Prevalence of brucellosis in cattle slaughtered in a local abattoir in Hammanskraal, South Africa

By

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Declaration

I declare that this thesis, which I hereby submit for the Masters in Tropical Animal Health in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, is my original work and has not been submitted by me for a degree to any other university.

SIGNED  DATE: 31 JANUARY 2020

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Summary

Prevalence of brucellosis in cattle slaughtered in a local abattoir in Hammanskraal, South Africa

Supervisor: Prof. Henriette van Heerden
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Department: Veterinary Tropical Diseases
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Background: Brucellosis is a contagious zoonotic bacterial disease of worldwide distribution and remains endemic in most low- and middle-income countries (LMICs), including South Africa. The current bovine brucellosis scheme in South Africa predominantly makes use of serological tests for diagnosis which lack 100% accuracy if the gold standard test (culturing) is not performed. Abattoirs can provide information on notifiable and zoonotic disease and can play a pivotal role in disease surveillance and monitoring. This study aimed at demonstrating the usefulness of abattoir surveillance for detection of *Brucella* spp. using serological, molecular and bacteriological methods.

Methods: Serum and tissue samples (liver, spleen and lymph nodes) were collected from slaughter cattle at a local abattoir in Hammanskraal, Gauteng Province in September 2018. A total of 122 serum samples were screened for *Brucella* antibodies while molecular and bacteriological methods were used to detect *Brucella* spp. from tissue samples of serological positive animals.

Results: The Rose Bengal test (RBT) revealed a seroprevalence of 22.1% (27/122, 95% CI = 15.1-30.5) and iELISA confirmed a seroprevalence of 9.0% (11/122, 95%CI = 4.6-15.6). Genus- specific 16S-23S rRNA interspacer region (ITS) - PCR detected *Brucella* DNA in 9 of the seropositive tissue samples of animals. From the 9 ITS-PCR positive animals, 44.4% (4/9) *Brucella* bacteria were isolated and confirmed to species level using the AMOS PCR assay. AMOS-PCR characterized the four *Brucella* isolates as *B. abortus* with one mixed culture consisting of *B. abortus* and *B. melitensis* in cattle.

Conclusion: The findings conclude that abattoirs are facilities that can provide invaluable information on disease surveillance and that the gold standard procedure can be performed on collected tissue samples that yield in a more accurate diagnosis.

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List of Abbreviations

AEC	Animal Ethics Committee
bv	Biovar
cELISA	Competitive ELISA
CFT	Complement fixation tests
DAFF	Directorate Animal Health of the Department of Agriculture, Forestry, and Fisheries
ELISA	Enzyme-linked immunosorbent assay
HRP	Horseradish peroxide
iELISA	Indirect ELISA
IL	Internal iliac
ITS	Interspacer region
OIE	World Organisation of Animal Health
OPS	O-polysaccharide
PCR	Polymerase chain reaction
RBT	Rose Bengal test
RB51	*Brucella abortus* strain RB51
RP	Retropharyngeal
S19	*Brucella abortus* strain 19
sLPS	Smooth lipopolysaccharide
SP	Supramammary
WHO	World Health Organization
LMIC	Low and middle income countries

CHAPTER 1 LITERATURE REVIEW

1.1 Introduction

Brucellosis is a contagious, communicable disease caused by the bacterial organism *Brucella.* The disease was first discovered by Dr. David Bruce in Malta; and in 1886, he isolated the causative agent of "Malta Fever" and named the bacterium *Micrococcus mellitensis* and then later renamed it *Brucella melitensis* (Rossetti et al., 2017, Hull and Schumaker, 2018). In 1905, Dr. Themistocle Zammit proved that this bacterium was present in infected goat milk and that infected goats transmitted the disease to humans through milk consumption (Rossetti et al., 2017). Bernard Bang described *B. abortus* as the pathogen causing abortions in cattle (named *Bacillus abortus* initially) (Hull and Schumaker, 2018).

Brucellosis is a disease of worldwide distribution and it is important in most developing (low to middle-income) countries. Brucellosis is ranked amongst the most economically important zoonoses globally and has been classified by the World Health Organization (WHO) and the World Organisation of Animal Health (OIE) as one of the world's leading neglected zoonotic diseases (Zhang et al., 2019, McDermott et al., 2013, Franc et al., 2018). The disease burden is mostly common in low to middle-income (LMICs) countries among humans, livestock and wildlife with related economic impacts (McDermott et al., 2013). Bovine brucellosis can cause substantial economic losses in infected cattle herds resulting in lower calving rates due to abortions or temporary infertility, increased replacement costs as well as reduced sale value of infected cows (Mcmahan et al., 1944, Mangen et al., 2002). The degree of production losses in animals is directly associated with the disease prevalence.

Research has demonstrated that high seropositive animals have a higher frequency of abortion, stillbirth, calf mortality, increased inter-calving period and infertility (McDermott et al., 2013). However these clinical signs are neither diseases specific or present in every infected animal (Cunningham, 1977). After infection, abortions are most likely to occur during the first pregnancy and less likely thereafter due to sustained immunity(Cunningham, 1977). Abortions storms are often a consequence of an infected animal introduced into an immunological naive herd (Ducrotoy et al., 2017). When individual prevalence stabilizes or decreases due to reduced exposure to *Brucella,* clinical signs become less discernible, and the disease becomes chronic in a herd, flock or farm (Ducrotoy et al., 2017). Brucellosis gains public health importance when the bacteria are transmitted to humans through the consumption of unpasteurized dairy products or through direct or indirect contact of infected material such as aborted foetuses (Young, 1995, Ducrotoy et al., 2014, Addis, 2015). Brucellosis, also known as "undulant fever" or "Malta fever" in humans, has an estimated annual occurrence of 500 000 human cases worldwide (Lita et al., 2016), and affects people of all age groups and both

sexes. The clinical signs exhibited by infected livestock are of economic significance to stakeholders (livestock farmers, meat and milk industry, and the human communities) and influence the livelihood of the persons exposed (Godfroid et al., 2010, Franc et al., 2018). The acute and chronic phase of the disease can result in severe economic losses and consequential inequality in the socioeconomic status of the infected persons (Ducrotoy et al., 2017).

Therefore, correct diagnosis is imperative for the control of the disease in animals, which in turn would contribute to eliminating the disease in animals and humans (Pappas et al., 2006). Currently, the detection of brucellosis is predominantly based on screening animals using various serological methods (Godfroid et al., 2010), however the true disease status of the herd is only achieved through culture and isolation of the *Brucella* spp. Serological tests are not 100% accurate and require two serological results and confirmation using the golden standard culturing test (Nielsen et al., 2005b), but serology remains the test of choice when diagnosing brucellosis. Moreover, to perform the gold standard diagnostic test (culturing), tissue samples such as lymph nodes are not available in live animals. Abattoirs can accord for collection of various samples (lymph nodes, liver, and spleen) in addition to whole blood to be tested, which is not possible in live animals. Abattoirs can provide information on notifiable and zoonotic disease and can play an important role in disease surveillance and monitoring (van Klink et al., 2015). Several countries that make use of “slaughter” surveillance have demonstrated its effectiveness in eradicating and control of diseases which lead to maintaining low prevalence levels of such diseases (Kaneene et al., 2006). Diagnostic surveys of slaughter animals can provide an early warning system for imminent epidemics or intervention program failures.

