Journal of Applied Microbiology 2005, **98,** 1270–1281 doi:10.1111/j.1365-2672.2005.02622.x

A REVIEW

Brucellosis – new aspects of an old disease

S.J. Cutler, A.M. Whatmore and N.J. Commander

Bacterial Zoonoses, Statutory and Exotic Bacterial Diseases, Veterinary Laboratories Agency, Surrey, UK

2004/0842: received 21 July 2004, revised and accepted 9 February 2005

1. SUMMARY

Clinical conditions synonymous with brucellosis have been described since the time of Hippocrates. Indeed, it has been suggested that organisms resembling brucellae have been detected in carbonized cheese from the Roman era (Capasso 2002). These observations highlight how both man and animals have lived with this disease since the days of early record keeping. Despite the numerous biotechnological improvements we now have, brucellosis remains a major worldwide zoonosis. Some countries have been successful in eradication of disease, while others, especially in the developing world still live with a huge disease burden. This review outlines some of the early significant historical milestones then brings the reader to the current challenges that face us in detection, microbial typing, taxonomy, vaccine development, understanding the host–microbial interactions and virulence mechanisms of these enigmatic bacteria.

2. INTRODUCTION

Brucellosis is a major bacterial zoonosis of global importance. The causative organisms are Gram-negative facultative intracellular pathogens that may affect a range of different mammals including man, cattle, sheep, goats, swine, rodents and marine mammals. In most host species, the disease primarily affects the reproductive system with concomitant loss in productivity of animals affected. In man, infection is associated with protean manifestations and characteristically recurrent febrile episodes that led to the description of this disease as 'undulant fever'. The disease is severely debilitating and protracted with several documented cases with signs associated with the disease lasting for over 30 years. It is believed that one such individual was Florence Nightingale, who endured over 25 years of ill health, personality changes (neurobrucellosis) and spondylitis which left her bedridden for 6 years of her life (Young 1995; Baly 1996; Dossey 1998).

The severity of this disease and lack of vaccines suitable for use in man has led to the investigation of *Brucella* as agents for bioterrorism. Indeed, the American military weaponized *Brucella suis* in 1954 (Greenfield *et al.* 2002), however, changing global politics resulted in abandonment of these efforts following the biological and toxic weapons convention in 1972.

Eradication of brucellosis has been a goal for many countries, with success in several countries in northern Europe such as the UK. Those countries that do eradicate infection cannot afford to be complacent as the threat of re-introduction is ever present through the movement of livestock. In order to control brucellosis, comprehensive surveillance, pre and postimport testing is of paramount importance (England *et al.* 2004).

Correspondence to: Sally J. Cutler, Bacterial Zoonoses, Statutory and Exotic Bacterial Diseases, Veterinary Laboratories Agency, Surrey KT15 3NB, UK (e-mail: s.cutler@vla.defra.gsi.gov.uk).

With the significant advances in postgenomics and proteomics, it was anticipated that we could finally gain an insight into the pathogenic mechanisms of the brucellae. Genomes of *Brucella melitensis* (DelVecchio *et al.* 2002), *B. suis* (Paulsen *et al.* 2002) and *Brucella abortus* (Sanchez *et al.* 2001) have been completely sequenced while *Brucella ovis* is currently in progress. Scrutiny of these sequences for classical virulence factors has been remarkably unsuccessful, suggesting that these microbes utilize highly specialized pathogenic mechanisms to subvert host cells.

The host susceptibility of the brucellae has similarly been a focus for research. How these highly homogeneous microbes show such distinct host associations has been questioned for many years. What is now becoming apparent is the ability of different brucellae to infect multiple host species, for example, the finding of *B. melitensis* infection in cattle (Godfroid and Kasbohrer 2002). It is possible that now our methods for microbiological typing are becoming more refined, that we can now gain new insights into the susceptible host ranges for brucellae.

The recent finding of *Brucella* spp. in marine mammals has also required us to readdress this genus. Taxonomy of these isolates remains unresolved although there is genetic evidence that these isolates may comprise a number of distinct groups (Cloeckaert *et al.* 2001b). The significance of these isolates remains to be established, both as potential zoonotic agents and as a source of infection for terrestrial mammals (Brew *et al.* 1999; Rhyan *et al.* 2001; Sohn *et al.* 2003).

This paper will review our knowledge of brucellosis with discussions on the impact of more recent findings on our understanding of this fascinating yet enigmatic microbe.

3. BACKGROUND

'*Micrococcus melitensis*' was first isolated by David Bruce in 1887 from spleen tissue of human cases that had died as a result of 'undulant fever'. The year 2004, marks the centenary of the formation of the Mediterranean Fever Commission, assembled to tackle the threat of brucellosis to British troops stationed in the Malta. Within 2 years its members had not only isolated the causative organism from goats, but also drawn the epidemiological link with consumption of goats milk. This led to the recommendation that all milk be boiled prior to consumption, which in turn reduced the incidence of brucellosis in man (Dalrymple-Champneys 1950a, 1950b).

The clinical presentations of brucellosis in man received much attention, with detailed descriptions not only of the undulant fever experienced by these individuals, but also, the joint manifestations, particularly spondylitis, neurological complications associated not only with personality changes (anxiety, amnesia, delusions, hallucinations, delirium, phobias and irritability), but also pronounced and prolonged frontal or occipital headaches, anorexia, abdominal pain and arthralgia (Dalrymple-Champneys 1950a).

Diagnostic tests were developed based on agglutination methods, complement fixation tests and later using enzyme-linked immunoassays. These assays have been plagued with problems of both sensitivity and specificity with the result of many modifications to the earlier tests (Nielsen 2002).

Lengthy campaigns have resulted in eradication of brucellosis from several countries in northern Europe; however, much of the developing world is still in the early stages of attempting to control this disease of significant economical and zoonotic impact. Furthermore, brucellosis is also considered a re-emerging problem in many countries such as Israel, Kuwait, Saudi Arabia, Brazil and Colombia, where there is an increasing incidence of *B. melitensis* or *B. suis* biovar 1 infection in cattle (Corbel 1997).

4. TAXONOMY OF THE BRUCELLAE

The debate of whether the brucellae should comprise a single monospecific genus or multiple species has been a source of much controversy. Differences in biochemical capabilities and susceptibilities to dyes and phages together with the differences observed in host preference, led to the division of the brucellae into six species, some of which could be further divided into biovars (this excludes those isolates from marine mammals to which formal names are yet to be approved). However, based on DNA–DNA hybridizations and with the advent of sequence-based approaches using the 16sRNA gene, the remarkable homogeneity among this group was revealed, and in consequence, the suggestion that they should be considered as a single species, *B. melitensis* (Verger *et al.* 1985, 1987). This has not been universally accepted, with many holding onto the previously used multiple species nomenclature on practical grounds, while others use nomenspecies. Other methods using restriction mapping and cross-hybridization to provide genomic indexing show agreement with the classical taxonomy of this genus (Verger *et al.* 1987; Michaux-Charachon *et al.* 1997). Large-scale sequencing studies are currently underway and should help resolve the taxonomic standing of the *Brucella* species and may provide sufficient data to allow a robust phylogeny of the group to be constructed.

5. POSTGENOMICS

Full genomic sequences have now been published for *B. melitensis* (DelVecchio *et al.* 2002) and *B. suis* (Paulsen *et al.* 2002), while the release of the completed genomic sequence for *B. abortus* is imminent. A further genome is currently being sequenced for *B. ovis*. This information will be of

huge value enabling identification of unique open reading frames (ORF) specific to these species. The possession of these unique species-specific ORF should however, be further validated by investigation of multiple isolates of brucellae both within species and between biovars. This could be easily facilitated through the use of either microarrays or through other hybridization or sequence-based approaches (Rajashekara *et al.* 2004). The caveat that of these approaches is that the gene of interest must be present, where this is not known, other approaches such as subtractive hybridization analyses may offer an alternative method for scrutinizing the differences between isolates and strains.

Comparative genomics of *B. melitensis* and *B. suis* has revealed remarkably few specific ORF compared with other similar microorganisms (Paulsen *et al.* 2002; Tsolis 2002). These studies revealed only 42 unique genes in *B. suis* and 32 for *B. melitensis* (Paulsen *et al.* 2002), which were largely associated with genomic islands suggesting a possible origin through lateral gene transfer (Rajashekara *et al.* 2004). It has been postulated that the relative lack of evidence for genetic exchange among the brucellae may result from their preference to persist within a protected intracellular niche, consequently, reducing opportunity for genetic exchange. This is further supported by the lack of plasmids or lysogenic phage within the brucellae.

