A COMPARISON BETWEEN MOLECULAR AND SEROLOGICAL METHODS IN THE DETECTION OF BOVINE *BRUCELLA* SPECIES

By

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APPROVAL FORM

This is to certify that that the dissertation entitled "a comparison between molecular and serological methods in the detection of bovine *Brucella*", submitted in partial fulfillment of the requirements for Bachelor of Science Honors Degree in Biological Sciences at Midlands State University, is a record of the original research carried out by Zanele Nkomo R121460g under my supervision and no part of the dissertation has been submitted for any other degree or diploma.

The assistance and the help received during the course of this research have been duly acknowledged. Therefore I recommend that I will be accepted as fulfilling the dissertation requirements.

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Name of supervisor

Signature……..

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ABSTRACT

Bovine brucellosis is endemic in Zimbabwe and is an important veterinary disease, responsible for large losses in livestock through abortions and still-born calves. It is also a zoonotic disease and as such poses a threat to public health. For this reason, it is important for every farmer to know the status of their herd. This study was carried out to compare the reliability of serological tests with molecular test in the detection of bovine brucellosis. A total of 17 whole blood and serum samples were collected from a herd of cattle in Matabeleland that had previously been vaccinated. This study was conducted in July 2015. The serum samples were tested for brucellosis serologically using the Complement Fixation test (CFT). DNA was extracted from the whole blood samples and the Polymerase Chain Reaction (PCR) was used to test for *Brucella* using *Brucella* specific primers. Test results from serological and molecular methods were then compared. Serological tests showed that all samples were positive for brucellosis using CFT. Using molecular methods, only 12 samples were found positive while 5 samples were negative for the *Brucella* bacteria. In this study, it was possible to differentiate the species as well as the biovars of *Brucella* using PCR which was not possible using serological techniques. Samples that were positive for *Brucella* were either *B. abortus* biovars (1, 2, 4) which produced a 100 bp and 498 bp bands or *B. abortus* biovars (3, 5, 6, 9) / *B. suis* (2, 3, 4, 5) which produced a 100 bp band. Further differentiation between *B. abortus* (3, 5, 6, 9) and *B. suis* (2, 3, 4, 5) could not be done due to a limitation in laboratory resources. The study confirms PCR to be a more reliable and specific tool for the detection of *Brucella* spp. when compared to the conventional CFT method of detecting brucellosis used in Zimbabwe. The use/adoption of PCR as a confirmatory test in the testing of Brucellosis is recommended in this study.

DEDICATION

In memory of, my loving father, the late Mr. Norman Nkomo. You are greatly missed.

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LIST OF ABBREVIATIONS

PBS – Phosphate Buffer Solution

TE – Tris-EDTA

TBE - Tris Borate-EDTA

PCR – Polymerase Chain Reaction

CFT- Complement Fixation Test

CHAPTER ONE: INTRODUCTION

1.1 Background of study

Brucellosis is a globally distributed and important zoonotic disease that is caused by a Gram-negative bacterium belonging to the genus *Brucella* (Corbel, 2006) . This bacterium can be easily transmitted among domesticated animals which include cattle, sheep, goats, pigs, dogs and buffalo (Corbel, 2006). Brucellosis has important effects on both animals and humans and the most common clinical symptoms of the disease in animals include abortions, placentitis, epididymitis and orchitis. In humans, brucellosis is an impairing and recurrent disease which can affect different organs. This has resulted in it being recognized as an occupational hazard for people who work closely with infected and susceptible animals and their products such as veterinarians, livestock breeders, laboratory technicians, abattoir workers (Gomo *et al.* 2012). As such, infection of animals with brucellosis has resulted in large economic losses in animal production as a consequence of abortion, sterility, and reduction in milk production as well as the cost of culling infected animals (Gwida *et al*. 2011).

The Office International des Epizooites (OIE) has classified brucellosis as an important disease because of its implications on international trade and public health (OIE, 2002). The surveillance and control of the disease are the first steps to be taken in its eradication and it has been hard to estimate the worldwide incidence of brucellosis because of its low levels of inspection and reporting. However, brucellosis was found to be endemic in Sub-Saharan countries, including Zimbabwe (Bevan, 1931).

The detection of brucellosis is usually done using a combination of different methods which include bacteriological, serological or molecular methods. Definitive diagnosis has been normally done by isolation and culture but this method is however both time-consuming and a

risk to laboratory personnel. Although serological techniques are normally preferred, they have the limitation that cross reactions as well as vaccinal antibodies can cause non specific diagnosis. Modern genetic characterization of *Brucella* using molecular DNA technology such as PCR have been developed but have not yet been implemented in most countries, including Zimbabwe. A comparison between the main detection method used in Zimbabwe i.e. serology and PCR, may therefore help to determine which test is most definitive and should be used (Akhtar *et. al*., 2008). This study was carried out in response to a farmer in Matabeleland North province in Zimbabwe, whose herd had serious cases of abortion although they had been previously vaccinated against brucellosis. This uncertainty in the herd's status poses a threat to the livestock and so another method of detection was used to confirm the results

1.2 Problem statement

Brucellosis is prevalent in all major livestock production systems throughout the world including sub-Saharan countries yet its presence is often unrecognized because of lack of awareness by veterinarians and health care members as well as little or no access to laboratory facility diagnostic facilities (Bishop *et. al*, 1994). This is a great cause of concern as *Brucella* is a threat to the economy and is also a life-threatening disease. Undiagnosed cases of brucellosis in animals result in an increase in the number of people being ignorantly infected by consuming the infected animals' by-products. Therefore, the provision of improved diagnostics is crucial to enable the investigation of brucellosis incidence in sub-Saharan counties including Zimbabwe (Matope *et. al*., 2010).

This study was conducted in response to a concern by a farmer in Matabeleland North in Zimbabwe, whose herd of cattle had serious cases of abortions and still-born calves even after being previously vaccinated against brucellosis. Apparently, the infection status of some

individual cows had been seen to be alternating between negative and positive. This has led to uncertainty and confusion concerning the health of the herd and poses a threat to the livestock herd since infected animals should be culled.

1.3 Justification of study

Brucellosis in dairy cattle is endemic in Zimbabwe and its prevalence continues to be monitored intensively. The incidence of various *Brucella spp.* within Zimbabwe is virtually unknown, despite the high sero positivity reported (Matope *et. al*, 2010). Bovine brucellosis, thus, requires public awareness and further epidemiological studies. As the disease seroprevalence increases in animals, humans are subjected to a greater risk therefore it is essential to monitor and control the *Brucella* pathogen (Matope *et. al*., 2010).

To date, studies that have been performed in the past on the prevalence of brucellosis in Zimbabwe have focused on serological as well as bacteriological detection. Some of the few studies such as those done by Matope (2010) and Gomo (2012) on the use of both techniques to confirm brucellosis diagnostics have in some instances, produced different results. For serological methods, the main disadvantage is the lack of specificity. However, a much more efficient method of detection and identification of the *Brucella* bacteria has been multiplex PCR. This method allows for the identification of *Brucella* at the species level as well at biovars level by amplifying *Brucella* DNA using a combination of primer pairs (Smirnova *et. al*, 2013).

