided of product, method of manufacture, in-process checks, quality control and quality assurance, and results of clinical studies.

14.4 Post-marketing surveillance

Even when in routine use, the quality of the vaccine should be monitored by examination of samples taken at the point of application and by periodic surveillance of the population (or a sub-set thereof) for evidence of efficacy (indicated by a reduction in disease incidence) and adverse effects.

14.5 Specific aspects

New brucellosis vaccines are likely to fall into one of the following groups, and each of these approaches raises different regulatory issues.

14.5.1 Live attenuated strains

These may include natural mutants, mutants produced by non-specific agents (e.g. chemicals, radiation), site-directed mutants or recombinant strains.

Key issues include:
i) Stability of strain.
ii) Maintenance of attenuation.

iii) Potency.
iv) Consistency.
v) Stability of presentation.

Information will need to be provided on the following aspects:
i) History of strain.
ii) Characterization of strain.
iii) Preparation of primary seed and working seed lots.
iv) Methods of production.
v) In-process controls.
vi) Final product testing.
vii) Specification.
viii) Reference material.
ix) Assignment of clinical trial batch(es) for reference.

Crucial factors include the establishment of markers of attenuation and of a suitable procedure for monitoring potency. The use of an appropriate animal model and a reference vaccine will generally be essential, particularly if the genetic basis of attenuation is unknown. Where the strain contains defined genetic modifications, *in vitro* methods such as pulsed field gel electrophoresis or PCR plus sequencing of relevant genes may be acceptable alternatives to the use of *in vivo* systems.

14.5.2 Non-living vaccines

These may include preparations of killed whole-cells, cell-free extracts, cell wall preparations, outer membrane proteins, outer protein membrane vesicles, purified sub-units from natural or rDNA strain, anti-idotypes such as synthetic peptides/mimeotopes, and will usually be based on purified proteins, carbohydrates or glicolipids. These may be used in their native form or after conjugation to specific protein carriers to enhance their immunogenicity. Antigens such as lipopolysaccharides may require detoxification to reduce inherent toxicity. The requirements will differ somewhat according to the nature of the material.

a) Purified protein or polysaccharide sub-units

Information is required on:
i) Seed lot system.
ii) Culture media.
iii) Inactivation/detoxification, if relevant.
iv) Purification.
v) Characterization - physical/chemical/biological/immunological; assay of potency; formulation; stability; consistency.
vi) Reference preparations. Similar requirements will apply to protein-protein or protein-polysaccharide conjugates but, in addition, information will be needed on the conjugation process, the subsequent purification and the characterisation procedures used to assure compliance with product specifications.

For all these preparations, a suitable potency assay will be necessary unless the clinical trial data show that a correlation can be demonstrated between efficacy and chemical composition.

b) Recombinant DNA Products

Regulatory requirements

General: Safety, quality, efficacy and consistency must be satisfied as for the natural product.

Specific: Factors specific to recombinant systems need to be addressed. These include:

i) Stability of foreign genes in new host.
ii) rDNA products may differ from natural counterparts.
iii) Effect of manufacturing process on impurity profile of final product.
iv) Unintentional expression of other genes through process variations.
v) Modification of product on "scale-up".
vi) DNA contamination of final product.

Information will be required on the following:

i) Development genetics (cloned gene, host, construct, properties and stability of the expression system).
ii) Control of seed lots.
iii) Fermentation; definition of batch, processing (single/multiple harvests).
iv) purification; methods of validation, justification of procedures, evidence of elimination of contaminants and impurities.
v) Control of seed lots, characterization of active ingredient; physico-chemical, structural, post-transitional modifications, conformational; bio-activity; potency; purity.
vi) Consistency and batch control; identify, purity, potency, safety.
vii) Specification and reference materials; assignment of clinically validated batch
viii) development of finished product formulation; justification of excipients. preservatives, etc.

The need for a suitable potency test will be as relevant as for sub-units produced from the natural strain. The WHO guidelines for rDNA products should be followed (WHO, 1991).

14.5.3 Genomic (nucleic acid) vaccines

These are based on DNA or RNA sequences encoding specific protective antigens. Usually they will be presented in an expression system which includes promoters for eukaryotic cells. So far most products have employed DNA sequences cloned into bacterial plasmids. These have raised considerable concerns with regulatory authorities. The main issues are:

i) Stability of construct.
ii) Fidelity of expression *in vivo.*
iii) Interaction with recipient chromosomes.
iv) Significance of persistence *in vivo.*
v) Oncogenic potential.
vi) Mutagenic potential.
vii) Genetic effects on recipients and offspring.
viii) Immunopathology.
ix) Environmental issues.