Comparative genomic analysis of *B. suis* and other members of the α-proteobacteria, in particular, belonging to the family *Rhizobiaceae*, has shown distinct similarities among these organisms both in genome structure and metabolic capabilities (Paulsen *et al.* 2002; Tsolis 2002). Of particular interest is the homology between two-component regulatory systems essential for intracellular replication (*BvrR/BvrS*), tumour formation in plants (*ChvG/ChvI*) and establishment of endosymbiosis (*ExoS/ChvI*) for *B. suis*, *Agrobacterium tumefaciens* and *Sinorhizobium meliloti* respectively (Cheng and Walker 1998; Sola-Landa *et al.* 1998; Lopez-Goni *et al.* 2002). Furthermore, the recent characterization of a type IV secretion system in *Brucella* that is essential for intracellular survival (O'Callaghan *et al.* 1999; Boschiroli *et al.* 2002) and their paralogs in *A. tumefaciens* essential for tumour formation in plants (*virB* genes) has highlighted the possible common mechanisms of pathogenesis utilized by these microbes (Tsolis 2002).

Scrutiny of genomes for classical virulence markers has shown a remarkable absence of these genes, such as capsules, fimbriae and toxins (Delrue *et al.* 2004). Of surprise was the identification of genes encoding putative flagellin components (Halling 1998). It was believed that these microbes were nonmotile and whether these genes are functional and expressed is a subject of intense debate. It is possible that these genes may have a role *in vivo*, but this remains to be established. Technological advances have now made it possible to selectively purify total RNA from intracellular brucellae enabling detailed study of the trancriptome of these pathogens (Covert *et al.* 2005).

The extreme uniformity among the brucellae may offer an opportunity to elucidate both pathogenicity-related genes and discovery of reliable epidemiological markers through comparative genomic analysis (Rajashekara *et al.* 2004). Whether these differences lie at the level of genes or single nucleotide polymorphisms (SNPs) remains to be resolved.

6. PROTEOMICS

Our understanding of the proteome of the brucellae is still in its infancy. Postgenomic approaches outlined above have much potential to identify differences in gene possession, or for the identification of SNPs; however, differential pathogenesis among these pathogens may result from post-translational modifications such as glycosylation. To date studies of the *Brucella* proteome have been limited. Recent investigations comparing proteomes of *B. melitensis* 16M and the vaccine strain, Rev1, have revealed differences in expression of the 31-kDa immunogenic outer membrane protein, regulatory proteins involved in the acquisition of iron, those utilized for sugar and amino acid binding, lipid degradation (Eschenbrenner *et al.* 2002; Mujer *et al.* 2002; Wagner *et al.* 2002). Insufficient data is currently available to conclude whether these differences are associated with the differences in virulence seen between these strains; however, it provides useful baseline information. As these studies were performed using organisms cultivated *in vitro*, the possible lack of expression of nonconstitutive virulence-associated genes must be considered. As biotechnology advances, so does the possibility of assessing the proteomics of these microbes in intracellular environments. Investigation of proteomics of *Brucella* in cell lines derived from different tissues, and from susceptible and nonsusceptible host species, should shed light on these elusive mechanisms central to host–microbial interactions.

7. HOST–MICROBIAL INTERACTIONS

Application of novel techniques is being used to help unravel details of the host–microbial interactions for brucellae. As yet the details of how these facultative intracellular pathogens interact with the host cells remain poorly understood (Moreno and Moriyon 2002). Most work has addressed the survival of *Brucella* within the macrophage where these bacteria can infect, persist and replicate for prolonged periods within their host. However, it must be remembered that these microbes have the ability to infect epithelial cell lines, both professional and nonprofessional phagocytic cells, respiratory tissue, neurones, male and

female reproductive tissues to name but a few (Moreno and Gorvel 2004). This extreme diversity of host–microbial interactions remains to be investigated.

Smooth brucellae gain entry into the host cells following interaction with cell surface microdomains known as lipid rafts (Watarai *et al.* 2002). These lipid rafts contain significant amounts of glycosylphosphatidylinositol anchored proteins, glycophingolipids and cholesterol and are believed to play a significant role not only for internalization, but also for intracellular replication of brucellae. Investigation of the cellular interactions following internalization within macrophages have demonstrated that brucellae reside within the acidified phagosome (Rittig *et al.* 2001). Indeed, the acidification of this environment is an important stimulus for expression of virulence genes of the *virB* operon, essential for expression of components needed for assembly of a type IV secretion system required for intracellular trafficking (Rouot *et al.* 2003). Once inside the host cell phagosome, and after being subjected to the initial oxidative burst, the internalized brucellae commence their subversion of the host phagosome through mechanisms as yet not fully elucidated. What is known is that the products of *vir*B operon interact with the endoplasmic reticulum in a way that the pH becomes neutral, nitrate ions may be utilized for anaerobic respiration and the brucellae undergo regulated multiplication (Celli *et al.* 2005). This modified phagosome has been termed as the 'brucellosome' and as yet is poorly understood, however, cellular interaction through the type IV secretion system and host endoplasmic reticulum play a crucial role (Kohler *et al.* 2003). One outcome of this cellular subversion is prevention of lysosomal fusion. The brucellae survive within this environment for prolonged periods, but must endure conditions of nutrient depletion and limited available oxygen (Moreno and Gorvel 2004).

Interaction with placental trophoblasts suggests that the ability to acquire iron is vital as the brucellae enter their acute replicative stage with placental disruption resulting in fetal loss or birth of weak and infected offspring. It is interesting to note that iron acquisition is altered in the attenuated vaccine strain of *B. melitensis*, Rev1 when compared with the virulent strain, 16M (see section 6; Eschenbrenner *et al.* (2002). Erythritol is believed to be important for determining tissue tropism for *B. abortus*. Indeed, *Brucella* uses this sugar alcohol in preference over alternatives and mutants unable to utilize erythritol are severely attenuated in ruminant hosts. *In vitro* data suggests that *Brucella* metabolizing erythritol have a heightened requirement for iron, scavenged through siderophores such as 2,3-dihydroxybenzoic acid or brucebactin (Gonzalez Carrero *et al.* 2002; Parent *et al.* 2002; Bellaire *et al.* 2003). This may be linked with the requirement for effective iron acquisition for virulence in ruminant hosts.

Novel iron regulatory systems have recently been proposed in other α-proteobacteriae, namely *Rhizobium*, but the role of these if any, among the closely related brucellae remains to be addressed (Chao *et al.* 2004; Platero *et al.* 2004; Yeoman *et al.* 2004).

8. VACCINES

The development of an efficacious vaccine for brucellosis has been a challenge for scientists for many years. Despite the availability of two smooth live vaccine strains, S19 for cattle and Rev1 for small ruminants and a further rough attenuated strain, RB51 for cattle, the search for improved vaccines has continued. This is in part through the remaining virulence of the smooth vaccine strains in human hosts (Hoover *et al.* 2004), their residual arbortifacient potential in pregnant animals (Blasco 1997) and their interference with conventional serological assays (Schurig *et al.* 2002). In addition, the Rev1 vaccine has suffered from a lack of coordinated standardization in production methods leading to considerable variability in efficiency of different preparations (Blasco 1997) and carries resistance to streptomycin, an antibiotic that is therapeutically useful in man (Cloeckaert *et al.* 2002). Some advantage has been offered through the availability of the rough vaccine strain, RB51. This is an attenuated rough mutant of *B. abortus* that has provided promising data for vaccination of cattle, however, results from vaccination of wildlife have given less encouraging results with failure to protect species including sheep, bison, reindeer or elk (Cutler and Whatmore 2003). It is generally acknowledged that all of the available vaccines are only efficacious in specific hosts, and cross-protection is not readily achieved. Currently, no vaccine is available for protection of swine from brucellosis; however, this area is being actively addressed. The RB51 vaccine has been assessed in swine, but with mixed results (Lord *et al.* 1998; Edmonds *et al.* 2001; Moriyon *et al.* 2004). Efficacy testing in large, well-controlled groups will be essential in order to evaluate performance of these candidate vaccines in swine. Despite the variable success of the RB51 rough vaccine, it may indeed have a valued role for booster immunization of livestock immunized during calf hood with smooth S19. Use in this manner would provide immunological stimulation, but without concomitant elevation of antibodies specific for the lipopolysaccharide (LPS) diagnostic antigen (Moriyon *et al.* 2004).

