PREVALENCE, HERD HEALTH AND ZOONOTIC IMPLICATIONS OF BRUCELLOSIS IN COMMUNAL AND SMALLHOLDER CATTLE FARMING AREAS IN NORTH-WEST PROVINCE, SOUTH AFRICA

by

BONTSI MARUMO

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SUPERVISOR: **Dr PN Kayoka**

CO-SUPERVISOR: **Dr Tiny Hlokwe**

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DECLARATION

Name: Bontsi Marumo

Student number: 41132920

Degree: Master of Science in Agriculture

PREVALENCE, HERD HEALTH AND ZOONOTIC IMPLICATIONS OF BRUCELLOSIS IN COMMUNAL AND SMALLHOLDER CATTLE FARMING AREAS IN NORTH-WEST PROVINCE, SOUTH AFRICA.

I declare that the above dissertation is my own work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references.

I further declare that I submitted the dissertation to originality checking software and that it falls within the accepted requirements for originality.

I further declare that I have not previously submitted this work, or part of it, for examination at Unisa for another qualification or at any other higher education institution.

24 February 2023

SIGNATURE DATE

DEDICATION

This dissertation is dedicated to the memory of my late mother and grandmother for their upbringing and for grooming me to be the woman that I am today. To my late father for encouraging me that I can do more than what I think I can. A special thanks to my daughter for loving and believing in me, for motivating and encouraging me to finish this study. Lastly, to God who gave me the power of mind, strength, and the will power to carry on.

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ABSTRACT

Brucellosis is recognized as one of the most important bacterial zoonosis as it is responsible for considerable economic losses in livestock and long-term chronic disease condition in humans, consequently a health-related burden worldwide. This study highlights the need to investigate the prevalence and distribution of brucellosis in the four main districts (Dr Ruth Segomotso Mompati, Dr Kenneth Kaunda, Bojanala platinum and Ngaka Modiri Molema) of the North-West province of South Africa, as well as its zoonotic implications. Specific objectives of this study were to determine the seroprevalence of Brucellosis in cattle though serological analysis, to isolate and identify *Brucella* from suspected specimens and to assess farm management, herd-health, and husbandry system. A total number of blood (*n*=770) samples from farmed animals (*n*=378) and abattoir-slaughtered (*n*=392) animals were collected for this study. The blood from abattoirs was accompanied by lymph nodes (*n*=392) corresponding to the blood sample of each animal. In addition to the above, milk samples (*n*=22) were collected from cows after farmers' permission. The Rose Bengal Plate Agglutination Test (RBT) was used to detect anti-*Brucella* antibodies in serum samples followed by confirmation of positive sera with the complement fixation test (CFT). Milk samples were screened by milk ring test (MRT) using *B. abortus* MRT antigen. Tissue samples corresponding to sero-positive samples were subjected to isolation and phenotypic characterisation of *Brucella* species by isolation onto Farrell's Medium with 5% bovine serum and penicillin. The same positive tissue samples were subjected to *Brucella spp*. molecular identification by amplification of the IS711, 16S rDNA and the internal transcriber subunit (ITS) gene fragments. Serological results indicated the overall prevalence for RBT positive samples to be 2% at 95% Confidence Interval (CI). All 770 samples were subjected to screening with the RBT, 18 out of 770 (2.3%) tested positive. Confirmation with CFT revealed that 16 (*n*=16) out of 18 samples were indeed positive. Sero-positive results were found in Ngaka Modiri Molema and Dr Ruth Mompati districts with the prevalence of 4.65% (95% CI: 2.61 – 8.11) and 2.34% (95% CI: 0.91-5.85) respectively. This resulted in the overall prevalence of 1.95% (95%CI: 1.14 – 3.12) for all four districts combined. Out of the 2.3% (18/770), only 0.90% (7/770) were from slaughtered animals. All 0.90% (7/770) tissue samples tested negative for PCR and cell culture. A questionnaire to determine the abattoir owners and farmers' general understanding and knowledge on zoonotic diseases was developed and interviews conducted. A multivariate analysis has shown a significant association between participants' statuses of literacy with the risk of brucellosis in a farm.

Key words: Brucellosis, *B. abortus*, RBT, CFT, MRT, PCR,

LIST OF ABBREVIATIONS

AP	-	Apparent Prevalence
ARC	-	Agricultural Research Council
bp	-	Base Pair
BSL3	-	Biosafety Level 3
BTA	-	Blood Tellurite Agar
CAES	-	College of Agriculture and Environmental Science
CDC	-	Centers for Disease Control
CI	-	Confidence Intervals
CF	-	Complement Fixation
CFT	-	Complement Fixation Test
CNS	-	Central Nervous System
COVID-19	-	Coronavirus Disease 2019
CO_2	-	Carbon Dioxide
CSF	-	Cerebrospinal Fluid
DAFF	-	Department of Agriculture Forestry and Fisheries
DALRRD	-	Department of Agriculture, Land Reformed and Rural Development
DNA	-	Deoxyribonucleic Acid
rDNA	-	Recombinant DNA
EDTA	-	Ethylenediaminetetraacetic acid
g	-	Grams
iELISA	-	Indirect Enzyme-Linked Immunosorbent Assay
EtBr	-	Ethidium Bromide

H_2S	-	Hydrogen sulfide
ITS	-	Internal Transcriber Subunit
IU	-	International Unit
LPS	-	Lipopolysaccharide
MRT	-	Milk Ring Test
OIE	-	Office International des Epizooties (World Organization for Animal Health)
OR	-	Odds Ratios
OBP	-	Onderstepoort Biological Products
OVR	-	Onderstepoort Veterinary Research
PCR	-	Polymerase Chain Reaction
PPE	-	Personal Protective Equipment
RBT	-	Rose Bengal Test
SANAS	`	South African National Accreditation System
SAT	-	Serum Agglutination Test
Se	-	Sensitivity
SOP	-	Standard Operational Procedure
Sp	-	Specificity
TAE	-	Tris-acetate-EDTA
UV	-	Ultraviolet
WHO	-	World Health Organization

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CHAPTER 1: INTRODUCTION

1.1 Specific work title

Prevalence, herd health and zoonotic implications of brucellosis in communal and smallholder farming areas in North-West Province, South Africa.

1.2 Background

It has been several decades since brucellosis is recognized as one of the important bacterial zoonosis as it is responsible for considerable economic losses in livestock and health-related burden (Adelakun *et al*., 2019). According to the World Health Organization (WHO), humans are accidental hosts who are mainly infected through the consumption of contaminated dairy products or by direct contact with infected animals. People can also be infected when they are exposed to 'infected excretions of cattle, foetuses, foetal membranes or with infected carcass materials in abattoirs' or by ingesting infected unpasteurized milk (WHO, 2016). The most effective way of reducing the impact of the disease in livestock and prevent human infection is to control this disease in the animal population.

In South Africa, Brucellosis is a notifiable medical condition in humans and a controlled disease in animals (Govindasamy, 2020). According to the South African legislation (Animal diseases Act. 35 of 1984 and the Animal Health Act 7 of 2002), all suspected and confirmed cases of abortion must be reported to the nearest State Veterinary office for zoo-sanitary actions as prescribed in the national Brucellosis control scheme. It is also stated in the Act 35 of 1984 that the responsible person must immunize heifers between the ages of 4 and 8 months in the Republic once with a remedy. The act further emphasizes on testing, isolation, branding and slaughtering of infected animals.

1.3 Problem Statement

Currently, there are very few publications on prevalence of brucellosis in the communal and smallholder farming areas in South Africa including North-West province. The disease is endemic in South Africa as it is also maintained in wild animals such sable antelope and lions (Simpson *et al*., 2021). The economic implications of brucellosis are a threat to the development of the agricultural sector, particularly in communities practising communal livestock management systems (Lokamar *et al.*, 2020). Its zoonotic nature makes brucellosis a burden to society. Most people rely on the consumption of livestock products such as milk and meat as source of proteins. The risk is assumed higher in rural communities where people have frequent contact with animals. Most of the livestock owners have little knowledge and awareness of brucellosis risk of infection and spread of the disease (Cloete *et al*., 2019). It is therefore essential to determine the prevalence and associated risk factors to prevent and control this disease. This will contribute towards a sustainable strategy for control of this zoonotic disease.

1.4 Hypothesis

The prevalence and distribution of brucellosis in the study area may not be different from other areas with similar zoo-epidemiological situations. The risk factors and zoonotic implications of brucellosis in the study area are not expected to be lower than the situation in commercial settings.

1.5 Aim and Objectives

The study aims at determining the prevalence of brucellosis in cattle in the North-West province of South Africa.

1.5.1 The specific objectives are:

- To determine the seroprevalence of Brucellosis in cattle using serological methods.
- To isolate and identify *Brucella* from suspected specimens from cattle.
- To assess farm management, herd-health, and husbandry system.

1.5.2 Research Questions

The research questions to address the objectives were the following:

- What is the prevalence of ruminant brucellosis in communal herds in North-West province?
- What are the potential sources of Brucella infections in communal herds in North-West province?
- Are the risk factors and zoonotic implications of Brucella in North-West province understood?

1.5.3 Significance and motivation of the study

The research output and the information generated produced comprehensive data on prevalence, distribution, risk factors and zoonotic implications of brucellosis in the study area, North-West province of South Africa. This will also assist in identifying potential risks posed to farm and abattoir workers to implement proper mitigation strategies to avoid infection. The risk factors associated with the survival and transmission of *Brucella* were identified for the formulation of control and prevention strategies.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Brucellosis is a disease caused by the bacterial genus *Brucella* (Morwal, 2017). *Brucella* is a Gram-negative, facultative and intracellular bacterium that is pathogenic to humans and animals (Poester *et al*., 2014). Brucellae organisms are shed in large numbers in the animal's urine, milk, placental and other fluids (Dauda *et al.,* 2015). A variety of *Brucella* species have been identified, of which four have moderate to significant pathogenicity to humans, and are named from the host source or features of the infection (Dauda *et al*., 2015). These species are *Brucella melitensis* (from sheep & goat), *Brucella suis* (from pigs), *Brucella abortus* (from cattle) and *Brucella canis* (from dogs). *B. melitensis* and *B. suis* have higher pathogenicity to humans while the latter have moderate pathogenicity (Poester *et al*., 2014). *B. abortus* and *B. melitensis* are the major causes of abortion, birth of weak offspring, stillbirth, retained placenta and infertility in cows, does and ewes. Small ruminants brucellosis is mostly caused by *B.melitensis* (Omer *et al.*, 2002). *Brucella ovis* is also an important cause of orchitis and epididymitis in rams but it is not recognized as a cause of natural infection in goats (Poester *et al*., 2014).

2.2 Signs and symptoms

2.2.1 In animals

Although other syndromes have been reported, the most common clinical signs in affected animals are abortions, placentitis, epididymitis and orchitis (Neta *et al*., 2010). However, in most cases, brucellosis is a chronic disease with acute phase and incubation period of two to three weeks (Hadush and Pal, 2013). Signs and symptoms are extensive and may develop over a period of weeks to months from the initial exposure (Poester *et al*., 2002). The initial phase of brucellosis in animals such as cattle, sheep, goats and other ruminants is often not apparent. The infection localizes in the reproductive system and may result in abortion, stillbirth and infertility (Givens and Marley, 2008). Other signs can include arthritis in cows and pigs, mastitis and lameness in goats, and oozing skin lesions in horses (Morwal, 2017). Some animals infected with brucellosis may exhibit swollen lymph nodes and testicles, difficulty walking and back pain (WHO, 2016).

2.2.2 In humans

Brucellosis clinical manifestations in humans include fever, sweats, fatigue, malaise, anorexia, weakness, weight loss and headache. These symptoms can be mild in certain individuals and other people may develop long-term chronic symptoms which can lead to disability in some people (Poester *et al*., 2014). Miscarriages and infant birth defects are common in pregnant women (Poester *et al*., 2014). Moreover, human brucellosis can occasionally be difficult to notice because its clinical signs and symptoms are similar with that of other illnesses (Neta *et al*., 2010). Due to this reason, infection with brucellosis can only be confirmed through laboratory testing (Neta *et al*., 2010). The WHO states that it is highly unlikely for brucellosis to affect the nervous system or eyes, (WHO, 2016).

2.3 Transmission

2.3.1 In animals

Amongst others, the *Brucella* species such as *B. abortus*, *B. melitensis*, *B. suis* and *B. canis* are usually transmitted between animals by contact with the placenta, fetus, fetal fluids and vaginal discharges from an infected animal" (Pappas *et al*., 2005). The spread of Brucellosis may be increased by the movement of infected animals from an infected herd into a non-infected herd (Dadar *et al*., 2021). Cross transmission of brucellosis can occur between animals causing stormy abortions and infertility in livestock (Dadar *et al*., 2021). Abortions and slaughtering of infected animals may result in significant economic losses to the livestock industry (Ntirandekura *et al.*, 2018). Research has proven that semen used for artificial insemination may pass on the disease if it comes from an infected animal; however, natural sexual transmission of brucellosis is unlikely to occur (Dauda *et al*., 2015).

2.3.2 In humans

In humans, brucellosis transmission is mainly through direct contact with infected animals, inhalation of aerosols or through the ingestion of infected food products (Corbel *et al*., 2006). Farm workers and laboratory personnel can acquire the bacteria through handling of infectious material, inhalation or accidental skin penetration (Hadush and Pal, 2013). In addition, human transmission could also be through ingesting contaminated food, direct contact with an infected animal, or inhalation of aerosols

(Hadush and Pal, 2013). However, the most common transmission in people is orally, by eating or drinking raw or unpasteurized milk products that contain the bacteria. Transmission between humans is possible through exchange of body fluids (Dauda *et al*., 2015). In their biosafety manual, the World Health Organization states that humans are susceptible to *B. abortus*, *B. melitensis* and *B. suis*, with *B. canis* causing mild disease (WHO, 2016). Although all these species are pathogenic, the first three species are reported to be more virulent and may cause serious complications in humans. According to Solera, various factors including the immune status, route of infection and the *Brucella* species determine the extent of infection (Solera, 2010).

2.4 Pathogenicity in non-human animals

Unlike many bacteria, *Brucella* genome consists of two circular chromosomes without plasmids (Christopher *et al.,* 2010). It is classified within the subdivisions of Proteobacteria, which includes *Agrobacterium, Rickettsia, Rhodobacter*, and *Rhizobium* (Christopher *et al*., 2010). The completion of genome sequences of *B. melitensis* (GenBank NC 003317 and NC 003318), *B. suis* (GenBank NC 002969), and *B. abortus* has assisted in the understanding of *Brucella* pathogenicity mechanisms (Delvecchio *et al*., 2002). Not only can *Brucella* species resist killing by neutrophils, but they can also replicate inside macrophages leading to multiplying and inhibiting phagosome–lysososme fusion (Cardoso *et al*., 2006). The major virulent factor of *Brucella* is the outer membrane which consists of Lipopolysaccharide (LPS) that has peculiar non-classical LPS as compared to other bacteria (Cardoso *et al*., 2006). These non-classical LPS changes the host immune response which provides resistance to anti-microbial attacks. The LPS O-chain is a key molecule for *Brucella* replication and survival as it provides protection from complement-mediated lysis, cellular cationic peptides, and oxygen metabolites (Cardoso *et al*., 2006). The genome sequences have shown similarities in sequence, structure and organization for *B. melitensis, B. suis*, and *B. abortus* (Halling *et al*., 2005).

