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New developments in the immunodiagnosis of brucellosis in livestock and wildlife

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Summary
Although relatively effective diagnostic tests for brucellosis have been in existence for more than 100 years, it remains a serious, embedded and also a re-emerging disease in many parts of the globe. There are many factors besides suboptimal diagnosis that impede the complete and sustained eradication of animal brucellosis. In this review a case for the continued improvement of diagnostic methods is made through identifying existing shortcomings and considering what impact these have upon control and eradication. The focus is on developments in immunodiagnostics as these seem more likely to yield the pragmatic solutions needed. Moreover, developments in DNA detection methods have been neatly and recently reviewed elsewhere. This article reviews issues such as test cost, mobility, sensitivity and specificity. Advances in low-cost materials, high-throughput testing, assay multiplexing and the quantification of pen-side tests are described and their relevance to disease control considered. Poor test specificity when resolving positive serology, due to infection with cross-reactive bacteria and vaccination with smooth *Brucella* strains, is also an impediment to efficient disease eradication. A case for the development of novel discrete epitope antigens to address this is presented alongside *in silico* methods of selection and tools that enable increased analytical sensitivity that may be required to detect relatively low, but potentially significant, analytes. References have been drawn from the study of brucellosis wherever possible. However, in some cases new technological developments worthy of discussion have been included via the use of pertinent alternative examples. In conclusion, despite developments and innovations the classical serological tests seem under no imminent danger of mass extinction but there is potential for significant improvement and supplementation.

Keywords
Brucella – Brucellosis – Detection – Homogeneous – Immunodiagnosis – Multiplex – Sensitivity – Specificity.

Introduction

Brucellosis is a global disease, endemic in many areas, and present in most countries. Even in regions that are free of the disease the threat of reintroduction into livestock or wildlife means that considerable costs continue to be incurred. The diagnosis of brucellosis in animals is complicated by the paucity of reliable clinical signs. This makes clinical diagnosis unreliable unless there is strong supporting epidemiological information; however, this is frequently absent. Bacterial culture plays an important part in confirming the presence of disease but, other than samples collected from an abortion event, the likelihood of obtaining a positive culture from material from a live infected animal is too low for reliable diagnosis. Furthermore, culture is not an appropriate technique for routine screening owing to the costs, difficulties and dangers that it presents. Alternative diagnostic approaches include DNA amplification and detection methods, but the effectiveness of such approaches

on readily available material such as blood, serum, swabs and milk has yet to be fully evaluated and existing information is conflicting (81).

Serology is the mainstay of diagnosis for brucellosis because the diagnostic material is relatively easily accessible, and the tests are relatively cheap, available and sensitive. Alongside many other crucial components (7), the currently available serodiagnostic assays have been shown to be capable of enabling the sustained eradication of brucellosis. However, the cost of the ongoing commitment to serology and infrastructural support is prohibitive and few countries have totally eradicated the disease – in many the situation is getting worse (69). What, if any, innovations in serodiagnosis would reduce the burden on the complementary resources that are needed for effective disease surveillance, control and eradication? What are the significant imperfections in immunodiagnosis to be addressed?

There are four main areas where improvements in serodiagnosis may significantly assist in the control and sustained eradication of brucellosis:

– reducing the cost
– increasing the specificity
– improving mobility
– developing a reliable assay that can differentiate infected from vaccinated animals (DIVA assay).

The relative importance of each of these will vary according to the situation to which the assays are to be applied. For example, in areas of moderate to low prevalence the ability to run consecutive vaccination and test and slaughter campaigns could reduce the time and effort required to eliminate the disease. Whether this is already possible is an area of some debate (52). In areas with limited veterinary infrastructure and unmonitored animal movements a reliable field assay may be of value. In regions where the disease has been eradicated but ongoing serological surveillance is required the positive predictive value of the assays is very low. Increasing specificity would reduce the need for costly and frequently unnecessary interventions. Effective surveillance for brucellosis often requires high-throughput testing, and any developments that would lead to a reduction in the cost of a serological result would leave more resources available elsewhere. Many of the routinely used serodiagnostic assays already have a high diagnostic sensitivity (25), yet methods leading to improvements in analytical sensitivity may yield improvements in specificity, as described later.

The Rose Bengal test (RBT) (56) in many respects sets a tough benchmark to improve upon. It is simple to perform, rapid, homogeneous, sensitive, the sample (serum) is accessible, the consumables are cheap, there is a low equipment requirement and the assay is standardised (59). It is a respectable assay (22). However, it is labour intensive, usually interpreted in a subjective manner (but not always), there is no multiplex capability, it is prone to false positive serological reactions (FPSRs), false negative results may occur due to prozoning (58) and it has limited mobility because it does not work with whole blood or plasma.

Reducing test cost

The most obvious way to reduce test costs is to reduce the cost of the test reagents and the cost of the equipment required. The RBT already sets a high standard here with its simplicity and relatively low cost. It may be that innovations in paper-based microfluidics will lead to a generation of tests that are equally simple to run and whose reagents and consumables are more affordable and easily stored (41, 51). Samples themselves, such as serum, can be stored on paper and the antibodies released for subsequent diagnostic analysis, as demonstrated for brucellosis (16). This may reduce the costs and risks associated with the distribution (to a confirmatory testing laboratory, for example) and storage of such samples.