In response to the uncertainty on the infection status of the sampled herd at a farm in Matabeleland, it would be essential to validate the infection status using PCR and then compare with serological results, which is the main method of brucellosis detection in Zimbabwe. This

would provide more confirmatory results as previous studies have shown PCR to have a higher sensitivity than most tests in the detection of brucellosis (Bishop, 2004).

1.4 Objectives of study

1.4.1 Main objectives

- To determine the infection status of a previously vaccinated herd of cattle showing signs of infection using the PCR technique as a diagnostic tool in comparison with conventional serological techniques.

1.4.2 Specific objectives

- To test for brucellosis in bovine serum samples serologically using the Complement fixation test.
- To test for the presence of *Brucella* DNA in the corresponding bovine whole blood samples using Polymerase Chain Reaction.
- To determine the *Brucella* species responsible for brucellosis in the area of study using PCR.
- To differentiate *Brucella* using *Brucella* specific primers at species and biovar level based on their different fragment sizes.

CHAPTER 2: LITERATURE REVIEW

2.1 Brucellosis

Brucellosis is a disease that was named after Sir David Bruce who first isolated *Micrococcus melitensis* from raw goat milk in 1886 (Charters, 1980). It became known as 'undulant fever', 'Mediterranean fever' or 'Malta fever' which is a zoonotic disease that is transmitted invariably by direct or indirect contact with infected animals as well as their products (Corbel, 2006). Other species of *Brucella* include *B. abortus* which was later isolated by Bang in 1897 and *B. suis* which was first described by Traum. These are the most common species of *Brucella* and are of great economic importance. More *Brucella spp.* were discovered which include B. canis, B. ovis, B. neomatae, B. microti as well as B. pinnipedialis and B. ceti which affect marine animals (Poester *et. al*., 2010).

Brucellosis is a chronic disease which affects different species of animals as well as humans. In sexually mature animals, the disease is mainly localized in the reproductive system. It may lead to placentitis and this is normally followed by abortions in pregnant females. This is usually in the last trimester of the pregnancy. It has also been seen to cause a reduction in milk production due to premature births. In males, it can lead to epididymitis and orchitis. In Africa, cattle infected with *brucella spp*. have to been reported to result in the formation of hygromas. This however does not seem to be a consistent symptom. Infection does not always lead to clinical symptoms (Corbel, 2006).

Brucella species are some-what host specific although cross-species infections are not rare especially with *B. melitensis* whose infection of dairy cows can result in severe economic and public health implications. This is because the udder is usually permanently infected especially in cows and goats. This then results in the frequent shedding of the pathogen in the milk (Corbel,

2006). The severity of brucellosis depends on different factors which include previous vaccinations, sex, age and management such as the size of the herd/flock as well as their densities. Abortions are common in unvaccinated animals.

2.1 Pathogenesis of *Brucella*

Bacteria of the genus *Brucella* are intracellular pathogens that are capable of surviving and replicating within phagocytic and non-phagocytic cells, among which macrophages are the target cells of mammalian hosts (Celli, 2005). Cattle are considered the most preferred host for B. abortus. This *brucella* species is classified into seven different biovars namely biovars 1-6 and 9 (Meador and Deyoe, 1989). B. abortus readily penetrates the mucous membrane such as those of the pharynx and the alimentary tract, where it survives and multiples. This occurs mostly in the reticulo-endothelial system (Enright 1990, Thoen and Enright 1986). Following this process, the *Brucella*-containing vacuoles of these cells fuse with the endoplasmic reticulum. Once in here, the bacteria can then establish chronic infection (Starr *et al*. 2008).

2.2 Clinical symptoms and transmission of Brucellosis

Bovine brucellosis is generally a disease that affects cattle. The organism can be isolated from the udder, the uterus and the lymphoid organs of infected animals (Poester et al. 2006, Kudi et al. 1997). Although abortion at the last trimester of the gestation period is considered the most common clinical symptom of the disease, infected cows normally abort once, or may give birth to weak or even healthy calves in the following gestations. Some cows may not have any symptoms of infection at all and give birth to normal calves (Nicoletti, 1980). *B.abortus*, like *B. melitensis*, may lead to a mild or moderate interstitial mastitis, resulting in the intermittent shedding of the pathogen in the milk (Xavier *et al*. 2009). In humans, infection presents itself

with general symptoms such as fever, malaise, sweats and lymphadenopathy. Chronic infection can be associated with arthritis, genitourinal changes, epididymitis and kidney abscesses (Hartigan *et al*. 1997, Colmenero *et al*. 2002).

Transmission of bovine brucellosis occurs mainly after abortion or at birth. Susceptible cattle may have contact with contaminated fetuses, fetal membrane and uterine secretions (Nicoletti, 1980). Infected bulls also play an important role in the transmission of the disease (Eaglesome *et. al.* 1992). The most effective transmission of *Brucella* via inhalation of contaminated dust or gas makes it one of the most common laboratory-acquired infections worldwide (Colmenero *et al*. 2002). Exposure of mucosa or skin bruises to fluid and tissues from aborted fetuses of infected animals or carcass is another important source of infection to humans (Fugier *et. al*. 2007, Hartigan *et al.* 1997).

2.3 Diagnosis of Brucellosis in animals

The diagnosis of *brucella* has been seen to be difficult and this is largely due to its similarity in clinical manifestations with other infections. This means that laboratory testing of the disease is a prerequisite for the proper diagnosis and confirmation of the disease (Maichamo *et. al*., 1998). Even so, the diagnosis of brucellosis in any species is not a trivial matter. The only finite diagnosis is actually the recovery of the causative agent from the host. Because of the inherent problems that come with bacterial isolation which include inefficiency, cost, danger to laboratory personnel and other factors, it seems important for laboratories to consider other more cost effective methods.

Serological methods for the diagnosis of brucellosis have advanced considerably since their inception by Wright and Smith in 1897, the only real problem being some uncertainties

regarding specificity. Molecular biology as a diagnostic tool is advancing and may soon be at a point of replacing bacterial isolation which is considered to be the 'gold standard' for brucellosis diagnosis. However, the perfect test has still not yet been established as the solution to the problems with accurate diagnosis will involve several tests for different functions of the immune response.

2.3.1 Serological methods of detecting Brucellosis

These are examples of indirect methods used in the detection of brucellosis. They are based on the detection of the immune response to bacterial infection. These methods were developed in 1897 and were initially for detection of brucellosis n cattle samples and then later for other domestic animals such as goats and sheep (Godfroid *et. al*., 2010). Serological tests detect antibodies based on the smooth surface LPS which are the immunodominant antigens of *Brucella*. For the specific detection of *B. ovis* and *B. canis*, antibodies against rough LPSs of *Brucella* are used. These methods include agglutination tests, complement fixation tests, precipitation tests as well as primary enzyme-linked immunosorbent assays (Poester *et. al*., 2010; Nielsen, 2002; Samartino *et. al*., 1999).