Some of these concerns may ultimately prove to be unjustified. Nevertheless they will need to be addressed if the vaccines are to gain acceptance from clinical application. Information will be required on:

i) Development of the DNA vaccine.
ii) Complete nucleotide sequence.
iii) Identification, source, isolation and sequence of the cloned gene.
iv) Fractional map of the plasmid with details of regions derived from eukaryotic sources.
v) DNA sequence homology check to exclude sequences encoding unintended biologically active molecules.
vi) Deletions of section markers.
vii) Evidence for stability of the construct *in vivo* especially in respect of possible rearrangements.
viii) Use of a cell bank and working cell bank.
ix) Consistent control of fermentation conditions, culture growth and plasmid yield,
x) Description of harvesting, extraction and purification procedures.
xi) Efficiency of removal of unwanted nucleic acid.
xii) Characterization of bulk purified plasmid (sequence, integrity, methylation etc.).
xiii) Levels of contaminants including protein, denatured DNA endotoxin.
xiv) Potency of the final vaccine.
xv) Details of adjuvants, excipients and mode of delivery.
xvi) Data on stability and batch-to-batch consistency.
xvii) Reference materials.

If multiple plasmids are to be used to produce a multi-component system, each must be fully characterized and possible interactions considered. If plasmids encoding cytokines are included, information will be required to establish the safety of these plasmids. Guidelines have been prepared for DNA vaccines and these should be followed (WHO, 1998; Robertson and Griffiths, 1997)**.**

15. WHAT DOES THE VACCINE INDUSTRY NEED FROM THE RESEARCH COMMUNITY?

In progressing from research concept to marketable product, the research community will inevitably focus on activities other than those of direct interest to the industry. However, it is crucial that both research organizations and companies understand each others needs. The diversity of research organizations and vaccine-producing companies makes it difficult to define precisely where the interactions occur between these two parties.

It is of crucial importance that researchers concentrate on research concepts and try to evaluate the possible applications of their research. One particular discovery could for example be exploited to develop a vaccine (one type of application) or a diagnostic tool (another type of application) or both.

Research and development (R&D) departments of veterinary pharmaceutical companies often concentrate on product development after deciding to use a scientific discovery in a particular application. Marketing departments develop concepts to bring products to the market. The route from research concept to marketing concept is not a one-way street. The reality of the market sometimes influences R&D activities, including fundamental research. Companies strongly recognize that one of the key elements in

determining success is the effectiveness of information exchange between marketing and R&D operatives.

Another essential element in determining outcome is the effectiveness of information exchange between companies and the research communities. This interaction may involve not only researchers and R&D managers from companies but also researchers and marketing managers.

15.1 *Brucella melitensis* infection as an example

B.melitensis infection is an important zoonosis worldwide. The disease occurs in sheep, goats, cattle and camels and is often a problem in poor countries. These factors strongly influence the product development strategy of companies. This process consists of choosing between the different research options and is divided into two parts. The first part consists of choosing the essential characteristics of the vaccine at the level of efficacy, safety, etc. In other words, an attempt is made to define a list of realistic criteria. To do this, it is necessary to know the specific features of the disease against which one wants to design a vaccine. The second part consists of choosing the type of vaccine that could meet these criteria and in defining the R&D plan. The involvement of all participants in the project, including research organizations, is desirable both at the level of the choice of criteria and at the stage of the design of the R&D plan. It is the quality of these choices that will dictate whether or not the R&D project will generate a marketable product.

The following are only a few examples of specific features of the disease followed by the criteria derived from them.

Because *B.melitensis* infection is a zoonosis:

i) Many countries will set up disease control or eradication policies which are managed at national level. This will influence the way companies market their products.
ii) A good vaccine will not only have to prevent disease symptoms such as abortion (in itself a source of economic loss), but will also have to prevent excretion of virulent *Brucella* organisms after natural challenge in order to prevent transmission of disease from animals to man.
iii) A live vaccine will have to be adequately attenuated so as not to cause disease in humans.
iv) A vaccine should confer protection to main species (e.g. sheep, goats, cattle, buffaloes and camels) subject to infection by *B.melitensis*.

Because the economic situation in many countries does not allow for expensive and complicated elimination programmes:

i) A vaccine must be cheap and easy to produce in large quantities.
ii) For vaccines which produce sero-conversion, the associated diagnostic test must be cheap and easy to perform.
iii) The vaccine should provide life-long immunity after only one administration.

