THE EVALUATION OF SEROLOGICAL TESTS IN THE DIAGNOSIS OF BOVINE BRUCELLOSIS IN NATURALLY INFECTED CATTLE IN KWAZULU-NATAL PROVINCE, SOUTH AFRICA

By

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Contents

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Thesis summary

The evaluation of serological tests in the diagnosis of bovine brucellosis in naturally infected cattle in Kwazulu-Natal Province, South Africa

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Bovine brucellosis is a disease affecting cattle worldwide and is mainly caused by *Brucella abortus.* Diagnosis of brucellosis is an important part of controlling bovine brucellosis in naturally infected cattle in South Africa. Serology remains the most practical method available to screen herds and confirm diagnosis. This study investigated the performance of Rose Bengal test (RBT), complement fixation test (CFT), serum agglutination test (SAT), competitive enzyme-linked immunoabsorbent assays (cELISA), indirect ELISA (iELISA) and *B. abortus* species specific (BaSS) PCR in the diagnosis of brucellosis in naturally infected cattle in Kwazulu-Natal Province of South Africa. Natural brucellosis infection status of animals was determined by culturing from abomasal fluid, milk, hygroma fluid, lymph nodes or uterine discharges samples. Some of the samples were used in the BaSS PCR assay whereas sera obtained from the same animals were tested using RBT, CFT, SAT, cELISA and iELISA. Diagnostic sensitivity (DSe) and diagnostic specificity (DSp) were determined for each test to assess each test performance to determine bovine brucellosis in naturally infected animals. Positive cultures were identified as *B. abortus* biovar 1. The iELISA and RBT had the highest DSe of 91.7% (95% confidence interval (CI): 80.0-97.7%) followed by cELISA with a DSe of 91.5% (95% CI: 79.6-97.6%) and the CFT and SAT with DSe values of 91.4% (95% CI: 79.6-97.6%) and 87.2% (95% CI: 74.3-95.2%) respectively. The BaSS PCR had the lowest DSe of 72.7% (95% CI: 39.0-94.0%), but highest DSp of 92.0% (95% CI:

79.1%-98.4%) followed by the RBT and iELISA DSp value of 86.7% (95% CI: 55.5%-98.3%). CFT and cELISA had a DSp of 81.3% (95% CI: 68%-89%) whilst the SAT had the lowest DSp of 68.8% (41.3%-89.0%). Although there was no statistically significant difference between the six tests in their ability to diagnose brucellosis, the RBT and indirect ELISA had the highest diagnostic sensitivity and specificity. The BaSS PCR diagnosed *B. abortus* at species level and could be used to confirm culture results.

Chapter 1: Literature Review

Bovine brucellosis

Brucellosis is caused by bacterium of the genus *Brucella* (Ficht, 2003, Azolra et al., 2008, Chaudhuri et al., 2010) consisting of *B. abortus, B. melitensis* and *B. suis,* which are smooth strains that are virulent and *B. ovis, B. canis* (naturally occurring virulent rough strains) and *B. neotamae* (Corbel, 2006). Newly described species include *B. ceti*, cetaceans as preferred hosts, *B. pinnipedialis,* seals as preferred hosts (Foster et al., 2007) and *B. microti* in rodents (Scholz et al., 2008). *B. inopinata* has been discovered recently from a human breast implant (Scholz et al., 2010).

Brucellosis is a zoonotic disease of both economic and public health importance worldwide. Bovine brucellosis affects cattle and is caused by a Gram-negative bacteria called *B. abortus*, less frequently by *B. melitensis* and rarely by *B. suis* (Bishop et al.,1994). The disease is responsible for annual losses in South African beef and dairy industries. *Brucella abortus* has 8 biotypes (subspecies) or biovars (bv) i.e. 1, 2, 3, 4, 5, 6, 7 and 9 (Alton et al., 1988, Bishop et al.,1994). *Brucella abortus* bv 7 has been considered to be a mix culture of biovar 3 and 5 (Alton et al., 1988, Bishop et al.,1994) and has been since deleted because no authentic isolate has been isolated for a long while (Alton et al., 1988).

Bovine brucellosis primarily affects the reproductive system by an inflammatory response that affects cells of the reticuloendothelial system of the liver, spleen, lymph nodes and the placenta during pregnancy (Ficht, 2003). Infection may result in death and expulsion of the fetus that will normally be experienced during the 5-8 months of gestation. Large numbers of *Brucella* organisms are released by infected animals during abortion that could infect other susceptible animals in the herd (McGiven et al., 2003, Culter et al., 2005).

Diagnostic tests

Control in South Africa is achieved by vaccination mostly with *B. abortus* S19 and RB51 vaccines, active surveillance programs/schemes, as well as a test and slaughter policy. In order to achieve effective surveillance programs/schemes reliable diagnostic test(s) are needed. Bacterial culture is the gold standard for

diagnosing brucellosis (Morgan, 1977, Sachse, 2003, Hinic et al., 2009). However, in some cases, culture and isolation yields negative results or are impractical, especially when dealing with large herds and huge numbers of animals. Furthermore, culture can take at least two weeks from sample submission to the production of a result. *Brucella* cultures also need to be handled by highly skilled staff in laboratories with appropriate biosafety measures. In the absence of bacterial culture, serological tests offer a more practical means of diagnosing brucellosis. However, more than one test is needed to confirm brucellosis since no single test absolutely identifies *Brucella* but a combination of tests are ideal (OIE, 2008, Matope et al., 2011).

Bacteriology

Isolation and identification of *Brucella* spp. is recognized as the 'gold standard' for definitive diagnosis of brucellosis (Alton et al., 1988; OIE, 2008) but is time consuming since it takes 10-14 days to culture the bacteria and complete 25 phenotypic traits used for species and biovar identification of cultured organisms (Alton et al., 1988, Whatmore, 2009). Furthermore absence of bacterial isolation does not rule out brucellosis since some positive animals yield negative culture results. Therefore, serological tests can be used as indirect methods to define the brucellosis status of animals (Gall and Nielsen, 2004).

Serology

Serology is the most practical and widely used method for the diagnosis of bovine brucellosis; defined as the measurement of specific antibody activity of immunoglobulin present in the serum (Thrusfeld, 2008). Diagnosis of bovine brucellosis, serologically, is normally performed by the traditional tests i.e. Rose Bengal test (RBT), serum agglutination test (SAT) and complement fixation test (CFT) (Van et al., 1984) that employ a whole cell antigen measure (Nielsen, 2002). The milk ring test (MRT) is used to screen dairy cattle herds for bovine brucellosis (Van et al., 1984). Milk samples are used to test for antibodies against bovine brucellosis.

Before reviewing the diagnostic tests, especially serological tests, it is useful to explain the antibody response to *B. abortus* in cattle. Antibody response to *B.*

abortus in cattle consists of an early immunoglobin M (IgM) isotype response usually in the first or second week following infection depending on the route of exposure, the dose of the inoculums and the health of the animal (Beh, 1974, Nielsen, 2002). This IgM response is followed, almost immediately, by the production of IgG1. antibody and later by small amounts of IgG2 and IgA (Nielsen, 2002). Since IgM antibodies are mainly produced during exposure to microorganisms other than *Brucella* species or environmental antigens, serological tests that measure IgM like SAT results in false positives and low assay sensitivity (Nielsen, 2002, Corbel, 2006). As IgG2 and IgA antibodies accumulate later after infection and are present in small amounts, the main isotype for testing for exposure to *Brucella* antigen is IgG1 and is the most useful (Nielsen, 2002).

Milk ring Test (MRT)

The MRT uses a whole cell antigen stained with hematoxylin (Huber and Nicoletti, 1986, Sutra et al., 1986). Antibodies present against brucellosis in the milk fat layer will attach to the fat globules via the Fc (fragment crysallizable region that is the tail region of the antibody and interacts with the cell surface receptors), a specific binding receptor of antibodies (Tizard, 1996, Nelsen, 2002). This will lead to the antibodies agglutinating with the antigen and as the fat globules rise a purple band appears at the top of milk. If no antibodies are present the fat band will appear buff colored (Nelsen, 2002). The MRT is used as a screening test in brucellosis negative dairy herds as a bulk milk test. MRT positive bulk milk samples results in the whole herd being tested using RBT, CFT and SAT.

