Field trial to evaluate the brucellin skin test in cattle in the Mpumalanga Province, South Africa

By

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Submitted in fulfilment of the requirements for the degree

Magister Scientiae (Veterinary Tropical Diseases)

in the Faculty of Veterinary Science, University of Pretoria

October 2012

Acknowledgements

I am deeply indebted and wish to express my sincere appreciation and gratitude to:

- Prof A L Michel, my supervisor, whose nurturing guidance, patience, wisdom and work ethic, I will forever cherish.
- Prof J Godfroid, the co-supervisor, from whose advice and mentorship I have benefitted immensely. I will always fondly remember the privilege.
- Dr M Hansen and Mr A Storm for the role played in the identification of the research farms, farmer liaison and for the long hours spent in field activities towards the fulfilment of this work.
- Dr A D Potts and his team at the Agricultural Research Council-Onderstepoort Veterinary Institute's Bacterial Serology section for the guidance and use of facilities, often after hours as well, while I did the laboratory work.
- Maria Mtsweni at the Faculty Library for very efficient assistance in sourcing reference material.
- Rina Serfontein and the entire Department of Veterinary Tropical Diseases for the very conducive learning environment.
- Dr D M Mtshali and my colleagues in the Department of Agriculture in KwaZulu-Natal for their consideration, understanding as well as encouragement.
- My wife, Loveness, and family for the love and support, without which life would be meaningless.
- The Dear Lord, from whom all blessing come.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AC	anti-complementary
BST	brucellin skin test
CFT	complement fixation test
CMI	cell-mediated immunity
CRS	composite reference standard
cELISA	competitive enzyme-linked immunosorbent assay
DAFF	Department of Agriculture, Forestry and Fisheries
ELISA	enzyme-linked immunosorbent assay
FPA	fluorescent polarisation assay
FPSR	false positive serological reaction
iELISA	indirect enzyme-linked immunosorbent assay
ICFTU	international complement fixation test units
Ig	immunoglobulin, e.g. IgM means immunoglobulin M
MAb	monoclonal antibody
mP	milli-polarisation unit
MRT	milk ring test
OD	optical density
OIE	World Organisation for Animal Health
OPS	O-polysaccharide
OVI	Onderstepoort Veterinary Institute
RBT	Rose Bengal test
rLPS	rough lipopolysaccharide
ROC	Receiver operating characteristic curve
SAT	serum agglutination test
sLPS	smooth lipopolysaccharide

Thesis Summary

Field trial to evaluate the brucellin skin test in cattle in the Mpumalanga Province, South Africa

by

Nhamo Nyanhongo

Promoter: Prof A L Michel
Co-promoter: Prof J Godfroid
Degree: MSc (Veterinary Tropical Diseases)

Brucellosis is a disease of socio-economic and zoonotic importance worldwide. In animals it is associated with the ingestion of feed that is contaminated with cyetic material from aborting herd-mates, while in humans it is associated with the consumption of unpasteurised milk and dairy products from infected animals. It may also be acquired from contact with infected material of animal origin by farmers, veterinarians, and abattoir and laboratory workers. Brucellosis was first reported in South Africa in the late nineteenth century. It is still present in the country today, with reported annual losses of at least R 300 million, and a national annual incidence of 5 000 cases in humans. The global incidence of human brucellosis is about half a million infections annually. As the incidence of human brucellosis is directly associated with prevalence in animals, control of animal brucellosis is emphasised.

Veterinary control is compromised by the chronic nature and the variable incubation period of the disease, with an estimated up to 15% of cattle in infected herds aborting before sero-conversion. Latency, which involves about 5% of calves born from infected dams, is also problematic as these infected animals often test seronegative, only to seroconvert in the peri-parturient period, thus allowing opportunity for disease spread within and between herds before diagnosis is made. In addition, the currently used serological tests are at times unable to distinguish brucellosis from cross-reacting antibodies from other infections or brucellosis vaccines.

It was the objective of this study to investigate, under South African conditions, the value of the brucellin skin test (BST) in improving the sensitivity and specificity of the currently used serological tests. It has proved a valuable additional test in diagnosing early and latent infections as well as in differentiating brucellosis from cross-reacting organisms in unvaccinated cattle in Europe. The study also evaluated the benefit of replacing some of the currently used serological assays with the fluorescence polarisation assay (FPA). The FPA, a rapid and homogenous serological test with only a few operational steps, has been validated and is in current use in Canada.

The study was carried out in Mpumalanga Province, on herds selected to reflect prevailing South African farming conditions. These herds were divided into certified *Brucella abortus*-negative herds (608 head) for the estimation of BST specificity, and confirmed *B. abortus*-infected herds (845 head) for the estimation of BST sensitivity.

The results obtained indicated the BST had a specificity of 99.18%, and a relative sensitivity of 42.86%. However, 65.38% of BST-positive animals were negative on serology. When the high specificity is considered, together with the experiences of other researchers who found that the skin test became positive earlier than serological tests, these animals may be assumed infected. It is concluded that the BST is a valuable addition to the panel of diagnostic tests currently used to identify infected herds and individuals in South Africa. The FPA, with a relative sensitivity of 93.65% and a specificity of 98.85%, can potentially be of use as a screening test under South African conditions.

Chapter 1
Introduction

Bovine brucellosis, caused by the Gram-negative facultative intracellular bacteria *Brucella abortus*, is an important international zoonosis responsible for heavy economic losses in the cattle industry (Alton *et al* 1988; Robinson 2003). It may occasionally be caused by *Brucella melitensis* in melitensis-endemic areas, or rarely by *Brucella suis* (OIE Terrestrial Manual 2009). *Brucella suis* has not been diagnosed in South Africa. *Brucella abortus*, which primarily infect cattle and buffalo, are small Gram-negative, non-motile aerobic coccobacilli, which are partially acid-fast. They require enriched media for primary isolation, e.g. Farrell's medium, although specimens from aborted foetuses or colostrum will grow on 5-10% blood agar. They require an atmosphere of 5-10% carbon dioxide for growth. In two to four days of incubation, *B. abortus* will produce small, 1-2 mm pale, honey-coloured, smooth and glistening colonies (Alton *et al*. 1988). In a cool shaded moist environment, *B. abortus* can survive for up to four months or longer, although they are killed within a few hours when exposed to direct sunlight.

In cattle, brucellosis is a herd problem where it spreads within a herd mostly by ingestion of feed contaminated with material from aborted foetuses. Infectious material includes placentae, foetal fluids, aborted foetuses, vaginal discharges, milk, semen and urine. Other routes of infection include the penetration of mucus membranes and conjunctiva, and inhalation or artificial insemination with *B. abortus* contaminated semen. Calves can be infected in utero or by suckling on infected dams. The incubation period, an important factor in the control of brucellosis, is variable from as short as two weeks to five months in a pregnant cow to as long as 18 months when it involves a calf infected in utero (Nicoletti 2010). Between-herds transmission is primarily through the introduction of chronically infected carrier animals (Robinson 2003). Transmission between buffalo (considered a possible reservoir of *B. abortus* in Southern Africa), and cattle is potentially possible where there is contact with domestic cattle (Madsen & Anderson 1995). Brucellosis affects mainly the reproductive system, with the resultant loss of productivity due to abortions and reduced milk production (Bercovich & Muskens 1999). In highly susceptible non-immune herds, signs of infection include high incidence of abortion, especially in the second half of gestation

with the majority in the third trimester. Subsequent pregnancies may be carried through to term although the cow will continue to shed the bacteria from the uterus and through milk and potentially infect naive herd mates. In less acutely infected herds, there is a high incidence of stillbirths or weak calves, accompanied by a high incidence of retained placentae with resultant metritis and impaired fertility. Infected bulls exhibit acute or chronic orchitis, epididymitis and seminal vasculitis. Arthritis and hygromata, mainly affecting the carpal joints, are typically seen in more chronically infected herds. Other losses are the cost of culling infected livestock, cost of administering eradication schemes, as well as lost trade opportunities. It is estimated that 14.7% of South African herds are brucellosis-infected resulting in annual losses of about R 300 million (Hesterberg *et al* 2008). Human brucellosis is largely a neglected disease in South Africa, with a paucity of recently published data (Blumberg 2011; Dean *et al* 2012), although incidences of 5 000 have been cited in the past (Schrire 1962). Human incidence is estimated at 500 000 globally (Pappas *et al* 2006, World Health Organisation 1975).

It is transmitted to humans through ingestion of unpasteurised milk or dairy products. It is an occupational hazard among farmers, veterinarians, and laboratory and abattoir workers through contact with infected material such as vaginal discharges, aborted foetuses and infected joints (Robinson 2003). Brucellosis in humans is a chronic debilitating disease with symptoms varying from undulant fever, headaches, depression, arthritis, and weight loss. There is associated loss of production because of reduced work capacity through sickness of the affected people (Mangen *et al* 2002).

As the incidence of human brucellosis is directly associated with prevalence in animals, control of animal brucellosis is emphasised in many countries. Several European countries have successfully eradicated brucellosis (Godfroid & Käsbohrer 2002) although they remain at risk of re-introduction through livestock movement, especially imports as well as from wildlife. These countries therefore; need to have a comprehensive surveillance; pre and post-import testing scheme in place (England *et al* 2004, as cited by Cutler *et al* 2005). South Africa's own Bovine Brucellosis Scheme was introduced in 1969, although, to date, the country is yet to achieve freedom status (Bosman 1980).

The success of a brucellosis eradication scheme depends heavily on the identification of infected herds as well as infected individual animals. Following identification, measures that include quarantine, surveillance, vaccination as well as test and slaughter are applied. The diagnosis of brucellosis is a challenging exercise as the clinical signs of abortion, epididymitis and orchitis are shared with other diseases such as trichomoniasis, vibriosis, leptospirosis, listeriosis and infectious bovine rhinotracheitis among others (Corbel *et al* 1979 as cited in the OIE Terrestrial Manual 2009).

Diagnosis has traditionally relied on serological detection of circulating antibodies against *Brucella* species followed by bacterial isolation and other tests. Serology relies on the use of serum, milk, whey or semen. Bacterial isolation and culture can be done on aborted foetal organs (abomasal contents, liver, spleen and lung), foetal membranes, vaginal secretions, milk, semen and joint synovial fluid. At post-mortem gravid or early post-parturient uterus, udder as well as the supra-mammary lymph nodes, mandibular lymph nodes, spleen and retropharyngeal lymph nodes can be sampled and cultured (Alton *et al* 1988). Culture, isolation and identification of *B. abortus* or its genetic material by polymerase chain reaction and nucleic acid recognition is the undisputed gold standard method of diagnosis. The only drawback to this diagnostic method is that it is expensive, time consuming and poses a hazardous risk due to its zoonotic nature. Direct microscopic examination of smears stained with modified Ziehl-Neielsen (Stamp's) stain from vaginal swabs or foetal tissue can also be used. *Brucellae* are partially acid-fast and stain red against a blue background. There is need to differentiate *Brucella* from organisms like *Chlamydophila abortus, Coxiella burnetti* and *Norcadia* (Ibironke *et al* 2008). Direct microscopy has the disadvantage of reduced sensitivity if there is a small number of bacteria in the tissue from which the smear is made. Immuno-staining may also be used to identify *Brucella* organisms in smears (Roop II *et al* 1987).

Serology can be used for a presumptive diagnosis of brucellosis or to screen herds (Nielsen 2002; OIE Terrestrial Manual 2009). No single test completely satisfies accurate diagnostic requirements. The milk ring test (MRT) is a sensitive, valuable and inexpensive screening test used on bulk milk samples. The added stained antigen is agglutinated by Brucella antibody/fat globule complex and all rise to form a coloured cream layer at the top. False positives may occur if the herd has a high proportion of mastitis, colostrum or cows that are drying off. It is an effective tool when adjustments

are made for herd size and herd followed up with serology to identify the individual positive animals (Alton *et al* 1988). It is only useful in dairy herds while the infected animal contributes to the bulk tank. The MRT cannot be applied on pasteurised, skimmed or sour milk.

Serum agglutination tests (rapid plate and standard tube agglutination tests) may be used on serum, whey, milk or vaginal mucus. They have rather low specificity and sensitivity (Alton *et al* 1988). Serum agglutination test (SAT) detect specific and non-specific antibodies from Brucella infection or vaccine. It is also the last test to reach diagnostically significant levels during disease incubation stage and after calving/abortion in cows or heifers. SAT also have the tendency to give false negative results in chronic disease owing to low serum agglutinins even when results of other tests may be positive. The slow tube agglutination test is sometimes used as a supplementary test along with Complement fixation test as it has value in that it detects IgM (immunoglobulin M), which is an indicator of new infections and the persistent and predominant immunoglobulin from S19 vaccination.

The Buffered Brucella Antigen Test (BBT) such as the card test or the Rose Bengal test (RBT), work on the principle that the non-specific IgM has reduced binding ability at low pH. They work optimally at temperatures of 22 °C plus or minus 4 °C. They are World Organisation for Animal Health (OIE)-prescribed tests for international trade. RBT is a simple rapid spot agglutination test used for large-scale screening of sera where the buffered antigen inhibits IgM but detects IgG and IgA. There will be false positives in cases of residual antibodies from vaccination or colostrum in calves and cross-reaction with Gram-negative bacteria such as *Yersinia enterolitica, Salmonella urbana, E coli, Xanthomonas maltophila* and *Vibrio* species (OIE Terrestrial Manual 2009).

The complement fixation test (CFT) is less sensitive than the RBT but is a more specific test and is usually used as a confirmatory test. It is an OIE-prescribed test for international trade. It is sensitive to IgG_1, making it a better indication of chronic infection than SAT as it is less sensitive to vaccinal antibodies due to S19. It also has the advantage of having a standardised system of units. It has the disadvantage of being complex to perform, requiring a sophisticated laboratory and well trained laboratory personnel (Alton *et al* 1988).

The indirect ELISA (iELISA) comes in a few variations employing different antigenic preparations, antibody-enzyme conjugates, and substrate/chromogens. They use whole cell, sLPS (smooth lipo-polysaccharide) or the O-polysaccharide (OPS) as antigen. The OIE supplies three standard sera for the purpose of national standardisation of the Brucella iELISA tests. The iELISA, which use the sLPS or OPS antigens, are highly sensitive but less specific due to problems of differentiating infection from S19 vaccinations. This problem may be partially solved by using rLPS or cytosol antigen. Another way of improving on specificity is the use of MAb (an anti-bovine immunoglobulin specific for IgG_1) in the conjugate. However, when used as a screening test, antigen rich in sLPS or OPS are used as the optimal antigen (OIE Terrestrial Manual 2009).