These are only a few criteria. The next step consists in the definition of the R&D plan.

It is the common task of researchers and companies to decide on the best route to follow between research concept and marketable product. Collaboration between these groups and other interested parties, such as regulators, is essential. Organizations such as WHO, FAO and OIE play an essential role in bringing them together.

16. CONCLUSIONS AND RECOMMENDATIONS

Recent progress in the development of new brucellosis vaccines has indicated a number of areas in which specific action is recommended to enhance prevention in animals and to achieve a workable vaccine for humans.

16.1 Animal vaccines

Correctly standardized Elberg 101 strain Rev.1 vaccine should continue to be considered as the basis of brucellosis control in small ruminants where vaccination is applied, until new safer and effective versions of *B.abortus* and *B.melitensis* vaccines, based on rough strains, are tested under controlled experimental and field conditions and shown to be at least equivalent to the Rev.1 vaccine.

Defined rough *Brucella* strains may prove to be a suitable alternative to Rev.1 for the protection of sheep and goats. Moreover, rough strains of *B.abortus*, e.g. RB51 and *rfbK* have been shown to be effective in preventing brucellosis in cattle without causing serological complications and abortion. Preliminary studies indicate that RB51 and *rfbK* may also protect goats against *B.melitensis*.

Additional research under controlled laboratory conditions (first phase) must to be performed to establish the protective value of the rough strains of *B.melitensis* or *B.abortus*, in comparison with Rev.1, against standard challenge with *B.melitensis* in small ruminants. If such additional experiments are carried out, it is essential to comply with the following conditions:

i) Protocols and place of performance to be discussed and agreed upon beforehand by the participating institutions.
ii) The cloned vaccine strains to be tested must be issued from one source.
iii) A statistically significant number of *Brucella*-free animals should be used in each experimental group.
iv) A reference group of Rev.1 vaccinated pregnant females should be included in each experiment. A batch of Rev.1 prepared from authentic seed and subjected to approved control testing should be used.
v) The virulence challenge must include conjunctival challenge with *B.melitensis* strain H38 (INRA) in doses of $5x10^6$ and $5x10^7$ cfu for goats and sheep respectively. Optimally, titrated challenge is recommended.

Once satisfactory results are obtained from laboratory experiments, the second phase of evaluation of safety and efficacy of rough strains should be implemented in field experiments, with unvaccinated control groups, and accompanied by careful

epidemiological, clinical (e.g. attack rate), serological and bacteriological investigations of animal and human populations.

The recommendations of the WHO working group meeting on brucellosis control and research (WHO, 1992) regarding the *B.suis* S2 vaccine are still valid: S2 might overcome some of the shortcomings of Rev.1 vaccine (S2 is more stable and less virulent). Additional research should therefore be carried out to study further the potential of S2 as an oral vaccine in areas where whole flock vaccination is the only possibility. However, in those countries currently using or considering the use of S2 it is strongly recommended that a protection experiment following the conditions outlined above be conducted to confirm the efficacy of the vaccine.

B.abortus S19 and RB51 are currently vaccines of choice for *B.abortus* prophylaxis in cattle. The problem of *B.melitensis* infection in cattle may be addressed by conducting controlled protection experiments with the authentic Rev.1 strain compared with S19 and RB51, and any other candidate vaccines. Eventually, adequately protective live vaccines developed from rough strains should be considered for adoption to allow for vaccinations compatible with eradication programmes.

Conventional vaccines as well as new rough and other vaccines should be evaluated in other susceptible animals species (e.g. camels, buffaloes, swine, yaks, etc). This is particularly relevant for developing countries.

16.2 Human Vaccines

Evaluation of candidate vaccines is an area of high priority for development and should begin without delay. Challenge experiments are unlikely to be feasible, and efficacy will need to be determined in the first instance on the basis of non-human primate studies, although field studies may be possible in certain populations. Placebo-controlled double blind trials would be ideal but may be limited by factors such as the incidence of the disease and by ethical considerations.

Initial candidate vaccines for study could include the *B.abortus* RB51, *rfbK* or equivalent *B.melitensis* live attenuated rough strains. Deletion or interruption mutants of *B.melitensis,* incorporating the *purE* deletion are also candidates for study.

Promising results have also been obtained with "O" polysaccharide-protein conjugate vaccines. These and the Russian acid-extracted polysaccharide also deserve further attention. DNA vaccines are potentially of interest although unlikely to reach clinical trial stage for some years.