1.2 Bovine brucellosis situation in South Africa

Brucellosis in South Africa is a controlled disease because of the health impact and the zoonotic risk that it poses (Animal Disease Act, Act 35 of 1984). The current scheme used is the bovine brucellosis scheme that was developed to detect and eradicate *B. abortus* in cattle but encountered several shortcomings. The objective of the bovine brucellosis scheme is to promote the eradication of bovine brucellosis for the advancement of animal and human health. The bovine brucellosis scheme has 7 test programs to achieve its objective, namely (DAFF, 2016): (1) the accreditation herd programme that has been discontinued due to its stringent requirements; (2) the maintenance program that accommodate herds that require annual negative certification; (3) the diagnostic herd program (herd surveillance program) to establish the prevalence of brucellosis in a herd in a certain area or local municipal area; (4) the diagnostic testing program conducts test upon special request from the owner to diagnose

one or more cattle in a herd but not the whole herd; (5) import program, where cattle imported are subjected to quarantine and a brucellosis test at that specific centre; (6) export program to meet the requirements of most importing countries that demand that cattle be subjected to brucellosis test; and lastly, (7) the infected herd which is determined when infection has been established either by serological test or isolation of *Brucella* organisms. Of the seven programs of the bovine brucellosis scheme, only the infected program is compulsory, while the first six are all voluntary.

The existing scheme only focuses on brucellosis control in cattle, which is biased as little attention is focused on other species such as pigs, sheep, goats and wildlife (DAFF, 2016, DAFF, 2017) Several shortcomings of the bovine brucellosis scheme in South Africa have been identified and a review of this policy is underway to implement corrective, self-sustainable and, cost-effective measures (DAFF, 2017). Initially the bovine brucellosis scheme achieved good control with very low occurrence of the disease with government funding and manpower. Since the late 1980s the responsibility of testing and vaccination became the responsibility of livestock owners (DAFF, 2017). Consequently, the submission of livestock owners with the bovine brucellosis legislation and the enforcement thereof by the government in South Africa is severely lacking (DAFF, 2017, Frean et al., 2018). In turn, this led to a gradual increase in the occurrence of the disease (DAFF, 2017).

In South Africa, infected or suspected positive cattle herds are screened using Rose Bengal test (RBT) and screened positive results are confirmed using complement fixation tests (CFT) (DAFF, 2016). Positive herds are classified as infected herds, and these herds are quarantined and the serologically-confirmed positive animals are issued with a red-cross permit for slaughter at a registered abattoir (Animal Disease Act, Act 35 of 1984). The infected herd remains under quarantine until the first negative test results and the herd is declared brucellosis-free following two negative test results with a 2-3 months interval between each test (DAFF, 2016). Culture is the gold standard diagnostic test and requires aborted material, milk or organs and the organism cannot be cultured from serum, which is the sample collected for serology diagnostics (Godfroid et al., 2013).

A test and slaughter policy is the recommended method to eradicate the disease however a study indicated that the feasibility of such a program requires the prevalence rate not to exceed 2% (Zamri-Saad and Kamarudin, 2016). In South Africa, the test and slaughter of test positive animals is being practiced in the bovine brucellosis scheme (DAFF, 2016, DAFF, 2017). However, the current system allows for “suspected” animals to remain indefinitely in the herd until tested positive, whereas international opinion tends to favour the stricter interpretation of test results in an infected herd with elimination of both infected as well as ‘suspect’ animals

from the herd as soon as possible in order to hasten the eradication (DAFF, 2017). Furthermore, the allowance of test-positive animals to remain in the herd until the end of their lactation period or until calving or the heifer calves of positive cows that are allowed to remain in the herd (DAFF, 2017) is counter to the test and slaughter program. Moreover, incentives required for farmers to present their animals for regular testing and identifying test-positive animals are lacking (DAFF, 2017).

Abattoir surveillance can be used to overcome the disadvantages of the brucellosis scheme in South Africa that requires serological testing which lack 100% accuracy. Facilities like slaughterhouses can avail animals for serum and tissue samples which will allow for serological, golden standard bacteriology and molecular testing. The aim of this study was to collect sera and tissues samples from slaughter cattle at an abattoir and determine the frequency of *Brucella* spp. detection. This approach will enable the government of South Africa to consider abattoir surveillance as one of the preventative and complementary cost-effective control measures that can be adopted and implemented in a measurable manner.

1.3 Aetiology

Brucellosis is caused by a Gram-negative, aerobic, facultative intracellular, non-motile, non-spore forming coccobacillus of the genus *Brucella* (Pappas et al., 2006). It is classified as an α-Proteobacteria (Pappas et al., 2006, Moreno et al., 2002) and it is divided into twelve species, each including several biovars (Young, 1995, Corbel, 1997). The *Brucella* spp. have strong host preference and classification was initially based on pathogenicity and host partiality, however cross species infections have been found to occur (Moreno et al., 2002). The lipopolysaccharide (LPS) in the bacterium cell wall appears to be the virulent factor of the *Brucella* spp. (Young, 1995). The major species affecting livestock and farm animals are *B. abortus* (biovars 1-6, and 9) that infects cattle, *B. melitensis* (3 biovars) in sheep and goats, *B. suis* (5 biovars) in pigs, *B. canis* in dogs and *B. ovis* in sheep (Moreno et al., 2002). According to Pappas et al. (2006) and Nicoletti (2010), the majority of cases are attributed by *B. melitensis* globally. Additionally, *B. abortus, B. melitensis* and *B. suis* are the main zoonotic species causing abortion (or abortion storms in immunological naïve heifers); whereas *B. ovis* is non-zoonotic and responsible for ram epididymitis (Nicoletti, 2010, Godfroid et al., 2010). The species that have the highest economic impact on domestic livestock productivity are *B. abortus* and *B. melitensis*; and of public health importance in human are *B. abortus, B. melitensis* and *B. suis* and in rare cases *B. canis* (Young, 1995, Pappas et al., 2006).

1.4 Epidemiology

Brucellosis is one of the most widespread infectious disease worldwide (Abdussalam and Fein, 1976). *Brucella* has a wide range of hosts, subsequently making identification difficult due to the variable picture it presents at individual and population level (Ducrotoy et al., 2017). In low and middle-income countries, misconceptions about the true incident of brucellosis often result from underreporting and insufficient surveillance information, lack of economic resources and capacity, and efforts between veterinarians and human doctors (Mangen et al., 2002, McDermott et al., 2013, Franc et al., 2018). Across Africa, the estimated brucellosis prevalence in ruminants is between 8.2% and 15.5%, and is comparable with the 16.0% prevalence in south Asia (Grace et al., 2012). In the southern African developed communities (SADC), a prevalence of 23.9%, 17.2%, 0.3% and 5.6% were reported in Zambia, Malawi and Zimbabwe respectively (Ahmadu et al., 1999, Matope et al., 2010, Bedard et al., 1993, Muma et al., 2007).

In 2013, a study conducted to estimate the economic impact of brucellosis across African nations and south/south east Asia recorded an average prevalence of 11.0% in the high-risk population such as the livestock owners, veterinarians and abattoir workers and 7% in-hospital patients (McDermott et al., 2013). In sub-Saharan Africa, human brucellosis is endemic and estimates of seroprevalence have been reported for many countries including 3.3% in the Central African Republic, 7.7% in Tanzania (John et al., 2010), 17% in Uganda (Tumwine et al., 2015) and 3.8% in Chad (Schelling et al., 2003). Due to common misdiagnosis in humans, discrepancies are often created between the reported number of cases and the actual number of cases in a region (Franc et al., 2018). The limited available brucellosis literature in Africa predominantly assess the seroprevalence of the disease and often lacks proof of epidemiological presence (Craighead et al., 2018).

In South Africa, brucellosis is a controlled disease and human brucellosis is a notifiable medical condition. A report from the Directorate Animal Health of the Department of Agriculture, Forestry, and Fisheries (DAFF) stated that bovine brucellosis occurs across all 9 provinces in South Africa, but infected cattle are especially concentrated in the central and highveld regions (Frean et al., 2018), including the Gauteng province. In cattle, the disease is mostly caused by *B. abortus*, although *B. melitensis* has recently been isolated from cattle in South Africa (Kolo et al., 2019, Godfroid et al., 2010). *B. abortus* biovar (bv)1 is responsible for 90% percent of infected cattle whilst the remaining 10% is due to *B. abortus* bv 2 in the country (Bishop et al., 1994, Chisi et al., 2017).