The first used serological test was the slow agglutination test (SAT). This test is based on the sedimentation of the complexes of IgM antibodies with *Brucella* cell antigens (Alton *et. al*., 1988). This is a slow test which requires overnight incubation of test samples. Although it is an inexpensive and easy test to perform, it lacks specificity and sensitivity. Another common test is the Rose Bengal test which is a simple agglutination test where drops of stained antigen and serum are mixed; any resultant agglutination signifies a positive result. It serves as an good screening test although it may prove to be overly sensitive for the diagnosis of individual animals, particularly those that have been vaccinated.

The complement fixation test provides the detection of anti-*Brucella* antibodies that are able to activate complement (Hill, 1963). It consists of a chain of complex proteins, which if activated by antigen-antibody complex, react to cause cell lysis. However, this test is very difficult to standardize and so this has seen the CFT being replaced by the primary enzyme-linked immunosorbent assay (ELISA). This test is based on the specific binding of antibodies in the test sample with immobilized antigen.

The smooth LPS antibodies that are used in these tests have a common disadvantage which is that the O-polysaccharides of *Brucella* are similar to those of bacteria known as *Yersinia enterocolitica* as well as other bacteria. This may lead to false positives and as such reduces the specificity of the test (Munoz *et. al*., 2005). This problem is partly solved in the competitive ELISA (cELISA) where there are specific epitopes of *Brucella* O-polysaccharides which serve as antigens.

The *Brucella* specific seropositive response is confirmation of infection but it does not provide any information about the type of *Brucella* species or that the animal is actually infected at the time of sample collection since antibody titer of the animal can be high for a long time, especially after the acute phase of the disease (Poester *et. al*., 2010).

2.3.2 Molecular methods of detecting brucellosis

In order to avoid difficulties with other methods used to test brucellosis, often the polymerase chain reaction (PCR) amplification has been used for the successful identification and biotyping of *Brucella spp*. (Yu *et. al*., 2010). The first and most crucial step involved in PCR is the correct and accurate isolation of DNA from biological samples. This is because the quality of the DNA has a significant impact on the sensitivity of the method (Baddour and Alkhalifa, 2008; Dauphin

et. al., 2009). Initially, PCR was developed for the determination of bacterial isolates but with time it has been used for the identification of *Brucella* species in human and animal clinical samples. The simplest method of identifying *Brucella* is PCR with single pairs of primers which are specific to bacterial DNA sequences such as 16S-23S rRNA operon, IS711 OF BCSP31 genes (Godfroid *et. al*., 2010; Baddour and Alkhalifa, 2008). A combination of primers such as BCSP31, OMP2B, OMP2A, OMP31 genes encoding for external membrane proteins can also be used to identify four *Brucella* species: *B. melitensis, B. abortus, B.canis* and *B.suis*(Imaoka *et. al*., 2007).

A more effective method of diagnosis and identification of *Brucella* is multiplex PCR known as AMOS-PCR. This method was developed in 1994 and allows identification of four *Brucella* species *B. abortus, B. melitensis, B. ovis* and *B. suis* hence the name AMOS-PCR for the first four letters of the species. With this method, it is possible to differentiate *B. abortus* biovars 1, 2 and 4, *B. melitensis*, *B. ovis* and *B. suis* biovar 1. This is all based on the polymorphism arising from species-specific localization of the insertion sequence IS711 in the *Brucella* chromosome (Bricker and Halling, 1994).

A new primer was later developed that allowed the identifying of isolates of biovars 3, 5, 6 and 9 of *B. abortus* using the IS711 AMOS primer (Ocampo-Sosa *et. al*., 2005). An improvement of this technique was made by incorporating additional strain specific primers into the primer mix for the identification of the most commonly used vaccine strains which are S19 and RB51(Bricker and Halling ,1995).

Other improvements have made it possible for the amplification and detection of DNA targets simultaneously through different Real-Time PCR methods. The results for this test are obtained

instantly, abbreviating the time taken by each test (Bricker *et. al*., 2004). However, the picture of PCR based tests is not yet complete as more research is needed in the molecular ground to improve the diagnostics of both animals and humans.

2.4 Epidemiology

No accurate figures are available on the prevalence of brucellosis in Southern Africa as most of them are not based on laboratory results. In 2001, the disease was reported to be widespread in Zambia and Malawi. No information on the number of brucellosis cases in Namibia and Mozambique. However, vaccination of 96389 animals in Namibia and 416 in Mozambique was reported by 2001. In the same year, 12 outbreaks involving 17 animals were reported in Botswana and 42675 animals were vaccinated. Due to the development of these countries, modern laboratory techniques are gradually being applied and seroprevalence of brucella is currently under more investigation (Bishop *et. al*., 2004).

In Zimbabwe, seropositivity in domestic as well as in wildlife in Zimbabwe has been recorded as high from previous studies. Surveys conducted in smallholder dairy cattle in Gokwe, Marirangwe, Mushangashe, Nharira, Rusitu and Wedza areas in Zimbabwe between September 2004 and November 2005 showed that of the 203 herds tested,52(25,95%) tested seropositive for brucellosis using Rose Bengal and confirmed by ELISA. Cattle samples from the Malipati and Pesvi in the Chiredzi district showed seroprevalence of 8.3% of the 700 samples collected based on RBT and ELISA. The prevalence of the individual communal areas were 9% (n = 490) and 6.7% (n = 210) in Malipati and Pesvi, respectively. Molecular techniques such as PCR are rarely used due to their relative expense in terms of the reagents required for the tests and as such results are not confirmed this way (Gomo *et. al*., 2013).

A study was carried out at the National Health Institute in 2008 which showed the feasibility of molecular methods as diagnostic tools for the detection of *Brucella* spp. in water buffalo. The results showed that of the 53 *Brucella* seropositive buffaloes, 37 were positive by culture and 33 were positive by PCR (Marianelli *et. al.,* 2008). The combined use of serological, bacteriological and molecular tools may be useful in the detection of *Brucella* spp.

Another study was performed to compare the efficiency of serological tests against PCR in order to identify the most sensitive, rapid and simple tests to detect *Brucella* infection in camels. A total of 895 serum samples were tested from Sudanese camels. Sudan is a well-documented endemic region for brucellosis in both animals and humans. Findings revealed that 84.8% (759/895) of the samples were positive for Brucella DNA by PCR, of which 15.5% (118/759) were serologically positive. A subpopulation of 6.8% of the animals was positive in serological tests but negative in PCR. Seroprevalence of brucellosis should be examined in confirmatory studies so as to evaluate the importance of the disease in both animals and humans (Gwida *et. al*., 2011).

2.5 Control of brucellosis

Although some countries have successfully controlled and eradicated brucellosis, its re-introduction remains a constant threat in many others, especially in developing countries. Despite tremendous efforts and financial investments, many European countries have still yet to eradicate this disease. Many factors contribute to the successful eradication of this disease especially the type of husbandry system that is being practiced. The transmission and spread of brucellosis is also affected by a variety of factors and as such good knowledge of these is essential to the success of a control policy (OIE, 2008). Generally, prevalence of brucellosis is usually higher

and control much more difficult in pastoral or migratory populations which is what is practiced by a significant proportion of agricultural populations in Africa.

Ideally, the effective control of brucellosis should be done through the use of a combination of improved methods of detection, vaccination and treatment together with increased measures of awareness as well as farm sanitation and hygiene. Collectively, this would help lessen the burden of the disease.