Another area of interest is the potential of 'carrier' organisms to serve as a delivery vehicle for antigens of other microbes. Organisms considered as 'safe' such as *Lactococcus lactis*, have been engineered to encode *Brucella* vaccine candidate genes, with partial success (Pontes *et al.* 2003). Despite the lack of detectable specific antibody a partial protective effect was demonstrable using this

microbial vehicle that did not deliver target antigens through the intracellular route. To overcome these criticisms and induce a good Th1 stimulation, an essential prerequisite for immunity against intracellular pathogens, RB51 has been assessed for its ability as a multivalent vaccine controlling not only brucellosis, but also bovine tuberculosis (*Mycobacterium bovis*) and Johne's disease (*Mycobacterium avium paratuberculosis*) (Vemulapalli *et al.* 2002). However, the less than optimal success of the current RB51 in wildlife would be a cause for concern if this live vaccine was shed for prolonged periods by vaccine recipients (Januszewski *et al.* 2001; Cook *et al.* 2002; Elzer *et al.* 2002).

One potential avenue for development of novel vaccines is 'nonliving vaccines'. Historically, successful vaccines against intracellular pathogens such as *Brucella* have consisted of live attenuated strains of the organism. Although the protective response generated by live vaccination is both efficacious and sustained, there is a trade-off to be made in terms of bacterial persistence within target host and potential transmission to unintended recipients (such as pregnant livestock or hosts in which the vaccine strains remain virulent), and the possibility of reversion to virulence. Nonliving brucellosis vaccines have been developed and used in the past, however, with mixed results (Ko and Splitter 2003). Vaccines utilizing killed preparations of *Brucella*, or antigenic fractions, had only limited success compared with the live attenuated strains. Vaccines based upon killed preparations of *Brucella*, have been shown to be protective but a single immunization is often insufficient to generate sustained protective immunity. Additional problems including local reactions at the inoculation site and production of antibodies complicating serodiagnosis, contributed to decline in use of such vaccines (Schurig *et al.* 2002). Recent advances in immunological understanding, particularly regarding adjuvant technology and delivery systems may be able to address some of the problems encountered with use of nonliving vaccines. Novel adjuvants and delivery systems could be utilized to both enhance and focus the immune response against subunit antigens. Use of poly-epsilon-caprolactone microparticles for the delivery of hot saline extracted antigens of *B. ovis* has shown protection against homologous challenge in the mouse model, however the response was less effective against challenge with smooth strains of *Brucella* (Murillo *et al.* 2001, 2002a, 2002b, 2002c).

DNA vaccination approaches offer the possibility of inducing both cellular and humoral responses. Approaches have varied from use of a whole library from *B. abortus* (Leclercq and Oliveira 2003), overcoming the need for prior knowledge and selection of specific antigens to selection of specific candidates and their subsequent evaluation as DNA vaccines against brucellosis. Various candidates have been explored for their value as DNA vaccines against brucellosis providing various levels of protective efficacy in the mouse model (Al-Mariri *et al.* 2001a, 2001b; Leclerq *et al.* 2002; Velikovsky *et al.* 2002; Onate *et al.* 2003; Munoz-Montesino *et al.* 2004). Disadvantages of the DNA vaccination approach are the amount of DNA required to elicit the required response, and the often disappointing results obtained following assessment of the vaccines in the target animal (Babiuk *et al.* 2003; van Drunen Littel-van den Hurk *et al.* 2004). Investigation of enhanced delivery mechanisms may overcome these issues.

The availability of the genome data and the use of postgenomic approaches to identify potential vaccine candidate antigens, together with the expanding knowledge of the protective immune response may yet provide an efficacious nonliving vaccine. Whether this type of vaccination approach could be cost-effective to deliver benefit where the disease has greatest impact, either following humanitarian upheavals or in areas of extreme poverty, remains an open question.

9. DIAGNOSTICS

9.1 Serodiagnosis

Diagnostic methods for brucellosis have primarily been based on serology with the LPS from smooth strains producing greatest immunological responses in various hosts. One of the major diagnostic problems results from the similarity of the O-antigenic side chain of LPS of *Brucella* with other microbes, in particular, with *Yersinia enterocolitica* 0 : 9 (Kittelberger *et al.* 1995). In *Brucella* this is composed of a linear polymer comprised of 4,6-dideoxy-4-formamido-α-D-mannose joined by 1, 2 or 1, 3 glycosidic bonds to the O-polysaccharide, forming the basis for differentiation by A and M epitope-specific monoclonal antibodies. This cross-reactivity between *Brucella* and other microbes has restricted the specificity of many diagnostic approaches. Diagnosis is further complicated by the use of smooth live vaccine strains (see section 8; Schurig *et al.* (2002). Despite these hurdles, modest improvements have been made with the introduction of assays such as the competition ELISA (Nielsen *et al.* 1995; Lucero *et al.* 1999). Furthermore, some assays have been modified to provide screening tools for bulk milk samples from livestock (1995; Vanzini *et al.* 2001; Funk *et al.* 2005). The various merits of serodiagnostic methods for brucellosis have been recently reviewed (Nielsen 2002).

Alternative antigens have been evaluated for their diagnostic potential and possible improvements in specificity, however, these have largely been less useful than the highly immunogenic O-antigen of LPS (Letesson *et al.* 1997; Cloeckaert *et al.* 2001a; Estein *et al.* 2002; Munoz *et al.* 2005).

Flourescence polarization assays (FPA) offer a valuable alternative to conventional serological tests. This assay measures the size of a fluorescently tagged molecule such as antigen in solution by its rotation. Rotational speed is increased if the size of the particle increases, for example through specific binding of antibody to the antigen. Consequently, this technique can be readily adapted to the detection of either antibodies or antigens through the concomitant change in rotational rate (Jolley and Nasir 2003). Ideally, antigens selected for this technique should be small (*c.* 20 kDa) to allow maximal change mediated through interaction with antibodies. Synthetic peptides derived from linear epitopes could be utilized to provide standardized antigen conjugated with fluorescein. Assays utilizing the O-side chain of LPS from *Brucella* spp. have been used with encouraging results (Nielsen *et al.* 1996, 2000, 2004).

Concern has been expressed over the detection of naturally rough strains of *Brucella* such as *B. ovis*, *Brucella canis* and the rough vaccine strain of *B. abortus*, RB51. Diagnosis of rough stains has been achieved using LPS extracts from rough strains either in ELISA or FPA assay formats (Vigliocco *et al.* 1997; Nielsen *et al.* 2004).

Immunoassays based upon cell mediated responses may have some application in the diagnosis of disease. The brucellin-based skin test, based upon intradermal inoculation of LPS free antigen preparations and subsequent monitoring of the delayed type hypersensitivity induced swelling at the inoculation site, offers advantage over the conventional serological assays for the specific diagnosis of bovine brucellosis, where possible cross-reaction in conventional serological assays is suspected (Pouillot *et al.* 1997; Saegerman *et al.* 1999; Bercovich 2000). However, the advantages gained in specificity are reduced following vaccination, and the necessity for two farm visits, delay between repeat tests, and subjective nature of result interpretation, make this type of assay impractical for high throughput diagnosis.

In vitro cell mediated immunity assays based upon proliferation or interferon (IFN)-γ production following specific antigenic stimulation, also offer advantages in specificity over serological assays (Kittelberger *et al.* 1997). Recent studies have indicated that the *in vitro* IFN-γ assay offers similar specificity advantages for diagnosis of porcine brucellosis, although again lacking the individual animal sensitivity and practicability of the conventional serological tests (S.J. Cutler, S.D. Brew, R. Thirlwell, L.L. Perrett, E. Young, N.J. Commander, P. MacMillan, T. Murphy, A.M. Whatmore, S. Shankster and P. Browning, unpublished data).