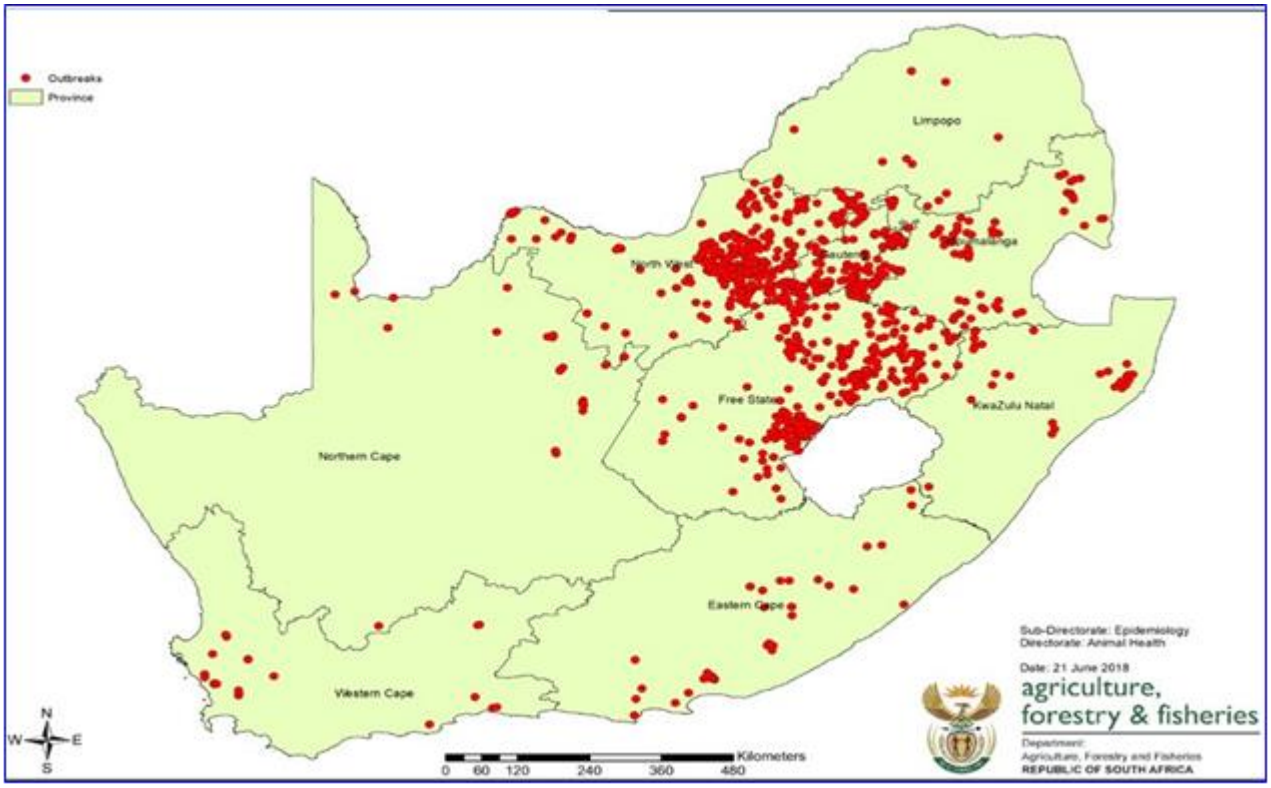

Figure 1. 1 Reported *Brucella abortus* animal outbreak across all nine provinces in South Africa from January 2015 and May 2018.Image courtesy: Sub directorate: Epidemiology of the Directorate Animal Health, DAFF (http://www.nicd.ac.za/wp-content/uploads/2018/12/Brucellosis)

The first isolation of *B. abortus* infection in cattle in South Africa was reported in 1913; and the first veterinary case of *B. melitensis* in sheep was documented in 1965 in the Transvaal province (Van Drimmelen, 1961, Van Drimmelen, 1965). However, it must be noted that in 1906, a serological survey was carried out in the southern Orange Free State province, northern part of Cape province and the eastern Transvaal province that showed clinical and some serological evidence that undulant fever was widely distributed in South Africa (Schrire, 1962). The confirmed diagnosis of *B. melitensis* in 231 human patients in Orange Free State province (Free State province) between 1906 and 1911 is evidence that the disease has been persistent for a long time in South Africa even before official documentation (Schrire, 1962). This is significant particularly because it is presumed that cattle and small ruminants are the primary source of human brucellosis infections (Caine et al., 2017).

Furthermore, in 1996, a 3.03% prevalence of *B. melitensis* in goats was diagnosed in the north-eastern Kwazulu-Natal province in South Africa (Emslie and Nel, 2002). Following the diagnosis of Malta fever in the same region, *B. melitensis* bv 1 was confirmed in native goats in three northern districts of the province of Kwazulu-Natal (Reichel et al., 1996). Cultured milk samples from serologically positive animal confirmed the diagnosis (Reichel et al., 1996). Despite goats being the primary host preference of *B. melitensis,* it has also been reported in

camels in Oman (Foster et al., 2018), dairy cattle in Spain (Álvarez et al., 2011). and in cattle in the African Mediterranean coast (Ducrotoy et al., 2017).

Furthermore, a recent study carried out in the Eastern Cape province was able to detect *Brucella* spp. isolates in raw milk, blood and lymph nodes of cattle and small ruminants in four municipal towns and provincial abattoirs (Caine et al., 2017). Of the 130 isolates recorded, 62.5% were from cattle, 25.4% from goats and 12.3% from sheep of which *B abortus* had the highest isolates (56.9%) from cattle compared to the 37.7% *B. melitensis* isolated from sheep and goats (Caine et al., 2017). In a more recent study, an estimated 5.5% prevalence of brucellosis was isolated from slaughtered cattle in abattoirs in the Gauteng province (Kolo et al., 2019). Prior to that, the last seroprevalence study conducted in an abattoir was in 1984 and a bovine brucellosis seroprevalence of 1.5% in cattle in KwaZulu-Natal was reported (Bishop, 1984). Furthermore, the first isolation of *B. melitensis* bv 2 and bv 3 in cattle in the Gauteng province was reported together with *B. abortus* bv 1 (Kolo et al., 2019). In sub-saharan Africa, extensive and pastoral grazing is the predominant system where many cattle exist in a mixed herd system (Ducrotoy et al., 2017). An outbreak of *B. melitensis* in cattle is often related to infected flocks of sheep and goats in the region (Álvarez et al., 2011) and mix rearing of cattle with small livestock and wildlife can explain the cross-species infection with *Brucella* spp. as suggested previously (Kolo et al., 2019).

According to (McDermott and Arimi, 2002) and (Ducrotoy et al., 2017), the limited data on brucellosis in small ruminants compared to cattle can explain the inadequate publications of *B. melitensis* isolates in sub-saharan Africa. This is because the investigation of brucellosis in southern Africa is generally restricted to cattle, excluding small ruminants, and largely based on serological surveys (Hesterberg et al., 2008, Njiro et al., 2011). Consequently, *B. abortus* is the most isolated species, probably due to a more frequent sampling of cattle than small ruminants (Ducrotoy et al., 2017). These bias protocols against other species can be found in the control schemes, including South Africa. The risk involved is the absence of epidemiological data, such as persistently infected herds of cattle or transmission of infection across species and confronts veterinarians and farmers with a big problem. The epidemiology of brucellosis is significant to government and the public health sector, especially among high-risk communities. Publication of epidemiological data of brucellosis in South Africa can encourage people to take protective measures at work and to actively engage in disease control programs, thereby helping to develop an effective policy for brucellosis control (Zhang et al., 2019). Moreover, ovine/caprine brucellosis due to *B. melitensis* is less commonly reported, however the cross-species infection of *B. melitensis* in cattle suggest that more efforts to control brucellosis in small ruminants is required (McDermott and Arimi, 2002).

