DISSERTATION

Validation of the Fluorescence Polarization Assay (FPA) for the diagnosis of Bovine Brucellosis

By

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DECLARATION

Name : **Banele Irene Skosana**

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Degree : **Master of Science in Agriculture**

Title of Dissertation : **Validation of the fluorescence polarization assay (FPA) for the diagnosis of bovine brucellosis**

I hereby certify that I am the sole author of this dissertation and that no part of this dissertation or the dissertation has been submitted for a degree to any other university or institute.

I declare that this is a true copy of my dissertation, including any final revisions, as approved by my dissertation's review committee.

SIGNATURE: Signed by Banele Irene Skosana **DATE:** 03 March 2021

DEDICATION

This dissertation is dedicated to the memory of my late sister Nomsa Sophie Skosana whom I promised to make proud by achieving my master's degree, to God who gave me the power of mind, strength, and mental wellbeing. To my family who motivated and encouraged me to finish this study your prayers got me where I am today.

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To my son Katlego, my pillar and source of strength, my bundle of joy, I thank you for being my number one motivator and my cheer leader.

ABSTRACT

Fluorescence polarization assay (FPA), a serological assay, was validated as an alternative test for the rapid and cost-effective diagnosis of bovine brucellosis, with the aim of improving the control of brucellosis in South Africa. The FPA is anticipated to distinguish between vaccinated and infected cattle, circumventing the challenge associated with the tests that are currently used. Positive cattle serum samples (*n* =420) confirmed by Complement Fixation Test were tested in conjunction with serum samples (*n* = 446) from non-infected cattle initially tested on Rose Bengal Test, CFT and compared with FPA. The optimum cut-off value that offers the highest diagnostic sensitivity (Dsn) and diagnostic specificity (Dsp) was determined as 87 mP with the use of ROC analysis. The Dsn and Dsp of FPA using this cut-off value was calculated at 99.09% - 100% and 68.09%- 76.61% respectively with a 95% confidence interval (cl). The area under curve (AUC) was calculated at 0.9842 with a 95% standard error (S.E) of 0.005532 with positive and negative likelihood ratio (+LR) (-LR) at 3.643 and 1.002, respectively. The FPA was found to be as effective as CFT and should be considered because of its accuracy and other advantages such as speed, high throughput and the objectivity of the interpretation of results that can be obtained electronically by the (PHERAstar) machine. The test should be included in routine serological diagnosis for brucellosis.

***Key words**: B. abortus, FPA, Cattle, Validation, Serology, RBT, CFT, sensitivity, specificity, reproducibility*

ISIFENGQO

I-Fluorescence polarization assay (i-FPA) ukuhlolwa kwe-serological okuqinisekiswe njengenye indlela yokuhlola ukuxilongwa okusheshayo nengabizi kwe-bovine brucellosis, okuzokwenza ngcono ukulawulwa kwe-brucellosis eNingizimu Afrika. Ngaphezu kwalokho, i-FPA kulindeleke ukuthi yehlukanise phakathi kwezinkomo ezigonyiwe nezithelelekile futhi lokhu kuzonciphisa inselelo ehambisana nokuhlolwa esetshenziswa njengamanje. Amasampula amahle avumayo we-serum ezinkomo (n
= 420) aqinisekiswa yi-CFT ahlolwe ngokuhlangana namasampula e-serum (n = 446) avela ezinkomeni ezingathelelekile ezahlolwa kuqala ku-RBT, CFT futhi kuqhathaniswa ne-FPA. Inani elinqunyiwe elikhulu elinikezela ukuzwela okuphezulu kokuxilonga (i-Dsn) kanye nokucaciswa kokuxilongwa (i-Dsp) kunqunywe njenge-87 mP kusetshenziswa ukuhlaziywa kwe-ROC. I-Dsn ne-Dsp ye-FPA esebenzisa leli nani elisikiwe libalwe ngama-99.09% - 100% no-68.09% - 76.61% ngokulandelana kwesikhathi sokuzethemba esingu-95% (cl). Indawo engaphansi kwe-Curve noma ijika thizeni (i-AUC) ibalwe ku-0.9842 enephutha elingu-95% elijwayelekile (SE) lika- 0.005532 elinezilinganiso ezinhle nezimbi ze-likehood (+ LR) (-LR) ngo-3.643 no- 1.002, ngokulandelana. I-FPA isebenza njenge-CFT futhi kufanele ibhekwe ngenxa yokunemba eneqiniso kwayo nezinye izinzuzo ezifana nejubane lokuthola imiphumela kanye nenhloso yokuchazwa kwemiphumela engatholakala ngomshini wekhomphuyitha (PHERAstar), i-FPA kufanele ifakwe ekuhlolweni okuvamile ngokujwayelekile kwe-serological ye-brucellosis.

Amagama abalulekile: *i-B. Abortus, i-FPA, izinkomo, ukuqinisekiswa, i-Serology, i- RBT, i-CFT, inzwelo thizeni, ukucaciswa, ukukhiqiza kabusha*

LIST OF ABBREVIATIONS

- ARC- Agricultural Research Council
- AUC - Area under curve
- AVPL – Allerton Veterinary Provincial Laboratory
- *B- Brucella*
- BCV- *Brucella*-containing vacuole
- cELISA- competitive Enzyme-Linked ImmunoSorbent Assay
- CFT- Complement fixation test
- DSe- Diagnosis sensitivity
- DSp- Diagnosis specificity
- ELISA- Enzyme-Linked ImmunoSorbent Assay
- FAO- Food and Agriculture Organization
- FPA- Fluorescence polarization assay
- iELISA- indirect Enzyme-Linked ImmunoSorbent Assay
- µl- Microliter
- MU- Measurement uncertainty
- mP- Milli-Polarization units
- OIE- Office International des Epizooties
- O-PS- Oligo-Polysaccharide.
- OVR-Onderstepoort Veterinary Research
- RBPT- Rose Bengal plate agglutination test
- RBT- Rose Bengal test
- RER- Replication endoplasmic reticulum
- ROC- Receiver operating characteristic
- SAT- Serum agglutination test

- S.E - Standard error
- SE- Sensitivity
- SP- Specificity
- *Spp*- Species
- SPSS – Statistical package for the social sciences
- SRBC- Sheep red blood cells
- STAT – Standard tube agglutination test
- WHO- World Health Organization

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CHAPTER 1: INTRODUCTION

1.1 Background

Bovine brucellosis is a zoonotic bacterial disease caused by *Brucella abortus*, which is a gram-negative, non-mobile, non-spore-forming, and rod-shaped bacterium (Mahmood *et al*., 2015). It is a non-capsulated (Jaff, 2016) and slow growing bacterium (Hendaus *et al*., 2015) with oxidative metabolic patterns (Gorofolo *et al*., 2017). It is an intra-cellular (occurs inside body cells) organism that can cause an extended and sometimes life-long, chronic disease in both human and animal (Preez and Malan, 2015; Chisi *et al*., 2017).

Brucella infection spreads rapidly in a cattle herd that is not vaccinated and causes abortions in pregnant cows during the late stage of pregnancy. Infected cows will abort once but the following pregnancy will be normal. This usually happens in a herd where *brucella* is endemic (Preez and Malan, 2015). Some cattle can withstand infection and a small percentage of infected cows recover spontaneously (Preez and Malan, 2015). Brucellosis is one of the most important re-emerging zoonotic diseases in many countries, in which infected animals are the source of infection (Mohammed, 2015). In South Africa, on 16 November 2020, the KwaZulu-Natal Agriculture Department confirmed an outbreak of brucellosis in the northern part of KwaZulu-Natal. There were more than 400 confirmed cases of brucellosis in cattle. During a television interview on eNews Channel Africa (eNCA), some farmers in rural areas confirmed a lack of knowledge about the disease and requested government intervention.

Control and prevention of brucellosis depends mainly on rapid and sensitive diagnostic tests (Trangadia *et al*., 2012) that allow the implementation of control measures such as isolation and “test and slaughter” policy. The conventional serological tests for the diagnosis of Brucellosis are subjected to certain limitations, for instance Rose Bengal Test has lower specificity and may result in strongly positive sera testing negative (OIE, 2009). Complement Fixation Test cannot distinguish between cattle that are vaccinated with *B. abortus* strain 19 from those exposed to cross-reacting microorganisms from cattle infected with *B. abortus* (Gall *et al*., 2000). Many serological tests are available for the diagnosis of Brucellosis. Among these, Rose Bengal plate test (RBPT), standard tube

agglutination test (STAT) and complement fixation test (CFT) have been used extensively to diagnose Brucellosis in animals (Trangadia *et al*., 2012).

1.1.1 History of Brucellosis

Brucellosis is diagnosed by isolation and identification of the causative organism. Bruce and a co-worker first reported this in 1887 when they isolated *Brucella melitensis* from the spleen of a soldier who died from acute brucellosis. This species is highly pathogenic for humans. The disease originated from infected goats (Nielsen, 2002). A study reported lytic lesions of the lumbar vertebrae in "southern Ape of Africa" (Australopithecus africanus) suggestive of brucellosis. The suspected source of infection could be the consumption of infected tissues from wild animals (Rossetti *et al*., 2017; D' Anastasios *et al*., 2009).

At the beginning of the 20th century, Benhard Bang identified *Brucella abortus* as the cause of abortions in cattle. It had been the main etiologic factor of brucellosis in animals and humans, also known as Bang's disease, and was isolated from aborting cattle for many years (Gorofolo *et al*., 2017). In 1914, Taum described *Brucella suis* causing disease mainly in swine but also in humans (Nielsen, 2002). *Brucella canis* was isolated from dogs and suspected to cause illness in humans. Carmichael who isolated the bacillus from the placentas, fetuses, and vaginal discharges of bitches that aborted first described it in 1966. The disease was diagnosed in Beagle dogs in United States. *Brucella neotomae* was isolated from rats in the United States while *Brucella ovis* infects sheep and goats. *Brucella marina* and *Brucella ceti* were found in sea mammals namely whales and seals in the Atlantic Ocean. *Brucella marina* and *Brucella pinnipedialis* were found in sea mammals. *Brucella microti* was isolated from the common vole (*Microtus arvalis*) in the Czech Republic, from the soil in the sea area and from mandibular lymph nodes of wild red foxes. Lastly, *Brucella inopinata* was isolated from the breast implant wound of a woman with clinical signs of brucellosis (Galinska and Zagorski, 2013).

Each of these Gram-negative bacteria infects a different group of land and aquatic mammals, including swine, cattle, goats, sheep, dogs, dolphins, whales, seals, and desert woodrats (de Figueiredo *et al*., 2015).

1.2 Problem statement

Brucellosis is one of the most neglected zoonotic bacterial diseases that can cause public health threats involving domestic animals, wild animals, and humans (Aworh *et al*., 2017). The economic implications of brucellosis in a cattle herd is huge worldwide, affecting mostly breeding animals with associated losses in productivity due to abortions, stillbirths, weak offspring, extended inter-calving period, unexpected deaths, and reduced milk production. The disease also causes major impediments for trade and export of livestock (Tasiame *et al*., 2016). The zoonotic aspect of this disease and the confusing clinical signs effects in humans raise a need to continuously control brucellosis (Dorneles *et al*., 2015). Currently used tests such as Rose Bengal Test (RBT), Serum Agglutination Test (SAT) and Complement Fixation Test (CFT) have shortcomings due to low sensitivity, false-negatives, and non-specific reactions, respectively.

The RBT has some disadvantages of low sensitivity mostly in chronic cases, relatively low specificity in endemic areas and prozones making strongly positive sera test negative (Geresu and Kassa, 2016). A false-negative reaction arises due to the prozones phenomenon (Trangadia *et al*., 2012). The SAT is unacceptable for international trade due to non-specific reactions (Delegates, 2016; OIE, 2004). The CFT has limitations with haemolysed serum samples, the anti-complement activity of some sera and the occurrence of the prozones phenomena false-negatives resulting from high antibody titre that interferes with the formation of the antigen-antibody complex (Geresu and Kassa, 2016). The CFT is extremely challenging, as it requires trained and qualified personnel to perform the test and serial dilutions are required for titrations prior testing of samples (OIE, 2004)

Handling aborted materials or attending to retained placentas or dystocia without protective gear is a common practice among most field veterinary assistants, abattoir workers, and in many rural pastoral settings. This may suggest that animal health workers and rural communities are also at great risk of contracting the disease if the disease is present in domestic animals (Luelseged *et al*., 2018).

1.3 Hypothesis

- The sensitivity and specificity of FPA are not lower than those of RBT and CFT for the diagnosis of brucellosis in cattle.
- The repeatability of FPA will not be more than 2 SD.
- The reproducibility of FPA will not be more than 2 SD.
- The Pearson correlation coefficient between FPA and standard tests such as RBT and CFT will not be more than 1.

1.4 Aim and Research objectives

The study aimed to validate the Fluorescence polarization assay (FPA) as an additional test for the rapid and cost cost-effective diagnosis of Brucellosis, which will improve the control of Brucellosis in South Africa.

The specific objectives are:

- To evaluate the specificity and sensitivity of FPA using cattle field sera.
- To determine the reproducibility of FPA
- To determine the repeatability of FPA
- To evaluate the correlation coefficient between FPA and standard tests such as RBT and CFT.

1.5 Significance and motivation of the study

Brucellosis is a zoonotic and controlled disease. The validation of FPA will result in improved diagnosis of affected cattle in South Africa. Validation is an ongoing activity that is crucial to all accredited laboratories. After the study is completed, the Serology laboratory will continue with validation especially if the test is approved to be implemented in South Africa, to gather more validation data prior to roll out in other laboratories. This test will contribute significantly to the improvement and efficiency of the eradication program due to its superior performance compared with the tests currently in use.

It will be helpful to laboratories seeking appropriate tests that do not require washing steps, longer incubation periods and are not time-consuming. The FPA may be preferred over other tests because of its characteristic cut-off adjustment useful in different epidemiological situations and its potential application in the field. Besides, the FPA is anticipated to differentiate vaccinated from diseased cattle and this will circumvent the challenge associated with the tests currently in use. The test can be automated and used to improve quality assurance processes, hence making it suitable for eradication programs, national serological surveys, and laboratories handling large volumes of brucellosis samples, as it is the case in South African laboratories (Kangumba, 2015). The FPA test has been used on stored whole blood samples and on haemolysed samples (Nielsen *et al*., 2001). It has a higher ability to detect low antibody titers unlike RBT (Muma *et al*., 2009). Adding to the abovementioned advantages, the diagnostic specificity and sensitivity of FPA are higher when compared to other tests used in different species (Nielsen *et al*., 2000).

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CHAPTER 2: LITERATURE REVIEW

2.1 Brucellosis in humans

2.1.1 Historical contexts

Human brucellosis was first confirmed in Iraq in 1938. Indeed, in Iraqi Kurdistan, brucellosis in humans is a widespread disease that remains a challenging health problem (Jaff, 2016). It has been reported in Southeast Asia, particularly Indonesia, Malaysia, Singapore and Myanmar, and Thailand. It was recognized as an emerging zoonotic disease in Southeast Asia and is frequently caused by *Brucella melitensis* (Zamri-Saad and Kamarudin, 2016).

Brucellosis is listed amongst the seven neglected zoonotic diseases by the World Health Organization (Ayoola *et al*., 2017; Aworh *et al*., 2017; Franc *et al*., 2018) considering the impact it has on both human and animal health (Njeru *et al*., 2016). Brucellosis is under-reported, often misdiagnosed even in higher-income countries, and is considered one of the main neglected zoonotic diseases (Norman *et al*., 2016).

Human brucellosis is endemic to certain regions and has been reported worldwide, with more than 500 000 cases reported annually (Mahmood *et al*., 2015; Aloufi *et al*., 2016; Jezi *et al*., 2019). Research evidence shows that brucellosis is more common in countries with poor standardized animal and public health programs and interventions (Jaff, 2016). Major endemic areas include countries of the Mediterranean basin, Middle East, Including Iraq and Iraqi Kurdistan, the Indian subcontinent, and parts of Mexico and Central and South America (Jaff, 2016).

It is estimated that the number of human brucellosis cases may be up to 26 times higher than the 500,000 cases reported annually (Jaff, 2016; Avijgan *et al*., 2019). The disease occurs naturally in the populations of developing areas of the world where numbers are thought to be severely underestimated. The developing countries

that are affected include Ethiopia, Chad, Tanzania, Nigeria, Uganda, Kenya, Zimbabwe, and Somalia where brucellosis in humans is related to infection of domestic cattle, camels, goats, and sheep (Yang *et al*., 2017).