9.2 Antigen detection

Detection of antigen rather than antibody has given promising results in some studies, however, numbers of brucellae present in the blood stream are often low, and usually only present during acute stages of infection. Controversy exists over whether whole blood or serum provide the better medium for amplification assays (Zerva *et al.* 2001; Vrioni *et al.* 2004; Queipo-Ortuno *et al.* 2005). Amplification of various gene targets has been used for diagnostic PCR, with these assays applied to samples of blood, milk, tissues and semen (Leal-Klevezas *et al.* 1995; Romero *et al.* 1995; Queipo-Ortuno *et al.* 1997; Gallien *et al.* 1998; Guarino *et al.* 2000; Amin *et al.* 2001; Zerva *et al.* 2001; Al Nakkas *et al.* 2002; Bricker 2002; Hamdy and Amin 2002; Navarro *et al.* 2002; Richtzenhain *et al.* 2002; Manterola *et al.* 2003). Antigen detection methods provide a rapid means of confirming the presence of *Brucella*, and can even be used for limited speciation (Bricker and Halling 1995; Bricker *et al.* 2003b). More recently, real-time PCR methods have been used for detection of *Brucella*, offering improvements in detection times and specificity through incorporation of hybridization probes (Newby *et al.* 2003; Al Dahouk *et al.* 2004; Probert *et al.* 2004). Of those methods published, some ask initially whether *Brucella* is present or not (usually with a *bcsp31* or IS*711* gene targets) and then follow this with molecular speciation (AMOS PCR). A combined approach offering genus detection of *bcsp31*and identification of *B. melitensis* or *B. abortus* through targeting an IS*711* sequence, can be achieved in a real-time multiplex format, however, at the cost of sensitivity with this assay only capable of 150-fg DNA detection limit (Probert *et al.* 2004).

Further benefit of PCR-based approaches has been demonstrated using known regions of gene deletion as the amplification targets. This permits differentiation of wild type and mutant strains by size differences in their amplicons and has proven particularly useful for differentiation of attenuated vaccine strains (see Fig. 1) (Sangari *et al.* 1994; Bricker and Halling 1995).

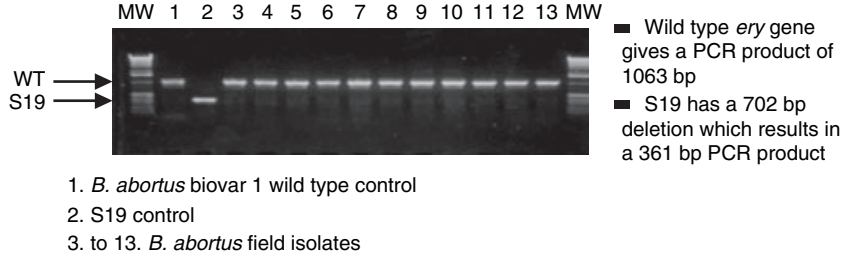

Fig. 1 The PCR differentiation of wild-type and S19 vaccine strains of *Brucella abortus*

The caveat to remember is that these microbes reside at intracellular locations, and are only present in readily collectable fluids such as blood, semen or CSF intermittently. Consequently, the application of antigen based methods is best restricted to acute outbreak situations, postdelivery, where both milk or abortion material can be sampled, or tissues and lymph nodes taken at the time of sacrifice. Once the host becomes pregnant, the brucellae multiply within the placental tissues resulting in abortion associated with liberation of large numbers of bacteria, and frequently facilitating transmission to others within the herd. At this point it is likely that tests will give their best performance.

9.3 Molecular typing

9.3.1 Outer membrane protein typing. A typing approach successfully used for differentiation of microbial groups has been PCR of a target gene with a high degree of diversity and its subsequent digestion with restriction endonucleases to produce restriction fragment length polymorphism (RFLP) profiles for differentiation based on polymorphic alleles. Several *Brucella omp* genes were considered as potential targets for typing schemes, however, the *Omp*2 locus [*Omp*2a and *Omp*2b (Cloeckaert *et al.* 2001b), 36-kDa porin proteins (Cloeckaert *et al.* 1995)] show greatest polymorphism among the brucellae. Consequently, these could be utilized to differentiate brucellae. Some additional value can be gained by including the more homogeneous *Omp*25 gene (Cloeckaert *et al.* 1996; Vizcaino *et al.* 2004). A further *Omp* gene, *Omp*31, is less useful as it is absent from all *B. abortus* biovars (Vizcaino *et al.* 2001); however, polymorphism at this *Omp31* locus successfully enabled differentiation of *B. canis* from *B. suis* (Vizcaino *et al.* 1997, 2004).

This method can differentiate isolates into nomenspecies, and in some cases, to biovar, however, resolution provided by this method was not generally sufficient to reliably identify biovars (Cutler *et al.*, unpublished data). Furthermore, this assay is not entirely *Brucella*-specific, with *Ochrobactrum* generating a product with the *Omp*25 PCR, albeit of smaller size. This method has proved itself of value for characterization of novel isolates recovered from marine mammals (Cloeckaert *et al.* 2001b; Vizcaino *et al.* 2004). Typically, classical biotyping is unable to differentiate these isolates, however, clear clustering is observed with the *omp* profiles of these strains.

9.3.2 IS711 typing. Chromosomal indexing of DNA polymorphisms can be achieved through use of the variable number and location of copies of a mobile insertion sequence believed to be specific for *Brucella*. Genomic DNA is digested with restriction endonucleases such as *Eco*R1, and resulting fragments probed with DIG-labelled IS*711*. This insertion sequence is present in all strains of brucellae tested to date, with copy number varying from four or five to more than 20 copies seen among different nomenspecies and isolates of *Brucella*, whilst being absent from closely related bacterial species. To this end, IS*711* provides a specific target able to generate data on DNA polymorphisms among the brucellae (Cloeckaert *et al.* 2000).

As with *omp* typing discussed above, IS*711* profiling of brucellae did not permit resolution of *Brucella* strains into the same groups as classical phenotype identification, although both methods, particularly when used in conjunction, are valuable tools for use in epidemiological studies (Cutler *et al.*, unpublished data). It could be argued that the IS*711* typing provides a more in depth method of indexing isolates as data is produced from the whole chromosome rather than a targeted gene. Although the lack of complete resolution may reflect biological reality it emphasizes the need for continued research and development into new epidemiological methods.

9.3.3 Amplified fragment length polymorphism. Amplified fragment length polymorphism (AFLP) has recently been heralded as a useful technique to differentiate numerous bacterial species. This technique provides a valuable method to index variation over the whole genome. The resolving power can be modified by the use of different combinations of enzymes and primers permitting assessment of varying levels of genomic diversity. The AFLP technique is based on the amplification of subsets of genomic restriction fragments using PCR. DNA is cut with restriction enzymes and double stranded adaptors ligated to the ends of DNA fragments to generate template DNA for PCR amplification. The sequence of the adaptors and adjacent restriction site serve as primer binding sites for subsequent amplification of the restriction fragments. Selective nucleotides can be included at the 3′-ends of the PCR primers, which therefore prime DNA synthesis from only a subset of the restriction sites. Labelling of one of the primers with a fluorescent dye permits visualization of a banding pattern following electrophoresis on an acrylamide gel.

This method clearly identified isolates as *Brucella* and, with some minor exceptions, produced adequate resolving power to designate isolates to one of the currently recognized nomenspecies (Whatmore *et al.* 2005). On occasions, isolates could be assigned to particular biovars or groups of biovars. Unfortunately, resolution proved inadequate for epidemiological tracing purposes (Whatmore *et al.* 2005). Although AFLP data may be of limited epidemiological significance, it should provide useful information on the taxonomy of *Brucella* and the relationship between the various nomenspecies and biovars.

9.3.4 Variable number tandem repeat typing. Use of the forensic based method of variable number tandem repeat typing has recently proved itself as a valuable approach for profiling highly homogeneous populations of microbes (Keim *et al.* 1999). This method has also been shown to provide high level of discriminatory power among the brucellae (Bricker *et al.* 2003a). This discriminatory power varies dependant on which loci are used. Deducing those loci best suited for epidemiological investigations is currently under investigation. Once the technique is refined, there will be a requirement for provision of a bank of data against which new profiles can be assessed.

10. WHERE DO WE GO FROM HERE?

We are still at a stage where we have far more questions than answers. Brucellosis remains a major worldwide zoonosis (Cutler and Whatmore 2003). Only 17 countries now claim to be free of disease, however, huge investment in surveillance is essential to maintain this status. Others still suffer as a result of enormous disease burden, not only through the loss of productivity and trade of livestock, but also as a result of severe and debilitating zoonotic disease in man. An estimated 500 000 human infections per year still occur worldwide. Global variation ranges from incidence of <1/100 000 population in UK, USA and Australia, to 20–30/100 000 in southern European countries such as Greece and Spain, and up to >70/100 000 in Middle Eastern countries, for example Kuwait and Saudi Arabia. Incidence data from many areas afflicted with extreme poverty is not available, however, is believed to be high. In order to eradicate or at least control disease, good surveillance, reliable tests and efficacious vaccines are of paramount importance. Current tests, although useful, are constrained through problems of both sensitivity and specificity. In consequence, Jacques Godfroid from Brussels disclosed that 'none of the conventional serological tests used for the diagnosis of porcine brucellosis are of use in the individual animal' (Cutler and Whatmore 2003). As outlined in the sections above, provision of these basic tools remains elusive, however, improvements are being offered through the gradual evolution of these methods.