1.5 Clinical signs

The most important clinical feature in cattle is abortion during the last gestation period. Infected females usually only abort once and remain infective for the remainder of their lives (Mcmahan et al., 1944, Godfroid et al., 2010, McDermott et al., 2013). The incubation period for brucellosis differs significantly in some infected animals, and has a very long incubation period (Adone and Pasquali, 2013). Clinical signs of economic importance exhibited include abortion, reduced fertility, and a significant decrease in milk production over the lifespan of an animal (Mcmahan et al., 1944, Mangen et al., 2002). Hygromas which usually manifest in chronically infected animals are considered an indirect indicator of the disease, however, this is not pathognomonic and needs to be verified with laboratory tests (Fensterbank, 1978, Musa et al., 1990).

The manifestation of brucellosis infection in humans is usually non-specific and present for long periods (Young, 1983). Most symptoms resemble those of malaria or influenza and are often misdiagnosed and underreported which may lead to wrong or delayed treatment and long-term disease complications due to intermitted fever and confusion with other acute febrile illnesses (Young, 1995, Dean et al., 2012). The human disease form is characterized by headaches, undulant fever, fatigue, sweating, chills, and loss of appetite, weight loss, muscular pain, joint pain, lumbar pain and arthritis (Young, 1995, Dean et al., 2012). Pregnant women may suffer the risk of miscarriages, congenital malformation, in utero foetal death or maternal death (Poole et al., 1972, Khan et al., 2001). In humans, the virulence of the disease correlates with the presence of rough or smooth lipopolysaccharide (Pappas et al., 2006, Franco et al., 2007).

1.6 Transmission

Large quantities of *Brucella* bacteria are mainly transmitted at the time of calving or abortion and is found in the aborted foetus, uterine fluid and the placenta (Mcmahan et al., 1944). Mucosal exposure to these infected materials is an important means of direct transmission between hosts/animals and animals commonly get infected through direct contact with the aborted material (Pappas et al., 2006). Cattle usually become asymptomatic after their first abortion and may become chronic carriers. Transmission of organisms to calves can be achieved through infected milk or through vertical transmission (Catlin and Sheehan, 1986). Other sources of infection within and between herds can be through contaminated forage, water or licking of calves from infected cattle (Richey and Harrell, 1997). Most probable route of brucellosis infection into a naive or disease-free herd is through the introduction of an infected animal (Ducrotoy et al., 2017).

The main source of infection in humans is through animals and their by-products. Most human cases arise from the consumption of unpasteurized dairy products or contact with infected animals, aborted material and through slaughtering within homesteads (Richey and Harrell, 1997, Pappas et al., 2006). This is a very important role in the direct transmission of brucellosis from animals to humans. Farmers, abattoir workers, pastoralists, veterinarians, animal health workers, laboratory staff as well as other people engaged in the livestock value chain are regarded to be the highest occupational risks groups (Pappas et al., 2006). The occupational or domestic exposure to livestock increases risk of transmission of *Brucella* through direct contact with placenta, fetus, fetal fluid, and vaginal discharges, particularly in areas that are endemic (Corbel, 1997, Pappas et al., 2006).

1.7 Diagnosis

The diagnosis of bovine brucellosis is complicated by the variable duration of the incubation period and its lack of clinical signs except abortion (McGiven et al., 2003, Ducrotoy et al., 2017). The dependence on clinical indications, which are polyphasic or not always present for diagnostic purposes make verification by laboratory tests results essential. Current diagnostic tests include direct tests that involve culture (bacterial isolation), DNA detection by polymerase chain reaction (PCR) based methods and indirect tests that involve serological tests (Godfroid et al., 2010, OIE, 2016).To serologically diagnose bovine brucellosis, antibodies attached to the bacterial cell wall O-polysaccharide (OPS) component of the smooth lipopolysaccharide (sLPS) must be detected (Corbel, 1997, Nielsen, 2002, McGiven et al., 2003). It is important to note that this molecule is also present in the vaccines and contributes to the protective efficacy of the vaccine (Goodwin and Pascual, 2016).

The gold standard for the diagnosis of brucellosis is through bacterial culture and identification (Nielsen, 2002). However, culture is a less sensitive test (Nielsen, 2002), and is often considered negative after 14-21 days of incubation (Godfroid et al., 2010, Godfroid et al., 2013). The use of culture is impractical for large herds (OIE, 2012). For this reason, serological tests are the preferred and most practical method of diagnosis. Presently, the South African bovine brucellosis scheme uses the RBT for screening suspicious or infected herds and CFT as a confirmatory test (DAFF, 2016).

RBT is a commonly used screening test due to its high sensitivity, ease and speed of use (Ruiz-Mesa et al., 2005, Nielsen, 2002). This rapid agglutination test depends on the reaction of the suspension of *B. abortus* antigen and the sampled cattle sera within 4 minutes (Alton et al., 1988) followed by the need for confirmatory tests (Nielsen, 2002). The CFT is a highly specific confirmatory test because of the IgG 1 isotype that fixes complement well (Nielsen, 2002, Padilla et al., 2010). CFT makes use of positive, negative or anti-complementary

controls in tested samples to determine the presence or absence of haemolysis. If the antibody is present in the serum and it is an isotype of IgG1, it will bind to the antigen and complement will be activated (Nielsen, 2002). The absence of haemolysis is recorded as a positive result because antibodies were present in the serum and complement was absent, hence erythrolysis could not occur (Nielsen, 2002). Although CFT is labour intensive because of the number of reagents needed for titration, it remains a valuable test in control or eradication programs (Nielsen, 2002). However, other diagnostic tests such as the enzyme-linked immunosorbent assay (ELISA) that are less labour intensive but are more costly, are equally efficient (Gall and Nielsen, 2004).

ELISA is a serological test that can be used as a screening test or a confirmatory test. ELISA tests are more sensitive and specific compared to other conventional tests and include the indirect ELISA (iELISA) which is the recommended test for screening according to OIE (OIE, 2016) and competitive ELISA (cELISA) which was developed to distinguish vaccine-induced antibodies from field strain-induced antibodies (Nielsen, 2002). The iELISA uses purified LPS or O-antigen as a diagnostic reagent (Nielsen, 2002) and detects antibodies (IgG and IgM) against the sLPS (Nielsen et al., 2005a). In contrast, the cELISA has a higher specificity and can be conveniently standardized by the use of purified smooth LPS antigen and monoclonal antibody for competition (Nielsen et al., 1999, Gall and Nielsen, 2004, McGiven et al., 2003), thus eliminating reactions due to residual vaccine (*B. abortus* strain 19 (S19)) induced-antibodies (OIE, 2009). Cut-off value adjustments of these assays are needed in endemic regions for specificity and can be useful in mass screening (Nielsen, 2002, Ulu Kilic et al., 2013).