The competitive ELISA (cELISA) employs a MAb specific for one of the epitopes of the Brucella OPS to increase specificity but has a lower sensitivity than the iELISA. This is achieved by selecting a MAb with a higher affinity than the cross-reacting antibody. This eliminates some but not all false positive serological reactions due to cross-reacting bacteria or S19 vaccinal antibodies (OIE Terrestrial Manual 2009). Both iELISA and cELISA are also prescribed by the OIE for international trade.

The fluorescence polarisation assay (FPA) measures the antigen-antibody interaction. The antigen is labelled with a fluorochrome before the addition of serum. If the serum contains antibodies to the antigen, there is a decrease in the rate of rotation due to an increase in the molecular weight of the antigen-antibody complex. It is this decrease, which enables the distinction between positive and negative. The antigen may be added to whole blood or serum; and a reading obtained in 15 seconds or two minutes respectively, using a fluorescent polarisation analyser. Controls must include a strong positive, weak positive, negative, as well as S19 vaccinate serum. The diagnostic sensitivity and specificity are identical to those of the cELISA. The specificity of FPA in FPSR is currently unknown (OIE Terrestrial Manual 2009). FPA can be used in the field as a cow-side test. It is also one of the OIE-prescribed tests for international trade.

The gamma interferon test involves the stimulation of lymphocytes in whole blood with brucellin and then measuring the gamma interferon production by capture ELISA (Weynants *et al* 1995).

The brucellin skin test (BST) is a cell-mediated immune-assay, which can be used for screening unvaccinated herds by the use of a purified and standardised antigen (free of sLPS). It is an OIE alternative test. The BST has such a high specificity that serologically negative unvaccinated animals, which are positive reactors, should be regarded as infected (Saegerman *et al* 1999). It has also been used as an aid in the interpretation of serological reactions thought to be FPSR due to infection with cross-reacting bacteria (Saegerman *et al* 1999). Results of BST should be supported by a reliable serological test to identify individuals in infected herds. BST allows farmers to observe the test results for themselves, but the major drawback is the need to conduct two farm visits for the injection and to read the resultant skin reaction 72 hours later. Compared to the tuberculin reaction, the brucellin reaction is of a rather limited intensity; leading to difficulties in interpreting the results (Saegerman *et al* 1999).

Chapter 2
Justification

2.1 Literature review

The first case of bovine brucellosis in South Africa was diagnosed in the then Orange Free State in 1915 (Asmare *et al* 2010) and the disease has been prevalent in the country ever since, with the period between 1996 and 2000 recording on average more than 300 outbreaks per year (Memish & Balkhy 2004). In order to enhance detection, control, and finally eradicate bovine brucellosis, the bovine brucellosis scheme was introduced in 1969 under Animal Health legislation. The scheme entails classification of cattle herds into accredited, brucellosis-free, maintenance, diagnostic, import/export or infected herds. A legal agreement is entered into between the state and the farmer before they can join the scheme. The accredited herd scheme has since been discontinued and farms only have to be certified brucellosis-free. A herd is certified brucellosis-free only after two successive negative serological tests two to three months apart, followed by a strictly CFT-negative test a year later. These tests must include all cattle over 18 months of age (which must be individually identified), and the herd kept closed. The brucellosis freedom certificate is valid for a year from the last bleed. Once certified, maintenance of freedom status may be done through an annual serological test or monthly MRT on bulk milk samples. Annual renewal of certification depends on all tests over the 12 months being negative. The maintenance herd programme is designed to cater for farmers who cannot meet the requirements of the brucellosis freedom declaration. In this case, all cattle above 18 months, which must be individually tagged, are serologically tested twice (at least two months apart). Where both tests are negative the farmer receives a declaration stating that the herd tested *Brucella*-negative. Thereafter the herd is maintained on the programme with an annual serological test or monthly bulk sample MRT. Should the herd fail any of these tests, it is then classified as an infected herd. The diagnostic herd status is used where farmers are unwilling to subject their herds to conditions of accreditation or maintenance but where the state may be interested in determining the prevalence of brucellosis in a given area. All animals over 18 months (whether tagged or not) are tested. Should there be positive reactors the herd is incorporated into the infected herd, and all cattle tagged and retested to identify and remove reactors. The tests are conducted once

every five years. Imported cattle are tested while in quarantine in accordance to the importation permit. Likewise, cattle destined for export are also tested in accordance with the importing country's conditions. An infected herd is one where infection has been determined by serology or by the isolation of *Brucella* bacteria. The herd is placed under official supervision and steps taken to eradicate the infection. In South Africa, brucellosis diagnosis is based on the RBT and SAT, with CFT as the confirmatory test. The MRT is used to monitor dairy herds once they are certified free of brucellosis. Many serological tests in current use rely on the detection of antibodies to the OPS on the surface of the *Brucella* bacteria (Cutler *et al* 2005; Munoz *et al* 2005). Although sensitive and proved successful in European schemes(Godfroid *et al* 2002), the serological tests tend to produce false-positive results in animals vaccinated with S-19 vaccine or those harbouring cross reacting bacteria such as *Yersinia enterolitica* O:9, *Salmonella urbana, E. Coli* O:157 and *Xanthomonas maltophila* among others (Munoz *et al* 2005). They have also proven unreliable in detecting latently infected calves which only sero-convert after parturition (Bercovich & Muskens 1999), a time when most transmission occurs.

Vaccination, when used in conjunction with other measures such as test and slaughter as well as animal movement controls, is an effective tool that lowers eradication costs (European Commission 2009). It increases resistance to infection, lowers abortion risk as well as reduces the excretion of *Brucella* species. These effects collectively lower human exposure and therefore reduce the incidence of human brucellosis. Strains S19 and RB51 are the current two live vaccines approved for use in bovine brucellosis. They both lead to positive skin reactions with the BST. Strain 19 is a smooth *Brucella* strain vaccine which induces long lasting immunity (Saegerman, *et al* 1999) but has the disadvantage of inducing abortion (when used in pregnant animals) and persistent residual antibodies (when used in adult animals) to classical serological tests like the RBT and CFT. Its use in South Africa is restricted to heifers between the ages of four and eight months. The RB51 vaccine, made from rough *Brucella* strain independent of the OPS, does not interfere with OPS-based serological tests, and can therefore be used in adult cattle. It will, however, interfere with the BST, and has a tendency to cause abortions when used during pregnancy (European Commission 2009). In South Africa the brucellosis vaccination policy states that all heifers be vaccinated with S19 (or alternatively RB51) between the ages of four and eight months, and any follow up

vaccinations be restricted to RB51. No bulls are to be inoculated as it may cause orchitis and reduced fertility.

It was an objective of this trial to investigate if the use of the brucellin skin test, which depends on cell-mediated immunity targeting bacterial protein and carbohydrates other than sLPS, can improve on the diagnosis of these cases of brucellosis. In Europe, the brucellin skin test has demonstrated the ability to identify false positive serological reactions (FPSR) as well as latently infected animals with a specificity of over 99 % (Bercovich & Muskens 1999; Bercovich 2000; Pouillot *et al* 1997; Saegerman *et al* 1999; Saegerman *et al* 2004). The investigation also assessed the ability of the BST to identify cattle previously vaccinated with RB51 and to use these data to estimate the length of time they may remain reactors after the last vaccination. Additionally, the trial assessed the performance of the fluorescence polarization assay in the South African environment. The FPA was validated in Canada, and has been assessed in the USA, Mexico and Argentina (Nielsen *et al* 1998). In Canada, the FPA was found to be highly sensitive and specific.

2.2 Hypothesis

The BST is useful in detecting bovine *Brucella* spp. infection in unvaccinated cattle herds and in latently infected animals prior to vaccination. It can hence improve the current diagnosis of brucellosis in South Africa. The BST is useful in demonstrating immune-reactivity in cattle herds that were exclusively vaccinated with RB51. The FPA discriminates between brucellosis-free and brucellosis-infected cattle with high specificity and sensitivity.

2.3 Benefits

- If proven effective under local conditions, the BST can significantly contribute to a more practical and sensitive diagnosis of bovine brucellosis, especially in early-unvaccinated young calves. It is important to re-iterate that in young heifers, infected prior to vaccination, the vaccine will not cure the infection and consequently, the heifers are likely to abort at their first gestation. Indeed, no curative vaccine against brucellosis currently exists, and under field conditions, vaccination of infected animals may generate a false sense of security. Conversely, vaccination failure is often attributed to the vaccine, whereas the vaccine was applied in infected animals

and thus was applied in inappropriate conditions. Consequently, in the absence of vaccination, every single young heifer classified positive in BST should be regarded as infected, and should not be vaccinated and such animals should be withdrawn from brucellosis-free herds.

- BST, if proven effective, can be used to improve on brucellosis diagnostic specificity when used in conjunction with the current serological assays. This should reduce the time taken to eradicate brucellosis from an infected herd.
- BST, if proven effective, can be used to identify animals that were vaccinated exclusively with RB51 brucellosis vaccine. The identification of RB51-vaccinated cattle herds is of importance in brucellosis-free countries (where it is illegal to vaccinate cattle against brucellosis).
- Since BST works on the principle of cell-mediated immunity, the main defense mechanism against brucellosis, it could be used to assess the efficacy or herd immunity in cattle vaccinated with the RB51 vaccine. Other researchers (Tittarelli *et al* 2008) found that serological antibodies induced after RB51 vaccination tended to be cleared after about 162 days, and therefore of limited use in this regard.

It was part of postgraduate training towards an MSc (Veterinary Tropical Diseases) for Nhamo Nyanhongo.

It was an opportunity to conduct and promote research relevant to South Africa and publish research findings as a joint undertaking between academic, research and state veterinary institutions.

2.4 Aims and Objectives

Validation of the brucellin skin test under South African conditions:

- To establish serological (RBT, CFT) bovine brucellosis status of the study population (negative for control group, positive for infected group).
- To determine the specificity of the BST in known brucellosis-negative herds to verify the false positive rate.
- To determine the sensitivity of the BST in calves latently infected with bovine brucellosis in known brucellosis-infected herds, in comparison with prescribed serological tests.

- To establish the BST reactor rate in known brucellosis-negative, exclusively RB51-vaccinated herds, and estimate, in the absence of infection, the minimum period for which they remain BST positive after the last vaccination (which would mean protected). In the case of S19 vaccination, such reactions have been documented at more than four years after vaccination (Saegerman *et al* 1999). This is the explanation why a single S19 vaccination provides lifelong protection. It is not known if a single RB51 vaccination will induce lifelong protection. Conversely, the benefits of multiple RB51 vaccinations have never been documented in scientific literature.

Validation of the fluorescence polarisation assay under South African conditions, using the same study population as the brucellin skin test:

- To determine the specificity of the FPA in a known brucellosis-free group of cattle and verify the false-positive rate.
- To determine the sensitivity of the FPA in a known brucellosis-infected and unvaccinated group of cattle.
- To determine the specificity of the FPA in known brucellosis-free, exclusively RB51-vaccinated vaccinated herds.

Chapter 3
Materials and Methods

3.1 Study area

The Province of Mpumalanga holds approximately 9.8% of the total cattle population of South Africa, estimated at 13.9 million (DAFF n.d), and accounts for 7.4% of the national milk production. The province was selected for study based on readily available brucellosis testing records at the office of the Provincial Brucellosis Co-ordinator.

With a total surface area of 79 490 km², Mpumalanga is the second smallest province in South Africa although it is the fourth largest economy (Mpumalanga Provincial Government n.d). It is bordered by Mozambique and Swaziland to the east, Gauteng Province to the west, Limpopo Province to the north, and KwaZulu-Natal and the Free State Provinces to the South. As shown on the map below (Figure 3.1), the province is divided into three district municipalities: Ehlanzeni, Gert Sibande and Nkangala. The district municipalities are in turn divided into local municipalities (18 in total). The study area comprised of six local municipalities in the districts of Gert Sibande and Nkangala.

Table 3.1, below, shows a summary of the occurrence of brucellosis in commercial herds in the study districts as at July, 2011.