Test costs may also be reduced when serological testing requirements are large, as they often are, by developing ever more efficient high-throughput methods. Where labour costs are high compared to reagent and equipment costs, systems that enable automation of assay performance, output and sample reporting provide long-term cost saving opportunities. Minimising the number of intervention steps in the diagnostic assay reduces 'hands on' time and automation requirements. Automation can be performed with basic assays such as the RBT (11, 28), but automation is much more readily available for assays in 96-well format, such as enzyme-linked immunosorbent assay (ELISA). Combining the automation with integrated sample tracking via a laboratory information management system can offer even greater efficiency savings (46). The efficiencies of ELISA, such as the application of a standard 96-well format and quantitative output, are also utilised by the fluorescent polarisation assay (FPA) and the time-resolved fluorescent resonance energy transfer (TR-FRET) formats, which have both been evaluated for brucellosis serology. These claim to offer similar diagnostic performance to ELISA (49, 60), the former being prescribed by the World Organisation for Animal Health (OIE) for international trade, and they are rapid, quantitative and homogeneous, such that the antigen–antibody reaction takes place in the liquid phase and is detected via fluorescent labels without subsequent washing or separation steps. The TR-FRET can also be used in a duplex format for the simultaneous detection of two analytes, e.g. antibodies to human immunodeficiency virus and hepatitis B surface antigen in human sera (57).

This minimalist interventional approach is also the theme of several other technologies that have been adapted for serodiagnostic purposes. Some of these, such as dynamic light scattering (DLS) and surface plasmon resonance (SPR), offer 'label-free' detection, although for immunodetection some element of bimolecular conjugation is required at some stage in the process. The DLS method is frequently used to measure nanoparticle size, such as during antibiotic delivery (70) and vaccine production (17), and it can also be used to measure the formation of polymers (3). The highly efficient light-scattering properties of gold nanoparticles, which may be conjugated with antigens or antibodies, enable these to be employed to create sensitive immunoassays where aggregation is measured by DLS. This has been demonstrated in the detection of influenza virus using antibody-coated gold nanoparticles (19). The DLS method is a one-step homogeneous quantitative method which, in the case of influenza virus detection, also offers an increase in analytical sensitivity of 1 to 2 orders of magnitude compared with commercially available kits.

Surface plasmon resonance has been offered as a detection platform from Biacore for several years and many studies relating to antigen–antibody binding have been performed and published, including for the detection of porcine serum antibodies to *Salmonella* lipopolysaccharide (LPS) (2). Surface plasmon resonance is essentially a means of measuring small changes in refractive index that occur in response to analyte binding at, or near, the metal film surface. Real-time antibody binding kinetics can be evaluated using this system and conjugated surfaces can be reused. However, the Biacore hardware is expensive and the throughput is limited by the number of flow cells, a maximum of eight per reader, although a sample read cycle is claimed to be as short as four minutes. A localised (L) SPR system that uses colloidal metal nanoparticles and is potentially more suited to high-throughput screening has more recently been developed (31). Based on this principle, PharmaDiagnostics NV has developed carboxyl-functionalised gold nanorods, which are now available as SoPRano™ technology to detect biomolecular interactions. The assay requires no separation steps, can be performed in 96- and 384-well formats, and data (Fig. 1) are collected via an absorbance plate reader capable of reading in the low to near infrared region, such as the POLARstar Omega from BMG Labtech. Preliminary work (Dr Vanessa Bonnard, PharmaDiagnostics, personal communication) using gold

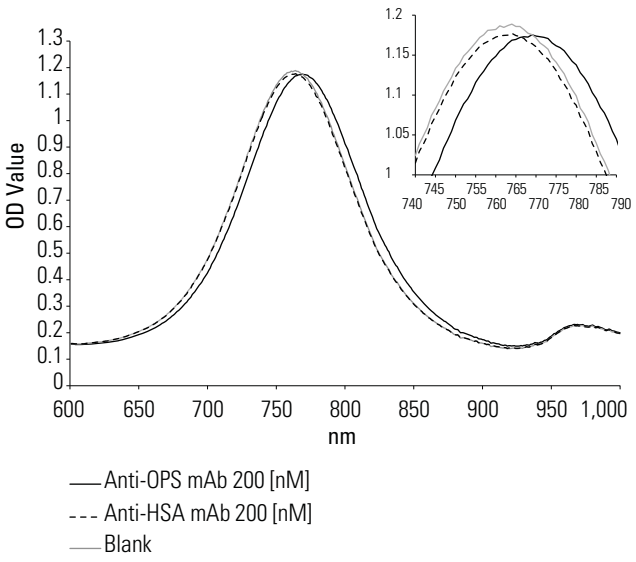

Fig. 1

Absorption spectrum from a localised surface plasmon resonance assay for the detection of anti-*Brucella* monoclonal antibody

A graph of optical density (OD) against wavelength (nm) for localised surface plasmon resonance of gold nanorods conjugated with *Brucella* O-polysaccharide (OPS) incubated with buffer only (Blank), anti-*Brucella* OPS monoclonal antibody at 200 nM concentration in buffer (anti-OPS mAb) and anti-human serum albumin monoclonal antibody (anti-hSA mAb) at 200 nM concentration. The embedded graph shows the OD at λmax with greater resolution and demonstrates the red shift in λmax that has occurred as a consequence of incubation with the anti-*Brucella* OPS mAb