McDermott and Arimi (2002) summarized epidemiological findings for brucellosis in sub-Saharan Africa. Brucellosis is more common in cattle than in other small ruminants. Bovine brucellosis prevalence rates range from 3.3% for Central African Republic to as high as 41% in Togo. Values within this range were reported for Chad, Sudan, Eritrea, Tanzania, Ghana, and Zimbabwe. More detailed investigations have shown that the seroprevalence of brucellosis in cattle is very closely related to the husbandry system with the greatest risk being for dairy cattle associated with mixed-breed herds in the state of Asmara in Eritrea (Omer *et. al*., 2000). Nomadism and traditionalism with sharing of males for breeding purposes as an example are also huge factors that contribute of the spread of the disease. Education level, disease knowledge, animal trade, as well as vaccination status have also been identified as factors in other studies (Mikolon *et. al*., 1998). The impact of brucellosis affects both public health and livestock and as such, effective control can be delivered through a unified approach that involves medics, scientists and veterinarians. It is important to target the reservoir of the disease in animals as it is a zoonotic disease. This will require the full cooperation of these parties so that benefits can be clearly demonstrated and communicated.

2.6 Vaccination

Bang, in 1906, observed that vaccinating cattle with live cultures could help protect them against brucellosis. Since then, three strains of *B. abortus* have been used in the preparation of vaccines which are, strain 19, a smooth strain used as a live attenuated vaccine; strain 45/20 (Bishop *et. al.,* 1994), a rough vaccine and more recently, strain RB 51, a rough live attenuated vaccine(Schurig *et. al*., 1991). The minimal requirements for vaccine production are to be followed and each batch of vaccine produced should conform to the minimum standards set by the OIE (OIE, 2013).

Strain 19 vaccine is relatively safe, potent and the practicality of its production and convenient use in cattle has seen it being the most widely used vaccine against bovine brucellosis (Nicoletti, 1990). Vaccination with stain 19 vaccine increases resistance to B. abortus by the animal but does not induce absolute immunity neither is it curative i.e. if an animal is already infected , vaccination will not cure the infection(Nicoletti, 1981). The increase in resistance that follows vaccination is termed 'relative immunity' since it is estimated to be only 70% effective against field challenge by preventing unrestricted multiplication of B. abortus in the uterus and mammary glands(Anon, 1986; Nicoletti, 1980). The main disadvantage of strain 19 vaccination is the induction of post-vaccinal antibodies that are detected in serological tests. There are no single individual tests that can be used to differentiate between antibodies induced by vaccination and those induced by infection (Nielsen and Gail, 2001). Strain 19 must be stored properly to retain its potency. Lyophilization has proved to be the best method of storage for this vaccine (Anon, 1986). The dose of strain 19 vaccine is administered subcutaneously to heifer calves at 4 months to eight months of age which contains $5x10^{10}$ viable *Brucella* cells. This provides life-long relative immunity although most heifers will have lost their antibody titers by

the age 16 to 18 months (Alexander et. al., 1981; Nicoletti, 1980). Vaccination of heifers helps to eliminate the occurrence of abortions in a herd. A reduced-dose vaccine containing $3x10^{8-9}$ organisms/dose in heifers of 4 to 12 months has been seen to provide the same degree of protection as the traditional dose (Anon, 1986; Nicoletti, 1980). The use of the strain 19 vaccine was prohibited in the US after RB51 was approved for use.

The RB51 rough strain has been the official vaccine used in the USA for the prevention of brucellosis since 1996 (Schurig, 2002). Many other countries have approved it as the official vaccine. The use of RB51 was approved for use in South Africa and surrounding countries in 2002. The RB51 strain is a rough rifampicin-resistant strain of *B. abortus* strain 2308(Schurig *et. al.*, 1991). Although this vaccine produces low levels of O-chain (Cloeckaert *et. al*., 1996), it does not usually induce the production of O-chain antibodies that can be detected in cattle serum during serological testing (Schurig, 2002). It has been reported that more than five million heifers have been administered with a dose of $1\text{-}4x10^{10}$ cells without noticeable side-effects. This vaccine has been seen to be the most preferred as it has reduced abortifacient effects in cows, although placentitis has been reported due to administration of full dosages. It is recommended that pregnant cows be vaccinated with a reduced dosage of 10^9 cells (Palmer *et. al*., 1997). Comparative studies on the potency of both vaccines have shown that strain RB51 induces a higher degree of protection that the strain 19 vaccine (Cheville *et. al*., 1996). Its advantage over S19 is that antibodies induced by it are not detected by the currently prescribed serological tests provided administration is done at the right time and at correct dosages.

CHAPTER 3: MATERIALS AND METHODS

3.1 Study site

Whole blood and serum samples were collected from a suspected seropositive herd of cattle from a farm in Matabeleland North.

Laboratory analyses of samples were carried out at the virology and molecular biology sections at the Central Veterinary Laboratories, Harare.

3.2 Sampling

Blood samples (10 ml) were collected from previously tested serologically positive cattle into EDTA treated plastic vacutainers and another batch (10 ml) into tubes without anticoagulant for serum separation. The samples were kept in an ice box with temperatures at approximately 4 °C until they were transported from the farm to the laboratory. Whole blood samples collected without coagulant were centrifuged at 6000 rpm for five minutes to obtain serum samples. The serum was kept at -20 °C for serological tests. Whole blood samples in EDTA tubes were also kept at -20 °C until they were used for molecular biology work.

3.3 Serology

3.3.1 Complement verification test

The complement verification test was done to validate the working dilution of complement before it was used in the test. Sheep red blood cells (SRBCs) were diluted to 2 % in Phosphate buffer solution (PBS). This was done by adding 1 ml of PBS buffer to 1 ml of blood cells. Haemolysin was diluted to 1/1000 and 1/3000 by adding 2 µl haemolysin to 1.998 µl PBS, and 1 ml was taken from the 1/1000 dilution and was mixed with 2 ml of PBS, respectively, for each solution. The mixtures were then vortexed for 5-6 seconds.

A volume of 1ml was taken from the 1/3000 haemolysin dilution and 2 ml of sensitized sheep red blood cells were added. The mixture was then incubated at 37 °C for 15minutes. Complement dilutions were made for different complement concentrations as shown in Table 3.1.

Table 3.1 Complement dilutions and concentrations

Complement concentration	Complement dilution
1/20	200 µl complement + 200 µl PBS buffer
1/25	200 µl complement + 300 µl PBS buffer
1/30	200 µl complement + 400 µl PBS buffer
1/35	200 µl complement + 500 µl PBS buffer

A 1/10 dilution of complement was made by adding 1ml complement to 1ml PBS. PBS (25 µl) was added to the last three wells of each 96 well microtitre plate. A volume of 25 µl of each dilution of complement according to their dilution ratios were added (25 µl of the first two) to each row. Solutions were then titrated from the 2nd well to the last well. The last three wells of each plate were labeled C, H, I for complement, haemolytic control system and blood. Volumes of 25 µl of PBS buffer were added to C and 75 µl PBS buffer to H and I. A 1 % dilution of SRBCs was made and 25 µl of the cells were added to well I.

