INTRODUCTION

In previous articles the distribution and diversity of Brucella sp infections has been covered. In addition the evasive mechanisms used by Brucella spp in avoiding the innate and acquired immune systems has been discussed in detail. This article will cover diagnostic tests and recent work on Brucella testing and the possible implications in strategies for diagnosis and control. The objective is to give the general veterinary practitioner some insight into this complicated subject.

Continued improvement of diagnostic methods, through identifying existing shortcomings, is essential and practitioners should be aware of the impact these may have upon control and eradication. Test cost, mobility, sensitivity and specificity are all factors which may have great influence and low-cost materials, high-throughput testing, assay multiplexing and the quantification of pen-side tests are therefore under continual investigation. Diagnostic tests for bovine brucellosis can be classified into three major groups – those which detect immunoglobulins, tests for the demonstration of Brucella organisms and those dependent on allergic reactions (Brucella skin test).

Diagnostic tests for Brucella sp may be employed to identify infections in individual animals, to diagnose the cause of abortion, to detect infected herds, to monitor and run eradication campaigns in infected herds, to run epidemiological surveys, to screen animal products preventing zoonotic spread and, lastly, as a requirement for movement of animals and animal products in local and international trade.
IMMUNOGLOBULINS AND ANTIBODY TESTING

Acquired immunity is initiated by initial contact with the foreign agent (immunization) which triggers a chain of events that leads to the activation of lymphocytes and the production of proteins (antibodies) with specificity against the foreign agent. Antibodies attach themselves to antigens and start the immune response cascade that leads ultimately to the neutralization of the foreign agent.

B lymphocytes proliferate in response to a particular antigen and differentiate into non-proliferating antibody secreting plasma cells. Antibody proteins are termed immunoglobulins (Ig). All immunoglobulin molecules have many common structural features and consist of protein "chains" linked together by chemical bonds. Based on the differences in their chains, immunoglobulin molecules are divided into five major classes: IgG, IgM, IgA, IgE, and IgD.

IgG is the predominant immunoglobulin of internal components such as blood, cerebrospinal fluid, and peritoneal fluid and is the only class of immunoglobulin that crosses the placenta, conferring the mother's immunity on the foetus. IgG makes up about 80% of the total immunoglobulin and is the smallest immunoglobulin thus it can readily diffuse out of the body's circulation into the tissues. The synthesis of IgG is largely governed by antigen stimulation, so that in germ free animals, IgG levels are very low but rise rapidly on transfer to a normal environment.

IgM is the largest immunoglobulin and because of its size, it is essentially "trapped" in the circulation. Elevated levels of IgM in normal individuals usually indicate recent infection or recent exposure to antigen.

IgA is a small immunoglobulin and is mainly concerned with defending the exposed external surfaces of the body against attack by micro-organisms. It is found selectively in the secretions of saliva, tears, nasal fluids, sweat, genito-urinary and gastro-intestinal tracts, secretions of the lungs, etc.

IgE is the antibody group responsible for most allergic reactions (e.g. hay fever). It is a very reaginic and efficacious antibody.

Classification of an animal as seropositive may be based on a threshold level above which an animal is considered positive. This categorisation allows for reduction in false positives due to non-specific reactions that may be diluted. The prevalence of detectable antibody depends on the rate of infection, the rate of antibody loss and the time at which these rates were effective.

Serological tests vary in their ability to detect antigenic differences and true positive result derived from an actual infection and false positive reactions may occur due to

- Group cross reactions (e.g. Yersinia sp cross reaction with Brucella abortus) and
- Non-specific inhibitors that may mimic the effects of antibodies.

True negative results indicate the absence of exposure to infection but false negatives may occur due to

- Natural or induced tolerance (e.g. BVD PI).
- Improper timing e.g. CFT may be negative for Brucella abortus before abortion.
- Unsuitable test.
• Non-specific inhibitors e.g. contaminated or haemolysed specimens may be anti-complimentary and result in false negative *Brucella abortus* CFT.
• Incomplete antibodies.
• Blocking antibodies. This may occur in *B. abortus* infection where excess IgG₁ may block IgG₂ which is the target of the CFT test.
• Insensitivity of the test (analytical sensitivity).