The ability of *Brucella spp*. to invade, replicate and survive for long period within the host is important for the course of the disease (Dauda *et al*., 2015). The long incubation period depends on factors such as age, gender, sexual maturity and stage of pregnancy (López-Santiago *et al*., 2019). Incubation period is longer in calves as they are are infected "in utero" and will shed the bacteria when they reach sexual maturity, whereas pregnant cows show symptoms as early as 14 days (Corbel *et al*., 2006).

Brucella spp. require a few critical steps during infection (Ackermann *et al*.,1988). They allow infection through mucosal surfaces and enter through digestive or respiratory tract (López-Santiago *et al*., 2019). After infecting the host, the bacteria are found in circulating granulocytes or polymorphonuclear, leucocytes and macrophages (López-Santiago *et al*., 2019). *Brucella* can survive intracellularly within phagocytic or non-phagocytic host cells once inside the host (López-Santiago *et al.*, 2019). Nevertheless, studies show that the outcome of infection is dependent on the species and host (Ahmed, Zheng and Liu, 2016). The *Brucella spp*. that infect livestock are host specific as mentioned earlier, *B. melitensis*, *B. abortus*, *B. suis* and *B. ovis* infect respectively small ruminants, cattle, pigs and sheep (WHO, 2005). However, *B. suis* and *B. melitensis* are zoonotic bacteria with the latter being highly pathogenic for humans (Moreno, 2014).

2.5 Diagnosis

Diagnosis of brucellosis is important to monitor the vaccination programme progress in animals and for epidemiological purposes. In its factsheet, the WHO reported that the history and research in epidemiological patterns are critical for clinical diagnosis of brucellosis (WHO, 2016). Diagnosis must be carried out on the whole herd because some infected animals show long incubation period and animals may stay serologically negative for a substantial period after infection (Ali *et al*., 2015). Therefore, one or two positives in a herd is enough evidence that there is an infection in the herd and that other animals may be in the incubation period.

The predicament with brucellosis diagnosis is that one specific method alone is not sufficient to conclude results. Consequently, diagnosis by serology is mostly accompanied by molecular or other supportive diagnosis (Negash and Dubie, 2021; Khan and Zahoor, 2018). In most cases, diagnosis by serological methods is used for the initial screening due to possible false negatives in the early days of infection (Khan and Zahoor, 2018).

Testing for detection of antibodies using serological methods, blood culture isolation and molecular identification of the bacteria can assist in making reliable diagnosis (Al Dahouk and Nöckler, 2011). As with human brucellosis, the disease is under diagnosed leading to inaccurate reporting in animals (Wojno *et al*., 2016). This is a concerning factor as brucellosis is a controlled disease in South Africa (SA), as in many parts of the world (DAFF, 2016c). For this reason, the South African Department of

Agriculture, Land Reform and Rural Development (DALRRD) has implemented the bovine brucellosis scheme which aims to improve disease control and its prevalence. South Africa is still run by the legislative framework (Bovine Brucellosis Scheme R.2483 of Dec 9 Dec 1988) which still emphasizes on vaccination and test- slaughter methods. This scheme is reported to have assisted in the reduction of brucellosis cases in South Africa, back in the 1980s. The number of cases started to increase again between late 1980 and 1994 when the responsibility of vaccinating heifers was handed over to livestock owners, as most of them did not comply with prescribed control measures.

Brucellosis diagnosis can be classified in two ways in which one demonstrates the presence of the bacteria and the other detect an immune response to its antigens (Poester *et al*., 2013). This is mainly because not all infected animals give a positive culture while the antibody detection provides only a provisional diagnosis (Poester *et al*., 2013). The techniques that can identify the causative agent through microscopic examination or culture, and molecular detection are referred to as the direct methods which are known to differentiate other *Brucella* species from the vaccine strain (Kang *et al*., 2011; WHO, 2005). Serological techniques for antibody detection includes the rose bengal test plate (RBT), the complement fixation test (CFT), the milk ring test (MRT), the standard tube agglutination test (SAT), the indirect enzyme linked immunosorbent assay (iELISA) and the 2-Mercaptoethanol test (2ME) methods that are used worldwide (Kaltungo *et al*., 2014). These methods are useful in herd screening, surveillance programs, controlling and eradication strategies in different geographical locations (Simpson *et al*., 2018). Conversely, the disadvantage with serological methods is that they cannot differentiate between true infections to vaccine strains such as S19 and RB51 (Madut *et al*., 2018). For this study, only the MRT, CFT and RBT methods were used. Both serological and molecular methods used for this specific study will be discussed in more details in the next chapter.

2.5.1 Direct diagnosis

2.5.1.1 Microscropy

Brucella identification is based on staining of the bacterial colony, observation of the morphology and colonial appearance under the microscope. *Brucella spp*. are Gram-negative, non-spore forming and non-motile facultative intracellular coccobacilli that is about 0,5–0,7 µm in width (Hadush and Pal, 2013). The biochemical identification comprises among others the production of hydrogen sulfide (H_2S) carbon dioxide (CO_2) requirements and the hydrolysis of urea (Kang *et al*., 2011). Other means of

identification are the sensitivty of the *Brucella* culture to agglutination with monospecific sera and phage typing (Bayram *et al*., 2011). This method requires isolation of *Brucella* microorganisms from body fluids or bone marrow biopsies since it has low sensititivity in milk and dairy products due to their presence in small amounts (OIE, 2016). *Brucella* appears as clumps of coccobacilli which makes it difficult to see under the microscope leading to bacterial culture and molecular diagnosis being considered. In most cases, the staining method is only useful if it is supported by other direct methods described below (Kang *et al*., 2011; McDonald *et al*., 2006).

2.5.1.2 Bacteriological culture

Although research suggests culturing as the preferred direct method because of its specificity, it is too risky to laboratory personnel and special cautionary measures such as working in the biosafety level 3 (BSL3) environments should be adhered to (Hadush and Pal, 2013). Working in a biosafety level 2 should be done with additional protection such as wearing of gowns, gloves, masks and googles (Govindasamy, 2020). The OIE terrestrial manual recommends that cultures should be isolated from samples such as uterine discharges, aborted fetuses, lymph nodes and tissues from reproductive organs from male and female animals in general (OIE 2016). Nonetheless, the choice of samples to collect for culturing is normally dependent on the clinical signs observed. In most cases, samples from aborted fetuses can include stomach contents, spleen, and lung. In sexually matured animals, semen and vaginal swabs have yielded good results (Lopes *et al*., 2014; Dahouk and Nöckler 2011). In addition to the above, preference is given to the mammary and genital lymph nodes including the uterus and the udder from dead animals (Mahajan *et al*., 2017).

Although regarded as the “golden” method of choice, culturing *Brucella* is time consuming and may take up to six weeks for the bacteria to grow in culture. Another challenge besides the rate of growth is that culturing *Brucella* is labour-intensive as repeated subculturing might be required (Wojno *et al*., 2016; Špičić *et al.,* 2010). However, it can take up to 7 days to grow *Brucella* in modern automated machines (WHO, 2016). It is also important to keep inoculated media for a longer period to avoid false negative results. Delivery time to the testing laboratory also plays a major role in the successful isolation of *Brucella* species. Therefore, fresh samples kept under required conditions are recommended (Hadush and Pal, 2013).

2.5.1.3 Molecular detection

The other direct method that is commonly used for *Brucella* identification is molecular detection using the polymerase chain reaction (PCR) (Poester *et al*., 2014). Apart from its speed and sensitivity, the PCR method has an advantage when it comes to safety of laboratory personnel. According to Gupta, this method is also popular for its rapidness in diagnosis, identification of genus or species differentiation as well as identification of other microorganisms (Gupta, 2014). In most cases, bacterial strain differentiation is essential where many biotypes have been detected in the population as this will assist proper identification (Hundal *et al*., 2016). Various PCR assays have been developed including assays where the *Brucella* species can be discriminated and differentiated in a single reaction. These include real time PCR assays that are more sensitive than the conventional PCR (Behera *et al.,* 2020). For PCR detection, deoxyribonucleic acid (DNA) needs to be extracted from different specimens either by manual or automated methods. The yield of DNA extracted is normally dependant on the technique or type of sample used. For animals, the DNA can be extracted from either whole blood, tissue, serum, cerebrospinal fluid or organs of aborted foetuses (Dadar *et al.,* 2021). Nonetheless, DAFF reported that the lungs produced more positives results as compared to the spleens, livers and bronchial lymph nodes (DAFF, 2016e). It is important that the extraction and PCR methods preferred must include a step that reduces inhibitors such as calcium and collagen in bone, milk and tissue, haematin in blood, that are known to decrease the efficiency of PCR by preventing the amplification of nucleic acids (Behera *et al*., 2020). Other PCR inhibitors include compounds such as proteins, fats, bile, calcium chloride, EDTA, heparin and ferric chloride (Thornton and Passen, 2004).

As with the previous direct detection methods already discussed, the PCR method has its disadvantages in that it requires expensive equipment for visualization, and the high risk of contamination is inevitable (Ko and Splitter, 2003). It is for this reason that the real time PCR system which is faster and less prone to contamination has been developed (Gwida *et al*., 2011).

2.5.2 Indirect diagnosis

2.5.2.1 Rose Bengal test (RBT)

The Rose Bengal Test is an agglutination test which detects anti-*brucella* antibodies in serum. It is a rapid test which uses suspensions of *B.abortus* cells stained with the rose bengal dye and buffered to

a low pH typically of 3.65 ± 0.05 (Godfroid *et al*., 2010). Our standard procedure in South Africa is that the rose bengal test is used for first round screening of samples before they are confirmed by the complement fixation test (Chisi *et al*., 2017). However, the sensitivity and specificity of RBT has been reported to be low especialy in chronic cases and a serial dilution (1:2 through 1:64) of the serum samples may be applied to increase specificity as the quality of antigens used is crucial (Christopher *et al*., 2010).

2.5.2.2 Complement fixation test (CFT)

The complement fixation is a test used to detect the presence of antibodies that does not form agglutination in serum when mixed with antigen (WHO, 2016). This test relies on the ability the complement to lyse erythrocytes in the absence of an antibody-antigen complex (Poester *et al*., 2014). The complement fixation test is widely used in diagnostic laboratories as a confirmatory test because of its increased specificity and sensitivity (Hadush and Pal, 2013). This method is used mostly for cattle, sheep, goat and other livestock animals and it has also been accepted for use with human sera samples (WHO, 2016). Due to its complexity, the CFT method is regarded as technically challenging as it uses different types of reagents, in addition to the test serum, this method uses *B. abortus* CFT antigen, complement, amboceptor, ovine erythrocytes and CFT buffer making it expensive and a well trained personnel is also needed (Yu and Nielsen, 2010). Although the CFT both sensitive and specific, it is largely affected by the misuse of strain 19 vaccine in cases where recent or repetitive vaccines have been administered in sexually mature heifers and cows (DAFF, 2016c). As a result, it is impossible to prescribe strict cut-off readings that indicate infection.

2.5.2.3 Milk ring test (MRT)

The milk ring test is slightly different from the serum agglutination tests in that *Brucella* cells are stained with haematoxylin mixed with whole milk or its cream (OIE, 2016). MRT is a simple and sensitive test that is used to detect the presence of *Brucella* antibodies in milk samples (Tekle *et al*., 2019). The test reaction involves adsorption of *Brucella* antibodies in the fat globules resulting in the antigen-antibody reaction (OIE, 2016). The sensitivity is expected at 95% probability of detecting an infected cow in a 100-cow herd if there are no sampling or recording or errors. Another reason could be at the time of sampling when the infected cow is contributing to the bulk supply of milk or there is an infected cow which might be excreting antibodies (DAFF, 2016a). A positive result in the tube test will be indicated

by the purple band around the milk layer meaning the *Brucella sp.* antibody is attached to the antigen. In the absence of *Brucella spp.*, the purple colour will be visible throughout the milk in the test tube (OIE, 2016). In conjunction with other methods, this method is very fast and inexpensive. The disadvantage is that false reactions may occur mostly because of the condition of the milk (WHO, 2016). That is the reason why milk should reach the testing laboratory within two days of sampling while kept at 4°C.

2.6 Treatment, prevention and control

2.6.1 In animals

According to the Animal diseases Act 35 of 1984, Brucellosis is not treated in animals. Instead, prevention and control of brucellosis is essential for proper management of its impact on the human health and social economic implications. To control the spread of brucellosis in animals, the world health organization recommends elimination of infected animals (Corbel *et al*., 2006). Together with the Food and Agriculture Organization (FAO) of the United Nations and the OIE, the WHO reported that although regulations for managing infected herd and flocks may vary in different countries; the reduction of exposure to *Brucella spp.* and the increase of the resistance to infection remains the basic principles (WHO, 2016). Other categories for prevention and control includes test and slaughter, occupational hygiene practices, control of movement and vaccination (WHO, 2016). As part of the surveillance programme, the OIE reported additional means of prevention as not drinking unpasteurised milk or eating dairy products from such milk, wearing of proper personal protective equipment (PPE) such as rubber gloves and overalls and regular testing (OIE, 2016).

The prophylaxis of brucellosis in cattle relies on vaccination of heifers between ages of 4 and 12 months of age with *B. abortus* S19 and RB51 vaccines (Dorneles *et al.*, 2015). The RB51 can be used again in female cattle after they have calved, and to heifers 2-3 months before mating (DAFF, 2016e). Conversely, the South African Act 35 of 1984 recommends immunisation of heifers between 4 and 8 months, which basically falls within the requirement of other countries across the world. This is mainly because vaccination in pregnant cows may cause abortion or low milk production, while bulls may become sterile (Dorneles *et al*., 2015). In general, a highly effective vaccine has not been developed. Despite the fact that the S19 and RB51 vaccines have been effective in controlling the state of

brucellosis in many countries, various challenges have been reported leading to an ongoing research to develop a vaccine without drawbacks (Dorneles *et al*., 2015). Amongst other challenges, the current vaccines have been reported to interfere with diagnosis of brucellosis in laboratories (Ducrotoy *et al*., 2017b). According to the South African Department of Agriculture Forestry and Fisheries the only success with the current vaccines will be if farmers co-operate with the veterinarians, animal health technicians and the laboratories (Dorneles *et al*., 2015). Due to its zoonotic nature and its negative impact on livestock and human health, research on a vaccine that will address the current challenges is vital.