A recent study compared serological tests in the diagnosis of bovine brucellosis in KwaZulu-Natal Province in South Africa and demonstrated that the RBT and CFT have the highest diagnostic specificity whereas the RBT and iELISA have the highest diagnostic sensitivity (Chisi et al., 2017). This study indicated there is no significant difference between the RBT, CFT, iELISA, and cELISA in the diagnosis of bovine brucellosis. However, several factors such as the age of the animal, incubation period of the disease, the pregnancy status, mechanism of infection and cross-reactive bacteria influence the immune response detected by serology (Gerbier et al., 1997, Adone and Pasquali, 2013). In serology, false positives may arise from antigenic similar cross-reactive gram-negative bacteria such as *Yersinia enterocolitica* 0:9, *Escherichia coli* (O: 157 and O: 116), Group N *Salmonella*, *Pseudomonas maltophilia* and *Vibrio cholerae* (Van Aert et al., 1984, Gerbier et al., 1997, McGiven et al., 2003). The presence of the *Brucella* OPS in vaccines results in similar antibody profiles in vaccinated and naturally infected animals and compromises serological diagnosis of the conventional serological test (RTB, SAT, CFT) and iELISA (Nielsen et al., 1996, Bundle and

McGiven, 2017). This emphasizes the reference to the gold standard of bacterial isolation for the classification of animals as *Brucella* infected or uninfected. Moreover, for the control of a disease to be successful, accurate and rapid diagnostic tools and results are required. *Brucella* spp. isolation and identification by PCR is the only tool that offers diagnostic certainty (Godfroid et al., 2010).

PCR based methods are efficient diagnostic tool for the identification of *Brucella* species. PCR methods are more practical and useful and require minimal biological samples to get results in a very short time (Yu and Nielsen, 2010). It is very sensitive and specific, low-cost and suitable for high volume demands (Yu and Nielsen, 2010). Amplification of *Brucella* DNA by PCR assay is achievable from biological tissues like aborted foetus, milk, serum, whole blood and can test *Brucella* culture directly (Yu and Nielsen, 2010, Dauphin et al., 2009). Current PCR methods make use of single- pair primers, multiplex primers and probes for identification. The assays available can identify *Brucella* at genus level or differential identification can be accomplished at the species level (Bricker, 2002, García-Yoldi et al., 2006). The 16S- 23S ribosomal DNA interspacer region (ITS) that identify genus-specific DNA (Keid et al., 2007) have been used to screen brucellosis initially in dogs (Keid et al., 2007) and later in livestock (Gomo et al., 2012). B*rucella* detection by PCR is more sensitive than culture using infected tissue samples, it can detect bacteria on low levels and pose less of a risk to laboratory staff and personnel (Yu and Nielsen, 2010, Bricker, 2002).

The multiplex AMOS PCR was developed to differentiate *B. abortus* bv 1, 2 and 4, *B. melitensis* bv 1, 2 and 3, *B. ovis*, and *B. suis* bv 1 and to distinguish between *B. abortus* field strains and *B. abortus* S19 and RB51 vaccine strains (Bricker and Halling, 1995, Ewalt and Bricker, 2003). AMOS is a multiplex using a reverse primer that anneals to the IS711 while each of the *Brucella* species-specific primers hybridizes to a species-specific nearby locus. *B. abortus* (biovars 1, 2, and 4) amplifies a 498-bp product, *B. melitensis* (all biovars) amplifies a 731-bp product, *B. ovis* amplifies a 976-bp product, and *B. suis* (biovar 1) amplifies a 285-bp product (Bricker and Halling, 1995).The Bruce-ladder multiplex PCR was developed to detect all *Brucella* species (Garcia-Yoldi et al., 2006). AMOS and Bruce-ladder PCR assays are primarily used to classify species from cultural colonies. PCRs are quick and easy tests that can be applied to detect *Brucella* DNA in tissue/samples or identify *Brucella* culture to species level. PCR require minimal manual labour and would be a reliable diagnostic tool especially in the national veterinary diagnostic laboratories of South Africa

1.8 Control

1.8.1 Prevention

Vaccination can be used as a cost-effective control measure to minimize the impact of the disease on human and animal health (Goodwin and Pascual, 2016). Vaccination is a significant control instrument especially when animal owners are not compensated for testing and slaughter. The *B. abortus* S19 vaccine is the most effective vaccine to date and is an important aspect for the control and elimination of the disease and is widely used (Goodwin and Pascual, 2016, Nielsen, 2002). However, elimination can be a challenge due to the *Brucella* spp. ability to infect multiple species and its presence in wild animals or feral livestock, even with a multi-faceted control strategy that includes vaccination and test and slaughter. Although (Bundle and McGiven, 2017) argue that current live vaccines do not provide protection across different animal host species, it is reported that *B. abortus* S19 vaccine protects cattle against *B. melitensis* even though this has not been proven for *B. abortus* RB51 vaccine (OIE, 2013a). However, these protective vaccines make it difficult to distinguish between infected and vaccinated animals through serology tests and this limits the fight against the disease (Nielsen et al., 1996). Nonetheless, a safe, low cost and effective vaccine mitigates disease outbreaks and avoid costly human infections (Corbel, 1997, Goodwin and Pascual, 2016, Maudlin and Weber, 2006).

In a systematic review of brucellosis awareness in communities, the data revealed that the pooled level of awareness of animal vaccination against brucellosis in the African population was 4.6% and that only the dairy industry (88.4%) had a high awareness of vaccination as a preventative measure (Zhang et al., 2019). Correspondingly, South Africa is faced with a similar challenge due to the voluntary entry of beef farmers and livestock owners in the brucellosis testing scheme. The brucellosis testing is only compulsory for dairy herd owners as they are mandated to test their herds annually (DAFF, 2016). In South Africa, the preferred vaccine is *B. abortus* S19 and the compulsory vaccination of heifers between the age of 4 and 8 months is with the government recommended dose 5×10^{10} of S19, however this not being adhered to due to poor owner compliance (DAFF, 2017).

The shortage of *B. abortus* S19 vaccine in South Africa has resulted in *B. abortus* RB51 to be the alternative vaccine choice that has similar efficacy to S19 (Olsen, 2000, Yang et al., 2013, DAFF, 2017). The lack of the O-side chain component in LPS in RB51 vaccine strain (Schurig et al., 1991) makes it possible to differentiate between naturally infected and vaccinated animals, which is particularly important if the vaccination purpose is to eliminate (Dorneles et al., 2015, Goodwin and Pascual, 2016). The lack of the O-side chain is advantageous because the vaccine can be administered once or multiple times without producing antibodies that interfere with conventional diagnostic test (Schurig et al., 1991). Furthermore, RB51's

recommended use is in 4-12month old heifer calves and can also be used in adult cows in selected high-risk situations (Olsen, 2000, Dougherty et al., 2013, Leal-Hernandez et al., 2005). Although RB51 is not completely safe to vaccinate pregnant cows particularly if a full dose is given (Palmer et al., 1996, Dougherty et al., 2013) several studies were able to record no abortions after vaccinating pregnant cows (Palmer et al., 1997, Samartino et al., 2000, Leal-Hernandez et al., 2005).

Generally, awareness and practice of vaccination will enhance the farmers' economic condition by minimizing the risk on livestock industries and international trade. For brucellosis to be eradicated, detection and slaughter of infected animals are required. Vaccination is the supreme controlled method against bovine brucellosis. In South Africa vaccination alone is not enough to eradicate brucellosis but can be used to bring the prevalence down to levels whereby the option to slaughter is not costly.

1.8.2 Abattoir surveillance

Surveillance is important because it serves as an early warning for outbreaks of diseases, measures prevalence, detect risks and protective factors and documentation of disease freedom and information on interventions (Häsler et al., 2011). *B. melitensis* and *B. abortus* were isolated in cattle in South Africa from a serological abattoir surveillance of cattle in Gauteng Province (Kolo et al., 2019) and the extracted data is of paramount importance for the brucellosis disease status in the country. Slaughtered animals at abattoirs can be tested for brucellosis to determine the strains circulating in the country and to get a more accurate prevalence of brucellosis in South Africa. Data evaluated from an abattoir help measure the relative importance of any disease event, determine evidence-based information for disease surveillance and can be efficient in frequent detection of notifiable diseases (Kaneene et al., 2006). Challenges that can arise from such surveillance is the underestimated prevalence due to the sampling bias associated with abattoir surveillance, lack of resource including laboratory and animal health personnel and inconsistency that can affect the epidemiological data.