Brucellosis is a common zoonotic infection in the Middle East, South and Central Asia, South and Central America, North and East Africa as well as in the Mediterranean countries of Europe (Hendaus *et al*., 2015). Eastern Mediterranean countries have shown an incidence of more than 100 cases per 100,000 persons/year, with the highest figures in Syria, Lebanon, Iraq, Saudi Arabia, Sudan, and Oman (Garcell *et al*., 2016). The disease continues to progress at high rates in Latin America, South East Asia, the Middle East, and the Persian Gulf (Banai *et al*., 2018).

Brucellosis is an overlooked infection of widespread geographic distribution associated with traveling and importing contaminated goods from endemic areas (Brehin *et al*., 2016). The entire human population is susceptible to contracting human brucellosis and it was stated that in prevalent areas, a quarter of the patients are younger than 14 years of age, with a rate of childhood infection of 11% to 56% (Bosilkovski *et al*., 2015). In France, a case of brucellosis in a 16-months-old patient hospitalized for an acute febrile illness was discovered; the child had eaten a cake made from unpasteurized goat's milk that was imported from Algeria (Brehin *et al*., 2016). A 27-year-old man from Western Cape, South Africa was diagnosed with *B. melitensis* infection after the laboratory confirmed the isolation of Gram-negative bacilli in 2014. After 3 days of incubation, an epidemiological investigation led to two other people being diagnosed with brucellosis after eating meat from a goat that had recently given birth on a farm (Wojno *et al*., 2016).

Shimol reported an outbreak of brucellosis acquired through camel milk affecting 15 members of an extended family in Israel (Shimol *et al*., 2012). An outbreak related to the consumption of raw cheese was described in Lebanon, as it was a cluster of six cases of breast brucellosis in Taif (Saudi Arabia) (Garcell *et al*., 2016).

2.1.2 Transmission of brucellosis to humans: consumption of contaminated animal products

Brucella spp is excreted in milk and may be present in the offal and meat of infected animals (de Glanville *et al*., 2017). Drinking unpasteurized milk and its products

(Aworh *et al*., 2017; Mahmood *et al*., 2015) and consuming untreated milk products leads to approximately a half-million cases of brucellosis in humans around the world every year (Bano and Lone, 2015). A number of publications have reported acquired infections through the consumption of unpasteurized goat's milk, camel milk, raw cheese and infected goat meat (Brehin *et al*., 2016; Wojno *et al*., 2016; Garcell *et al*., 2016 and Shimol *et al*., 2012).

In humans, the disease is often caused by *Brucella melitensis*, which is the species associated with more severe disease (Wojno *et al*., 2016) as the most pathogenic species, followed by *Brucella suis*, while *Brucella abortus* is considered the mildest type of brucellosis (Galinska and Zagorski, 2013; Norman *et al*., 2016 and Wojno *et al*., 2016). Brucellosis may develop after a prolonged incubation period of up to several months, and manifestations are versatile often making a diagnosis challenging (Norman *et al*., 2016). Since there is no human vaccine, livestock vaccination is an effective method of preventing human infections (Jezi *et al*., 2019) and the eradication of brucellosis is a major public health challenge in many countries (Jaff, 2016).

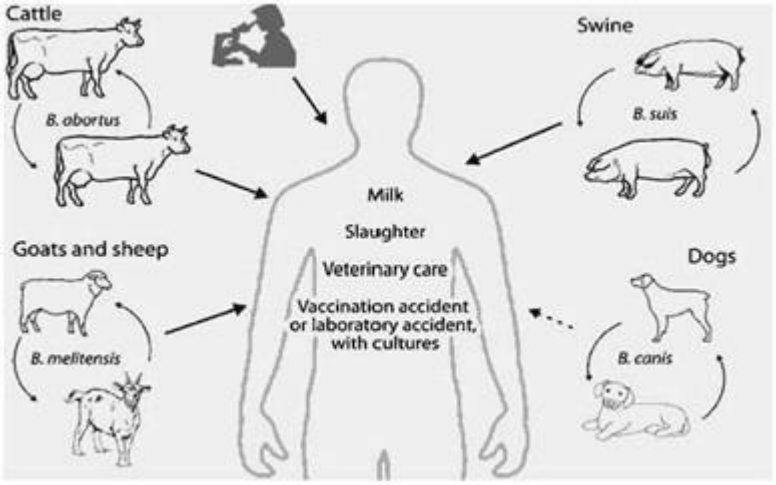

Figure 2.1: Transmission of *Brucella* to humans (Luelseged *et al*., 2018)

2.1.3 Reservoirs of *Brucella*

The natural reservoirs of *Brucella* are domestic animals, primarily cattle, sheep, goats as well as wild animals (Galinska and Zagorski, 2013). After penetration into the body, the bacilli proliferate in the lymphatic system, mainly in the lymph nodes, and subsequently break through the protective barrier to penetrate various organs. (Galinska and Zagorski, 2013). Once in the lymphatic system, the bacteria first multiply in the regional lymph nodes and is later carried by the lymph and blood to different organs (Bano and Lone, 2015). *Brucellae* quickly translocate across the mucosal epithelium layer *in vivo* and are endocytosed by mucosal macrophages and dendritic cells (de Figueiredo *et al*., 2015). The type of Brucella spp. to which an individual is exposed determines the risk of the disease and its severity. This will be influenced by the species of host animal acting as a source of infection (Dr Corbel, 2006).

2.1.4 Transmission of brucellosis to humans: via direct contamination

Animal to human transmission is caused by direct contamination with infected animals, placenta, and aborted fetuses (Jaff, 2016; Zamri-Saad and Kamarudin, 2016) as well as by direct contact with the infected animal through skin abrasions or mucous membrane (Njeru *et al*., 2016). The transmission can also be caused by direct contact with secretions from infected animals, inhalation of contaminated animal feces, and infected animal products (Alsaif *et al*., 2018; Ayoola *et al*., 2017). People working in close contact with infected materials like abattoir workers, veterinarians, and laboratory workers are easily infected through skin abrasion (Mantur and Amarnath, 2008). Tuon documented human-to-human transmission of brucellosis via blood transfusion, bone marrow transplantation, sex, transplacental or perinatal exposure, and breast milk in humans (Tuon *et al*., 2017). Contact with contaminated substances of animal origin as well as inhalation of aerosolized organisms are primary causes of brucellosis (Ayoola *et al*., 2016).

2.1.5 Virulence factors of *Brucella*

The *Brucella* bacteria has several virulence factors for invasion and many mechanisms for evading cellular immunity, which enables them to survive the human immune responses and replicate within intracellular niches (Amjadi *et al*., 2019). Recently a urease enzyme was identified as an important determinant of virulence as this enzyme protects *Brucella* bacteria in their passage through the stomach by the oral route, which is the major way of infection in human brucellosis (Bano and Lone, 2015).

2.1.6 Symptoms of human Brucellosis

Clinical signs and symptoms of brucellosis are not specific, and the diagnosis mostly relies on incorporating clinical, epidemiological, and serologic findings. Serologic tests play a fundamental role in the diagnosis of this disease (Avijgan *et al*., 2019). The most frequent symptoms of human brucellosis are fever, chills or shaking rigors, malaise, generalized aches, pain all over the body, joint and low back pain, headaches, anorexia, easy tiredness, nausea, loss of appetite, and general weakness (Jezi *et al*., 2019; Khazaei *et al*., 2016). The incubation period of brucellosis normally is 1 to 3 weeks, but it can be several months before any sign of infection is detected (Roushan and Ebrahimpour, 2015). Symptoms observed can include recurrent fevers, arthritis, swelling of the testicles, enlargement of the heart, neurologic symptoms (in up to 5% of all cases), chronic fatigue, depression, and hepatomegaly and/or splenomegaly (Jaff, 2016). Nonspecific influenza-like symptoms observed in humans include pyrexia, diaphoresis, fatigue, anorexia, myalgia, and arthralgia (de Figueiredo *et al*., 2015). The great variety of clinical shreds of evidence of human brucellosis makes the diagnosis relatively difficult. This absence of characteristic symptoms makes it challenging to differentiate brucellosis from numerous febrile diseases that commonly appear in the same regions making laboratory testing very important for diagnosis (Zakaria, 2018). Besides, in the absence of human brucellosis vaccine, the most effective strategy to control human brucellosis is to control brucellosis in animal populations. In recent decades, the development of an effective vaccine for brucellosis control/eradication has been a challenge for researchers (Jezi *et al*., 2019).

2.1.7 Treatment of human Brucellosis

Treatment of human brucellosis is frequently not successful due to reoccurrence. Double or triple treatments with an aminoglycoside (doxycycline- streptomycin/gentamicin or doxycycline-rifampicin-streptomycin/gentamicin) suggestively decrease treatment letdown and setbacks proportion, as indicated in table 1 and table 2. They are presently preferred as first-line treatment procedures. The period of treatment is a minimum of six weeks for doxycycline, and rifampicin up to two weeks for aminoglycoside therapy (daily intramuscular injections), see Tables 2.1 and 2.2 for details. Patients require prolonged follow-up to monitor further complications or relapses (Frean *et al*., 2018).

The patients are treated with combination of either two or three antimicrobials. When used in combination, tetracycline, doxycycline, trimethoprim/sulfamethoxazole, and rifampin are administered for at least 45 days. Streptomycin is used for the first 14-30 days, and gentamicin for the first 7-10days. In patients that manifested neurobrucellosis, myocarditis, or therapeutic failure, treatment duration should be 60-180 days (Bosilkovski *et al*., 2015)

The treatment course of brucellosis in children is longer and is argued that rare treatment failure is high among children. Children older than 8 years are treated with doxycycline for 45 days to 8 weeks plus gentamicin for 5 days or 7 days respectively or doxycycline for 45 days and streptomycin for 14 days (Khazaei *et al*., 2016; Roushan and Ebrahimpour, 2015). Treatment of complications such as spondylitis, osteomyelitis, neurobrucellosis and *Brucella* endocarditis may require prolonged therapy for at least 8 weeks (Bano and Lone, 2015).

Table 2. 1: Treatment regimens for Brucellosis (Frean *et al*., 2018).

Form of brucellosis infection	Recommended antibiotic regimen	Duration	Administration and dosage
Uncomplicated adults	Doxycycline	6 weeks	Oral doxycycline, 1000-2000mg/day
	Plus, streptomycin or gentamicin (preferred regimen)	1-2 weeks	Intramuscular gentamicin 5mg/kg. day
	Or doxycycline plus rifampicin	6 weeks Intramuscular gentamicin 5mg/kg. day	
Uncomplicated children <8y	Cotrimoxazole plus rifampicin	4-6 weeks	
≥8y	Doxycycline plus rifampicin	4-6 weeks	
Focal - adults	Doxycycline	12 weeks	Oral doxycycline, 1000-2000mg/day
Spondylitis	Plus, streptomycin or gentamicin	2 weeks	Intramuscular gentamicin 5mg/kg. day
	Or doxycycline plus rifampicin	12 weeks	
	Or doxycycline plus ciprofloxacin	12 weeks	
Neurobrucellosis	Doxycycline plus rifampicin plus (ceftriaxone OR Cotrimoxazole)	Prolonged, until CSF normalises.	
Endocarditis	Doxycycline plus rifampicin plus streptomycin or gentamicin Surgery if indicated	6 weeks to 6 months, depending on clinical response	Intramuscular gentamicin 5mg/kg. day
Focal – children <8y	Cotrimoxazole	6 weeks at least	
	Plus, streptomycin or gentamicin	2 weeks	
≥8y	Doxycycline	6 weeks at least	Oral doxycycline 10-200mg/day

	Plus, streptomycin or gentamicin	2 weeks	Intramuscular streptomycin 20-25mg/kg
	Rifampicin can be added to either regimen	6 weeks at least	Oral rifampin 15-20 mg/kg. day
Brucellosis in pregnancy	Rifampicin with/without Cotrimoxazole (avoid in the last week before delivery, risk of kernicterus.	6 weeks	
Complex focal, relapsed, or refractory infection, or antibiotic toxicity/resistance	Consider adding quinolone or Cotrimoxazole as second line to doxycycline or rifampicin; triple therapy has better cure rates.		Oral Cotrimoxazole 10-20 mg/ (kg. day)/50-60mg/ (kg. day)

Table 2. 2: Antibiotic dosage for Brucellosis treatment (Frean *et al*., 2018).

Cotrimoxazole	Trimethoprim 10 mg/kg/d (max. 480 mg/d), sulfamethoxazole 50mg/ kg/d (max/ 2g/d)	In 2 doses/day
Doxycycline	2-4 mg/kg/d (max 200mg/d)	In 2 doses/day
Rifampicin	15-20 mg/kg/d) max 2g/d)	In 1 or 2 doses/day
Gentamicin	5 mg/kg/d	In 1 to 3 doses/day
Streptomycin	20-40 mg/kg/d (max 1/d)	In 2 doses/day
Ciprofloxacin	1 g/d	In 2 doses/day
Ofloxacin	400 mg/d	In 2 doses/day

2.2 Brucellosis in animals

2.2.1 Brucellosis in pigs

Brucella suis is a Gram-negative coccobacillus in the family Brucellaceae (class Alphaproteobacteria). *Brucella suis* biovars 1, 2, and 3 are the *Brucella species* usually found in pigs (The center for food science and public health, 2018). Brucellosis in swine is a disease caused by infection with intracellular bacteria from the genus *Brucella*, and a disease of economic importance with worldwide distribution (Olsen and Tatum, 2017) and has a high economic impact in pig farms (Dieste-Perez *et al*., 2015). Although the disease is associated with reproductive losses in swine worldwide, its primary importance is related to its zoonotic capability of causing clinical symptoms in humans (Olsen & Tatum, 2017).

Brucella suis is common among domesticated pigs in parts of Latin America and Asia. Control programs have eliminated or nearly eliminated this organism in some countries including several European nations, the U.S., Canada, and Australia. However, *B. suis* is still maintained in feral pigs or wild boar in many of these regions, resulting in sporadic transmission to domesticated swine. Infected pigs have occasionally been documented in some African nations, but surveillance is limited (The center for food science and public health, 2018). In Europe, *B. suis* biovar 2 has been isolated mainly from some immune-compromised patients, it is a major cause of abortions, infertility, and economic losses in pig farms (Dieste-Perez *et al*., 2015).

2.2.2 Transmission

Most domesticated pigs are thought to acquire *B. suis* when they ingest feed or water contaminated by birth products (e.g., fetus, placenta, fetal fluids) or vaginal discharges from an infected sow, or eat dead fetuses and fetal membranes. Pigs also shed this organism in milk, urine, and semen. Both symptomatic and asymptomatic boars can excrete bacteria, and venereal transmission is common in swine. Piglets get infected during nursing or in *utero* (The center for food science and public health, 2018). Although the disease is associated with reproductive losses in swine worldwide, its primary concern is related to its zoonotic capability of causing clinical illness in humans. Addressing brucellosis in livestock reservoirs is the most efficient and economical approach for reducing human infections (Kalleshamurthy *et al*., 2019).

2.2.3 Clinical Signs

In pigs, the most common clinical signs are reproductive losses, which may include abortions (The center for food science and public health, 2018), stillbirths, the birth of weak piglets (which may die early in life) from 100 to 110 days of gestation, and decreased litter size (Olsen and Tatum, 2017). Other signs include infertility, orchitis, inflammatory lesions in the joints, reproductive organs, paralysis of posterior limbs, and lameness (Kalleshamurthy *et al*., 2019). Metritis, infertility, and placental retention may be observed in infected sows (Olsen and Tatum, 2017).

2.2.4 Treatment, prevention, and control

Further studies need to be conducted since only one study reported that to clear *B. suis* one can use a combination of oxytetracycline and tildipirosin (The center for food science and public health, 2018). No commercial vaccine is currently used to control brucellosis in swine. The most effective approach although not always practical is the whole-herd depopulation (Olsen and Tatum, 2017). In an infected herd, the placenta, abortion products, and contaminated bedding should be removed and destroyed. Contaminated fomites should be disinfected. Programs to eradicate this organism from a country also include movement controls on infected herds, surveillance, and tracing of infected animals (The center for food science and public health, 2018).

2.3 Brucellosis in cattle

Brucella abortus, the etiological agent, is a Gram-negative non-motile and non- sporulating bacterium that forms coccobacilli with oxidative metabolism (Gorofolo *et al*., 2017). *Brucella abortus* is found worldwide in cattle, with rare exceptions such as Iceland. Eradication programs in several European nations, Canada, Australia, New Zealand, Japan, and Israel have eliminated this organism from domesticated animals. The U.S. is also *B. abortus* free. Sporadic cases may be reported in travelers and immigrants in *B. abortus*-free countries (The center for food security and public health, 2018).