2.6.2 In humans

In humans, antibiotics are provided as a means of treatment. Since clinical signs of brucellosis are similar to those of other illnesses, treatment with relevant antibiotics may be delayed leading to some patients not fully recovering (Corbel *et al*., 2006). If treated within a month of experiencing symptoms, most patients have been reported to have fully recovered (Corbel *et al*., 2006). As a result, people who have been in contact with an infected *Brucella* animal are encouraged to report to the nearest health care provider for early detection and monitoring for about six months, even when symptoms have disappeared (WHO, 2005). The following antibiotics that are normally prescribed to treat brucellosis separately or combined include Doxycycline, Streptomycin, Ciprofloxacin, Rifampin and Tetracycline. Antibiotics are to be taken for many weeks to prevent the recurring of the disease and for full recovery.

2.7 Bovine brucellosis distribution in South Africa

Brucellosis is distributed worldwide and affects livestock as well as people (Godfroid *et al*., 2010). According to Moreno, brucellosis is still prevalent in most parts of the world with an extensive variety of hosts (Moreno, 2014). In South Africa, bovine brucellosis is reported as endemic in all nine provinces of the republic with the Highveld regions concentrated (Cloete *et al*., 2019). The recent publication on characterization of *Brucella* species and biovars in South Africa indicated that animal brucellosis is widespread in the country (Matle *et al*., 2021). Studies on brucellosis knowledge and zoonotic implications have shown that most farming communities lack knowledge, therefore, farming communities in rural areas are more at risk (Cloete *et al*., 2019). This normally results in misdiagnosis and inappropriate treatment for the people infected (Simpson *et al*., 2018b).

Another recent publication was on the seroprevalence between cattle handlers and variables surrounding the sero positivity of the herds (Govindasamy, 2020). This study was conducted from 2014 – 2016 in the Gauteng province of South Africa. The study identified herd risk factors associated with cattle handlers and veterinary officials exposure to *Brucella* (Govindasamy, 2020). This animal and human association might help in further epidemiological studies regarding this disease.

Due to the intensity and distribution of brucellosis in the country, the former Department of Agriculture, Forestry and Fisheries (DAFF) established control measures that are currently under discussion for ensuring that the farming industry works together with the government to facilitate an effective management of the disease (DAFF, 2016e).

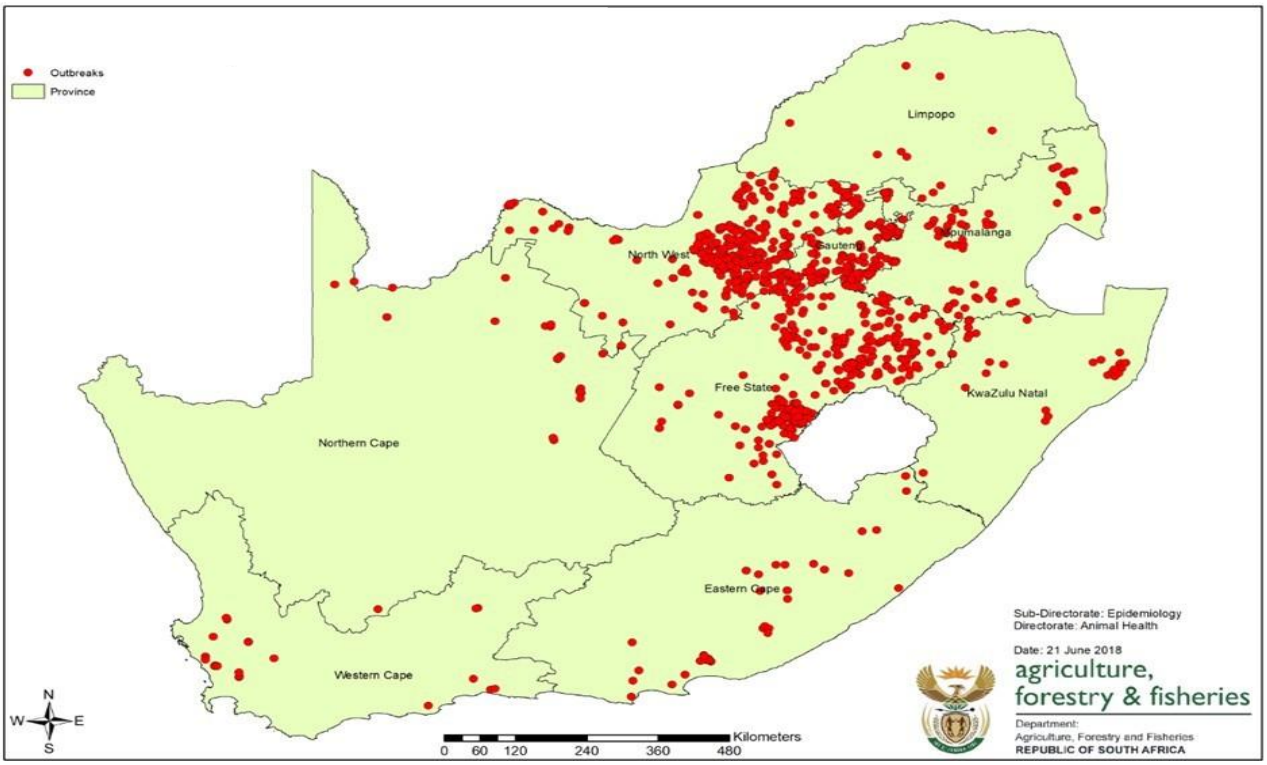

Figure 2.1: Outbreaks reported in animals from January 2015 to May 2018 across all nine provinces of South Africa.

Image courtesy of Department of Agriculture, Forestry and Fisheries Sub-Directorate: Epidemiology of the Directorate Animal Health.

CHAPTER 3: MATERIALS AND METHODS

3.1 Ethical clearance and confidentiality

The College of Agriculture and Environmental Science (CAES) and the Onderstepoort Veterinary Research (OVR) animal ethics guidelines and regulations were adhered to. The CAES ethics clearance was approved (2020/CAES_AREC/123) for this research (Appendix A). The ethical clearance letter was also granted by the OVR ethics committee (Appendix B). Permission to conduct this study and have access to samples was authorized by the director for North-West veterinary services (Appendix C). As a requirement, section 20 permit was issued by the Director of Animal Health from the Department of Agriculture Forestry and Fisheries (now called the Department of Agriculture, Land Reform and Rural Development) in 2018 (Appendix D). Approval for sampling in abattoir facilities was granted by the North-West director of veterinary services. All participants signed a consent letter which clearly stated confidentiality of this study, prior to the interview (Appendix E).

3.2 Study area, design, and sampling strategy

3.2.1 Study area

This study was conducted in selected communal, commercial, and non- commercial farms of the North-West province. Samples were collected from twenty (*n*=20) abattoirs and forty (*n*=72) farms in all four major districts of the North-West (NW) province namely: (Dr Ruth Segomotso Mompati, Dr Kenneth Kaunda, Bojanala platinum and Ngaka Modiri Molema) under the supervision of a veterinarian. Both beef and dairy cattle were sampled from thirty-one (*n*=31) communal, twenty-four (*n*=24) commercial, and seventeen (*n*=17) non-commercial farms.

3.2.2 Study design and sampling strategy

This prospective study design used is cross-sectional with a multistage sampling strategy. The sampling frame included all sub-districts that are more rural in communal production setting in the selected areas. Villages/dip tanks in those municipalities were selected randomly. Study villages/dip tanks were selected in collaboration with the provincial Department of Agriculture (Veterinary services) based on accessibility, livestock population, perceived history of zoonoses and collaboration from

communities. The animals included in this study were also randomly selected at the time of visit at each villages/dip tank. Figure 3.1 below indicates the sampled area per district in the province.

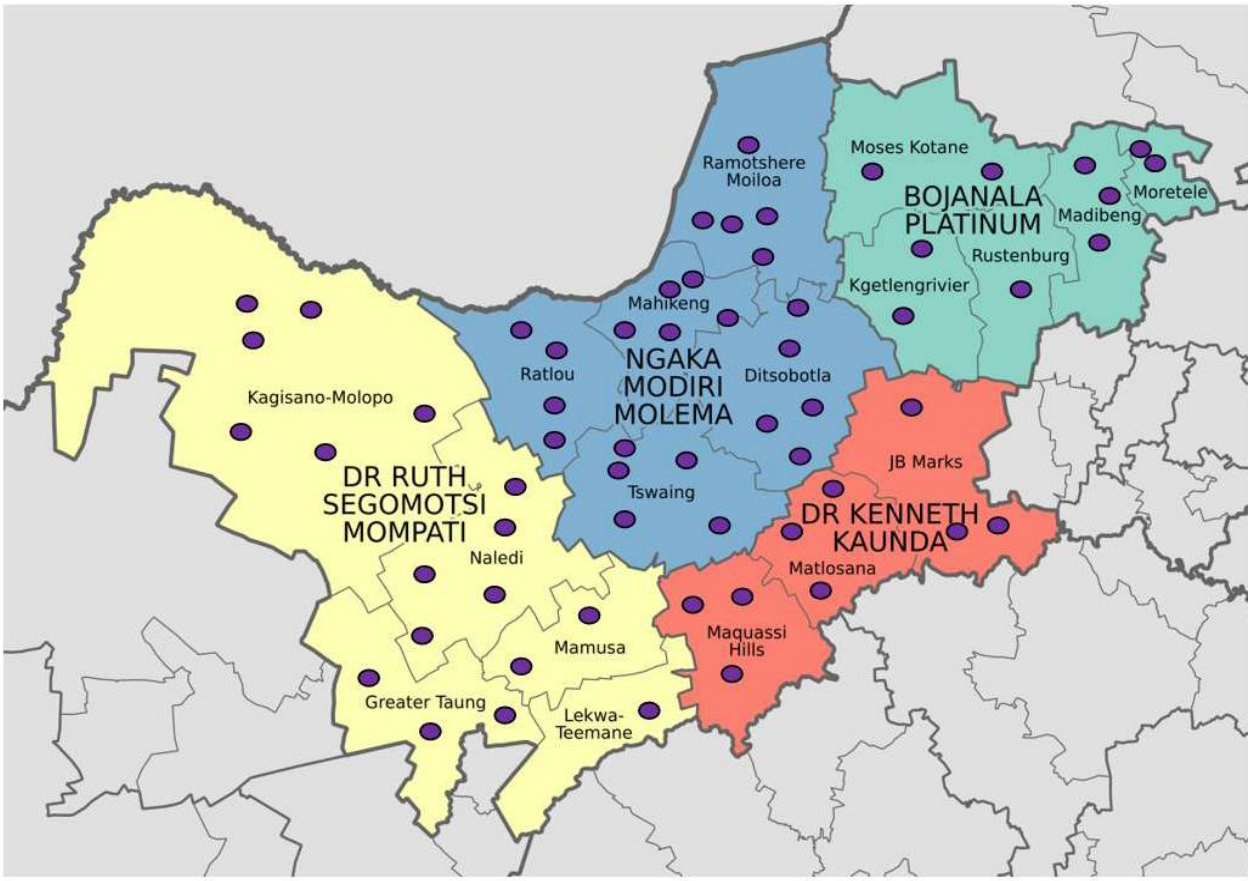

Figure 3.1: Sample collection site (indicated in purple-coloured marks).

3.3 Source of samples and population

The study population included all cattle above the age of 6 months which were selected on random bases during site visits. In all farms, animals that have recently aborted or have the history of abortion were sampled, otherwise animals were selected randomly. Blood, tissue, and milk samples were collected for this study.

For abattoirs, consent from the managers of low-throughput and rural abattoirs was obtained prior to the visits. On the day of the visit to the abattoir, all condemnations data and reason for condemnation were obtained from the meat inspector on duty. Retrospective historical information was also assessed

from the previous records. The animals slaughtered on the day of the visit were identified as to the type of livestock, breed (if, possible), sex, age, the origin, and management system. Farmed animals were restrained using collecting crushes and fences for a short period to allow for blood collection. The veterinarian on site was observing any injuries that might arise during sampling and ensure comfort of the animals.

Figure 3.2 Animal Heath Technician collecting blood from restrained cattle on a farm.

A total of seven hundred and seventy (*n*=770) animals were sampled were one thousand one hundred and eighty-four (*n*=1184) samples were collected from farmed (blood & milk) and slaughtered animals (blood & tissue). Table 3.1 and Appendix F clearly indicates the number and type of sample collected from the animals (*n*=770) per district. The reason for collecting corresponding tissue and blood samples from same animal was for comparative evaluation of brucellosis testing methods.

Table 3.1: Number and types of samples collected per district in the North-West Province.

Type of samples	Name of district sampled				Total per sample type
	Ngaka Modiri Molema	**Dr. Kenneth Kaunda**	**Dr Ruth Mompati**	**Bojanala Platinum**	
Blood samples from abattoirs	113	93	66	120	**392**
Blood samples from Farms	124	103	105	46	**378**
Tissue samples from abattoirs	113	93	66	120	**392**
Milk samples from farms	5	3	5	9	**22**
Total per district	**355**	**292**	**242**	**295**	**1184**

3.4 Sample size determination

The number of animals that were sampled at farms and abattoirs were determined using the epidemiological formula as described by Thrusfield (2007) and The EPITOOLS software for calculations (Thrusfield, 2007). The values used in the calculation included estimated prevalence at 0.05 since it is unknown, desired precision at 0.05, confidence level at 0.95 with an estimated population size of 10 000. The total sample size calculated was (*n*= 770). Therefore, the project aimed to sample at least 385 farmed animals as well as 385 slaughtered animals. However, the number of samples collected from farms could not reach 385 hence more samples were collected from abattoir animals to get to the required sample size.

3.5 Data collection

Samples were collected by animal health technicians under the supervision of the state veterinarians before being tested at Onderstepoort Veterinary Research. All COVID-19 protocols were followed, with the research team wearing appropriate personal protective equipment (PPE) and frequently using the

sanitizer. Social distancing between participants and the research team was also maintained during questionnaire administration.

3.5.1 Blood samples

Approximately, 7.5 ml of blood was collected in red top vacutainer tubes from the jugular and coccygeal veins of the farmed animals (Table 3.1). A total of blood samples (*n* = 770) was collected. After blood collection, the vacutainer tubes were kept in suitable cooling boxes in a standing position to allow blood clotting prior to transportation to Onderstepoort Veterinary Research (OVR) institute laboratory for serological testing. In the laboratory, tubes were centrifuged at 1500 X g for 15 minutes; sera were decanted in sterile tubes and stored at 4 °C for short term storage before processing and at -20°C for long term storage.

3.5.2 Tissue samples

Lymph nodes comprised of mesenteric lymph nodes, retropharyngeal lymph nodes, supramammary lymph nodes (*n*=392) were collected for the isolation of *Brucella* species (Table 3.1). Sealable sterile plastic bags were used to collect tissue samples. Since no aborted materials were found during sampling, only the corresponding lymph nodes to the blood samples that tested positive with CFT, were used for culture isolation and PCR detection of *Brucella* species.

3.5.3 Milk samples

Milk samples were collected from individual lactating cows upon the farmer's consent. However, most farmers were reluctant to allow milk sampling. The milk samples (*n*=22) were collected in sterile screw-capped bottles and transported on ice to OVR institute for analysis.

