Sero-prevalence of brucellosis in Cattle in Swaziland

By

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LIST OF ABBREVIATIONS

AMOS	*Brucella abortus, melitensis, ovis, suis*
CFT	complement fixation test
CO_2	carbon dioxide
DNA	deoxyribonucleic acid
cELISA	competitive enzyme-linked immunosorbent assay
EDTA	ethylene diaminetetraacetic acid
EFTA	ethylene glycol-bis-(B-aminoethyl ether) N,N,N',N'.-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FPA	fluorescent polarisation assay
H_2S	hydrogen sulphide
HIV	human immunodeficiency virus
iELISA	indirect enzyme-linked immunosorbent assay
Ig	immunoglobulin, e.g. IgM means immunoglobulin M
mP	milli-polarisation unit
OBP	Onderstepoort Biological products
OD	optical density
OIE	World Organisation for Animal Health
OPS	O-polysaccharide
PCR	polymerase chain reaction
RBT	Rose Bengal test
rLPS	rough lipopolysaccharide
SAT	serum agglutination test

SCVL	Swaziland Central Veterinary Laboratory
sLPS	smooth lipopolysaccharide
YO9	*Yersinia enterocolitica* O:9

THESIS SUMMARY

Tittle: Sero-prevalence of brucellosis in cattle in Swaziland

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Degree: Master of Science (Animal/Human/Ecosystem Health)

This study aimed at establishing the first cross-sectional sero-prevalence study of bovine brucellosis in the cattle population in Swaziland as well as technology transfer regarding the ELISA technique to allow an extended serological diagnosis of bovine brucellosis in the country. Twenty diptanks out of 778 functional diptanks were randomly selected for the cross-sectional brucellosis survey across all the four regions of Swaziland. A total of 1623 bovine sera from individual cattle out of an estimated bovid population of 622 715 was tested for the presence of *Brucella* antibodies using the Rose Bengal test (RBT) and indirect Enzyme Linked Immunosorbent Assay (iELISA). The iELISA proved to be more sensitive than the RBT, overall. However, in some cases the RBT yielded more positive reactors than the iELISA and vice versa. The serological prevalence of bovine brucellosis at diptank level ranged from 0.0% to as high as 53.3% and 50.9%, as determined by the RBT and iELISA tests, respectively. Regional sero-prevalence ranged from 3.05% to 53.05% by RBT and by iELISA, the sero-prevalence ranged from 3.90% to 52.84% and in both tests Lubombo region had the highest sero-prevalence and Hhohho region the least. Based on the findings of this study sero-prevalence of bovine brucellosis across all sampled regions was 16.14% and 17.38% in RBT and iELISA test, respectively. However, to achieve maximum sensitivity in this survey a parallel interpretation of the test results achieved by the two tests was used and an overall sero-prevalence of 21.50% was established. The western parts of the country exhibited a lower bovine brucellosis sero-prevalence, however, a systematic national bovine brucellosis survey is recommended.

CHAPTER 1

1.1. Introduction

Brucellosis is caused by Gram-negative bacteria of the genus *Brucella*, which are facultative intracellular cocco-bacilli, non-spore forming and non-encapsulated. *Brucella* species are non-motile; however, they carry all the genes except the chemotactic system which is necessary to assemble a functional flagellum. They belong to the alpha-2 subdivision of the Proteobacteria (Araj 2010, CarvalhoNeta et al. 2010, Seleem et al. 2010).

There are ten species recognized within the genus *Brucella*, of which six of the species are classical species and these are; *Brucella melitensis* (*B. melitensis*), *Brucella abortus* (*B. abortus*), *Brucella suis* (*B. suis*), *Brucella ovis* (*B. ovis*), *Brucella canis* (*B. canis*) and *Brucella neotomae* (*B. neotome*) (Carvalho-neta et al. 2010, Gofroid et al. 2010). However, in addition to the six *brucella* species there have been marine isolates from cetaceans and pinnipedialis and these are *Brucella ceti* (*B. ceti*) and *Brucella pinnipedialis* (*B. pinnipedialis*) respectively (Carvalho-neta et al. 2010, Godfroid et al. 2011). There is also *Brucella microti (B. microti)* which infects rodents (*microtus arvalis*) and *Brucella inopinata* which was originally isolated from a human patient, but its preferential host is not known (Godfroid et al. 2010, da Silver Mol et al. 2012). Three of these *brucella* species can be subdivided into biotypes (da Silver Mol et al. 2012). Therefore, three biotypes have been identified (1-3) in *B. melitensis*; eight biotypes (1-7, 9) in *B. abortus*; and five biotypes (1-5) in *B. suis* (da Silver Mol et al. 2012, Godfroid et al. 2011, OIE 2009). This classification is based mainly on differences in pathogenicity and host preference (Godfroid et al. 2013, Godfroid et al. 2010). Currently distinction between species and between biovars of a given species is performed using differential tests based on phenotypic characterization of lipopolysacharides (LPS) antigens, phage typing, dye sensitivity requirement for carbon dioxide (CO_2), H_2S production, and metabolic properties (Godfroid et al. 2010, Poester et al. 2010, OIE 2009).

Each *Brucella* species has a preferred natural host that serves as a reservoir of infection (Silva et al. 2000, Bandara and Mahipala 2002). *B. melitensis* is the most important species in sheep and goats (Bandara and Mahipa 2002). Cattle and buffalo are liable to catch *B. melitensis* and *B. suis* rarely in regions where these species are prevalent (Muñoz et al. 2005). *B. suis* infects swine and a range of wild animals, *B. ovis* infects sheep, and *B neotomae* is a pathogen for desert and wood rats (Doosti and Dehkordi 2011). *B. melitensis*, *B. suis*, *B. abortus*, and *B. canis* are also human pathogens (Araj 2010, Doosti and Dehkordi 2011). *B. abortus* usually causes brucellosis in cattle, bison and buffalo.

Despite their host preferences, all *Brucella* species may also infect wild species (Godfroid et al. 2010). Classical *Brucella* species have been isolated from a great variety of wildlife species such as bison, elk, feral swine, wild-boar, fox, hare, African buffalo, reindeer, and caribou. A very important consideration with regard to terrestrial brucellosis in wildlife is to distinguish between a spillover of infection from domestic animals and a maintenance host in wild species. The probability of brucellosis becoming established and being sustainable in a species depends on a combination of factors including host susceptibility, infective dose, contact with infected animals, management and environmental factors. Hence, the development of the game farming industry appears to have contributed to the re-emergence of brucellosis as an international concern for both livestock and wildlife, by increasing the density of potentially infected game species and by the introduction of artificial feeding (Godfroid et al. 2013, Godfroid et al. 2010, Godfroid 2002). Wildlife and livestock ranges overlap, and feed, water and mineral sources are shared between species on many farms and ranches (Godfroid et al. 2013). Brucellosis infection of wildlife reservoirs, although a lesser threat for causing human infections, can be a source for the reintroduction of infection into domestic livestock, which are more likely to transmit brucellosis to humans (Olsen 2013).

Brucellosis is a disease of world-wide economic and public health concern, which is endemic in sub-Saharan Africa (Muma et al. 2007) and it is one of the important livestock and human diseases in many developing countries (Jergefa et al. 2009). However, in developing countries, like Swaziland, the actual rate of *Brucella* infection is often unknown in both livestock and humans. This may be due to the fact that public awareness of this zoonotic disease is very low and lack of sufficient knowledge of the disease among physicians in addition to the absence of effective prevention and management strategies contributes to the widespread occurrence of the disease. This is a concern as it poses a high risk of transmission to the human population especially with the added burden of increased susceptibility as a result of the impact of the human immunodeficiency virus (HIV) in Africa.

Brucellosis is endemic in Swaziland as evidenced by the annual reports (2009-2011) from the Swaziland Central Veterinary laboratory (SCVL) and control measures are hardly practiced. There are vaccination programmes put in place by Government but they lack implementation. Brucellosis vaccination is voluntary by individual farmers. Data available is obtained from farmers' submission of blood samples from animals suspected to have brucellosis infection and the country is dependent on that information concerning the disease status of the country. The Swaziland Disease Act of 1965, section 48, states that in the case of an outbreak of bovine brucellosis amongst stock, all infected animals shall be isolated and treated in a manner prescribed by the Director of Veterinary Services. It also states that no person shall move or otherwise dispose of any stock which is infected with brucellosis unless the purchaser has

received written notification of the stock's condition at the time of sale and the seller has obtained written permission from the Director of Veterinary Services to sell the stock. It also states that all stock that may have been in contact with an animal that is infected with bovine brucellosis shall be treated in a manner prescribed by the Director of Veterinary Services. However, despite these guidelines and regulation, removal of suspect animals from herds is voluntary; there are no guidelines and policies that compel farmers to eliminate positive suspects.

Since there is almost no control of bovine brucellosis in Swaziland, there is a risk that the disease may be spreading within and between cattle herds. Very little is known about the prevalence of brucellosis in Swaziland, which is essential information for the prioritization and implementation of the diseases control schemes. This study will therefore provide data on the prevalence of the disease, and suggest control measures to be adopted. As a country it is important to control bovine brucellosis, to protect the health of the human population and to ensure productivity of the cattle herds. This will ensure that the country continues to export to the lucrative European market.

1.2. Literature Review

Bovine brucellosis is a contagious infectious disease usually caused by *Brucella abortus*, less frequently by *B. melitensis* and rarely by *B. suis* (McDermott and Arimi 2002, Kebede et al. 2008, Carvalho-Neta et al. 2010). Although eight biovars of *B. abortus*, the causative agent of bovine brucellosis, have been identified, biovar 1 is mostly frequently isolated from cattle in countries where biovar prevalence has been studied (Carvalho-Neta et al. 2010). *B. suis*, more especially biovars 1 and 3, are capable of infecting cattle, however, infection is usually not associated with clinical signs (Seleem et al. 2010). Importantly, *B.suis* and *B. melitensis* infection in cattle interfere with serological diagnosis of *B. abortus* infection (Carvalho-Neta et al. 2010). Susceptibility of an animal to brucellosis depends on its natural resistance, age, sex, level of immunity, environmental stress, route of infection and virulence of the infective strain (Kebede et al. 2008, Carvalho-Neta et al. 2010).