Rose Bengal test (RBT)

RBT belongs to a set of serological tests known as buffered tests and utilizes whole cell antigen. The test is simple to perform, inexpensive and suitable for screening individual animals. IgG1 is the immunoglobulin that is active in the RBT even though it can detect IgM and IgG_2 isotypes. However, RBT more effectively detects IgG1 than IgM and IgG2 (Nielsen, 2002, Cadmus et al., 2008). The RBT antigen is buffered at a pH of 3.65 which ensures that non-specific agglutins are destroyed. This low pH of 3.65 inhibits agglutination with IgM and instead encourages agglutination with IgG1 (Allan et al., 1976). This effectively reduces non-specific binding and improves the specificity of RBT (Cadmus et al., 2008). The antibodies in

the serum agglutinate when they come into contact with the test antigen. False positives normally occur due to the presence of IgM as a result of S19 vaccination and some cross reacting antibodies (Nelsen, 2002, Bishop et al.,1994).

One drawback of the RBT is a phenomenon known as *prozone formation* (Tizard, 1996) which is mostly the cause of false negatives (Tizard, 1996). Prozone formation occurs when a high concentration of antibodies are added during the test resulting into inhibition of agglutination. The presence of incomplete antibodies is another cause of prozone formation (Plackett and Alton, 1975). Prozones are more likely to occur in pregnant cows because the ratio of IgG2: IgG1 is increased since most of the IgG1 is transferred to the mammary glands while IgG2 remains high. Other reasons for prozone formation is cross linking which occurs due to deep sited antibodies (Tizard, 1996) or the antibodies are only capable of restricted movement in the hinge region causing them to be functionally monovalent. The RBT is widely used as a rapid and simple screening test. However, this test requires confirmation by other serological tests due to false positive results that may occur in endemic areas.

Gall and Nielsen (2004) examined the sensitivity and specificity values of serological assays detecting the exposure to *B. abortus* in more than 50 publications. These authors compared the different serological tests by giving the averaged sum of the sensitivity and specificity values for each test to give a performance index (PI, with a 200 score being perfect). Based on the PI, RBT (PI = 167.6) ranked lower than the other conventional tests like SAT (PI=171.6) and CFT (PI = 172.5) but slightly higher than MRT (PI=164). The mean RBT sensitivity and specificity was 81.2% and 86.3%, respectively (Gall and Nielsen, 2004).

Serum agglutination test (SAT)

SAT utilizes the process of adding *Brucella* cells (whole cell antigen) to dilute serum and observing the pattern of the cell pellets formation after a set incubation time (Corbel, 1972). The SAT has been reported to be useful in detecting new infections as early as two weeks but its use in chronically infected herds is limited (Bishop et al.,1994). Some chronically infected animals will be classified as negative when they are in fact infected (Bishop et al.,1994) since SAT predominantly targets the

immunoglobulin IgM in an early infection that might not be detectable in chronic infections when IgG dominates (Saegerman et al., 1999). Some veterinarians in South Africa use the SAT titres as a proxy measure to determine *B. abortus* S19 vaccination since IgM is the predominant immunoglobulin after vaccination with strain S19 (Bishop et al.,1994). It is thought that that high SAT titres and low CFT titres are indicative of a vaccine reaction rather than a field infection.

McGiven et al. (2003) compared the diagnostic sensitivity and specificity of various serological tests with one another (which included 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% (McGiven et al., 2003). These authors indicated that the performance of SAT is relatively substandard if compared to FPA, cELISA and iELISA (McGiven et al., 2003). 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 (2008).

Complement Fixation Test (CFT)

The CFT takes advantage of the immune phenomenon that involves the complement system (Tizard, 1996). Activation of the complement takes place when an antigen invades the animal's body and forms a complex with the animals antibody (Tizard, 1996). The final product of this activation is the generation of membrane attack complexes (MAC) which "puncture holes" in the antigen's membrane resulting into disruption and eventually death of the antigen (Tizard, 1996). In the CFT, the antigen is replaced by sheep red blood cells resulting into haemolysis of the red blood cells (RBCs) when antibodies are absent in the serum. This effectively is known as the indicator system of the CFT (Tizard, 1996). The principle of the CFT is that diluted animal sera , whole cell antigen and a pre-titrated amount of guinea pig compliment are mixed together (Nielsen, 2002). The complement in the test serum is inactivated at a temperature of 37 ˚C (Alton et al., 1988 , Nielsen, 2002). An antigen-antibody complex is created when antibodies are present in the serum which results in the complement being activated. When the indicator system consisting of sheep RBCs is added and antibodies are present in the serum, the complement is not available as it

has been already consumed by the antigen-antibody complex. Therefore, lysis of RBCs does not take place as a result, compared to when antibodies are absent in the test serum, lysis of RBCs by the complement takes place (Nielsen, 2002).

The CFT is widely used for the diagnosis of brucellosis in cattle, sheep and goats. It is relatively insensitive to antibody produced in response to vaccination but highly sensitive and specific in naturally brucellosis infected animals (Nielsen, 2002). The CFT detects specific antibodies of the IgM and IgG1 types. This test is more sensitive against the IgG1 type than that of IgM type (Nielsen, 2002). However, CFT gives false negative results with the IgG2 type antibodies (MacMillan et al 1990, McGiven et al., 2003). The CFT is technically challenging to perform due to a large number of reagents, controls used and reagent titrations are necessary (Nielsen, 2002). Furthermore, this test requires good laboratory facilities and trained staff.

Prozone formation and cross reactions are problems encountered with CFT (Hall et al., 1971, Godfroid et al., 2002). Another phenomenon encountered with CFT is anti-complementary activity i.e. this is when substances other than *Brucella* antigen fix the complement (Plackett and Alton, 1975). This can arise when there is competition for the antigen with non-complement fixing antibodies (Hall et al., 1971). Some studies have demonstrated that IgG2 immunoglobulin can attach complement leading to anti-complementary activities during the test (Plackett and Alton, 1975). 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 (Nielsen, 2002, OIE, 2008).

As indicate previously Gall and Nielsen (2003) ranked the CFT based on PI at the bottom with other conventional tests like MRT (PI=164), RBT (PI=167.6) and SAT (PI=171.6). The mean sensitivity and specificity of CFT was 89% and 83.5%, respectively (Gall and Nielsen, 2004). McGiven et al. (2003) reported the diagnostic sensitivity and sensitivity of CFT to be 91.8% and 99.9%, respectively. The performance of the diagnostic sensitivity of CFT (as well as SAT) was found to be inferior compared to FPA, iELISA and cELISA in this study (McGiven et al., 2003). RBT and CFT are used in series by many reference and state laboratories as the confirmation tests for bovine brucellosis in cattle and cattle herds. Therefore utilizing

the results of RBT and CFT as serial tests increases the specificity compared to viewing individual test results as RBT has a higher sensitivity than CFT whereas CFT has a higher specificity (McGiven et al., 2003, Gall and Nielsen, 2004).

Primary binding assays

In contrast to the traditional tests that utilize whole cell antigen as the diagnostic reagent, primary binding assays use lipopolysaccharide (LPS) or O-side chain. LPSs are very large molecules consisting of a lipid and a polysaccharide joined by a covalent bond (Lapaque et al., 2005). They are found in the outer membrane of Gram-negative bacteria. They act as endotoxins and elicit strong immune responses in animals. LPS consists of the O-side chain (Lapaque et al., 2005) that varies in the amount of homopolymer amongst the Gram-negative bacteria. The homopolymer of O side chain is 1, 2-linked N-acylates 4-amino-4, 6-dideoxy-α-d-mannopyranosyl residue (Caroff et al., 1984). Bacteria with the O-side chain are called smooth whilst those without are called rough strains.

The FPA and ELISA are examples of primary binding assays. These assays are based on the detection of antibodies to the LPS antigen of the smooth *Brucella* strains (McGiven et al., 2003, Porte et al., 2003). One of the major diagnostic problems that result from the use of the O-antigen is false positive results with bacteria possessing similar O-antigenic side chains of the LPS such as *Yersinia enterocolitica* O:9 (McGiven et al., 2003, Nielsen and Ewalt, 2008). ELISAs were developed to be more sensitive and specific alternatives to conventional tests and are therefore robust, fairly simple to perform with a minimum of equipment.

Indirect ELISA (iELISA)

The iELISA utilizes smooth LPS (S-LPS) as diagnostic antigen and a mouse monoclonal antibody specific to an epitope on bovine IgG1 conjugated to horse radish (Vanzini et al., 1998, Nielsen, 2002). The immunodominant epitope of S-LPS is the O-chain (McGiven et al., 2003). The iELISA is more sensitive than the conventional serological test. However, it is not as specific as the other tests (Nielsen et al., 1996). Another drawback is that it cannot distinguish vaccinal antibodies from antibodies produced from a field infection (Nielsen et al., 1996).