Bovine brucellosis is a widespread zoonotic disease, endemic in some regions of the world namely Latin America, the Middle East, Africa, and Asia, and accountable for big economic losses due to animal and human health problems (Dorneles *et al*., 2015). The Italian

government has conducted a countrywide eradication program for cattle brucellosis since 1994. Animal cases are currently limited to seven regions of southern Italy, with the highest prevalence of infection in areas of Sicily, Calabria, and Apulia. This eradication program, along with strict regulations on cattle movements, has reduced the prevalence and geographic distribution of brucellosis in Italy (Gorofolo *et al*., 2017). Bovine brucellosis is endemic in all nine provinces of South Africa but particularly focused in the central and Highveld zones (Frean *et al*., 2018). Figure 2.2 shows the *B*. *abortus* outbreaks from January 2015 to May 2018 as reported to the Directorate Animal Health of the Department of Agriculture, Forestry and Fisheries (DAFF). A recent outbreak was reported in KwaZulu Natal in November 2020 (DAFF).

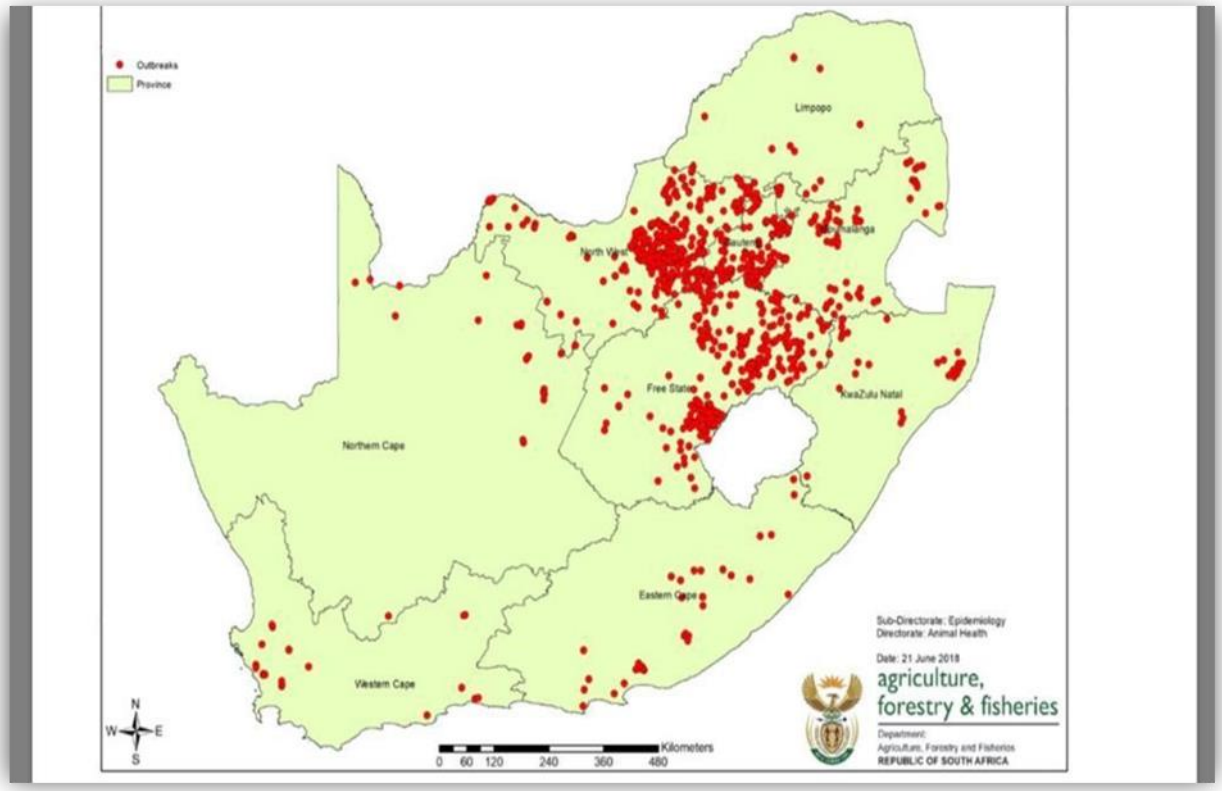

Figure 2. 2: Reported *Brucella abortus* outbreaks in animals from January 2015 to May 2018 across all nine provinces of South Africa. Image courtesy of the Epidemiology Sub-Directorate of the Directorate Animal Health, Department of Agriculture, Forestry, and Fisheries (DAFF) (Frean *et al*., 2018).

2.3.1 Transmission

Cattle often acquire *B. abortus* by contact with organisms in vaginal discharges and birth products (e.g., placenta, fetus, fetal fluids) from infected animals. Ingestion and transmission through mucous membranes are the major routes, but organisms can also enter the body via broken skin (The center for food security and public health, 2018). *Brucella abortus* is a significant zoonotic agent, and humans are typically infected by consumption of raw dairy products, exposure to aerosolized bacteria at slaughter, or during veterinary care and animal husbandry (Gorofolo *et al*., 2017).

2.3.2 Clinical Signs

Abortions (typically during the second half of gestation), stillbirths, and the birth of weak offspring are the predominant clinical signs in cattle. Weak calves may die soon after birth. Signs of illness do not usually accompany uncomplicated reproductive losses; however, retention of the placenta and secondary metritis are possible complications. Epididymitis, seminal vesiculitis, orchitis, or testicular abscesses are seen in bulls. Infertility or reduced fertility occurs occasionally in both sexes, due to metritis or orchitis/epididymitis. Arthritis and hygromas may be seen, especially in long-term infections (The center for food security and public health, 2018). In cattle, the disease presents primarily as reproductive disorders such as abortion, resulting in substantial economic losses to agriculture (Dorneles *et al*., 2015).

2.3.3 Treatment, prevention and control

There is no treatment for bovine brucellosis currently. Vaccination is an important inexpensive tool used in the control, management, and elimination of brucellosis, Nevertheless, vaccination alone is not sufficient for controlling and preventing brucellosis. It should be associated with test and slaughter policies, pasteurization of dairy products, surveillance, and hygiene procedures (Jezi *et al*., 2019). In general, the control programs of *Brucella* infection in cattle rely mainly on vaccination with live attenuated *B. abortus* strain 19. This provides good levels of protection against *B. abortus* in cattle (Gwida *et al*., 2016). The improvement of a successful vaccine to eliminate brucellosis is still a task for scientists for years ahead. Regardless of the huge improvements with the development of current vaccines namely *B. abortus* S19 and RB51 vaccines, the hunt for better vaccines continues

(Dorneles *et al*., 2015). Vaccination of animals is the most effective way of controlling and eradicating this zoonotic disease especially in high prevalence regions. Not consuming undercooked meat and unpasteurized dairy products and using protective gear when handling tissues are the best ways of preventing infection in humans (Jaff, 2016).

The slaughtering and proper disposal of seropositive animals to decrease the incidence of infection in healthy animals and effective vaccination and hygienic practices would reduce the spread of the disease in/from endemic regions (Khan and Zahoor, 2018).

2.4 Brucellosis in sheep and goats

In sheep and goats, brucellosis is caused by *Brucella melitensis*, a Gram- negative coccobacillus or short rod in the family Brucellaceae (class Alphaproteobacteria) (The center for food security and public health, 2018). This organism is a facultative intracellular bacterium mostly associated with reproductive failure (Fiasconaro *et al*., 2015; Saxena *et al*., 2018). *Brucella melitensis* contains three biovars (biovars 1, 2, and 3). All three biovars cause disease in small ruminants, but their geographic distribution varies (Saxena *et al*., 2018). *Brucella melitensis* is the most common and more virulent species causing human disease (Chota *et al*., 2016); with some estimates suggesting that, it is responsible for 70% of all infections. Most people acquire this organism by direct contact with infected animals or their tissues, or by the ingestion of contaminated dairy products. *Brucella melitensis* was eradicated in some countries, but it continues to cause significant losses from decreased productivity and lost trade in much of the developing world. In *B. melitensis*-free nations, the cost of surveillance to prevent its reintroduction is significant. There are also concerns that this organism is be used in a bioterrorist attack (The center for food security and public health, 2018). Infections in sheep and goats spill over into wild ruminants. However, there is no evidence that these animals serve as reservoir hosts for domesticated sheep and goats (Saxena *et al*., 2018).

This organism is absent from domesticated animals in northern and central Europe, Canada, the U.S., Australia, New Zealand, Japan, and some other countries. Sporadic cases are sometimes reported in traveler and immigrants in *B. melitensis* - free nations (The center for food security and public health, 2018). *Brucella melitensis* is particularly common in the Mediterranean. It also occurs in the Middle East, Central Asia, around the Persian Gulf (also known as the Arabian Gulf), and in some countries of Central America.

This organism has been reported from Africa and India, but it does not seem to be endemic in Northern Europe, North America (except Mexico), Southeast Asia, Australia, or New Zealand. Biovar 3 is the predominant biovar in the Mediterranean countries and the Middle East, and biovar 1 predominates in Central America. In the U.S., cases have mainly been reported in imported goats and rarely in cattle (Saxena *et al*., 2018).

2.4.1 Transmission

Brucella melitensis is shed when the animal aborts or carry the pregnancy to term, and reinvasion of the uterus can occur during subsequent pregnancies (The center for food security and public health, 2018). In animals, *B. melitensis* is transmitted by contact with the placenta, fetus, fetal fluids, and vaginal discharges from infected animals. Small ruminants are infectious after either abortion or full-term parturition. Goats usually shed *B. melitensis* in vaginal discharges for at least 2 to 3 months but shedding usually ends within three weeks in sheep. The organism is found the milk and semen; shedding in milk and semen can be prolonged or lifelong, particularly in goats. Kids and lambs that nurse from infected dams may shed *B. melitensis* in the feces (Saxena *et al*., 2018).

2.4.2 Clinical Signs

The predominant clinical signs in sheep and goats are abortions (most often during the last trimester), stillbirths, and the birth of weak offspring. Most animals abort only once, and subsequent pregnancies are usually normal. Reductions in milk yield are common. While mastitis was reported in small ruminants experimentally infected with large doses of *B. melitensis*, clinically apparent mastitis is uncommon in the field. (The center for food security and public health, 2018). Animals that abort may retain the placenta. Sheep and goats usually abort only once, but reinvasion of the uterus and shedding of organisms can occur during subsequent pregnancies (Alsaif *et al*., 2018). Some infected animals carry pregnancy to term but shed the organism. Milk yield is significantly reduced in animals that abort, as well as in animals whose udder become infected after a normal birth. However, clinical signs of mastitis are uncommon. Acute orchitis and epididymitis can occur in males and may result in infertility. Arthritis is seen occasionally in both sexes (Saxena *et al*., 2018). The infection causes inflammation of the male reproductive organs the epididymis in rams, resulting in infertility and sterility in some affected rams. In some flocks, over 50 percent of rams are affected. Ovine Brucellosis (OB) has occasionally been associated with abortion

in ewes, and increased perinatal mortality (Animal Biosecurity and welfare, 2017.).

2.4.3 Treatment, prevention, and control

Antibiotics can mitigate the clinical signs, and a few studies have reported that treatment may have eliminated *Brucella* from small ruminants or cattle. However, even when the organisms seem to have disappeared, they might persist in lymph nodes or other tissues and re-emerge. In addition, none of the published treatments have been extensively evaluated (The center for food security and public health, 2018). There is no vaccine or other preventive treatment available, and infected rams cannot be cured. Eradication of the disease from infected flocks requires identification of infected rams and culling them (Animal Biosecurity and welfare, 2017).

The control programs of *Brucella* infection in animals rely mainly on vaccination with live attenuated *B. melitensis* Rev 1. This provides good levels of protection against *B.melitensis* in sheep and goats (Gwida *et al*., 2016). This vaccine also interferes with serological tests, particularly when injected subcutaneously, but conjunctival administration to lambs and kids between the ages of 3 and 6 months minimizes this problem (Saxena *et al*., 2018). Programs to eradicate this organism from a country also include movement controls on infected herds, surveillance, and tracing of infected animals (The center for food security and public health, 2018).

2.5 Brucellosis in dogs

Brucella canis is a gram-negative coccobacillus bacterium that primarily causes reproductive failure in dogs (Hensel *et al*., 2018). *Brucella canis* is an important cause of reproductive failure in dogs, especially in kennels (The center for food security and public health, 2018). The organism is an intracellular bacterium found in breeding kennels. *Brucella canis* infection causes a zoonotic disease that can infect humans (Bramlage *et al*., 2015). It is a worldwide disseminated disease and highly resistant to antibiotic therapy mainly due to its intracellular location (Pujol *et al*., 2019). However, human infections with this organism may be under-diagnosed, as the symptoms are nonspecific, diagnostic suspicion among physicians is low, and obtaining a definitive diagnosis may be difficult (The center for food security and public health, 2018).

Brucella canis appears to be widely distributed and was reported in North, Central, and

South America and parts of Asia, Africa, and Europe. New Zealand and Australia appear to be free of *B. canis* (The center for food security and public health, 2018). It has a ubiquitous distribution and is reported in the United States, Canada, Central, and South America, Tunisia, South Africa, Nigeria, Madagascar, Malaysia, India, Korea, Japan, and China among others (Ayoola *et al*., 2016). Few studies have been conducted to evaluate disease occurrence and distribution in the United States, so the status is unknown. However, in the past 2 decades, serologic studies of dogs have been published from countries in Africa, Asia, and South America and have reported moderate to high seroprevalence, ranging from 6% to 35% (Hensel *et al*., 2018).

2.5.1 Transmission

Thie organism is shed in urine, and low concentrations of bacteria has been detected in saliva, nasal and ocular secretions, and feces. In dogs, *B. canis* primarily enters the body by ingestion and through the genital, oronasal, conjunctival mucosa, and transmission through broken skin may be possible. Most cases are thought to be acquired venereally (including via artificial insemination) or by contact with the fetus and fetal membranes after abortions and stillbirths (The center for food security and public health, 2018). *Brucella canis* infection in dogs occurs predominantly through ingestion, inhalation, or contact with aborted fetuses or placenta, vaginal secretions, or semen. Infected dogs intermittently shed low concentrations of bacteria in seminal fluids and non-estrus vaginal secretions (Hensel *et al*., 2018). Transmission of *Brucella* infection in dogs occurs via ingestion of contaminated materials or venereal routes. It can be easily transmitted among dogs reared intensively in breeding kennels or where owners rear two or more dogs (Ayoola *et al*., 2016). *Brucella canis* organisms are shed in the highest numbers in aborted material and vaginal discharge. Infected females transmit canine brucellosis during estrus, at breeding, or after abortion through oronasal contact of vaginal discharges and aborted materials (Bramlage *et al*., 2015).

2.5.2 Clinical Signs

In females, early embryonic death may lead to resorption and result in perceived conception failure whereas in males, *B. canis* usually infects the prostate, testicles, and epididymis, and clinical signs are associated with these areas. Enlargement of one or both testicles may be seen (Bramlage *et al*., 2015). The typical manifestation in females is mid

- to late-term abortion (days 45–59 of pregnancy), followed by an odourless, brown-to-yellow vaginal discharge for 1–6 weeks. Another manifestation is embryonic death with resorption, which appears as conception failure after successful mating (Hensel *et al*., 2018). The clinical manifestation of the disease in dogs includes abortion, infertility, orchitis, epididymitis, and testicular atrophy (Ayoola *et al*., 2016). Infected dogs have a history of lameness, spinal pain, neurologic dysfunction, muscle weakness, or any combination of these signs, caused by vertebral osteomyelitis and intervertebral disc infection (Hensel *et al*., 2018). Most abortions occur during the last trimester, especially between 45 and 55 days, and typically have no significant premonitory signs. Abortion is usually followed by a mucoid, serosanguinous, or gray-green vaginal discharge that persists for several weeks. Early embryonic deaths and resorption are usually reported a few weeks after mating and may be mistaken for failure to conceive. Reproductive losses recur during subsequent pregnancies in some dogs, but not in others. Such recurrences may be intermittent (The center for food security and public health, 2018).

2.5.3 Treatment, prevention, and control

Antimicrobial drug treatment alone after signs of reproductive failure is usually unsuccessful because of the ability of the bacteria to sequester intracellularly for long periods and cause episodic bacteraemia. The recommended course of treatment is multimodal and includes surgical sterilization and antimicrobial drugs (Hensel *et al*., 2018). No treatment is certain to eliminate *B. canis*, and recrudescence is possible. Even when this organism seems to have disappeared, it may persist in tissues such as the lymph nodes, spleen, uterus, and prostate. For this reason, euthanasia of infected animals is often recommended in kennels, and this option should be discussed when *B. canis* is found in a pet (The center for food security and public health, 2018).

In infected kennels, brucellosis is controlled by sanitation and infection control measures, together with the euthanasia, isolation, or removal of infected dogs. Housing in individual cages reduces the spread of the organism (The center for food security and public health, 2018).