A volume of 25 µl of the lowest dilution of complement i.e. 1/25 was added to well C and 25 µl of SRBCs from the incubator were added to wells C and H.

3.3.2 Serum Plating

Bovine serum samples were used for the test. In the Complement fixation test (CFT), antigen was prepared to a 1 in 12.5 dilution. This was done by adding 6 ml of PBS to 480 ml of antigen. Serum samples (25 µl) were placed into two wells per column of a microtitre plate. Complement was prepared according to working dilutions from the complement verification test. The diluted antigen (25 µl) was added to the test wells as well as 25 µl of buffer. Complement was then added into each well at a volume of 25 µl. Positive and negative controls were included in the test. Volumes of 25 µl PBS, 25 µl CFT antigen and 25 µl complement were added to well C, 50 µl PBS and 25 µl complement were added to well H and 75 µl PBS buffer were added to well I. Finally, 25 µl of haemolysin with blood cells were added into each well.

3.4 Molecular Characterization

3.4.1 DNA extraction from whole blood

The DNA from bovine whole blood was extracted using a Master Pure™ Complete DNA Extraction kit (Epicenter, USA). An aliquot of 200 µl of each of the whole blood samples in EDTA tubes was transferred to micro centrifuge tubes and 600 µl of red cell lysis solution were added. The tubes were then inverted three times to mix the contents and the bottom of the tube flicked to suspend any remaining material. Each sample was incubated at room temperature for five minutes before they were vortexed briefly. The samples were then incubated at room temperature for an additional five minutes after which they were briefly vortexed to mix. The white blood cells were pelleted by centrifuging for 25 s in a micro centrifuge at 3000 rpm. Most of the supernatant was removed, leaving approximately 25 µl of liquid which was vortexed and mixed to re-suspend the pellet. The white blood cells were re-suspended in 300 µl of tissue and cell lysis solution by pipetting the cells up and down several times. RNase (1 µl) was added to

the sample and mixed thoroughly. The samples were incubated at 37 °C for 30 minutes. The samples were placed on ice for three to five minutes and then processed for DNA precipitation.

A volume of 150 µl of protein precipitation reagent was added to 300 µl of lysed sample and the samples were vortexed vigorously for ten seconds. The samples were centrifuged for 10 minutes at 10,000rpm in a micro centrifuge. An additional 25 µl of protein precipitation reagent was added, mixed and centrifuged for all samples with small or loose pellets. The supernatants were then transferred to clean micro centrifuge tubes and the pellets were discarded. A volume of 500 µl Isopropanol was added to the recovered supernatant. The tubes were inverted for 30-40 times. The DNA was pelleted by centrifugation at 4 °C for ten minutes in a micro centrifuge at 3000rpm. The supernatant was then poured off carefully without dislodging the DNA pellet. The samples were then rinsed twice with 75 % ethanol, being careful not to dislodge the pellet. Centrifugation was repeated briefly if the pellet was dislodged. All of the residual ethanol was removed with a pipette. The DNA was re-suspended in 35 µl of Tris-EDTA (TE) buffer.

3.4.2 Gel Electrophoresis

DNA molecules were separated according to their size and charge by electric current. The concentration of the agarose gel used influences the migration of the DNA molecules during electrophoresis (OIE, 2008).

3.3.2.1 Preparation of agarose gel

Agarose powder, 1.0 g, was weighed using an analytical balance and placed into a schotts bottle; 100 ml of 1× Tris Borate EDTA was added. The mixture was heated in a microwave for two minutes to dissolve the agarose until the solution was clear. The solution was allowed to cool

down by placing the bottle under running water until the temperature reached around 55 °C . Ethidium bromide (5 µl) was added. The gel was then poured into the gel casting apparatus and allowed to solidify by letting it stand for thirty minutes. After solidifying, the gel was transferred to an electrophoresis tank and covered with Tris Borate-EDTA (TBE) buffer. The comb was gently removed from the gel. DNA samples were mixed with loading dye before the samples (30 µl) were carefully loaded into the 'wells' of the electrophoresis gel. The gel was run at 120V for one hour. After which it was visualized under UV transillumination using the Gel Logic 100 Documentation system.

3.4.3 Detection of *Brucella* by conventional PCR

PCR mix was prepared as a multiple of the number of samples including the negative and positive controls as shown on table 3.2. Four controls were used which were, 1 negative control and 2 positive controls (*B. abortus* 1, 2, 4 and *B. abortus* 3, 5, 6, 9) and another negative control which was used to counter for pipetting error. Commercial PCR master mix was used which was diluted using Promega water. Optimization was done by reducing the amount of water free nuclease and increasing the amount of DNA for the reaction. Extracted DNA was not quantified.

Table 3.2 Reaction setup for conventional PCR

Component	Volume	Final concentration
Primer AMOS abortus 10ppm	21 µl	0.2ppm
PrimerAMOSmelitensis10ppm	21 µl	0.2ppm
Primer AMOS ovis 10ppm	21 µl	0.2ppm
Primer AMOS suis 10ppm	21 µl	0.2ppm
Primer AMOS RB51 10ppm	21 µl	0.2ppm
Primer AMOS ery1 10ppm	21 µl	0.2ppm
Primer AMOS ery2 10ppm	21 µl	0.2ppm
Primer AMOS IS711 30ppm	21 µl	0.6ppm
DNA	5 µl	-
Water nuclease free	12 µl	-
PCR master mix 2x	25 µl	1x
Total volume	777 µl	

After preparation, the PCR mix was then mixed by vortexing before it was aliquoted into reaction tubes. A volume of 45 µl of PCR mix and 5 µl of DNA sample was made for each reaction tube before the samples were run by conventional PCR.

Table 3.3 Primer sequences

Code	Sequence
Primer Abortus	GAC GAA CGG AAT TTT TCC AAT CCC(24)
Primer Melitensis	AAA TCG CGT CCT TGC TGG TCT GA(23)
Primer Ovis	CGG GTT CTG GCA CCA TCG TCG(21)
Primer Suis	GGCG CGG TTT TCT GAA GGT TCA GG(23)
Primer RB51	TTA AGC GCT GAT GCC ATT TCC TTC AC(26)
Primer Ery1	GCG CCG CGA AGA ACT TAT CAA(21)
Primer Ery2	CGC CAT GTT AGC GGC GGT GA(20)
Primer IS711	TGC CGA ACA CTT AAG GCG CTT CAT(24)

3.3.4 PCR Amplification

DNA samples with PCR mix were added into the reaction tubes and mixed by pipetting. They were then placed into the Thermocycler/PCR machine where they were run, uninterrupted, using a Gene Amp Thermal PCR System 2400 with the following programme; initial denaturation: 94 °C/5minutes; 33 cycles of: 94 °C/30seconds (Denaturation), 60 °C/30seconds (Annealing), 72 °C/30seconds (Elongation) and 72 °C/7minutes (Final extension). The samples were stored at 4 °C for further use.