As discussed in the previous article when deciding on an appropriate serological test, and when interpreting the results of any diagnostic serological test, its specificity, sensitivity and predictive values should be considered. **Specificity** refers to the probability that the test will identify all non-infected animals compared to **sensitivity** of a test which is an indication of the probability that the test will identify all infected animals within a given population. There is no ideal test and no single test has both 100% sensitivity and specificity. Generally specificity and sensitivity are inversely related to each other. As sensitivity increases, specificity decreases, resulting in an increased number of false-positive results and vice versa when sensitivity decreases, the proportion of false negatives increases.

The positive and negative predictive values are an indication of the probability that positive or negative results will correspond to truly infected or healthy animals, respectively. Both will be influenced by the prevalence of a disease within a herd. As prevalence decreases, the predictive value of a positive test also declines increasing the number of false-positive reactions. When confronted with a low prevalence, the number of false positives may exceed the number of true positives.

Sensitivity, specificity and predictive value calculations are illustrated in the following table

<table>
<thead>
<tr>
<th>Test Status</th>
<th>True Disease status</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>diseased</td>
<td>not diseased</td>
</tr>
<tr>
<td>positive</td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td>negative</td>
<td>c</td>
<td>d</td>
</tr>
<tr>
<td>Totals</td>
<td>a+c</td>
<td>b+d</td>
</tr>
</tbody>
</table>

• Sensitivity = a/(a+c)  
• Specificity = d/(b+d)  
• The predictive value of a positive test = a/(a+b)  
• The predictive value of a negative test =d/(c+b)

Typically screening tests are selected with a high diagnostic sensitivity at the expense of specificity because these are required to detect as many cases as possible and a high proportion of false positives are not critical. In practice this means that when the prevalence is high, a test with adequate sensitivity but high specificity is a better choice, in order to allow for detection of the majority of diseased animals and herds (allowing for the minimum number of false-positive reactors). Once in the final stages of eradication, a sufficiently specific but highly sensitive test is recommended.

**SEROLOGICAL TESTS**
There are a number of serological tests available which show different performances under different conditions.
The milk ring test (MRT) is used as a screening test in brucellosis negative dairy herds on a bulk milk sample. Identification of a positive bulk milk sample will result in the whole herd being subjected to further individual testing using other screening tests such as the RBT, CFT and SAT.

The serum agglutination test (SAT) has been recognized as being useful in detecting new infections as early as two weeks, however, its use in chronically infected herds is limited as some chronically infected animals may test negative when they are in fact infected.

The Rose Bengal test (RBT) is simple to perform and is a rapid, homogeneous and sensitive standardized assay. In addition the sample (serum) required is easily accessible, consumables are cheap and require little equipment. On the negative side it is labour intensive, interpretation may be subjective, it has no multiplex capability, it is prone to false positive serological reactions and false negative results may occur due to a prozone effect. As a test it has limited mobility as it cannot be performed on whole blood or plasma samples. Despite a high sensitivity the specificity can be disappointingly low and as consequence the positive predictive value of the test is low and a positive test result requires confirmation by a more specific test.

The complement fixation test (CFT) is commonly used for the diagnosis of brucellosis in cattle, sheep and goats and reported to be relatively insensitive to antibody produced in response to vaccination. However, it is highly sensitive and specific in naturally infected animals. CFT detects specific antibodies of the IgM and IgG1 types and it is more sensitive to the IgG1 type than IgM type. Drawbacks are false negative results with the IgG2 type antibodies and it is technically challenging to perform as a large number of reagents, controls and reagent titrations are required. This test requires good laboratory facilities and trained staff whilst prozone formation, cross reactions anti-complementary activity are problems encountered with CFT. The CFT is recommended by the OIE as the test prescribed for international trade and is often used as a secondary test for confirmation of RBT positive samples.

The Indirect ELISA (iELISA) is more sensitive than the conventional serological test. However, it is not as specific as the other tests and it cannot distinguish vaccine stimulated antibodies from antibodies produced from a field infection.

The Competitive ELISA was developed to differentiate vaccine antibodies of B. abortus S19 from natural infection antibodies. It is simpler to perform than the CFT and may be readily standardized by the use of purified S-LPS antigen and monoclonal antibody for competition. However, the cELISA cannot completely eliminate cross reactions from other bacteria like Y. enterocolitica O:9.

The Fluorescence Polarisation Assay (FPA) is a very simple and rapid assay that does not require a solid phase but disadvantages of the FPA include a shorter history of use and it has not been established in the testing routine of most National Brucellosis Reference Laboratories. It has not been validated in African conditions yet.