Competitive ELISA

The cELISA uses the O-side chain of LPS as the diagnostic antigen (Nielsen et al., 1995, 1996). A monoclonal antibody specific to the O chain of LPS (Nielsen et al., 1995) provides competition to antibodies present in positive sera for specific epitope sites on the O chain of the LPS (Nielsen, 2002). Since traditional serological tests and iELISA cannot differentiate vaccinal antibodies from field infection antibodies, the cELISA was developed to differentiate vaccine antibodies of *B. abortus* S19 from natural infection antibodies (Chin et al., 1989, Nielsen, 2002). The cELISA is simpler to perform than the CFT and may readily be standardized by the use of purified S-LPS antigen and monoclonal antibody for competition (Claes et al., 1990). However, the cELISA is not a full prove test either as it has its own limitations since it cannot completely eliminate cross reactions from other bacteria like *Y. enterocolitica* O:9 (Van et al., 1984, Godfroid et al., 2002).

Fluorescence Polarisation Assay (FPA)

The FPA utilizes the principle of fluorescent molecules rotating randomly in solution (McGiven et al., 2003, Muma et al., 2007). The speed of rotation is inversely related to the size of the molecule i.e. a smaller molecule will rotate faster than a bigger one. This principle is exploited in the FPA. Positive *Brucella* sera will contain antigen bound to antibodies and will thus rotate at a slower speed than negative sera that contains no antibodies (McGiven et al., 2003, Muma et al., 2007). This allows for the difference in speed to be measured and thus classify sera as positive or negative. FPA is a very simple and rapid assay that does not require a solid phase (McGiven et al., 2003). Disadvantages of the FPA include, a shorter history of use , is not established in the testing routine of most National Brucellosis Reference Laboratories and has not being validated in African conditions as yet (McGiven et al., 2003., Matope et al., 2011).

Diagnostic sensitivity and specificity of primary binding assays

McGiven et al. (2003) compared the diagnostic sensitivity and specificity (DSp + DSe) of CFT, SAT, FPA, cELISA and iELISA and the results were in the following order of magnitude: FPA>iELISA>cELISA>CFT>SAT. The order of magnitude based only on diagnostic sensitivity of these tests were iELISA>FPA> cELISA>CFT>SAT (McGiven et al., 2003). With the PI (averaged sum of the

sensitivity and specificity values) determined by Gall and Nielsen (2004) for brucellosis serological tests ranked identical to the diagnostic sensitivity and specificity (DSe+DSp) found by McGiven et al. (2003) in the following order of magnitude: FPA>iELISA>cELISA>CFT>SAT. From these comparative studies (McGiven et al., 2003, Gall and Nielsen, 2004), it seems that the primary banding assays were more sensitive and specific than the traditional serological tests which include RBT and CFT that are currently being used as serial tests for brucellosis in South African laboratories.

Polymerase chain reaction (PCR)

Serology and culture thus far discussed for detecting brucellosis infection have their limitations and there has been increased attention in developing rapid accurate methods for detecting *Brucella* species from bovine samples. PCR assays are rapid and specific tests which can be used effectively in the diagnosis of bovine brucellosis (Azolra et al., 2008). A number of conventional PCR's have been developed that can diagnose brucellosis at genus level i.e. 16S rRNA (Bricker, 2002), 16S-23S intergenic spacer region (Fox et al., 1998), omp2 (Leal-Klevezas et al., 1995) and BCSP31 (Cortez et al., 2001, Hinic et al., 2009). The specificity of PCR varies depending on the target region and the sensitivity varies depending on the infected material and stage of infection. The 16S rRNA amplifies *Ochrobacterium anthropi* which is a close relative of *Brucella* (Bricker, 2002). The 16S-23S intergenic spacer region is a highly variable region in bacteria and does not amplify *O. anthropi*. The BCSP31 PCR amplifies the BCSP gene which encodes a protein with unknown function (Baily et al., 1992, Bricker, 2002) in *Brucella* species except *B. ovis* (Halling et al., 1993). The PCR assay that detects a homologous region in the omp2a and 2b genes provides twice the number of target per bacterium and has been applied to blood and found to be more sensitive than serology and culture (Leal-Klevezas et al., 1995).

Multiplex PCR assays have been developed that enable identification and differentiation of *Brucella* at the species / biovar level from culture or infected tissue. These include the AMOS-PCR that utilises specific primers amplifying a locus containing a copy of the genetic element IS711 (Bricker and Halling, 1995) that distinguish *B. abortus* bv 1, 2 and 4, *B. melitensis*, *B. ovis, B. suis* bv 1 and vaccine

strains due to the different copies of the element in each of these species / biovars (Bricker and Halling, 1994, 1995). However, in order to implement effective epidemiological trace backs and eradication programs, species specific assays are needed (Bricker, 2002).

The *B. abortus* species specific (BaSS) PCR was developed from the AMOS PCR specifically for cattle isolates (Bricker et al., 2003). The BaSS PCR assay identifies and discriminates *B. abortus* field strains (wild-type biovars 1, 2, and 4) from *B. abortus* vaccine strains, as well as other *Brucella* species and non-*Brucella* bacteria. Since it was developed from the AMOS-PCR, the BaSS PCR is based on the amplification of the genetic element IS711 and exploits the pleomorphosis exhibited by the species specific localization of the IS711 (Bricker and Halling, 1994). The BaSS PCR assay consists of seven unique primers that have the ability to target and amplify four different loci of the *B. abortus* genome (Bricker et al., 2003, Sachse, 2003). This includes 16S rRNA gene primers that amplify a 800 bp conserved region in most bacteria (internal control), the *B. abortus* specific primers that amplify a 498 bp product consisting of the IS711 adjacent to the *Alk*B gene that is specific to *B. abortus* (Bricker et al., 2003), primers that amplify IS711 inserted within the *wbo*A gene that appears only in *B. abortus* vaccine strain RB51, primers that amplify the erythritol (eri) catabolic region with a 702 bp deletion from the S19 vaccine strain (Bricker and Halling, 1995, Bricker et al., 2003, Sachse, 2003). The sensitivity and specificity of the BaSS PCR using bacterial isolates as sources of DNA has been estimated in the range of 67 -100% whilst specificity was estimated at 93.2-99.7% (Bricker et al., 2003).

Control, monitoring and surveillance scheme in South Africa

The brucellosis scheme is used to detect, control, combat and eventually eradicate bovine brucellosis in South Africa (Bosman, 1980). Various test programs have been devised to attain eradication of bovine brucellosis in South Africa. These include accreditation, maintenance, diagnostic herd test, ordinary diagnostic import, export and infected test programs (Bosman, 1980). Joining a test program is voluntary except for infected and import programs. All the above programs require an accurate and practical means of detecting or excluding infection in a herd. In dairy herds the

MRT is used to screen dairy herds (Bosman, 1980). Bulk milk samples are collected and tested for antibodies against bovine brucellosis using the MRT. MRT positive milk samples result in the testing of the whole herd using official screening tests namely RBT and CFT. The herd testing positive on both serological tests is regarded as infected (Bosman, 1980). Therefore, brucellosis eradication programs are based, almost exclusively, on serological screening of herds to detect and remove infected animals using the MRT in dairy herds and serological tests for diagnosing the disease in individual animals.

Study objectives and hypothesis

Allerton Provincial Veterinary Laboratory (APVL) in Pietermaritzburg in Kwazulu-Natal Province in South Africa plays an important role in the brucellosis eradication scheme since this laboratory provides a diagnostic service to Kwazulu-Natal. *Brucella* infection is confirmed either by use of culturing or serological tests at this laboratory. An animal or herd is regarded as infected when animals test positive on both RBT and CFT serological tests and/or when *B. abortus* organisms are isolated from a suspected animal. It is known that the RBT produce false positives and that the CFT is technically challenging (Nielsen, 2002) and requires good laboratory facilities and trained staff. Since literature indicates that primary binding tests have a better performance than the currently used RBT and CFT, the diagnostic sensitivities and specificities of cELISA, CFT, iELISA, RBT and SAT and their performance were measured. The use of BaSS PCR was also included to evaluate the performance of the rapid PCR method against serological tests. Samples with known bacteriology status were used to measure diagnostic sensitivities and specificities of these tests. The null hypothesis of this study was “There is no difference in the performance of the BaSS PCR, cELISA, CFT, iELISA, RBT and SAT in the diagnosis of bovine brucellosis in naturally infected cattle in Kwazulu-Natal”, compared to the alternative hypothesis that indicated “There is a difference in the performance of the BaSS PCR, cELISA, CFT, iELISA, RBT and SAT in the diagnosis of bovine brucellosis in naturally infected cattle in Kwazulu-Natal”.