2.6 Pathogenesis of brucellosis

Brucella spp cause the disease but requires a significant process during infection (Poester *et al*., 2013). Macrophages in the intestine are a portal of entry for *Brucella spp*. Epithelial cells can be invaded by *Brucella spp* and allow an infection to go through mucosal surfaces. Once the *Brucella spp* succeed in invading, they can survive intracellular in phagocytic or non-phagocytic host cells (Luelseged *et al*., 2018). *Brucella spp* are capable of interfering with intracellular traffic, preventing the fusion of the *Brucella*-containing vacuole (BCV) with lysosome markers, and directing the vacuole to a section that has rough endoplasmic reticulum (RER), which is extremely tolerant to intracellular duplication of *Brucella* (Poester *et al*., 2013). A successful entry of *Brucella* into the host is a crucial step in establishing infection. Considering that the digestive tract is the main entrance route of *Brucella*, some studies investigated possible virulence factors involved in successful infection through the digestive tract (Xavier *et al*., 2010).

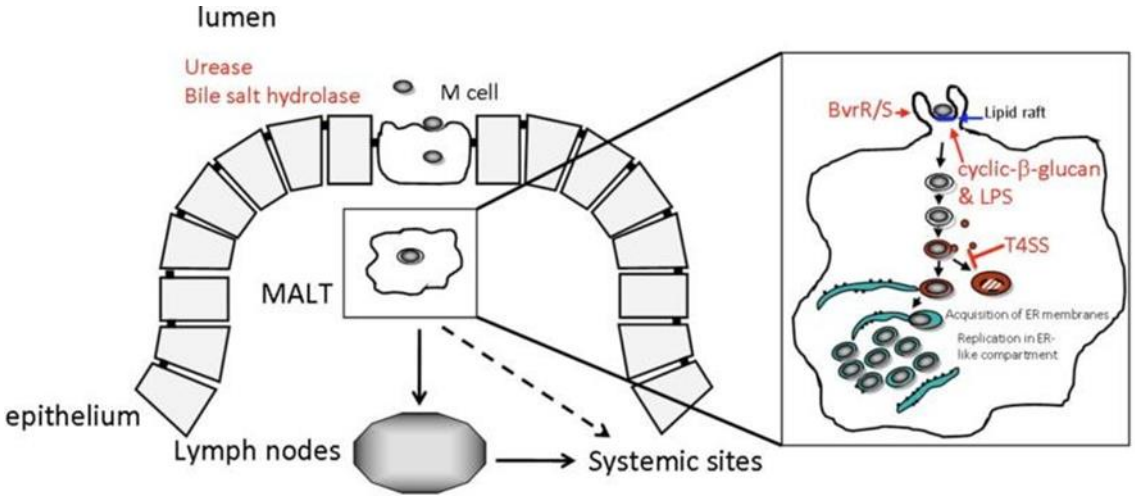

Figure 2. 3: A schematic representation of *Brucella* invasion through the digestive tract.

As shown in Figure 2.3, the entry of *Brucella* bacteria is through macrophages and subsequently, the bacteria are taken up by macrophages of the mucosa-associated lymphoid tissue (MALT). These macrophages transport the bacteria to the lymph nodes and on to systemic sites. Blown-up macrophage shows trafficking within the macrophage from entry via lipid rafts, through the endosomal pathway to the ER-like compartment in which *Brucella* replicates. In red are *Brucella* virulence factors that are involved in establishing infection (Xavier *et al*., 2010).

Table 2. 3: Animals affected by *Brucella* species (DAFF, 2016).

Host	*B. abortus*	*B. melitensis*	*B. suis*	*B. canis*	*B. ovis*
Cattle	+	+	+(rare)	-	-
Buffaloes	+	+	-	-	-
Bison	+	-	-	-	-
Sheep	+(rare)	+	+(possible)	-	+
Goats	+(rare)	+	-	-	-
Swine	+(rare)	+	+	-	-
Dogs	+	+	+(rare)	+	-
Camels	+(rare)	+	-	-	-
Caribou/Reindeer	-	-	+(biovar 4)	-	-
Elk	+	-	-	-	-
Horses	+	+(rare)	+(rare)	-	-
Rodents	+(rare)	+(rare)	+(biovar 5)	-	-

The recently recognized types associated with marine animals, *B. ceti* and *B*. *pinnipedialis*, may also have the capacity to cause human disease (Amjadi *et al*., 2019). Recent isolates from humans (*B. inopinata*), aquatic mammals (*B. pinnipedialis* and *B. ceti*), and a common vole (*B. microti*) are recognized as new species, bringing the current number to 10 species in the genus (de Figueiredo *et al*., 2015). *Brucella* bacterium is divided into two categories, rough colony, and smooth colony, based on lipopolysaccharide (LPS) expression listed in table 2.4. Smooth species, fully expressing the O-chain, are more virulent than the rough species with little or no O- chain (Amjadi *et al*., 2019).

Table 2. 4: Genus *Brucella* (Young, 2012).

Species	Biovar(s)	Host(s)	Human pathogen
B. melitensis	1-3	Goats, sheep	Yes
B. abortus	1-6,9	Cattle	Yes
B. suis[a]	1 and 3	Swine	Yes
	4	Caribou, reindeer,	Yes
	5	Rodents	Yes
B. neotomae	-	Desert wood Rats	No
B. ovis[a]	-	Sheep	No
B. canis	-	Dogs	Yes

[a] signifies naturally rough strains (lacking O-polysaccharide).

2.7 Diagnostic assays for brucellosis

2.7.1 The Rose Bengal Test

The Rose Bengal Test is used as a rapid screening test and is considered a reliable test in the diagnosis of brucellosis (Saxena *et al*., 2018). It is a simple spot agglutination test using antigen with Rose Bengal dye and buffered to a low pH, usually 3.65 ± 0.05 (WHO, 2013). The RBT can show false-positive results because of S19 vaccination or non-specific serological reactions (Kangumba, 2015). It is quick, cheap, and easy to perform (Delegates, 2016). According to a study conducted by Geresu and Kassa (2016), RBT has some disadvantages of low sensitivity particularly in chronic cases, relatively low specificity in endemic areas and prozones that make strongly positive sera test negative. The Rose Bengal Test is an agglutination test that detects anti-*Brucella* antibodies in serum. It is a rapid and sensitive test and acts as a good screening test for bovine serum samples (Konstantinidis *et al*., 2007).

2.7.2 Complement fixation test

The Complement fixation test is based on the ability of complement (obtained from guinea pig serum) to lyses erythrocytes (traditionally sheep erythrocytes sensitized with hemolysin) in the absence of an antibody-antigen complex (Pérez-Sancho *et al*., 2015). The CFT is widely used but it is complex to perform, the accuracy of titration and maintenance of reagents require enough specialized personnel to perform the test in the laboratory facility (WHO, 2016). The test is used for the diagnosis of various bacterial and protozoal diseases. When *Brucella*-specific antibodies are present in the serum being analysed, they bind to the *Brucella* antigens (whole cells) provided externally, forming antigen-antibody complexes that then bind to the complement (Perez-Sancho *et al*., 2015). The CFT is sensitive, specific, and used as a confirmatory test for testing of individual animals (Minas A *et al*., 2005).

2.7.3 Fluorescence polarization assay

Fluorescence Polarization Assay is established on the principle of spinning molecules in a liquid medium correlating with their mass. The method is rapid and depends on serological instrument that provides an alternative to conventional serology and has been included in routine testing elsewhere (Mahmood *et al*., 2015). The FPA test is simple, quick, and easy to perform and gives rapid results. Furthermore, it is expected to be highly reproducible across laboratories and instruments and reduces human error and variability that may occur during the reading of agglutination tests result such as the card test, the standard plate test, and other similar tests (Kangumba, 2015).

The mechanism of the assay is based on the use of a fluorescence polarization analyzer to measure the reduced rate of rotation of large molecules in solution, such as antigen-antibody complex, which may be present in blood sera from infected animals (Fiasconaro *et al*., 2015). A fluorochrome-labeled antigen of small molecular weight is added to serum or other fluid to be tested. If the antibody is present, attachment to the labelled antigen will cause its rotational rate to decrease and this decrease can be measured.

CHAPTER 3: MATERIALS AND METHODS

3.1 Source of samples

Blood samples that were collected by Gauteng Department of Agriculture and Rural Development were used for the project. Blood samples were from known positive and negative herds and submitted to the Bacterial Serology laboratory at the Agricultural Research Council (ARC), Onderstepoort Veterinary Research (OVR) in Pretoria. These samples were from cattle reared by commercial and non- commercial farmers. The period of collection covered September 2018 to April 2019.

3.2 Ethical clearance

The College of Agriculture and Environmental Science and Health Research Ethics committee of the University of South Africa provided ethics clearance (2018/CAES/075) for this research (Appendix A). The Onderstepoort Veterinary Research issued an approval letter for the research project and ethical clearance letter (Appendices B and C). The permission to access samples collected by Gauteng veterinary service was obtained from the Gauteng Department of Agriculture and Rural Development, State Veterinary Pretoria (Appendix D). Section 20 permit to purchase the *Brucella* FPA test kit was issued by the Director of Animal Health from the Department of Agriculture Forestry and Fisheries in 2018 (Appendix E).

3.3 Study population and sampling

The study was conducted in Pretoria, the administrative capital of South Africa, located in Gauteng province, with an estimated population of 15, 488,137 (2020). Samples were tested at the Agricultural Research Council (ARC) laboratory, Onderstepoort Veterinary Research. The study population comprised vaccinated and unvaccinated cattle from commercial and non-commercial farmers. Herds that had many serological reactors with several high titers were classified as serologically positive while those with no reactions on RBT and SAT were classified as negative. Samples were categorized into positive and negative sera and stored separately. The serum samples were stored in the refrigerator at 4°C ± 2 ºC until processed. In total, 493 positive serum samples and 532 negative serum samples from 1025 female cattle were used for this study.

Table 3. 1: The total number of positive samples collected was 493.

Farm name	District/Municipality	Samples collected
Herd 1	Kungwini Bronkhorstspruit local municipality	108
Herd 2	Randfontein local Municipality	13
Herd 3	Ekurhuleni Boksburg local Municipality	98
Herd 4	Kungwini Bronkhorstspruit local municipality	9
Herd 5	Kungwini Bronkhorstspruit local municipality	140
Herd 6	Randfontein Krugersdorp local municipality	29
Herd 7	Merafong local municipality	38
Herd 8	Ekurhuleni local municipality	58
Total		493

Table 3. 2: The total number of negative samples collected was 532.

Farm name	District/municipality	Samples collected
Herd A	Sedibeng local municipality	116
Herd B	Kungwini Bronkhorstspruit local municipality	117
Herd C	Kungwini Bronkhorstspruit local municipality	112
Herd D	Merafong fochville local municipality	60
Herd E	Kungwini Bronkhorstspruit local municipality	63
Herd F	Pretoria Tshwane local Municipality	11
Herd G	Randfontein Krugersdorp local municipality	31
Herd H	Midvaal local municipality	22
Total		532

3.4 Preparation of blood samples for analysis

Blood samples were centrifuged for 10 minutes at 3220-rpm speed to get the clear serum. The serum samples were then marked, aliquoted in Nunc tubes and stored at 4°C ± 2 ºC in the Serology laboratory refrigerator.

3.5 Data analysis

All data were entered manually into a Microsoft Excel spreadsheet, and data cleaning and analysis were carried out using a statistical package for the social sciences (SPSS) version 26 and Graph pad Prism version 9 software.

The cut-off value for FPA that gives the highest sum of DSn and DSp values and the area under curve (AUC) and their 95% CI were determined from the analysis of results of negative reference sera (532 sera) and positive reference sera (493 sera). Receiver operator characteristic (ROC) analysis of the results were carried out using the same software.

The DSn, DSp, Youden's J value and AUC and their 95% confidence interval (CI) of every test used in the study were calculated with cross-tabulation and ROC analysis of the results for reference sera classified negative based on negative reactions on RBT and SAT and for reference sera classified positive based on a large number of

positive reactors on serological testing with several high titers. The agreement of test results was assessed with the analysis of the results of all sera tested in brucellosis free and infected flocks (*n=1025*), by Kappa analysis and calculation of Kappa (K) statistic. The performance of the tests used were compared by Friedman test for repeat measurements.

3.6 Serological tests

3.6.1 Rose Bengal Test

The Rose Bengal reagent used for the study contains *Brucella abortus* biovar 1 (Weybridge 99 strain) inactivated by heat and phenol, coloured with Rose Bengal stain, and diluted in an acidified buffer. The *Brucella abortus* Rose Bengal Test Onderstepoort Biological Products antigen (obtainable from Onderstepoort Biological Products, VLA, and IDEXX Pourquier) was stored at 4 ±3˚c. An equal volume (25 µl) of Rose Bengal antigen and serum were mixed in haemagglutination plates (180x180 mm containing 100 hemispherical wells 15mm in diameter). The plates were tapped gently on each side to ensure that the serum mixes properly with the antigen in the wells. The plates were placed on the shaker; set at 40-rpm speed for 4 minutes. The source of positive control was Onderstepoort National Standard manufactured by Onderstepoort Biological Products. The method for preparation of the positive control was already done according to the OIE Terrestrial Manual 2019. The lifespan of the prepared positive control is 1 year. The serum was stored at -20-˚C ±10˚C in small aliquots and brought to room temperature before use as per OIE procedure (2013).

The plates were read using the UV lightbox (Figure 3.1)

The well was read as positive in the presence of any ringing or agglutination or as negative in the absence of any ringing or agglutination and the results were recorded in the BR5 form.

Figure 3. 1: Reading and recording of RBT results on the BR5 form.

3.6.2 Complement Fixation Test

The following reagents were used for CFT:

- *Brucella abortus* complement antigen (supplied by Onderstepoort Biological Products)
- *Brucella abortus* standardized positive control (supplied by Onderstepoort Biological Products)
- *Brucella abortus* negative control (made in the laboratory)
- Guineapig Complement for CFT (supplied by Virion serian product – Kat medical)
- Amboceptor from rabbit (supplied by supplied by Virion serian product – Kat medical)
- CFT buffer (made in the laboratory)
- Sheep red blood cells (Bled by the laboratory and send to Western Cape laboratory to test and used if negative).
- Test sera (supplied by different animal health technicians from the State veterinarian)
- Positive and negative control sera were inactivated together at 58 ºC ± 2 ºC for 30 minutes.

Procedure: The 96 well U-bottom microtitre plates were marked according to the standard layout provided in the laboratory at OVR. One hundred µl of each serum was dispensed into the respective wells according to the lab numbers. The whole H- row was for the positive and negative controls. One hundred µl of the positive and 100 µl of negative control was added in the appropriate wells starting with the positive control.

Then plates were sealed using a plastic film. Test sera, positive and negative controls were inactivated by heating at 58 ºC ± 2 ºC for 30 minutes in a hot-air oven. The prepared plates were separated into two and marked as anti-complementary plates and as test plates.

Twenty-five µl of buffer was dispensed in each well of both anti-complementary and test plates. Serum dilution was performed by taking 25µl of serum and diluting in the anti-complementary, and then diluting again to the test plate discarded. The anti-complementary had 25µl of buffer and 25µl complement. The test plate had 25µl of diluted serum, 25µl of antigen, and 25µl of complement. The test plate consists of antigen and anti-complementary. The test plate and anti-complementary plates were incubated for 30 minutes at 38 ± ºC. After 30 minutes, 3% of 50µl formalitic system blood was added to the test plate and incubated for 30 minutes in the shaker. After the process was done, the test and anti-complementary plates were centrifuged at 450-rpm speed for 3 minutes. Using a magnifying mirror or lightbox, the plates were read, and results recorded in the *Brucella abortus* CFT daily form and in the CA5 form (OIE Terrestrial Manual, 2013). CFT results were read as follows.

1. For negative result, there was no sedimentation of SRBC, only complete hemolysis.
2. For positive result, there was sedimentation of SRBC as a distinct button at the bottom of the well. The sedimentation was scored between 1 and 4. The final dilution was determined as the dilution showing more than 0% hemolysis and was converted to international units/ml (IUmr1).

3. The anti-complementary well should be negative, otherwise, the test for the serum must be repeated (with and without antigen) or the results recorded as anti-complementary.

4. The positive control on each plate should give the correct titer (± 2 IU titers on each side are still acceptable). The negative control should always be negative.