3.5.4 Questionnaire administration

The questionnaire to determine the abattoir owners and farmers' general understanding and knowledge on zoonotic diseases was developed (Appendix G). This questionnaire was first pre-tested on a pilot group of 5 farmers to prevent biasness and assess the relevancy of questions and amended

accordingly. The research team members in close collaboration with the provincial animal health and extension services assisted in interviewing farmers. The researcher explained the content of the questionnaire and completed it while the respondent answered. In most cases, the local language Setswana was used to explain to participants who did not understand English.

The questionnaire was divided into three sections. The first section consisted of general information such as the area and district of the farm, the gender and age of the farm owner or worker, the duration of farming and the size and origin of the animals. The second section determined the farmer's knowledge regarding zoonotic diseases. Amongst others, this included determining if the farmer had experienced abortions among their cattle and if so, at what stage of pregnancy. This section also addressed the handling of aborting dams and aborted foetuses. In the last section, preventive measures and practices against brucellosis were assessed.

3.6 Laboratory test methods

3.6.1 Serological methods

Serological test methods that were used in this study were validated by the OVR bacteriology serology laboratory using proficiency testing samples. The sensitivity and specificity values obtained from validation are indicated in the table below:

Table 3.2: Sensitivity and specificity values for serological methods used.

	RBT	CFT	MRT
Sensitivity	99.15% (95.37% - 99.98%)	99.03% (94.71% - 99.98)	100% (95.85% - 100.00%)
Specificity	100.00% (94.56% - 100.00%)	100.00% (93.94% - 100.00%)	100% (87.66% - 100.00%)

3.6.1.1 Rose Bengal Test

The Rose Bengal Test was used to detect anti-*Brucella* antibodies in serum samples. This rapid and sensitive agglutination test was used as a screening test for cattle serum and it utilised *B. abortus* Rose Bengal Test antigen [purchased from Onderstepoort Biological Products (OBP), Pretoria, South Africa] and stored at 4 ± 3 °C. The serum was placed at room temperature for about 30 minutes before the test commenced. In white porcelain haemagglutination plates, 25 µl of serum and 25 µl of antigen were

dispensed into each well according to the plate numbering (the plates used were 15mm in diameter and contain 100 hemispherical wells which were 180x180 mm). This was followed by gently tapping of the plate to allow the two to mix before incubating for 4 minutes while placed on the shaker set at 40 rpm. The results were observed on the ultraviolet (UV) light box and positive sera were subjected to the complement fixation test (CFT) for brucellosis confirmation.

3.6.1.2 Complement Fixation Test

The complement fixation test (CFT) was used for the diagnosis of brucellosis though the detection of antibodies in the serum. This method consisted of two stages where the first stage involved the antigen mixed with the serum of a guinea pig which is referred to as the complement. If the test serum contains antibodies to the antigen, the complement would not get fixed and would not react in the second stage. The second stage consisted of addition of sheep red blood cells which have been mixed with anti-sheep red blood cell antibody. If all the complement has been fixed in the first stage, no haemolysis would occur. This means that the test serum contained antibody to *Brucella* and was considered positive. If haemolysis of the red blood cells took place, this means that the antigen was not fixed in the first stage because the serum contained no antibodies. The test was recorded as negative. Except for the complement, which is heat labile, reagents were allowed to reach room temperature at 22 ^{o}C ± 3 ^{o}C before testing commenced. The procedure was carried out according to the Bacteriology serology laboratory's standard operational procedure. This procedure involves inactivation and serial dilutions of sera, reagent dispensing and relevant incubation at different phases.

3.6.1.3 Milk Ring Test

Milk samples were screened following milk ring test (MRT) using *B. abortus* MRT antigen (purchased from Onderstepoort Biological Products, Pretoria, South Africa) stored at 6 ^{o}C ± 2 ^{o}C. The milk samples were collected last during sampling and transported on ice to OVR to avoid heating which can lead to the loss of *Brucella* antibodies. Upon reaching the laboratory, milk samples were refrigerated at 5 ^{o}C ± 3 ^{o}C for at least 12 hours before testing. Homogenised, pasteurised, or sour milk were not used as it can interfere with the results.

The antigen that was sufficient for the day's test was mixed thoroughly into another bottle and kept at room temperature with the test samples for at least one hour before testing. The procedure involved

dispensing 30 µl of antigen, before adding 1ml of the milk into plastic non-sterile tubes (1 - 5 ml) with screw tops. The tubes were then inverted ± 5 times to ensure thorough mixing and incubated at 37 ^{o}C ± 2 ^{o}C for 1 hour before reading the results.

3.6.2 Isolation and phenotypic characterization of *Brucella* species

In a biosafety cabinet class II (BSL II), tissue samples were streaked onto Farrell agar plates which were semi-restrictive to *Brucella* with 5% bovine serum and antibiotics (Matle *et al*., 2021; De Miguel *et al.*, 2011). The cultures were then incubated at 37 °C in a 10% CO_2 environment for up to 14 days and checked after every 48 hours. A known positive control *Brucella* culture was included for quality control purposes. Suspicious bacterial colonies resembling *Brucella* were stained with Stamp's and Gram's stains and further sub-cultured and tested with specific biochemical substrates (Geresu *et al*., 2016). Identification process included biochemical tests namely oxidase, catalase, urease, and microscopic morphological examination of the individual colonies. Further identification through biotyping of Brucella colonies, was carried out based on the biochemical test results (Madut *et al*., 2018). *Brucella* colonies on Farrell's medium are small (1 mm diameter), round, translucent with smooth margins and have a pale honey colour. Colonies are small, round, grey and non-haemolytic on Blood agar (Geresu *et.al*., 2016). If identification of Brucella not completely confirmed after all the above procedures, PCR test was used as an alternative method for further confirmation.

Figure 3.3: Suspect/presumptive *Brucella* colonies on Farrell's agar plates.

3.6.3 Molecular identification and differentiation of *Brucella* species by PCR

Brucella species identification at genus level was conducted by amplification of the IS*711*, 16S recombinant DNA (rDNA) and the internal transcriber subunit (ITS) gene fragments using polymerase chain reaction (PCR), (Hinić *et al*., 2009). This technique can also be used for differentiation between the *B. abortus* wild type strain and RB51 and S19 vaccines following DNA extraction from clinical specimen (Hamidi *et al*., 2016). The method of DNA extraction depends on the type of specimen to be extracted. For this study, DNA was extracted from the tissue samples whose corresponding serum sample was confirmed positive with the CFT.

3.6.3.1 DNA extraction

3.6.3.1.1 DNA extraction from homogenised tissues

DNA extraction for homogenised tissues was carried out using the QIAamp® DNA Mini and Blood Mini Handbook kit, according to the manufacturer's instructions. This purification kit uses components that are guaranteed to be RNase-free.

3.6.3.1.2 DNA extraction from culture

A total of 200µl distilled water was pipetted into an eppendorf tube. An inoculation loop was used to streak out a loop-full of the culture from the BTA plates into the distilled water. The mixture was then vortexed briefly before being put in a heating block at 95°C for 10 minutes. This step was followed by centrifugation at 15 000 rpm for 5 minutes before starting with PCR.

3.6.3.2 Polymerase Chain Reaction

Preparation of the PCR master-mix was carried out in lab 1 (nucleic acid free laboratory) which is reserved for such and is regarded to as the clean area. The preparation was carried out in a laminar flow cabinet using a sterile 1.5 ml eppendorf tube. The master-mix preparation for *Brucella* species identification and detection is the same with that of RB51 and S19 vaccines. The difference was related to the primers used and PCR programmes in the machine. Depending on the number of samples, the reagents were used as indicated in table 3.3.

Table 3.3: Master-mix preparation for *Brucella species* identification by polymerase chain reaction.

Reagent	**Volume** (19 µl per reaction)			
	1 x reaction	**5 x reaction**	**10 x reaction**	**30 x reaction**
Water	5.5	27.5	55	165
ISP1/F4 (20µl)	0.5	2.5	5	15
ISP2/R2 (20µl)	0.5	2.5	5	15
Phusion Flash Master-Mix	12.5	62.5	125	375

After putting the required volume (19 µl) into tubes, the mixture was vortexed followed by brief (1 second) centrifugation. The total amount of 19 µl was liquated into pre-labelled 0.2ml thin-walled microfuge tubes followed by 6 µl of the DNA template. The *B. abortus* positive control (Ref: 2012-D-10059), water, and an unrelated sample (*E. coli* 0157:H7ATCC43888) was added for quality control purposes.

The mixture was placed on a thermocycler and exposed to the following cycling conditions:

- Initial denaturation: - 94 ◦C for 2 minutes
- Denaturation: - 95 ◦C for 20 seconds
- Annealing: - 55.5 ◦C for 20 seconds
- Extension: - 72 ◦C for 30 seconds
- Number of cycles: - 31
- Cool to 4 ◦C

3.6.3.3 Gel electrophoresis

After PCR amplification was completed, samples were run electrophoretically on a 1.5 % agarose gel containing 10 µl of 1% ethidium bromide. Five microliter of loading dye was added to each tube containing the PCR product and 15 µl of the mixture was loaded on the gel. A 100bp marker was loaded and used to determine the size of the bands (PCR products). The gel was run for approximately

60 minutes at 100 voltages. The gel was then visualised under the Gel Doc [Aplegen Omega Fluor Gel Documentation System, San Francisco (USA)] and results observed and analysed.

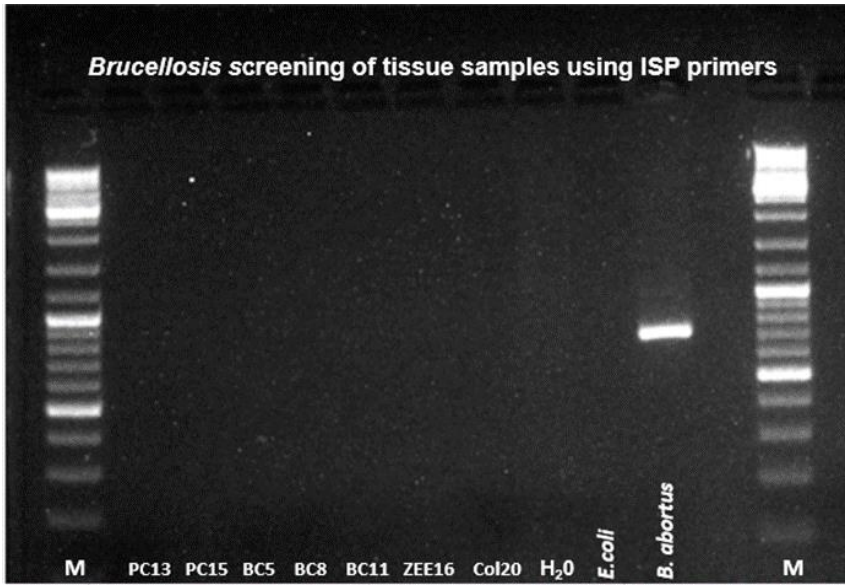

Figure 3.4: Screening of tissue samples using ISP primers

The presence of a 650 bp (ISP primers) or 905 bp (F4; R2 primers) band in a sample lane is considered as a positive result for *Brucella spp*. for this sample. The absence of this band indicates that Brucella *spp*. could not be detected in the sample and the result is reported as negative. The above-mentioned primer sequences are indicated in table 3.4 below.

Table 3.4: Primer sequence for ISP, F4 and R2 primers.

Primer	Sequence
ISP	Forward ISP 1 5'-GGTTGTTAAAGGAGAAGAGC-3'
	Reverse ISP 2 5'-GACGATAGCGTTTACACTTG-3'
F4/R2	F4 primer 5'-TCGAGCGCCCGCAAGGGG-3'
	R2 primer 5' –AACCATAGTGTCTCCACTAA-3'

3.7 Statistical data analysis

The data obtained was entered into Microsoft Excel® (Microsoft, USA) database and descriptive statistics generated. The association between different variables and knowledge on farm practices regarding zoonoses was assessed by chi-square ($\chi 2$) test. Odds ratios (OR) and confidence intervals

(CI, 95%) were calculated to assess potential risk indicators associated with brucellosis seroprevalence in a univariate logistic regression model.

An equation for apparent prevalence was used to calculate the percentage of positive animals (% positive) where:

Apparent prevalence was= $\frac{\textbf{Number of positive animals}}{\textbf{Number of animals tested}}\mathbf{100}\%$. The true prevalence estimate was calculated using an equation Var (AP) = $\frac{\mathbf{AP(1-19)}}{\mathbf{n(se+sp)2}}$adopted from Cameron (2007) which relates to sensitivity and specificity (Cameron, 2007). Var (AP) was estimated of variance for the apparent prevalence, AP was used for apparent prevalence, Se for sensitivity of CFT test and Sp specificity, for serology assays. The same formula was used to calculate the prevalence based on the culture and molecular assays. The calculation of the 95%confidence interval for the true prevalence was performed using the following equation:

$AP - (Z\alpha \text{ X } \sqrt{\text{var}(AP)})$; $AP+Z\alpha \text{ X } \sqrt{\text{var}(AP)})$

Where: Zα at a 95% confidence level is 1.96.

3.7.1 Univariate analysis

For the screening of variables against diseases exposure, the univariate logistic regression model was used at the individual level. The Chi square test (P value ≤ 0.05) was used to test all the variables individually for unconditional association during the initial analysis. Any variables with high p value ≥ 0.05 were excluded and those with p values of ≤ 0.05 were used in developing a multilevel logistic regression model for each exposure.

3.7.2 Multivariable analysis

The multivariable logistic regression model was used to analyse the amount of the outcome variables, based on the variables (factors) found to be significantly associated with the univariable analysis of p-value ≤ 0.05. The logistic model was reduced by stepwise elimination removing variables with p ≥ 0.05. The process was repeated until the model with the lowest Akaike's second-order information criterion was identified. The odd ratios with 95% confidence level interval results were recorded. The results of the data obtained, and analysis will be discussed in detail in the next chapter.

CHAPTER 4: RESULTS

4.1 Sample collection and distribution

4.1.1 Blood samples

A total of seven hundred and seventy (*n*=770) blood samples were collected from abattoirs and farms in all four major districts of the North-West province. The district distribution and gender of the animals as well as abortion status of the cows from which samples were collected in communal, commercial, and non- commercial farms of the North-West province are presented below (Table 4.1 and Figure 4.1).

Table 4.1: Summary of district distribution, gender and abortion status of animals sampled and tested for brucellosis.

Variable	*Level*	*Ngaka Modiri Molema*	*Dr Kenneth Kaunda*	*Dr Ruth Mompati*	*Bojanala Platinum*	*Total*
Distribution	*No. of Samples*	237	196	171	166	**770**
	Percentage	30.78%	25.45%	22.21%	21.56%	**100%**
Gender	*Male*	56	76	51	63	**246**
	Female	181	120	120	103	**524**
Abortion	*Abortion*	8	10	5	5	**28**
	No Abortion	85	93	74	14	**266**
Districts	*Abattoir*	113	93	66	120	**392**
	Farm	124	103	105	46	**378**

The above table shows an overall animal participation of 30.78% (237/770) in the Ngaka Modiri Molema district, 25.45% (196/770) from Dr Kenneth Kaunda district, 22.21% (171/770) from Dr Ruth Mompati and 21.56% (166/770) from Bojanala Platinum districts.