Table 3.1 Occurrence of bovine brucellosis in commercial herds in the six municipalities selected for the study

Local Municipality	Number of brucellosis-infected herds	Number of brucellosis-free herds	Total commercial herds
Dipaleseng	51	52	103
Emalahleni	43	209	252
Govan Mbeki	86	60	146
Lekwa	59	165	224
Msukaligwa	73	90	163
Steve Tshwete	15	77	92
Total	**327**	**653**	**980**

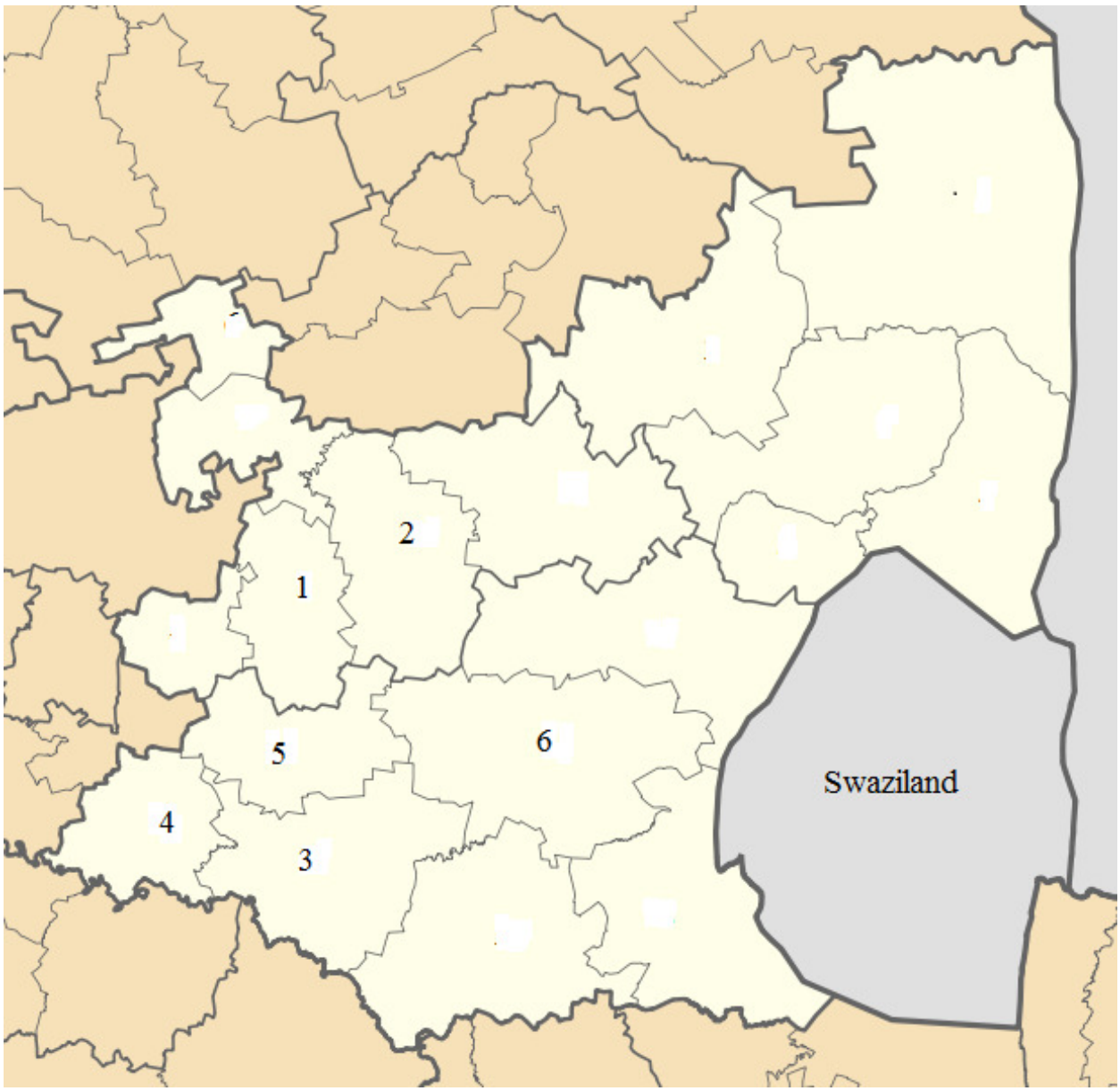

Figure 3.1 District map of Mpumalanga Province showing study area

(Adapted from http://en.wikipedia.org/wiki/List_of_municipalities_in_Mpumalanga, accessed 10/09/12)

Key to map

District		Local Municipality
Nkangala	1	Emalahleni
	2	Steve Tshwete
Gert Sibande	3	Lekwa
	4	Dipaleseng
	5	Govan Mbeki
	6	Msukaligwa

3.2 Experimental Design

3.2.1 Study animals

3.2.1.1 Brucellosis-negative control group

The negative control group were selected from known brucellosis-free herds on the basis of available test records in the office of the brucellosis co-ordinator. They were defined as herds which had been certified brucellosis free in accordance with the national Brucellosis Control Scheme regulations for a minimum uninterrupted period of three years.

Briefly, certified brucellosis free herds are kept as closed herds, accepting additional animals from other certified herds. Animals are identifiable with individual tags. As a pre-requisite to the issuance of brucellosis freedom certificate, all breeding cattle in the herd (cows, heifers and bulls) over the age of 18 months are serologically tested for brucellosis (screening with the RBT and confirming the positive reactions with the CFT). Following a negative first herd test, a second test is conducted two or three months later. Only where both tests are negative is a brucellosis freedom certificate issued by the Area State Veterinarian. The brucellosis freedom certificate is subsequently maintained through annual whole herd serological testing, or alternatively through regular bulk milk ring tests in the case of dairy farms.

In order to re-emphasise the negative control group's brucellosis freedom status, blood was collected from all the study animals, and the serum subjected to the RBT, CFT and iELISA tests.

3.2.1.2 Herds infected with *B. abortus*

Brucella abortus exposed animals were sourced from known brucellosis-infected herds on the basis of available records of positive serological reactions on routine surveillance testing. These were supported by bacteriological isolation and identification of *B. abortus* bacteria from some of the reactor animals.

3.2.2 Sampling

3.2.2.1 Brucellosis-negative control group

A total of 608 calves between the ages of three and nine months were selected. The minimum age of three months was selected in order to minimise the effect of maternal antibodies on serological tests, while the maximum age was influenced by the age of vaccination for brucellosis in each herd. Only animals that had not yet been vaccinated for brucellosis qualified for the study as brucellosis vaccination is well documented as eliciting positive BST (brucellin skin test) reaction. The number of animals was chosen to match available funds for the purchase of antigen as well as provide a statistically significant number for the assessment of BST specificity in brucellosis-free animals.

The farms were selected to represent a cross-section of farm management systems and cattle breeds in South Africa. Table 3.2 below shows selected farms and some management aspects related to brucellosis management.

Table 3.2 Distribution of brucellosis-free study herds by district, breed, herd-size, and brucellosis vaccine management

Herd	District	Breed	Number tested	Average Age (months)	Herd vaccine type	Vaccination regime
A	Lekwa	Holstein-Friesland	23	4	RB51	6 mths, 12 mths, then annually
A	Lekwa	Drakensburger	269	4	RB51	6 mths, 12 mths, then annually
B	Lekwa	Drakensburger	16	4	RB51	6 mths, 12mths
C	Lekwa	Drakensburger	145	6	RB51	8 mths, 12 mths, then annually
D	Govan Mbeki	Brahman cross	155	6	RB51	8 mths, at 1st bulling, then every 3 yrs
Total			**608**			

3.2.2.2 RB51-vaccinated adult cows

The RB51-vaccinated cows were selected from farms that were certified brucellosis-free (the same farms as the negative control for calves). In addition the animals had to have been exclusively vaccinated with RB51 brucellosis vaccine (to the exclusion of S19 vaccine) so that any BST reactions observed could be attributed to RB51 vaccine. In this case, wild strain brucellosis was excluded by subjecting all the animals to serology (RBT, CFT and iELISA). Any animal with a positive serological reaction was assumed to be infected with wild strain *Brucella* spp. A total of 424 adult cows ranging in age from one to ten years were selected from farms A, B and C and renamed herds P, Q and R respectively.

3.2.2.3 Herds infected with *B. abortus*

A total of 846 animals between the ages of three and eleven months were selected from 13 known brucellosis exposed herds on the basis of serological reactions supported by bacteriological isolation and identification of *B. abortus*. Table 3.3 below shows a list of the selected farms.

Table 3.3 Distribution of brucellosis-infected study herds by district, breed, herd-size, age, vaccine management and character of abortions attributable to brucellosis

Herd	District	Breed	Number tested	Average Age (months)	Herd vaccine type	Vaccine Regimen	History of abortion in herd (3 years)
E	Dipaleseng	Drakens-burger	348	9	none	N/A	Sporadic
F	Dipaleseng	mixed	27	6	RB51	*once	Yes
G	Dipaleseng	mixed	28	6	RB51	**8 mths, 12 mths, at bulling	Sporadic
H	Dipaleseng	mixed	84	6	RB51	8 mths, 12 mths, at bulling	Sporadic
I	Msukaligwa	mixed	5		RB51	8 mths, at bulling	Sporadic
J	Emalahleni	mixed	3		RB51	8 mths, at bulling	Sporadic
K	Lekwa	mixed	85	6	RB51	12 mths, then annually	Only weak calves
L	Steve Tshwete	Mixed	91	6 - 9	S19, RB51 combination	S19 at 3-4 mths, RB51 at 8mth, 12 mths	Sporadic
M	Lekwa	mixed	101	7	RB51	8 mths, at bulling	Only in bought in cattle
M (bull calves)	Lekwa	mixed	72	7			Only in bought in cattle

*had only just started to use vaccine in herd
**had been vaccinating for two years only

The number tested was chosen to match available resources. BST testing took place between October, 2010, and June, 2011.

3.3 Test Methods/Procedure

3.3.1 Animal identification

Where calves were not individually tagged, animal identification was done by means of placing individually numbered ear-tags on each animal before commencement of testing. Although it had earlier been envisaged to identify each calf with its dam for the purposes of follow-up on positive BST reactors, the lack of cow-calf records on most of the participating farms led to the abandonment of the idea.

3.3.2 Serum collection

Once the animals were individually identifiable, approximately 10 ml of blood was collected from each individual animal by venipuncture of either the jugular or the median caudal vein into Vacutainer™ tubes without anti-coagulant. The blood was transported to Vryheid Regional Veterinary Laboratory, where it was allowed to clot and serum separated and stored at -20 ℃ until the time of serological testing.

Blood collection took place on the participating farms, together with BST testing; starting in October, 2010, and ending in June, 2011.

3.3.3 The brucellin skin test (BST)

The procedure was carried out as described earlier (Saegerman *et al* 1999, OIE Terrestrial Manual 2009). An area of approximately ten square centimetres of healthy skin on the side of the neck was clipped with a pair of scissors and the measurement of normal skin thickness taken with a springmeter (Hauptner). With the aid of a tuberculin syringe coupled to a 4 mm, 25 gauge needle, 100 µl of brucellin (batch 10 0001, MEGACOR diagnostic, Austria) was injected intradermally to leave a visible pea-sized nodule at the injection site. The reaction was assessed approximately 72 hours post-injection primarily by sight and palpation followed by measurement with a spring meter. A positive reaction was assessed qualitatively as either a firm well circumscribed induration or as a soft oedematous induration. The same operator took both pre and post injection measurements to minimise variation.

Although we had initially aimed at identifying every calf with its dam in order to establish if there was a relationship between the brucellosis serological status of the dam and the BST reaction of the calf, the state of most of the farm records was not solid enough for the establishment of this relationship. The idea was subsequently abandoned.

3.3.4 The Rose Bengal test (RBT)

The tests were conducted at the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI), Bacterial Serology laboratory between January and March, 2012. The procedure was carried out as described earlier (Alton *et al* 1988). Test sera and antigen were allowed to reach room temperature (22 ± 4 ℃), before 25 µl of test serum and 25 µl of antigen (Onderstepoort Biological Products, batch 146) were added respectively and mixed on a WHO haemoagglutination (HA) plate so that they covered the well bottom. The plate was subsequently placed on a rotary agglutinator (Heidolph polymax 2040, Heidolph, Germany) for four minutes and the results read immediately as positive or negative based on the presence or absence of any agglutination respectively.

3.3.5 The complement fixation test (CFT)

The CFT was carried out according to the procedure of Alton and others (1988) together with the standard operating procedures used at ARC-OVI, by way of the warm fixation method. The tests were carried out between December, 2011, and March, 2012.

Briefly, test as well as positive and negative control sera were inactivated in a microtitre plate in a hot air oven at 58 ± 2 ℃ for 30 minutes and allowed to cool down before proceeding with the test. The test was carried out in 96-well u-bottomed microtitre plates. First, 25 µl of CFT buffer was added to all the wells of the test as well as anti-complementary (AC) plates. The next step was to take 25 µl of test serum and add to AC plate to achieve a 1:2 dilution. The subsequent step was to transfer 25 µl of this dilution to the first row of the test plate and take 25 µl to the second row and so on to the sixth row to achieve a 1:128 final dilution (discarding the last 25 µl). Control sera received the same treatment. Subsequently, 25 µl of antigen (lot 5, Onderstepoort Biological Products, South Africa), diluted to working strength, was added to the test plate but not to the AC plate (where 25 µl of CFT buffer was added instead). The next step was to add 25 µl of complement (batch 303 284, Siemens, Germany) at working

dilution to all wells. The plates were incubated at 37 ℃ for 30 minutes in a hot air oven (taking care to avoid stacking of plates) after which 50 µl of sensitised sheep red blood cells (amboceptor lot 302 183, Siemens, Germany) was added to each well. The plates were again incubated at 37 ℃ on a shaker for another 30 minutes. The plates were removed from the incubator and centrifuged at 450 rcf for three minutes, after which they were read over a magnifying mirror by comparing the haemolysis to standards corresponding to 0-4 (0=100% lysis while 4=0% lysis) in the last dilution with a reaction. The results are scored with the help of the International CFT Units/ml table (ignoring all trace reactions of greater than 75% haemolysis), i.e. only results above 20 ICFTU were considered positive. The result was read in conjunction with the corresponding AC plate result. Where there was more than a trace reaction on the AC plate, the result was recorded as anti-complementary unless it was negative.

3.3.6 The indirect ELISA (iELISA)

The tests were carried out at the University of Pretoria, Faculty of Veterinary Sciences, Department of Veterinary Tropical Diseases' laboratories between December, 2011, and March, 2012. The iELISA assay was carried out according the manufacturer's instructions using the Chekit™ (batch BAT 1132 220 - X101, Idexx Laboratories, Switzerland). The procedure was carried out at room temperature (18-25 ℃) by dispensing 90 µl of reconstituted wash buffer (supplied in kit) into each well of the microtitre plate (supplied pre-coated with antigen). This was followed by the addition of 10 µl of test sera (including positive and negative controls) into their respective microtitre plate wells. The plate was shaken for three minutes on a microtitre plate shaker to mix the contents. The plate was subsequently covered and incubated at 37 ± 2 ℃ in a humid chamber for one hour. It was washed three times in wash buffer, after which 100 µl Chekit-brucellose-anti-ruminant-Ig-PO conjugate was added. This was followed by an incubation and washing step similar to the one above. This was followed by the addition of 100 µl Chekit-TMB substrate and incubation at room temperature for 15 minutes. Chekit-Stop solution (100 µl) was added to each well and the plate read at 450 nm in a microtitre plate reader (Powerwave X52, BioTek, USA) with the aid of Gen 51.11 software (BioTek). The results were analysed using the formula: Sample OD% = (OD sample - OD negative control) * 100 / (OD positive control - OD negative control). Test sera with optical density values equal to or greater than 80% were considered positive.

3.3.7 The fluorescence polarisation assay (FPA)

The tests were performed at the ARC-OVI, Bacterial Serology laboratory between January and March, 2012, according to the manufacturer's instructions.