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Chapter 2: The evaluation of serological tests in the diagnosis of bovine brucellosis in naturally infected cattle in Kwazulu-Natal Province

Introduction

Brucellosis is a zoonotic disease of economic and public health importance with a global distribution. The disease is caused by an intra-cellular, facultative, Gram negative bacterium that belongs to the genus *Brucella* (Ficht, 2003, Azolra et al., 2008, Chaudhuri et al., 2010). The species in this genus consists of *B*. *abortus*, *B. melitensis* and *B. suis,* which are smooth strains that are virulent to animals and humans and *B. ovis, B. canis* (naturally occurring virulent rough strains) and *B. neotamae* (Corbel, 2006*).* Newly described species include *B. ceti* (cetaceans as preferred hosts), *B. pinnipedialis* (seals as preferred hosts) (Foster et al., 2007), *B. microti* from rodents (Scholz et al*.,* 2008) and *B. inopinata* (isolated from a human breast implant) (Scholz et al*.,* 2010).

Bovine brucellosis is a disease affecting cattle worldwide and is often caused by *B. abortus*, less frequently by *B*. *melitensis*, and rarely by *B*. *suis* (Bishop et al., 2004). There are eight recognized biovars (bv, sub species) of *B. abortus* (Alton et al., 1988, Bishop et al., 2004). In South Africa, 90% of infections in cattle are due to *B. abortus* bv 1 and 10% are due to biovar 2 (Bishop et al., 2004). The prevalence of brucellosis in rural cattle in Kwazulu-Natal Province is approximately 1.45% according to a survey done in rural Kwazulu-Natal communal areas (Hesterberg et al., 2008).

Control of cattle brucellosis in South Africa is achieved by vaccination mostly with *B. abortus* S19 and RB51 vaccines, active surveillance programs as well as test and slaughter policy. In order for the surveillance to be effective, reliable diagnostic test(s) needs to be used. Bacterial culture and identification is the gold standard for diagnosis of *B. abortus*, (Morgan, 1977, Nielsen, 2002, Sachse, 2003, Azolra et al., 2008). However, in some cases, culture yields negative results or are impractical with large herds and huge numbers of animals (OIE, 2008). Furthermore, culturing and identification takes at least two weeks from sample submission and *Brucella*

cultures need to be handled by trained staff in laboratories with appropriate biosafety. In the absence of bacterial culture, serological tests offer a more practical means of diagnosing brucellosis. However, more than one test is used to confirm brucellosis since no single test absolutely identifies *Brucella* i.e. combinations of culturing, serological test(s) and / or PCR can give a definitive diagnosis.

Serology remains the most practical method available to screen herds and confirm diagnosis. In South Africa, veterinary laboratories have traditionally used Rose Bengal test (RBT), compliment fixation test (CFT), serum agglutination test (SAT) and milk ring test (MRT) in brucellosis diagnosis. These tests use a whole cell antigen as the diagnostic reagent (Van et al., 1984, Alton et al., 1988). RBT and CFT are used, in combination to confirm bovine brucellosis in many countries (Nielsen, 2002, OIE, 2008). RBT is used for its higher sensitivity while CFT is used for its higher specificity (Nielsen, 2002, OIE, 2008). Combination of CFT and SAT serological tests are unreliable since SAT is regarded as an unreliable test and "being unsatisfactory for the purpose of international trade" (OIE, 2008). It is essential to identify diagnostic tests that are reliable, specific, cost effective and easy to perform that will ensure that no uninfected animals are destroyed due to misclassification.

The primary binding assays which include indirect ELISA (iELISA), competitive ELISA (cELISA) and the fluorescent polarization assay (FPA) employ purified lipopolysaccharide (LPS) or O-antigen as the diagnostic reagent (Nielsen, 2002). ELISA test were developed to be more sensitive and specific alternatives to conventional tests (Gall and Nielsen, 2004). The tests are robust, fairly simple to perform with a minimum of equipment and the iELISA is recommended by the OIE as a suitable screening test (OIE, 2008). The conventional serological tests (SAT, RBT and CFT) and iELISA are however unable to distinguish between vaccine strain S19 and naturally infected animals (Nielsen et al., 1995). The cELISA was developed to overcome this problem and is fairly rapid to perform and cross-reacts less with other antigens (or antibodies) than the conventional tests. Further, the cELISA is simpler to perform than the CFT and may readily be standardized by the use of purified S-LPS antigen and monoclonal antibody for competition (McGiven et al. 2003, Gall and Nielsen, 2004). One of the major diagnostic problems that result

from the use of the O-antigen is false positive results with bacteria possessing similar O-antigenic side chains of the LPS such as *Yersinia enterocolitica* O: 9 (Van et al., 1984, McGiven et al., 2003).

PCR is a very rapid, highly specific and sensitive test (Azola et al., 2008). Various regions have been identified in the *Brucella* genome and are used in PCR assays using blood (Leal-Klevezas et al., 1995, Ilhan et al., 2008), milk (Leal-Klevezas et al., 1995), tissue (O'Leary et al., 2006) as well as stomach content and aborted foetuses (Centinkaya et al., 1999, Cortez et al., 2001). However, for diagnostic purposes blood, milk, hygroma fluid and abomasal fluid samples need to be investigated but there may be problems of sensitivity with some samples since the stage of infection may influence the number and location of *Brucella* organisms in white blood cells and lymphoid tissue glands (bacteriemia may not be constant) (O'Leary et al., 2006). Therefore, PCR cannot be used as a diagnostic tool using, especially, blood since the bacteriemia is not constant, but can be used to confirm identification of *Brucella* at species level from culture (O'Leary et al., 2006). A PCR assay developed to diagnose *Brucella* at species level includes AMOS PCR (Bricker and Halling, 1994, 1995, Bricker, 2002, Hinic et al., 2009). The *B. abortus* species specific (BaSS) PCR assay was developed from the AMOS PCR specifically for cattle isolates (Bricker et al., 2003). The BaSS PCR assay identifies and discriminates *B. abortus* field strain cultures (wild-type biovars 1, 2, and 4), vaccine strains, as well as, other *Brucella* species and non-*Brucella* bacteria (Bricker et al., 2003).

Current surveillance and control scheme in South Africa use serological tests consisting of RBT as a screening test and CFT as a confirmatory test. Bleeding and testing of beef and dairy cattle in commercial and communal areas are carried out within defined intervals by state and private veterinarians. Specimens used for the isolation of *Brucella* organisms include milk, hygroma fluid and lochia from live female animals, lymph nodes from carcasses of slaughtered adult animals, foetus and placenta tissues. Laboratory test results are sent to the state veterinarian, who is responsible for liaising with the animal owner, for further/appropriate actions on the farm depending on the findings. All animals reacting positive to RBT and CFT are dealt in accordance with the applicable control measures (Animals Diseases Act 35 of 1984), which include branding and slaughtering. If all animals in a herd, test

negative on both RBT and CFT, the herd will be declared free of brucellosis by the state veterinarian and a brucellosis free certificate will be issued to dairy herds.

In this study, *Brucella* bacterial culture status was determined at Allerton Provincial State Veterinary Laboratory (APVL) from milk, abomasal fluid from aborted feotuses, uterine discharges and hygroma fluid samples from suspected brucellosis infected cattle from commercial and communal herds in Kwazulu-Natal, as well as lymph nodes (nodes collected from abattoirs that slaughtered known brucellosis seropositive cattle). Serum samples of some of these cattle with known bacteriological status were obtained and evaluated to determine the diagnostic sensitivity (DSe) and specificity (DSp) of the RBT, CFT, SAT, cELISA and iELISA tests. Furthermore, the serological tests were evaluated on brucellosis free herd samples. The DSe and DSp of the PCR-BaSS assay was determined as part of a preliminary study using milk, abomasal fluid from aborted feotuses, lymph node and hygroma fluid samples submitted to APVL.

Materials and methods

Brucella negative herds

This study was conducted at APVL, Kwazulu-Natal Province in South Africa. Serum samples were collected from *Brucella* negative cattle (n=186) from 21 herds that were declared negative by the state veterinarian of the responsible area in Kwazulu-Natal based on RBT and CFT test results.