Our understanding of the biology of the brucellae has made significant progress over recent years, however, our comprehension of pathogenicity mechanisms remains evasive. Despite genome sequencing and provision of advanced biotechnology, we still seek to understand the details of host–microbial interactions. Certainly, tools such as confocal microscopy and time-lapse video microscopy have been valuable aides in the study of microbial and host–cell interactions, and we look forward to their application to unravelling details of infection processes among brucellae. Results from molecular and proteomic investigations should eventually lead to a greater understanding of the mechanisms employed for the highly adapted pathogenesis of these microbes.

11. ACKNOWLEDGEMENTS

Thanks to the *Brucella* group at VLA Weybridge and those in the *Brucella* community for helpful advice and discussions. We also thank Defra who have funded much of our brucellosis work and the EU FP5 programme (QLRT-1999-00014) for their support in our vaccine studies. We are indebted to COST Action 845 on Brucellosis in Man and Animals and to MedVetNet for facilitating meetings and collaborative links.

12. REFERENCES

Al Dahouk, S., Tomaso, H., Nockler, K. and Neubauer, H. (2004) The detection of *Brucella* spp. using PCR-ELISA and real-time PCR assays. *Clin Lab* **50**, 387–394.

Al Nakkas, A.F., Wright, S.G., Mustafa, A.S. and Wilson, S. (2002) Single-tube, nested PCR for the diagnosis of human brucellosis in Kuwait. *Ann Trop Med Parasitol* **96**, 397–403.

Al-Mariri, A., Tibor, A., Mertens, P., De Bolle, X., Michel, P., Godfroid, J., Walravens, K. and Letesson, J.J. (2001a) Induction of immune response in BALB/c mice with a DNA vaccine encoding bacterioferritin or P39 of *Brucella* spp. *Infect Immun* **69**, 6264–6270.

Al-Mariri, A., Tibor, A., Mertens, P., De Bolle, X., Michel, P., Godfroid, J., Walravens, K. and Letesson, J.J. (2001b) Protection of BALB/c mice against *Brucella abortus* 544 challenge by vaccination with bacterioferritin or P39 recombinant proteins with CpG oligodeoxynucleotides as adjuvant. *Infect Immun* **69**, 4816–4822.

Amin, A.S., Hamdy, M.E. and Ibrahim, A.K. (2001) Detection of *Brucella melitensis* in semen using the polymerase chain reaction assay. *Vet Microbiol* **83**, 37–44.

Babiuk, L., Pontarollo, R., Babiuk, S., Loehr, B. and van Drunen Littel-van den Hurk, S. (2003) Induction of immune responses by DNA vaccines in large animals. *Vaccine* **21**, 649–658.

Baly, M.E. (1996) Florence Nightingale's fever. *Br Med J* **312**, 1040.

Bellaire, B.H., Elzer, P.H., Baldwin, C.L. and Roop, R.M. II (2003) Production of the siderophore 2,3-dihydroxybenzoic acid is required for wild-type growth of *Brucella abortus* in the presence of erythritol under low-iron conditions in vitro. *Infect Immun* **71**, 2927–2932.

Bercovich, Z. (2000) The use of skin delayed-type hypersensitivity as an adjunct test to diagnose brucellosis in cattle: a review. *Vet Q* **22**, 123–130.

Blasco, J.M. (1997) A review of the use of *B. melitensis* Rev 1 vaccine in adult sheep and goats. *Prev Vet Med* **31**, 275–283.

Boschiroli, M., Ouahrani-Bettache, S., Foulongne, V., Michaux-Charachon, S., Bourg, G., Allardet-Servent, A., Cazevieille, C., Lavigne, J. *et al.* (2002) Type IV secretion and *Brucella* virulence. *Vet Microbiol* **90**, 341–348.

Brew, S.D., Perrett, L.L., Stack, J.A., MacMillan, A.P. and Staunton, N.J. (1999) Human exposure to *Brucella* recovered from a sea mammal. *Vet Rec* **144**, 483.

Bricker, B.J. (2002) PCR as a diagnostic tool for brucellosis. *Vet Microbiol* **90**, 435–446.

Bricker, B.J. and Halling, S.M. (1995) Enhancement of the *Brucella* AMOS PCR assay for differentiation of *Brucella abortus* vaccine strains S19 and RB51. *J Clin Microbiol* **33**, 1640–1642.

Bricker, B.J., Ewalt, D.R. and Halling, S.M. (2003a) *Brucella* 'HOOF-prints': strain typing by multi-locus analysis of variable number tandem repeats (VNTRs). *BMC Microbiol* **3**, 15.

Bricker, B.J., Ewalt, D.R., Olsen, S.C. and Jensen, A.E. (2003b) Evaluation of the *Brucella abortus* species-specific polymerase chain reaction assay, an improved version of the *Brucella* AMOS polymerase chain reaction assay for cattle. *J Vet Diagn Invest* **15**, 374–378.

Capasso, L. (2002) Bacteria in two-millennia-old cheese, and related epizoonoses in Roman populations. *J Infect* **45**, 122–127.

Celli, J., Salcedo, S.P. and Gorvel, J.-P. (2005) From the cover: *Brucella* coopts the small GTPase Sar1 for intracellular replication. *Proc Natl Acad Sci* **102**, 1673–1678.

Chao, T.-C., Becker, A., Buhrmester, J., Puhler, A. and Weidner, S. (2004) The *Sinorhizobium meliloti* fur gene regulates, with dependence on Mn(II), transcription of the sitABCD operon, encoding a metal-type transporter. *J Bacteriol* **186**, 3609–3620.

Cheng, H.-P. and Walker, G.C. (1998) Succinoglycan production by *Rhizobium meliloti* is regulated through the ExoS-ChvI two-component regulatory system. *J Bacteriol* **180**, 20–26.

Cloeckaert, A., Verger, J., Grayon, M. and Grepinet, O. (1995) Restriction site polymorphism of the genes encoding the major 25 kDa and 36 kDa outer-membrane proteins of *Brucella*. *Microbiology* **141**, 2111–2121.

Cloeckaert, A., Verger, J., Grayon, M. and Vizcaino, N. (1996) Molecular and immunological characterization of the major outer membrane proteins of *Brucella*. *FEMS Microbiol Lett* **145**, 1–8.

Cloeckaert, A., Grayon, M. and Grepinet, O. (2000) An IS711 element downstream of the bp26 gene is a specific marker of *Brucella* spp. isolated from marine mammals. *Clin Diagn Lab Immunol* **7**, 835–839.

Cloeckaert, A., Baucheron, S., Vizcaino, N. and Zygmunt, M.S. (2001a) Use of recombinant BP26 protein in serological diagnosis of *Brucella melitensis* infection in sheep. *Clin Diagn Lab Immunol* **8**, 772–775.

Cloeckaert, A., Verger, J., Grayon, M., Paquet, J., Garin-Bastuji, B., Foster, G. and Godfroid, J. (2001b) Classification of *Brucella* spp. isolated from marine mammals by DNA polymorphism at the omp2 locus. *Microbes Infect* **3**, 729–738.

Cloeckaert, A., Grayon, M. and Grepinet, O. (2002) Identification of *Brucella melitensis* vaccine strain Rev.1 by PCR-RFLP based on a mutation in the rpsL gene. *Vaccine* **20**, 2546–2550.

Cook, W.E., Williams, E.S., Thorne, E.T., Kreeger, T.J., Stout, G., Bardsley, K., Edwards, H., Schurig, G. *et al.* (2002) *Brucella abortus* strain RB51 vaccination in elk. I. Efficacy of reduced dosage. *J Wildl Dis* **38**, 18–26.

Corbel, M. (1997) Brucellosis: an overview. *Emerg Infect Dis* **3**, 213–221.

Covert, J., Eskra, L. and Splitter, G. (2005) Isolation of *Brucella abortus* total RNA from *B. abortus*-infected murine RAW macrophages. *J Microbiol Methods* **60**, 383–393.

Cutler, S. and Whatmore, A. (2003) Progress in understanding brucellosis. *Vet Rec* **153**, 641–642.

Dalrymple-Champneys, W. (1950a) Undulant fever a neglected problem. *Lancet* **1**, 429–435.

Dalrymple-Champneys, W. (1950b) Undulant fever a neglected problem. *Lancet* **1**, 477–485.