The FPA was carried out in 96-well flat-bottomed black polystyrene microtitre plates (Greiner). The reagents (Meridian Life Science, USA, batch 8115) and test sera were allowed to reach room temperature (23 ± 2 ℃). Test and control sera were pipetted into the wells of the micro-titre plate at 20 µl per well (negative controls into wells A1, B1 and C1, with positive control in D1). This was followed by the addition of 180 µl of reconstituted dilution buffer (supplied as a 25 x concentrate). The buffer and serum were mixed by incubating on a rotating micro-titre plate shaker (Labinco LD145, The Netherlands) at 600 rpm for three minutes, followed by a stationary one minute (both at room temperature). A background reading was then taken in the Pherastar™ FPA reader (BMG Labtech, Germany). Each well then received 10 µl of antigen-FITC conjugate (supplied with kit), followed by three minute incubation on the plate shaker at 600 rpm at room temperature. The second reading was taken.

The reader, which had previously been calibrated to accept results only if the mean negative control was in the range 70-95 millipolarisation units (mP) and the positive control 120-250 mP, automatically subtracted the background reading and calculated the polarisation value per well. The test result was interpreted as follows: negative if the sample reading was less than 10mP, suspect if between 10-20 mP, and positive when above 20 mP. Positive and suspect samples were confirmed by retesting with the same protocol, except the samples were tested in duplicate. Where both samples read below 10 mP, the sample was reported as negative. If any of the retests read 10-20 mP, the sample was considered suspect, and only considered positive if both read above 20 mP.

Chapter 4
Results

4.1 Determination of panels of known *B. abortus* free and infected calves

4.1.1 *B. abortus*-negative control group

When the 608 unvaccinated calves from the four brucellosis-free herds were tested with the RBT, CFT and the iELISA, 607 of the calves tested negative to all three assays (Table 4.1). One calf tested positive to the RBT, but was negative to the other two assays. The RBT positive result was deemed a false positive based on the absence of test agreement with the other two assays. Based on the above results, and the brucellosis history of the herds, as certified brucellosis-free with negative annual serological tests (or negative monthly MRT for the dairy herd) supported by the absence of unexplained abortions, it was concluded that *B. abortus* was absent in all 608 calves, and they subsequently served as the known *B. abortus*-negative control group for the determination of the specificity of the BST and the FPA.

Table 4.1 Brucellosis antibody response to RBT, CFT and iELISA among 608 unvaccinated calves from four *B. abortus*-free herds

Assay	Negative	Positive	Total tested	Brucellosis reactor rate (%)
RBT	607	1	608	0.16
CFT	608	0	608	0
iELISA	608	0	608	0

4.1.2 Determination of the *B. abortus*-positive control group

In order to determine and compare the diagnostic test performance of the BST and FPA with the currently accepted serological tests, the number of truly infected animals had to be determined. This was achieved by test agreement of at least two assays among the RBT, CFT and iELISA (Jacobson 1998; Alonzo & Pepe 1999; Greiner & Gardner 2000;

Banoo 2010), when applied to the 845 unvaccinated calves from the ten *B. abortus*-infected herds in the study. The herds were deemed *B. abortus*-infected based on the history of *B. abortus* bacterial isolation, supported by brucellosis-positive serological reactions to routine herd testing. When the 845 unvaccinated calves from these herds were tested with the RBT, CFT and iELISA (Table 4.2 and Figure 4.1), 63 calves were classified as truly *B. abortus*-infected based on test agreement of at least two of the three assays. These 63 calves subsequently served as the *B. abortus*-positive control group for the determination of the sensitivity of the BST and the FPA. The apparent brucellosis prevalence among the 845 calves in the ten *B. abortus* infected herds was 7.46% (95% CI: 5.69% - 9.23%).

Table 4.2 Brucellosis antibody response to RBT, CFT and iELISA among 845 unvaccinated calves from ten *B. abortus*-infected herds

Assay	Negative	Positive	Total tested	Brucellosis reactor rate (%)
RBT	792	53	845	6.27
CFT	787	58	845	6.86
iELISA	719	126	845	14.91

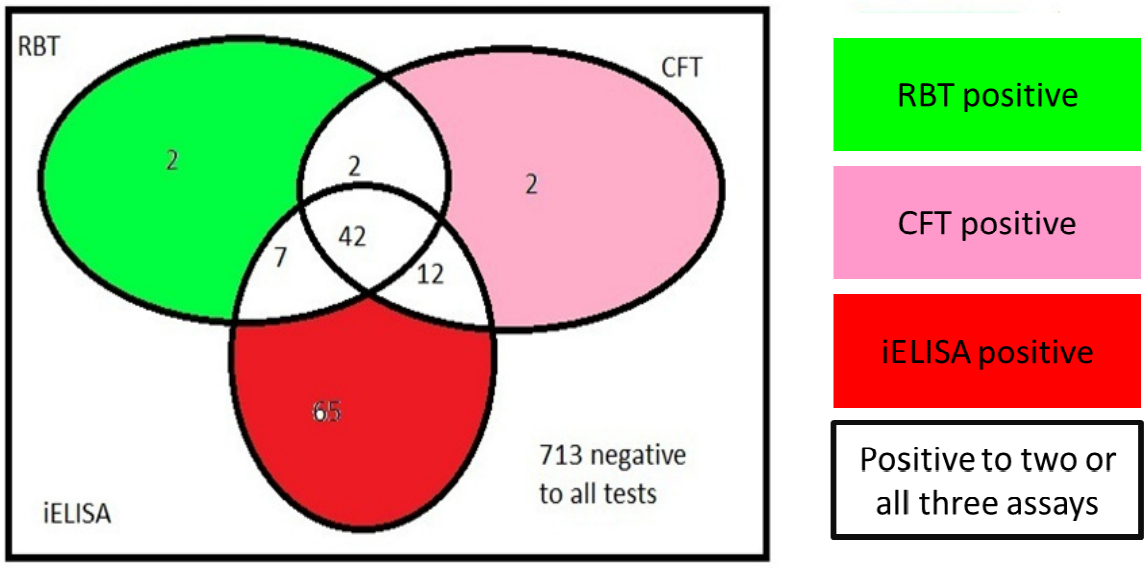

Figure 4.1 Overview of immune response profile of three serological tests among 845 unvaccinated calves in ten brucellosis-infected herds

4.2 Diagnostic test performance of assays for the detection of *B. abortus* infected cattle

4.2.1 The Rose Bengal test

The RBT was applied to 608 unvaccinated calves from four brucellosis-free herds, 423 RB51-vaccinated cows from three brucellosis-free herds, as well as to 845 unvaccinated calves from ten brucellosis-infected herds. The results are summarized in Tables 4.3, 4.4 and 4.5.

Table 4.3 Brucellosis antibody response to the RBT among 608 unvaccinated calves from four brucellosis-free herds

Herd	Negative	Positive	Total tested	Brucellosis reactor rate (%)
A	293	0	293	0
B	16	0	16	0
C	144	1	145	0.69
D	154	0	154	0
Total	**607**	**1**	**608**	**0.16**

Table 4.4 Brucellosis antibody response to RBT among 423 RB51-vaccinated cows from three *B. abortus*-free herds

Herd	Negative	Positive	Total tested	Brucellosis reactor rate (%)
P	23	0	23	0
Q	26	0	26	0
R	374	0	374	0
Total	**423**	**0**	**423**	**0**

The time period from last vaccination of the animals to serum collection ranged from three months (herd P) to one year (herd Q). Most of the animals had been vaccinated multiple times (Table 3.2; section 3.2.2.2).

Table 4.5 Brucellosis antibody response to RBT among 845 unvaccinated calves from ten *B. abortus*-infected herds

Herd	Negative	Positive	Total tested	Brucellosis reactor rate (%)
E	315	33	348	9.48
F	25	2	27	7.41
G	28	0	28	0
H	72	12	84	14.29
I	2	3	5	60
J	3	0	3	0
K	87	0	87	0
L	90	1	91	1.10
M (Heifers)	98	2	100	2.00
N (Bulls)	72	0	72	0
Total	**792**	**53**	**845**	**6.27**

The above table shows there was one brucellosis positive reactor among the 608 unvaccinated calves in the *B. abortus*-free group (specificity of 99.84%, 95% CI: 99.52% - 100%).

There were no brucellosis positive reactors among the 423 RB51-vaccinated cows in the *B. abortus*-free group (100% specificity).

There were 53 brucellosis positive reactors among the 845 calves tested in *B. abortus*-infected herds. The brucellosis reactor rate in this group was 6.27%.

4.2.2 The complement fixation test (CFT)

When the CFT was applied to the 608 unvaccinated calves from the four brucellosis-free herds, the 423 RB51-vaccinated cows from the three brucellosis-free herds, and the 845 unvaccinated calves from the ten brucellosis-infected herds, the results summarized in Tables 4.6, 4.7 and 4.8 were observed.

Table 4.6 Brucellosis antibody response to complement fixation test among 608 unvaccinated calves from four brucellosis-free herds

Herd	Negative (< 20 ICFTU*)	Positive (> 20 ICFTU)	Total tested	Brucellosis reactor rate (%)
A	293	0	293	0
B	16	0	16	0
C	145	0	145	0
D	154	0	154	0
Total	**608**	**0**	**608**	**0**

*ICFTU means International complement fixation units

Table 4.7 Complement fixation test brucellosis antibody responses among 423 RB51-vaccinated cows from three brucellosis-free herds (period from vaccination to bleeding ranged from three months to one year)

Herd	Negative (< 20 ICFTU)	Positive (> 20 ICFTU)	Total tested	Brucellosis reactor rate (%)
P	23	0	23	0
Q	26	0	26	0
R	374	0	374	0
Total	**423**	**0**	**423**	**0**

Table 4.8 Complement fixation test brucellosis antibody response among 845 unvaccinated calves in ten brucellosis-infected herds

Herd	Negative (< 20 ICFTU)	Positive (> 20 ICFTU)	Total tested	Brucellosis reactor rate (%)
E	315	33	348	9.48
F	24	3	27	11.11
G	28	0	28	0
H	68	16	84	19.05
I	2	3	5	60.00
J	2	1	3	33.33
K	87	0	87	0
L	90	1	91	1.10
M (Heifers)	99	1	100	1.00
N (Bulls)	72	0	72	0
Total	**787**	**58**	**845**	**6.86**

The tables above shows there were no brucellosis positive reactors among the *B. abortus*-free herds, both unvaccinated calves and exclusively RB51-vaccinated cows (100% specificity). There were 58 brucellosis positive reactors among the *B. abortus*-infected herds. The brucellosis reactor rate among the infected herds was 6.86%.

4.2.3 The indirect ELISA

The iELISA was applied to 608 unvaccinated calves from four brucellosis-free herds, 423 RB51-vaccinated cows from three brucellosis-free herds, as well as to 845 unvaccinated calves from ten brucellosis-infected herds. The results are summarized in Tables 4.9, 4.10 and 4.11.

Table 4.9 Antibody response detected by the indirect ELISA among 608 unvaccinated calves from four brucellosis-free herds

Herd	Negative*	Positive	Total tested	Brucellosis reactor rate (%)
A	293	0	293	0
B	16	0	16	0
C	145	0	145	0
D	154	0	154	0
Total	**608**	**0**	**608**	**0**

*< 80% of the absorbance of positive control, Positive means > 80% of the absorbance of positive control

Table 4.10 Antibody response detected by the indirect ELISA among 423 RB51-vaccinated cows from three brucellosis-free herds (period from vaccination to bleeding ranged from three months to one year)

Herd	Negative	Positive	Total tested	Brucellosis reactor rate (%)
P	23	0	23	0
Q	26	0	26	0
R	373	1	374	0.27
Total	**422**	**1**	**423**	**0.24**

Table 4.11 Antibody response detected by the indirect ELISA among 845 unvaccinated calves from ten brucellosis-infected herds

Herd	Negative	Positive	Total tested	Brucellosis reactor rate (%)
E	275	73	348	20.98
F	21	6	27	22.22
G	28	0	28	0
H	48	36	84	42.86
I	2	3	5	60.00
J	1	2	3	66.67
K	87	0	87	0
L	91	0	91	0
M (Heifers)	95	5	100	5.00
N (Bulls)	71	1	72	1.39
Total	**719**	**126**	**845**	**14.91**

There were no brucellosis positive reactors among the *B. abortus*-free unvaccinated calves (100% specificity). There was one brucellosis positive reactor among the *B. abortus*-free, exclusively RB51-vaccinated cows (Table 4.10). The specificity in this group was 99.76% (95% confidence interval of 99.29% - 100%).

There were 126 brucellosis positive reactors among the 845 calves tested in the ten *B. abortus*-infected herds (Table 4.11), a brucellosis reactor rate of 14.91%.

4.3 Evaluation of the performance of the brucellin skin test for the detection of *B. abortus* infected cattle

4.3.1 Evaluation of the brucellin skin test in the *B. abortus* negative and positive control groups

The mean skin reaction size in the *B. abortus*-free unvaccinated animals was 0.149 mm (95% CI: 0.123 - 0.174 mm) with a standard deviation of 0.320 mm and a range of zero to 4.2 mm. The skin reactions in 99.18% of the *B. abortus*-free unvaccinated animals were below 1.1 mm. Less than 1% of *B. abortus*-free unvaccinated animals showed a skin reaction above 1.5 mm.