Predisposing factors to high and variable prevalence in extensive livestock production systems include large herd sizes, extensive movement of cattle and mingling with other herds at common grazing and watering points (McDermott et al. 2002). According to Berhe et al. (2007) large herd sizes enhance the exposure potential especially following abortion though increased contact and common feeding and watering points promoting transmission of *Brucella* organisms. According to Godfroid et al. (2001) the prevalence and epidemiology of brucellosis

in livestock production is influenced by factors like production systems, agro-ecological zones, husbandry practices, contact with wildlife and management factors. Silva et al. (2000) in a study in Sri Lanka concluded that agro-ecological zones do have an effect on brucellosis prevalence as there was no substantial difference in sero-prevalence rates in cattle and buffaloes.

Bovine brucellosis, in highly susceptible herds, is characterized primarily by abortion in late pregnancy, particularly, from the 5th to the 8th month of gestation. After abortion retention of foetal membranes and endometritis may follow impairing the fertility of the animal in subsequent pregnancies. In bulls the disease usually causes epididymitis, orchitis, seminal vesiculitis and sterility. Arthritis and hygromas, especially of the carpal joints, may occur in chronic infections in both males and females (Kebede et al. 2008, Carvalho-Neta et al. 2010, Mekonnen et al. 2010, Hegazy et al. 2011). In subsequent pregnancies, the fetus is usually carried to full term but shedding of bacteria from the uterus and milk continues. However, second or even third abortion may occur in the same animal. In less acute infections, cattle may give birth to weak calves or stillbirths, or merely show a retained placenta. Other signs of brucellosis infection in a herd may be decreased milk yield (Olsen and Tatum 2010).

The disease is transmitted among animals and causes disease in many different mammals. Sources of infection for the transmission of bovine brucellosis are aborted fetuses, foetal membranes, vaginal discharges and milk from infected animals. The most common route of transmission is the gastro-intestinal tract following ingestion of contaminated feed, fodder, water, and licking after-birth, aborted fetuses and infected new-born calves, all of which may contain a large number of the organisms and constitute a very important source of infection (Kebede et al. 2008). Infection may also occur through injured or intact skin, mucosa of the respiratory system and conjunctiva (Silva et al. 2000, Kebede et al. 2008). Calves may be infected *in utero* or by suckling of infected dams (Kebede et al. 2008). In pregnant animals, the uterus is the preferred site of infection where it leads to a necrotizing placentitis. In non-pregnant animals, the first infection often occurs in the udder followed by the infection of the uterus later after the onset of pregnancy. Infected adult males may develop orchitis and become infertile, however they are most unlikely to transmit the disease via semen containing *Brucella* organism but the chance of spread from the bull is very high if the semen is used for artificial insemination (Kebede et al. 2008, Carvalho-Neta et al. 2010).

1.3. Zoonotic aspect of brucellosis

Brucellosis is a zoonosis of both public health and economic significance in most developing countries (Abdoel et al. 2008). Brucellosis infection has been attributed to at least four of the six recognized *Brucella* species in terrestrial mammals, *B. ovis* and *B. neotomae* being the

exceptions (Godfroid et al. 2005). According to Seleem et al (2010) and Godfroid et al (2005) *B. melitensis* is by far, the most important zoonotic agent, but *B. abortus* and *B. suis* also contribute a significant threat. *B*. *canis* can also cause disease in humans, although this is rare even in countries where the infection is common in dogs. Godfroid et al. (2005), further state that, recently, human brucellosis has also been attributed to marine mammal strains. Human – human transmission by tissue transplantation or sexual contact has been reported but is insignificant in epidemiological terms (Godfroid et al. 2005, Corbel 1997).

The World Health Organization (WHO) laboratory biosafety manual classifies *Brucella* in risk group III (OIE 2009). Brucellosis is readily transmissible to humans, causing acute febrile illness-undulant fever, which may progress to a chronic form resulting in serious complications affecting the musculo-skeletal, cardiovascular, and central nervous systems. The established mode of transmission of zoonotic *Brucella* species to man is usually by direct contact with infected animals i.e. infected uterine contents, discharges and their carcasses, through ingestion of raw or unpasteurized infected milk or dairy products and inhalation of contaminated dust (Silva et al. 2000, Bandara and Mahipa 2002, Hesterberg et al. 2008). Some specific occupational groups including farm workers, veterinarians, laboratory workers, ranchers, and meat-packing employees are considered at higher risk of contracting brucellosis (Seleem et al. 2010). Precautions should be taken to prevent human infection and to minimize occupational exposure to this zoonotic agent, laboratory manipulation of live cultures and/or contaminated material from infected animals must be done under containment level 3 (biosafety level 3) conditions (OIE 2009). Prevention of human brucellosis depends on the control of the disease in animal hosts (Olsen 2010, Godfroid et al. 2005), the exercise of hygienic precautions to limit exposure to infection through occupational activities, and the effective heating of dairy products and other potentially contaminated foods. It has a great impact on economic development, both in terms of direct losses (morbidity and mortality) and indirect losses, such as those due to the costs of ineffective control measures (McDermott and Arimi 2002).

Worldwide, millions of individuals are at risk, especially in developing countries where the infection in animals has not been brought under control (Kebede et al. 2008). Heat treatment procedures of milk e.g. pasteurization are not routinely applied whereas consumption of raw milk and poor hygienic conditions favour human brucellosis infection (Falenski et al. 2011). Brucellosis in humans is a multi-systemic, acute to chronic, disease characterized by fever, headache, joint pains, musculo-skeletal pains, night-sweats, malaise and body wasting. These rather non-specific signs have caused tremendous problems with the clinical diagnosis of brucellosis in sub-Saharan Africa, leading to it being constantly misdiagnosed as malaria, a

disease which is very prevalent (Godfroid et al. 2011, Seleem et al. 2010, McDermott and Arimi 2002).

The majority of Africans, in both pastoral and mixed farming systems, live closely with livestock populations having a high incidence of brucellosis and thus are at high risk of infection. In Africa, livestock play a crucial role in the livelihoods of the majority of people (McDermott and Arimi 2002). Livestock are a critical resource, providing milk, meat, traction, manure and cash income. Livestock also have vital social roles in many cultural traditions.

In countries where bovine brucellosis is uncontrolled, like Swaziland, most human cases occur due to improper handling of infected tissues and blood, handling or drinking of unpasteurized milk and dairy products and from inhalation of contaminated aerosols when in close contact with infected animals (Silva et al. 2000, Hesterberg et al. 2008, Kebede et al. 2008, Falenski et al. 2011). Bovine brucellosis is present in animals in most developing countries where surveillance and control activities are often inadequate and/or not implemented or unavailable (McDermott and Arimi 2002).

1.4. Brucellosis diagnosis

Abortion is usually the only clinical sign of *B. abortus* infection in bovines and a definite diagnosis is obtained by isolation and identification of the organism (Silva et al. 2000). Due to large numbers of samples involved, surveys through culture may be impractical, hence, the preference for serological tests to detect sero-positive animals. Testing of livestock for brucellosis can be done by culture and serology including testing milk samples (Abdoel et al. 2008).

A precise diagnosis of *Brucella* species infection is important for the control of the disease in animals and consequently in man. Clinical diagnosis is based usually on the history of reproductive failures in livestock, but it is a presumptive diagnosis that must be confirmed by laboratory methods (da Silver Mol et al. 2012).

Brucella species, except for *B. ovis* and *B. canis*, contain smooth lipopolysaccharide (SLPS) in their outer cell wall. Smooth lipopolysaccharide contains an immunodominant O-polysaccharide (OPS) which has been chemically defined as a homopolymer of 4, 6-dideoxy-4-formamide-alpha-D-mannose linked via glycosidic linkages (Nielsen and Yu 2010, Poester et al. 2010, Schurig et al. 2002). *Brucella ovis* and *B. canis* lack the OPS component and as a result, their outer surface contains only rough lipopolysaccharide (RLPS) and protein antigens (Nielsen and Yu 2010,

Schurig et al. 2002, Jiménez DE Bagüés et al. 1994). Because all smooth species share common epitopes in the OPS, virtually all serological tests for antibody to these bacteria use *B. abortus* antigen (Godfroid et al. 2013) while RPLS is commonly used as the main antigen for the detection of antibody to *B. ovis* and *B. canis* (Poester et al. 2010).

Brucellosis diagnostic methods include direct and indirect tests. The direct tests involve microbiological analysis or DNA detection by polymerase chain reaction (PCR)-based methods. Indirect tests may be applied either in vitro (mainly to milk and blood) or in vivo (allergic test). The choice of a particular testing strategy depends on the prevailing epidemiological situation of brucellosis in susceptible animals in a country or region (Godfroid et al. 2010).

Routine diagnosis of bovine brucellosis is done by Rose Bengal test (RBT) in Swaziland. However, according to Silva et al. (2000) difficulties may occur when cattle with long-standing infection remain serologically negative as a result of catabolism of antibodies with time. Such animals become serologically positive during or after pregnancy (Silva et al. 2000) and therefore, sero-prevalence may indicate currently infected animals or a successful immune response by the host to a previous infection. About 60% of blood samples submitted by farmers to the Swaziland Central Veterinary Laboratory (SCVL) test positive in RBT. However, no follow-up is carried out on the RBT positive reactors and the diagnosis remains unconfirmed.

1.4.1. Direct techniques

Several techniques are available to identify *Brucella* species. The staining of smears from pathological material is still often used, and can provide useful information from abortive material. Bacterial isolation is nevertheless always preferable and often for genotyping strains. New PCR techniques allowing the identification and typing of *Brucella* have been developed and are being implemented in specialized diagnostic laboratories, however the performance of these molecular methods for direct diagnosis of brucellosis from field samples still needs to be improved (Godfroid et al. 2013).