The table further indicates that Ngaka Modiri Molema district had the most 30.78% (237/770) blood samples collected as compared to the others. Of the 237 samples, 52.32% (124/237) were collected

from twenty-seven (*n*=27) farms and 47.67% (113/237) blood samples abattoirs. Animal history for Ngaka Modiri Molema district records indicated that 76.37% (181/237) samples were from cows; with 3.37% (8/237) having a history of abortion. Only 23.62% (56/237) samples were collected from bulls.

The Kenneth Kaunda district had 52.55% (103/196) farmed animals (*n*=14 farms) and 47.44% (93/196) animals from abattoirs. Animal history in this district indicated 5.10% (10/196) abortion cases from 61.22% (120/196) cows tested. The number of bulls for this district was 38.77 (76/196).

Out of the171 samples collected from the Dr Ruth Mompati district, 61.40% (105/171) samples were from farms (*n*=16) and 38.59% (66/171) from abattoirs. The animal history indicated that only 2.92% (5/171) abortion cases of the 70.17% (120/171) females tested. The number of bulls tested for this district was 29.82% (51/171).

The percentages within the Bojanala Platinum district were 27.71% (46/166) and 72.29% (120/166) farmed and abattoir animals respectively. Samples were collected from (*n*=15) farms and the history of abortion was also 3.01% (5/166) cases with 62.05% (103/166) cows and 37.95% (63/166) bulls tested. A clear overall distribution of samples tested is shown in the pie chart below (Figure 4.1).

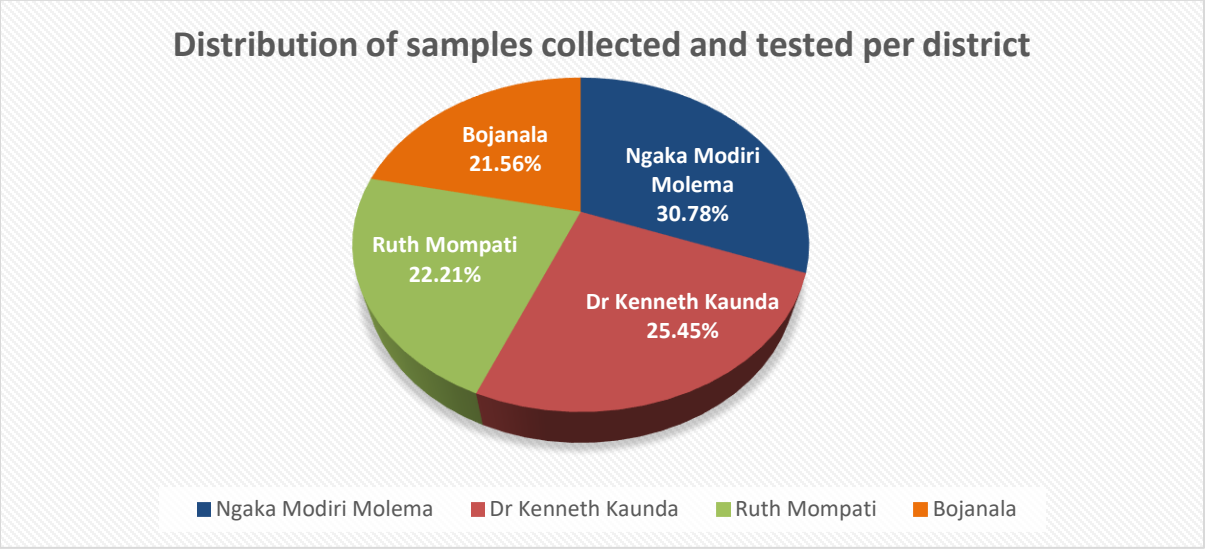

Figure 4.1: Pie chart indicating the overall distribution of sample collected per district in the North-West province.

4.1.2 Tissue samples

In all the districts, each blood sample from abattoirs was accompanied by a tissue sample from the same animal. In total, additional 392 tissue samples accompanied the 392 abattoir blood samples. The tissue sample distribution was 28.82% (113/392) in Ngaka Modiri Molema, 23.72 % (93/392) for Dr Kenneth Kaunda, 16.84% (66/392) in Dr Ruth Mompati and 30.61% (120/392) in the Bojanala Platinum district.

4.1.3 Milk samples

Out of the 770 cattle sampled, milk was collected from only 2.86% (22/770) of the animals (lactating cows). The Bojanala Platinum district had the greatest number of samples at 40.90% (9/22), followed by Ngaka Modiri Molema and Ruth Mompati at 22.73% (5/22) each. The district with the least milk samples collected was Dr Kenneth Kaunda at 13.63% (3/22). The distribution of milk samples per district is indicated in figure 4.2 below.

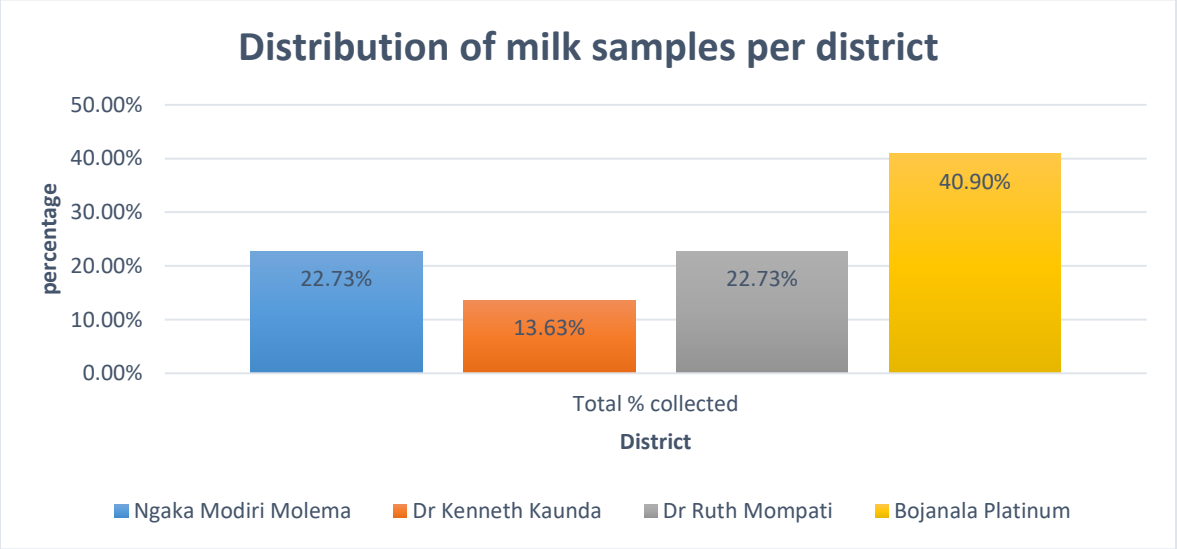

Figure 4.2: Distribution of milk samples collected per district.

4.1.4 Gender, abortion, and farm status

Overall, the total number of female animals tested were 68.05% (*n*= 524) and 31.94% (*n*=246) were males. There was a significant difference in the sex of animals sampled between districts with more female cattle sampled (*p = 0.002, DF = 3, X^2 = 14.85*).

The abortion status could be determined only for 294/524 (56.10%) farmed cows and abortions were reported in 5.34% (28/524) cases. The least number of abortion cases were reported in both Dr Ruth Mompati and Bojanala Platinum districts at 1.70% (5/294) and 1.70% (5/294) respectively. Dr Kenneth Kaunda district had 3.40% (10/294) cases followed by Ngaka Modiri Molema with 2.72% (8/294). The results showed no significant difference in abortion statuses amongst districts (p = *0.064, DF = 3, X^2 = 7.25*). A clear indication of the gender, abortion, and place of sampling per district is presented in the chart below (Figure 4.3).

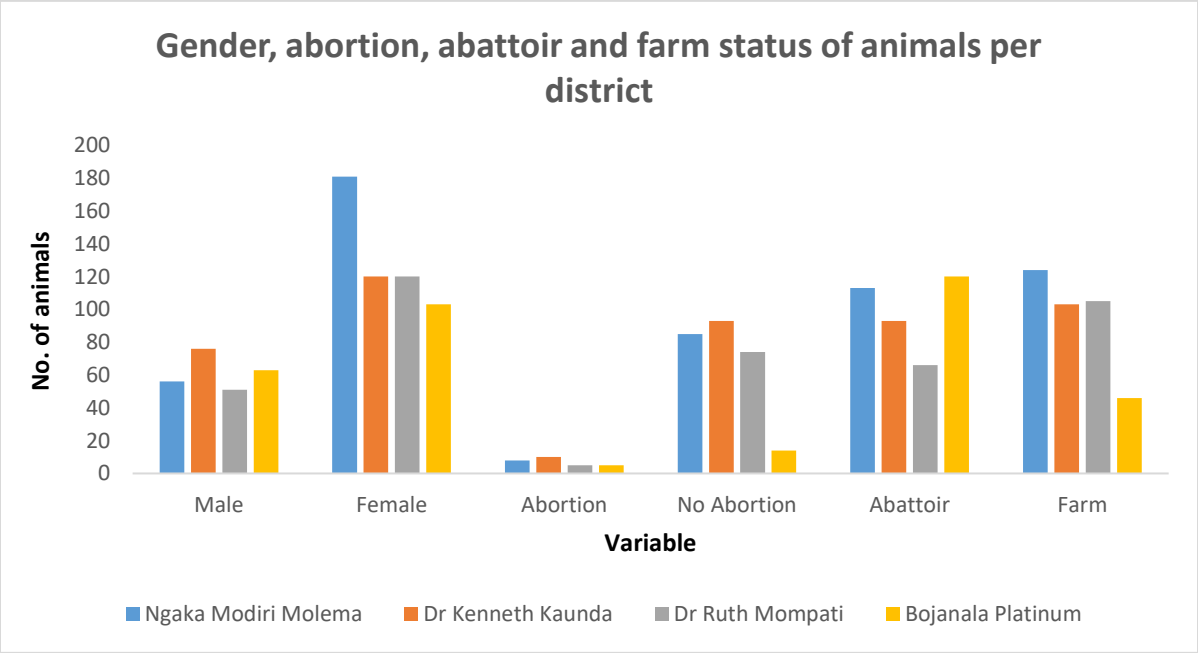

Figure 4.3: Pie chart indicating cattle gender, abortion status, and sampling place (abattoir/farm) of the animals per district in the North-West Province.

In addition to figure 4.3 above results indicated that there was a significant difference between the hygiene status of abattoir and farm sample distributions within districts, with more samples collected from abattoirs in Ngaka Modiri Molema district than any other districts.

A total of eight cattle breeds were sampled for this study. The most samples were collected from Bonsmara at 41.43% (319/770), followed by Nguni at 25.32% (195/770). Other breeds were New Jersey 13.40% (103/770), Brahman 7.64% (59/770), Mixed breed 6.62% (51/770), Afrikaner 3.77% (29/770) and Mixed Brahman 1.30% (10/770). The least sampled breed was the Holstein Friesian at 0.52% (4/770).

Table 4.2: Types of cattle breeds sampled and tested for brucellosis in different districts in Northwest Province.

Breed	***Total (n)***	***Percentage (%)***
Afrikaner	29	3.77%
Holstein Friesian	4	0.52%
Bonsmara	319	41.43%
Brahman	59	7.64%
Mixed Brahman	10	1.30%
Mixed	51	6.62%
New Jersey	103	13.40%
Nguni	195	25.32%
Total	770	100%

4.2 Laboratory tests results

4.2.1 Rose Bengal Test (RBT)

A screening test using Rose Bengal Test (RBT) was performed on all seven hundred and seventy (*n*=770) sera samples as per laboratory procedure. Sera from three hundred and seventy-eight (n = 378) farm-based and three hundred and ninety-two (*n*=392) abattoir-slaughtered animals were tested. Only 2.3 % (18/770) samples tested positive for antibodies against *Brucella abortus*, which was indicated by agglutination. This agglutination was observed in sera from 3.17% (12/378) farmed animals and 1.53% (6/392) abattoir- slaughtered animals. The overall sero-prevalence for RBT positive was found to be 2% at 95% Confidence Interval (CI). Table 4.3 below summarizes the RBT results at 95% CI.

Table 4.3: Rose bengal test results at 95% confidence interval.

Districts	*n*	*Positive*	*%*	*95% CI range*
Ngaka Modiri Molema	237	11	5 %	(2.60 – 8.12)
Dr Ruth Mompati	171	5	3 %	(2.60 – 8.12)
Dr Kenneth Kaunda	196	2	1 %	(0.28 – 3.64)
Bojanala Platinum	166	0	0 %	(0 – 2.26)
Total	770	18		

Individually, the most positive reactors were from Ngaka Modiri Molema district at 4.64% (11/237), followed by Dr Ruth Mompati and Dr Kenneth Kaunda at 2.52% (5/171) and 1.02% (2/196) respectively. No positive RBT results were identified from the Bojanala Platinum district. As a result, a total of 97.6% (*n*=752) sera tested negative as no agglutination was observed.

A clear representation of the RBT prevalence of 2% at 95% confidence interval is indicated in the figure below (Figure 4.4).

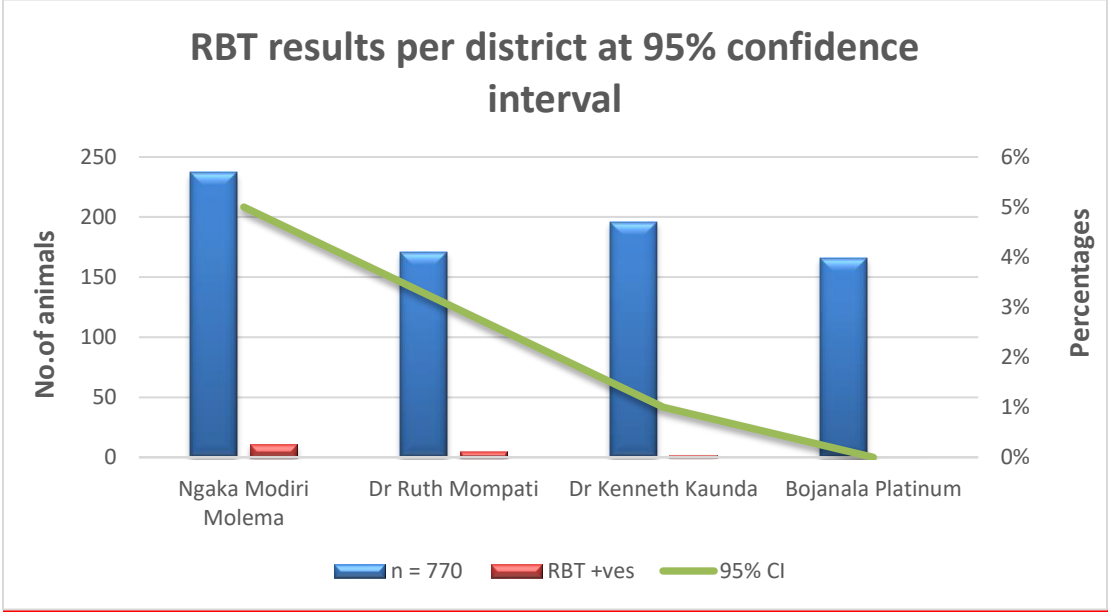

Figure 4.4: Rose bengal test results from sampled cattle per district at 95% confidence interval.