Internationally accepted serological tests for bovine brucellosis within the parameters described by the OIE include:
• the Rose Bengal test
• the buffered plate agglutination test
• the complement fixation test (a prescribed test for international trade)
• enzyme-linked immunosorbent assays (ELISAs), both indirect (I-ELISA) and competitive (prescribed tests for international trade)
• the fluorescence polarisation assay (a prescribed test for international trade)

Other tests which are not recognised for the purposes of international trade but which can be used in combination with officially approved standardised tests include:
• the brucellin skin test
• the serum agglutination test
• the native hapten and cytosol protein-based tests
• milk tests (the milk I-ELISA, and milk ring test)
• the interferon gamma test.

OTHER DIAGNOSTIC ASSAYS
There are a number of diagnostic assays which may be used alone, or in various combination with any other test, dictated by the specific circumstances pertaining to each scenario.

Microscopic examination using Stamp’s modification of the Ziehl-Neelsen stain is not specific for Brucella spp. as Coxiella burnetii, Chlamydophila abortus and Nocardia spp. also stain weakly acid-fast. Nocardia spp can be differentiated from these organisms on morphological grounds, but it is extremely difficult to differentiate C.abortus and C. burnetii from Brucella spp. beyond any doubt.

Culture of Brucella abortus from cattle with positive titres, or aborted foetuses is required to confirm the diagnosis. This becomes increasingly important when the incidence of disease in a region declines, as the number of false-positive serological reactions may then increase. In order to get valuable epidemiological information, isolated organisms have to be typed into species and biovars.

The specimens of choice for culture in aborted foetuses and full term calves include the foetal membranes, lungs, stomach content, liver and spleen. When dealing with live cows the uterine discharge, milk or colostrum may specimens of choice. Other specimens may also include the uterus, milk, udder tissue, fluid aspirated from hygromas, male accessory sex glands and testes, semen or seminal plasma.

The causative organism is most frequently isolated from the retropharyngeal, supramammary and internal iliac lymph nodes (in that order) in naturally-infected cattle and more specifically in older animals. It was reported that the head lymph nodes most frequently gave positive isolates in heifers, followed by the iliac and supramammary nodes.

The sensitivity of the culture methods used may be influenced by the number of viable organisms present and long incubation times can result in overgrowth of B. abortus by contaminants. It is not usually feasible to collect a large number of tissues from animals at slaughter.
Polymerase chain reaction assays are rapid and specific tests which can be used effectively in the diagnosis of humans, production animal and wildlife brucellosis. A number of conventional PCRs have been developed that can diagnose brucellosis at genus level. The specificity of the PCR varies depending on the target region and the sensitivity varies depending on the infected material and stage of infection. Multiplex PCR assays have been developed that enable identification and differentiation of Brucella at the species and biovar level from culture or infected tissue. In order to implement effective epidemiological tracebacks and eradication programs, species specific assays are needed.

In his study Chisi (2013) found the BaSS PCR developed by Bricker et al. (2003) was suitable to diagnose B. abortus at species level from abomasal fluid and bacterial colonies. He therefore recommended that the BaSS PCR should be used to confirm B. abortus at species level during the waiting period for culture identification. The BaSS PCR is able to identify B. abortus from abomasal fluid within two days.

COMPARATIVE STUDIES
The findings of a number of different studies have been reported in which the sensitivities and specificities of different diagnostic tests were compared for more than one Brucella sp and in a number of different domestic animals, wildlife species and humans

McGiven et al. (2003) compared the diagnostic sensitivity and specificity of various serological tests with one another (which included SAT, CFT, fluorescence polarization assay (FPA), enzyme-linked immunoabsorbent assays (ELISA, indirect and competitive ELISA (iELISA and cELISA)). The diagnostic sensitive value of SAT was the lowest at 81.5% compared to other tests ranging from 91.8-97.2% and these authors indicated that the performance of SAT is relatively substandard if compared to FPA, cELISA and iELISA. Furthermore, SAT is susceptible to false positive reactions by cross reacting antibodies from organisms with similar antigenic structure to Brucella and its use is discouraged by the OIE.

O’Grady et al recently investigated the usefulness of culture for the confirmation of brucellosis in cattle. In this study involving 248 animals from four actively infected dairy herds paired supramammary, retropharyngeal, and internal iliac lymph nodes were cultured. Results were compared with those from five serological tests which included a microserum agglutination test (MSAT), complement fixation test (CFT), the indirect (iELISA) and competitive ELISA, and the fluorescence polarisation assay (FPA). 