3.3.5 Electrophoresis of PCR products

Agarose gel was prepared to a concentration of 2 % by adding 2 g of Agarose powder into 100ml of 1 X TE buffer. A volume of 25 µl of the PCR product was loaded into the wells of the gel. GeneRuler 100bp plus DNA ladder was included so as to approximate the product size. Agarose gel electrophoresis was then performed at 100 V for 1hour 30 minutes. The gel was visualized under UV transillumination using the Gel Logic 100 Documentation system. Table 3.4 shows the assay used identification of the DNA bands viewed according to their band sizes.

Table 3.4 *Brucella* species and band sizes identification

***Brucella* species**	**DNA Bands size (bp)**
B. abortus biovars 1,2,4	498bp + 100bp
Brucella RB51	498bp+ 364bp+ 100bp
Brucella S19	498bp
B. abortus biovars 3,5,6,9	100bp
B. melitensis biovars 1,2,3	731bp+100bp
Brucella suis biovars 1	285bp+ 100bp
Brucella suis biovars 2,3,4,5	100bp
Brucella ovis	976bp +100bp or 364bp +100bp

(Method source: Manual of standards for diagnostic tests and vaccines (2008) Paris: Office International des Epizooites O.I.E)

CHAPTER 4: RESULTS

4.1 Serology

4.1.1 Complement verification test

There was complete haemolysis on the 1/20 dilution. Partial haemolysis was seen on the 1/25 dilution from the third well as well as on the 1/30 and 1/35 dilutions. Table 4.1 shows all results obtained for the complement verification test. Haemolysis showed the strength of the complement so that the most suitable working dilution could be obtained. A key is given for the interpretation of the results obtained in the test on table 4.2.

Table 4.1 Complement titration

Haemolysin dilution:	**1/20**				**1/25**				**1/30**				**1/35**			
Record of haemolysis:	0	0	0	0	0	0	+	2+	0	0	2+	4+	0	trace	3+	4+
	0	0	0	0	0	0	+	2+	0	0	2+	4+	0	trace	3+	4+
	0		4+	4+												
	C		**HS**	**I**												

Key: C- complement
HS- haemolytic control system
I-blood

Table 4.2 Recording of results

Visual appearance	Degree of haemolysin	Record
Button same as HS	No haemolysis	4+
Minimal haemolysis	25 % haemolysis	3+
Half button	50 % haemolysis	2+
Tiny button	75 % haemolysis	+
Complete haemolysis	100 % haemolysis	0

4.1.2 Complement fixation test

Complement fixation test on bovine sera samples showed that cattle tested were all positive for brucellosis. The cattle from which blood samples were collected were of different ages which ranged from 18-84 months and had all been vaccinated at different ages using either the S19 or the RB51 *Brucella* vaccine. Table 4.3 gives the results obtained from the CFT including the age, vaccination and the vaccine type of each animal.

Table 4.3 Complement Fixation Test

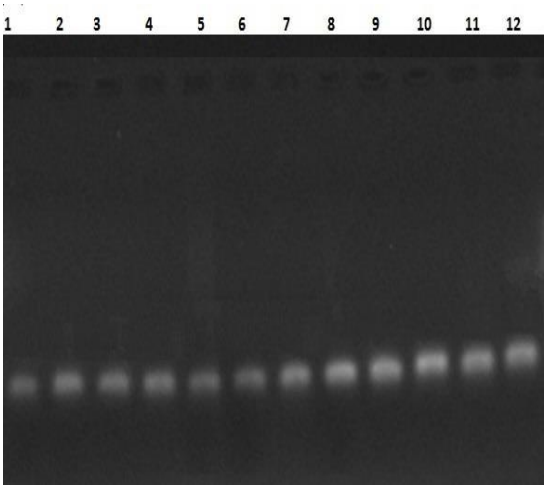
Sample	Animal age (months)	Vaccination age (months)	Vaccine type	Infection Status
1	52	4	S19	+
2	36	9	RB51	+
3	60	7	S19	+
4	72	12	S19	+
5	48	12	RB51	+
6	72	9	S19	+
7	24	4	RB51	+
8	36	4	RB51	+
9	48	4	RB51	+
10	54	4	S19	+
11	84	6	S19	+
12	72	9	S19	+
13	48	6	RB51	+
14	36	10	RB51	+
15	18	5	RB51	+
16	24	12	RB51	+
17	18	9	RB51	+

Key: (+) positive result

4.2 Molecular Characterization

4.2.1 DNA extraction

Bovine DNA was successfully extracted from the 17 whole blood samples and was seen as visible bands from lane 1-17 as seen on Fig 4.1a and 4.1b.



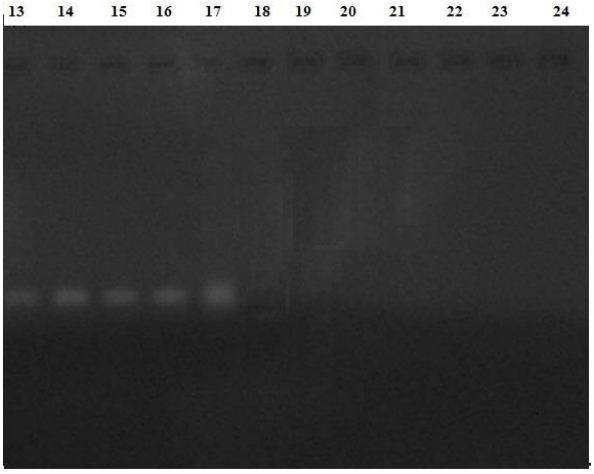


Fig 4.1a **Fig 4.1b**
Bovine genomic DNA extracted from whole blood samples.

4.2.2 Polymerase Chain Reaction

Results showed that 12 samples were positive for Brucella and five samples were negative. Positive results were seen by visible bands of different sizes showing different types of Brucella. Samples 2, 3, 4, 6 and 13 (Fig 4.2a, lanes 2, 3, 4, 6) showed the same banding patterns with visible bands of 100bp + 498bp each. Samples 5, 9, 10, 12, 14, 16 and 17 (Fig. 4.2a and 4.2b lanes 5, 9, 10, 12, 14, 16, 17) also had the same banding pattern which showed visible bands of 100bp each. Positive controls for *B. abortus* biovars 1, 2, 4 and *B. abortus* biovars 3, 5, 6, 9 gave expected bands of 100bp + 498bp and 100bp respectively (Fig 4.2b lanes 19, 20). Negative

control showed no band as there was no DNA present in it (Fig 4.2b lane 18). Bands of sizes 100bp + 498bp were characteristic for B. abortus strain of biovar 1, 2, 4. Band sizes of 100bp were characteristic for B. abortus strain biovar 3, 5, 6, 9 or B. suis as these strains show the same banding pattern. Primer dimers were seen as faint bands at the front of each lane with bovine DNA.