Several research centres should be involved in these studies and links with vaccine manufactures should be established.

Brucella vaccines are not a priority for the WHO Global Programme on Vaccines and Immunization (GPV), the advice of this group on the design of pre-clinical and clinical evaluation studies should be sought.

16.3 Genetic resistance to brucellosis

It has been demonstrated that genetic resistance to brucellosis in livestock can be selected for. Furthermore, the frequency of this trait can be enhanced in herd populations. Therefore, in order to implement genetic selection in *Brucella*-exposed animals, suitable samples should be collected and stored for retrospective studies. Prospective studies should be performed to evaluate the effects of selection on the incidence of brucellosis in cattle and small ruminants.

The specific recommendations are:

i) Collect and store total leukocytes and/or DNA from cattle, sheep or goats naturally challenged and confirmed to be culture positive for *Brucella spp* for subsequent NRAMP1 genotyping by PCR-based single stranded conformational analysis (PCR-SSCA) for association with resistance or susceptibility to brucellosis. This should be done in an international reference or resident countries laboratory.

ii) Genotype by PCR-SCCA for NRAMP-resistant allele, DNA from cattle, sheep or goats that are being considered for importation to improve other production traits.

iii) Genotype DNA by PCR-SCCA from sires, and if possible dams, from national or private reproduction resource centres (i.e. studs) to increase the frequency of the resistant allele and improve potential vaccine responses against brucellosis and increase the composite herd brucellosis resistance.

iv) Evaluate the effects of NRAMP1 resistance allele selection on the incidence of brucellosis in cattle, sheep and goats under field conditions.

v) Pursue basic research to discover and characterize additional brucellosis resistance genes in cattle and small ruminants.

16.4 Identification of protective antigens and virulence factors

Very few protective *Brucella* antigens have been identified until now. Identification of these antigens is essential for developing new generation *Brucella* vaccines for animals and humans. Putative candidate antigens should be screened for protection *in vivo* and in immunological assays *in vitro.*

16.5 Mucosal immunity

The role of mucosal immunity in brucellosis is not well understood. Research should be conducted to elucidate the role of mucosal immunity and to identify ways of inducing it either alone or in combination with systemic immunity.

16.6 Killed Vaccines

Killed vaccines are of lower priority for animals, but may not be do for humans. They may be useful for special situations in which live attenuated vaccines cannot be used, and may be used for revaccination purposes. Additional research should be carried out on selection of suitable adjuvants and on optimization of the killing (irradiation, chemical, heat, etc.) and formulation process to be applied.

Non-replicating live (irradiated) vaccines should also be considered as candidates. They contain metabolically active bacteria which may express antigens relevant to *in vivo* growth. They are usually more effective than killed vaccines but less so than live vaccines.

16.7 General recommendations

The role of WHO in promoting international collaboration in brucellosis research has extended over half a century. This should be continued and strengthened, in collaboration with other international organizations such as the FAO and OIE and the reference laboratories.

The participants stressed the need for enhancing public understanding of brucellosis, and therefore recommended that: a) an international conference on brucellosis, covering all areas of research and field aspects, should be organized under the auspices of the relevant International Organizations as soon as possible, and b) an International Society for Brucellosis should be established on the model of the International Society for Leptospirosis.

ANNEX 1

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ANNEX 2

Acronyms

Bcg	Bacille Calmette-Guérin
BCSP	*Brucella* Cell Surface Protein
CFT	Complement-fixation test
cfu	colony forming units
CMI	cell mediated immune mechanisms
GM-CSF	granulocyte macrophage-colony stimulating factor
HS	hot saline extract
IFN-gamma	gamma interferon
IL-1	interleukin-1
INRA	Institute National de la Recherche Agronomique
MAb	monoclonal antibody
MHC	major histocompatibility complex
OM-PG	outer membrane peptidoglycan
Omps	outer membrane proteins
O-PS	O-polysaccharide
pfu	plaque forming units
R&D	research and development
rfbD	TDP-rhamnose synthetase gene
rfbK	phosphomannomutase gene
R-LPS	rough-lipopolysaccharide
SDS-I CW	sodium dodecyl sulphate-insoluble cell wall
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
S-LPS	smooth-lipopolysaccharide
SSCA	single stranded conformational analysis
S2	live attenuated vaccine *B.suis* strain 2
S2	*B.suis* strain 2 vaccine
Th1	T-helper 1
Th2	T-helper 2
TNF-alpha	tumour necrosis factor alpha