1.4.1.1. Staining

Brucella organisms have the shape of coccobacilli, measuring 0.6µm to 1.5µm long and 0.5 µm to 0.7 µm wide. Morphologically, they present as individual bacteria, but occasionally they can be observed in clumps of two or more organisms (Godfroid *et al*. 2013). *Brucella* is a Gram-negative bacterium that can resist weak acid treatment and therefore appears red after Stamp

staining (Godfroid et al. 2010, Poester et al. 2010). However, this staining is not specific enough and other abortive pathogens such as *Chlamydophila abortus* and *Coxiella burnetti*, also stain red in the same way and have a similar morphology (Godfroid et al. 2013, Godfroid et al. 2010, Poester et al. 2010).

1.4.1.2. Culture

Isolation of the organism is considered the gold standard diagnostic method for brucellosis since it is specific and allows bio-typing of the isolate (da Silver Mol et al. 2012, Araj 2010, Godfroid et al. 2013, Godfroid 2002). However, in spite of its high specificity, culture of *Brucella* species is challenging. *Brucella* species is a fastidious bacterium and requires enriched media for primary cultures (da Silver Mol et al. 2012). Furthermore, its isolation requires a large number of viable bacteria in clinical samples, proper storage and quick delivery to the diagnostic laboratory (da Silver Mol et al. 2012). Contamination of clinical samples may complicate *Brucella* species isolation, therefore, nutrient-rich media supplemented with antibiotics are used to inhibit overgrowth of contaminants that may prevent isolation of *Brucella* species (da Silver Mol et al. 2012, Doosti et al. 2011, Poester et al. 2010).

Other limiting factors for culturing *Brucella* species are the requirements for appropriate laboratory biosafety conditions and well-trained personnel who are able to perform the procedure safely (da Silver Mol et al. 2012). The World Health Organization laboratory biosafety manual classifies *Brucella* species as a biosafety level 3 organism, whose manipulation should be performed in biosafety level 3 laboratories (da Silver Mol et al. 2012, OIE 2009).

For definitive diagnosis of *Brucella*, the choice of sample depends on the clinical signs observed (Godfroid et al. 2013, Godfroid et al. 2010). Samples for *Brucella* species isolation from cattle include fetal membranes, particularly the placental cotyledons where the number of organisms tend to be very high (da Silver Mol et al. 2012). Additionally, fetal organs such as the lungs, bronchial lymph nodes, spleen and liver, as well as fetal gastric contents, milk and vaginal secretions and semen are samples of choice for isolation (da Silver Mol et al. 2012, Godfroid et al. 2010). Vaginal secretions should be sampled after abortion or parturition, preferably using a swab in transport medium, allowing isolation of the organism up to six weeks post parturition or abortion (da Silver Mol et al. 2012). Vaginal swabs, semen and seminal fluid have low numbers of viable organisms, and therefore isolation is more difficult, often resulting in false negative results. Use of enriched media containing selected antibiotics can improve sensitivity in these cases (da Silver Mol et al. 2012). Milk samples should be pooled from all four mammary

glands. Non pasteurized dairy products can also be sampled for isolation (da Silver Mol et al. 2012).

Samples of choice in slaughterhouses include mammary, iliac, pharyngeal, parotids and cervical lymph nodes, and spleen. Samples must be immediately sent to the laboratory, preferably frozen at -20 ˚C, and they must be labeled as suspect *Brucella* samples (da Silver Mol et al. 2012, Godfroid et al. 2010, Poester et al. 2010).

Brucella colonies are elevated, transparent, and convex, with intact borders, smooth, and a brilliant surface. The colonies have a honey colour under transmitted light. Optimal temperature for culture is 37 ˚C, but the organism can grow under temperatures ranging from 20 ˚C to 40 ˚C , whereas optimal pH ranges from 6.6 to 7.4. Some *Brucella* species like *B. abortus* wildtype (biovars 1-4) require carbon dioxide (CO_2) for growth whereas others such as *B. abortus* S19 vaccine strain, *B. melitensis*, and *B. suis* do not (da Silver Mol et al. 2012, Godfroid et al. 2010). Typical colonies appear after 2 to 30 days of incubation, but a culture can only be considered negative when there are no colonies after 2 to 3 weeks of incubation. False negative results should be considered in the absence of bacterial growth since the sensitivity of culture is low (da Silver Mol et al. 2012, Godfroid et al. 2010). The identification of *Brucella* species is based on morphology, staining and metabolic profile (Godfroid et al. 2013, Godfroid et al. 2010).

1.4.1.3. Polymerase Chain Reaction (PCR)

Molecular diagnostic techniques for detection of *Brucella*, which are simpler, faster and less hazardous and usually more sensitive have been developed (Doosti et al. 2011). These are important tools for diagnosis and epidemiological studies, providing relevant information for identification of species and biotypes of *Brucella* species (da Silver Mol et al. 2012). The best validated techniques are based on the recognition of specific DNA sequences of *Brucella* species, such as the 16S-23s genes, the IS711 insertion sequence or the bcsp31 gene encoding a 31-kDa protein (Godfroid et al. 2013, Godfroid et al. 2010). Molecular detection of *Brucella* species can be done directly on clinical samples without previous isolation of the organism (da Silver Mol et al. 2012).

Polymerase chain reaction and its variants, based on amplification of specific genomic sequences of the genus, species or even biotypes of *Brucella* species, are the broadly used molecular techniques for brucellosis diagnosis (da Silver Mol et al. 2012).The technique is

chosen based on the type of biological sample and the goal, i.e. diagnosis or molecular characterization or epidemiological survey. Most of the molecular diagnostic methods for brucellosis have sensitivities ranging from 50% to 100% and specificities between 60% and 98% (da Silver Mol et al. 2012). The DNA extraction protocol, type of clinical sample, and detection limits of each protocol, are factors that can influence the efficacy of the technique (da Silver Mol et al. 2012, Godfroid et al. 2010).

For typing of *Brucella* species, the multiplex AMOS PCR, named for its applicability to "*abortus, melitensis*, *ovis*, *suis*" species is often used. PCR protocols derived from it allow discrimination between *Brucella* species and between vaccine and wild type strains; however, they do not allow discrimination among all the biovars of a given *Brucella* species (Godfroid et al. 2013, Godfroid et al.2010). A newly developed multiplex PCR assay named Bruce-ladder can identify and differentiate between all the *Brucella* species and the vaccine strains in the same test (Godfroid et al. 2013).

1.4.2. Indirect techniques

Antibody response to *B. abortus* infection in cattle consists of early IgM isotype production, appearing usually 5-15 days after exposure but may be delayed (Nielsen and Yu 2010, Poester et al. 2010). The IgM antibody response is followed very shortly by production of IgG_1 isotype of antibody and subsequently by IgG_2 and IgA (Nielsen and Yu 2010, Poester et al. 2010). Because of the early onset of IgM antibody production, theoretically it would be best to measure this isotype as an indicator of exposure. However, a number of other microorganisms contain antigens with epitopes similar to those of OPS and the main antibody response to these cross reacting antigens is IgM (Nielsen and Yu 2010, Poester et al. 2010, Corbel 1997). Therefore, the measurement of IgM antibody sometimes gives false positive reactions in serological tests leading to low assay specificity. Production of IgG_2 and IgA isotypes occurs later in infection and as a result, measurement of these antibodies would generally lower assay sensitivity. Therefore, the most useful antibody measurement for serological test for brucellosis is IgG_1 (Nielsen and Yu 2010, Poester et al. 2010).

In addition to cross reactions, vaccinal antibodies sometimes cause diagnostic inaccuracies. *Brucella abortus* S19 is a widely used vaccine (Schurig et al. 2002). This organism is antigenically indistinguishable from pathogenic strains of *B. abortus* (Godfroid et al. 2013, Poester et al. 2010, Schurig et al. 2002). However, administration of the vaccine to heifers, usually between 4 and 12 months of age, generally allows the antibody response to wane sufficiently to eliminate

some diagnostic problems by the time animals reach sexual maturity and are tested for brucellosis (Poester et al. 2010, Stevens et al. 1994). However, some animals were found to have residual antibody leading to higher antibody levels in vaccinated animals (Poester et al. 2010). Development of improved serological tests and development of a live vaccine which contains no OPS (*B. abortus* RB51), have largely overcome most of the problems (Godfroid et al. 2013, Schurig et al. 2002).

1.4.2.1. Serological methods

Serological diagnosis is presumptive evidence of infection and is crucial for laboratory diagnosis of brucellosis since most control and eradication programs rely on these methods (da Silva Mol et al. 2012, Poester et al. 2010). Although these methods have been extensively improved with regard to sensitivity and specificity, they have some limitations as they are indirect methods, and in most cases lack the possibility to differentiate vaccinated from infected animals (da Silver Mol et al. 2012). There are considerable differences in the accuracy of the various serological tests and it is common to use a panel of tests and use the majority results as an indicator of exposure (Poester et al. 2010).

Inactivated whole bacteria or purified fractions are used as antigens for detecting antibodies generated by the host during infection. Antibodies against smooth *Brucella* species cross-react with antigen preparations from *B. abortus*, whereas antibodies generated against rough *Brucella* species cross react with antigen preparations from *B. ovis* (da Silver Mol et al. 2012, Godfroid et al. 2010).

Serological tests are generally divided into three categories: the classical or conventional tests, primary binding assays and developing technology (Nielsen and Yu 2010, Poester et al. 2010). All conventional tests rely on the antibody performing a secondary function, for instance fixation of complement while for primary binding assays the only function of the antibody is attachment to an immobilized antigen (Nielsen and Yu 2010, Poester et al. 2010).