1.9 Aim and objective

The aim of this study was to determine the frequency in the detection of brucellosis in slaughtered cattle at a low-throughput abattoir. This will shed light on whether the abattoir can be used as one of the complementary control measures in South Africa through providing both sera and tissue samples for both direct (culture and PCR) and indirect (serology) diagnostic test. If all objectives are attainable, data from an abattoir will be able to assist with the geographic epidemiology of brucellosis in South Africa and can aid in preventive actions by targeting specific "infected" farm areas and surrounding locations. Results from this study will also establish whether surveillance strategies can be used to eliminate the disease including

testing of bulls and cows at slaughter and on farm testing of whole herds. Furthermore, the different diagnostic approach will be able to establish the true health status of the slaughter cattle as either infected or non-infected.

1.9.1 Study Objectives

The objectives of this study were to determine brucellosis detection from slaughtered cattle at an abattoir and to characterize the *Brucella* spp. of seropositive animals. This was achieved as follows:

1. To determine the frequency of brucellosis detection from serum collected from slaughtered cattle at an abattoir in the study area.
2. To perform serological test using the RBT and iELISA
3. To collect corresponding tissue samples (lymph nodes, spleen, liver)
4. To detect *Brucella* DNA from collected tissue samples of positive animals using ITS-PCR
5. To culture and isolate *Brucella* spp. from the harvested tissues of seropositive animals

CHAPTER 2

2.1 MATERIALS AND METHODS

This cross-sectional study was an extension of a larger research project, the main aim of which was to determine the seroprevalence and characterize the *Brucella* spp. in slaughtered livestock (cattle, sheep and pigs) in abattoirs across Gauteng Province in South Africa (Fig 2.1 A). This current study focused on screening cattle for brucellosis in a local abattoir in the north of Gauteng Province.

2.2 Study Area

The study site was at an abattoir located in Hammanskraal, which is a small town in the Pretoria district located in the northern part of Gauteng province of South Africa (Fig 2.1 B). Gauteng Province is situated on southern Africa great interior plateau (Highveld) and is the smallest province of the nine provinces in South Africa (Fig 2.1 A). The province is largely urbanized with some farming practices (horticulture and farming). Gauteng Province is the economic hub of South Africa; the province is the most densely populated and houses approximately 23.7% of the country's population. Hammanskraal is situated at 25°24'S and 28°17'E is home to about 21,345 residents with a population density of 2,800/km^2. The local abattoir is a multi-species low through put facility that slaughters cattle, sheep and pigs.

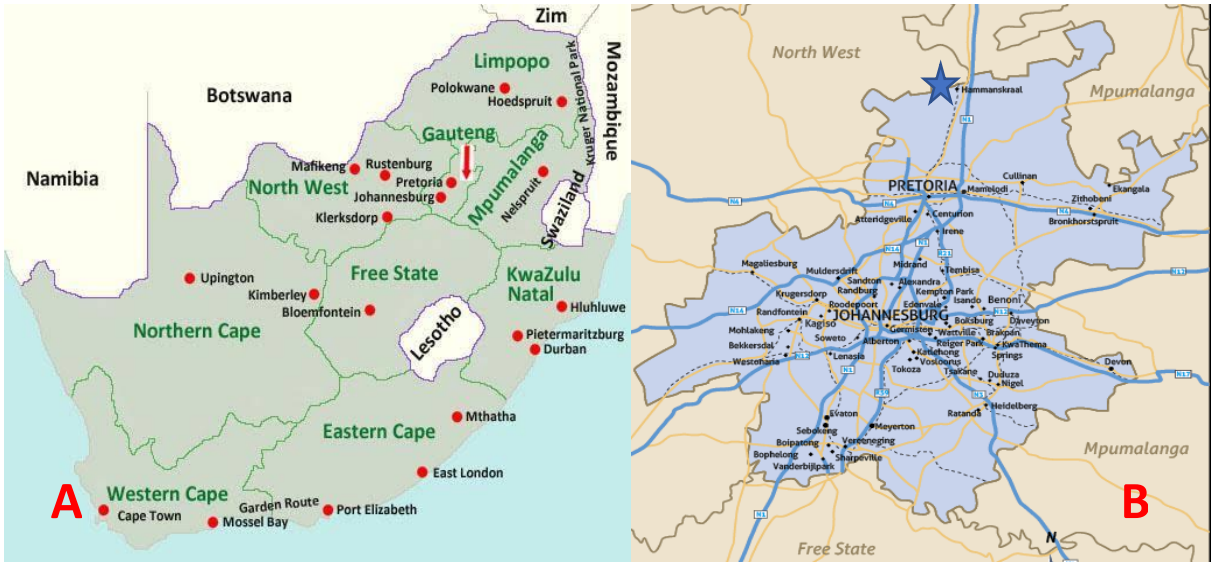

Figure 2. 1 (A) map of South Africa with Gauteng province indicated by the red arrow (image from https://showme.co.za/facts-about-south-africa/the-maps-of-south-africa/). (B) Map of Gauteng Province in blue with Hammanskraal in northern part of Gauteng province (image from https://www.globalafricanetwork.com/2017/10/12/company-news/gautengs-metros-are-driving-growth-and-investment/).

2.3 Study Design

A cross-sectional study was conducted and unclotted blood samples were collected from slaughtered cattle at the abattoir. Testing of *Brucella* spp. was performed by serological assays (Rose Bengal test (RBT) and indirect enzyme linked immunoassay (iELISA)) for the detection of humoral-mediated immunity in the cattle serum samples. Tissues of the slaughtered animals that were seropositive were assayed using polymerase chain reaction (PCR) for the detection of *Brucella* DNA using the genus-specific 16S-23S rRNA interspacer region (ITS) PCR assay (Keid et al., 2007). Positive tissues were subjected to culture and isolation for *Brucella* spp. (OIE, 2018). *Brucella* isolates were identified to species level using the AMOS multiplex PCR assay that targets the IS711 gene for speciation of *B. abortus* bv1, 2 and 4; *B. melitensis* bv. 1, 2 and 3; *B. ovis* and *B. suis* bv 1 (Bricker and Halling, 1995) and Bruce-ladder assay that differentiate most of the *Brucella* species (García-Yoldi et al., 2006).

2.4 Sample size

A convenience simple sampling method was used to recruit a total of 122 cattle. The convenience sampling method was used as a result of inconsistency of animals slaughtered daily at the abattoir. The throughput range varied significantly between 6 to 25 cattle on any given day. An average number of 20 cattle per day were sampled within a two-week period in September 2018. In cases where the maximum required number of animals were not available, sampling was carried out on the available group of no less than 10 animals.

2.5 SAMPLING PROCEDURE

2.5.1 Blood/Sera collection

Each animal was identified by age through the (dental formula), sex, species, breed and farm origin/address. This constituted the animal demographic information. During the head examination, the age of the animal was determined using the dental formula and therefore animals were marked as adults when there were two permanent incisors or as young when the permanent teeth have not erupted (Eubanks, 2012). The vaccination status of the animals was not available.

A 50 ml sterile sampling container was used to collect blood from the jugular vein of the animal at the point of slaughter and was dispensed immediately into an 8 ml serum (yellow capped) tube with serum separator. Blood collection tubes were labelled with the animal's demographic data (serial number, sex and breed). All collected blood samples were placed on ice and transported to the laboratory as soon as possible. The blood collected was centrifuged (3000 rpm, for 5 min, 5°C) and sera were collected and stored in the freezer at -20˚C until the testing procedure.