Naturally infected animals

Brucellosis status using culturing (gold standard, n=94, Table 1), was determined from milk, abomasal fluid from aborted fetuses, uterine discharges and hygroma fluid samples from suspected brucellosis infected cattle from commercial and communal herds in Kwazulu-Natal submitted to APVL. These samples also included lymph nodes that were collected at abattoirs from brucellosis seropositive animals with high CFT titers (392 or greater CFT IU) that were slaughtered under supervision of a state veterinarian according to the law (Act 35 of 1984). The *Brucella* status (negative or positive) of these animals was established through bacterial culture from lymph nodes. The samples used in this study were collected between 2009 and 2012.

Bacteriology

The abomasal fluid, milk, hygroma fluid, uterine discharges and lymph nodes samples were inoculated onto *Brucella* selective medium and blood agar (BA) (Quinn et al., 1994) and incubated at 37 °C (with 6% O_2, 10% CO_2 and 84% N_2). Plates were examined daily for the first 7 days and discarded as negative after 14 days. Suspected *Brucella* colonies were stained with modified Ziehl Nielsen (Stamp) and oxidase, urease and catalase tests were performed (Quinn et al., 1994). Suspected colonies that grew in CO_2 and tested positive for urease, catalase, oxidase and stained positive for Ziehl Nielsen were considered positives. Speciation of *Brucella* colonies were done at Agriculture Research Council-Onderstepoort Veterinary Institute (ARC-OVI) reference laboratory at Onderstepoort in South Africa as indicated by Alton et al. (1988) and OIE (2008).

Serology

Serum samples were collected from cattle with known or suspected brucellosis status by state veterinarians, animal health technicians (AHT) and/or private veterinarians in Kwazulu-Natal Province and sent to APVL. Serum samples consisting of naturally infected animals and certified *Brucella* negative herds were tested for *B. abortus* antibodies using RBT, CFT and SAT as part of the brucellosis surveillance program. In this study, samples were further tested using iELISA and cELISA.

RBT

Sera from bovine were tested for antibodies against *Brucella* antigen using RBT. This was done at APVL as described by Alton et al. (1988). The whole cell antigen and positive control were procured from Onderstepoort Biological Products (OBP). Briefly, the test was performed on 100 well plastic plates. Equal volumes (50µl) of serum and stained Rose Bengal antigen (pH 3.65) were mixed on a shaker for five minutes. Agglutination was determined by visual inspection. Any agglutination was regarded as positive and all doubtful reactions were recorded as negative. Positive and negative controls were included for each batch of sample tested.

CFT

This test was done according to the procedure described by Alton et al. (1988) that test for antibodies against *B. abortus* in bovine serum. In brief, the test was carried out on a 96 well microtitre plate. Sera were first pre-heated at 56 °C for 30 minutes in order to deactivate the complement. The sera dilution in the CFT extended from undiluted sera in row A of microtitre plate to 1/128 sera dilution (row G) using veronal buffer from row B onwards (as indicated in Herr and Te Brugge, 1985). The plate consisting of dilution of sera and complement was placed in row B and the complement/antigen mixture was placed in row C to H and the plate was incubated at 37 °C for 30 minutes. Sheep red blood cells were added to the plate and incubated for a further 30 minutes at 37 °C. The plate was read using a viewing mirror after centrifuging the plate at 2500 rpm for 5 minutes. Positive, anti-complementary and negative controls were included in each test as controls. If haemolysis was present the sample was recorded as negative and a lack of haemolysis, indicated by the formation of a red button at the centre of the well, was recorded as positive. A titre of 60 CFT IU or higher was considered positive.

SAT

The SAT was done according to the procedure described by Alton et al. (1988) where 20 µl of test serum were mixed with 80 µl of veronal buffer on a microtitre plate. Serial dilutions were done and the plate was incubated at 37 °C for 90 minutes and centrifuged at 1500 rpm for 5 minutes. The plate was then viewed under a light viewing box and a positive result was declared when agglutination was present. Those samples with titres greater than 161 SAT IU were considered positive. Positive and negative controls were included.

Indirect ELISA (iELISA)

The bovine brucellosis iELISA kit (Institut Pouquirer) was used according to the manufacturer's instruction. Test buffer (190 µl) was added to microtitre plate coated with S-LPS antigen followed by 10 µl of test sera and incubated for 1 hour at room temperature (18-25 °C). The plate was rinsed three times with PBS buffer and after conjugate was added it was incubated at room temperature (18-25 °C) for 30 minutes. Finally, a substrate was added and incubated for 20 minutes at room temperature (18-25 °C). Weak and strong positive as well as negative controls were

included. Optical density (OD) at 450 nm was measured. The sample results were calculated as positive percentage relative to the strong positive control serum (S/P) using the formula:

S/P%= 100 X [(Sample OD – negative control OD) / (Mean OD of positive control – negative control)]

A sample was considered positive if the S/P value was greater than 120%.

Competitive ELISA (cELISA)

The *Brucella* cELISA kit (Svanovir) was used according to the manufacturer's instruction. In this test, the sera samples (5 µl) were exposed to *B. abortus* S-LPS coated plates on a 96 well microtitre plate simultaneously with a mouse monoclonal antibody specific for an epitope on the O-polysaccharide portion of S-LPS. The plate was incubated for 30 minutes, at room temperature, followed by PBS buffer wash. The goat anti-mouse IgG antibody conjugate with horseradish peroxidase was added to the wells which bound to any monoclonal antibody bound to the S-LPS on the plate and was incubated for 30 minutes at room temperature and washed as described above. Substrate was added and the plate incubated in a dark place for 10 minutes. The controls consisted of a negative, positive (weak and strong) and a conjugate. Sample dilution buffer was placed in two wells and acted as the conjugate control. The OD measurements were obtained at 450 nm using a micro plate reader (BioTek ELx808) and reactivity was calculated as percentage inhibition (PI), using the formula:

PI=100 – [(Mean OD of sample/Mean OD of conjugate control) x 100]

A sample was considered positive if the PI value was greater than 30%.

BaSS PCR

DNA was extracted from 500 µl abomasal fluid, milk, hygroma fluid and uterine discharges using the Qiagen DNeasy blood and tissue extraction kit according to the manufacturer's instructions (Qiagen). The primers (Bricker et al., 2003) used in the PCR reaction consisting of 16S-F (5'GTG-CCA-GCA-GCC-GCC-GTA-ATA-C3'), 16S-R (5'TGG-TGT-GAC-GGG-GGG-TGT-GTA-CAA-G3'), *B. abortus* specific (5'GAC-GAA-CGG-AAT-TTT-TCC-AAT-CCC3'), RB51 (5' GCC-AAC-CAA-CCC-AAA-TGC-TCA-CAA3'), eriF (5' GCG-CCG-CGA-AGA-ACT-TAT-CAA3'), eriR (5'CGC-CAT-GTT-AGC-GGC-GGT-GA3') and IS711 (5' TGC-CGA-TCA-CTT-AAG-

GGC-CTT-CAT-TGC-CAG3'). The PCR reaction (25 µl) consisted of 1X PCR buffer, 1.5 mM $MgCl_2$, 0.02mM dNTP's, 0.2 µM of each of the 7 primers (indicated above), 1 U *Taq* DNA Polymerase (Promega) and 1 µl of extracted DNA. The PCR conditions were an initial denaturation cycle of 95 °C for 5 minutes, followed by 40 cycles of 95 °C for 15 seconds, 52 °C for 30 seconds and 72 °C for 90 seconds. The amplified products were separated using 2% agarose gel in 1X TBE buffer. The PCR products were observed by UV light in the presence of ethidium bromide (10 mg/ml). *Brucella melitensis* as negative control and *B. abortus* as positive control were included.

Table 1. Results and test cut off values of serological tests including Rose Bengal test (RBT), complement fixation test (CFT), serum agglutination test (SAT), competitive ELISA (cELISA), indirect ELISA (iELISA) and the *Brucella abortus* species specific (BaSS) PCR as well as sample information and culture status.