Table 4.12 Distribution of BST reactivity among 608 calves in the four *B. abortus*-free control herds

Herd	Brucellin skin test reaction [increase in skin thickness (mm)]					Total tested
	< 0.5	0.51-1.0	1.1-1.49	1.5-2.0	> 2.0	
A	277	13	0	0	2	292
B	11	4	0	1	0	16
C	135	9	0	1	0	145
D	154	0	0	0	1	155
Total	**577**	**26**	**0**	**2**	**3**	**608**

Table 4.13 Distribution of BST reactivity in 63 brucellosis-infected, positive control animals

Herd	Brucellin skin test reaction [increase in skin thickness (mm)]					Sero-positive*
	< 0.5	0.51-1.0	1.1-1.49	1.5-2.0	> 2.0	
E	13	3	0	2	17	35
F	0	0	1	1	2	4
G	0	0	0	0	0	0
H	10	3	1	1	2	17
I	2	0	0	0	1	3
J	1	0	0	0	0	1
K	0	0	0	0	0	0
L	1	0	0	0	0	1
M	0	1	0	0	1	2
N	0	0	0	0	0	0
Total	**27**	**7**	**2**	**4**	**23**	**63**

*Sero-positive defined as serum positive to two or more antibody tests including RBT, CFT and iELISA in series

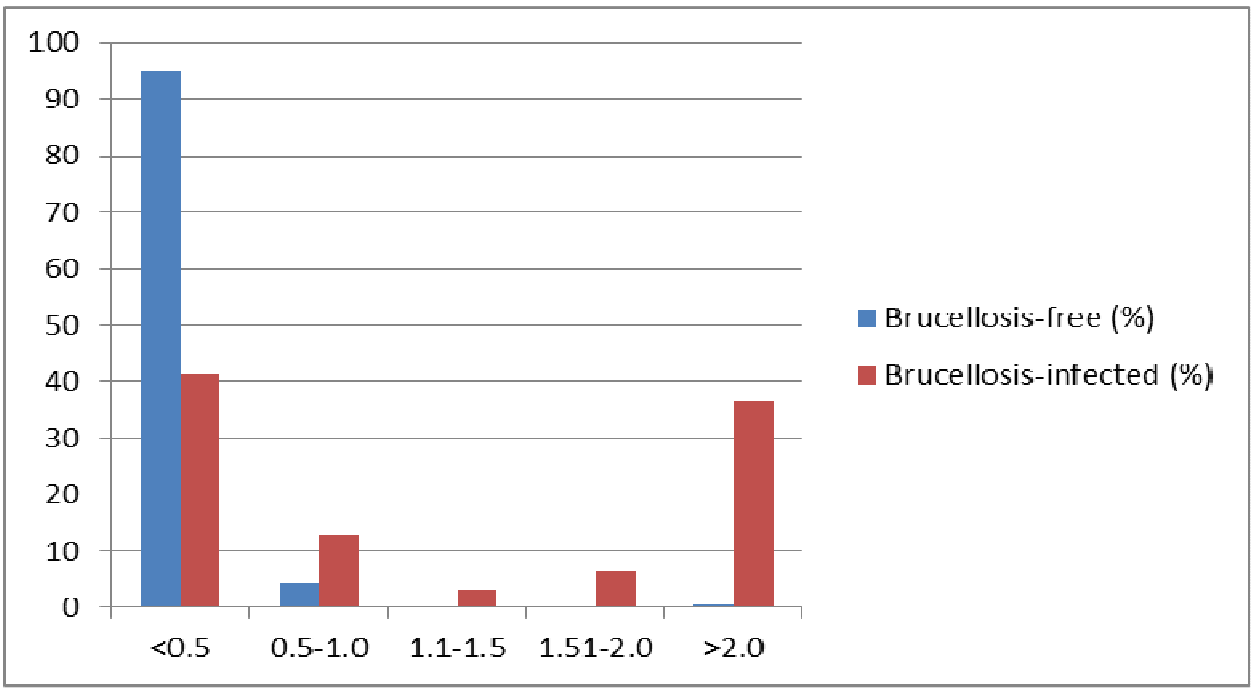

Figure 4.2 Distribution of BST reactivity in brucellosis-free negative control group (n=608) and brucellosis-infected positive control group (n=63)

(The horizontal axis shows the increase in skin thickness (mm), while the vertical axis shows the number of animals in each category as a percentage)

4.3.2 Determination of the brucellin skin test cut-off value

The data from Tables 4.12 and 4.13, as well as Figure 4.2, show a considerable overlap in the distribution of the BST reaction sizes between the *B. abortus*-free control group and the *B. abortus*-infected control group. However, the data in Table 4.12 and Figure 4.2 show that 99% of the BST reactions in the *B. abortus*-free negative control group lie below 1.1 mm. The same data set shows that less than 1% of the BST reactions in the negative control group lie above 1.5 mm. The higher cut-off value of 1.5 mm was selected for improved specificity.

When the data in Table 4.12 were evaluated at the 1.5 mm cut-off value, 603 of the 608 brucellosis-free negative control animals were classified as *B. abortus*-free while five were classified as infected (false positive).

An evaluation of the *B. abortus*-infected positive control group data (Table 4.13) at the BST cut-off value of 1.5 mm classified 27 of the 63 brucellosis-infected positive control animals as *B. abortus*-infected.

The performance of the BST was subsequently summarized in the 2 X 2 contingency table as shown in Table 4.14 below.

Table 4.14 Summary of BST performance in 63 brucellosis-infected positive control animals and 609 brucellosis-free animals

		Brucellosis- infected		**Brucellosis-free**		**Total**	
BST	Positive	(a)	27	(c)	5	**(a+c)**	**32**
	Negative	(b)	36	(d)	603	**(b+d)**	**639**
	Total	**(a+b)**	**63**	**(c+d)**	**608**	**(a+b+c+d)**	**671**

Relative sensitivity = a / (a+b) = 42.86% (95% CI: 30.46% - 55.95%)
Specificity = d / (c+d) = 99.18% (95% CI: 98.09% - 99.73%)
Apparent brucellosis prevalence = (a+b) / (a+b+c+d) = 9.39% (95% CI: 7.29% - 11.85%)
At this prevalence:
Positive predictive value = a / (a+c) = 84.38% (95% CI: 67.21% - 94.72%)
Negative predictive value = d / (b+d) = 94.37% (95% CI: 92.29% - 96.02%)

4.4 The field performance of the brucellin skin test

4.4.1 Performance of the brucellin skin test in the identification of RB51 vaccinated cattle

In order to be certain that any BST-positive reaction among the RB51-vaccinated cows was only due to the vaccine, the brucellosis status of the herd of origin had to be determined. This was achieved by subjecting all the 423 cows in the three herds to the RBT, CFT and the iELISA. The data in Tables 4.15 show that among the 423 RB51-vaccinated cows in the three brucellosis-free herds, 422 tested negative to all three assays (RBT, CFT and iELISA). One cow tested positive to the iELISA. The positive iELISA result was classified as a false positive as there was no assay agreement with the other two assays. Based on these results, supported by the herd history (absence of any epidemiological evidence suggestive of brucellosis, supported by regular testing for a minimum period of three years), all 423 cows were classified brucellosis-free.

Table 4.15 Brucellosis antibody response to RBT, CFT and iELISA among 423 RB51-vaccinated cows from three *B. abortus*-free herds (period between vaccination and performance of BST ranged from three month to one year)

Assay	Negative	Positive	Total tested	Brucellosis reactor rate (%)
RBT	423	0	423	0
CFT	423	0	423	0
iELISA	422	1	423	0.24

The data in Table 4.15 show that 422 cows gave negative brucellosis-antibody reactions to all three assays used. The one cow with a positive brucellosis-antibody reaction to the iELISA was classified a false-positive as there was no assay agreement with the other two assays (RBT and CFT).

Table 4.16 is a summary of the BST reactions (mm) among 422 *B. abortus*-free, RB51-vaccinated cows. One cow was excluded from the BST performance analysis due to injury on the brucellin injection site. The period between the last vaccination and the performance of the BST ranged from three months in herd P to one year in herd Q.

Table 4.16 Summary of the BST reactions (mm) among 422 brucellosis-free, RB51 vaccinated cows in three herds

Herd	RB51 vaccination age (months)	Brucellin skin reaction [increase in skin thickness (mm)]					Total tested	Brucellosis reactor rate (%)
		< 0.5	0.51-1.0	1.1-1.49	1.5-2.0	> 2.0		
P	6, 12 then annually	14	3	3	1	2	23	13.04
Q	6, 12 then annually	14	3	2	0	7	26	26.92
R	8, 12 then annually	161	33	13	22	144	373	44.50
Total		**189**	**39**	**18**	**23**	**153**	**422**	**41.71**

There were three, seven and 166 reactors in herds P, Q and R respectively. Figure 4.3 shows the distribution in size of the BST positive reactions among the 422 cows in the three RB51-vaccinated herds.

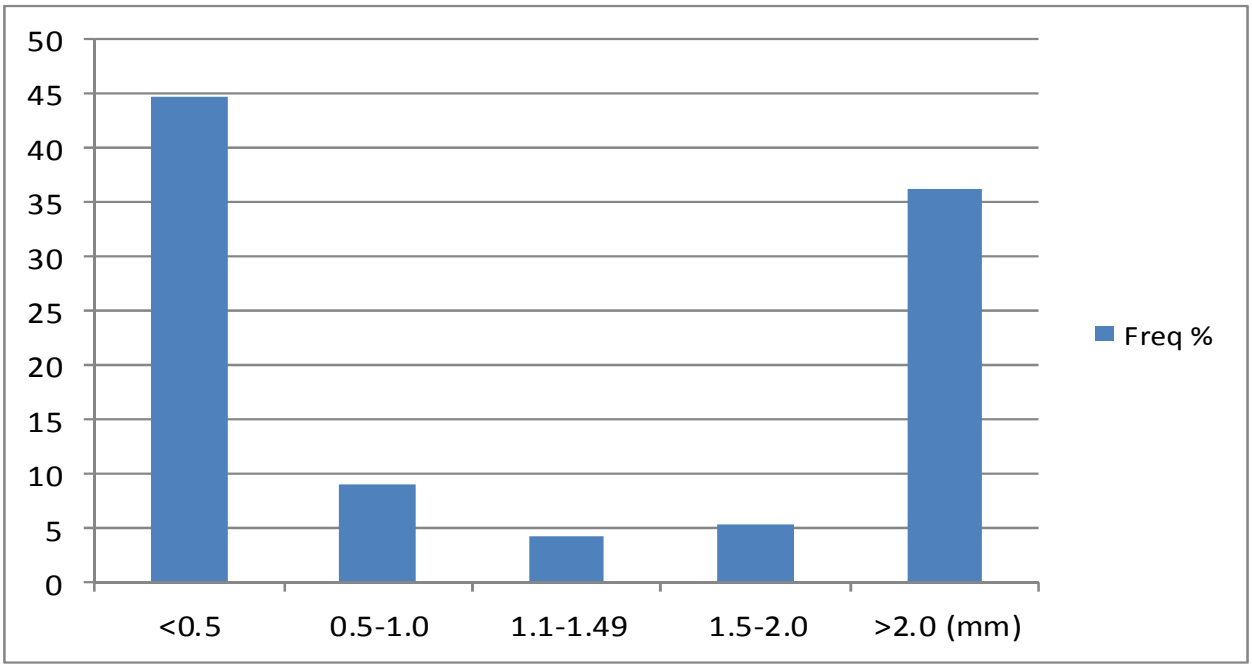

Figure 4.3 Distribution of the brucellin skin test reactivity size (mm) among 422 RB51-vaccinated cows in three herds (the horizontal axis shows the increase in skin thickness (mm), while the vertical axis shows the number of animals in each category as a percentage)

The data in Figure 4.3 show a brucellosis-positive quantitative BST reactivity rate of 41.7%.

The BST reactivity in RB51-vaccinated cows along with data from BST performance in brucellosis-free control group is summarized in a 2 x 2 contingency Table 4.17 below.

Table 4.17 Summary of BST performance in 422 *B. abortus*-free, RB51-vaccinated cows and 609 *B. abortus*-free negative controls

		RB51-vaccinated		**Brucellosis negative controls**		**Total**	
FPA	Positive	(a)	176	(c)	5	**(a+c)**	**181**
	Negative	(b)	246	(d)	603	**(b+d)**	**849**
	Total	**(a+b)**	**422**	**(c+d)**	**608**	**(a+b+c+d)**	**1030**

Persistence of measurable cell mediated immunity (CMI) = a / (a+b) = 41.71% (95% CI: 36.96% - 46.57%)
Specificity = d / (c+d) = 99.18% (95% CI: 98.09% - 99.73%)
Prevalence of vaccination = (a+b) / (a+b+c+d) = 40.93% (95% CI: 37.91% - 44.00%)
Positive predictive value = a / (a+c) = 97.24% (95% CI: 93.67% - 99.09%)
Negative predictive value = d / (b+d) = 71.06% (95% CI: 67.88% - 74.09%)

The persistence of CMI as measured by the BST in the RB51 vaccinated group is similar to the relative sensitivity in the brucellosis-infected control group (Table 4.13).

4.4.2 The performance of the brucellin skin test in *B. abortus*-infected herds

The data in Table 4.18 show a brucellosis positive reactor rate of 9.25% among the 843 unvaccinated calves in the *B. abortus* infected herds.

Table 4.18 BST reactivity among 843 unvaccinated calves in ten *B. abortus* infected herds as measured by the increase in skin thickness (mm)

Herd	Brucellin skin reaction (mm)					Total tested	Brucellosis reactor rate (%)
	< 0.5	0.51-1.0	1.1-1.49	1.5-2.0	> 2.0		
E	273	15	7	7	45	347	14.99
F	22	0	1	1	3	27	14.81
G	27	0	1	0	0	28	0
H	66	6	3	3	5	83	9.64
I	2	1	0	0	2	5	40.00
J	2	1	0	0	0	3	0
K	75	3	3	3	3	87	6.90
L	87	1	1	1	2	91	3.30
M (Heifers)	93	4	0	0	3	100	3.00
N (Bulls)	71	1	0	0	0	72	0
Total	**718**	**32**	**16**	**15**	**63**	**843**	**9.25**

4.5 Evaluation of the performance of the fluorescence polarization assay (FPA)

4.5.1 Evaluation of the fluorescence polarization assay in brucellosis negative and positive control groups

The mean FPA reactivity was -9.556 mP (95% CI of -11.408 mP to -7.704 mP), with a standard deviation of -23.251 mP, and a range of -212 mP to 348 mP. About 99% of the brucellosis-negative control calves registered brucellosis FPA reactivity below 20 mP.