Source: Data courtesy of Vanessa Bonnard from PharmaDiagnostics NV

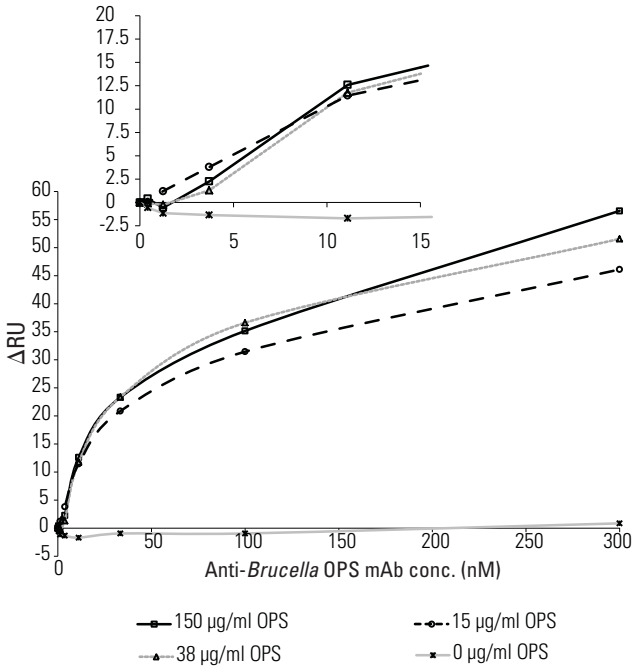

Fig. 2

Detection of anti-*Brucella* O-polysaccharide monoclonal antibody by localised surface plasmon resonance assay

This graph shows changes in ΔRU (obtained from a ratiometric analysis defined as the OD at λmax[blank] + 80 nm / OD at λmax) of *Brucella* OPS conjugated gold nanorods (γ-axis) against the concentration of co-incubated anti-*Brucella* OPS monoclonal antibody (mAb; x-axis) for four different nanorod preparations (conjugated with 150, 38, 15 or 0 µg/ml of *Brucella* OPS, all capped with polyethylene glycol [PEG]). The embedded graph shows the same data with greater resolution in the range of 0 to 15 nM of mAb

Source: Data courtesy of Vanessa Bonnard from PharmaDiagnostics NV

nanorods conjugated to the O-polysaccharide (OPS) from *B. melitensis* has demonstrated that the system is capable of specifically detecting an anti-*Brucella* OPS monoclonal antibody within the nM concentration range (Fig. 2). This puts the analytical sensitivity in the realm of that required for a serodiagnostic assay (49).

Other particle-based detection platforms can also be adapted to separation-free serodiagnosis. These include the AlphaLISA™ (Perkin Elmer) system, based on luminescent oxygen channelling (74), which has been shown to be effective for the detection of anti-*Brucella* antibodies in ruminant serum (47). An alternative separation-free system is the 96-well format system from MesoScaleDiscovery (MSD). This applies electrochemiluminescence (ECL) to derive a proximity-based antibody-dependent signal and has been successfully used for the detection of anti-*Brucella* smooth lipopolysaccharide (sLPS) antibodies in serum (72). The ECL excitation energy is provided via the plate surface, where the antibody–antigen interaction takes place in much the same manner as in an ELISA. This means that the system can also be used, like ELISA, with a more 'traditional' separation-based protocol. This may in theory increase the analytical sensitivity and dynamic range of the assay – but not always (72).

Multiplexing

An alternative strategy to reduce the expense of gathering serological data is to share the costs by creating multiplex assays that detect antibodies to antigens from several different pathogens simultaneously within the same reaction vessel. Such assays may be in suspension 'flow-cytometer' or planar formats (37). The previously mentioned MSD platform can be used for multiplex testing in planar form (as well as the separation-free application described above). This capability has been demonstrated by the simultaneous detection of bovine serum antibodies against four antigens for different respiratory diseases (53). A bovine cytokine multiplex has also been developed for this platform (13) and applied to the study of bovine tuberculosis. In theory, it should be possible to perform separation-free multiplex assays on this platform, and the manufacturers state that such assays have been developed by some of their pharmaceutical clients but not published.

A programme to monitor a range of infectious diseases in European wildlife (www.wildtechproject.com/wildtech/) is taking a multiplex approach to serodiagnosis in order to develop and apply efficient means to estimate seroprevalence. One of the platforms selected is Luminex®. This is a suspension array 96-well format which uses different populations of beads that can each be specifically conjugated and fluorescently identified. As part of the antigen selection process several *Brucella* antigens have been conjugated to specific bead populations, including *B. suis* 1330 (whole cell), *B. abortus* rough LPS, recombinant BP26 (48), as well as a *Yersinia enterocolitica* O:9 (whole cell) antigen. Preliminary data from this study are encouraging, and supportive of the multiplex approach (Drs Liljana Petrovska and Antonia Touloudi, personal communication). The application of the Luminex® platform to the detection of antibodies against some viral antigens of veterinary significance has already been published (5).