Sample No[a]	***Brucella* status**[b]	**Sample type**[c]	**Serological test**[d]					**BaSS PCR**[e]
			RBT	**CFT**	**SAT**	**cELISA**	**iELISA**	
Test cut off values			**Pos /Neg**	**>60 CFT IU**	**161 SAT IU**	**30% PI**	**120%**	**Pos/Neg**
B-1	Pos	abofluid	pos	pos	pos	pos	pos	ND
B-2	Neg	abofluid	neg	neg	pos	neg	neg	ND
B-3	Neg	abofluid	neg	neg	neg	neg	neg	ND
B-4	Neg	abofluid	neg	neg	neg	neg	neg	ND
B-5	Neg	abofluid	neg	neg	pos	neg	neg	ND
B-6	Pos	Uterine Dis	pos	ND	ND	ND	ND	pos
B-7	Pos	Uterine Dis	pos	ND	ND	ND	ND	neg
B-8	Pos	abofluid	pos	pos	pos	pos	pos	pos
B-9	Pos	abofluid	pos	pos	pos	pos	pos	pos
B-10	Pos	abofluid	pos	pos	pos	pos	pos	ND
B-11	Pos	abofluid	neg	pos	pos	pos	pos	ND
B-12	Pos	abofluid	pos	pos	pos	pos	neg	ND
B-13	Pos	abofluid	pos	pos	pos	pos	pos	ND
B-14	Neg	abofluid	neg	neg	neg	neg	neg	neg
B-15	Pos	abofluid	ND	ND	ND	ND	ND	pos
B-16	Neg	abofluid	ND	ND	ND	ND	ND	neg
B-17	Neg	abofluid	ND	ND	ND	ND	ND	neg
B-18	Neg	abofluid	ND	ND	ND	ND	ND	neg
B-19	Neg	abofluid	ND	ND	ND	ND	ND	neg
B-20*	Neg	abofluid	ND	ND	ND	ND	ND	neg*
B-21*	Neg	abofluid	ND	ND	ND	ND	ND	neg*
BA-1	Pos	abofluid	pos	pos	pos	pos	pos	ND
BA-2	Pos	abofluid	pos	pos	pos	pos	pos	ND
BA-3	Pos	lymphno	pos	pos	pos	pos	pos	ND
BA-4	Pos	lymphno	pos	neg	neg	pos	neg	ND
BA-5	Pos	abofluid	neg	neg	neg	neg	pos	ND
BA-6	Neg	abofluid	ND	ND	ND	ND	ND	neg
BA-7	Pos	abofluid	ND	ND	ND	ND	ND	neg
BA-8	Neg	milk	ND	ND	ND	ND	ND	neg

Sample No[a]	*Brucella* status[b]	Sample type[c]	Serological tests[d]					BaSS PCR[e]
			RBT	CFT	SAT	cELISA	iELISA	
Test cut off vlaues			Pos/neg	>60 CFT IU	161 SAT IU	30% PI	120%	Pos/neg
BD-1	Neg	abofluid	ND	ND	ND	ND	ND	neg
BR-1	Neg	abofluid	ND	ND	ND	ND	ND	neg
CD-1	Neg	abofluid	ND	ND	ND	ND	ND	neg
E-1	Neg	abofluid	ND	ND	ND	ND	ND	neg
H-1	Neg	abofluid	neg	neg	neg	neg	neg	neg
HS-1	Neg	abofluid	neg	neg	neg	neg	neg	ND
HT-1	Neg	abofluid	ND	ND	ND	ND	ND	neg
HU-1	Neg	abofluid	ND	ND	ND	ND	ND	neg
L-1	Pos	lymphno	pos	pos	pos	pos	pos	ND
M-1	Pos	hygroma	pos	pos	pos	pos	pos	neg
ML-1	Pos	lymphno	pos	pos	pos	pos	pos	ND
ML-2	Pos	lymphno	pos	pos	pos	pos	pos	ND
ML-3	Pos	lymphno	pos	pos	pos	pos	pos	ND
ML-4	Pos	lymphno	pos	pos	pos	pos	pos	ND
MS-1	Neg	abofluid	neg	neg	neg	neg	neg	ND
MS-1	Pos	lymphno	pos	pos	pos	pos	pos	ND
MS-2	Pos	lymphno	pos	pos	pos	pos	pos	ND
MS-3	Pos	lymphno	pos	pos	pos	pos	pos	ND
MS-4	Pos	lymphno	pos	pos	pos	pos	pos	ND
MS-5	Pos	lymphno	pos	pos	pos	pos	pos	ND
MS-6	Pos	lymphno	pos	pos	pos	pos	pos	ND
MS-7	Pos	lymphno	pos	pos	pos	pos	pos	ND
MS-8	Pos	lymphno	pos	pos	pos	pos	pos	ND
MS-9	Pos	lymphno	pos	pos	pos	pos	pos	ND
MT-1	Neg	abofluid	neg	neg	neg	neg	neg	neg
N-1	Pos	lymphno	pos	pos	pos	pos	pos	ND
N-2	Pos	hygroma	pos	pos	pos	pos	pos	ND
N-3	Pos	hygroma	pos	pos	neg	pos	pos	ND
N-4	Pos	hygroma	pos	pos	neg	neg	pos	ND
N-5	Pos	hygroma	pos	pos	pos	pos	pos	ND
N-6	Pos	hygroma	pos	pos	pos	pos	pos	ND
N-7	Pos	hygroma	pos	pos	pos	pos	pos	ND
N-8	Pos	hygroma	pos	pos	pos	pos	pos	ND
N-9	Pos	hygroma	pos	pos	pos	neg	pos	ND
N-10	Pos	hygroma	pos	pos	pos	pos	pos	ND

Sample No[a]	*Brucella* status[b]	Sample type[c]	Serological tests[d]					BaSS PCR[e]
			RBT	CFT	SAT	cELISA	iELISA	
Test cut off values			Pos/neg	>60 CFT IU	161 SAT IU	30% PI	120%	Pos/neg
NT-1	Neg	milk	ND	ND	ND	ND	ND	neg
P-1	Neg	abofluid	neg	neg	neg	neg	neg	neg
P-2	Neg	lymphno	pos	pos	pos	pos	pos	pos
P-3	Neg	lymphno	pos	pos	pos	pos	pos	pos
R -1	Pos	lymphno	pos	neg	pos	pos	pos	ND
R -2	Pos	lymphno	pos	pos	neg	pos	pos	ND
RB-1	Neg	milk	ND	ND	ND	ND	ND	neg
RB-2	Neg	milk	ND	ND	ND	ND	ND	neg
RB-3	Neg	milk	ND	ND	ND	ND	ND	neg
RB-4	Neg	milk	ND	ND	ND	ND	ND	neg
RB-6	Neg	milk	ND	ND	ND	ND	ND	neg
RB-B	Neg	milk	ND	ND	ND	ND	ND	neg
S-1	Pos	lymphno	pos	pos	pos	pos	pos	ND
S-2	Neg	abofluid	ND	ND	ND	ND	ND	neg
SM-1#	Neg	abofluid	pos	pos	pos	pos	pos	neg#
ST-3	Pos	abofluid	pos	pos	pos	pos	pos	pos
STN-1	Neg	abofluid	ND	ND	ND	ND	ND	neg
SVNC-1	Pos	abofluid	pos	pos	pos	pos	pos	pos
T-1	Neg	abofluid	neg	neg	neg	neg	neg	neg
TP-1	Neg	abofluid	ND	ND	ND	ND	ND	neg
TR-1	Neg	abofluid	ND	ND	ND	ND	ND	neg
V-1	Pos	abofluid	pos	pos	pos	pos	pos	pos
V-2	Pos	abofluid	pos	pos	pos	pos	pos	pos
W-1	Neg	abofluid	neg	neg	neg	neg	neg	neg
WD-1	Neg	abofluid	ND	ND	ND	ND	ND	neg
WG-1	Pos	lymphno	pos	pos	pos	pos	pos	ND
WG-2	Pos	lymphno	pos	pos	pos	pos	pos	ND
WTH-1	Neg	abofluid	ND	ND	ND	ND	ND	neg
WV-1	Neg	abofluid	neg	neg	neg	neg	neg	neg

[a] The letters of the samples indicated specific herds and numbers indicate the amount of samples from each herd.

[b] Culture status where (pos) indicate *Brucella abortus* isolated and identified and (neg) indicate no *Brucella* isolated.

[c] Abofluid: abomasal fluid from aborted foetus, lymphno: lymph node, hygroma: hygroma fluid, uterine Dis: Uterine discharge.

[d] Serological tests results from serum samples where (pos) indicate seropositive and (neg) indicate seronegative results for *B. abortus* antibody and ND indicate not determined according to each serological test cut off value.