Brucella abortus was isolated from 86.8% of animals in at least one of the lymphnodes. Retropharyngeal lymphnodes (RP) were culture positive in 90.5% of the identified animals followed by Supra mammary (SM) (84.4%) and internal iliac (IL) (56.4%). All three lymphnodes were positive in 51.7% of cases. Both the RP and SM lymphnodes were positive in 75.4% of culture positive cases. SM alone were culture positive in 13.7% of cases and RP alone in 6.2% of cases.

The MSAT was the most sensitive test (71.8%) in detecting culture-positive animals followed by FPA (68.8%), iElisa (62.0%), cElisa ((59.5%) and the CFT (59.5%), however, a significant percentage of infected animals were undetectable using these standard serological assays ..
O’Grady et al concluded that latently infected sero-negative but culture positive animals remain a risk for transmission of infection and that RP and SM lymphnodes are a sensitive test in confirming the presence of B abortus in herds with active infections.

Chisi SL (2013) recently evaluated a number of tests in his thesis for the evaluation of serological tests in the diagnosis of bovine brucellosis in KZN. The sensitivity and specificity of five serological tests namely the RBT, CFT, SAT, iELISA, cELISA were determined in 63 naturally infected cattle with known bacterial culture status and his results are illustrated below.

<table>
<thead>
<tr>
<th>TEST</th>
<th>% SENSITIVITY (95% CI)</th>
<th>% SPECIFICITY (95% CI)</th>
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<tbody>
<tr>
<td>RBT</td>
<td>91.7 (80.0-97.7)</td>
<td>86.7 (55.5-98.3)</td>
</tr>
<tr>
<td>CFT</td>
<td>91.4 (79.6-97.6)</td>
<td>81.3 (68.0-89.0)</td>
</tr>
<tr>
<td>i ELISA</td>
<td>91.7 (80.0-97.7)</td>
<td>86.7 (55.5-98.3)</td>
</tr>
<tr>
<td>c ELISA</td>
<td>91.5 (79.6-97.6)</td>
<td>81.3 (68.0-89.0)</td>
</tr>
<tr>
<td>SAT</td>
<td>87.2 (74.3-95.2)</td>
<td>68.8 (41.3-89.0)</td>
</tr>
<tr>
<td>BaSS PCR</td>
<td>72.7 (39.0-94.0)</td>
<td>92.0 (79.1-98.4)</td>
</tr>
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</table>

95% CI intervals computed by Chisi would include the average sensitivities and specificities from other authors for RBT and CFT.

Results of this study indicate that the performance of traditional tests used to diagnose bovine brucellosis in South Africa, namely RBT and CFT but especially RBT, is similar to primary binding assays (iELISA and to a lesser extent cELISA) in diagnosing naturally infected cattle. RBT and CFT performed better than the cELISA and iELISA in Brucella free herds with a DSp of 100%. The costs of the ELISA kits prohibit their use in third world countries, despite reports by McGiven et al. (2003) and others that ELISAs are automated, rapid, easy tests that renders themselves easily to quality control measures. Due to this fact the RBT and CFT therefore are considered the best combination to detect Brucella infections at the Allerton Provincial Veterinary Laboratory (APVL) in Kwazulu-Natal.

**COST SAVING STRATEGIES**

There are continual attempts at costs saving strategies which will be briefly mentioned here but not all will be discussed in detail.

*Reducing costs* may be achieved by innovations such as paper-based microfluids resulting in a generation of tests that are simple to run and whose reagents and consumables are more affordable and easily stored, and others such as automation and the development of more efficient high-throughput methods. Developing tests that would be suitable to use on different sample types, such as serum, blood, body fluids may also be beneficial.
**Multiplexing** is an alternative cost reducing strategy by creating multiplex serological assays that detect antibodies to antigens from several different pathogens simultaneously within the same reaction vessel. In one such developmental study several *Brucella* antigens including *B. suis* 1330 (whole cell), *B. abortus* rough LPS, recombinant BP26 (48), as well as a *Yersinia enterocolitica* O:9 (whole cell) antigen had been evaluated. And it has been reported that the preliminary data is encouraging, and supportive of the multiplex approach. In another recent study a multiplex assay was for instance capable of detecting *B. ovis*, *A. seminis*, and *H. somni* DNA simultaneously from semen samples from experimentally infected rams. The method was highly specific since and urine can be used as an alternative to semen samples.