4.2.2 Complement fixation test (CFT)

All (*n*=18) samples recorded as positive for RBT were subjected to the Complement Fixation Test (CFT) which was used as a confirmatory serological diagnosis test for detecting the presence of *Brucella* antibodies (antibodies against *B. abortus*). The CFT results indicated that out of the 2.3% (18/770) samples that tested positive for RBT, only two (*n*=2) samples were negative which was observed by complete hemolysis. The negative results from this confirmatory test were from abattoirs in the Dr Kenneth Kaunda district. This resulted in a total of 2.07% (16/770) samples that tested positive for CFT which were identified by the absence of haemolysis in wells.

The overall positive results were detected only in Ngaka Modiri Molema [1.42% (11/770)] and Dr Ruth Mompati [0.64% (5/770)] districts. The CFT results showing the overall prevalence of 1.95% at 95% confidence interval are summarized in the table below (Table 4.4).

Table 4.4: Complement fixation test results districts at 95% confidence interval.

Districts	***n***	***Positive***	***%***	***95% CI range***
Ngaka Modiri Molema	237	11	5%	(2.61 – 8.11)
Dr Ruth Mompati	171	5	2.%	(0.91-5.85)
Dr Kenneth Kaunda	196	0	0%	(0.0 – 0.52)
Bojanala	166	0	0%	(0.0-- 0.79)
Total	770	16		

A clear view of the overall CFT prevalence of 1.95% (95%CI: 1.14 – 3.12), CI is graphically presented in the figure below (Figure 4.5).

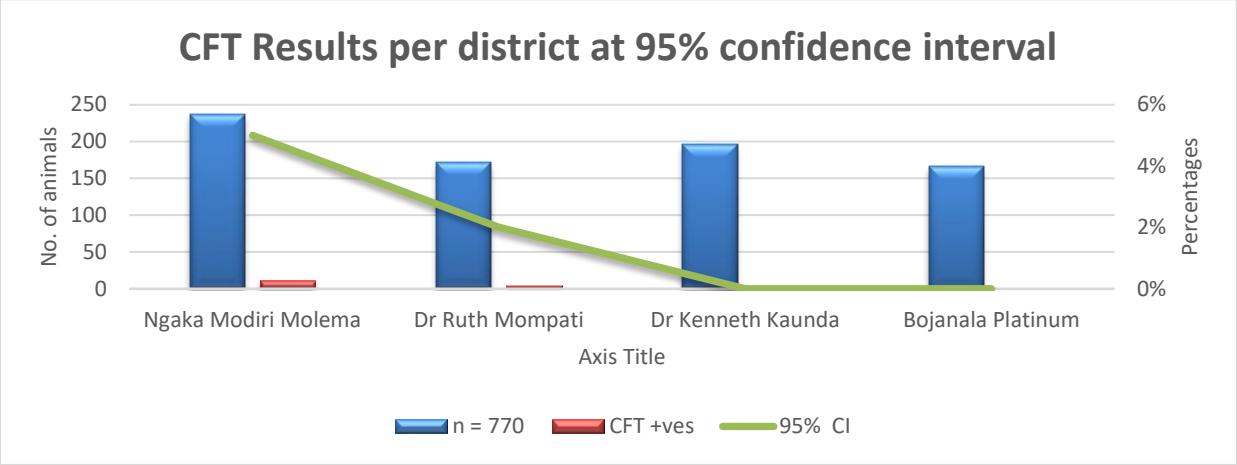

Figure 4.5: Complement fixation test results per district at 95% confidence interval.

Of the 1.9% (*n*=16) CFT positive samples, 0.90% (*n*=7) samples were collected from abattoirs and 1.17% (*n*=9) where from farmed animals.

Table 4.5: Results of the complement fixation test showing the values of antibody level (Titres).

District	Village/Abattoir	Titres (IU ml $^{-1}$)	Results
Ngaka Modiri Molema	Abattoir 1	784	Positive
	Abattoir 2	30	Positive
	Village A	344	Positive
	Village A	784	Positive
	Village B	30	Positive
	Village B	30	Positive
	Village C	784	Positive
	Village C	43	Positive
	Village D	784	Positive
	Village E	784	Positive
	Village E	98	Positive
Dr Ruth Mompati	Abattoir 3	784	Positive
	Abattoir 4	784	Positive
	Abattoir 4	344	Positive
	Abattoir 4	290	Positive
	Abattoir 4	784	Positive
Dr Kenneth Kaunda	Abattoir 5	-	**Negative**
	Abattoir 5	-	**Negative**

(-): Indicates no titre obtained.

Interpretation was obtained using the titres indicated in the table below.

Table 4.5b: Interpretation of titres in bovine brucellosis.

Vaccination history	IU ml $^{-1}$	Interpretation
Unvaccinated, calf hood (‹ 8 months) or unknown	≤ 15 18-24 ≥ 30	Negative Suspicious Positive
Adult vaccinated (› 8 months)	≤ 24 30-49 ≥ 60	Negative Suspicious Positive

4.2.3 Milk Ring Test (MRT)

The Milk Ring Test (MRT) was conducted on samples collected from lactating cows. Milk samples could be obtained from only 5.82% (22/378) cows during random sampling. All collected milk samples reacted negatively to the MRT. A lighter shade cream layer was observed on the milk which is an indication of the negative results. The results and distribution of milk samples per district are presented in table 4.6 below.

Table 4.6: Milk ring test results and distribution of milk samples per district (*n*=22).

District	Total collected	Total % collected	Results
Ngaka Modiri Molema	5	22.73%	Negative
Dr Kenneth Kaunda	3	13.63%	Negative
Ruth Mompati	5	22.73%	Negative
Bojanala	9	40.90%	Negative
Total	22	100%	

4.2.4 Isolation and phenotypic characterisation of *Brucella* species

Tissue samples 43.75% (7/16) corresponding with the sixteen (*n*=16) sera that tested positive in the CFT were processed for bacterial culture and 42.85% (3/7) yielded suspicious colonies of *Brucella* species on Farrell medium. The suspicious *Brucella* were isolated from samples collected from Ngaka Modiri Molema and Dr Ruth Mompati districts.

The results on Farrell's medium were recorded as suspicious as the bacterial colonies appeared small, round, and translucent. A positive control (*Brucella abortus* strain) was included to compare colonies. Gram staining revealed coccobacilli arranged in a small group, hence regarded as suspects.

The suspicious colonies were subjected to oxidase, catalase, and urea hydrolysis tests. All 42.85% (3/7) suspects were oxidase and catalase positive and reacted negative to hydrolysis with urea. The positive control used turned pink in colour for urea hydrolysis test. Results of hydrolysis with urea is indicated in figure 4.6 below. A summary of the biochemical tests conducted is highlighted in table 4.7.

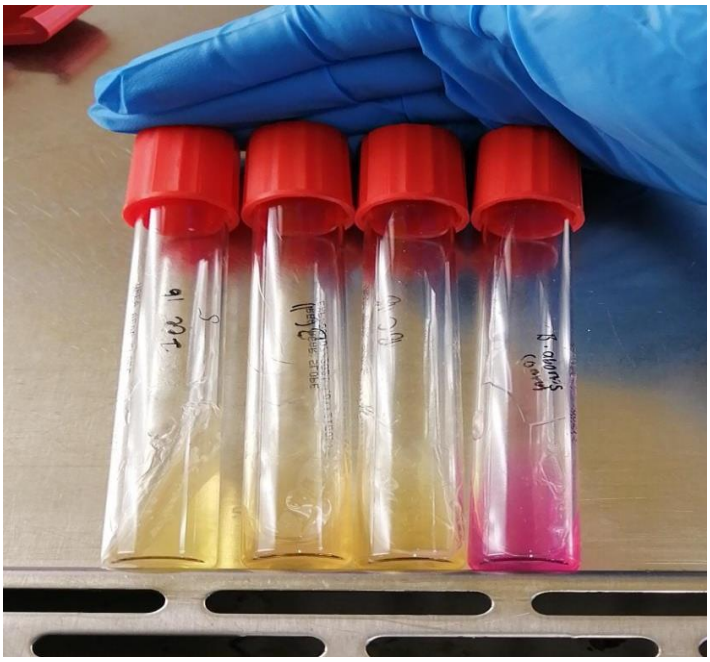

Figure 4.6: Urea hydrolysis of all three suspect *Brucella* colonies.

Table 4.7: Stamp staining, Gram staining and biochemical tests conducted for the three suspect *Brucella* colonies from tissue samples.

Test	***Reaction***
Stamp staining	Suspect
Farrell's medium	Suspect
Gram's stain	Suspect
Oxidase test	Positive
Catalase test	Positive
Urea hydrolysis	Negative

4.2.5 Molecular identification and differentiation of *Brucella* species using PCR

Polymerase Chain Reaction (PCR) was carried out on the seven (7/770) tissue samples that were subjected to culturing. A total of 0.90% (7/770) for positive CFT sera samples subjected to molecular identification for further confirmation. This technique was used for differentiation between the *B. abortus* wild type strain RB51, and S19 vaccines. All samples tested negative for the PCR technique.

This was indicated by the absence of the PCR product with the corresponding band size on the agarose gel. Positive controls were indicated by the presence of a 650 base pair PCR product. The PCR results are tabulated below:

Table 4.8: Polymerase chain reaction results for tissue samples corresponding with positive complement fixation test sera.

District	*Abattoir*	*Results*
Ngaka Modiri	Abattoir 1	Negative
Molema	Abattoir 2	Negative
Dr Ruth Mompati	Abattoir 3	Negative
	Abattoir 4	Negative
	Abattoir 4	Negative
	Abattoir 4	Negative
	Abattoir 4	Negative

4.2.6 Questionnaire

The study animals from the livestock population were randomly selected at each villages/dip tank during the visits. Blood and milk (from lactating animals) samples were collected upon the farmer's agreement. This resulted in a total of 62 participants in the study and the subsequent questionnaire survey. The demographic data of respondents is presented as follows:

Table 4.9: Demographic data of respondents (*n* = 62).

Variable	Bojanala Platinum (*n*= 17)	Dr Kenneth Kaunda (n = 16)	Dr Ruth Mompati (n = 11)	Modiri Molema (*n*=18)	P-value
Gender					
Female	0 (0.00)	2 (12.50)	0 (0.00)	0 (0.00)	NA
Male	17 (100)	14 (87.50)	11 (100)	18 (100)	
Marital status					
Single	9 (52.94)	8 (50.00)	1 (9.10)	10 (55.56)	
Married	5 (29.41)	4 (25.00)	7 (63.64)	5 (27.78)	NA
Divorced	2 (11.74)	3 (18.75)	2 (18.20)	0 (0.00)	
Widowed	1 (5.88)	1 (6.25)	1 (9.10)	3 (16.67)	
Relationship to farm					
Herdsman	8 (47.10)	3 (18.75)	0 (0.00)	8 (44.44)	
Owner	8 (47.10)	9 (56.25)	9 (81.82)	5 (27.78)	NA
No. of household members					
One person	4 (23.53)	6 (37.50)	1 (9.10)	6 (33.33)	
Two people	5 (29.41)	5 (31.25)	2 (18.20)	3 (16.67)	0.284
Three people	1 (5.88)	5 (31.25)	7 (63.64)	8 (44.44)	
More than 3 people	7 (41.17)	0 (0.00)	1 (9.10)	1 (5.56)	
Status of employment					
Employed	13 (76.47)	11 (68.75)	6 (54.55)	17 (94.44)	NA
Unemployed	4 (23.53)	5 (31.25)	5 (45.45)	1 (5.56)	
Occupation					
Business	4 (23.53)	0 (0.00)	0 (0.00)	0 (0.00)	
Cattle farming	8 (47.10)	6 (37.50)	0 (0.00)	8 (44.44)	NA
Company employed	0 (0.00)	1 (6.25)	4 (36.36)	4 (22.22)	
Meat Inspector	1 (5.88)	4 (25.00)	2 (18.20)	5 (27.78)	
Status of literacy					
No School	3 (17.65)	1 (6.25)	0 (0.00)	5 (27.78)	NA
Grade 1 – 6	4 (23.53)	1 (6.25)	0 (0.00)	3 (16.67)	
Grade 7 – 12	5 (29.41)	6 (37.50)	0 (0.00)	1 (5.56)	
Grade 12 +	5 (29.41)	8 (50.00)	11 (100)	9 (50.00)	

Table 4.10: Respondents' farm management practices and husbandry system.

Question	*Variables*	*Respondents (%)*	*P-value*
Why do you keep animals?	**N = 50/62**	**80.65%**	NA
	Food and Trading	96.00%	
	Trading	4.00%	
How long have you been keeping animals?	**N = 50/62**	**80.65%**	NA
	2-5 years	18%	
	5 years +	82%	
Breeds of animals kept?	**N = 62/62**	**100%**	NA
	Brahman	3.22%	
	Mixed breed	91.94%	
	Nguni	4.84%	
Number of cattle kept?	**N = 50/62**	**80.65%**	NA
	10 animals	10%	
	10 – 20 animals	34%	
	20 – 30 animals	36%	
	30 + animals	20%	
Type of farming practiced.	**N = 62/62**	**100%**	NA
	Beef production	19.35%	
	Beef and Dairy	80.65%	
Breeding practice?	**N = 50/62**	**80.65%**	NA
	Natural	100%	
Grazing practice?	**N = 50/62**	**80.65%**	NA
	Free grazing	90%	
	Partial grazing	10%	
Any lactating cows?	**N = 50/62**	**80.65%**	NA
	Lactating cow present	50%	
	No lactating cows	50%	
What do you do with the milk from animals?	**N = 25/62**	**40.32%**	NA
	Selling and household	76%	
	Household consumption	24%	
What do you do with the milk before consumption?	**N = 26/62**	**41.94%**	NA
	Nothing	96%	
	Sour	4%	

Note: N = total number of respondents out of 62 participants.

The questionnaire had sections that were not applicable to other respondents, therefore data was analyzed based on the number of respondents for the relevant section (Appendix G). For an example, if only 50 participants responded for the applicable section, the percentage will be calculated out of 50.