Delrue, R., Lestrate, P., Tibor, A., Letesson, J. and De Bolle, X. (2004) *Brucella* pathogenesis, genes identified from random large-scale screens. *FEMS Microbiol Lett* **231**, 1–12.

DelVecchio, V.G., Kapatral, V., Redkar, R.J., Patra, G., Mujer, C., Los, T., Ivanova, N., Anderson, I. *et al.* (2002) The genome sequence of the facultative intracellular pathogen *Brucella melitensis*. *Proc Natl Acad Sci* **99**, 443–448.

Dossey, B.M. (1998) Florence Nightingale. Her crimean fever and chronic illness. *J Holist Nurs* **16**, 168–196 (discussion 197–201).

van Drunen Littel-van den Hurk, S., Babiuk, S. and Babiuk, L. (2004) Strategies for improved formulation and delivery of DNA vaccines to veterinary target species. *Immunol Rev* **199**, 113–125.

Edmonds, M.D., Samartino, L.E., Hoyt, P.G., Hagius, S.D., Walker, J.V., Enright, F.M., Schurig, G.G. and Elzer, P. (2001) Oral vaccination of sexually mature pigs with *Brucella abortus* vaccine strain RB51. *Am J Vet Res* **62**, 1328–1331.

Elzer, P.H., Smith, J., Roffe, T., Kreeger, T., Edwards, J. and Davis, D. (2002) Evaluation of *Brucella abortus* strain RB51 and strain 19 in pronghorn antelope. *Ann N Y Acad Sci* **969**, 102–105.

England, T., Kelly, L., Jones, R., MacMillan, A. and Wooldridge, M. (2004) A simulation model of brucellosis spread in British cattle under several testing regimes. *Prevent Vet Med* **63**, 63–73.

Eschenbrenner, M., Wagner, M.A., Horn, T.A., Kraycer, J.A., Mujer, C.V., Hagius, S., Elzer, P. and DelVecchio, V.G. (2002) Comparative proteome analysis of *Brucella melitensis* vaccine strain Rev 1 and a virulent strain, 16 M. *J Bacteriol* **184**, 4962–4970.

Estein, S.M., Baldi, P.C. and Bowden, R.A. (2002) Comparison of serological tests based on outer membrane or internal antigens for detecting antibodies to *Brucella ovis* in infected flocks. *J Vet Diagn Invest* **14**, 407–411.

Funk, N.D., Tabatabai, L.B., Elzer, P.H., Hagius, S.D., Martin, B.M. and Hoffman, L.J. (2005) Indirect enzyme-linked immunosorbent assay for detection of *Brucella melitensis*-specific antibodies in goat milk. *J Clin Microbiol* **43**, 721–725.

Gallien, P., Dorn, C., Alban, G., Staak, C. and Protz, D. (1998) Detection of *Brucella* species in organs of naturally infected cattle by polymerase chain reaction. *Vet Rec* **142**, 512–514.

Godfroid, J. and Kasbohrer, A. (2002) Brucellosis in the European Union and Norway at the turn of the twenty-first century. *Vet Microbiol* **90**, 135–145.

Gonzalez Carrero, M.I., Sangari, F.J., Aguero, J. and Garcia Lobo, J.M. (2002) *Brucella abortus* strain 2308 produces brucebactin, a highly efficient catecholic siderophore. *Microbiology* **148**, 353–360.

Greenfield, R.A., Drevets, D.A., Machado, L.J., Voskuhl, G.W., Cornea, P. and Bronze, M.S. (2002) Bacterial pathogens as biological weapons and agents of bioterrorism. *Am J Med Sci* **323**, 299–315.

Guarino, A., Serpe, L., Fusco, G., Scaramuzzo, A. and Gallo, P. (2000) Detection of *Brucella* species in buffalo whole blood by gene-specific PCR. *Vet Rec* **147**, 634–636.

Halling, S. (1998) On the presence and organization of open reading frames of the nonmotile pathogen *Brucella abortus* similar to class II, III, and IV flagellar genes and to LcrD virulence superfamily. *Microb Comp Genomics* **3**, 21–29.

Hamdy, M.E. and Amin, A.S. (2002) Detection of *Brucella* species in the milk of infected cattle, sheep, goats and camels by PCR. *Vet J* **163**, 299–305.

Hoover, D.L., Nikolich, M.P., Izadjoo, M.J., Borschel, R.H. and Bhattacharjee, A.K. (2004) Development of new *Brucella* vaccines by molecular methods. In *Brucella: Molecular and Cellular Biology* ed. Lopes-Goni, I. and Moriyon, I. pp. 369–402. Norfolk, UK: Horizon Bioscience.

Januszewski, M.C., Olsen, S.C., McLean, R.G., Clark, L. and Rhyan, J.C. (2001) Experimental infection of nontarget species of rodents and birds with *Brucella abortus* strain RB51 vaccine. *J Wildl Dis* **37**, 532–537.

Jolley, M. and Nasir, M. (2003) The use of fluorescence polarization assays for the detection of infectious diseases. *Comb Chem High Throughput Screen* **6**, 235–244.

Keim, P., Klevytska, A., Price, L., Schupp, J., Zinser, G., Smith, K., Hugh-Jones, M., Okinaka, R. *et al.* (1999) Molecular diversity in *Bacillus anthracis*. *J Appl Microbiol* **87**, 215–217.

Kittelberger, R., Hilbink, F., Hansen, M.F., Ross, G.P., Joyce, M.A., Fenwick, S., Heesemann, J., Wolf-Watz, H. *et al.* (1995) Serological crossreactivity between *Brucella abortus* and *Yersinia enterocolitica* 0 : 9 II the use of *Yersinia* outer proteins for the specific detection of *Yersinia enterocolitica* infections in ruminants. *Vet Microbiol* **47**, 271–280.

Kittelberger, R., Reichel, M.P., Joyce, M.A. and Staak, C. (1997) Serological crossreactivity between *Brucella abortus* and *Yersinia enterocolitica* 0 : 9. III. Specificity of the in vitro antigen-specific gamma interferon test for bovine brucellosis diagnosis in experimentally *Yersinia enterocolitica* 0 : 9-infected cattle. *Vet Microbiol* **57**, 361–371.

Ko, J. and Splitter, G.A. (2003) Molecular host-pathogen interaction in brucellosis: current understanding and future approaches to vaccine development for mice and humans. *Clin Microbiol Rev* **16**, 65–78.

Kohler, S., Michaux-Charachon, S., Porte, F., Ramuz, M. and Liautard, J. (2003) What is the nature of the replicative niche of a stealthy bug named *Brucella*. *Trends Microbiol* **11**, 215–219.

Leal-Klevezas, D.S., Martinez-Vazquez, I.O., Lopez-Merino, A. and Martinez-Soriano, J.P. (1995) Single-step PCR for detection of *Brucella* spp. from blood and milk of infected animals. *J Clin Microbiol* **33**, 3087–3090.

Leclercq, S.Y. and Oliveira, S.C. (2003) Protective immunity induced by DNA-library immunization against an intracellular bacterial infection. *J Drug Target* **11**, 531–538.

Leclerq, S., Harms, J.S., Rosinha, G.M., Azevedo, V. and Oliveira, S.C. (2002) Induction of a Th1-type of immune response but not protective immunity by intramuscular DNA immunisation with *Brucella abortus* GroEL heat-shock gene. *J Med Microbiol* **51**, 20–26.

Letesson, J.J., Tibor, A., van Eynde, G., Wansard, V., Weynants, V., Denoel, P. and Saman, E. (1997) Humoral immune responses of *Brucella*-infected cattle, sheep, and goats to eight purified recombinant *Brucella* proteins in an indirect enzyme-linked immunosorbent assay. *Clin Diagn Lab Immunol* **4**, 556–564.

Lopez-Goni, I., Guzman-Verri, C., Manterola, L., Sola-Landa, A., Moriyon, I. and Moreno, E. (2002) Regulation of *Brucella* virulence by the two-component system BvrR/BvrS. *Vet Microbiol* **90**, 329–339.

Lord, V.R., Cherwonogrodzky, J.W., Schurig, G.G., Lord, R.D., Marcano, M.J. and Melendez, G.E. (1998) Venezuelan field trials of vaccines against brucellosis in swine. *Am J Vet Res* **59**, 546–551.

Lucero, N.E., Foglia, L., Ayala, S.M., Gall, D. and Nielsen, K. (1999) Competitive enzyme immunoassay for diagnosis of human brucellosis. *J Clin Microbiol* **37**, 3245–3248.