Table 4.19 Brucellosis antibody FPA reactivity among 608 unvaccinated calves from the *B. abortus* negative control group (as measured by the decrease in the rate of molecular rotation, in mP units)

Herd	FPA reading (mP units)					Total
	< 10	10-19	20-30	31-40	> 40	
A	272	13	2	2	2	291
B	16	0	0	0	0	16
C	141	3	0	0	1	145
D	156	0	0	0	0	156
Total	**585**	**16**	**2**	**2**	**3**	**608**

Table 4.20 Distribution of brucellosis antibody FPA reactivity among 63 *B. abortus* positive control calves from ten herds (as measured by the decrease in the rate of molecular rotation, in mP units)

FPA reading (mP)	< 10	10-19	20-30	31-40	> 40	Total
Number of animals	2	2	3	1	55	63

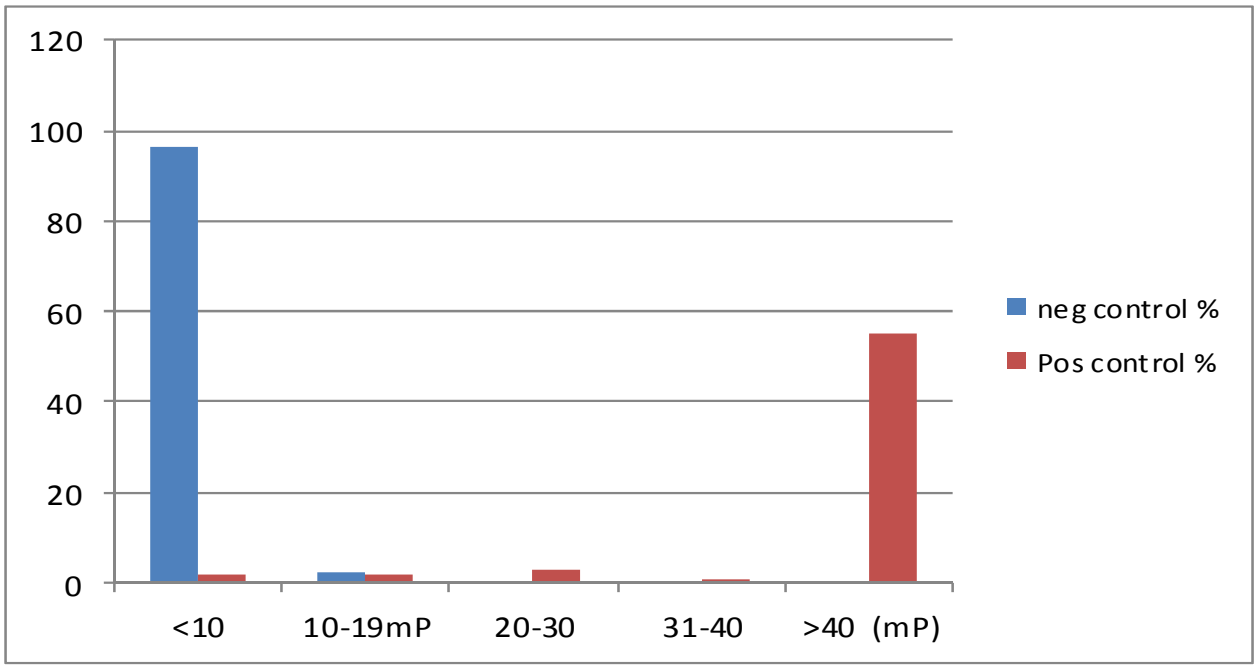

Figure 4.4 Comparison of the distribution of brucellosis antibody responses between 608 brucellosis-free calves with 63 brucellosis-infected calves as measured by the FPA [the X-axis shows the FPA reactivity (mP units); the Y-axis shows the number of animals in each category as a percentage]

Figure 4.4 above shows 99% of the 608 brucellosis-negative control calves and 6.35% of the brucellosis-positive control calves registered brucellosis FPA reactivity below 20 mP.

4.5.2 Determination of the fluorescence polarization assay cut-off value

Estimation of the optimum cut-off value was done by receiver-operator characteristic (ROC) analysis using StatsDirect version 2, 7, 8 software, as shown in Figure 4.5.

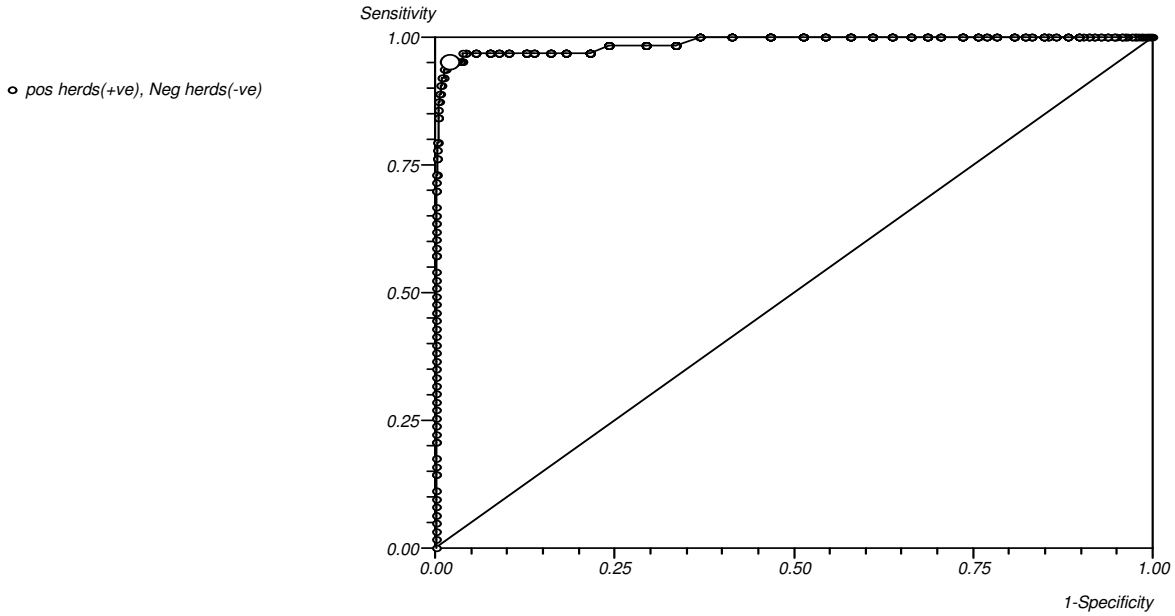

Figure 4.5 ROC analysis of the fluorescence polarization assay from 63 brucellosis-infected and 608 brucellosis-free control sera

The area under the ROC curve was 0.987534. The optimum cut-off value selected by the software was 18 mP. As this cut-off value was considered close enough to the manufacturer's recommended cut-off value of 20 mP; the optimum cut-off value of 20 mP units was selected for analysis of results. At 20 mP cut-off value, the performance of the FPA was as summarized in Table 4.21.

Table 4.21 Contingency table of FPA performance among 63 *B. abortus*-infected positive control and 608 *B. abortus*-free negative control animals

		Controls					
		Brucellosis infected		**Brucellosis free**		**Total**	
FPA	Positive	(a)	59	(c)	7	(a+c)	66
	Negative	(b)	4	(d)	601	(b+d)	605
	Total	**(a+b)**	**63**	**(c+d)**	**608**	**(a+b+c+d)**	**671**

Relative sensitivity = a / (a+b) = 93.65% (95% CI: 84.52% - 98.20%)

Specificity = d / (c+d) = 98.85% (95% CI: 97.64% - 99.53%)

Apparent prevalence = (a+b) / (a+b+c+d) = 9.39% (95% CI: 7.29% - 11.85%)

At this prevalence:

Positive predictive value = a / (a+c) = 89.39% (95% CI: 79.35% - 95.61%)

Negative predictive value = d / (b+d) = 99.34% (98.31% - 99.82%)

4.6 The field performance of the fluorescence polarization assay

4.6.1 Performance of the fluorescence polarization assay in RB51-vaccinated herds

Table 4.22 shows a summary of results obtained from testing 421 samples from RB51 vaccinated cows. One sample could not be tested as the serum got finished prior to testing.

Table 4.22 Distribution of brucellosis antibody FPA reactivity among 421 brucellosis-free, RB51-vaccinated cows in three herds (as measured by decrease in rate of molecular rotation in mP units)

Herd	FPA reading (mP units)					Total tested	Reactor rate (%)
	< 10	10-19	20-30	31-40	> 40		
P	22	1	0	0	0	23	0
Q	26	0	0	0	0	26	0
R	370	0	0	0	2	372	0.54
Total	**418**	**1**	**0**	**0**	**2**	**421**	**0.48**

Table 4.22 and Figure 4.6 show 99.52% of the brucellosis FPA reactivity in the brucellosis-free, RB51-vaccinated group lies below 20 mP.

4.6.2 The performance of the fluorescence polarization assay in *B. abortus*-infected herds

A total of 839 sera originating from ten brucellosis-infected herds were tested with the FPA as shown in Table 4.23. Four samples out of the original 843 could not be tested after the serum got exhausted prior to testing.

Table 4.23 Distribution of brucellosis antibody FPA reactivity among 839 unvaccinated calves from ten brucellosis-infected herds (as measured by decrease in rate of molecular rotation in mP units)

Farm	FPA reading (mP units)					Total tested	Reactor rate (%)
	< 10	10-19	20-30	31-40	> 40		
E	297	4	1	2	44	348	13.51
F	18	4	1	0	4	27	18.52
G	28	0	0	0	0	28	0
H	49	5	7	4	19	84	35.71
I	1	1	1	0	2	5	60.00
J	2	0	1	0	0	3	33.33
K	81	0	0	0	0	81	0
L	90	1	0	0	0	91	0
M (Heifers)	89	8	0	0	3	100	3.00
N (Bulls)	70	1	0	0	1	72	1.39
Total	**725**	**24**	**11**	**6**	**73**	**839**	**10.73**

Table 4.23 above shows a brucellosis FPA positivity rate of 10.73 among the 839 calves in the ten brucellosis-infected herds.

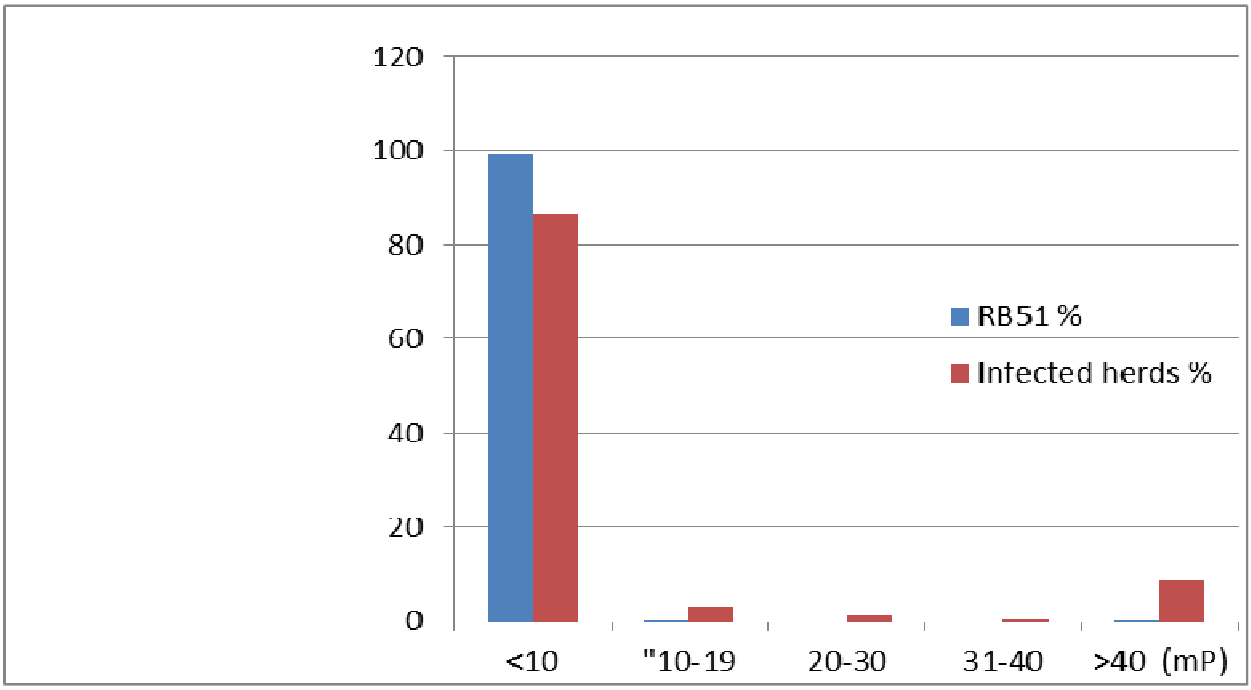

Figure 4.6 Comparison of the brucellosis antibody FPA reactivity between 421 RB51-vaccinated cows from three brucellosis-free herds and 839 calves from ten brucellosis-infected herds [the X-axis represents FPA reactivity (mP units); Y-axis represents the number of cows in each category as a percentage]

Figure 4.6 shows a marked distinction in the distribution of brucellosis FPA antibody reactivity between vaccinated and infected animals in RB51-vaccinated and infected herds. Vaccinated animals are classified as *B. abortus* free (FPA reactivity below 20 mP).

4.7 Assessment of assay agreement between the brucellin skin test and serological assays

An assessment of the test agreement among the 843 sera from brucellosis-infected herds yielded the outcome summarized in Table 4.24. Only 839 corresponding individual calves per assay were considered for all assessments that involved the FPA.

Table 4.24 Assay agreement between BST and serology among 843 calves in ten brucellosis-infected herds

BST	RBT		CFT		iELISA		FPA*	
	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
Positive	21	57	23	55	52	26	38	40
Negative	32	733	35	730	74	691	52	709
Total	**53**	**790**	**58**	**785**	**126**	**717**	**90**	**749**
Kappa	**0.266**		**0.281**		**0.447**		**0.416**	
Total positive (parallel testing)	**110**		**113**		**152**		**130**	

*Only 839 animals were considered for the assessment involving the FPA

The data in Table 4.24 show that the level of assay agreement between BST on one hand, and RBT, CFT and FPA on the other, as measured by the Kappa statistic, was fair. Assay agreement between BST and iELISA was moderate (Viera & Garret 2005). Parallel testing that involved the BST and iELISA identified the highest number of animals as *B. abortus*-infected.

4.8 Assessment of assay agreement between the fluorescence polarization assay and currently accepted serology

Test agreement between the FPA on one hand, and the RBT, CFT and iELISA on the other, involving 839 sera, was carried out. The results are summed up in Table 4.25.