The 'Wildtech' project is also developing a planar serological array using the ArrayStrip™ (CLONDIAG) platform (21), where up to 441 different antigens in a 21 × 21 array may be printed in discrete locations within a test well. This is a highly proprietary platform with a relatively constrained business model. A more open approach is to print antigens in a discrete array directly into wells of standard plastic 96-well ELISA plates, and to capture antibody-dependent chemiluminescence using a digital camera. Printing can be done, for example, using a nanolitre printer such as those available from BioDot and results recorded using a digital imaging system such as that from Quansys Biosciences. This system has been used to create the 'Enferplex' assay for the detection of antibodies to multiple antigen targets from *Mycobacterium bovis* (71, 82). In this case the strategy has been to measure the antibody response simultaneously to a range of antigens from the same pathogen. The objective is to increase the sensitivity and specificity of the overall diagnostic output, via the use of an algorithm incorporating the response to each antigen, in a manner that is cost effective and therefore practicable.

Simultaneous immunoassay multiplexes have also been developed through the application of surface enhanced Raman scattering (SERS) technology. A 'proof of concept' study simultaneously and differentially detected human, mouse, rat and rabbit immunoglobulin (Ig)G (77). Multiplexing of SERS assays is possible owing to the tuneable fluorescent wavelengths and narrow fluorescent bandwidths of the nanoparticles used. Developments in this technology are rapidly emerging, and the advent of a dual SERS–fluorescence coding approach seems to offer the potential to massively increase the number of analytes differentially detected in one go (79). Yet this is not the most exciting aspect of this technology – it is the exquisite analytical sensitivity that delivers the radical opportunities.

Increasing analytical sensitivity

The application of colloidal metal nanoparticles to Raman Scattering to develop SERS has enhanced the sensitivity of this approach by many orders of magnitude, up to a staggering 1,014 times, to the point where the method is capable of detecting a single antigen–antibody

interaction (63). This approach has been applied to the antibody-mediated detection of *Mycobacterium avium* subsp. *paratuberculosis* with reported detection limits in milk of less than 1,000 colony-forming units (cfu)/ml (83). In conjunction with immunomagnetic separation, a SERS immunoassay for *Escherichia coli* reported a limit of detection of 8 cfu/ml (30). Thus, immunoassays, including those with multiplexing capability, have moved into the realms of analytical sensitivity only previously available through DNA detection.

Increasing analytical sensitivity does not directly correlate with increases in diagnostic sensitivity or specificity. In many cases increased sensitivity leads to a decrease in specificity. Why should those with an interest in the diagnosis of brucellosis be interested in this? Some antibodies, such as those raised against the *Brucella* OPS, are preponderant, and existing immunoassays such as the sLPS indirect (i)ELISA already have good diagnostic sensitivity. Increasing the analytical sensitivity of such assays may lead to reductions in specificity, as antibodies generated against similar structures from other Gram-negative bacteria may generate FPSRs (15). Other antigens of interest to the diagnostician may elicit a lower level of antibody response, and increasing the capability to detect these may be of value. Increasing sensitivity to enable the detection of antibodies against less immunodominant, but more specific, antigens may yield assays with improved specificity.

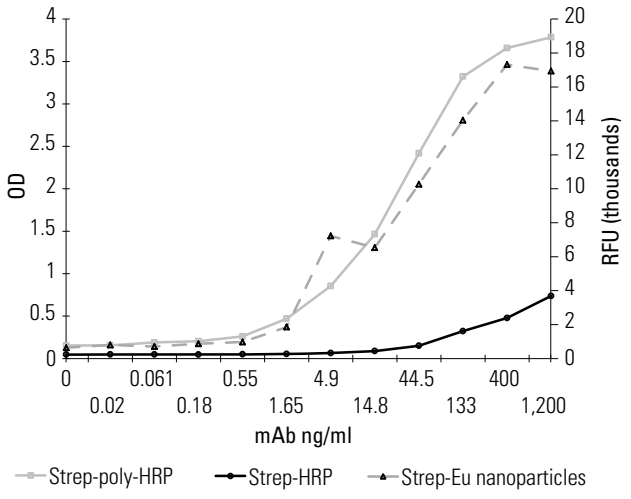

Fig. 3
Detection of anti-*Brucella* O-polysaccharide monoclonal antibody using three different streptavidin conjugates
The line graph shows the signal response in optical density (OD) and relative fluorescent units (RFU) on the y-axis against the concentration of a biotinylated anti-*Brucella* OPS monoclonal antibody (mAb) added to a *Brucella* smooth lipopolysaccharide-coated indirect enzyme-linked immunosorbent assay plate. The signals for three different streptavidin conjugates: horseradish peroxidase (HRP), poly-HRP and Europium nanoparticles (each used at 0.4 µg/ml) are shown