Manterola, L., Tejero-Garces, A., Ficapal, A., Shopayeva, G., Blasco, J.M., Marin, C.M. and Lopez-Goni, I. (2003) Evaluation of a PCR test for the diagnosis of *Brucella ovis* infection in semen samples from rams. *Vet Microbiol* **92**, 65–72.

Michaux-Charachon, S., Bourg, G., Jumas-Bilak, E., Guigue-Talet, P., Allardet-Servent, A., O'Callaghan, D. and Ramuz, M. (1997) Genome structure and phylogeny in the genus *Brucella*. *J Bacteriol* **179**, 3244–3249.

Moreno, E. and Gorvel, J.-P. (2004) Invasion, intracellular trafficking and replication of *Brucella* organisms in professional and non-professional phagocytes. In *Brucella Molecular and Cellular Biology* ed. Lopes-Goni, I. and Moriyon, I. pp. 287–312. Norfolk, UK: Horizon Bioscience.

Moreno, E. and Moriyon, I. (2002) *Brucella melitensis*: a nasty bug with hidden credentials for virulence. *Proc Natl Acad Sci USA* **99**, 1–3.

Moriyon, I., Grillo, M., Monreal, D., Gonzalez, D., Marin, C., Lopez-Goni, I., Mainar-Jaime, R., Moreno, E. *et al.* (2004) Rough vaccines in animal brucellosis: structural and genetic basis and present status. *Vet Res* **35**, 1–38.

Mujer, C.V., Wagner, M.A., Eschenbrenner, M., Horn, T., Kraycer, J.A., Redkar, R., Hagius, S., Elzer, P. *et al.* (2002) Global analysis of *Brucella melitensis* proteomes. *Ann N Y Acad Sci* **969**, 97–101.

Munoz, P.M., Marin, C.M., Monreal, D., Gonzalez, D., Garin-Bastuji, B., Diaz, R., Mainar-Jaime, R.C., Moriyon, I. *et al.* (2005) Efficacy of several serological tests and antigens for diagnosis of bovine brucellosis in the presence of false-positive serological results due to *Yersinia enterocolitica* O:9. *Clin Diagn Lab Immunol* **12**, 141–151.

Munoz-Montesino, C., Andrews, E., Rivers, R., Gonzalez-Smith, A., Moraga-Cid, G., Folch, H., Cespedes, S. and Onate, A.A. (2004) Intraspleen delivery of a DNA vaccine coding for superoxide dismutase (SOD) of *Brucella abortus* induces SOD-specific CD4+ and CD8+ T cells. *Infect Immun* **72**, 2081–2087.

Murillo, M., Grillo, M.J., Rene, J., Marin, C.M., Barberan, M., Goni, M.M., Blasco, J.M., Irache, J.M. *et al.* (2001) A *Brucella ovis* antigenic complex bearing poly-epsilon-caprolactone microparticles confer protection against experimental brucellosis in mice. *Vaccine* **19**, 4099–4106.

Murillo, M., Gamazo, C., Goni, M., Irache, J. and Blanco-Prieto, M. (2002a) Development of microparticles prepared by spray-drying as a vaccine delivery system against brucellosis. *Int J Pharmacol* **242**, 341–344.

Murillo, M., Gamazo, C., Irache, J.M. and Goni, M.M. (2002b) Polyester microparticles as a vaccine delivery system for brucellosis: influence of the polymer on release, phagocytosis and toxicity. *J Drug Target* **10**, 211–219.

Murillo, M., Goni, M.M., Irache, J.M., Arangoa, M.A., Blasco, J.M. and Gamazo, C. (2002c) Modulation of the cellular immune response after oral or subcutaneous immunization with microparticles containing *Brucella ovis* antigens. *J Control Release* **85**, 237–246.

Navarro, E., Escribano, J., Fernandez, J. and Solera, J. (2002) Comparison of three different PCR methods for detection of *Brucella* spp. in human blood samples. *FEMS Immunol Med Microbiol* **34**, 147–151.

Newby, D.T., Hadfield, T.L. and Roberto, F.F. (2003) Real-time PCR detection of *Brucella abortus*: a comparative study of SYBR Green I, 5′-exonuclease, and hybridization probe assays. *Appl Environ Microbiol* **69**, 4753–4759.

Nielsen, K. (2002) Diagnosis of brucellosis by serology. *Vet Microbiol* **90**, 447–459.

Nielsen, K.H., Kelly, L., Gall, D., Nicoletti, P. and Kelly, W. (1995) Improved competitive enzyme immunoassay for the diagnosis of bovine brucellosis. *Vet Immunol Immunopathol* **46**, 285–291.

Nielsen, K., Gall, D., Jolley, M., Leishman, G., Balsevicius, S., Smith, P., Nicoletti, P. and Thomas, F. (1996) A homogeneous fluorescence polarization assay for detection of antibody to *Brucella abortus*. *J Immunol Methods* **195**, 161–168.

Nielsen, K., Lin, M., Gall, D. and Jolley, M. (2000) Fluorescence polarization immunoassay: detection of antibody to *Brucella abortus*. *Methods* **22**, 71–76.

Nielsen, K., Smith, P., Conde, S., Draghi de Benitez, G., Gall, D., Halbert, G., Kenny, K., Massengill, C. *et al.* (2004) Rough lipopolysaccharide of *Brucella abortus* RB51 as a common antigen for serological detection of *B. ovis*, *B. canis*, and *B. abortus* RB51 exposure using indirect enzyme immunoassay and fluorescence polarization assay. *J Immunoassay Immunochem* **25**, 171–182.

O'Callaghan, D., Cazevieille, C., Allardet-Servent, A., Boschiroli, M., Bourg, G., Foulongne, V., Frutos, P., Kulakov, Y. *et al.* (1999) A homologue of the *Agrobacterium tumefaciens VirB* and *Bordetella pertussis* Ptl type IV secretion systems is essential for intracellular survival of *Brucella suis*. *Mol Microbiol* **33**, 1210–1220.

Onate, A.A., Cespedes, S., Cabrera, A., Rivers, R., Gonzalez, A., Munoz, C., Folch, H. and Andrews, E. (2003) A DNA vaccine encoding Cu, Zn superoxide dismutase of *Brucella abortus* induces protective immunity in BALB/c mice. *Infect Immun* **71**, 4857–4861.

Parent, M.A., Bellaire, B.H., Murphy, E.A., Roop, R.M. II, Elzer, P.H. and Baldwin, C.L. (2002) *Brucella abortus* siderophore 2,3-dihydroxybenzoic acid (DHBA) facilitates intracellular survival of the bacteria. *Microb Pathog* **32**, 239–248.

Paulsen, I.T., Seshadri, R., Nelson, K.E., Eisen, J.A., Heidelberg, J.F., Read, T.D., Dodson, R.J., Umayam, L. *et al.* (2002) The *Brucella suis* genome reveals fundamental similarities between animal and plant pathogens and symbionts. *Proc Natl Acad Sci* **99**, 13148–13153.

Platero, R., Peixoto, L., O'Brian, M.R. and Fabiano, E. (2004) Fur is involved in manganese-dependent regulation of mntA (sitA) expression in *Sinorhizobium meliloti*. *Appl Envir Microbiol* **70**, 4349–4355.

Pontes, D.S., Dorella, F.A., Ribeiro, L.A., Miyoshi, A., Le Loir, Y., Gruss, A., Oliveira, S.C., Langella, P. *et al.* (2003) Induction of partial protection in mice after oral administration of *Lactococcus lactis* producing *Brucella abortus* L7/L12 antigen. *J Drug Target* **11**, 489–493.

Pouillot, R., Garin-Bastuji, B., Gerbier, G., Coche, Y., Cau, C., Dufour, B. and Moutou, F. (1997) The brucellin skin test as a tool to discriminate false positive serological reactions in bovine brucellosis. *Vet Res* **28**, 365–374.

Probert, W.S., Schrader, K.N., Khuong, N.Y., Bystrom, S.L. and Graves, M.H. (2004) Real-time multiplex PCR assay for detection of *Brucella spp.*, *B. abortus*, and *B. melitensis*. *J Clin Microbiol* **42**, 1290–1293.

Queipo-Ortuno, M., Morata, P., Ocon, P., Manchado, P. and Colmenero, J. (1997) Rapid diagnosis of human brucellosis by peripheral-blood PCR assay. *J Clin Microbiol* **35**, 2927–2930.

Queipo-Ortuno, M., Colmenero, J., Baeza, G. and Morata, P. (2005) Comparison between lightcycler real-time polymerase chain reaction (PCR) assay with serum and PCR-enzyme-linked immunosorbent assay with whole blood samples for the diagnosis of human brucellosis. *Clin Infect Dis* **40**, 260–264.