Data analysis for table 4.10 above indicated that 96.00% (48/50) of respondents kept animals for food and trading, while only 4.00% (2/50) of respondents kept animals strictly for trading. At least 18% (9/50) participants stated that they kept animals for 2-5 years, while 82% (41/50) of the respondents kept animals for over a period of 5 years. The results further indicated that 91.94% (57/62) kept mixed breed with 18 (36%) respondents keeping at least 20 – 30 animals. Only 20% (10/50) keep more than 30

animals. Most respondents [64.52% (40/62)] get their animals from the local markets and only 35.48% (22/50) breed their own animals. A total of 80.65% (50/62) respondents are farming for both beef and dairy productions while 19.35% (12/62) are involved in beef production only. All [100% (50/62)] respondents who answered this question confirmed practicing natural breeding. Most [90% (45/50)] respondents preferred free grazing with only 10% (5/50) practicing partial grazing.

During the interview, 50% (25/50) of respondents said they had lactating cows and 50% (25/50) had no lactating cows. A total of 76% (19/25) respondents used milk for household and selling for direct consumption while 24% (6/25) using for household consumption only. When asked what they do with milk before consumption, 96% (25/26) of respondents that they do nothing.

Table 4.11: Predictors of knowledge, attitude, and practice of brucellosis control among respondents.

Question	*Variables*	*Respondents (%)*
Experience of abortions in herd?	**N = 48/62**	**77.42%**
	Abortion	47.92%
	No abortion	52.08%
Stage of pregnancy at which abortions were observed?	**N = 24/48**	**50%**
	Early	29.17%
	Late	33.33%
	Mid	37.50%
What happens to the aborted foetus and after birth materials?	**N=24/48**	**50%**
	Bury	70.83%
	Leave on field	25%
	Other	4.17%
How do you handle aborted materials?	**N = 24/48**	**50%**
	Bare hand	83.33%
	Glove	4.17%
	Plastic	12.50%
Do you handle aborted material or assist in delivery when with wounds or cuts?	**N= 24/48**	**50%**
	Handle	79.17%
	Not handle	20.83%
Do you wash hands after handling aborted materials?	**N = 24/48**	**50%**
	Wash	100%
What do you use to wash their hands?	**N = 24/48**	**50%**
	Water and soap	54.17%
	Water only	45.83%
Do you know that you can get diseases from animals?	**N =61/62**	**98.38%**
	Yes	78.68%
	No	21.31%
Ever heard of brucellosis?	**N= 62/62**	**100%**
	Yes	32.26%
	No	67.74%
Do you know which species of animals are affected with brucellosis?	**N = 20/62**	**32%**
	Cattle, sheep, pig, goat	30.00%
	Cattle	12.00%
	Cattle, sheep, and goat	5.00%
	Do not know	53.00%
Do you know that brucellosis can be transmitted to humans?	**N = 20/62**	**32%**
	Yes	33.87%
	No	66.13%
Do you know how the disease is transmitted?	**N = 20/62**	**32%**
	Contact, Raw milk,	25.00%
	Do not know	5.00%
	Drinking raw milk	40.00%
	Touching body of animals	30.00%
Would you like to know the brucellosis status of your animals?	**N = 62/62**	**100%**
	Would like to know	100%
	Don't want to know	0%

When asked about experience of abortions in the herd, 77.42% (48/62) responded and 52.08% (25/48) of the respondents had not experienced abortions on their farms while 47.92% (23/48) of the participants experience abortions on their farm. Abortion was experienced during mid stage of pregnancy by 37.50% (9/24), 33.33% (8/24) late stage and 29.17% (7/24) early in the pregnancy. When asked what happens to the aborted foetus and after birth materials, majority of the respondents 70.83% (17/24) buried the material while only a few left the material on the field. On handling aborted materials, 83.33% (20/24) of respondents used bare hands, 12.50% (3/24) used plastic and only 4.17% (1/24) used gloves. A total of 79.17% (19/24) respondents handled aborted material or assist in delivery even when they had wounds or cuts. All respondents washed their hands after handling aborted materials, with 54.17% (13/24) using soap and 45.83% (11/24) using plain water.

When asked if they know that they can get diseases from animals, 98.38% (61/62) participated. Most [78.68% (48/62)] knew that they can get diseases from animals and 21.31% (13/61) did not know. Of the 62 respondents, only 32.26% (20/62) have ever heard of brucellosis. The ones who knew about brucellosis were able to name the species of animals affected as indicated in table 13 above. All 62 (100%) respondents were willing to know the brucellosis status of their animals. When asked how the disease is transmitted only 32% (20/62) answered this question. At least 25% (5/20) of respondents said through contact and consumption of raw milk, 40% (8/20) said by drinking raw milk, 30% (6/20) said by touching body of animals and 5% (1/20) did not know.

On symptoms experienced in the last ten years, all 100% (62/62) of the respondents answered as indicated on the chart below (Figure 4.7), with majority of the participants (63% (39/62) having experienced more that one of the brucellosis related symptoms. This number was followed by 14% (9/62) who had no symptoms at all, 8% (5/62) experienced muscle pains, 6% (4/62) mentioned tiredness has been experienced, 5% (3/62) had joint pains, 2% (1/62) reported loss of appetite and another 2% (1/62) reported night sweats.

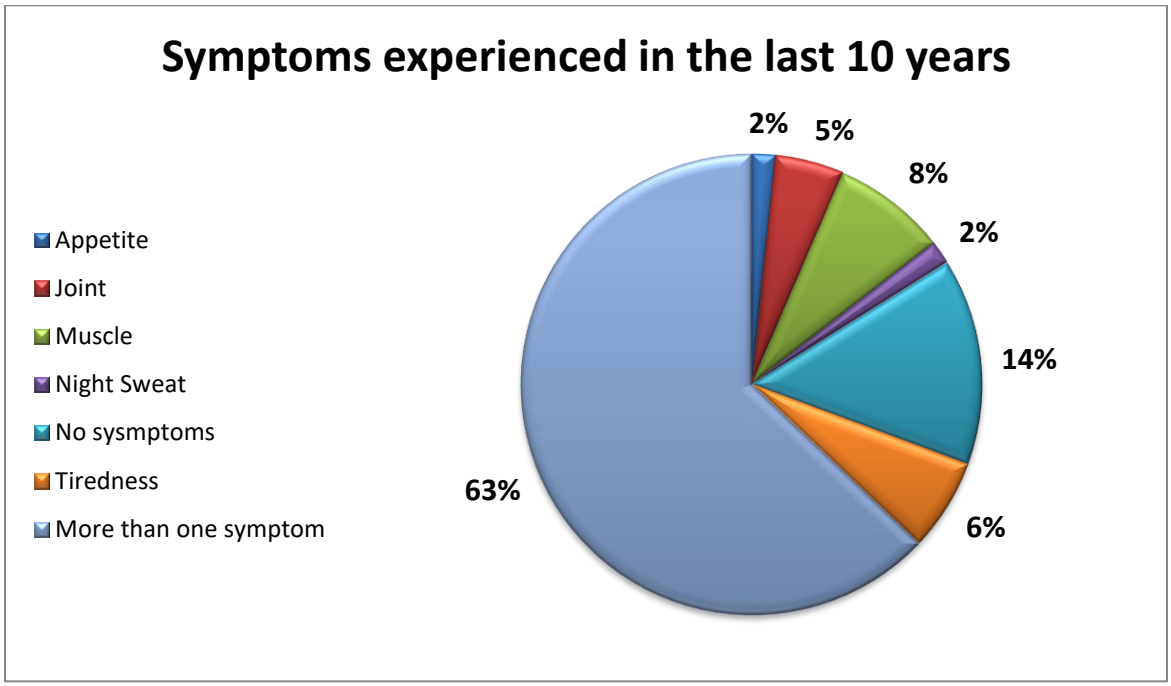

Figure 4.7: Pie chart presenting symptoms experienced in the last 10 years.

When asked what they do when they experience such problems, all 62 (100%) of the respondents answered the question where 66% (41/62) said they went to the clinic, 32% (20/62) were self-medicating while only 2% (1/62) consulted traditional healers.

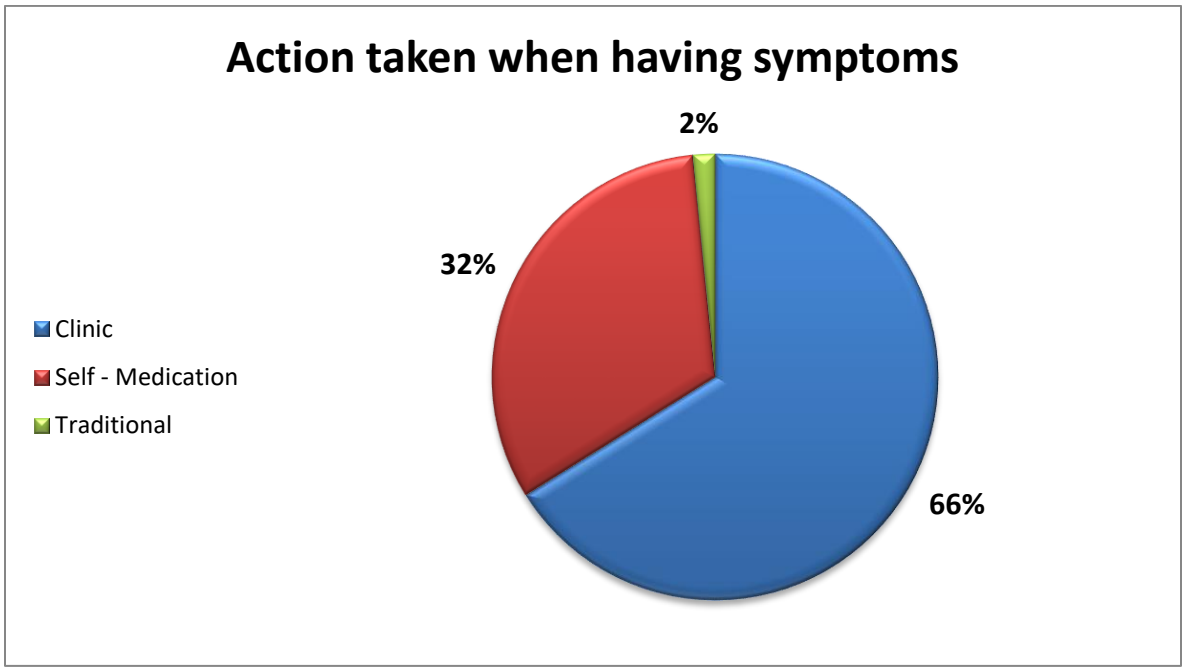

Figure 4.8: Pie chart presenting action taken when having symptoms.

Table 4.12: Preventive and control measures.

Questions	*Variables*	*Respondents*
Where do you buy animals?	**N = 50/62**	**80.65%**
	Auction and Local market	90%
	Auction	10%
Do you check the health status of the animals you purchase?	**N = 50/62**	**80.65%**
	Yes	100%
	No	0%
How do you identify animals?	**N = 50/62**	**80.65%**
	Branding	38%
	Tagging	62%
What signs do you observe to determine the health status of your animals?	**N = 62/62**	**100%**
	Abortion or weak calf	51.61%
	Abortion	14.52%
	Don't know	33.87%
Are you aware of the availability of vaccines against brucellosis?	**N = 62/62**	**100%**
	Aware	95.16%
	Not aware	4.84%
Where do you get such information regarding vaccines?	**N = 59/62**	**95.16%**
	Veterinary Office	100%
Do you vaccinate animals against brucellosis?	**N = 46/62**	**74.19%**
	Not sure	54.35%
	Vaccinate	45.65%
Which vaccine do you use against brucellosis?	**N = 21/62**	**33.87%**
	S19	14.28%
	S19 / RB51	85.72%
Any reasons for not vaccinating your herd? (Those not vaccinating)	**N = 23/62**	**37.09%**
	Other reason	**100%**
Do you know the brucellosis status of your herd?	**N = 46/62**	**74.19%**
	No status	43.48%
	Yes status	56.52%
How do you know the brucellosis status of your herd?	**N = 20/46**	**43.47%**
	AHT/VET	100%
What would you do if you know that your animals have brucellosis?	**N = 62/62**	**100%**
	Keep	11.29%
	Sell	32.25%
	Slaughter	56.45%

The above table shows that 90% (45/50) of animal keepers responded that they buy their cattle from auction and local market. All 100% (50/50) of the respondents checked the health status of animals they purchase and 62% (31/50) used tagging while 38% (19/50) used branding. Regarding signs respondents observe when animals are infected, all 100% (62/62) of the respondents participated. Most of the respondents [51.61% (32/62)] said abortion or weak calf. Although 14.52% (9/62) said abortion is experienced, 33.87% (21/62) respondents said they did not know.

All the 62 (100%) of the respondents answered the question on knowledge on vaccine availability and 95.16% (59/62) said they were aware and only 4.84% (3/62) were not aware. All respondents obtained information regarding vaccines from the veterinary office. However, results show that only 45.65% (21/46) respondents vaccinate their animals. The rest 25 (54.35%) were not sure of the animal's vaccination status as they were not owners. For those who vaccinated their animals, 85.72% (18/21) used either S19 or RB5 while only 14.28% (3/21) used S19.

4.2.7 Multivariate analysis

In addition to the above, chi-square and odds ratios were used to determine the association between the status of literacy, occupation, and farm management practices.

Table 4.13: Multivariate association of the status of literacy and occupation of respondents with their knowledge and attitude on brucellosis.

Variable A	Variable B	Status of literacy (*P*-value)	Occupation (*P*-value)
Status of literacy & Occupation	What do you do with the milk before consumption?	**0.3360**	**0.0.1124**
	What happens to the aborted foetus and after birth materials?	<0.001	0.0035
	How do you handle aborted materials?	0.0005	<0.0001
	Do you handle aborted materials or assists delivery when you have wounds or cuts on your hands?	0.0002	0.0001
	Have you ever heard of brucellosis?	0.0029	<0.0001
	Do you vaccinate your animals against brucellosis?	0.0002	<0.0001
	What are the reasons that you do not vaccinate your herd?	<0.001	<0.001

The results indicated that the status of literacy and occupation were not statistically significant with what participants did with the milk before consumption [(p=0.3360 status of literacy); (p=0.01124 occupation)]. Nonetheless, the association of the status of literacy and occupation with other variables were statistically significant as indicated in table 4.13 above.

CHAPTER 5: DISCUSSION

5.1 Seroprevalence of Brucellosis in cattle using serological methods

5.1.1 Rose Bengal Test (RBT) and Complement Fixation Test (CFT)

In the current study, the RBT and CFT serological tests were used to determine the prevalence of brucellosis in communal and small holder cattle farming areas in the North-West province. Serological results obtained indicated the overall prevalence for RBT positive samples to be 2% at 95% Confidence Interval (CI) [95%CI: 1.48 – 3.66]. According to research, RBT could demonstrate false- positive results because of non-specific serological reactions or vaccination with the S19, hence all RBT reactors should be confirmed by CFT (DAFF, 2016e). Of the 2,07% (16/770) samples that tested positive with CFT, three [(*n*=3) 3/16] samples had low titres but were still within the required titres (Table 4.5). According to the Department of Agriculture, Land Reform and Rural Development's bovine brucellosis manual, antibody titre values ranging from ≥30 IU/ml are regarded as positive (Godfroid *et al*., 2014). Of the four districts, the samples that yielded positive results originated from Ngaka Modiri Molema and Dr Ruth Mompati with the prevalence of 4.65% (95% CI: 2.61 – 8.11) and 2.34% (95% CI: 0.91-5.85) respectively. Looking at the distribution and number of samples tested, there is a possibility that the other two districts (Dr Kenneth Kaunda and Bojanala Platinum) are not necessarily free of brucellosis as the sample numbers were much lesser than the other two districts. Only 12.16% (46/378) samples could be obtained from the Bojanala Platinum farmers and 23.72% (93/392) from Dr Kenneth Kaunda abattoirs. This was mainly because participation in the study was voluntary and only a few farmers were interested in contributing to the study in these districts. This resulted in the overall brucellosis sero prevalence of 1.95% (95%CI: 1.14 – 3.12), which is presented graphically in Figure 4.5.