Table 4.25 Assessment of test agreement between the FPA, and RBT, CFT and iELISA among 839 unvaccinated calves from ten brucellosis-infected herds

FPA	RBT		CFT		iELISA		Total
	Positive	Negative	Positive	Negative	Positive	Negative	
Positive	52	38	51	39	84	6	90
Negative	1	748	7	742	42	707	749
Total	**53**	**786**	**58**	**781**	**126**	**713**	**839**
Kappa	**0.703**		**0.661**		**0.746**		

The data in Table 4.25 shows the level of agreement between the FPA, and the other serological tests as measured by the kappa statistic, was substantial (Viera & Garret 2005).

Table 4.26 Comparison of the performance of the FPA against other serological assays in *B. abortus* infected positive control, and free negative control animals

	RBT	**CFT**	**FPA**	**iELISA**
Sensitivity (%) (n=63)	80.95 (70.25-90.65)	88.89 (81.13-96.65)	93.65 (87.63-99.67)	96.83 (88.98-99.61)
Specificity (%) (n=608)	99.84 (99.52-100)	100	98.85 (98.00-99.77)	100
Performance index	180.79	188.89	192.50	196.83

(Confidence interval in brackets)

Because the confidence intervals in Table 4.26 show some overlap, the McNemar-Liddel test for the significance of the differences (P values, where a $P < 0.05$ means the difference between the assays is significant) was performed between the various assays. The results in Table 4.27 were obtained.

The FPA was more sensitive when compared to the RBT ($P = 0.0117$), but was inferior to the iELISA ($P = 0.0129$). There was no significant difference between the FPA and CFT ($P = 0.2891$).

The FPA specificity was inferior compared to all three tests (RBT, CFT and iELISA), ($P = 0.0156$).

Table 4.27 McNemar-Liddel test for the comparison of differences between test diagnostic sensitivity (top half) and specificity (bottom half) of the FPA and currently accepted serology by *P* values

Test	RBT	CFT	FPA	iELISA
RBT		0.3593	0.0117	0.0129
CFT	> 0.9999		0.2891	0.1797
FPA	0.0156	0.0156		> 0.9999
iELISA	> 0.9999	> 0.9999	0.0156	

Chapter 5
Discussion

The trial set out to re-establish the brucellosis status of the study population to enable the establishment of reference brucellosis-free and brucellosis-infected groups for the evaluation of the specificity and sensitivity of the assays under investigation, the BST and FPA. The other objectives were to establish the persistence of BST-measurable CMI in brucellosis-free, exclusively RB51-vaccinated animals as well as find out if it could identify latent *B. abortus* infection in brucellosis-infected herds under South African field conditions. The trial also aimed at establishing the specificity of the FPA in brucellosis-free, RB51 vaccinated herds, as well as its reactor rate in *B. abortus*-infected herds.

In order to assess accuracy, the sensitivity and specificity of a new test are evaluated against a reference standard test (often called the gold standard) which is used to identify truly infected individuals as well as uninfected individuals (Knottnerus, Van Weel & Muris 2002; Banoo 2010). The use of an imperfect reference test biases the accuracy estimates of the index test, and is often the case in the absence of a recognised reference standard test (Alonzo & Pepe 1999). In the case of brucellosis, bacterial culture is the widely accepted reference standard although it incorporates spectrum bias, given that sera from culture-positive animals are not representative of the target population in which the index test will be applied, as the target population may include early and latent infections which may not be detected by culture (Jacobson 1998). The limited sensitivity of culture leads to the misclassification of some truly infected individuals as uninfected (type II error), implying that many false positives by the index test (relative to culture) are in fact true positives, or gold standard test misses (Alonzo & Pepe 1999). Clinically defined reference standards are not usually appropriate either, especially when the clinical presentation is neither sensitive nor specific (Banoo 2010). This, indeed, is the case with brucellosis, where the disease is often chronic, with variable incubation periods, rendering clinical detection of infected animals difficult. When apparent: the clinical features of bovine brucellosis are varied (abortion, infertility, retained placentae, orchitis and epididymitis) and non-specific as they are shared with numerous other diseases. In addition, no single diagnostic test has the ability to detect all stages of brucellosis with high sensitivity and specificity.

Thus, the choice of the reference standard is crucial for the legitimacy of comparison. In such situations, where no single suitable reference standard test exists, or where the index test is thought to be more sensitive than the existing gold standard test, a composite reference standard, CRS, (where two or more assays are combined) may be used (Jacobson 1998; Alonzo & Pepe 1999; Greiner & Gardner 2000; Banoo 2010). Where the assays in the CRS are highly specific but with relatively poorer sensitivity, the assays in the CRS may be used in parallel (where a positive outcome is defined as being positive to either of the tests). However, where the assays in the CRS are highly sensitive, a positive outcome is defined as one that is positive to two or more of the assays (and negative as negative by two or more of the assays) (Banoo 2010). The performance indices of the assays which made up the CRS in this study place them in the latter group (OIE Terrestrial Manual 2009); and it was on this basis that a truly infected individual as well as the uninfected individual were defined. Thus, with the CRS serological panel which consisted of the RBT, CFT and iELISA, as the gold standard method, an individual animal was classified either brucellosis free or infected on the basis of test agreement of at least two of the three assays used. This was supported by the bacterial isolation of *B. abortus* from at least one animal in each infected herd, or proven epidemiological absence in the case of brucellosis free herds.

The use of multiple tests increases the confidence in a negative result when there is assay agreement on the result (Griner *et al* 1981; Jacobson 1998). The results obtained from applying the RBT, the CFT, and the iELISA, Table 4.1, to 608 unvaccinated calves from *B. abortus*-free herds, re-enforce their classification as brucellosis-free. The 608 calves had been sourced from herds that were officially certified brucellosis-free for a minimum uninterrupted period of three years, whose freedom status was maintained through regular annual serological tests (or regular monthly milk ring tests for the dairy farm). In addition, the herds had shown no epidemiological evidence, such as abortions, that could lead one to suspect the presence of *B. abortus*. On this basis, the 608 calves formed the *B. abortus*-free (negative control) group that was used in the analysis of the specificity of the BST and the FPA. Similarly, the 423 *B. abortus*-free, exclusively RB51-vaccinated cows were sourced from herds that were officially certified *B. abortus*-free for a minimum of three years, re-enforced through regular annual serological tests. There was similarly no evidence of suspicious abortions during the period of official brucellosis freedom. All 423 cows were tested with the RBT, CFT and iELISA, Table 4.15, with *B. abortus*

negative results. Wild strain *B. abortus* was deemed absent from all 423 cows, and any subsequent positive reaction to the BST was assumed to be due to the RB51 vaccine (Cheville *et al* 1994). In the case of the FPA, it was assumed to be a false positive reaction.

The use of multiple tests allows for the confirmation of infection when there is test agreement on positive reactions (Griner *et al* 1981). The results obtained from applying the RBT, the CFT and the iELISA to 845 unvaccinated calves from ten *B. abortus*-infected herds yielded 63 truly *B. abortus*-infected animals by test agreement as illustrated in Table 4.2 and Figure 4.1. The high degree of confidence in the infection status of the 63 calves stems from their origin; in herds from which *B. abortus* had previously been isolated, and which continued to show positive brucellosis reactors to routine testing. The 63 animals were subsequently used as the reference *B. abortus*-infected (positive control) animals for the analysis of the sensitivity of the BST and FPA.

The performance of the BST was evaluated in *B. abortus*-free calves, *B. abortus*-infected calves, in *B. abortus*-free, exclusively RB51-vaccinated cows as well as in infected herds under field conditions. The data presented in Table 4.13 and Figure 4.2 show that over 99% of the skin reactions among the negative control group lay below 1.1 mm. This finding is in agreement with the findings of earlier researchers who selected 1.1 mm as the suitable cut-off value (Saegerman *et al* 1999). This study further found that still less than 1% of the skin reactions in the negative control group lay above 1.5 mm, which was subsequently selected as a suitable cut-off value for increased specificity. At this cut-off value, the specificity of the BST among 608 unvaccinated brucellosis-free calves was 99.18% (95% CI: 98.09% - 99.73%), with five false positive reactions. The specificity was largely similar to that of 99.83% (95% CI: 99.40% - 100%) by Saegerman and others (1999) albeit at a lower cut-off value of 1.1 mm. In France, at a cut-off value of 2 mm, other researchers observed a similar BST specificity of 99.83% (Pouillot *et al* 1997). In New Zealand, MacDiarmid and Hellstrom (1987) observed a BST specificity of 99.98% on a large sample of 5 064 animals at a cut-off value of 2 mm. Saegerman and others (1999) observed some false-positive reactions attributable to *E coli* O157, *Salmonella urbana* and *Xanthomonas maltophila.* The false positive BST reactions observed in this study were neither visible to the naked eye nor perceptibly palpable. They were established only by the objective quantitative assessment of the increase in skin thickness. The study did

not establish the cause of the false positive reactions. The relative sensitivity of the BST observed in this study, 42.86% (95% CI: 30.46% to 55.95%), was much lower when compared to that of between 78% and 93% (Saegerman *et al* 1999), 81% (Bercovich & ter Laak 1992), or 64% (Pouillot *et al* 1997), or that of between 52% and 61% by MacDiarmid and Hellstrom (1987). It may be worth noting that Bercovich and ter Laak (1992) defined a truly infected animal through bacterial culture while Saegerman and others derived their sensitivity from experimentally infected animals, and observed that the sensitivity tended to decrease with increasing post-infection period. It is possible the later phenomenon contributed to the lower sensitivity value observed in this study. This study employed animals whose ages ranged from three to nine months. As these animals were sourced from brucellosis-infected herds, it could not be established precisely when each individual was infected as animals were either infected via ingestion after birth, or in-utero (Nicoletti 2010). Indeed, the reported BST sensitivity has been known to be higher when bacterial culture or experimental infection was used to define an infected animal for the assessment of sensitivity (Sutherland 1983). Other workers in Cameroon observed a much lower sensitivity of 33% relative to the serology (Martrenchar *et al* 1993). It must be noted, however, that the latter used parallel testing with the SAT, the RBT and the CFT (were positive meant an individual positive to any of the three assays) to define an infected animal. This meant their gold standard indicator of infection may have been more sensitive and perhaps less specific than the one used in this study. In comparison, this may have led to a lower estimate of the observed BST sensitivity in their study when compared to this one.

The observed BST persistence of measurable CMI in brucellosis-free, RB51-vaccinated cows was 41.71% (95% CI: 36.96% - 46.97%), Table 4.17. This level of measurable CMI was quite similar to the relative sensitivity observed in the same study in the *B. abortus*-infected group. As we were highly confident that all the 423 RB51-vaccinated cows were exposed to the RB51 strain of *B. abortus*; this group could as well be considered a parallel positive control group. In the event, the similarity in the BST sensitivity between the two groups (i.e. the 63 positive control calves and the 423 RB51-vaccinated cows) lends credence to the value of the gold standard of multiple tests in the identification of truly infected individual calves. The similarity in the observed sensitivities between the two groups may be considered to add some value to the repeatability of the observed BST sensitivity in the South African cattle population. It was also similar to that of 40% (95% CI: 16.7% - 69.2%) observed by other workers in

Italian cattle (De Massis *et al* 2005) on a similar trial, but with much fewer animals (n=10). Since we are certain that nearly all 423 cows were vaccinated (exposed to the RB51 strain of *B. abortus*), it may be concluded that the BST is not an ideal indicator of vaccine exposure, or cell mediated immunity, at individual animal level. These observations corroborate those of De Massis and others (2005) who also concluded that while the BST offered a low sensitivity in the identification of vaccinated individual animals; it was a useful screening test to identify vaccinated herds. In their study, De Massis and others (2005) observed that the anamnestic effect following brucellin injection could be followed up with a RB51-specific CFT between days 9 and 20 post brucellin injection to identify individual vaccinated animals with between 90 and 100% sensitivity. Thus, parallel testing, first with the BST followed by RB51-specific serology 9-20 days later, would offer a more comprehensive assessment of vaccine efficacy as both humoral and CMI are evaluated. This study did not verify the delay period of 9-20 days under South African conditions. In addition, smooth *Brucella* antigen serology could be performed, only this time to identify latent truly infected individuals, in which case the positive reaction is known to persist beyond three weeks (Lapraik 1982; Sutherland 1983; Cheville *et al* 1994).

When applied to *B. abortus*-infected herds (Table 4.18); the BST detected all brucellosis-infected herds in the study as brucellosis-positive, with the exception of herd J which consisted of only three animals. Herd N, which consisted of bull calves from the same farm as herd M, was classified brucellosis-free through serological test agreement. The BST also classified this herd as brucellosis-free. However, in this herd, one calf showed brucellosis sero-positivity to both the FPA and iELISA (Tables 4.11 and 4.23). The true status of the animal could not be established as follow-up slaughter and bacteriology was not attempted owing to budgetary constraints.

The BST (9.25%), (Table 4.18), and iELISA (14.91%), (Table 4.11), depicted comparatively higher reactor rates in the infected herds, compared to RBT (6.27%), (Table 4.5) and CFT (6.86%), (Table 4.8). Out of the 78 animals which tested positive to the BST, only 27 (34.62%) were in agreement with our CRS. Agreement between diagnostic tests, as measured by the kappa coefficient, was only fair between BST on one hand, and RBT (0.266) and CFT (0.281) on the other, Table 4.24. This may be explained by the fact that they measure different types of the immune response, cell-mediated immunity for BST and humoral antibodies for serology. The kappa statistic

between BST, and the FPA (0.416) and the iELISA (0.447) was a moderate (Viera & Garret 2005). As the 63 *B. abortus* positive control animals were determined from positive response to serology, it follows the relative BST sensitivity in this study may be an underestimate of the true sensitivity, especially when the high specificity of 99.18% and the relatively higher reactor rate (in comparison to the RBT and CFT) in *B. abortus*-infected herds under field conditions are considered. The high BST specificity observed in this study implies a similarly high positive predictive value for this assay (McKenna & Dohoo 2006). In these circumstances, it is highly likely the 65.38% discrepancy between the BST and the CRS is a reflection of the gold standard misses (Alonzo & Pepe 1999), and therefore represents truly infected individuals. This observation may be explained by a gold standard which was biased towards animals that had mounted a strong humoral, but not necessarily a strong CMI response to infection. In other words, the CRS employed in this study did not eliminate spectrum bias. Irrefutable evidence Vis a Vis the status of the animals which tested positive on BST but negative on serology might only have been obtained through slaughter and bacterial isolation, which was beyond the budget of this study. Alternatively, the animals which tested positive on BST but negative by the CRS could be followed up with serology to establish the number which subsequently maintained persistent sero-positivity attributable to BST-induced anamnestic response (provided they were isolated from the infected herd immediately after the second BST reading prevent further transmission). These findings are consistent with those of others (Kolar 1984; Fensterbank 1977) who found the BST to detect infection early (before circulating antibodies), as well as in the chronic latent stages when serologic tests are often negative. That iELISA is reputed to detect early brucellosis infections (Saegerman *et al* 2004), may partly explain the better agreement between BST and iELISA. The study also noted that 36 animals were sero-positive, but negative on BST. Of these, 15 animals showed CFT reactions above 344 ICFTU (seven of these were 784 ICFU). This may be due to anergy as suggested by other studies (Cunningham *et al* 1980). Of the remainder, eight had CFT titres of 60 ICFTU and below.