Although SERS techniques currently seem to represent the gold standard in immunoassay sensitivity, the platform is beyond the means of most diagnostic laboratories and the same might be said of metal nanoparticle DLS. Alternative means of increasing analytical sensitivity are more accessible. Immuno-polymerase chain reaction (PCR) is an interesting technique for antigen and antibody detection that has recently been reviewed (50), and a homogeneous analogue would be the proximity ligation assay (29), reagents for which are available from OlinkTM. Time-resolved fluorescence (TRF) using long lifetime lanthanide fluorophore biomarkers is also reported to increase assay sensitivity over standard fluorescence techniques. The incorporation of these fluorophores in conjugative microspheres further increases the sensitivity by multiplying the number of fluorophores that signal each binding event. These microspheres also increase the analytical sensitivity of homogeneous TR-FRET reactions (75). The use of streptavidin poly-HRP (horseradish peroxidase) is probably the easiest means of increasing assay sensitivity for those laboratories most familiar with ELISA. To demonstrate this, the results of a simple immunoassay using a *B. melitensis* sLPS-coated plate incubated with a biotinylated anti-OPS monoclonal antibody and three different conjugates were compared. The results (Fig. 3) showed that the streptavidin poly-HRP and the streptavidin-coated Europium beads (used via TRF) enabled an approximately 30-fold increase in analytical sensitivity compared with standard streptavidin HRP conjugate (unpublished data).

Improving diagnostic mobility

Nanoparticle TRF can also be exploited in a lateral flow (LF) immunoassay format where analytical sensitivity may be increased between 7- and 300-fold (35). Other formulas to increase analytical sensitivity include the use of up-converting phosphor technology, which has been applied in LF format for the detection of *Brucella* (66). In general, mobile field or 'pen-side' assays for brucellosis could be of considerable value (1, 9). In many endemic areas, uncontrolled animal movement and a veterinary infrastructure that is insufficient to supply and deliver consistently on the outcomes of a centralised testing laboratory are major obstacles. These problems might be partially bypassed by the application of field tests. To be effective in this context the tests must be extremely reliable, to give the user confidence that they can take rapid, possibly even immediate, action on the outcome of the test result. The quantification of data from such devices as an aid to interpretation would be a useful step in this direction. Lateral flow devices based upon a competitive format, developed by using an anti-*Brucella* OPS monoclonal antibody (mAb) conjugated with colloidal gold, have been developed in a fashion analogous to that of the competitive (c)ELISA. A reduction of intensity for the test line, a printed

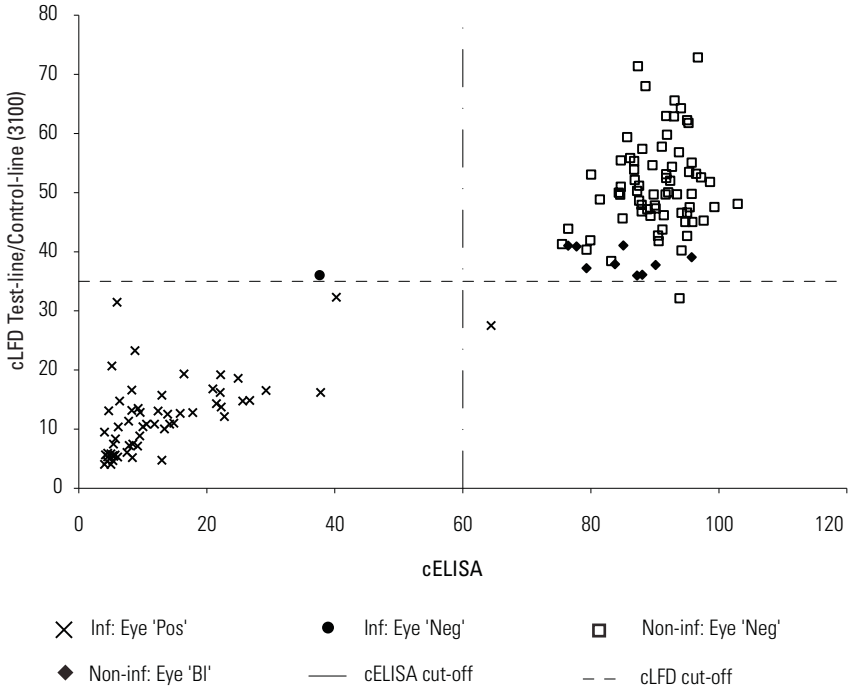

Fig. 4

Quantitative output from a competitive lateral flow device for the detection of anti-*Brucella* antibodies in bovine sera

The scatter plot shows the quantitative data from a *Brucella* smooth lipopolysaccharide (sLPS) competitive enzyme-linked immunosorbent assay (cELISA; *x*-axis) against the data from a homologous competitive lateral flow device (cLFD) for bovine sera from *B. abortus*-infected animals (x and •) and non-infected animals (□ and ◆). The positive/negative cut-offs for each assay are shown by dashed lines. The differentiation of the data points within each infection status shows what the visual only (by 'Eye') interpretation of the cLFD result was for each sample. Samples from infected animals interpreted by eye as positive are shown as '×', those interpreted as negative by eye are shown as '•'. Samples from non-infected animals interpreted by eye as negative are shown as '□' those whose status was indeterminate by eye are shown as '◆'

line of sLPS antigen, indicates the presence of competing serum antibody and a positive response (unpublished data). A control line was also printed which consisted of an anti-monoclonal antibody. Within the context of this review the main points to consider are: the ability to print and interrogate multiple lines on the device, the ability to read the output by eye and by a hand-held quantitative reader, and the fact that the quantitative output gave more certainty to the interpretation (Fig. 4).