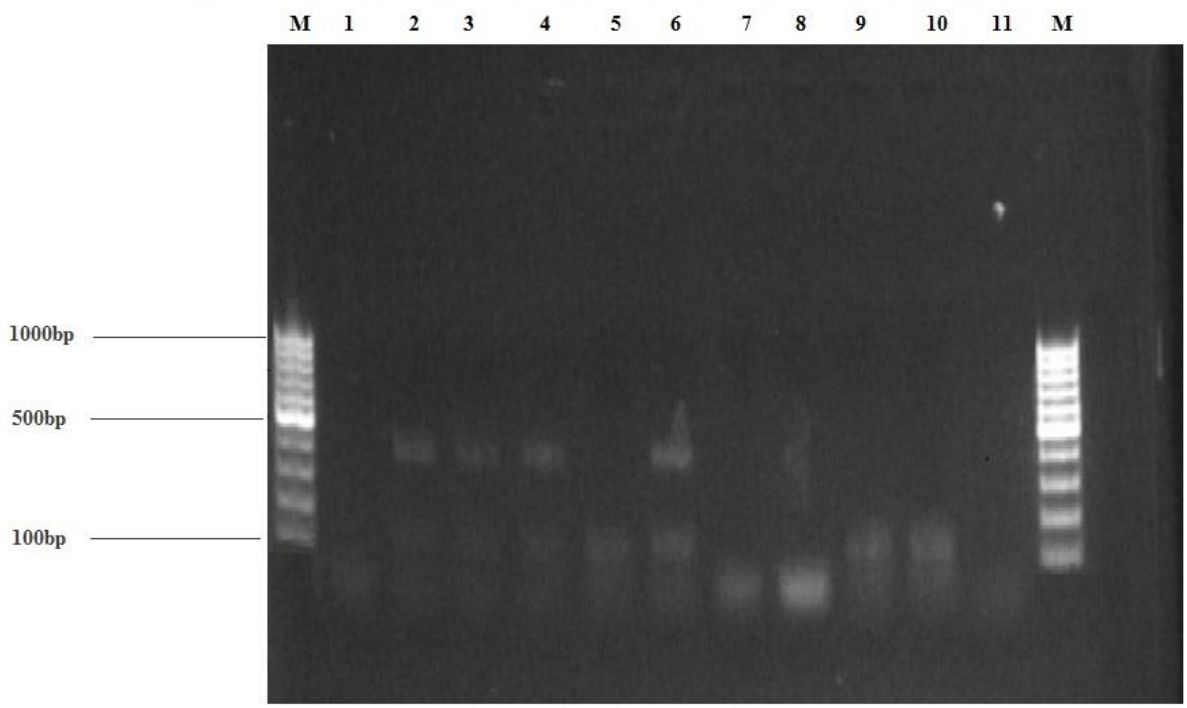


Fig 4.2a Identification and differentiation of *Brucella* isolated from bovine whole blood samples using multiplex PCR. Lane M: Fermentas GeneRuler 100bp plus DNA ladder; Lane 1-11: bovine DNA from seropositive cattle.

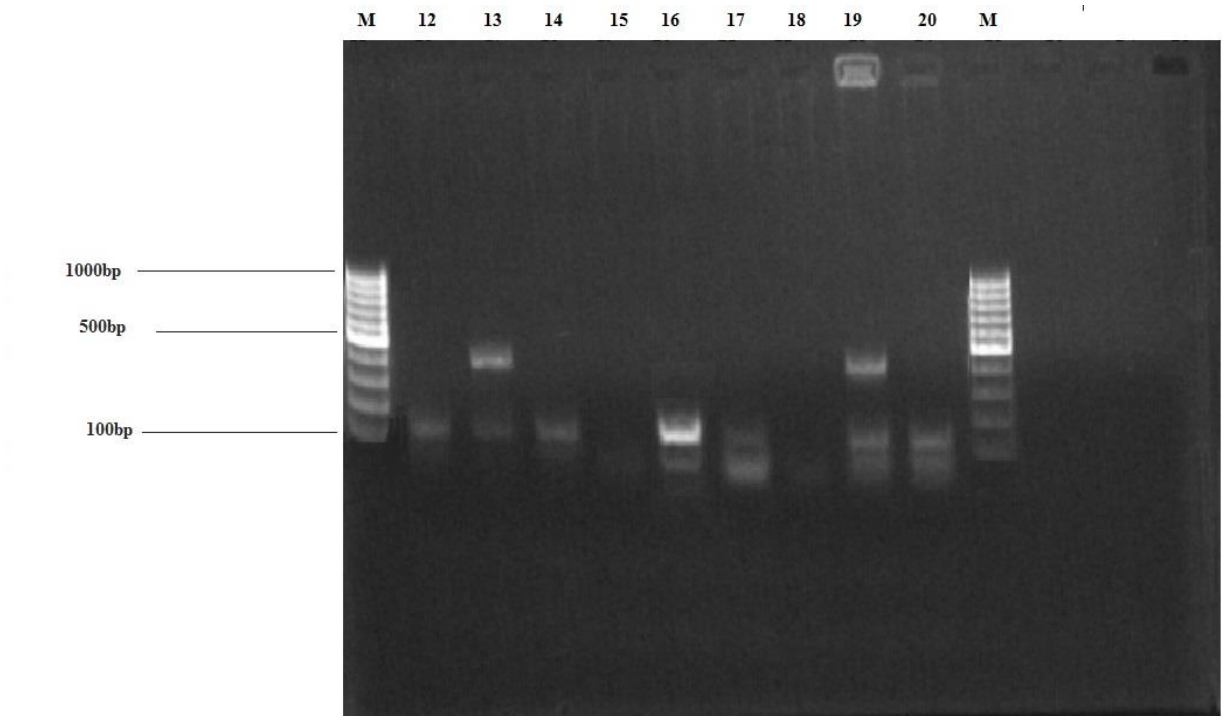


Fig 4.2b Identification and differentiation of *Brucella* isolated from whole blood samples using multiplex PCR. Lane M: Fermentas GeneRuler 100bp plus DNA ladder; Lane 12-17: bovine DNA from seropositive cattle; Lane 18: negative control; Lane 19-20: Positive controls.

4.3 Serological and molecular analyses

A comparison of serological and PCR results showed that all serum samples were positive for brucella using the CFT method while only 12 were positive for brucella using multiplex PCR. Table 4.4 shows an analysis of these results. PCR results showed that only samples 1, 7, 8, 11and 15 were negative for *Brucella* while samples 2, 3, 4, 5, 6, 9,10,12,13,14,16 and 17 were positive for *Brucella*. Positive samples were of either *B. abortus* biovars 1, 2, 4 or biovars 3,5,6,9 or *B. suis* biovars 2,3,4,5.

Table 4.4 Serological and Molecular methods results

Sample number	Serological result	PCR result
1	+	-
2	+	+
3	+	+
4	+	+
5	+	+
6	+	+
7	+	-
8	+	-
9	+	+
10	+	+
11	+	-
12	+	+
13	+	+
14	+	+
15	+	-
16	+	+
17	+	+

Key: (+) positive (-) negative

CHAPTER 5: DISCUSSION

5.1 Serology

5.1.1 Complement fixation test

Test results from serology and PCR showed a difference in five samples. Serology results had 17 positive samples out of the 17 serum samples tested showing 100% seropositivity of the herd while PCR results showed 12 positive samples out of the 17 whole blood samples tested, showing 70.58% positivity. The difference in results from the two methods of detection can be attributed to different factors that are concerned with the method of detection used. Although CFT is the main method currently being used for the detection of brucellosis is Zimbabwe, the results may be confused by the extensive use of vaccines, which can give rise to antibody responses similar to those resulting from the actual infection (Nielsen, 2002).