Interpretation of CFT test results:

1. 0 = 100% haemolysis of SRBC. No button/pellet of SRBC at bottom, uniformly red supernatant.
2. 1 = 75% haemolysis of SRBC. Small button/pellet of SRBC at bottom of well, lighter supernatant.
3. 2 = 50% haemolysis of SRBC. Bigger button/pellet of SRBC medium red supernatant midway between 0 and 4.
4. 3 = 25% haemolysis of SRBC. Still bigger/pellet of SRBC still lighter, almost clear supernatant.
5. 4 = 0% haemolysis of SRBC. Large button/pellet of SRBC, supernatant clear, like water.

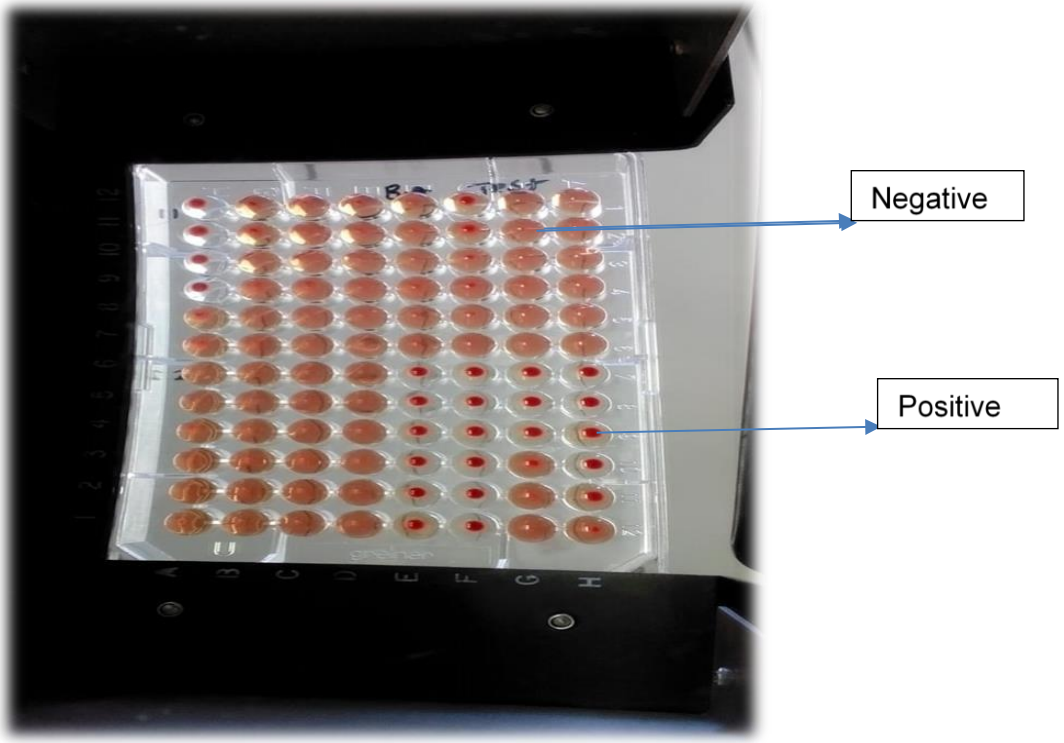

Figure 3. 2: CFT testing plate displaying negative and positive samples. Positive result is where sedimentation of SRBC is present as a distinct button at the bottom of the well.

negative result is where no sedimentation of SRBC is present meaning that there is complete haemolysis.

3.6.3 Fluorescence polarization assay

The Fluorescence Polarization assay was performed using a *Brucella abortus* antibody test kit which uses Fluorescence Polarization technology designed to determine the presence of antibodies to *Brucella abortus* in bovine serum. The reagents used in this assay were manufactured by Diachemix LCC, DOO biotehnika IVD Kraljevo Serbia, and supplied by Prionics AG Wagistrasse 27A CH- 8952 Schlieren, Switzerland. The entire FPA assay was done in 96 well black flat-bottom microtiter plates (type COS96fb manufactured by Corning USA) with no precipitation or washing steps and the adding of only one reagent, the conjugate, was required.

Initially, 20 µl of the controls and samples were dispensed to the wells of a black 96- well microtiter plate. The negative controls were dispensed to wells A1, B1, C1, the positive control to well D1, and the test samples to the remaining wells. A volume of (180 µl) of diluted reaction buffer (1/24) was added to all wells containing controls and samples. The dilution buffer was provided by the manufacturer in 25x concentrate and was diluted by adding 24 parts of distilled water to 1 part of 25x concentrate. The diluted reaction buffer was free of particles and was stored at room temperature for one month. Buffer and serum samples were mixed, in the microplate on a rotating microplate shaker set at 600 rpm for 2 minutes at room temperature. This was followed by letting the plate to stand for a minute.

After the initial mixing, a background reading on samples and controls was taken at fluorescence polarization mode by a multi-mode Microplate reader (Prionics AG-PHERAstar Fluorescence polarization reader) connected to a desktop computer. Then 10 µl of the conjugate (tracer) was added to all the wells containing controls and samples, followed by mixing on a microplate shaker at 600 rpm for 2 minutes at room temperature. After letting the plate stand for a minute, a second reading was taken using the FPA reader. The reader automatically subtracted the background reading and calculated a value for every sample in millipolarization units (mP) refers appendix F.

The fluorescence polarization reader was calibrated using blank and low polarized standards, which were included in the kit together with the antigen. The calibration was done automatically; the instrument takes the value of the low polarized standard (fixed at 25 mP) and calculates an internal compensation factor (G-factor 0.850) which is utilized in the equation for calculating the results. The results of each microplate measurement were accepted if the mP value of the positive control was 120-250 mP and the negative control read between 70– 90 mP.

Equipment used included FPA PHERAstar Fluorescence polarization reader (96-well format), 96-well black flat-bottom microtiter plates, pipettes (10µl, 20µl, 180µl) disposable tips, Microtiter plate shaker, and timer displayed in figure 3.3 below.

Figure 3. 3: FPA equipment and reagent

The interpretation of the test is as follows:

Negative: < 10mP

Suspect: 10-20mP

Positive: > 20 mP

The yellow block indicates that the reaction of the test sample is suspect. The white indicates a negative reaction. All the remaining blue ones are positive reactors as show in figure 3.4.

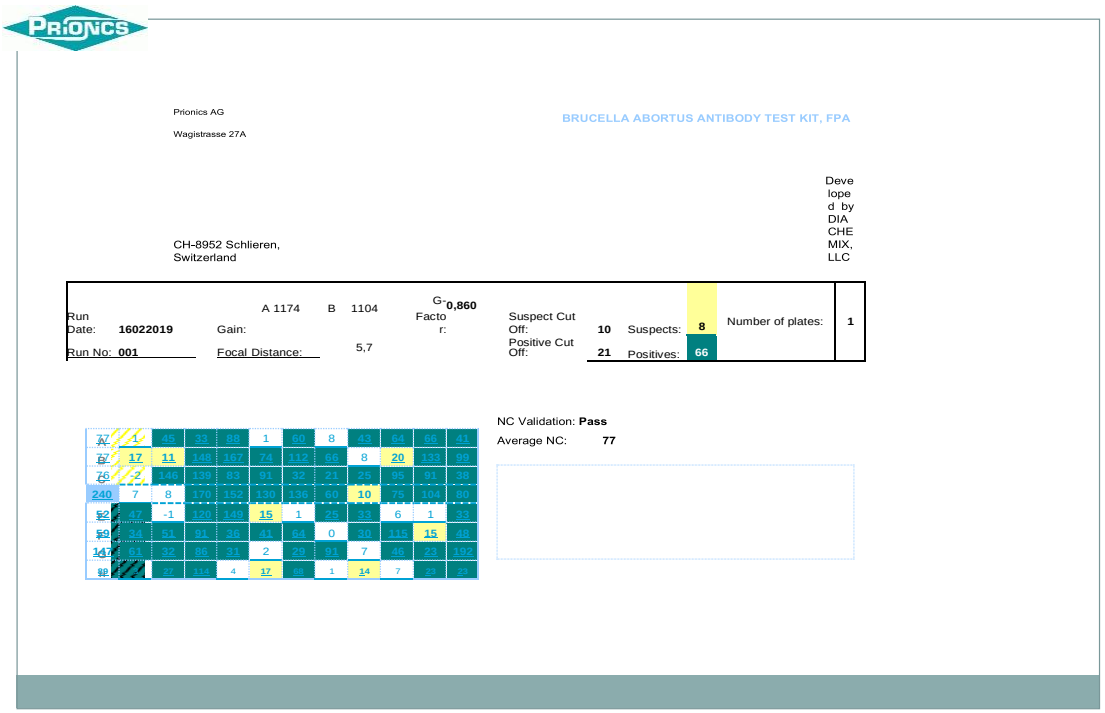

Figure 3. 4: Results display in an excel worksheet.

3.7 Determination of the specificity of the FPA

The specificity of the FPA is the number of animals without the disease who will have a negative test result out of the total number tested, that is,

DSp = [(True Negatives (TN) / (TN + False Positives (FP))] x 100. Specificity was estimated in *Brucella*-free herds by testing animal populations known to be free of the disease and testing negative, that is Dsp = animal testing negative/total number of animals tested. Specificity is the fraction of those without the disease that had a negative test result.

3.8 Determination of the sensitivity of the FPA

The sensitivity of FPA is the number of animals with the disease who will have a positive test result out of the total number tested, that is, DSe= [(True Positives (TP) / (TP + False Negatives (FN))] x 100. Sensitivity was estimated in *Brucella* positive known herds by testing animal population known to have the disease and testing positive, that is, DSe = animal testing positive/total number of animals tested. Sensitivity is the fraction of those with the disease that had a positive test result.

3.9 Determination of the reproducibility of FPA

Reproducibility was determined through inter-laboratory testing by testing the same samples with a different laboratory and comparing them to see if they can produce the same results. Allerton Provincial Veterinary laboratory (APVL) participated in the inter-laboratory testing. Onderstepoort veterinary research sent samples and instructions to Allerton Provincial Veterinary Laboratory. Samples were packaged in Nunc tubes to avoid damage during transportation. The time between dispatch of samples and receipt of laboratory results was limited to one month. Allerton Provincial Veterinary Laboratory used the same approach as described under the section on determination of repeatability (3.11).

After testing APVL sent their excel results sheet to OVR. All results were received and recorded. OVR was responsible for the data entry and checking for the preparation of the interim and final reports. The calculation was determined through Friedman and kappa tests.

3.10 Determination of repeatability of FPA

For repeatability, positive cattle serum samples *(n = 15*) and negative cattle serum samples (*n=5*) that were submitted for routine testing by Pretoria and Germiston State Veterinary offices were used. The sera included a variety of titers from low to high. The ranges for the low samples were from 24-49 IU per ml, medium 60-199 IU per ml and high from 240-784 IU per ml. Three analysts tested the samples on three different days. The calculations were determined through kappa and Friedman tests.

3.11 Determination of RBT and CFT limit of the lowest detection

Rose Bengal was carried out by dispensing 25 µl of buffer into all wells. Twenty-five

microliters of positive control were added, and dilution started from the first row until the last well. Twenty-five microliters of negative control were added in row 2 and diluted until the last well. Twenty-five microliters of serum were dispensed from the third row to the fifth row and diluted as with the controls. New tips were used for each dilution. Twenty-five microliters of *B. abortus* Rose Bengal Test antigen was added to all wells and the plates put on a shaker; set at 40-rpm speed for 4 minutes. Agglutination was an indication of positive reaction.

For CFT the dilution was carried out as normal by picking up 25 µl of serum from the test plate using a hand-held 8-channel micropipette and delivering into row of the A/C plate. Twenty-five microliters from row 1 of the A/C plate was picked up and delivered to row 1 of the test plate. Twenty-five microliters from row 1 of the test plate row 1 was picked up and delivered to row 2 of the test plate. Serial dilutions were continued to row 6 of the test plate. Twenty-five microliters from row 6 was picked and discarded. This accomplished a ½ serial dilution of ¼, 1/8, 1/16, 1/32, 1/64 and 1/126 into row 6. The procedure was repeated for row 2 of the sera, delivering to row 2 of the A/C plate and diluting row 7-12 of the test plate. New tips for each batch of 8 sera were used. Fifty µl component/ CFT diluent (C'/2) was dispensed into the A/C plate. Fifty microliters C'/Ag was dispensed into the test plates. The plates were tapped gently to ensure mixing and incubated at 37˚C ± 2˚C 30 minutes.

Fifty microliters haemolytic system (mixed well) was dispensed into all wells of both the test plate and the A/C plate and incubated at 37˚C ± 2˚C for 30 minutes on a shaker, avoiding stacking of the plates. The plates were removed from the incubator and centrifuged at a maximum of 450 RCF ±15 RCF for 3 minutes. Reading took place by recording the sedimentation of the SRBC as positive and complete haemolysis as negative.

3.12 Determination of FPA turnaround time

The turnaround time was calculated from the time used to set up an assay until test results were obtained. The time used by the different experiences of individuals in the same setting was compared. Forty positive cattle serum samples were used in this experiment. A routine maintenance and quality control evaluation was conducted before the initiation of sample tests run. The researcher after having been trained was able to carry test runs for FPA, RBT, and CFT. The average time to complete each test was considered as the turnaround time.

CHAPTER 4: RESULTS

A total number of 493 positive samples were from herds that had many serological reactors with several high titres and were classified as serologically positive herds. Among 493 only 400 tested positive for RBT, 19 tested negative and 74 samples were invalid, 368 tested positive for CFT, 51 tested negative and 74 samples were invalid. The number of samples testing positive with FPA was 379, with 23 testing negative and the other 17 were suspects. Furthermore, 74 samples were invalid due to insufficient tracer dispensed in the wells. The serology gold standard for brucellosis, the complement fixation test CFT was compared with FPA as a confirmatory serological diagnosis of brucellosis. Tables 4.1 and 4.2 below show summarized results of serological examination.

4.1 Brucellosis tests per herd for the positive and negative samples

Table 4. 1: Results for each test per herd for the positive samples (N=493)

Name of herd	No. of animals tested	RBT+ve	RBT -ve	CFT+ve	CFT-ve	FPA+ve	FPA -ve
Herd 1	108	87	21	55	32	74	34
Herd 2	13	13	0	13	0	13	0
Herd 3	98	98	0	98	0	95	3
Herd 4	9	9	0	9	0	9	0
Herd 5	140	140	0	140	0	137	3
Herd 6	29	29	0	29	0	26	3
Herd 7	38	38	0	38	0	38	0
Herd 8	58	58	0	58	0	25	33
Total	493	400	19	368	51	379	23

A total number of 532 negative samples from herds that were classified negative based on negative results on RBT and SAT. Among 532 only 445 tested negatives for RBT, and 87 samples were invalid. Four hundred and eighteen tested negatives with FPA, 10 were positive 17 were suspects and 87 were invalid samples.

Table 4. 2: Results for each test per herd for the negative samples (N=532).

Name of herd	No. animals tested	RBT+ve	RBT -ve	FPA +ve	FPA –ve
Herd 1	116	0	116	9	107
Herd 2	117	0	117	13	104
Herd 3	112	0	112	0	112
Herd 4	60	0	60	1	59
Herd 5	63	0	63	0	63
Herd 6	11	0	11	0	11
Herd 7	31	0	31	0	31
Herd 8	22	0	22	1	21
Total	532	0	445	10	418

4.2 Sensitivity of the FPA

The sensitivity of FPA was determined by the ROC curve analysis.

Table 4. 3: Positive Roc analysis.

		FPA			
		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Suspect	17	4,1	4,1	4,1
	Negative	23	5,5	5,5	9,5
	Positive	379	90,5	90,5	100,0
	Total	419	100,0	100,0	

Area under the ROC curve		
Area		0,9842
Std. Error		0,005532
95% confidence interval	0,9733 to 0,9950	
P value	<0,0001	

Data	
Controls (mpValue)	419
Patients (Results)	419
Missing Controls	74
Missing Patients	74

Youden J statistic
72.6(68.0% to 76.0%)

	Sensitivity %	95% CI	Specificity %	95% CI	Likelihood ratio
> -0.5000	95,94	93,60% to 97,45%	0,000	0,000% to 0,9085%	0,9594
> 0.5000	90,45	87,26% to 92,91%	100,0	99,09% to 100,0%	

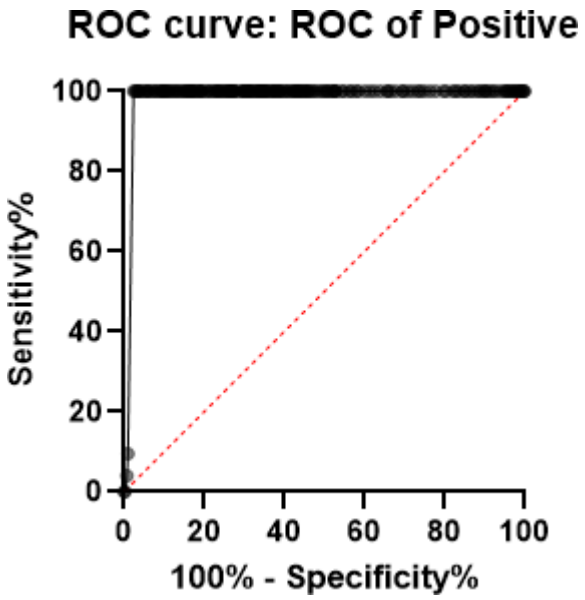

Figure 4. 1: ROC curve diagram of FPA using positive reference sera originating from animals that were confirmed positive by RBT, and CFT.