The samples that tested positive in the RBT screening but negative upon CFT confirmation were collected from cattle originating from Dr Kenneth Kaunda district. All confirmed positive reactors from Dr Ruth Mompati were collected from cattle in one abattoir hence it must be noted that this abattoir had challenges regarding cleanliness and general hygiene. The abattoir was very small with animals touching each other, and workers were wearing dirty overalls from the previous' day slaughtering. According to Wang *et al.*, proper waste disposal and cleanliness have a major impact on the transmission of *Brucella* between animals and humans (Wang *et al*., 2014). It could be that there was

cross contamination between the four samples during bleeding and slaughtering. Research has indicated that contamination can occur during slaughtering of infected animals, which has a significant on the economy (Ntirandekura *et al*., 2018).

The eleven [11 (1.43%)] positive reactors from Ngaka Modiri Molema districts were collected from two abattoirs with one positive cattle each, four farms with 2 positive cattle each and another farm with only one positive cattle. This prevalence was low and in agreement with most research already conducted in North-West. A study that took place in Mafikeng, a small town in the Ngaka Modiri Molema district reported 0.23% prevalence of *B. abortus* in buffaloes of Mafikeng game reserve (Nyirenda *et al*., 2016). Another study conducted from 2007-2015, recorded 6.31% of cattle brucellosis prevalence in the North-West province (Kolo *et al*., 2019). Although the study included other animal species such as sheep, goats, pigs, cattle had the highest occurrence in all nine provinces of South Africa (Kolo *et al*., 2019). The study by Kolo *et.al* was conducted on samples submitted over a 9-year period at the Onderstepoort Veterinary Institute bacterial serology laboratory. This study reported 6.31% bovine brucellosis cases in 9 years, making it less than 1% sero-positive results tested per year. Another retrospective study which was conducted between 2009 and 2013 in the Bojanala district, revealed overall herd prevalence of 33.33% and 3.18% individual prevalence in dairy, commercial and communal cattle (McCrindle *et al*., 2020). The latter had the low individual prevalence considering the number of years for the study. It should also be noted that only one district of the North-West province was used which might have been bigger if all districts were included. This prevalence is consistent with (Modisane, 2019) findings of 7.7% prevalence in seven years from Mabeskraal village (Modisane, 2019). This percentages are supporting the hypothesis for this study which states that the prevalence distribution may not be different from other areas with similar zoo-epidemiological situations.

5.1.2 Milk Ring Test (MRT)

In addition to RBT and CFT, the MRT was carried out on milk samples obtained from lactating cows during sampling. Most farmers were reluctant to allow milk sampling and those who were willing had no lactating cows, hence there was a limited number of milk samples in this study. All 5.82% (*n*=22) of the milk samples reacted negative to antibodies against *B. abortus* during the MRT. The results of the MRT agreed with the results of the RBT and CFT of sera from the same (corresponding) animals. The advantage of MRT is that it is inexpensive as milk can be pooled from several cows from one farm

(OIE, 2018). However, this method has the disadvantage that the milk: antigen ratio in bulk samples often makes it difficult to detect a small number of animals in a large herd (DAFF, 2016f). Another challenge with the MRT is that late lactation cycle may produce false reactions for cows that are vaccinated by S19 in less than 4 months before testing (Ducrotoy *et al*., 2017b).

5.2 Isolation and identification with cell culture and PCR

Culture of *Brucella* species has been extensively used in research and is regarded as the preferred direct method because of its specificity. In our study, only tissue samples corresponding with sera that tested positive in the CFT were subjected to bacterial culture and PCR amplifications with the aim to identify the *Brucella* species involved. Unfortunately, only seven (*n*=7) of such tissues were available as the other positives were from live farmed animals. Suspect *Brucella* species colonies were observed in only 42.8% (3/7) samples on Farrell's medium agar plates (a selective bacteriological medium for *Brucella* species). In addition, direct DNA extraction using QIAamp® DNA Mini and Blood Mini Handbook extraction kit was carried out on the tissue samples. Our PCR results (using cell lysates and extracted DNA using kit) showed the absence of *Brucella* species from the collected tissue samples.

Tissue culture colonies observed in our study were Gram-negative as well as oxidase and catalase positive, and urease negative, hence suspect of *Brucella* species. Negative species confirmation PCR results could mean that there is a possibility of other bacteria with the same characteristics as *Brucella* that were picked from colonies observed on the Farrell's medium. Research proved that isolates of *Brucella* are often confused with other bacteria such as *Psychrobacter phenylpyruvicus*, *Psychrobacter immobilis*, and *Bordetella bronchiseptica* (Declercq, 2018). According to the South African department of Agriculture, isolation of *Brucella* species from lung samples produced more positive results as compared to samples from the spleen, liver and bronchial lymph nodes (DAFF,2016a). In our study, mesenteric, retropharyngeal and supramammary lymph nodes were cultured. Perhaps samples confirmed positive serologically were collected from previously vaccinated animals or animals exposed to *Brucella* species, hence resulting in development of antibodies against the disease. Another reason could be that the sensitivity and specificity for serological tests can be lower than the range for suspects (table 4.5b and section 2.5.2), therefore giving inappropriate results (Christopher *et a*l., 2010). Considering that tissue samples were from abattoirs with some negative

samples from the same farm, the latter could be the main reason. The World Health Organization stated that the use of vaccines may stimulate production of antibodies, therefore interfering with serological diagnosis (WHO, 2004). Therefore, a combination of indirect and direct methods such as cell culturing and PCR are advised to be able to isolate and identify the *Brucella* species if present (Poester *et al*., 2013). However, direct detection also comes with its limitations. With cell culture, bacterial growth may take up to 21 says to grow and handling requires biosafety level 3 environment for the protection of laboratory personnel and highly skilled personnel (Bricker *et al*., 2000). Our study used both direct and indirect methods for the detection of brucellosis.

5.3 Farm management, general knowledge, and husbandry system

The last objective of the study was to assess farm management, herd-health, and husbandry system through a questionnaire with a view to establish the herd health and zoonotic implications of brucellosis in communal and small holder cattle farming areas of the province. The questionnaire had sections that were only applicable to other respondents, percentages were calculated based on the total respondents for that section.

Overall, a total of 62 respondents gave consent to participate in the study, with majority of the participants being males 96.8% (60/62). This figure is probably because cattle farming and livestock ownership has always been associated with males and has been characterised by traditional beliefs which limit women's access to ownership (Visser and Ferrer, 2015). This therefore creates a scenario where women farmers tend to own smaller herds, which can be sold when under pressure and allow them to engage in other livelihoods such as small businesses as a complementary strategy (Motiang, 2017). Another credible explanation for the lack of women participation is that women face time consuming household responsibilities which end up limiting their participation in forums, auctions, cattle dipping activities and all other valuable farming activities.

The findings indicated that 90% (45/50) of the respondents preferred free grazing for their animal feeding and many 82% (41/50) have been keeping cattle for over 5 years mostly for food and trading purposes (beef and dairy production). It is a norm for Africans to breed cattle for food and trading purposes mainly because of the access to wage income in big cities (DAFF, 2016c). Other farmers

breed cattle as a supplementary income to be able to care for the household due to the economic crisis is South Africa that has drastically increased during the Covid-19 pandemic.

Our results showed a concerning high number of participants 96% (25/26) who indicated that they do not pasteurise nor boil their milk before consumption. Strains of *B.abortus* and *B.melintesis* have been detected and isolated from people who travelled to countries where these strains are common and drank contaminated milk (Hadush and Pal, 2013). Some of the participates highlighted that they have experienced abortion cases in their cattle herds with abortion occurring during either early, middle, and late stage of pregnancy and mostly buried the aborted materials, unfortunately with materials mainly handled with bare hands, even when they and wounds or cuts. At least all participants who handled aborted materials washed their hands thereafter, with over half of them using both water and soap Although 78.68% (48/62) of respondents admitted knowing that they can get diseases from animals, it is apparent from this study that herdsmen and a few farm owners' did not know how the disease is transmitted or how to handle aborted materials, hence the risk of transmission of the disease from animals to humans through handling of aborted may be high and worsen when the aborted material is handled without wearing gloves. Handling of aborted or infected materials put farm workers and laboratory personnel at risk of acquiring the bacteria (Hadush and Pal, 2013). This is an interesting finding proving that cattle handlers have limited knowledge on handling Brucellosis. Upon hearing about the disease, participants were interested in knowing the brucellosis statuses of their cattle herds.

The results further indicated that general knowledge on brucellosis was dependent on the respondents' status of literacy. This study has shown a significant association between the statuses of literacy with the risk of brucellosis in a farm. This was confirmed by the lower levels of p-values when the status of literacy and occupation of participants were used against knowledge on herd-health and husbandry system (Table 4.13). The fact that the number of abortion cases experienced in the past was at 47.92% (23/48) is very alarming. This finding together with how respondents handled aborted materials is a risk on its own as the respondent's knowledge is limited. Lack of knowledge on the disease has a substantial public health implication and this can interfere with control and possible eradication of the disease.

Despite 78.68% (48/62) of respondents knowing that they can get diseases from animals, at least a small percentage 32.26% (20/62) responded that they have heard of Brucellosis before. In addition, 53.00% of the 20 respondents confirmed not to know the type of species affected by Brucellosis.

Looking at the low prevalence rate, the high number of abortions could be caused by other microorganisms than *Brucella*. This finding was supported by the significant association observed between the status of literacy ($p=0.0029$) and occupation ($p<0.0001$) to the question whether respondents have ever heard of brucellosis before.

The study revealed that 90.00% (45/50) of farmers bought their cattle from auctions and local market. This percentage is excluding abattoir workers because the animals are only brought to them for slaughtering. Introduction of cattle alone poses a risk of bringing the disease into the herd, thereby infecting farm workers and castle handlers. Although 100% (62/62) of the participants indicated that they observe the health status of the animals by its appearance, infected animals could be missed as clinical signs take time to show. Signs and symptoms are said to develop over a period of weeks to months from the initial exposure (Poester *et al*., 2014).

This study has shown a significance association between status of literacy and vaccination of animals at $p=0.0002$. Of the 46 respondents, only 45.65% (21/46) confirmed that they vaccinated their animals. However, the respondents were not sure if the vaccine was for brucellosis or other veterinary diseases. Only (37.09%) 23/62 respondents indicated that they indeed do vaccinate against brucellosis, and they all got information regarding vaccination against brucellosis from the local veterinary office. This low percentage is evidence that awareness campaigns are indeed needed in communal and smallholder farming areas in the North-West province of South Africa.

Our study revealed that most of the participants 63% (39/62) have experienced more that one of the symptoms associated with brucellosis. Although symptoms of human brucellosis are often confused with other diseases (Poester *et al*., 2014), it is imperative to conduct a study on human brucellosis in the four districts under study and the further emphasis on training. This will also assist communities in knowing what actions to take when such symptoms are experience and therefore getting proper treatment of the disease.

5.4 Limitations of the study

This study had some limitations namely the limited number of abattoirs and farms that agreed to participate in this project, some abattoir owners declined the request for sampling due to the fear of

possible COVID-19 contamination/infection that might occur. The funds allocated to this project were available for a defined period, hence, Covid 19 pandemic did prevent the complete smooth running of the project.

A limited number of milk samples were collected, due to the belief of farmers that sampling lactating cows will affect milk production and make their calves sick.

Abortion materials were immediately discarded by farmers, hence not available during sampling. Only mesenteric, retropharyngeal and supramammary lymph nodes were collected from slaughtered cattle at the abattoirs for bacterial isolation and detection and species identification by PCR. According to Mahajan, aborted fetuses, stomach contents such as spleen and lung, semen and vaginal swabs have proved to give good results (Mahajan *et al*., 2017). With the latter in mind, there is a high possibility that the type of samples collected had an impact on the outcome of the study.

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

The economic implications of brucellosis are a threat to the development of the agricultural sector, particularly in communities practising communal livestock management systems (Lokamar *et al*., 2020). Although the prevalence of brucellosis was found to be low at 1.95% (95% CI: 1.14 – 3.12) in the four main districts of North-West province under study, the possibility of undetected cases of brucellosis cannot be ruled out, especially in the Bojanala Platinum and Dr Kenneth Kaunda districts where a limited number of cattle were sampled. This prevalence indicates that Brucellosis is present in the North-West Province. Due to its chronic nature, if not controlled the spread of brucellosis will continue. Our study highlights the need to thoroughly investigate the presence of brucellosis in the North-West province as well as other areas of South Africa.

The current study further revealed that the risk and spread of brucellosis is highly dependent on disease knowledge by livestock keepers. The risk of infection is increased through handling of aborted materials by ignorant farm workers and cattle owners. Education regarding brucellosis and zoonotic diseases causing abortion in animals should be mandatory on all farms. On-going training and awareness campaigns are recommended to assist in the control and possible eradication of this disease in the country. Awareness campaigns should include engagement with cattle farmers and forceful implementation of complementary measures such as improved and standardised data collection including monitoring of formulated policies to reduce the risks associated with the spread of pathogens causing brucellosis infection (Kolo *et al*., 2019).

Despite the availability of the bovine brucellosis legislation which articulates the current approach pertaining to the control of bovine brucellosis, brucellosis remains endemic in South Africa *(*Pappas *et al*., 2005). According to the DAFF (2016) bovine brucellosis discussion paper, this is mainly influenced by lack of compliance to regulatory requirements by livestock owners (DAFF, 2016e). However, the observation from this study was that most livestock owners were willing to comply if information was made available to them.

Our study further recommends the implementation and monitoring of strict biosecurity measures regarding the cattle-human interface. This has always been a major setback and the risk is assumed higher in rural communities where people have frequent contact with animals as also observed in other

studies (Govindasamy, 2020). Hence, the outcome of the current study agrees with the suggestion by the world health organization that cattle handlers, including veterinary officials and their families also be occasionally screened for early detection and treatment (DAFF, 2016e). To have a sustainable strategy for controlling brucellosis, the study also recommends full enforcement by the South African government for compliance to the legislation which includes vaccination of heifers, test and slaughter and compulsory testing before selling cattle. Indeed, the effective implementation of brucellosis control as a priority of the South African Veterinary Strategy plan (2006-2016) is crucial.

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