The complement fixation test (CFT) is a technically challenging test as it requires a number of reagents which should be titrated as well as a large number of controls. However since only the IgG1 isotypes of antibodies fixes complement well, the sensitivity of the test is quite high. The worldwide use of strain 19 vaccine, which induces a persisting antibody response, led to the development of tests that could solve or at least reduce the problem of the interference of vaccination in order to differentiate vaccinated animals from infected ones (Bishop, 2004). Unfortunately, the CFT does not discriminate *B. abortus* S19 derived antibodies (Nicoletti *et. al*., 1981, Sutherland *et. al*., 1982). This problem led to the development of the RB51 vaccine that shows negligible interference in classical serological brucellosis tests (Alton, 1990). Tested cattle had all been previously vaccinated at different ages and with either the RB51 or the S19 vaccines yet most were still aborting while some were reported both negative and positive at alternating times. Sample numbers 1 and 11 (Table 4.3 sample 1, 11) tested positive serologically while

results were negative using PCR (Fig. 4.2 lane 1, 11). These were cattle that had been previously vaccinated using the S19 live vaccine. The inability of the test to differentiate between vaccine antibodies and actual infection may be the reason for the false positive results obtained in the serological tests.

On the other hand, sample numbers 7, 8 (Fig 4.2a lane 7, 8) and 15 (Fig 4.2b lane 15) tested positive serologically while PCR predicted them as negative. These cattle had previously been administered intravenously with the B. *abortus* strain RB51 vaccine at different ages. It has been reported that the RB51 vaccine when administered intravenously can induce severe placentitis and placental infection in most vaccinated animals. Other studies have shown that it can induce abortion in some cases when applied to pregnant cattle. These cattle were vaccinated at 12 months of age. This late vaccination may be another strong reason for the false positive results obtained serologically.

Another issue concerning serology is the cross-reactions which occur between *Brucella* species and other gram-negative bacteria such as Yersinia enterocolitica O: 9; Franciella tularensis; Escherichia coli O: 157, Salmonella Urbana N.; Vibrio Cholarae and Strenotrophomonous maltophilia. This is due to the presence of the O-chain of the smooth lipopolysaccharide (S-LPS) present on the surface of these bacterial cells which show great similarity with smooth *Brucella spp.* except for *B. ovis* and *B. canis*. Because all smooth species share common isotopes in the O-polysaccharide (OPS), all serological tests use *B. abortus* S-LPS antigen (Godfroid *et. al*., 2002, Munoz *et. al*., 2005, Al Dahouk *et. al*., 2003). False positive results in this study (Table 4.3 sample 1, 7, 8, 11 and 15) show the limitations of serological tests.

The presence of anti-Brucella antibodies suggests exposure to Brucella spp. but does not indicate which Brucella species induced the production of these antibodies. Moreover, seropositivity does not necessarily mean that the animals have current or active infection at the time of sampling (Celli, 2005).

5.2 Molecular characterization

5.2.1 Polymerase Chain Reaction

In this study, whole blood samples were used as a convenient source of material to obtain *Brucella* infection information using PCR. This method is sensitive, specific and rapid compared to CFT. The PCR assay used employed the use of primers that are multiplexed to amplify strain-specific targets thus, reducing the risk of false-positive reactions due to mis-priming. The assay also includes strain specific primers for the two commonly used vaccine strains; S19 and RB51 and this allows for discrimination between vaccinated and field infected animals. This assay, known as AMOS-PCR, allowed the identification and differentiation of *Brucella* species as well as their biovars (Bricker and Halling, 1994).

Only 12 samples tested for brucellosis and 5 samples tested negative. Of these 12 samples, 5 samples i.e. sample 2, 3, 4, 6 and 13 (Fig 4.2a/Fig 4.2b lane 2, 3, 4, 6, 13) showed *B. abortus* (1, 2,4) which had band sizes of 100bp and 498bp (Table 3.4) while the remaining 7 samples i.e. sample 5, 6, 9,10, 12, 14, 16 and 17 (Fig 4.2a/4.2b lane 5, 6, 9, 10, 12, 14, 16, 17) showed that *Brucella* species were either *B. abortus* (3,5,6,9) or *B. suis* (2.3.4.5) as seen on Table 3.4. Further determination of the exact species of *Brucella* could not be done due to limitations in test reagents.

The CFT is the highest ranking of the confirmatory tests as recommended by the World Organization for Animal Health (OIE) and Food and Agriculture Organization (FAO). However, many samples appear to give a doubtful result. Many factors prevent the use of CFT in the routine diagnosis of brucellosis, including the difficulty handling samples, lengthy procedure, requirement for highly skilled technicians and cross-reactivity. Reliance on serological tests alone for the diagnosis of brucellosis can be misleading and thus other tests may be used to confirm serological test results. Studies conducted by Hinic and colleagues in 2009 and by Ning and colleagues in 2012 on the comparison of serological and molecular methods in detecting *Brucella* propose the use of PCR as a method of confirmation. Even so, PCR may not be the perfect test as the presence of large amounts of bovine genomic DNA may have inhibitory effects on the assay and the DNA extraction method used may also be crucial in determining the ability of PCR to detect the *Brucella* bacterium. However, PCR can be complementary to serological tests for the detection of *Brucella* species infections (Guarino *et. al*., 2000).

5.4 Conclusion

From the results obtained it can be concluded that there were false positive results from serology which may have resulted from the above mentioned factors that affect this method of detection. While PCR directly detects the presence of *Brucella* DNA, serology is dependent on the variable titers of antibodies in different stages of infection of the animal (Carpenter, 1975). It can therefore be confirmed that it is important to use more than one type of diagnostic techniques for the detection of brucellosis.

5.5 Recommendations

False positive serological reactions represent a major problem that requires governing from veterinary authorities. From the results obtained, it is clear that serological methods that are currently being used in Zimbabwe are not reliable. PCR is a more specific and reliable method which should be used as the standard method. This would provide a more realistic estimate of the disease in Zimbabwe and as such avoiding unnecessary culling of non-infected animals.

It is also important to educate farmers on the correct administration of vaccines. It is also advisable that farmers administer the same type of vaccine to their animals as recommended by veterinarians and health inspectors. The S19 vaccine interferes with serological methods and this may result in false positive results being reported. Late vaccinations may also result in the live vaccine antibodies being persistent in the systems of vaccinated animals; as such livestock breeders must be educated about the disease and its effects as well as proper vaccination methods to help control brucellosis.

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APPENDIX

Preparation of reagents

1 x TE (Tris EDTA) buffer

For 500ml working solution, measure;

2,5ml (10mM) from 2M Tris-HCl

1ml (1nM) from 0,5M EDTA

Make up to 500ml with distilled water.

Adjust pH with HCl to pH 8 and autoclave.

1xTBE (Tris Borate EDTA) buffer

54g Tris Borate

27.5g Boric acid

20ml 0.5M Na-EDTA pH 8

Bring to 1liter with double distilled water and ensure complete dissolution of added solutes (discard buffer that develops precipitates). To make 1x TBE working solution, mix 1.5 parts of 5x TBE buffer with 9 parts distilled water. Store buffer in a glass at 4°C.