Table 4. 4: DSn and DSp of FPA at different cut-off values calculated by ROC analysis using as positive reference sera with positive results in two serological tests in parallel.

mpValue	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
< 79.00	100	99,09% to 100,0%	73,51	69,08% to 77,51%	3,775
< 81.50	100	99,09% to 100,0%	73,27	68,83% to 77,28%	3,741
< 83.50	100	99,09% to 100,0%	73,03	68,59% to 77,06%	3,708
< 85.00	100	99,09% to 100,0%	72,79	68,34% to 76,83%	3,675
< 87.00	100	99,09% to 100,0%	72,55	68,09% to 76,61%	3,643
< 88.50	100	99,09% to 100,0%	72,32	67,84% to 76,38%	3,612
< 90.00	100	99,09% to 100,0%	71,84	67,35% to 75,93%	3,551
< 92.00	100	99,09% to 100,0%	70,64	66,11% to 74,80%	3,407
< 93.50	100	99,09% to 100,0%	70,41	65,87% to 74,58%	3,379

4.3 Specificity of the FPA

The specificity of the FPA is the fraction of samples from animals without the disease, which test negative. Specificity was estimated in *Brucella*-free herds by testing animal populations *(n=415*) had tested negative and were known to be free of the disease *(n=25)* using a ROC curve analysis.

Table 4. 5: ROC Negative analysis.

FPA

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Suspect	17	3,8	3,8	3,8
	Negative	418	93,9	93,9	97,8
	Positive	10	2,2	2,2	100,0
	Total	445	100,0	100,0	

Area under the ROC curve		
Area		0,9888
Std. Error		0,004082
95% confidence interval	0,9808 to 0,9968	
P value	<0,0001	
Data		
Controls (Control Variable)		445

Patients (FPA)	445
Missing Controls	87
Missing Patients	87

Youden J statistic
97.8 (96.0.0% to 98.8%)

	Sensitivity %	95% CI	Specificity %	95% CI	Likelihood ratio
< -0.5000	3,82	2,399% to 6,032%	100	99,14% to 100,0%	
< 0.5000	97,75	95,91% to 98,77%	100	99,14% to 100,0%	

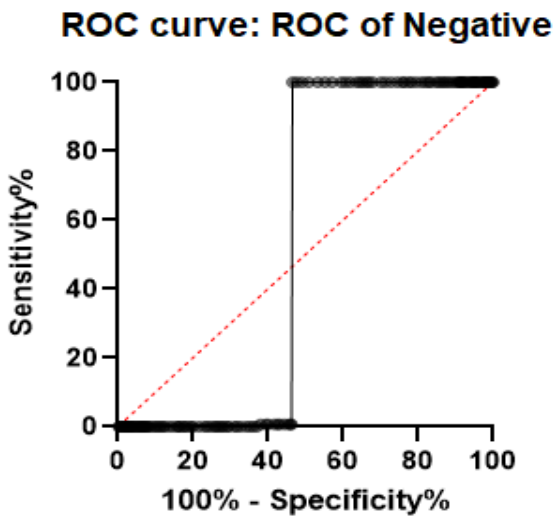

Figure 4. 2: ROC curve diagram of FPA using negative reference sera originated from animals that were confirmed negative in RBT and SAT.

4.4 Reproducibility of FPA

Reproducibility was determined through inter-laboratory testing between Allerton laboratory and Onderstepoort Veterinary laboratory. Allerton Provincial Veterinary Laboratory tested their samples using a Synergy H1 Microplate instrument with injectors using *Brucella* FPA kit, which seems to be more rapid. Dispensation into wells was done automatically as well as calculations by Gen 5 software, while Onderstepoort ran the test using the previous version of the PHERAstar plate reader connected to the computer desktop, hence, there were differences in the plate readers, pipetting technique and

calculation of results. Table 4.6 shows how the results were interpreted after subtraction of the negative mP value from the sample mP value. Delta mP gives the final reading that determines whether the sample is positive or negative.

Table 4. 6: Reproducibility of FPA Positive and negative.

Allerton * OVR Crosstabulation

					Total
Allerton					5
		Expected Count	1,5	3,5	5,0
	1.00	Count	1	14	15
		Expected Count	4,5	10,5	15,0
Total		Count	6	14	20
		Expected Count	6,0	14,0	20,0

Symmetric Measures

		Value	Asymptotic Standard Error[a]	Approximate T[b]	Approximate Significance
Measure of Agreement	Kappa	0,875	0,121	3,944	0,000
N of Valid Cases		20			

Table 4. 7: Positive and negative Friedman Test.

Descriptive Statistics

	N			
Allerton	20	0,2500	1,0000	1,0000
OVR	20	0,0000	1,0000	1,0000

Friedman Test: Ranks

	Mean Rank
Allerton	1,53
OVR	1,48

Test Statistics[a]

N	20
Chi-Square	1,000
df	1
Asymp. Sig.	0,317

a. Friedman Test

4.5 Repeatability of FPA

For repeatability, positive cattle serum samples *(n = 15)* and 5 negative samples that were stored at Onderstepoort were used. The sera included a variety of titters from the lowest to the highest titter. The ranges for the low samples ranged from 24-49 IU per ml, medium 60-199 IU per ml and high from 240-784 IU per ml. Three analysts analyzed the same samples on the same day under the same conditions. The test was performed on 3 different days using the same reagents every time.

Table 4. 8: Repeatability of FPA Positive and negative Onderstepoort Analyst 1 day 1, Analyst 1 day 2 and Analyst 1 day 3.

Table Analyzed	Repeated Combined
Friedman test	
P value	0,2636
Exact or approximate P value?	Approximate
P value summary	Ns
Are means significantly different? (P < 0.05)	No
Number of groups	3
Friedman statistic	2,667
Data summary	
Number of treatments (columns)	3
Number of subjects (rows)	20

ns not significant

Multiple comparison of Analyst 1 day 1, Analyst 1 day 2 and Analyst 1 day 3

Multiple comparison	
Number of families	1
Number of comparisons per family	3
Alpha	0,05

Dunn's multiple comparisons test	Rank sum diff,	Significant ?	Summary	Adjusted P Value	
Analyst 1 day 1 vs. Analyst 1 day 2	0	No	ns	>0,9999	A-B
Analyst 1 day 1 vs. Analyst 1 day 3	-3	No	ns	>0,9999	A-C
Analyst 1 day 2 vs. Analyst 1 day 3	-3	No	ns	>0,9999	B-C

Test details	Rank sum 1	Rank sum 2	Rank sum diff,	n1	n2	Z
Analyst 1 day 1 vs. Analyst 1 day 2	39	39	0	20	20	0
Analyst 1 day 1 vs. Analyst 1 day 3	39	42	-3	20	20	0,4743
Analyst 1 day 2 vs. Analyst 1 day 3	39	42	-3	20	20	0,4743

Table 4. 9: Analyst 2 day 1, Analyst 2 day 2 and Analyst 2 day 3.

Table Analyzed	Repeated Combined
Friedman test	
P value	0,0408
Exact or approximate P value?	Approximate
P value summary	*
Are means significantly different? (P < 0.05)	Yes
Number of groups	3
Friedman statistic	6,4
Data summary	
Number of treatments (columns)	3
Number of subjects (rows)	20

**. Correlation is significant at the 0.01 level (2-tailed).

Multiple comparison of Analyst 2 day 1, Analyst 2 day 2 and Analyst 2 day 3

Multiple comparison	
Number of families	1
Number of comparisons per family	3
Alpha	0,05

Dunn's multiple comparisons test	Rank sum diff,	Significant?	Summary	Adjusted P Value	
Analyst 2 day 1 vs. Analyst 2 day 2	6	No	ns	>0,9999	D-E
Analyst 2 day 1 vs. Analyst 2 day 3	6	No	ns	>0,9999	D-F
Analyst 2 day 2 vs. Analyst 2 day 3	0	No	ns	>0,9999	E-F

Test details	Rank sum 1	Rank sum 2	Rank sum diff,	n1	n2	Z
Analyst 2 day 1 vs. Analyst 2 day 2	44	38	6	20	20	0,9487
Analyst 2 day 1 vs. Analyst 2 day 3	44	38	6	20	20	0,9487
Analyst 2 day 2 vs. Analyst 2 day 3	38	38	0	20	20	0

Table 4. 10: Analyst 3 day 1, Analyst 3 day 2 and Analyst 3 day 3.

Table Analyzed	Repeated Combined
Friedman test	
P value	>0,9999
Exact or approximate P value?	Approximate
P value summary	Ns
Are means significantly different? (P < 0.05)	No
Number of groups	3
Friedman statistic	0
Data summary	
Number of treatments (columns)	3
Number of subjects (rows)	20

Multiple comparison of Analyst 3 day 1, Analyst 3 day 2 and Analyst 3 day 3

Multiple comparison	
Number of families	1
Number of comparisons per family	3
Alpha	0,05

Dunn's multiple comparisons test	Rank sum diff,	Significant?	Summary	Adjusted P Value	
Analyst 3 day 1 vs. Analyst 3 day 2	0	No	ns	>0,9999	G-H
Analyst 3 day 1 vs. Analyst 3 day 3	0	No	ns	>0,9999	G-I
Analyst 3 day 2 vs. Analyst 3 day 3	0	No	ns	>0,9999	H-I

Test details	Rank sum 1	Rank sum 2	Rank sum diff,	n1	n2	Z
Analyst 3 day 1 vs. Analyst 3 day 2	40	40	0	20	20	0
Analyst 3 day 1 vs. Analyst 3 day 3	40	40	0	20	20	0
Analyst 3 day 2 vs. Analyst 3 day 3	40	40	0	20	20	0

Table 4. 11: Analyst 1 day 1, Analyst 2 day 1 and Analyst 3 day 1.

Table Analyzed	Repeated Combined
Friedman test	
P value	0,0067
Exact or approximate P value?	Approximate
P value summary	**
Are means significantly different? (P < 0.05)	Yes
Number of groups	3
Friedman statistic	10
Data summary	
Number of treatments (columns)	3
Number of subjects (rows)	20

Multiple comparison of Analyst 1 day 1, Analyst 2 day 1 and Analyst 3 day 1

Multiple comparison	
Number of families	1
Number of comparisons per family	3
Alpha	0,05

Dunn's multiple comparisons test	Rank sum diff,	Significant?	Summary	Adjusted P Value	
Analyst 1 day 1 vs. Analyst 2 day 1	-7,5	No	ns	0,707	A-D
Analyst 1 day 1 vs. Analyst 3 day 1	0	No	ns	>0,9999	A-G
Analyst 2 day 1 vs. Analyst 3 day 1	7,5	No	ns	0,707	D-G

Test details	Rank sum 1	Rank sum 2	Rank sum diff,	n1	n2	Z
Analyst 1 day 1 vs. Analyst 2 day 1	37,5	45	-7,5	20	20	1,186
Analyst 1 day 1 vs. Analyst 3 day 1	37,5	37,5	0	20	20	0
Analyst 2 day 1 vs. Analyst 3 day 1	45	37,5	7,5	20	20	1,186

Table 4. 12: Analyst 1 day 2, Analyst 2 day 2 and Analyst 3 day 2.

Table Analyzed	Repeated Combined
Friedman test	
P value	0,6065
Exact or approximate P value?	Approximate
P value summary	Ns
Are means significantly different? (P < 0.05)	No
Number of groups	3
Friedman statistic	1
Data summary	
Number of treatments (columns)	3
Number of subjects (rows)	20

Multiple comparison of Analyst 1 day 2, Analyst 2 day 2 and Analyst 3 day 2

Multiple comparison

Number of families	1
Number of comparisons per family	3
Alpha	0,05

Dunn's multiple comparisons test	Rank sum diff,	Significant?	Summary	Adjusted P Value	
Analyst 1 day 2 vs. Analyst 2 day 2	-1,5	No	ns	>0,9999	B-E
Analyst 1 day 2 vs. Analyst 3 day 2	0	No	ns	>0,9999	B-H
Analyst 2 day 2 vs. Analyst 3 day 2	1,5	No	ns	>0,9999	E-H

Test details	Rank sum 1	Rank sum 2	Rank sum diff,	n1	n2	Z
Analyst 1 day 2 vs. Analyst 2 day 2	39,5	41	-1,5	20	20	0,2372
Analyst 1 day 2 vs. Analyst 3 day 2	39,5	39,5	0	20	20	0
Analyst 2 day 2 vs. Analyst 3 day 2	41	39,5	1,5	20	20	0,2372

Table 4. 13: Analyst 1 day 3, Analyst 2 day 3 and Analyst 3 day 3.

Table Analyzed	Repeated Combined
Friedman test	
P value	0,4724
Exact or approximate P value?	Approximate
P value summary	Ns
Are means significantly different? (P < 0.05)	No
Number of groups	3
Friedman statistic	1,5
Data summary	
Number of treatments (columns)	3
Number of subjects (rows)	20

Multiple comparison of Analyst 1 day 3, Analyst 2 day 3 and Analyst 3 day 3

Multiple comparison	
Number of families	1
Number of comparisons per family	3
Alpha	0,05

Dunn's multiple comparisons test	Rank sum diff,	Significant?	Summary	Adjusted P Value	
Analyst 1 day 3 vs. Analyst 2 day 3	1,5	No	ns	>0,9999	C-F
Analyst 1 day 3 vs. Analyst 3 day 3	3	No	ns	>0,9999	C-I
Analyst 2 day 3 vs. Analyst 3 day 3	1,5	No	ns	>0,9999	F-I

Test details	Rank sum 1	Rank sum 2	Rank sum diff,	n1	n2	Z
Analyst 1 day 3 vs. Analyst 2 day 3	41,5	40	1,5	20	20	0,2372
Analyst 1 day 3 vs. Analyst 3 day 3	41,5	38,5	3	20	20	0,4743
Analyst 2 day 3 vs. Analyst 3 day 3	40	38,5	1,5	20	20	0,2372

4.6 Correlation between FPA and standard tests such as RBT and CFT

Table 4. 14: Correlation of Positive samples.

Correlations

		RBT	CFT	FPA
RBT	Pearson Correlation	1	.585**	.371**
	Sig. (2-tailed)		0,000	0,000
	N	419	419	419
CFT	Pearson Correlation	.585**	1	.476**
	Sig. (2-tailed)	0,000		0,000
	N	419	419	419
FPA	Pearson Correlation	.371**	.476**	1
	Sig. (2-tailed)	0,000	0,000	
	N	419	419	419

**. Correlation is significant at the 0.01 level (2-tailed).

Table 4. 15: Correlation of Negative samples.

Correlations

		RBT	SAT	FPA
RBT	Pearson Correlation	.[a]	[a]	.[a]
	Sig. (2-tailed)			
	N	446	446	446
SAT	Pearson Correlation	.[a]	.[a]	.[a]
	Sig. (2-tailed)			
	N	446	446	446
FPA	Pearson Correlation	.[a]	.[a]	1
	Sig. (2-tailed)			
	N	446	446	446

a. Cannot be computed because at least one of the variables is constant.

4.7 Determination of RBT, and CFT limit of the lowest detection

The lowest detection of RBT was at row 12. The plates were read under the RBT reading UV light box. Agglutination was visible, meaning a positive reaction was visible until row 12. The lowest detection of CFT was at row 11 where there was sedimentation of SRBC observed was positive prior to complete haemolysis.

Table 4. 16: Lowest detection of RBT, CFT and FPA.

Sample id (17)	Day 1	Day 2	Day 3
RBT	$^{2}/_{512}$	$^{1}/_{256}$	$^{1}/_{4096}$
CFT	$^{2}/_{2048}$	$^{1}/_{512}$	$^{4}/_{4096}$
FPA	-	-	-

The lowest concentration of FPA in serum was not performed due to the computers at Onderstepoort being replaced with the new ones, which caused a loss of FPA software. The dilution of sample 17 increased every day for RBT and CFT.