1.4.2.1.1. Rose Bengal Test

The Rose Bengal Test (RBT) is a rapid and inexpensive screening test with high sensitivity (>99%), performed on individual serum, however the specificity can be disappointingly low (Smits et al. 2005, Gibbs and Bercovich 2002). The RBT uses *B. abortus* S99 or S1119.3 whole

cells stained with Rose-Bengal stain (Poester et al. 2010, Smits et al. 2005, Nielsen 2002). It is an agglutination test using the stained antigen at a pH of 3.65 which help to visualize the result and to eliminate some non-specific reactivity. It is used at a pH of 3.65 which is low enough to prevent agglutination by IgM and encourages agglutination by IgG_1 hence the reduction of non-specific interactions (Poester et al. 2010, Muma et al. 2007, Nielsen 2002).

This test is considered a screening test only for the detection of *Brucella* agglutinins; however, false negative reactions occur rarely, mostly due to prozoning and can sometimes be detected by diluting the serum sample or retesting after 4-6 weeks (OIE 2009). The RBT's low specificity is its principal limitation as it also detects antibody resulting from *B. abortus* S19 vaccination and some cross reacting antibodies. Due to this test's low specificity, its positive predictive value is low and thus a positive test result require confirmation by a more specific test (OIE 2009, Smits et al. 2005, Gibbs and Bercovich 2002, Nielsen 2002). The negative predictive value of the RBT is high and a negative test result excludes active brucellosis with a high degree of certainty (Smits et al. 2005). To increase the specificity and the positive predictive value of the test, it may be applied to a serial dilution (1:2 through 1:64) of the serum sample. The specificity of the RBT increases when higher dilutions agglutinate and titres of 1:8 or 1:16 and above may be regarded as positive. This approach, however, inevitably results in lower sensitivity (Smits et al. 2005)

Generally, equal amounts of antigen and serum are mixed and agglutination is graded N, 1+, 2+, 3+ depending on the number of agglutinated cells (Muma et al. 2007, OIE 2009). The presence and absence of visible agglutination indicates the presence or absence of *Brucella* antibodies in the samples tested (OIE 2009). Appearance of agglutination must be within the specified time i.e. four minutes. If incubated for longer periods, false reactions may sometimes occur due to the formation of fibrin clots (Poester et al. 2010).

1.4.2.1.2. Complement Fixation test (CFT)

The complement fixation test is highly specific and therefore accepted worldwide (da Silver Mol et al. 2012). Due to its high accuracy, complement fixation is used as a confirmatory test for *B. abortus, B. ovis,* and *B. melitensis* infections (da Silver Mol et al. 2012, Poester et al. 2010), and therefore it is the reference test recommended by the OIE for the international transit of animals (da Silver Mol et al. 2012, Godfroid et al. 2010, OIE 2009). However, this method has some disadvantages such as high cost, complexity of execution, and the requirement for special equipment and trained personnel. In addition, the test presents limitations with hemolysed

serum samples or serum with anti-complementary activity, and the occurrence of prozoning phenomena (da Silver et al. 2012, Poester et al. 2010).

The principle for the complement fixation test is based on dilutions of serum from cattle, sheep or goats, antigen and a precipitated amount of complement being added together (Nielsen 2002). If antibody is present in serum, it will bind to the antigen activating a complement cascade. Cattle immunoglobulins (Ig) that can activate bovine compliment are IgG and IgM (Godfroid et al 2010, Niesen and Yu 2010). An indicator system which consists of sheep erythrocytes sensitized with rabbit antibody is then added. If the test serum contained antibody, complement is not available and lyses of the erythrocytes will not take place. This test may not be highly sensitive but it shows an excellent specificity (da Silver Mol et al. 2012, Godfroid et al. 2010).

1.4.2.1.3. Serum Agglutination Test (SAT).

The principle of this test is to detect agglutinin antibodies directed against *Brucella* species (Godfroid et al. 2010). At an optimum concentration of antigen and antibodies, large antigen – antibody complexes form and precipitate at the bottom of a test tube. Agglutination in the tubes is examined visually and it is facilitated by the addition of a dye that stains the cells (Araj 2010, Godfroid 2010, Godfroid et al. 2010). The reaction is relatively slow as compared to the rapid agglutination tests, it requires an overnight incubation at 37°C (Araj 2010, Godfroid et al. 2010). The relative lack of sensitivity and specificity of this test has often been presented as a major drawback (Godfroid et al. 2010). However, the specificity of the test is increased by treating the serum with a chelating agent such as EDTA, which reduces cross reactions due to IgM (Godfroid et al. 2010). The highest serum dilution showing more than 50% agglutination is considered the agglutination titer (Araj 2010). Commercial cell suspensions could vary in quality and thus each cell lot should be quality controlled with known positive and negative standard sera before accepting it for diagnosis. SAT measures total *Brucella* antibody (IgG, IgM and IgA) (Araj 2010).

1.4.2.1.4. Indirect Enzyme-linked immunosorbent Assays (iELISA)

A large number of variations of the indirect ELISA have been described employing different antigen preparations, anti-globulin-enzyme conjugates, and substrate/chromogens (Nielsen 2002, OIE 2009). The most common format uses *B. abortus* smooth lipopolysacharide antigen coated passively onto a polystryrene matrix (Nielsen 2002). Diluted serum is added, the diluting

buffer usually contains a detergent such as Tween 20 and divalent cation chelating agents ethylene diaminetetra-acetic acid disodium salt (EDTA) and ethylene glycol-bis-(B-aminoethyl ether) N,N,N',N'.-tetraacetic acid (EGTA) to reduce non-specific binding of serum proteins (Vanzini et al. 1998, Nielsen 2002). It is followed by an antiglobulin reagent specific for or cross reacting with the test species specific immunoglobulin, conjugated with an enzyme, usually horseradish peroxidase or alkaline phosphatase. A strong positive, a weak positive and negative serum control is included to easily monitor assay performance and quality control. The iELISAs that use smooth lipopolysaccharides or the O-polysaccharides as antigens are highly sensitive (92% - 100% sensitivity) for the detection of anti-*Brucella* antibodies, but they are also more susceptible to non-specific reactions, notably due to YO_9 infection (Godfroid et al. 2010, Poester et al. 2010, OIE 2009). Another disadvantage is their inability to differentiate vaccinal antibody resulting from *B. abortus* S19 or *B. melitensis* Rev 1 from antibody induced by pathogenic strains (Nielsen 2002, OIE 2009).

1.4.2.1.5. Competitive Enzyme-linked immunosorbent Assays (cELISA)

The cELISA with smooth *Brucella* LPS as antigen is used for the detection of anti-*Brucella* in serum samples from cattle, sheep, goats and pigs (da Silver Mol et al. 2012). The competitive ELISA was developed to complement serological tests because of their inability to differentiate between vaccinal antibody and infection antibody. This test is capable of differentiating vaccine antibody response from actual infections. The specificity of the cELISA is very high, however, it is slightly less sensitive than the iELISA (Nielsen and Yu 2000). The sensitivity of the cELISA varies from 92% to 100%, whereas its specificity ranges from 90% to 99% in vaccinated populations (da Silver Mol et al. 2012, Poester et al. 2010). The main rationale for this assay was that vaccine induced production of antibodies are of lower affinity due to the shorter exposure to antigen as a result of immune elimination. In contrary, antibody production due to field infection, in which antigen persists, results in increased antibody affinity (Nielsen and Yu 2010, Poester et al. 2010, Nielsen 2002). A competing antibody is selected to inhibit binding of vaccinal but not field strain induced antibodies. This assay is an excellent confirmatory assay for the diagnosis of brucellosis in most mammalian species (Nielsen and Yu 2010, Poster et al. 2010). Competitive assay kits are available commercially from various sources.

1.4.2.1.6. Fluorescence polarization Assay (FPA)

The fluorescence polarization assay (FPA) for detection of antibodies has been validated for use for the serological diagnosis of *Brucella* species containing O-polysaccharide in its outer wall in cattle , pigs, bison and various species of deer (Nielsen et al. 2001). The FPA is based on the following physical principle: a molecule in solution rotates randomly at a rate inversely proportional to its size (Godfroid et al. 2010, Minas et al. 2007). The rate of rotation can be measured in the horizontal and vertical planes using a fluorescent label and polarized light. This test has the potential of being performed outside the diagnostic laboratory using a portable FPA reader (Minas et al. 2007, Nielsen et al. 2001, Dajer et al. 1999, Nielsen et al. 1998). The sensitivity of the FPA varies between 87.5% and 100%, and specificity from 84% to 100% (da Silver Mol et al. 2012).

The rotational speed of a small labeled antigen molecule will be altered if an antibody is attached to it and these changes in speed of rotation can be measured using polarized light. The result is a measurement of the time it takes the molecule to rotate through a given angle and the degree of depolarization is recorded in milli-polarization units (mP) (Godfroid et al. 2010, Nielsen and Yu 2010, Poester et al. 2010).

During brucellosis testing, serum samples are incubated with a tracer i.e. a specific antigen of *B. abortus* consisting of an OPS fragment, approximately 22 kDa in size, labeled with fluorescein isothiocyanate. In the presence of antibodies against *Brucella* species, large fluorescent complexes are formed. In negative samples, the antigen remains uncomplexed. These smaller molecules spin more quickly and therefore cause greater depolarization of the light than do the sample positive for *Brucella* species (Godfroid et al. 2010, Poester et al. 2010).

The FPA is a homogenous assay, requiring no washing steps or removal of unreacted components. It can be performed in a 96-well format or in a tube format. The tube format can be used in the field for rapid diagnosis. The serum or milk incubation time is a minimum of 2 minutes while the whole blood assay requires only 15 seconds of incubation. Since only 2 reagents, antigen and diluent buffer are required, the test is technically simple and relatively inexpensive.