Rajashekara, G., Glasner, J.D., Glover, D.A. and Splitter, G.A. (2004) Comparative whole-genome hybridization reveals genomic islands in *Brucella* species. *J Bacteriol* **186**, 5040–5051.

Rhyan, J.C., Gidlewski, T., Ewalt, D.R., Hennager, S.G., Lambourne, D.M. and Olsen, S.C. (2001) Seroconversion and abortion in cattle experimentally infected with *Brucella* sp. isolated from a Pacific harbor seal (*Phoca vitulina richardsi*). *J Vet Diagn Invest* **13**, 379–382.

Richtzenhain, L.J., Cortez, A., Heinemann, M.B., Soares, R.M., Sakamoto, S.M., Vasconcellos, S.A., Higa, Z.M., Scarcelli, E. *et al.* (2002) A multiplex PCR for the detection of *Brucella spp.* and *Leptospira* spp. DNA from aborted bovine fetuses. *Vet Microbiol* **87**, 139–147.

Rittig, M.G., Alvarez-Martinez, A.T., Porte, F., Liautard, J.P. and Rouot, B. (2001) Intracellular survival of *Brucella* spp. in human monocytes involves conventional uptake but special phagosomes. *Infect Immun* **69**, 3995–4006.

Romero, C., Gamazo, C., Pardo, M. and Lopez-Goni, I. (1995) Specific detection of *Brucella* DNA by PCR. *J Clin Microbiol* **33**, 615–617.

Rouot, B., Alvarez-Martinez, M.-T., Marius, C., Menanteau, P., Guilloteau, L., Boigegrain, R.-A., Zumbihl, R., O'Callaghan, D. *et al.* (2003) Production of the type IV secretion system differs among *Brucella* species as revealed with VirB5- and VirB8-specific antisera. *Infect Immun* **71**, 1075–1082.

Saegerman, C., Vo, T.K., De Waele, L., Gilson, D., Bastin, A., Dubray, G., Flanagan, P., Limet, J.N. *et al.* (1999) Diagnosis of bovine brucellosis by skin test: conditions for the test and evaluation of its performance. *Vet Rec* **145**, 214–218.

Sanchez, D.O., Zandomeni, R.O., Cravero, S., Verdun, R.E., Pierrou, E., Faccio, P., Diaz, G., Lanzavecchia, S. *et al.* (2001) Gene discovery through genomic sequencing of *Brucella abortus*. *Infect Immun* **69**, 865–868.

Sangari, F.J., Garcia-Lobo, J.M. and Aguero, J. (1994) The *Brucella abortus* vaccine strain B19 carries a deletion in the erythritol catabolic genes. *FEMS Microbiol Lett* **121**, 337–342.

Schurig, G., Sriranganathan, N. and Corbel, M. (2002) Brucellosis vaccines: past, present and future. *Vet Microbiol* **90**, 479–496.

Sohn, A.H., Probert, W.S., Glaser, C.A., Gupta, N., Bollen, A.W., Wong, J.D., Grace, E.M. and McDonald, W.C. (2003) Human neurobrucellosis with intracerebral granuloma caused by a marine mammal *Brucella* spp. *Emerg Infect Dis* **9**, 485–488.

Sola-Landa, A., Pizarro-Cerda, J., Grillo, M., Moreno, E., Moriyon, I., Blasco, J., Gorvel, J. and Lopez-Goni, I. (1998) A two-component regulatory system playing a critical role in plant pathogens and

endosymbionts is present in *Brucella abortus* and controls cell invasion and virulence. *Mol Microbiol* **29**, 125–138.

Tsolis, R.M. (2002) Comparative genome analysis of the alpha-proteobacteria: relationships between plant and animal pathogens and host specificity. *Proc Natl Acad Sci* **99**, 12503–12505.

Vanzini, V.R., Aguirre, N.P., Valentini, B.S., Torioni de Echaide, S., Lugaresi, C.I., Marchesino, M.D. and Nielsen, K. (2001) Comparison of an indirect ELISA with the *Brucella* milk ring test for detection of antibodies to *Brucella abortus* in bulk milk samples. *Vet Microbiol* **82**, 55–60.

Velikovsky, C.A., Cassataro, J., Giambartolomei, G.H., Goldbaum, F.A., Estein, S., Bowden, R.A., Bruno, L., Fossati, C.A. *et al.* (2002) A DNA vaccine encoding lumazine synthase from *Brucella abortus* induces protective immunity in BALB/c mice. *Infect Immun* **70**, 2507–2511.

Vemulapalli, R., He, Y., Sriranganathan, N., Boyle, S.M. and Schurig, G.G. (2002) *Brucella abortus* RB51: enhancing vaccine efficacy and developing multivalent vaccines. *Vet Microbiol* **90**, 521–532.

Verger, J., Grimont, F., Pad, G. and Grayon, M. (1985) *Brucella*, a monospecific genus as shown by deoxyribonucleic acid hybridization. *Int J Syst Bacteriol* **35**, 292–295.

Verger, J., Grimont, F., Grimont, P. and Grayon, M. (1987) Taxonomy of the genus *Brucella*. *Ann Inst Pasteur Microbiol* **138**, 235–238.

Vigliocco, A.M., Silva Paulo, P.S., Mestre, J., Briones, G.C., Draghi, G., Tossi, M. and Nielsen, K. (1997) Development and validation of an indirect enzyme immunoassay for detection of ovine antibody to *Brucella ovis*. *Vet Microbiol* **54**, 357–368.

Vizcaino, N., Verger, J., Grayon, M., Zygmunt, M. and Cloeckaert, A. (1997) DNA polymorphism at the omp-31 locus of *Brucella* spp.: evidence for a large deletion in *Brucella abortus*, and other species-specific markers. *Microbiology* **143**, 2913–2921.

Vizcaino, N., Cloeckaert, A., Zygmunt, M.S. and Fernandez-Lago, L. (2001) Characterization of a *Brucella* species 25-kilobase DNA fragment deleted from *Brucella abortus* reveals a large gene cluster related to the synthesis of a polysaccharide. *Infect Immun* **69**, 6738–6748.

Vizcaino, N., Caro-Hernandez, P., Cloeckaert, A. and Fernandez-Lago, L. (2004) DNA polymorphism in the omp25/omp31 family of *Brucella* spp.: identification of a 1·7-kb inversion in *Brucella cetaceae* and of a 15·1-kb genomic island, absent from *Brucella ovis*, related to the synthesis of smooth lipopolysaccharide. *Microbes Infect* **6**, 821–834.

Vrioni, G., Gartzonika, C., Kostoula, A., Boboyianni, C., Papadopoulou, C. and Levidiotou, S. (2004) Application of a polymerase chain reaction enzyme immunoassay in peripheral whole blood and serum specimens for diagnosis of acute human brucellosis. *Eur J Clin Microbiol Infect Dis* **23**, 194–199.

Wagner, M.A., Eschenbrenner, M., Horn, T.A., Kraycer, J.A., Mujer, C.V., Hagius, S., Elzer, P. and DelVecchio, V.G. (2002) Global analysis of the *Brucella melitensis* proteome: identification of proteins expressed in laboratory-grown culture. *Proteomics* **2**, 1047–1060.

Watarai, M., Makino, S., Michikawa, M., Yanagisawa, K., Murakami, S. and Shirahata, T. (2002) Macrophage plasma membrane cholesterol contributes to *Brucella abortus* infection of mice. *Infect Immun* **70**, 4818–4825.

Whatmore, A.M., Murphy, T.J., Shankster, S., Young, E., Cutler, S.J. and Macmillan, A.P. (2005) Use of amplified fragment length polymorphism to identify and type *Brucella* isolates of medical and veterinary interest. *J Clin Microbiol* **43**, 761–769.

Yeoman, K.H., Curson, A.R.J., Todd, J.D., Sawers, G. and Johnston, A.W.B. (2004) Evidence that the *Rhizobium* regulatory protein RirA binds to cis-acting iron-responsive operators (IROs) at promoters of some Fe-regulated genes. *Microbiology* **150**, 4065–4074.

Young, D.A. (1995) Florence Nightingale's fever. *Br Med J* **311**, 1697–1700.

Zerva, L., Bourantas, K., Mitka, S., Kansouzidou, A. and Legakis, N.J. (2001) Serum is the preferred clinical specimen for diagnosis of human brucellosis by PCR. *J Clin Microbiol* **39**, 1661–1664.