4.8 RBT, CFT and FPA turnaround time

RBT: The turnaround time for RBT is as follows: 30 min for samples and reagents to reach room temperature, 3 min plate marking, 30 min for 100 sample and antigen dispensing, 4 min on shaker and 5 min sample interpretation. The total amount of time it would take to perform RBT would be 72 minutes maximum to test 100 samples. The overall assay would depend on how many samples the laboratory tested including signing of the results and sending them back to the State veterinarian.

CFT: Based on turnaround time CFT is time-consuming as you have to do the blood washing, preparing of the reagents, incubating the inactivated sera which is 30 minutes at 58 ºC ± 2 ºC in a hot-air oven. The test plates and anti-complementary plates will be incubated for another 30 minutes at 37 ºC. After 30 minutes, 50µl haemolytic system was added into the test plate and anti-complementary plates then incubated again for 30 minutes in the shaker. The breakdown for CFT is as follows: 90 min sample preparation, 60 min blood wash and antigen and buffer preparation, 30 min incubation, 15 min titration and antigen dispensing, 30 min incubation, 10 min HS dispensing, 30 min incubation, 3 min centrifuge and 15 min interpretation of the results. The total amount of time it would take to perform CFT would be 14 hours. The process of performing the test normally takes 2 days to complete. Recording of the results and capturing them in the system will take another day. The overall assay would take about a week including signing of the results and sending them back to the State veterinarian.

FPA: The breakdown for the FPA was as follows: 10 min sample preparation, 5 min buffer preparation, 5 min sample and buffer dispensing, 6 min shaking and incubation, 4 min blank reader, 2 min tracer and 3 min sample interpretation.

The total amount of time it would take to perform the FPA test would be 35 minutes' maximum. The kit's manufacturer has informed the researcher and the supervisors about the latest automated version of the PHERAstar reader (BMGPHERAstar) which seems to be more rapid. You only dispense 25 µl of sample into the 96 well plate, load the plate into the machine. The FPA reader adds the sample diluent and tracer. Results are calculated by Gen 5 software. With this system, it would take approximately 10-15 minutes to perform the whole FPA test.

CHAPTER 5: DISCUSSION

In the present study, six samples that tested positive with RBT and CFT tested negative with FPA. This may indicate false positive with RBT and CFT as false positive can occur related to the condition of the serum samples or vaccination status of animals. Fifteen samples that tested negative with all three tests. Currently, the Rose Bengal test is used as a rapid screening test. Only samples testing positive on RBT are then processed further to be confirmed with CFT. A study by Kangumba (2015) reported that the RBT could show false- positive results because of S19 vaccination or non-specific serological reactions (Kangumba, 2015). A study by Geresu and Kassa (2016) reported that RBT has some disadvantages of low sensitivity particularly in chronic cases, relatively low specificity in endemic areas and prozones that cause strongly positive sera to test negative. Therefore, positive samples are missed from the onset when relying on RBT as a screening test that determines the confirmation with CFT. Furthermore, eight samples that tested positive with RBT and CFT reacted as suspect with FPA.

Two hundred and sixty-two positive samples tested positive in four serological assays (RBT, SAT, CFT, FPA). Only one sample from known negative herd tested positive for all three serological tests. Strongly positive sera can lead to false negative on RBT. That could explain why some samples that tested negative on RBT tested positive with FPA.

The optimum cut-off value for FPA offering the highest ‘‘performance index’’ (sum of DSn and DSp) was determined by ROC analysis in two ways, one using as positive reference sera originating from positive animals as confirmed by CFT *(n=347)* and sera from negative herds testing negative on RBT and SAT (*n=446*). Using the two different panels of positive and negative reference sera, the accuracies of all tests were assessed from AUC values calculated by ROC analysis, as well as their performance from determination of Youden’s J value, which is the indicator of the test’s DSn and DSp sum, by cross-tabulation (Armitage and Berry, 1994). The FPA cut-off value used as positive (originated from positive animals confirmed by CFT) was determined at 87.25 mP, this value is almost similar with the one determined by (Nielsen *et al*., 2005) in an evaluation study for FPA’s performance on goat sera. At this cut-off, DSp and DSn were determined at 99.09% - 100% and 68.09% - 76.61% respectively with a 95% confidence interval (cl) respectively.

The DSp did not differ significantly from DSp of 98.6, 99.4 and 98.9% found in sheep and goats studies (Nielsen *et al*., 2001and Nielsen *et al*., 2005), while DSn is higher than 91.5, 94.9 and 88.7% found in the same studies.

The lowest concentration of FPA in serum was not performed due to the computers at Onderstepoort being replaced with the new ones that caused the loss of FPA software and the dilution of FPA was not performed. All data were entered manually into a Microsoft Excel spread sheet for data cleaning analysis and it was carried out using a Graph pad prism and Statistical package for the social science that reduced the number of positive sera (*n=493 to n=347*) and negative sera (*n=532 to n= 446*) due to insufficient tracer dispensed in wells.

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

The prevention and control of brucellosis in domestic livestock is by vaccination. The use of *B. abortus* strain S19 that remain the reference vaccine in cattle, and it has extremely reduced its incidence in many endemic areas. Vaccination of livestock increases the value, productivity and induces good immunity of the animal. It does not only improve the health status of the animals but also is an important step to reduce the risk of severe illness and the transmission to human population (Bano and Lone, 2015)

According to our criteria for positive samples, there should be at least one culture positive animal from the herd, but it was difficult to get samples from known positive herds confirmed by *Brucella* bacterial isolation. It appeared that the job of animal health technicians most of the time ends after branding the positive serological reactors. Consultations with Dr. Grobler, the State veterinarian in Pretoria regarding the negative herds that did not show reaction for the past 1 year (as per our criteria) were unfruitful because it was difficult to trace back the animals as some of the farmers had sold those animals and introduced new animals in the herd.

Due to these challenges, it was decided to evaluate the status of the herds according to the serological profile. Herds that had many serological reactors with several high titres were classified as serologically positive herds. Negative herds were classified based on negative RBT and SAT results.

The turnaround time of FPA is unrivalled for speed, with test completion in as little as 35 minutes, requiring no washing steps or long incubation. As a one-step solution, it can be performed in a laboratory or field setting with few reagents required. FPA has a leading performance yielding quantitative results. The sensitivity of FPA was greater and specificity was lower than those of the tests currently in use.

Future perspectives

- Collaboration with general bacteriology will focus on tracing animals that have been identified as positive after isolation of *Brucella spp*.
- At least one publication in an accredited journal is expected from this study.
- The kit's manufacturer has informed us about the latest automated version of the

PHERAstar reader (BMGPHERAstar) which seems to be more rapid as it is fully automated as mentioned in 4.8 above, turnaround time of FPA.

- Fluorescence Polarization Assay should FPA be given a chance as a diagnostic test in South Africa, it will be an asset to brucellosis control programs. The FPA is relatively preferable compared to the other test that were used and has other advantages, including its accuracy, less time to perform the assay, electronic data entered into an excel worksheet meaning quick results will be obtained, and a record of results can be printed and sent directly to the state veterinary (Gall *et al*., 2001).
- Validation is an ongoing activity that is crucial to all accredited laboratories, and after the completion of this study, laboratories will continue with validation especially if the test is approved to be used in South Africa.

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8. APPENDICES

Appendix A

UNISA university of south africa

UNISA CAES ANIMAL RESEARCH ETHICS COMMITTEE

Date: 11/06/2018

NHREC Registration # : N/A
REC Reference # : 2018/CAES/075
Name : Ms BI Skosana
Student #: 46317848

Dear Ms Skosana

Decision: Ethics Approval from 08/06/2018 to 31/05/2019

Researcher(s): Ms BI Skosana
46317848@mylife.unisa.ac.za

Supervisor (s): Dr P Kayoka-Kabongo
kaponpnk@unisa.ac.za; 011-471-2949

Prof FT Tabit
tabitft@unisa.ac.za; 011-471-2080

Dr A Potts
PottsA@arc.agric.za; 012-529-9396

Working title of research:

Validation of the fluorescence polarization assay (FPA) for the diagnosis of bovine brucellosis

Qualification: MSc Agriculture

Thank you for the application for research ethics clearance by the Unisa CAES Animal Research Ethics Committee for the above mentioned research. Ethics approval is granted for a one-year period, **subject to submission of the Section 20 approval**. After one year the researcher is required to submit a progress report, upon which the ethics clearance may be renewed for another year.

Due date for progress report: 31 May 2019

Please note the following for further action:

1. The research requires Section 20 approval and this must be submitted to the Committee once obtained.

University of South Africa
Preller Street, Muckleneuk Ridge, City of Tshwane
PO Box 392 UNISA 0003 South Africa
Telephone: +27 12 429 3111 Facsimile: +27 12 429 4150
www.unisa.ac.za

*The **medium risk application** was **reviewed** by the CAES Animal Research Ethics Committee on 08 June 2018 in compliance with the Unisa Policy on Research Ethics and the Standard Operating Procedure on Research Ethics Risk Assessment.*

The proposed research may now commence with the provisions that:

1. The researcher(s) will ensure that the research project adheres to the values and principles expressed in the UNISA Policy on Research Ethics.
2. Any adverse circumstance arising in the undertaking of the research project that is relevant to the ethicality of the study should be communicated in writing to the Committee.
3. The researcher(s) will conduct the study according to the methods and procedures set out in the approved application.
4. Any changes that can affect the study-related risks for the research participants, particularly in terms of assurances made with regards to the protection of participants' privacy and the confidentiality of the data, should be reported to the Committee in writing, accompanied by a progress report.
5. The researcher will ensure that the research project adheres to any applicable national legislation, professional codes of conduct, institutional guidelines and scientific standards relevant to the specific field of study. Adherence to the following South African legislation is important, if applicable: Protection of Personal Information Act, no 4 of 2013; Children's act no 38 of 2005 and the National Health Act, no 61 of 2003.
6. Only de-identified research data may be used for secondary research purposes in future on condition that the research objectives are similar to those of the original research. Secondary use of identifiable human research data require additional ethics clearance.
7. No field work activities may continue after the expiry date. Submission of a completed research ethics progress report will constitute an application for renewal of Ethics Research Committee approval.

Note:
*The reference number **2018/CAES/075** should be clearly indicated on all forms of communication with the intended research participants, as well as with the Committee.*

Yours sincerely,

University of South Africa
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Telephone: +27 12 429 3111 Facsimile: +27 12 429 4150
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Prof EL Kempen

Chair of CAES Animal REC

E-mail: kempeel@unisa.ac.za

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Prof MJ Linington

Executive Dean : CAES

E-mail: lininmj@unisa.ac.za

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Appendix B

ARC • LNR
Excellence in Research and development

Dr. A Potts
Tel: Enquiries Navrae
Fax: (012) 5299429
Email: PottsA@arc.agric.za

Ref. no. / Verw. nr.

ARC-ONDERSTEPOORT VETERINARY INSTITUTE
LNR-ONDERSTEPOORT VEEARTSENYKUNDE-INSTITUUT
Private Bag / Privaatsak X05, Onderstepoort 0110, South Africa / Suid-Afrika
Tel: (012) 529-9111 · Fax: (012) 565-6573 (Int: +27 12)
E-Mail: ovi-info@arc.agric.za · Web site: www.arc.agric.za

30 August 2018

The Director: Animal Health

Re: Amendment to Section 20 approved project: Validation and improvement of methods for diagnosis of bovine brucellosis (Ref. No.: 12/11/1/19

I would like to apply for an amendment to the above project as follows:

Researchers

Dr Evelyn Madoroba to be removed. Dr AD Potts (Senior Researcher: ARC-OVI), Dr PN Kayoka-Kabongo (Senior Lecturer: UNISA) and Ms I Skosan (UNISA MSc student) to be added.

Specific objectives

While the aim of the study - to improve the diagnosis of brucellosis in South Africa through the validation of the Fluorescence Polarization Assay (FPA) - remains the same, Objective 3 of the study, to compare FPA results to those obtained using phage display random peptides library and/or Enzyme Linked Immunosorbent Assay, will no longer be done.

Sampling

Blood will no longer be collected from all nine provinces of South Africa, but several provinces including Gauteng and Northwest. The number of samples to be collected will be as follows: 500 samples from positive herds and 500 from negative herds.

Testing

The SAT will no longer be used as a comparative test to the FPA only the RBT and CFT.

Yours sincerely

Dr AD Potts
HOL: Bacterial Serology Laboratory
For Senior Manager Research: Animal Health and Protection

Appendix C

AEC 6.16

APPROVED

ARC • LNR
Excellence in Research and Development

ONDERSTEPOORT VETERINARY INSTITUTE
ANIMAL ETHICS COMMITTEE

Application for clearance to use vertebrate animals (including their embryos and foetuses) for an experimental study or in a standard operating procedure for diagnostic purposes

Project No: 30/02/D007

Project Leader: Dr Evelyn Madoroba

Project Title: Validation and improvement of methods for diagnosis of bovine brucellosis

Where clearance is sought for a standard operating procedure (SOP), a separate clearance form must be completed per procedure

NOTE:

- Please read the Animal Ethics Categories form before you complete this application.
- This application must be typed.
- It must be signed by the Principal Investigator (the applicant) and other persons who are vouching for specialised aspects of the experimental design (i.e. statistician, safety officer, and persons responsible for supervising the use of scheduled medicinal substances).
- The application needs to be written simply but include all relevant detail.
- A score sheet must accompany all applications.
- If any animal during the experimental period gets sick/dies due to causes not related to the experimental work, a morbidity/mortality report must be submitted to the AEC accompanied by a full post mortem report.
- Once the experimental work starts a copy of the score sheet and a summary of the experimental work conducted must be visible at the experimental facilities in case of inspections by the AEC.
- Submitting applications:
  - An electronic copy of the application should be emailed to LopezL@arc.agric.za and a signed hard copy mailed to Dr Laura Lopez, Chairperson of the Onderstepoort Veterinary Institute – Animal Ethics Committee (OVI-AEC), Diagnostic Registration (Tel: 012-5299272).
  - **Deadline** for researchers to submit protocols will be the **1st of each month**. Late protocols go through to the next month.
    - If all the relevant signatures are present on the hard copies they will be distributed electronically to all AEC members for review and comments. Comments from AEC members will be submitted to Dr Lopez by the 10th of the month.
    - Dr Lopez will collate all the comments and send them back to the AEC members and applicants after the monthly AEC meeting.
    - Revised applications must be submitted by the 20th of the month.
    - AEC meetings will be held on the third Thursday of each month (except December) where all protocols and comments will be discussed, and final committee approval (or not) given, and communicated to applicants asap.
- Telephone enquiries on any animal ethics related matters may be directed to the Chairperson, Dr Laura Lopez (Tel: 012-529 9272, or LopezL@arc.agric.za).

Appendix D

agriculture and
rural development
Department: Agriculture and Rural Development
GAUTENG PROVINCE

Diagonal Building, Diagonal Street, Johannesburg
P O Box 8769, Johannesburg, 2000
Telephone: (011) 240 2526
Email: gdard@gauteng.gov.za
Website: http://www.gdard.gpg.gov.za

CAES Research Ethics review committee

RE: APPROVAL FOR BLOOD SAMPLES TO BE USED FOR STUDENT MASTER'S PROJECT ENTITLED: "VALIDATION OF THE FLUORESCENCE POLARIZATION ASSAY (FPA) FOR THE DIAGNOSIS OF BOVINE BRUCELLOSIS

The validation of fluorescence polarization assay is a national project to improve the current diagnosis of Brucellosis in the country.

Routine samples collected are submitted to Onderstepoort Veterinary Institute, Serology laboratory, which is the OIE reference laboratory for Brucellosis.

A Master's degree award has been one of the outcomes of this project during which a student will learn the laboratory techniques.

I have no objection that Ms B. I. Skosana, registered with UNISA, will be allowed to use samples collected by Gauteng veterinary services for her project. It should be under the control of the OVI (Dr Potts).