[e] The (pos) indicates 498 bp BaSS-PCR fragment obtained with *B. abortus* specific and IS711 primers indicating natural infections and (neg) indicates no BaSS-PCR fragment obtained from natural infection or vaccine strain detected and ND indicate not determined. * BaSS-PCR results indicate *B. abortus* RB51 vaccine strain (350 bp fragment amplified by RB51 and IS711 primers) and # BaSS-PCR results indicates *B. abortus* S19 vaccine strain detected (180 bp fragment amplification by eriF and eriR primers lacking/absent).

Statistical analysis

The two by two contingency table (Table 2) was used to arrange data so that the DSe and DSp were calculated to determine the performance of each of the serological tests and BaSS PCR using bacterial culture as the gold standard. Kruskal-Wallis equality of populations rank test (non-parametric analysis of variance) was used to assess if there were any statistically significant differences amongst the various tests in diagnosing brucellosis in this study.

Table 2. The two by two contingency table used to arrange data to calculate the diagnostic sensitivity and specificity of each serological test and the *Brucella abortus* species specific (BaSS) PCR using bacterial culture as the gold standard.

	Bacterial culture		
Test Result	Positive (Infected)	Negative (Not infected)	**Totals**
Positive	True positive (a)	False Positive (b)	**(a+b)**
Negative	False Negative (c)	True Negative (d)	**(c+d)**
Totals	**True positive + False Negative (a+c)**	**False Positive + True Negative (b+d)**	**(a+b+c+d)**

The diagnostic sensitivity and specificity was calculated as follows:

Diagnostic sensitivity (DSe) = true positive/true positive + false negatives= a/a+c

Diagnostic specificity (DSp) = true negative/true negative + false positive= d/b+d

DSp in *Brucella* free herds was estimated by testing animal populations known to be free of the disease i.e. DSp = animals testing negative / total number of animals tested.

Results

The *Brucella* bacterial culture status of 94 samples from milk, abomasal fluid from aborted fetuses, uterine discharges and hygroma fluid from cattle of commercial and communal herds in Kwazulu-Natal, as well as lymph nodes (nodes collected from abattoirs that slaughtered known brucellosis seropositive cattle) is indicated in Table 1. Of the 94 samples, 49 (52%) *Brucella* cultures were isolated. The isolates had the microscopic and bacteriological characteristics typical of the *Brucella* genus namely Gram-negative coccobacilli, non-motile, positive for modified Ziehl-Neelsen staining with oxidase and catalase production (Alton et al., 1988, Quinn et al., 1994). The *Brucella* isolates were identified as *B. abortus* bv 1 at the ARC-OVI laboratory, Onderstepoort, South Africa.

The DSe and DSp of the five brucellosis serological tests RBT, CFT, SAT, iELISA, cELISA with known bacterial culture status were determined in naturally infected cattle using 63 samples (Table 1). The iELISA and RBT had the highest DSe of 91.7% (95% CI: 80.0-97.7%) followed by cELISA that had a DSe of 91.5% (95% CI: 79.6-97.6%) whilst CFT and SAT had a DSe of 91.4% (95% CI: 79.6-97.6%) and 87.2% (95% CI: 74.3-95.2%), respectively. Of the serological test iELISA and RBT had the highest DSp of 86.7% (95% CI: 55.5-98.3%), followed by CFT and cELISA with a DSp of 81.3% (95% CI: 68%-89%) whilst the SAT had the lowest DSp of 68.8 % (41.3-89.0%). The DSe and DSp total for each test were calculated and established RBT and iELISA (178.4) as the most sensitive and specific tests (Table 3).

Table 4 reports results regarding diagnostic specificity of the serological tests for brucellosis for 186 individual cattle in *Brucella* free herds. The DSp of RBT and CFT

was 100% whilst that for SAT was 97% (95% CI: 93-98%), cELISA 95% (95% CI: 90-97%) and iELISA 93 % (87-95%).

Table 3. Diagnostic sensitivity (DSe) and diagnostic specificity (DSp) results for serological tests including Rose Bengal test (RBT), complement fixation test (CFT), serum agglutination test (SAT), competitive ELISA (cELISA), indirect ELISA (iELISA) and the *Brucella abortus* species specific (BaSS) PCR with known bacterial culture status.

Tests	**Number of samples (n)**	**Diagnostic sensitivity, DSe % (CI at 95%)***	**Diagnostic specificity, DSe % (CI at 95%)***	**DSe+DSp (Performance index)**
RBT	63	91.7 (80.0-97.7)	86.7 (55.5-98.3)	178.4 (135.5-196)
SAT	63	87.2 (74.3-95.2)	68.8 (41.3-89.0)	156.0 (115.6-184.2)
CFT	63	91.4 (79.6- 97.6)	81.3 (54.4-96.0)	172.7 (134-175.6)
cELISA	63	91.5 (79.6-97.6)	81.3 (54.4-96.0)	172.8 (134-175.6)
iELISA	63	91.7 (80.0-97.7)	86.7(55.5-98.3)	178.4 (135.5-196)
BaSS-PCR	50	72.7 (39.0-94.0)	92.0 (79.1-98.4)	164.7 (118.1-192.4)

* Confidence interval (CI) at 95%.

Table 4 Information regarding diagnostic specificity (DSp) of *Brucella* free herds for serological tests namely Rose Bengal test (RBT), complement fixation test (CFT), serum agglutination test (SAT), competitive ELISA (cELISA) and indirect ELISA (iELISA).

Serological tests	**RBT**	**CFT**	**SAT**	**cELISA**	**iELISA**
Total number of sera tested	186	186	186	180	186
Number of negative samples	186	186	180	171	172
Number of positive samples	0	0	6	9	8
Diagnostic specificity (DSp %)	**100**	**100**	**96.8**	**95.0**	**92.5**
Confidence interval (at 95%)	-	-	93.6-98.8	90.7-97.7	88.3-96.2

The DSe and DSp of the BaSS-PCR assay using abomasal fluid, milk, hygroma fluid and uterine discharges samples with known bacterial culture status were determined to be 72.7% (95% CI: 39.0-94.0) and 92% (95% CI: 79.1-98.4%), respectively using 50 samples (of which 18 had known serology status). The BaSS-PCR assay determined bacterial infection with a 800 bp fragment amplified by 16S primers, *B. abortus* infection with 500 bp *B. abortus* specific IS711 repeat element fragment amplification in *alk*B gene and the two vaccine strains were distinguished by 350 bp fragment of the *wbo*A gene present in RB51 and absence of 180 bp eri gene in S19 (Bricker et al., 2003, Figure 1). BaSS PCR could not amplify 180 bp fragment of eri gene of the sample SM-1 indicating it as *B. abortus* vaccine S19 (Figure 1, Figure 1). Samples B-20 and B-21 amplified the 350 bp fragment unique to *B. abortus* RB51 strain (Figure 1, Table 1). *Brucella* cultures could not be isolated from lymph node samples (P1, P2, and P3) from brucellosis infected animals, whereas the BaSS PCR amplified *B. abortus* specific fragments from all three samples (Table 1). These samples arrived at APVL slightly decomposed resulting in heavy overgrown plates upon culturing. The Kruskal Wallis analysis of variance was done and a *P* value of 0.9 at 95% confidence interval was calculated using RBT, SAT, CFT, cELISA, iELISA and the BaSS PCR results.