The FPA is very accurate and sensitivity: specificity can be manipulated by altering the cutoff value between positive and negative reactions to provide a very sensitive screening test as well as a highly specific confirmatory test. Control sera of strong positive, weak positive and negative, as well as S19 vaccinate serum should always be included (OIE 2009) thus the FPA is also capable of distinguishing vaccinal antibody in most vaccinated animals and it can eliminate

cross reactions as well (Nielsen and Yu 2010, Poester et al. 2010). The assay performance of FPA equals that of a competitive enzyme immunoassay that was originally used for its increased sensitivity and specificity (Nielsen et al. 1998).

1.5. Justification

The rural areas of Swaziland are regarded as resource-poor areas with a high unemployment rate and, as such, subsistence farming dominates commercial agricultural activities. Livestock keeping constitutes the main livelihood strategy, provides nutrition (meat and milk), traction and crop manure. People live in close proximity with their animals under poor hygienic conditions. This increases the threat of disease transmission such as brucellosis, which is an important zoonosis, as their level of awareness of such diseases is very low. Brucellosis has a negative impact on economic development in terms of direct loss through abortions. Currently, there is a vaccination programme in Swaziland which is partially implemented by Government .Bovine brucellosis is present in the cattle population in Swaziland, as evidenced by the results from the serological screening test (i.e. RBT) performed at the Central Veterinary Laboratory. However, there is no detailed epidemiological investigation of the disease in Swaziland because of limitations in performing confirmatory tests such as CFT, ELISA and culture, which is essential in determining the true disease status. This project aimed at establishing the sero-prevalence of bovine brucellosis in the cattle population in Swaziland, as well as technology transfer regarding the ELISA technique to allow and extend serological diagnosis of bovine brucellosis in Swaziland.

1.5.1. Hypothesis

The sero-prevalence of bovine brucellosis in the cattle population of Swaziland at dip tank level is higher than 20%.

1.6. Objectives

- To establish the sero-prevalence of bovine brucellosis in the cattle population in Swaziland.

CHAPTER 2

2.1. Methodology

2.2.1. Sampling design

Swaziland is divided into four administrative regions i.e. Manzini, Shiselweni, Lubombo and Hhohho. Swaziland has an estimated cattle population of 622 715 and 778 functional diptanks according to the last census and statistics in 2012. For the purpose of this study only 20 diptanks (Figure 2.1) were sampled across the country, whereby within each diptank, each kraal was represented. The 20 diptanks of study included 12 diptanks that were sampled during a recent bovine tuberculosis (BTB) survey. Out of the 20 diptanks used in this survey, seven diptanks were sampled from the Lubombo region, seven in Manzini, four in Shiselweni and two in the Hhohho region as per cattle population, with the Lubombo region having the highest and Hhohho the least. The number of diptanks selected represented a compromise between the above number, laboratory capacity and funds available for the survey. The diptanks were selected with assistance of regional veterinary officers.

Figure 2.1: Distribution of the sampled diptanks across the four regions of Swaziland.

2.1.2. Study animals

The target population for the study was cattle, both males and females aged 18 months and above. From each diptank, each kraal was sampled and consent was sought from all concerned farmers from the identified diptanks prior to sampling. The farmers also cooperated in the sampling. Sampling was done during routine national herd health management interventions. Animal handling was restricted to using diptank races and ropes. Every tenth animal was sampled, without giving any preference to any animal. The identification of cattle (name or number, age, kraal number and sex), vaccination status and abortion history, was noted on the sampling data sheet where possible. However, for the samples taken for the BTB survey, only the animal identification (name or number, kraal number, age and sex) was available on the data sheet. A total of 1623 animals was sampled for this study.

2.1.3. Collection, transport and storage of samples

About 10ml of blood was collected from either the jugular or coccygeal vein, using vacutainer tubes without anti-coagulant and 18 gauge hypodermic needles. Blood samples were labeled with serial numbers corresponding with information on the data sheet and transported to the Swaziland Central Veterinary laboratory in Manzini within 12 hours of collection. Blood was allowed to clot overnight at room temperature. The serum was separated and harvested into 2ml serum storage tubes, labeled with diptank number and serial number, and then stored at -20 ˚C. Samples were then transported frozen and on ice to the University of Pretoria, Department of Veterinary Tropical Diseases laboratory where they were subjected to serological testing.

2.1.4. Serological testing

2.1.4.1. Rose Bengal Test

The RBT was performed as described in the OIE Manual of diagnostic tests and vaccine for terrestrial animals, 2009. RBT is a rapid agglutination test using stained *B. abortus* rose bengal antigen at pH of 3.65 which helps to visualize the result and to eliminate some non-specific reactivity. The low pH of the assay prevents agglutination with IgM and encourages agglutination by IgG1, thereby reducing non-specific interaction (Muma et al. 2007).

As the manual states, 25µl of serum samples were dispensed into an appropriately labeled well of a 100-well disposable hemagglutination plate. An equal amount of undiluted *B. abortus* Rose

Bengal antigen was added to the serum samples. Immediately after the last drop of antigen had been added, the serum and the antigen were mixed by gentle tapping the edges of the plate. The mixture was then agitated gently for 4 minutes at ambient temperature on a rocker. Results were obtained by viewing the mixture for agglutination on X-ray light box. Agglutination was either graded as N for no visible agglutination and that was regarded as a negative result or, depending on the number of agglutinated cells, the agglutination was graded 1+ for least number of agglutinated cells up to 3+ for most agglutinated cells and these were regarded as positive results.

For each plate run, there was a positive and a negative control. The controls were treated in the same way as the samples. Different dilutions for the positive control were used i.e. 1/1, 1/10, 1/25 and 1/35 of the positive serum with a known negative serum. The Onderstepoort Biological Products (OBP) standard was adopted for this test and it states that the *B. abortus* Rose Bengal antigen should give a clear positive reaction with 1/25 dilution, but not with 1/35 dilution. The antigen as well as the positive control were sourced from OBP. The negative control was sourced from ARC-OVI, Bacteriology Serology department.

2.1.4.2. iELISA test

Chekit Brucellose Serum kits (Idexx laboratories) were used to detect antibodies against *Brucella abortus* as per the manufacturer's instructions. Microtiter plates are supplied precoated with inactivated antigen. 90µl of wash solution is dispensed into each well of the microtiter plate and 10 µl of serum sample is then added. The dilutions of the samples are incubated in the well of these plates. Antibodies specific for *B. abortus* bind to the antigen in the wells and form an antigen/antibody complex on the plate well surface. Unbound material is removed from the wells by washing. A peroxidase-labeled anti-ruminant IgG conjugate is added, which binds to the ruminant antibodies complexed with *B. abortus* antigen. Unbound conjugate is removed by washing and the TMB containing substrate is added to the wells. The degree of colour that develops (optical density measured at 450nm) is directly proportional to the amount of antibody specific for *B. abortus* present in the sample. The diagnostic relevance of the results is obtained by comparing the optical density (OD) that develops in wells containing the samples with the OD from wells containing the positive controls.

The optical densities of the samples were measured at 450nm in an ELISA reader. IDEXX software was used to calculate means and percentage (%) values and provided data summaries.

The following formulae were used for the calculations:

Positive control: $OD_{pos} - OD_{neg}$; NB: The OD values of duplicates were averaged.

Sample: $OD_{sample} - OD_{neg}$

For a valid assay, the minimum OD of the positive control should be 0.800 and should not exceed 2.000 and the OD of the negative control should not exceed 0.500. The difference between the positive and the negative control must be ≥0.300.

Finally: $$\text{Value (\%)} = \frac{OD_{sample} - OD_{neg}}{OD_{pos} - OD_{neg}} \times 100$$

The results were then interpreted as follows

Value	<80 %	≥80%
Interpretation	Negative	Positive

2.2. Data analysis

Sampling data and laboratory results were entered and stored in a Microsoft Excel spread sheet and analysis was done using standard software programs. A map to show the distribution of sampled diptanks as well as distribution of bovine brucellosis prevalence was drawn based on the geographical coordinates of the sampled diptanks using Arc View GIS. Statistical comparisons: to compare results a binomial test (non-parametric) at confidence interval of 95% was used. The binomial test was used to test whether the observed proportions are significantly lower or higher that the 20% national prevalence.

The following formulae (Thrusfield 2007) were used to calculate prevalence:

$$\text{Percentage (\%) of reactive animals} = \frac{\text{the number of reactive animals per diptank}}{\text{The total number of animals sampled per diptank}} \times 100$$

$$\text{Serial interpretation} = \frac{\text{positive reactors in both RBT and ELISA}}{\text{Number of cattle sera tested per diptank}} \times 100$$

Parallel interpretation = positive reactors in either RBT or ELISA X 100

Number of cattle sera tested per diptank

Agreement between RBT and ELISA was determined by the following formula (Thrusfield 2007):

$$\% \text{ agreement} = \frac{\text{number of cases where both tests gave the same result}}{\text{Total number of cases}} \times 100$$

CHAPTER 3

3.1. Results

3.1.1. Study animals

A total of 1623 (0.26%) bovine sera was examined from across the four regions of Swaziland out of an estimated bovid population of 622 715. The national beef herd in Swaziland comprises both *Bos taurus* and *Bos indicus* cattle. The breeds range from pure, hybrids to compound breeds in some instances. There are 778 functional diptanks in Swaziland distributed in all the four regions. A total of 20 diptanks was sampled across the country. Seven diptanks each were sampled in the Lubombo region as well as Manzini and four and two diptanks were sampled in the Shiselweni and Hhohho regions, respectively. The breakdown of diptanks per region and number of animals sampled per diptank is summarized in Table 3.1**.**

Table 3.1: Total number of animals sampled per diptank in the four regions

Region	**Diptank name**	**Total number of animals sampled**
Lubombo	Mkhiwa	86
	Thunzini	58
	Langkraal	32
	Mpaka	140
	Siphofaneni	131
	Siteki	96
	Matata	45
Manzini	Croydon	75
	Sigombeni	94
	EIE Ranch	17
	Mtilane	86
	TM Corporation	31
	Dvudvusini	129
	Mbangamadze	75
Shiselweni	Gege	132
	Matsanjeni	116
	Pienaar	80
	Luphala	66
Hhohho	Elukwatini	60
	Ludzibini	74
Total		**1623**

3.1.2. Serological testing

3.1.2.1. Serological findings per test

A breakdown of the results per diptank obtained by RBT is indicated in Table 3.2., which indicates that 262 (16.14%) out of 1623 animals tested positive for bovine brucellosis by RBT whilst 1361 (83.86%) animals were non-reactors. Sero-prevalence ranged from 0% (Elukwatini, TM-corporation and Dvudvusini) to as high as 53.49% recorded at Mkhiwa diptank.