Yours sincerely

Dr J Walters
Deputy Director Animal Health
Veterinary Services
Gauteng

Date: 07/05/2018

Appendix E

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1

Dr Prudence Kayoka-Kabongo
ARC-OVR
Onderstepoort
Tel: 011 471 2949
E-mail: Kabonpnk@unisa.ac.za
Cc: Dr Andrew Potts - PottsA@arc.agric.za

RE: AMENDMENT OF SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) FOR: "VALIDATION AND IMPROVEMENT OF METHODS FOR DIAGNOSIS OF BOVINE BRUCELLOSIS"

An amendment is hereby granted on the Section 20 approval that was issued for the above mentioned study on 14 January 2016, together with the amendment dated 17 September 2018:

i) We have taken note and approved of the amendments made to the project in the letter dated 13 December 2018 regarding the following:
   a. Objective 3 of the study to "compare FPA results to those obtained using phage display random peptides library and/or Enzyme Linked Immunosorbent Assay" will be included;
   b. The SAT will also be included as a comparative test to the FPA in addition to the RBT, CFT and ELISA;

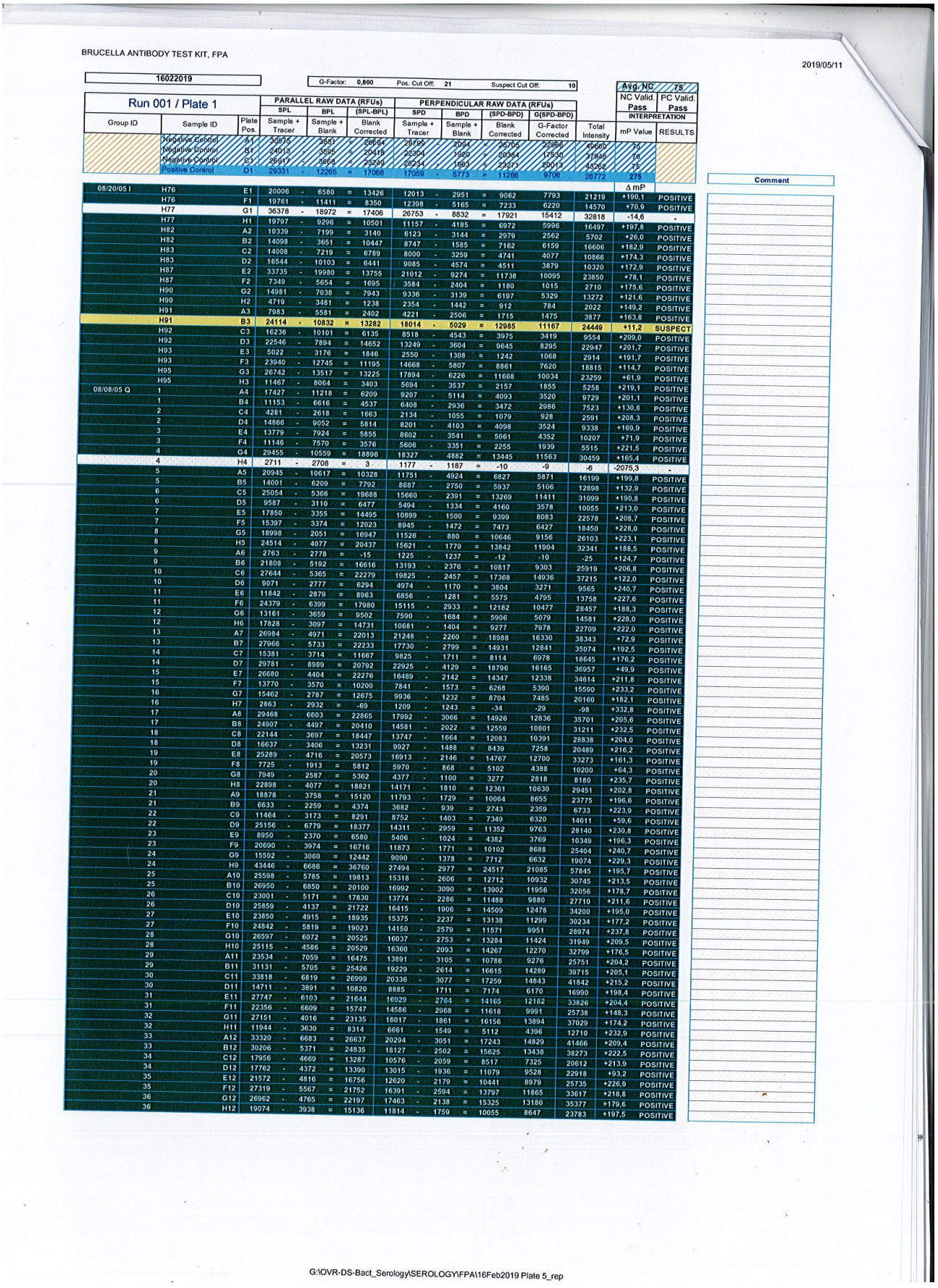
ii) As requested, brucellosis FPA and iELISA kits may be utilized for the study on the condition that a valid veterinary import permit is obtained and the conditions stipulated therein are strictly adhered to;

iii) All conditions as specified in the Section 20 approval of 14 January 2016 and the amendment of 17 September 2018 remain in effect.

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2018 -12- 1 4

Appendix F



BRUCELLA ANTIBODY TEST KIT, FPA

2019/05/11

16022019

G-Factor: 0,860 Pos. Cut Off: 21 Suspect Cut Off: 10

Avg. NC 75

NC Valid. Pass | PC Valid. Pass

Run 001 / Plate 1

Group ID	Sample ID	Plate Pos.	PARALLEL RAW DATA (RFUs) SPL Sample + Tracer	BPL Sample + Blank	(SPL-BPL) Blank Corrected	PERPENDICULAR RAW DATA (RFUs) SPD Sample + Tracer	BPD Sample + Blank	(SPD-BPD) Blank Corrected	G(SPD-BPD) G-Factor Corrected	Total Intensity	INTERPRETATION mP Value	RESULTS
	Negative Control	A1	30875 - 5581 = 25294			28795 - 2094 = 26705			22866	45560	75	
	Negative Control	B1	24013 - 3595 = 20418			22304 - 1940 = 20364			17530	37948	76	
	Negative Control	C1	26917 - 3668 = 23249			25234 - 1693 = 23271			20013	43262	75	
	Positive Control	D1	29331 - 12265 = 17066			17059 - 5773 = 11286			9706	26772	275	

Comment

												Δ mP	
08/20/05 I	H76	E1	20006 - 6580 = 13426			12013 - 2951 = 9062			7793	21219	+190,1	POSITIVE
	H76	F1	19761 - 11411 = 8350			12398 - 5165 = 7233			6220	14570	+70,9	POSITIVE
	H77	G1	36378 - 18972 = 17406			26753 - 8832 = 17921			15412	32818	-14,6	-
	H77	H1	19797 - 9296 = 10501			11157 - 4185 = 6972			5996	16497	+197,8	POSITIVE
	H82	A2	10339 - 7199 = 3140			6123 - 3144 = 2979			2562	5702	+26,0	POSITIVE
	H82	B2	14098 - 3651 = 10447			8747 - 1585 = 7162			6159	16606	+182,9	POSITIVE
	H83	C2	14008 - 7219 = 6789			8000 - 3259 = 4741			4077	10866	+174,3	POSITIVE
	H83	D2	16544 - 10103 = 6441			9085 - 4574 = 4511			3879	10320	+172,9	POSITIVE
	H87	E2	33735 - 19980 = 13755			21012 - 9274 = 11738			10095	23850	+78,1	POSITIVE
	H87	F2	7349 - 5654 = 1695			3584 - 2404 = 1180			1015	2710	+175,6	POSITIVE
	H90	G2	14981 - 7038 = 7943			9336 - 3139 = 6197			5329	13272	+121,6	POSITIVE
	H90	H2	4719 - 3481 = 1238			2354 - 1442 = 912			784	2022	+149,2	POSITIVE
	H91	A3	7983 - 5581 = 2402			4221 - 2506 = 1715			1475	3877	+163,8	POSITIVE
	H91	B3	24114 - 10832 = 13282			18014 - 5029 = 12985			11167	24449	+11,2	SUSPECT
	H92	C3	16236 - 10101 = 6135			8518 - 4543 = 3975			3419	9554	+209,0	POSITIVE
	H92	D3	22546 - 7894 = 14652			13249 - 3604 = 9645			8295	22947	+201,7	POSITIVE
	H93	E3	5022 - 3176 = 1846			2550 - 1308 = 1242			1068	2914	+191,7	POSITIVE
	H93	F3	23940 - 12745 = 11195			14668 - 5807 = 8861			7620	18815	+114,7	POSITIVE
	H95	G3	26742 - 13517 = 13225			17894 - 6226 = 11668			10034	23259	+61,9	POSITIVE
	H95	H3	11467 - 8064 = 3403			5694 - 3537 = 2157			1855	5258	+219,1	POSITIVE
08/08/05 Q	1	A4	17427 - 11218 = 6209			9207 - 5114 = 4093			3520	9729	+201,1	POSITIVE
	1	B4	11153 - 6616 = 4537			6408 - 2936 = 3472			2986	7523	+130,8	POSITIVE
	2	C4	4281 - 2618 = 1663			2134 - 1055 = 1079			928	2591	+208,3	POSITIVE
	2	D4	14866 - 9052 = 5814			8201 - 4103 = 4098			3524	9338	+169,9	POSITIVE
	3	E4	13779 - 7924 = 5855			8602 - 3541 = 5061			4352	10207	+71,9	POSITIVE
	3	F4	11146 - 7570 = 3576			5606 - 3351 = 2255			1939	5515	+221,5	POSITIVE
	4	G4	29455 - 10559 = 18896			18327 - 4882 = 13445			11563	30459	+165,4	POSITIVE
	4	H4	2711 - 2708 = 3			1177 - 1187 = -10			-9	-6	-2075,3	-
	5	A5	20945 - 10617 = 10328			11751 - 4924 = 6827			5871	16199	+199,8	POSITIVE
	5	B5	14001 - 6209 = 7792			8687 - 2750 = 5937			5106	12898	+132,9	POSITIVE
	6	C5	25054 - 5366 = 19688			15660 - 2391 = 13269			11411	31099	+190,8	POSITIVE
	6	D5	9587 - 3110 = 6477			5494 - 1334 = 4160			3578	10055	+213,0	POSITIVE
	7	E5	17850 - 3355 = 14495			10899 - 1500 = 9399			8083	22578	+208,7	POSITIVE
	7	F5	15397 - 3374 = 12023			8945 - 1472 = 7473			6427	18450	+228,0	POSITIVE
	8	G5	18998 - 2051 = 16947			11526 - 880 = 10646			9156	26103	+223,1	POSITIVE
	8	H5	24514 - 4077 = 20437			15621 - 1779 = 13842			11904	32341	+188,5	POSITIVE
	9	A6	2763 - 2778 = -15			1225 - 1237 = -12			-10	-25	+124,7	POSITIVE
	9	B6	21808 - 5192 = 16616			13193 - 2376 = 10817			9303	25919	+206,8	POSITIVE
	10	C6	27644 - 5365 = 22279			19825 - 2457 = 17368			14936	37215	+122,0	POSITIVE
	10	D6	9071 - 2777 = 6294			4974 - 1170 = 3804			3271	9565	+240,7	POSITIVE
	11	E6	11842 - 2879 = 8963			6856 - 1281 = 5575			4795	13758	+227,6	POSITIVE
	11	F6	24379 - 6399 = 17980			15115 - 2933 = 12182			10477	28457	+188,3	POSITIVE
	12	G6	13161 - 3659 = 9502			7590 - 1684 = 5906			5079	14581	+228,0	POSITIVE
	12	H6	17828 - 3097 = 14731			10681 - 1404 = 9277			7978	22709	+222,0	POSITIVE
	13	A7	26984 - 4971 = 22013			21248 - 2260 = 18988			16330	38343	+72,9	POSITIVE
	13	B7	27966 - 5733 = 22233			17730 - 2799 = 14931			12841	35074	+192,5	POSITIVE
	14	C7	15381 - 3714 = 11667			9825 - 1711 = 8114			6978	18645	+176,2	POSITIVE
	14	D7	29781 - 8989 = 20792			22925 - 4129 = 18796			16165	36957	+49,9	POSITIVE
	15	E7	26680 - 4404 = 22276			16489 - 2142 = 14347			12338	34614	+211,8	POSITIVE
	15	F7	13770 - 3570 = 10200			7841 - 1573 = 6268			5390	15590	+233,2	POSITIVE
	16	G7	15462 - 2787 = 12675			9936 - 1232 = 8704			7485	20160	+182,1	POSITIVE
	16	H7	2863 - 2932 = -69			1209 - 1243 = -34			-29	-98	+332,8	POSITIVE
	17	A8	29468 - 6603 = 22865			17992 - 3066 = 14926			12836	35701	+205,6	POSITIVE
	17	B8	24907 - 4497 = 20410			14581 - 2022 = 12559			10801	31211	+232,5	POSITIVE
	18	C8	22144 - 3697 = 18447			13747 - 1664 = 12083			10391	28838	+204,0	POSITIVE
	18	D8	16637 - 3406 = 13231			9927 - 1488 = 8439			7258	20489	+216,2	POSITIVE
	19	E8	25289 - 4716 = 20573			16913 - 2146 = 14767			12700	33273	+161,3	POSITIVE
	19	F8	7725 - 1913 = 5812			5070 - 868 = 5102			4388	10200	+64,3	POSITIVE
	20	G8	7949 - 2587 = 5362			4377 - 1100 = 3277			2818	8180	+235,7	POSITIVE
	20	H8	22898 - 4077 = 18821			14171 - 1810 = 12361			10630	29451	+202,8	POSITIVE
	21	A9	18878 - 3758 = 15120			11793 - 1729 = 10064			8655	23775	+196,6	POSITIVE
	21	B9	6633 - 2259 = 4374			3682 - 939 = 2743			2359	6733	+223,9	POSITIVE
	22	C9	11464 - 3173 = 8291			8752 - 1403 = 7349			6320	14611	+59,6	POSITIVE
	22	D9	25156 - 6779 = 18377			14311 - 2959 = 11352			9763	28140	+230,8	POSITIVE
	23	E9	8950 - 2370 = 6580			5406 - 1024 = 4382			3769	10349	+196,3	POSITIVE
	23	F9	20690 - 3974 = 16716			11873 - 1771 = 10102			8688	25404	+240,7	POSITIVE
	24	G9	15502 - 3060 = 12442			9090 - 1378 = 7712			6632	19074	+229,3	POSITIVE
	24	H9	43446 - 6686 = 36760			27494 - 2977 = 24517			21085	57845	+195,7	POSITIVE
	25	A10	25598 - 5785 = 19813			15318 - 2606 = 12712			10932	30745	+213,5	POSITIVE
	25	B10	26950 - 6850 = 20100			16992 - 3090 = 13902			11956	32056	+178,7	POSITIVE
	26	C10	23001 - 5171 = 17830			13774 - 2286 = 11488			9880	27710	+211,6	POSITIVE
	26	D10	25859 - 4137 = 21722			16415 - 1906 = 14509			12478	34200	+195,0	POSITIVE
	27	E10	23850 - 4915 = 18935			15375 - 2237 = 13138			11299	30234	+177,2	POSITIVE
	27	F10	24842 - 5819 = 19023			14150 - 2579 = 11571			9951	28974	+237,8	POSITIVE
	28	G10	26597 - 6072 = 20525			16037 - 2753 = 13284			11424	31949	+209,5	POSITIVE
	28	H10	25115 - 4586 = 20529			16360 - 2093 = 14267			12270	32799	+176,5	POSITIVE
	29	A11	23534 - 7059 = 16475			13891 - 3105 = 10786			9276	25751	+204,2	POSITIVE
	29	B11	31131 - 5705 = 25426			19229 - 2614 = 16615			14289	39715	+205,1	POSITIVE
	30	C11	33818 - 6819 = 26999			20336 - 3077 = 17259			14843	41842	+215,2	POSITIVE
	30	D11	14711 - 3891 = 10820			8885 - 1711 = 7174			6170	16990	+198,4	POSITIVE
	31	E11	27747 - 6103 = 21644			16929 - 2764 = 14165			12182	33826	+204,4	POSITIVE
	31	F11	22356 - 6609 = 15747			14586 - 2968 = 11618			9991	25738	+148,3	POSITIVE
	32	G11	27151 - 4016 = 23135			18017 - 1861 = 16156			13894	37029	+174,2	POSITIVE
	32	H11	11944 - 3630 = 8314			6661 - 1549 = 5112			4396	12710	+232,9	POSITIVE
	33	A12	33320 - 6683 = 26637			20294 - 3051 = 17243			14829	41466	+209,4	POSITIVE
	33	B12	30206 - 5371 = 24835			16127 - 2502 = 15625			13438	38273	+222,5	POSITIVE
	34	C12	17956 - 4669 = 13287			10576 - 2059 = 8517			7325	20612	+213,9	POSITIVE
	34	D12	17762 - 4372 = 13390			13015 - 1936 = 11079			9528	22918	+93,2	POSITIVE
	35	E12	21572 - 4816 = 16756			12620 - 2179 = 10441			8979	25735	+226,9	POSITIVE
	35	F12	27319 - 5567 = 21752			16391 - 2594 = 13797			11865	33617	+218,8	POSITIVE
	36	G12	26962 - 4765 = 22197			17463 - 2138 = 15325			13180	35377	+179,6	POSITIVE
	36	H12	19074 - 3938 = 15136			11814 - 1759 = 10055			8647	23783	+197,5	POSITIVE

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