Increasing diagnostic specificity

Novel platforms, whether laboratory- or field-based, are interesting and welcome, but the biological components of the assay remain the key to the outcome. To improve the specificity of the serodiagnosis of brucellosis the challenge has been to find a reliable alternative antigen to the OPS or the sLPS. The current reliance upon the OPS creates the confounding problem of false-positive results, caused either by infection of the host with Gram-negative bacteria in possession of OPS similar in structure to that possessed by smooth *Brucella* (15) or by vaccination with the smooth strains *B. melitensis* Rev.1 or *B. abortus* S19.

Many potentially diagnostically useful protein antigens have been reported, too many to list here, but some examples are Lumazine synthase (27), copper/zinc superoxide dismutase (Cu/Zn SOD) L7/L12 (4), and malate dehydrogenase (45). Perhaps the most frequently cited is BP26 – also known as outer membrane protein (OMP)28 (12, 18, 40, 44, 67). Despite the preponderance of publications extolling the virtues of one antigen or another, no antigen has been universally acclaimed as a solution. Thus, although the search goes on, it seems most unlikely that screening assays will be based on anything other than the OPS. However, an independent confirmatory assay built on a non-OPS antigen and not expressed by an effective vaccine would be extremely useful.

The increased availability of fragmentation mass spectrometry protein identification systems, combined with genome data and refinement of two-dimensional gel electrophoresis (2DGE) methods, makes the identification of proteins derived from immunoselection procedures more accessible. As such, new potential diagnostic and vaccine candidates for brucellosis continue to be identified (4, 14, 39, 62, 84). A downside with this approach is that the proteins under investigation are only those expressed under the particular laboratory circumstances within which

the *Brucella* cells were grown and from which the proteins were derived. An alternative approach that, in theory, enables the immunoassessment of all proteins within the genome is to express these proteins via a recombinant gene expression library and then probe the library with immune sera (45) to identify the recombinant clones from which the sequence of the protein of interest can be determined. A third approach is to synthesise recombinant antigens from all the open reading frames (ORFs) and probe all of these against antisera via microarray (42). Yet there are technical challenges to the recombinant approaches, not least of which is the uncertainty regarding whether all the proteins are in their native configuration.

Unlike a repeating polysaccharide, such as the *Brucella* OPS, proteins contain many different epitopes. Some of these may be immunodominant and others not. Some may be unique to *Brucella*, and others, even though the protein as a whole is unique, may not be. Therefore, investigation of the immune response at the peptide rather than the protein level may be the way forward (6). In support of this, several papers have demonstrated that truncated BP26 antigens may be more effective diagnostic antigens (68, 73). A recent study (65) has separately evaluated individual and overlapping peptides from the BP26 protein using monoclonal antibodies and evaluated the most promising peptides as diagnostic antigens in iELISA, with some modest success. Mapping peptide epitopes from *B. burgdorferi* has led to the discovery of specific peptides that enabled differentiation, via the antibody response, between active and past disease (10). This approach, eliminating non-specific antigens and operating at the epitope level, is ripe for enhancement by systems, such as those described above, that increase analytical sensitivity and enable the efficient combination of several antigens.

Whole proteome immuno-screening is a challenge; screening all peptide sequences within the proteome magnifies this challenge many fold. Bioinformatics may help to reduce the workload by predicting which proteins are the most likely to generate a significant immune response. A recent study used the complete human serum antibody response to the *B. melitensis* proteome to predict what types of protein are likely to be immunogenic candidates (43). It may be unsurprising that properties such as membrane association and secretion were positively associated with antigen recognition by antibodies. Indeed, one-third of the proteome contained such enriching features, and they accounted for 91% of the antigens recognised by the humoral immune system after *B. melitensis* infection. This type of approach was used with some success to identify peptides that could aid the diagnosis of bovine tuberculosis. By predicting the proteins that are secreted by *M. bovis*, using experimental and theoretical data, the proteome was reduced to 119 DIVA candidates. From these, 4,162 20-mer (with 12-mer overlap) peptides were evaluated for their ability to stimulate the memory T-cell response in infected cattle (33). From among these, in turn, a cocktail of nine peptides was identified that reacted in 54% of infected animals, with no reaction from animals vaccinated with bacille Calmette–Guérin (BCG) (34).

Once candidate proteins have been identified, the peptides within may be screened *in silico* for their potential to elicit an immune response. Two programmes for the identification of T-cell epitopes were tested against existing data on the immune response to 105 peptides derived from a number of proteins from *M. bovis* (32). The selection processes identified a panel that contained a higher proportion of reactive peptides than the initial set but some good sequences were lost. Selection software tools for antibody peptide epitopes are widespread, although they are more directly targeted towards highlighting good potential vaccine candidates and targets for monoclonal antibody generation than for the identification of potential diagnostic antigens (78). To narrow the scope of *in vitro* investigation further, a peptide BLAST search would identify sequences that are insufficiently unique to the organism of interest and may have poor specificity.