2.5.2 Tissue collection

For the isolation and/or detection of *Brucella* spp., tissue samples were aseptically collected from the liver, spleen and lymph nodes, from each animal post-slaughter. The retropharyngeal, and internal iliac lymph nodes were collected from each animal. The tissue samples excised from the carcasses were placed into sterile Ziploc bags with animal data. The tissue samples were transported on ice and stored in the freezer at -20˚C until they were processed. The tissue samples were processed in the bio-safety laboratory level (BSL) 2 plus at the University of Pretoria, Department of Veterinary Tropical Diseases according to the set laboratory protocols. Each of the tissue samples was cut into smaller pieces and placed in sterile phosphate buffered saline (PBS) in 2.0 ml screw cap eppendorf tubes and stored at -80°C for DNA extraction and bacterial isolation.

2.5.3 Serological Test Methods

The RBT and iELISA tests were used for the serological procedure. For the RBT, a commercial IDEXX *Brucella* antigen stained with Rose Bengal was used as per the standard procedure consisting of 50 µl RBT antigen and 50 µl serum placed in the Rose Bengal plate by micropipette. The plate was placed on a shaker for 4 min at room temperature and the level of agglutination recorded immediately after the 4 min of shaking. Visible agglutination was recorded as positive and no agglutination was recorded as negative.

For iELISA, the commercial ID Screen® Brucellosis Serum Indirect Multi-Species antibody test kit (IDvet) was used to test the sera according to the manufacturer's instructions and the cut-off value was 120%. According to the protocol, the wells were coated with purified *B. abortus* LPS. The samples to be tested and the control were added to the microwells diluted at 1/20. Anti-*Brucella* antibodies, if present formed an antibody-antigen complex. A multispecies horseradish peroxide (HRP) conjugate was added to the microwells that fixated to the anti-*Brucella* antibodies and formed an antigen-antibody-conjugate-HRP complex. After washing to eliminate the excess conjugate, the substrate solution (TMB) was added. The resulting blue coloration depended on the quantity of specific antibodies present in the specimen and became yellow after the addition of stop solution. In the absence of antibodies, no coloration appeared. The microplate was read at 450 nm. Samples with a sample to positive ratio (S/P %) are classified as follows: Less than or equal to 110% were considered negative; greater than 110% but less than 120% were considered doubtful and; greater than or equal to 120 % were considered positive. The iELISA was conducted on all animal sera regardless of the RBT results.

2.5.4 Bacterial isolation from tissue samples

Bacterial isolation from tissue was performed at the BSL 2 plus laboratory at the Department of Tropical Veterinary Diseases, University of Pretoria according to (OIE, 2016). The tissues in PBS were homogenized with a Precellys 24 homogenizer (Bertin Technologies). The homogenate (200 µl) from each tissue (lymph nodes, spleen and liver) was inoculated onto Farrell's and modified CITA (OIE, 2016) media respectively and incubated at 37˚C with 5.0% CO_2 (OIE, 2016). Plates were observed for 10 to 14 days for bacterial colony growth. *Brucella* organisms were presumptively morphologically identified by using the Stamp's modified Ziehl-Neelsen staining method (OIE, 2016) and suspect *Brucella* isolates were identified using the ITS-PCR, speciated using the AMOS PCR (Bricker and Halling, 1995) and differentiated from vaccine strains using the Bruce-ladder (García-Yoldi et al., 2006) assays.

2.5.5 Molecular methods

2.5.5.1 Genomic DNA Extraction and PCR assays

Genomic DNA was extracted from the respective tissue samples and from *Brucella* cultures using the Purelink® Genome DNA Kits (Thermo Fisher Scientific) according to the manufacture's protocol. DNA extraction was conducted on tissue samples of animals that tested positive on serology tests (RBT or iELISA).

The 16S-23S rRNA interspacer region (ITS) PCR: The genus-specific *Brucella* ITS-PCR assay as described by (Keid et al., 2007) that amplifies a 214 bp fragment using primers (ITS66: ACATAGATCGCAGGCCAGTCA and ITS279: AGATACCGACGCAAACGCTAC) were used to identify *Brucella* DNA in the tissue samples and also in the isolates. Primers were titrated at a final concentration of 0.5 µM with 1x DreamTaq Green PCR Master Mix (Thermo Fisher Scientific) and 2 µl of DNA from the respective tissues (liver, spleen or lymph nodes) in a 15 µl PCR reaction. The ITS-PCR cycling condition consisted of 95°C for 3 min, followed by 35 cycles of 95°C for 1 min, 60°C for 2 min, 72°C for 2 min and a final extension of 72°C for 5 min. The PCR products were analysed by electrophoresis using a 2.0% agarose gel stained with ethidium bromide and viewed under UV light.

AMOS PCR: The multiplex AMOS PCR assay identifying and differentiating *B. abortus* bv. 1, 2 and 4, *B. melitensis* bv. 1, 2 and 3, *B. ovis* and *B. suis* bv. 1 as previously described by (Bricker and Halling, 1995) was conducted using DNA extracted from cultures. Four species-specific forward primers were used at a final concentration of 0.1 µM with 0.2 µM reverse primer IS*711* (Table 2.1) with 1x MyTaq^{TM} Red PCR Mix (Bioline, South Africa) and 2 µl of template DNA in 25 µl PCR reaction. PCR cycling conditions were initial denaturation at 95°C for 5 min followed by 35 cycles at 95°C for 1 min, 55.5°C for 2 min, 72°C for 2 min and a final extension step at

72°C for 10 min. Electrophoresis was used to analyse PCR products using a 2.0% agarose gel stained with ethidium bromide and viewed under UV light.

Table 2. 1: Sequences and characteristics of the oligonucleotide primers used for different *Brucella* species in the AMOS PCR assay.

	Primer name	**Sequence (5´- 3´)**	**DNA Targets**	**Amplicon (bp)**	**Concentration (µM)**	**Reference**
AMOS	***B. abortus***	GAC GAA CGG AAT TTT TCC AAT CCC	*IS711*	498	0.1	Bricker and Halling, (1994)
	B. melitensis	AAA TCG CGT CCT TGC TGG TCT GA		731	0.1	
	B. ovis	CGG GTT CTG GCA CCA TCG TCG GG		976	0.1	
	B. suis	GCG CGG TTT TCT GAA GGT GGT TCA		285	0.1	
	IS711	TGC CGA TCA CTT AAG GGC CTT CAT			0.2	

Bruce-ladder: A multiplex Bruce-ladder PCR assay was performed to classify and differentiate between *Brucella* spp. field isolates and the vaccine strains (García-Yoldi et al., 2006) (Table 2.2). Eight species-specific forward and reverse primers were used at a final concentration of 6.25 µM with 1xMyTaq^{TM} Red PCR Mix (Bioline, South Africa) and 2 µl of template DNA in a 25 µl PCR reaction. The PCR cycling conditions included an initial denaturation cycle at 95°C for 5 min followed by 25 cycles at 95°C for 30 sec, at 64°C for 45 sec, and at 72°C for 3 min and a final extension step at 72 °C for 10 min. PCR products were analysed by electrophoresis using a 2.0% agarose gel stained with ethidium bromide and viewed under UV light.

Table 2. 2: Sequences and characteristics of the Bruce-ladder PCR assay primers used in the study from Lopez-Goni et al. (2006).