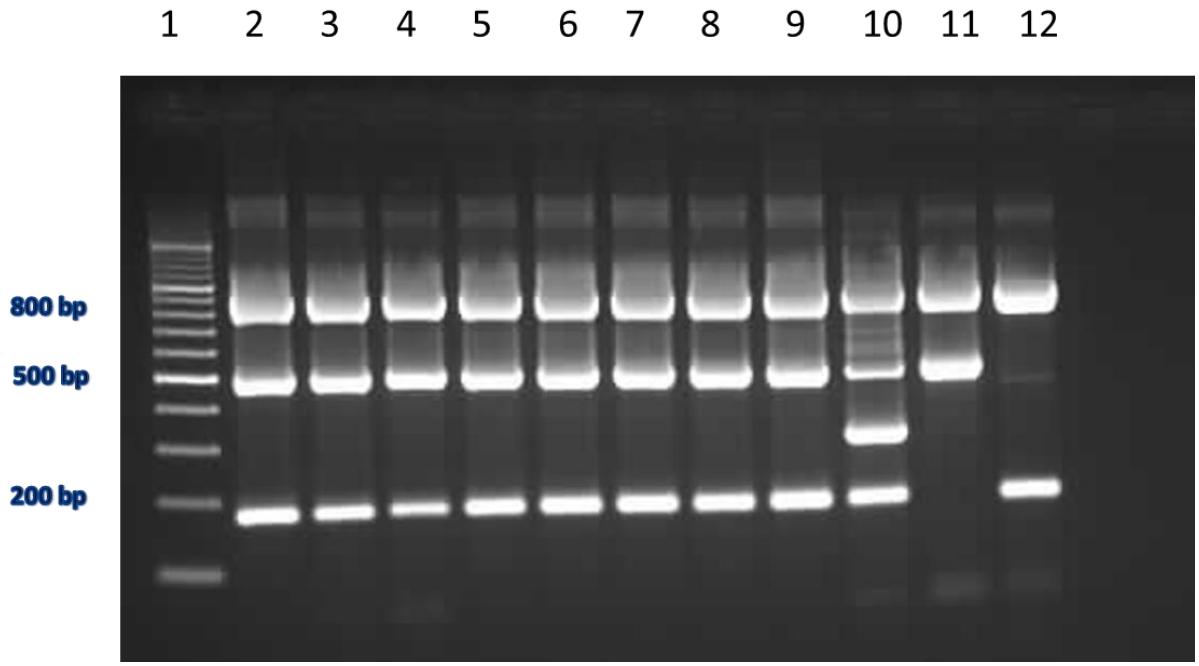

Figure 1. PCR (BaSS) amplified products on 2% agarose gel of samples collected from commercial and communal herds in Kwazulu-Natal in South Africa using *Brucella abortus* species specific PCR (BaSS PCR) assay. Lane 1: 100 bp molecular marker, lane 2-9: *B. abortus* bv 1 isolates from natural infections isolated in this study, lane 10: *B. abortus* RB51 isolated from sample SM-1, lane 11: *B. abortus* S19 isolated from sample B-20 and, lane 12: *B. melitensis* isolate (negative control).

Discussion

Currently, South African brucellosis surveillance strategy uses both RBT and CFT to establish brucellosis status of animals and herds. However, literature indicates that primary binding tests have a better performance than the currently used RBT and CFT (Paweska et al., 2002, McGiven et al., 2003, Gall and Nielsen, 2004, Muma et al., 2007). Therefore, the performance of the diagnostic sensitivities and specificities of cELISA, iELISA, CFT, RBT and SAT were evaluated. The results indicated that iELISA and RBT had the highest diagnostic sensitivity and specificity (Table 3) for bovine brucellosis in naturally infected cattle in Kwazulu-Natal Province of all the serological tests.

No statistically significant difference in the performance of RBT, SAT, CFT, cELISA, iELISA and the BaSS PCR in the diagnosis of bovine brucellosis in naturally infected cattle in Kwazulu-Natal was found in this study. This was indicated by the statistical analysis of results from the five serological tests and the BaSS PCR using Kruskal Wallis analysis of variance with a *P* value of 0.9 at 95% confidence interval. The samples size of the BaSS PCR (n=50) was slightly smaller than the serological tests (n=63). However the Kruskal Wallis analysis of variance test does not take into account that most of the BaSS-PCR samples (34/50, 68%) were culture negative which could cause a bias and could therefore not be a true reflection of the performance of the test. Despite this, the BaSS PCR did verify the culture status of all isolates as *B. abortus.*

A meta-analysis study to investigate the performance of serological tests based on sensitivity and specificity values established that the RBT had a mean sensitivity and specificity of 81.2% and 86.3%, respectively (Gall and Nielsen, 2004). The DSe and DSp performance of the RBT test in this study were 91.7 % and 86.7% respectively, which represents a better performance than what has been reported by other studies (Paweska et al., 2002, McGiven et al., 2003, Gall and Nielsen, 2004, Muma et al., 2007). The DSp using brucellosis free herds of 100% was in agreement with Gall and Nielsen (2004).

The DSe of CFT (91.4%) in this study was slightly higher than the 83% and 89% found by Paweska et al. (2002) and Gall and Nielsen (2004), respectively. The DSp of 81.3% in our study was similar to the mean DSp of 83.5% (Gall and Nielsen, 2004) and 83% (Paweska et al., 2002). The 100% DSp of CFT from brucellosis free herds was in agreement with the study by Paweska et al. (2002) conducted in South Africa. Based on these findings this study demonstrated that the CFT is a highly sensitive and specific test contrary to what McGiven et al (2003) found out, that it can underperform. McGiven et al (2003) indicated that using this test should be carefully considered based on the underperformance of CFT together with its technical complexity, anticomplementary reactions and inability to work on haemolysed samples.

Findings from a Canadian study found the DSe for SAT using infected cattle confirmed by culture to be 93.3% at a cut off of 80 SAT IU (Dohoo et al., 1986). Whereas the DSe for SAT using infected cattle also confirmed by culture was 87.2% in this study. The lower DSe value most probably is due to the cut off of 161 SAT IU compared to 80 SAT IU used by Dohoo et al. (1986). However, the DSp of 68.8% from infected herds was lower than reported by Gall and Nielsen (2004) but could be due to the fact that some samples came from chronically infected herds. The predominant immuglobulin reported in literature from chronically infected herds is IgG1 (Saegerman et al., 1999, Nielsen 2002) that SAT cannot detect as it mostly detects IgM, the dominant immunoglobulin in an early *B. abortus* infection (Saegerman et al., 1999). The SAT has been reported to be unsuitable to other tests (McGiven et al., 2003, OIE, 2008).

A number of studies have reported that the primary binding assays i.e. cELISA and iELISA have higher DSe and DSp, therefore deeming these assays to perform better than the conventional tests (Paweska et al., 2002, McGiven et al., 2003, Gall and Nielsen, 2004, Muma et al., 2007). In our study, the iELISA had the joint highest DSe of 91.7% with the RBT. ELISAs have been reported to be easy to perform, less labour intensive and reliable. However, in this study, the researcher encountered difficulties with the cELISA. The cELISA controls were out of range and the test had to be repeated to obtain reliable results on a number of runs. This could be attributed to the in experience of the researcher or the test kit was sensitive to temperature fluctuations. These technical difficulties were not experienced using the iELISA test kit.

The BaSS PCR was performed on field samples namely milk, abomasal fluid from aborted foetuses, lymph node and hygroma fluid samples submitted to the laboratory for culturing. The DSe and DSp of 72.7% and 92.6%, respectively, reported in our study are in agreement with previous reported DSe of 66.7-100% and DSp of 93.2-99.8%, using cultures (Bricker et al., 2003). However, DNA isolated from abomasal fluid had a DSe of 100% (n=7) whilst the DSp improved to 96.3%. These results indicate that abomasal fluid could be the sample of choice from aborted animals. When other sources of DNA were used i.e. milk and hygroma the sensitivity dropped to 78% as the BaSS PCR failed to detect *Brucella* DNA in these samples. All

attempts to extract DNA from sera and whole blood from known *B. abortus* positives failed as previously reported by O'Leary et al. (2006)

The BaSS PCR successively differentiated *B. abortus* wild strain from RB51 and S19 *B.abortus* strains. Differentiating vaccine strains from field infections is very important because of the drastic control measures that are imposed on the farmer i.e. quarantine of farm and slaughter of affected animals. The BaSS PCR assay detected vaccine strains RB51 (B-20 and B-21) and S19 (SM-1) from abomasal fluid (Table 1). Serological tests on serum sample of SM-1 indicated the animal to be *Brucella* seropositive. However, the cELISA that differentiates between natural infections and vaccine strains did not detect the vaccine in this sample. Failure of the cELISA to detect the vaccine strain might be that the sera was tested within six months of vaccination, a window period required for cELISA to be able to differentiate vaccine strain from wild strain as is indicated by the Brucella cELISA kit, manufacturer (Svanovir). The BaSS PCR also diagnosed *B. abortus* RB 51 vaccine from two foetuses (B-20 and B21) from brucellosis confirmed positive farm experiencing abortions. These abortions occurred shortly after the farmer vaccinated the herd with RB 51 vaccine strain.

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 cost of the ELISA kits prohibits it use in third world countries, despite reports by McGiven et al. (2003) and others that ELISAs are an automated, rapid, easy test that renders itself easily to quality control measures. Due to this fact the RBT and CFT therefore remains the best combination to detect *Brucella* infections at the APVL in Kwazulu-Natal Province. The BaSS PCR developed by Bricker et al. (2003) diagnosed *B. abortus* at species level from abomasal fluid and bacterial colonies. It is 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.

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