It is likely that despite *in silico* analysis many peptides from a full proteomic investigation would still require *in vitro* analysis. High-throughput solutions for peptide microarray are becoming ever more available through a number of commercial suppliers and it is now commonplace to be able to put up to 10^4 peptides on a single array (55). The feasibility of the approach is presented by a study into the antibody response against peptides derived from 61 proteins from *M. tuberculosis*. This was conducted using a high content microarray containing 7,446 individual peptides (23) and the results were informative, although it should be said that the data generated required powerful and considered interpretation. Perhaps more impressive than this was the application of the same array to the identification of peptides that bound to recombinant major histocompatibility complex (MHC) antigens and thus may be possible T-cell epitopes (24).

Developments in the use of the cellular immune response as a means to diagnose brucellosis have been comparatively few. This is in contrast to the development of interferon-gamma (IFNg) assays for bovine tuberculosis, for example, where specific proteins, peptides and 'negative' antigens have been extensively evaluated as stimulators of the T-cell response (76). There is obviously a greater need for such development for bovine tuberculosis owing to the unavailability of an established serological assay. Although the skin test is favoured by some as a means to diagnose brucellosis (26), the value of *in vitro* cytokine assays for this purpose seems stymied by the complex and variable cytokine response to infection (20).

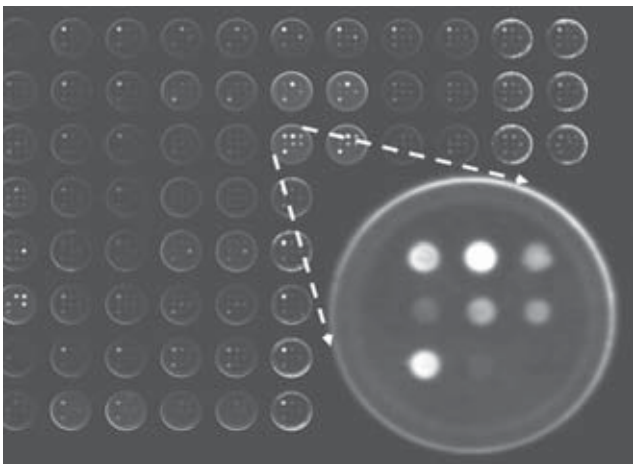

Fig. 5
A captured image from a multiplex enzyme-linked immunosorbent assay array for the simultaneous detection of anti-*Brucella* antibodies against several antigens
An example image from a serological 96-well chemiluminescent multiplex iELISA assay for the detection of bovine antibodies to smooth lipopolysaccharide (sLPS) derived from *B. abortus* (strain S99), *B. melitensis* (strain 16M), *Yersinia enterocolitica* O9, recombinant protein BP26, Brucellergene™ and rough (r)LPS derived from *B. abortus* (strain RB51). Each spot within the well relates to a specific antigen or dilution thereof

Proteins and peptides are not the only source of structural specificity within *Brucella*. All *Brucella* OPS antigens possess within them unique epitopes, as demonstrated by monoclonal antibodies and structural investigations (54). It was remarked upon some time ago that the use of these specific epitopes, some of which have been laboratory synthesised (8), as diagnostic antigens may yield diagnostics with improved specificity (80), especially as there is evidence of polyclonal antibodies within *Brucella*-infected cattle that possess the required specificity (38). At the time of writing there were no data publicly available to demonstrate the efficacy, or otherwise, of this approach. More mainstream is the application of rough LPS as a diagnostic antigen. Antigens dominated by rough LPS have been used successfully for some time in the serodiagnosis of infections with *B. ovis* (64) and *B. canis* (36). Rough LPS has not been used for routine serological detection of infection by smooth *Brucella* species, but there does appear to be some potential for its use as part of a testing algorithm (48, 61).

Researchers at the Animal Health Veterinary Laboratories Agency in the United Kingdom have attempted to bring together the potential diagnostic benefits from multiplexing several different antigens and increasing analytical sensitivity via the use of streptavidin poly-HRP. The objective was to obtain a serodiagnostic output with improved specificity with the aim of resolving FPSRs. Several *Brucella*-derived antigens were printed within an array within each well of a 96-well ELISA plate using a BioDot printer (Fig. 5). This included sLPS (from *B. abortus* S99 and *B. melitensis* S19), recombinant protein BP26 (48), native whole protein extract Brucellergene™ and rough LPS derived from *B. abortus* RB51. The results from the antigens within the multiplex were similar to those from the equivalent separate individual assays for each antigen that were run in parallel. However, despite demonstrating a statistically significant difference between the mean response to the non-OPS- dominated antigens for serum populations from *Brucella*-infected cattle and cattle presenting FPSRs, the difference was not sufficient for a useful diagnostic assay. Combining the data from the three non-OPS antigens together in an algorithm increased the overall diagnostic performance above that of the best individual non-OPS assay on its own, but only by a narrow margin (manuscript in preparation). This project was admittedly modest in its scope and did not achieve its key aim of resolving FPSRs. However, several refinements are possible and there is ample opportunity to incorporate additional antigens in the future, whether lipid, carbohydrate, protein, peptide or combinations thereof.

That this review has focused on the developments in immunodiagnosis rather than methods of detecting specific DNA or *Brucella* itself is more a reflection of the limitations of the author rather than developments in direct detection. Furthermore, a review covering much of this subject has recently been published (81). The author defers to expertise elsewhere on the aforementioned topic, but remains of the opinion that the interpretation of immune mediators will be the linchpin of the diagnosis of animal brucellosis for many years to come. There has also been insufficient space to do justice to the specific diagnostic issues surrounding vaccination; suffice to say that much of the antigen discovery research described above has been driven by the desire to create an elegant and effective DIVA solution.