Table 3.2.: RBT serological results at diptank level

Region	**Diptank name**	**Number of animals tested**	**No. and % of negative and positive animals**	
			Negative	**Positive**
Lubombo	Mkhiwa	86	40 (46.51%)	46 (53.49%)
	Thunzini	58	50 (86.21%)	8 (13.79 %)
	Langkraal	32	21 ()65.62 %	11 (34.48 %)
	Mpaka	140	117 (83.57 %)	23 (16.43 %)
	Siphofaneni	131	101 (77.1 %)	30 (22.9 %)
	Siteki	96	76 (79.17 %)	20 (20.83 %)
	Matata	45	44 (97.78 %)	1 (2.22 %)
Manzini	Croydon	75	61 (81.33 %)	14 (18.67 %)
	Sigombeni	94	85 (90.43 %)	9 (9.57 %)
	EIE Ranch	17	14(82.35 %)	3 (17.65 %)
	Mtilane	86	75 (87.21)	11 (12.79)
	TM Corporation	31	31 (100 %)	0 (0%)
	Dvudvusini	129	129 (100 %)	0 (0 %)
	Mbangamadze	75	55 (73.33 %)	20 (26.67 %)
Shiselweni	Gege	132	128 (96.97 %)	4 (3.03 %)
	Matsanjeni	116	76 (65.52 %)	40 (34.48%)
	Pienaar	80	76 (95 %)	4 (5 %)
	Luphala	66	56 (84.15 %)	10 (15.15 %)
Hhohho	Elukwatini	60	60 (100 %)	0 (0 %)
	Ludzibini	74	66 (89.19 %)	8 (10.81 %)
Totals		**1623**	**1361 (83.86%)**	**262 (16.14%)**

Out of the 1623 animals tested, a total of 1341 (82.62%) animals were non-reactors in the iELISA test, whilst 17.38% animals were reactors. Bovine brucellosis sero-prevalence obtained by the iELISA ranged from 0% (Elukwatini and TM-coporation diptanks) to the highest of 50.86% which was recorded at Matsanjeni diptank. A breakdown of the iELISA results of each diptank per region is indicated in Table 3.3.

Table 3.3.: Brucellosis serology results determined by iELISA at diptank level

Region	**Diptank name**	**Number of animals tested**	**No. and % of negative and positive animals**	
			Negative	**Positive**
Lubombo	Mkhiwa	86	52 (60.47 %)	34 (39.53 %)
	Thunzini	58	52 (89.66%)	6 (10.34 %)
	Langkraal	32	24 (75%)	8 (25 %)
	Mpaka	140	113(80.71%)	27 (19.29 %)
	Siphofaneni	131	90 (68.70%)	41 (31.30 %)
	Siteki	96	65 (67.71%)	31 (32.29 %)
	Matata	45	43 (95.56%)	2 (4.44 %)
Manzini	Croydon	75	58 (77.33)	17 (22.67 %)
	Sigombeni	94	88 (93.62%)	6 (6.38 %)
	EIE Ranch	17	13 (76.47%)	4 (23.53 %)
	Mtilane	86	79 (91.86 %)	7 (8.14 %)
	TM Corporation	31	31 (100%)	0 (0 %)
	Dvudvusini	129	129 (99.22%)	1 (0.78 %)
	Mbangamadze	75	58 (77.33%)	17 (22.67 %)
Shiselweni	Gege	132	125 (94.70%)	7 (5.30 %)
	Matsanjeni	116	57 (49.14%)	59 (50.86%)
	Pienaar	80	78 (97.5%)	2 (2.5 %)
	Luphala	66	64 (96.97%)	2 (3.03%)
Hhohho	Elukwatini	60	60 (100%)	0 (0 %)
	Ludzibini	74	63 (85.14%)	11 (14.86 %)
Totals		**1623**	**1341 (82.62%)**	**282 (17.38%)**

The overall prevalence of bovine brucellosis, at diptank level is presented in Table 3.4. The sero-prevalence of bovine brucellosis at diptank level ranged from 0% to 53.5% and 0% to 50.9% in the RBT and iELISA tests, respectively. Bovine brucellosis was found to be prevalent in all the diptanks sampled in the Lubombo and Shiselweni regions, whilst in the Manzini and Hhohho regions there was one diptank each which had no serological-reactors in neither of the two tests.

Table 3.4.: The overall prevalence of bovine brucellosis, at diptank level

Region	**Diptank name**	**Number of animals tested**	**No. and % of positive animals (prevalence)**	
			RBT	**ELISA**
Lubombo	Mkhiwa	86	46 (53.5%)	34 (39.5%)
	Thunzini	58	8 (13.8%)	6 (10.3%)
	Langkraal	32	11 (34.4%)	8 (25%)
	Mpaka	140	23 (16.4%)	27 (19.3%)
	Siphofaneni	131	30 (22.1%)	41 (31.3%)
	Siteki	96	20 (20.8%)	31 (31.3%)
	Matata	45	1 (2.2%)	2 (4.4%)
Manzini	Croydon	75	14 (18.7%)	17 (22.7%)
	Sigombeni	94	9 (9.6%)	6 (6.4%)
	EIE Ranch	17	3 (17.6%)	4 (23.5%)
	Mtilane	86	11 (12.8%)	7 (8.1%)
	TM Corporation	31	0 (0%)	0 (0%)
	Dvudvusini	129	0 (0%)	1 (0.8%)
	Mbangamadze	75	20 (26.7%)	17 (22.7%)
Shiselweni	Gege	132	4 (3.0%)	7 (5.3%)
	Matsanjeni	116	40 (34.5%)	59 (50.9%)
	Pienaar	80	4 (5%)	2 (2.5%)
	Luphala	66	10 (15.2%)	2 (3.0%)
Hhohho	Elukwatini	60	0 (0%)	0 (0%)
	Ludzibini	74	8 (10.8%)	11 (14.9%)
Totals		**1623**	**262 (16.14%)**	**282 (17.38%)**

A summary of bovine brucellosis sero-prevalence from all the four regions is presented in Table 3.5. Bovine brucellosis was found to be prevalent in all the four regions of Swaziland. The Lubombo region recorded the highest sero-prevalence by both RBT and iELISA i.e. 23.5% and 25.2%, respectively and Hhohho had the lowest sero-prevalence of 5.9% and 8.2% by RBT and iELISA, respectively. Out of the 1623 sera examined, 216 (16.14%) were seropositive by RBT while 281 (17.38%) sera tested positive by the iELISA test.

Table 3.5: A summary of the serological test results for bovine brucellosis per region

Region	**Cattle population per region**	**Number of animals tested**	**Number and % of RBT positive animals**	**Number and % of ELISA test positive animals**
Lubombo	172 927	588 (0.34%)	139 (23.5%)	149 (25.2%)
Manzini	175 108	507 (0.29%)	57 (11.25)	52 (10.2%)
Shiselweni	150 594	394 (0.26%)	58 (14.7%)	70 (17.8%)
Hhohho	124 086	134 (0.11%)	8 (5.9%)	11 (8.2%)
Total	**622 715**	**1623 (0.26%)**	**262 (16.14%)**	**282 (17.38%)**

3.1.2.2. Interpretation of results

Sero-prevalence results by both RBT and iELISA were interpreted in series. An animal was considered seropositive when both RBT and iELISA results were positive. An overall bovine brucellosis sero-prevalence of 12.01% was obtained by serial interpretation of results by both RBT and iELISA. A breakdown of the sero-prevalence results obtained by serial interpretation is indicated in Table 3.6.

Table 3.6.: Serial interpretation of RBT and iELISA results

Region	**Diptank name**	**Number of animals tested**	**Reactive animals in both RBT and iELISA**	
			number	**Percentage (%)**
Lubombo	Mkhiwa	86	28	32.56
	Thunzini	58	5	8.62
	Langkraal	32	8	25
	Mpaka	140	17	12.14
	Siphofaneni	131	26	19.85
	Siteki	96	18	18.75
	Matata	45	1	2.22
Manzini	Croydon	75	13	17.33
	Sigombeni	94	5	5.32
	EIE Ranch	17	2	11.76
	Mtilane	86	5	5.81
	TM Corporation	31	0	0
	Dvudvusini	129	0	0
	Mbangamadze	75	17	22.67
Shiselweni	Gege	132	3	2.27
	Matsanjeni	116	37	31.90
	Pienaar	80	0	0
	Luphala	66	2	3.03
Hhohho	Elukwatini	60	0	0
	Ludzibini	74	8	10.81
Total		**1623**	**195**	**12.01**

The bovine brucellosis serological results were further interpreted in parallel. An animal was considered seropositive when either RBT or iELISA result was positive. An overall sero-prevalence of 21.50% was obtained through application of parallel interpretation of results. A breakdown of sero-prevalence results obtained through parallel interpretation is indicated in Table 3.7.