Although the BST specificity was quite high (99.18%), the low relative sensitivity (42.86%) precludes the use of the BST as the sole confirmatory test at individual level. However, it may be of good use when employed in parallel with serology, when both the cell-mediated immunity and the humoral antibody responses to *B. abortus* are assessed (Pouillot *et al* 1997). The data from this study support this view (Table 4.24) as 65.38%

of BST-positive animals were sero-negative despite the high specificity of the BST. It is with the removal of these *B. abortus*-infected animals that are latent to serology that the advantage of parallel testing (BST and serology) would be realized. Bercovich and others (1992) observed that brucellosis-infected herds were cleared of brucellosis when both seropositive and BST positive cattle were removed. The highest parallel-assay brucellosis positive reactor rate among brucellosis-infected herds was found when the BST was used in parallel with the iELISA. Since both assays exhibited high discriminatory power in this trial, they may be combined in parallel (the 'or' rule) to increase the sensitivity of the diagnostic algorithm (Weinstein, Obuchowski & Lieber 2005), and allow for early identification and consequent elimination of infected animals. The parallel use approach offers the potential to cleanse an unvaccinated *B. abortus*-infected herd fast as both the serological reactors as well as the latent carriers are identified early. The same strategy offers potential application with calves from vaccinated herds, prior to routine vaccination for brucellosis. The BST could still potentially be used to screen unvaccinated animals in an infected herd or suspect infected herd, followed by serological testing two to three weeks later to take advantage of the anamnestic sensitizing effect of the brucellin to identify otherwise latent carriers, with the resultant increase in the overall sensitivity of the testing protocol. A similar strategy could be employed in the assessment of brucellosis vaccination efficacy or herd immunity in *B. abortus*-free herds by initially screening the herd with the BST. The initial screen will determine the vaccination status (alternatively if the herd has some immunity) as well as sensitise any animals that may not have been identified by the BST but were otherwise effectively vaccinated. The vaccination efficacy or level of herd immunity is subsequently confirmed at individual level by routine brucellosis serology in the case of S19 vaccine or alternatively the RB51-CFT in the case of the RB51 vaccine, two to three weeks after the initial BST.

Before a new test can replace an established one, the new assay must either out-perform the old one, or at least be as good in addition possessing some advantages over the old one (Nielsen 2005). In this study we investigated performance of the FPA under South African conditions and compared it to the assays in current use to see if the FPA could offer any advantages. We used the same panel of *B. abortus*-free and infected control groups as well as the *B. abortus*-free, RB51-vaccinated cows to assess the FPA specificity, sensitivity as well as specificity in RB51-vaccinated cows, as was used for the BST.

The data in Tables 4.19 and 4.20, as well as Figure 4.4 show that 98.85% of the *B. abortus*-free (negative control group) calves as well as 6.35% of the *B. abortus*-infected (positive control group) calves registered brucellosis FPA reactivity below 20 mP. When the FPA readings from the brucellosis-free group as well as the brucellosis-infected control group were analysed with the Receiver Operator Characteristic (ROC) curve (Figure 4.5), the observed area under the ROC curve (an indication of the test's accuracy) was 0.9875. These data imply the FPA is able to distinguish brucellosis-free from brucellosis infected animals 98.75% of the time. When the cut-off value of 20 mP was selected, it translated to a test specificity of 98.85% (95% CI: 97.64% - 99.53%) and a relative sensitivity of 93.65% (95% CI: 84.52% - 98.20%). The FPA specificity observed in this study fell within the range of 94.9% - 100% observed in other studies in the Americas (Nielsen *et al* 1998). Another study (McGiven *et al* 2003) observed a specificity of 99.1% in bovine. The sensitivity observed in our study also fell within the similar range of 87.5% - 100% observed in sera from culture positive animals (Nielsen *et al* 1998). In their European study, McGiven and others observed a specificity of 96.6%. Among brucellosis-free, RB51-vaccinated cows, our study observed a FPA specificity of 99.52% (98.29% - 99.93%), Table 4.22 and Figure 4.6. In addition the data showed the FPA was able to distinguish RB51-vaccinated individual animals from *B. abortus* infected animals, implying that the performance of the test was not adversely affected by RB51 vaccination (Figure 4.6). The brucellosis positivity rate in *B. abortus*-infected herds was 10.73% (Table 4.23). This positive reactivity rate was higher than the RBT (6.27%), the CFT (6.86%) and the BST (9.25%), but lower than the iELISA (14.91%). When compared to other serological tests (RBT, CFT and iELISA) there was substantial agreement among all of them (kappa coefficient > 0.661), Table 4.25. When the performance of the FPA was compared with the other assays (Table 4.26), the FPA (192.50) showed the second highest performance index after the iELISA (196.83), and was better than the RBT (180.79) and the CFT (188.89). The FPA was significantly superior in sensitivity to the RBT (P= 0.0117), was not significantly different from the CFT (P = 0.3593), but was inferior to the iELISA (P = 0.129). It was significantly inferior in specificity to the three assays (P = 0.0156), Table 4.27.

Our study has shown that the FPA compares favourably with the currently used tests. Owing to its superior sensitivity, the FPA has the potential to replace the RBT as the brucellosis screening test in South Africa. Although the iELISA offers superior

sensitivity to the FPA, it is more expensive and a little more complex as it has multiple washing steps and takes longer to perform. The FPA offers additional advantages of being an objective test which is free from the variability due to human error when reading agglutination tests such as the RBTor SAT. It is free from the pro-zoning disadvantages that can affect the RBT. Like the RBT, it is a homogenous assay that is quick and easy to perform, and is relatively inexpensive once the initial capital outlay for the equipment is done. It is not affected by haemolysis in serum, nor is it affected by anti-complementary activities of poor quality serum that affect the complement fixation test. This study did not evaluate the performance of the FPA in S19-vaccinated cattle, under South African conditions.

The foregone discussion provided evidence that the BST can add value to the currently used brucellosis diagnostic panel in South Africa. It has demonstrated the BST's potential to detect early latent brucellosis infections in conjunction with the currently used serology. This study brought forth the BST's potential role in the assessment of brucellosis herd immunity (or alternatively a tool with which to assess brucellosis vaccine efficacy). This study has demonstrated the potential role of the FPA in improving South Africa's brucellosis diagnostic capacity. The improved diagnostic capacity will in turn help reduce the social and economic impact of brucellosis from the current annual level of 5 000 human cases and economic losses of R300 million, to more sustainable levels.

Chapter 6
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Appendices

Brucellin skin testing procedure

Figure 1 Animal identification by means of an ear-tag

Figure 2 Blood collection by veni-puncture of the median caudal vein (tail vein)

Figure 3 Clipping of hair at injection site with pair of scissors prior to injecting brucellin

Figure 4 Measurement of normal skin thickness with a springmeter prior to injecting brucellin

Figure 5 Intradermal injection of brucellin with a tuberculin syringe

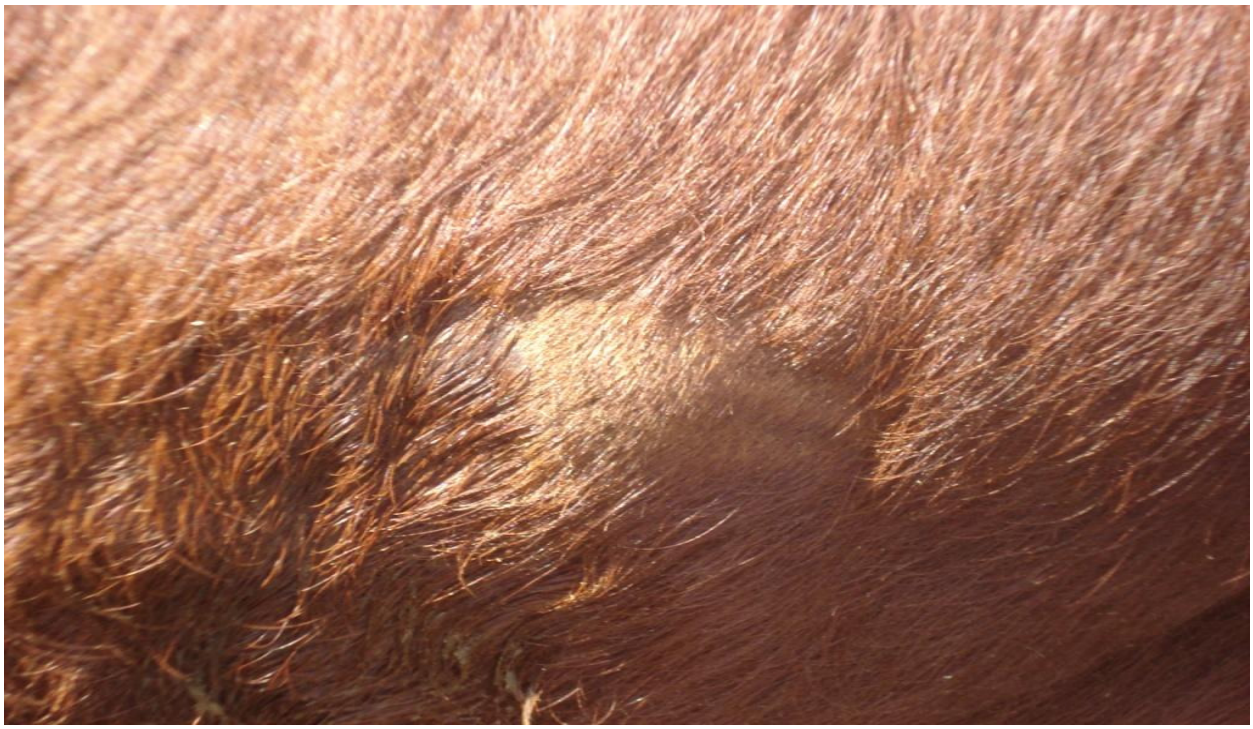

Figure 6 Positive BST response showing allergic skin thickening 72 hours post brucellin injection

Equipment used in the fluorescence polarisation assay

Figure 7 Rotating micro-titre plate shaker for the incubation of test sera and FPA reagents

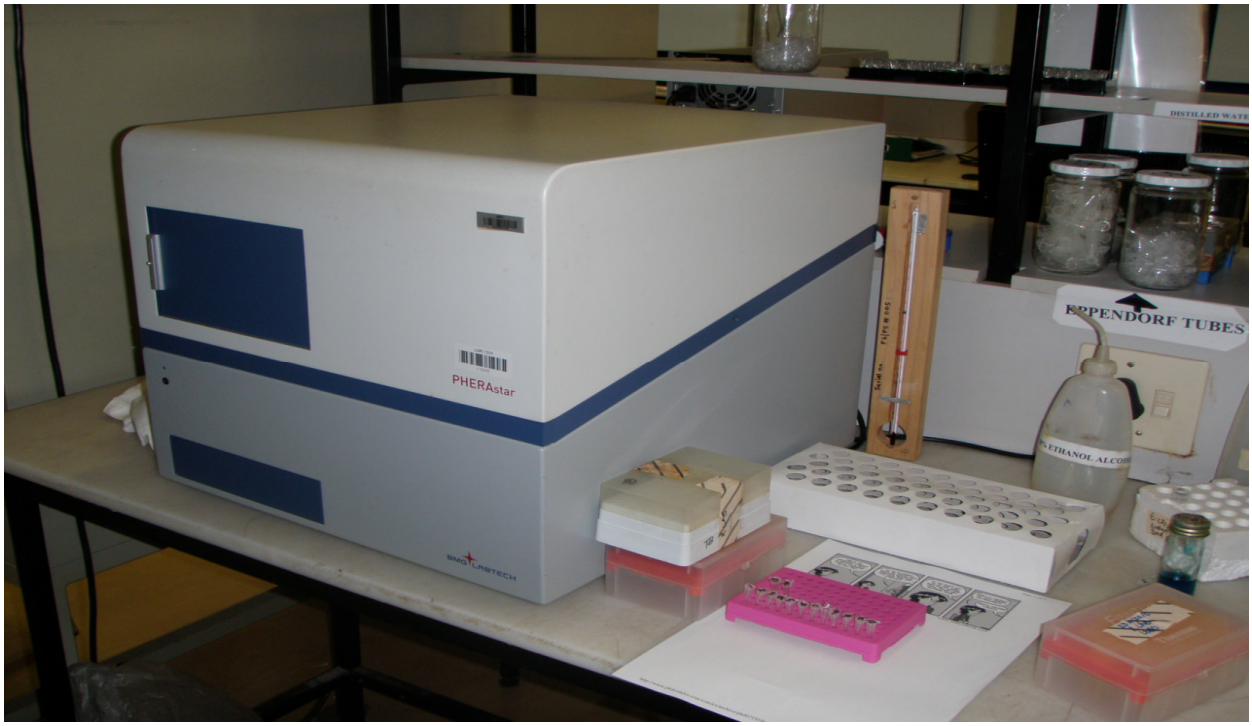

Figure 8 Pherastar™ FPA reader

Interpretation of the kappa statistic

Table 1 Interpretation of Kappa

Kappa	Agreement
< 0	Less than chance agreement
0.01-0.20	Slight agreement
0.21-0.40	Fair agreement
0.41-0.60	Moderate agreement
0.61-0.80	Substantial agreement
0.81-0.99	Almost perfect agreement

(Table 1 above was adapted from Viera & Garret 2005)