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Les récentes innovations du diagnostic immunologique de la brucellose chez les animaux d'élevage et dans la faune sauvage

J.A. McGiven

Résumé

Bien que des tests diagnostiques relativement efficaces soient disponibles depuis plus d'un siècle pour détecter la brucellose, celle-ci demeure une maladie grave et bien établie dans certains endroits du monde, voire ré-émergente. Outre l'insuffisance des capacités diagnostiques, de nombreux autres facteurs font obstacle à l'éradication complète et durable de la brucellose animale. L'auteur plaide en faveur d'une amélioration continue des méthodes diagnostiques, en identifiant les défaillances actuelles et en analysant leurs conséquences sur les opérations de contrôle et d'éradication. L'accent est mis sur les innovations introduites dans le domaine du diagnostic immunologique, car ces méthodes offrent les meilleures perspectives de solution concrète aux problèmes rencontrés. Par ailleurs, les méthodes de détection recourant à l'ADN sont déjà décrites en détail ailleurs. Le présent article aborde divers aspects tels que le coût, les possibilités d'utilisation sur le terrain, la sensibilité et la spécificité des tests. La mise au point de matériels diagnostiques peu onéreux et de techniques à haut débit, la construction d'épreuves multiplexes et la quantification des tests rapides de terrain sont examinées en analysant leur pertinence pour la lutte contre la maladie. Lors de la détermination des sérologies positives, une sensibilité insuffisante des épreuves due à des réactions croisées avec d'autres bactéries ou à la présence de souches vaccinales lisses de *Brucella* constitue également un frein aux efforts d'éradication. Pour résoudre ce problème, l'auteur préconise de mettre au point de nouveaux déterminants antigéniques discrets et de recourir à des méthodes de sélection *in silico* et à des outils capables d'améliorer la sensibilité analytique afin de détecter des concentrations d'analytes relativement faibles mais potentiellement significatives. Dans la mesure du possible, l'auteur se réfère à des études dédiées spécifiquement à la brucellose. Quelques innovations technologiques intéressantes sont également présentées en recourant à des exemples pertinents relatifs à d'autres pathologies. Les avancées et innovations récentes ne menacent pas, dans l'immédiat, les épreuves sérologiques classiques, mais il existe toutefois un véritable potentiel d'amélioration et de complémentarité.

Mots-clés

Brucella – Brucellose – Détection – Diagnostic immunologique – Épreuves multiplexes – Homogène – Sensibilité – Spécificité.

Novedades en el inmunodiagnóstico de la brucelosis en el ganado y la fauna salvaje

J.A. McGiven

Resumen

Aunque desde hace más de 100 años existen pruebas relativamente eficaces para diagnosticar la brucelosis, en muchas partes del mundo esta sigue siendo una enfermedad grave, pertinaz y a veces también reemergente. Muchos son los factores que subyacen a esta situación de insuficiente diagnóstico, que impide la erradicación completa y duradera de la brucelosis animal. El autor aboga por una continua mejora de los métodos de diagnóstico, señalando las actuales deficiencias y exponiendo los efectos que tienen sobre el control y la erradicación de la enfermedad. El autor se concentra en las novedades en el terreno del diagnóstico inmunológico, entendiendo que estos métodos encierran el mayor potencial de ofrecer las soluciones prácticas que se necesitan, teniendo en cuenta además que últimamente ya se han expuesto en otra parte, con toda claridad, las novedades en materia de métodos de detección por el ADN. Se examinan aquí cuestiones como el costo, la movilidad, la sensibilidad y la especificidad de las distintas pruebas. También se describen los avances obtenidos en lo tocante a materiales de bajo costo, pruebas de elevado rendimiento, ensayos múltiples o cuantificación de las pruebas rápidas de terreno, y se expone la relación que todo ello guarda con la lucha contra la enfermedad. Otro impedimento para lograr erradicar la brucelosis reside en la escasa especificidad de las pruebas a la hora de resolver una serología positiva, debido a la infección con bacterias que presentan reactividad cruzada y a la vacunación con cepas lisas de *Brucella*. Para soslayar este problema el autor preconiza la obtención de nuevos determinantes antigénicos discretos, junto con herramientas y métodos de selección *in silico* que permitan alcanzar la sensibilidad analítica requerida para detectar sustancias que quizá estén presentes en cantidad escasa, pero aun así significativa. Siempre que ha sido posible se han tomado las referencias de estudios referidos a la brucelosis. En ciertos casos, sin embargo, se han empleado otros ejemplos alternativos para exponer ciertas novedades tecnológicas que merecía la pena examinar. El autor concluye que, pese a todas las novedades e innovaciones, las pruebas serológicas clásicas no parecen amenazadas de extinción masiva, aunque sí hay margen para perfeccionarlas sensiblemente y complementarlas.

Palabras clave

Brucella – Brucelosis – Detección – Ensayo multiplex – Especificidad – Homogéneo – Inmunodiagnóstico – Sensibilidad.

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