Table 3.7.: Parallel interpretation of RBT and iELISA results

Region	**Diptank name**	**Number of animals tested**	**Reactive animals in either RBT or iELISA**	
			number	**Percentage (%)**
Lubombo	Mkhiwa	86	52	60.47
	Thunzini	58	9	15.52
	Langkraal	32	11	34.38
	Mpaka	140	33	23.57
	Siphofaneni	131	45	34.35
	Siteki	96	33	34.38
	Matata	45	2	4.44
Manzini	Croydon	75	18	24
	Sigombeni	94	10	10.64
	EIE Ranch	17	5	29.41
	Mtilane	86	13	15.12
	TM Corporation	31	0	0
	Dvudvusini	129	1	0.78
	Mbangamadze	75	20	26.67
Shiselweni	Gege	132	8	6.06
	Matsanjeni	116	62	53.45
	Pienaar	80	6	7.50
	Luphala	66	10	15.15
Hhohho	Elukwatini	60	0	0
	Ludzibini	74	11	14.86
Total		**1623**	**349**	**21.50**

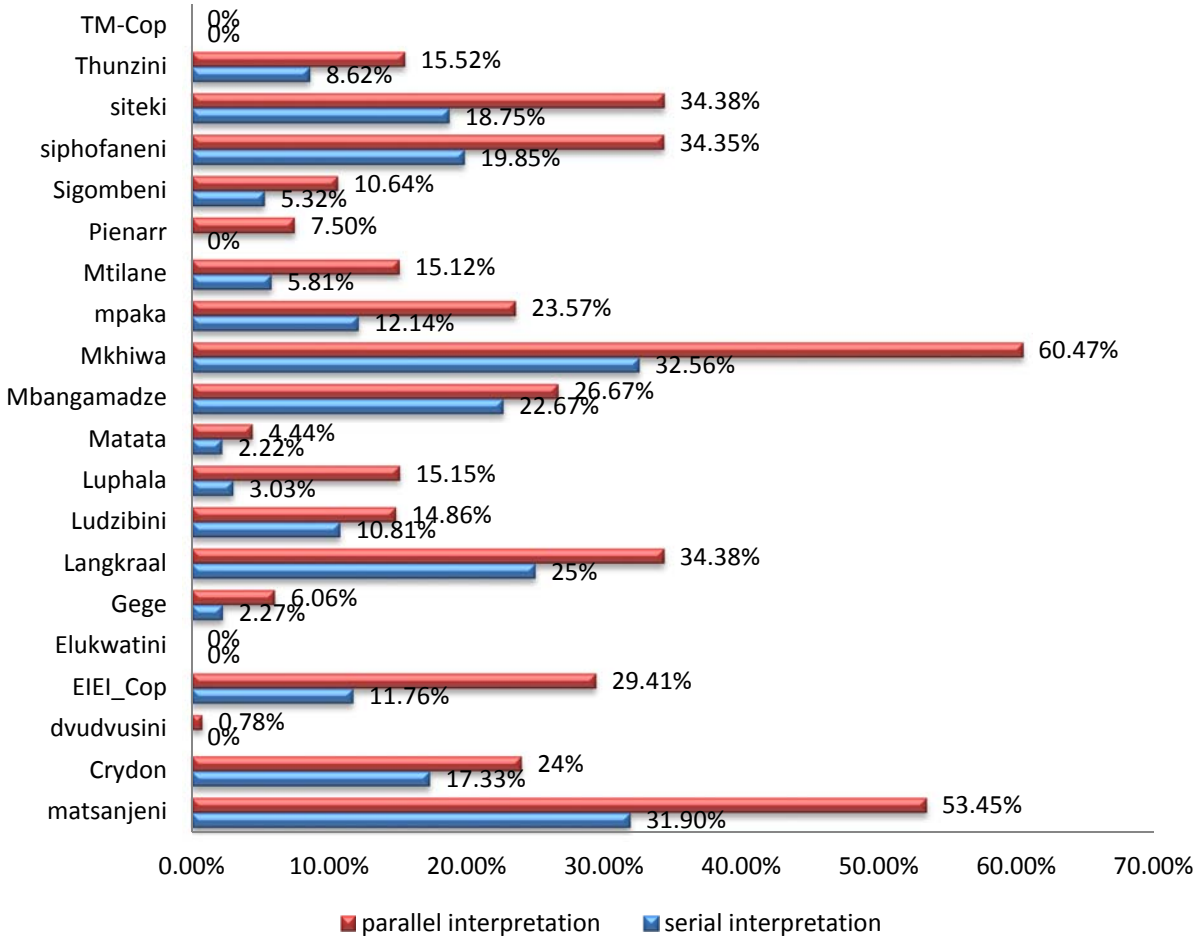

Figure 3.1.: Serial vs parallel interpretation of RBT and ELISA results.

An overall bovine brucellosis sero-prevalence of 21.50% was obtained from parallel interpretation of results from the tests that were employed for this study and i.e. RBT and iELISA (Table 3.7), whereas an overall bovine brucellosis sero-prevalence of 12.01% was obtained by serial interpretation of results from both RBT and iELISA (Table 3.6). Generally, a higher bovine brucellosis sero-prevalence was observed with parallel interpretation of the results. Prevalence ranged from 0% at Elukwatini and TM-corporation diptank to the highest prevalence of 60.47% at Mkhiwa diptank. Nine out of twenty diptanks exhibited prevalences higher than 20% when results were interpreted in parallel i.e. Matsanjeni 53.45%, Crydon 24%, EIEI-corporation 29.41%, Langkraal 34.38%, Mbangamadze 26.67%, Mkhiwa 60.47%, Mpaka 23.57%, Siphofaneni and Siteki with 34.35% and 34.38%, respectively (Figure 3.1).With serial interpretation, prevalence also ranged from 0% at Elukwatini and TM-corporation to the highest of 53.45% observed at Matsanjeni diptank (Figure 3.1). Out of the twenty diptanks sampled, only four diptanks revealed a prevalence of above 20% when results were interpreted

in series i.e. Matsanjeni 31.90%, Langkraal 34.38%, Mbangamadze and Mkhiwa with 26.67% and 32.56%, respectively. A comprehensive breakdown of the sero-prevalence interpretations at diptank level indicated in Table 3.6, Table 3.7 and Figure 3.1. Figure 3.2 reveals that even though bovine brucellosis is prevalent in most parts of the country, the western part is least affected as it has diptanks that were not affected and/or exhibited the lowest sero-prevalence. The distribution of all the diptanks tested for bovine brucellosis sero-prevalence across the four regions of Swaziland is indicated in Figure 3.2**.**

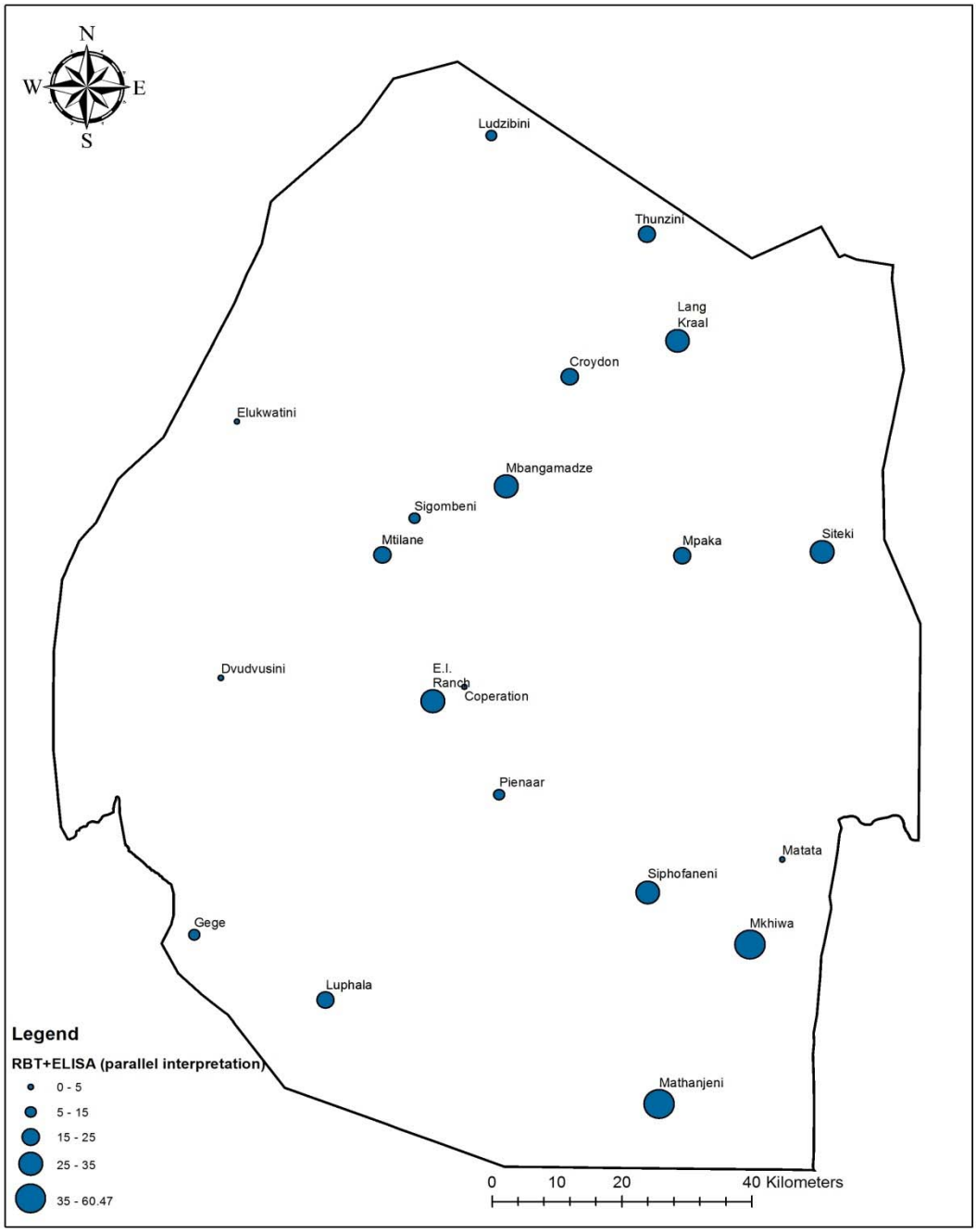

Figure 3.2: Map of Swaziland showing the distribution and bovine brucellosis sero-prevalence at sampled diptanks.