**Increasing test mobility** In general, mobile field or ‘pen-side’ assays for brucellosis could also be of considerable value. It has been reported that it is possible to achieve, in a lateral flow (LF) immunoassay format, an increased analytical sensitivity by between 7- and 300-fold by means of **manoparticle (?)** TRF. Other ways include the use of up-converting phosphor technology, which has been applied in LF format for the detection of *Brucella sp.* Increasing the diagnostic specificity and analytical sensitivity of assays becomes more technical and beyond the scope of this article. For those who may be interested it is suggested to read the article by as listed below in the references.

**CONCLUSION**

The use and understanding of different tests for Brucellosis under different circumstances is necessary due to the complicated nature of the infection and its innate and acquired immune response.

Brucellosis infected individuals may have variable incubation periods and may often be subclinical in nature, and therefore a definitive diagnosis should be based on the isolation and identification of *B. abortus* and on positive serological results. The latter may be based on detection of antibodies in samples such as blood, milk, whey, vaginal mucus, or seminal plasma. The phenomenon of culture-positive and seronegative animals, some of which may be latently infected, would imply that serology alone is not a completely reliable indicator of the risk posed by individual animals in spreading infection.

When evaluating the available tests in various scenarios, such as the occurrence of brucellosis at various stages of detection surveys and eradication programmes the actual epidemiological situation of what the true incidence and prevalence rates are should be taken into consideration. The predictive values of tests vary according to the progress of the eradication programme and criteria other than positive serology may also be important in an eradication programme.

The “ideal” diagnostic test should meet the following criteria:
- it should detect infection early and during the long and variable incubation period;
- it should not be influenced by the presence of “non-specific” antibodies;
- it should detect carriers;
- it should be able to differentiate between responses to vaccination and those due to field infection.
As there is no single available test that completely conforms to these requirements, it will always result in multiple tests being employed, and it may be necessary that one no longer puts emphasis on intrinsic values of a test, but rather its positive predictive value. The latter will then be related to the clinical utility of the result. Eradication programmes should not necessarily have a “zero seropositivity” situation as the objective but rather the absence of infection, even if seropositive animals may be present.

REFERENCES

QUESTIONS:

1. Which of the following immunoglobulins are most common in the blood stream
   a. Immunoglobulin G
   b. Immunoglobulin M
   c. Neither of the above
   d. Immunoglobulin E
   e. B and d above

2. The Compliment fixation test is
   a. Prone to false negative results from IgG2 effects
   b. Technically challenging
   c. Complicated by haemolysed samples
d. Possibly false negative in recently aborted animals
e. All of the above

3 Microscopic examination of correctly stained samples is
a. Specific for Brucella spp
b. May confuse Brucella spp with campylobacter bovis
c. Can differentiate between Nocardia spp and Brucella spp
d. Possibly affected by other acid fast organisms
e. Not useful in diagnosing Brucella spp

4 Positive cultures for Brucella spp in actively infected herds are most likely from
a. Internal iliac lymphnodes
b. Retropharyngeal lymph nodes
c. Prescapular lymph nodes
d. Supramammary lymph nodes
e. None of the above

5 The BaSS PCR is
a. Unsuitable for testing for Brucella spp
b. Useful for identifying Brucella spp in aborted material
c. A slow and tedious test
d. Not able to differentiate Brucella spp
e. High in diagnostic sensitivity

6 The serum agglutination test
a. May give false negative tests in chronically infected animals
b. Is useful in detecting early infections
c. May be used to identify individual seropositive animals following identification of positive herds by milk ring test
d. Has low specificity
e. All of the above

7 The following tests are internationally accepted tests by the OIE
a. Rose Bengal test
b. CFT
c. ELISA
d. Buffered plate agglutination tests
e. All the above

8 Multiplexing of PCR screen is
a. A cost reducing strategy
b. Able to detect multiple antigens at once
c. Only been documented in human diagnostics
d. a + b
e. all of the above

9 Ideal diagnostic tests should meet the following criteria
a. Should be able to detect infection early
b. Be influenced by “non specific” antibodies
c. Should be able to detect carrier animals
d. Does not need to be able to differentiate between vaccine and field stains
e. None of the above

10 A suitable test for the final stage of disease eradication
   a. Should preferably be highly specific
   b. Should be preferably be highly sensitive
   c. Should preferably be sufficiently specific
   d. a + c
   e. b + c