Table 3.8 indicates that out of a total of 1623 animals tested using both RBT and iELISA, 1469 (90.51%) yielded the same results in both tests. There were 1274 (78.50%) non-reactive animals in both RBT and iELISA and 195 (12.01%) reactive animals in both tests. Only 154 (9.49%) of the animals tested, yielded opposite results in either RBT or iELISA.

Table 3.8.: Tests agreement between RBT and iELISA

Region	**Diptank name**	**Number of animals tested**	**Tests agreement**		**Tests disagreement**
			negative	**positive**	
Lubombo	Mkhiwa	86	34	28	24
	Thunzini	58	49	5	4
	Langkraal	32	21	8	3
	Mpaka	140	107	17	16
	Siphofaneni	131	86	26	19
	Siteki	96	63	18	15
	Matata	45	43	1	1
Manzini	Croydon	75	57	13	5
	Sigombeni	94	84	5	5
	EIE Ranch	17	12	2	3
	Mtilane	86	73	5	8
	TM Corporation	31	31	0	0
	Dvudvusini	129	128	0	1
	Mbangamadze	75	55	17	3
Shiselweni	Gege	132	124	3	5
	Matsanjeni	116	54	37	25
	Pienaar	80	74	0	6
	Luphala	66	56	2	8
Hhohho	Elukwatini	60	60	0	0
	Ludzibini	74	63	8	3
Total			**1274 (78.50%)**	**195 (12.01%)**	
Grand total		**1623**	**1469 (90.51%)**		**154 (9.49%)**

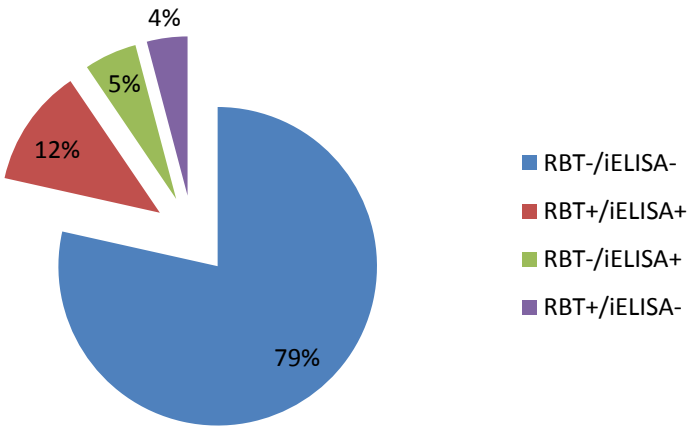

Figure 3.3.: Proportions of tests agreement between RBT and iELISA

Figure 3.3 indicates that 79% of the animals tested negative in both RBT and iELISA, and 12% of the animals were positive in both RBT and iELISA. Only 4% of the animals tested were positive in RBT and negative in iELISA and the remaining 5% were non-reactors in RBT and reactors in iELISA.

CHAPTER 4

Discussion

The overall sero-prevalence of bovine brucellosis was determined using both the serial and parallel interpretation of test results. An overall sero-prevalence of 12.01% was determined by the serial interpretation of results whereas with parallel interpretation, a sero-prevalence of 21.50% was observed. According to Zhou et al (2011), series testing has a higher specificity and decreased sensitivity as each case has to be positive in both tests to be considered a true positive whereas with parallel testing, a case has to be positive in either one of the tests to be considered positive i.e. parallel testing increases the probability to identify true positives and also false positives thus increasing sensitivity but decreasing specificity (Thrusfield 2007). Because RBT and iELISA used in this study are both screening tests, and neither RBT nor iELISA is 100% accurate, and serological diagnosis is presumptive evidence of infection, the use of the combined positive result as an indicator of exposure is more useful. Therefore, parallel interpretation of results was considered the best approach in this study. In parallel testing, disease is less likely to be missed, thus increased test sensitivity is obtained but also more false positives are obtained, hence, there is a decrease in test specificity. The overall improved sensitivity in parallel testing increases the chance of obtaining the most probable true prevalence status of a disease. However, for the purpose of obtaining true prevalence status of a disease, the use of combined tests with high sensitivity and high specificity is desirable.

There was a slight variation of sero-prevalence between the two tests used for this study. The iELISA detected 17.38% overall prevalence whilst the RBT detected an overall prevalence of 16.14%. The variation was also observed at regional as well as at diptank level. Even though the iELISA presented a higher overall sero-prevalence than the RBT, at regional and diptanks level, there were cases where RBT detected a higher prevalence than the iELISA.

Even though, the tests detected varied prevalences in the regions, a higher prevalence by one test in one region did not translate to the same outcome at diptank level. There were still variations of prevalence by each test at diptank level per region. The serological prevalence of bovine brucellosis at diptank level varied from 0% in both RBT and iELISA to as high as 53.3% and 50.9% in RBT and iELISA, respectively. Despite the fact that bovine brucellosis is prevalent in Swaziland, the western parts of the country, according to this study, proved to be less affected by the disease. The lowest sero-prevalence or 0% prevalence was obtained in diptanks inclined to the western parts. The serological prevalence of bovine brucellosis in this part of the country is surprisingly low considering the fact that there is no formal control programme in

place, and there is free trade and movement of animals between diptanks and regions. Internal livestock movement does not require testing for brucellosis antibodies and prohibition of movement of positive animals. The most probable reason might be that herds in this part of the country are stable and farmers rarely buy and /or sell cattle outside their communities.

This sero-prevalence of bovine brucellosis in cattle in the extensive management system in this study disagrees with reports from other countries with similar husbandry systems and the same rural setting. From this study, bovine brucellosis sero-prevalence ranged from 0% to as high as above 50% in some diptanks. In other studies, in extensive cattle management system in Ethiopia, a prevalence figure varied with ranges from 0.77% to 8.2% (Berhe et al. 2007). In another study, according to Hesterberg et al. (2008), in the north-eastern parts of KwaZulu-Natal, South Africa, the mean prevalence ranged from 2.4% to 15.5%.

Factors associated with high and variable prevalence in extensive livestock production systems are large herd sizes, extensive movement of cattle and mingling with other herds at common grazing and watering points (Hesterberg et al. 2008, Berhe et al.2007, McDermott and Arimi 2002). These factors contribute to the variation in sero-prevalence of bovine brucellosis between herds in Swaziland. Sharing of grazing pastures contributes to the spread of infection. Cattle in the rural areas of Swaziland are generally grazed on communal pastures and can move over distances of several kilometers which could lead to contamination of large areas as calving is not restricted to a specific place, such as pens. This could function as source of infection for other groups within that community using the same pasture (Smits 2013). Omer et al (2000) and Berhe et al (2007), further argue that migrations increase the chances of the spread of infection. This factor is important for Swaziland as internal livestock movement does not require testing for brucellosis antibodies and prohibition of movement of positive animals.

The RBT and ELISA results correlated positively in 90.5% of the cases under investigation in this study. This may be attributed to the fact that both tests have similar characteristics; they are both screening tests, both with high sensitivity and limited specificity. However, according to literature, the indirect ELISA, which has been used in this study, generally has a higher sensitivity and specificity and detects all the *Brucella* antibody isotypes in serum as compared to RBT with low pH which discourages agglutination by IgM but encourages agglutination by IgG_1 (Poester et al. 2010, Muma et al. 2007, Nielsen 2002, Uzal et al. 1995).This slight variation in test attributes contributed to the 9.49% variation of results observed in this study. According to previous studies, the differences in the sensitivity and specificity of serological tests used are important factors that contribute to variation in sero-prevalence estimates of *Brucella* infection (McDermott and Arimi 2002, Mangen et al. 2002).

According to Nielsen and Yu (2010) another factor that might have contributed to the variation may be genetic diversity of populations, some animals will respond with low antibody levels to

exposure to *Brucella* species, resulting in false negative results. Other animals will respond with very high levels of antibody which may cause prozoning in some assay types like RBT. High responders may also have elevated antibody levels to naturally occurring antibody caused by exposure to cross-reacting microorganisms such as *Yersinia enterocolitica* 0:9 (Muñoz et al. 2005, Pouillot et al. 1998)

CHAPTER 5

Conclusion and Recommendations

In conclusion, the results obtained from this study indicate an overall sero-prevalence of bovine brucellosis of 21.50% in Swaziland and significantly higher infection rates (more than 50%) at some diptanks. This figure is considerably higher than the hypothesized 20% sero-prevalence of bovine brucellosis at diptank level. A further investigation has to be carried out across the country and more diptanks investigated. Also, a confirmatory test for the purpose of identifying true positives is recommended. Furthermore, the serological tests that were used in this study are unable to infer which smooth *Brucella* species induced antibodies in the animal host. Therefore, more research is recommended to determine which smooth *Brucella* species is causing infection in the cattle population of Swaziland.

Moreover, due to the zoonotic nature of brucellosis an attempt to decrease the impact of the disease in the local human population through awareness campaigns is critical. Furthermore, owing to the relatively non-specific symptoms in humans and a frequent lack of information on zoonotic diseases, it is important to inform and collaborate with public health services to increase the likelihood of correct, prompt diagnosis and treatment, as well as, to advocate disease prevention through precautionary measures. For further prevention and early diagnosis of disease, a human health survey at diptanks with high prevalence of *Brucella* infection is recommended.

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APPENDIX

Sero-Survey of Brucellosis in Swaziland- Template data form

Serial no.	Animal Id.	Kraal no.	Sex	Colour	Aborted yes/no	Vaccinated yes/no	Age (months)
1							
2							
3							
4							
5							
6							
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8							
9							
10							
11							
12							
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BTB survey- Template data sheet

Serial no.	Kraal no.	Animal id.	Sex	Colour
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