PCR Name	Primer name	Sequence (5´- 3´)	DNA Targets	Amplicon (bp)	Concentration (µM)
Bruce-ladder	BMEI0998f BMEI0997r	ATC CTA TTG CCC CGA TAA GG GCT TCG CAT TTT CAC TGT AGC	*wboA*	1682	6.25
	BMEI0535f BMEI0536r	GCG CAT TCT TCG GTT ATG AA CGC AGG CGA AAA CAG CTA TAA	*bp26*	450	6.25
	BMEII0843f BMEII0844r	TTT ACA CAG GCA ATC CAG CA GCG TCC AGT TGT TGT TGA TG	*omp31*	1071	6.25
	BMEI1436f BMEI1435r	ACG CAG ACG ACC TTC GGT AT TTT ATC CAT CGC CCT GTC AC	Deacetylase	794	6.25
	BMEII0428f BMEII0428r	GCC GCT ATT ATG TGG ACT GG AAT GAC TTC ACG GTC GTTCG	*eryC*	587	6.25
	BR0953f BR0953r	GGA ACA CTA CGC CAC CTT GT GAT GGA GCA AAC GCT GAA G	ABC Transporter	272	6.25
	BMEI0752f BMEI0752r	CAG GCA AAC CCT CAG AAG C GAT GTG GTA ACG CAC ACC AA	*rpsL*	218	6.25
	BMEII0987f BMEII0987r	CGC AGA CAG TGA CCA TCA AA GTA TTC AGC CCC CGT TAC CT	CRP Regulator	152	6.25

2.6 DATA ANALYSIS

Data of each animal were captured in Microsoft Excel and Epi-Info 7 version 10 was used to analyse data. An animal was defined as seropositive for brucellosis when it tested positive on both RBT and iELISA as prescribed by (OIE, 2016), but the estimated seroprevalence results were based on RBT and/or iELISA seropositive results. ITS-PCR was done on RBT and/or iELISA seropositive tissue, as Kolo et al. (2019) obtained *Brucella* culture from slaughtered cattle tissue samples that were RBT and/or iELISA positive. Epi- Info 7-version 10 was used to conduct descriptive analysis through analysis of frequency with a 95% confidence interval. The overall seroprevalence was calculated and stratified according to age, sex and breed seroprevalence with a confidence interval of 95%. To estimate the association between animal demography and seroprevalence, a 2x2 contingency tables, the odds ratio and Chi square tests were calculated. The significance was set at $P< 0.05$, Fishers exact test. Charts plotting was done for corresponding seropositive results.

2.7 ETHICAL APPROVAL

The protocol of this study was approved by the University of Pretoria Animal Ethics Committee (AEC), project V054-18 (see appendix: A). This study was an extension of a larger project and used the Section 20 approved by the Directorate of Animal Health according to Act 35 of 1984.

Chapter 3

3.1 Results

A total of 122 animals were sampled from the Hammanskraal abattoir. Of the total, 60.66% (74/122) were females and 39.34% (48/122) were males. There was a 50:50 (61:61; young: adult) distribution of each age group within the sample population. The distribution stratified by breed was Afrikaner, 12.3% (15/122), Angus, 4.1% (5/122), Bonsmara, 37.7% (46/122), Brahman 14.8% (18/122), Friesian, 4.9% (6/122), Nguni, 19.7% (24/122) and mixed breed 6.6% (8/122) (Fig 3.1).

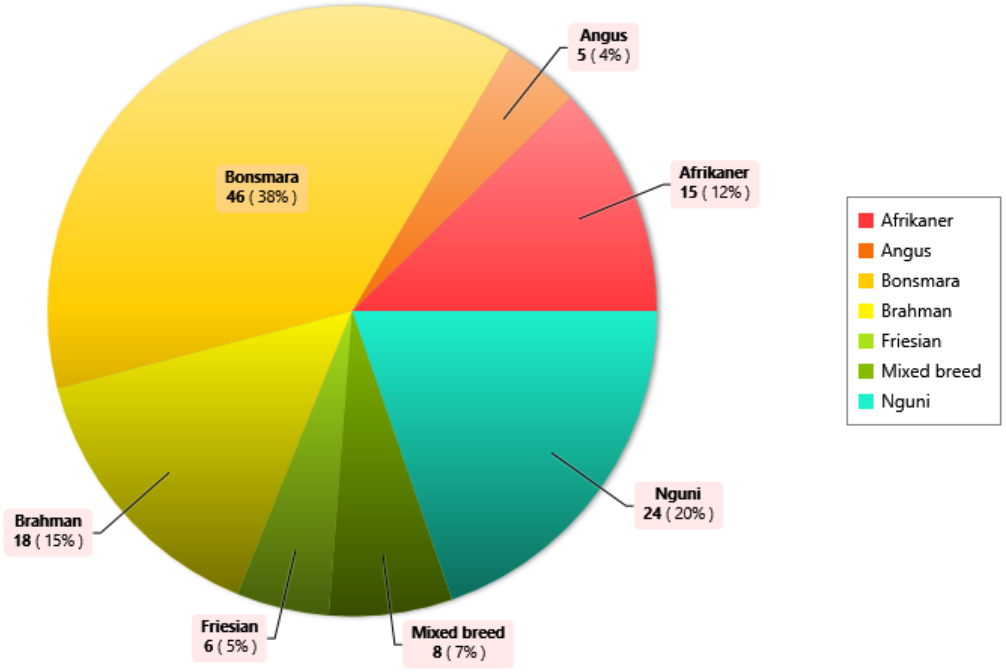

Figure 3. 1. Breed distribution of cattle sampled at the Hammanskraal abattoir during this study.

The RBT and/or iELISA seropositive animals resulted in a 25.41% (31/122) brucellosis seroprevalence in the slaughtered cattle of which 27.03% (20/74) females and 22.92% (11/48) males were positive. With RBT, 22.1% (27/122, 95%CI: 15.1-30.5) of the cattle were seropositive and iELISA detected a seroprevalence of 9.02% (11/122, 95% CI: 4.6-15.6) in the cattle sera. Of the 27 positive RBT results, 25.93% (7/27) were confirmed positive on iELISA. Thus, the overall brucellosis estimated seroprevalence (RBT and iELISA) in the slaughtered cattle was 5.74% (7/122) (Table 3.1). With RBT, 22.97% (17/74) females and 20.83% (10/48) males were positive, while10.81% (8/74) females and 6.25% (3/48) males were positive with iELISA (Table 3.1).

The age category with the highest seroprevalence in the RBT and/ or iELISA seropositive tests was demonstrated in the adult group consisting of 32.79% (20/61), whereas 18.03% (11/61)

was demonstrated in the young group. The sex with the highest seroprevalence was displayed in females compared to males. The RBT and/or iELISA brucellosis seropositive results show that there was no significant difference in the age (P= 0.06) and sex (P=0.61) (Table 3.1). Of the RBT positives confirmed with iELISA (RBT and iELISA), 71.43% (5/7) were adults and female and 28.57% (2/7) were young and male (Table 3.1). The seroprevalence of the confirmed seropositive animals show there was no statistical difference in the age (P=0.24) and sex (P=0.55).

Table 3. 1: Prevalence of brucellosis seropositive animals in this study using Rose Bengal test (RBT) and iELISA based on all the animals, sex and age.

Animal demography	**Serology positives % (RBT and/or iELISA)**	**OR (95%CI)**	**Chi(x^2)**	**P-value**
Age				
Young	18.03% (11/61)	0.5 (0.19-1.05)	3.5	0.06
Adult	32.79% (20/61)			
Sex				
Female	27.03% (20/74)	0.8 (0.34-1.87)	0.3	0.61
Male	22.92% (11/48)			
Animal demography	**RBT positives %**	**OR (95%CI)**	**Chi(x^2)**	**P-value**
Age				
Young	16.39% (10/61)	0.5(0.21-1.22)	2.3	0.127
Adult	27.87% (17/61)			
Sex				
Female	22.97% (17/74)	0.8(0.37-2.13)	0.7	0.78
Male	20.83% (10/48)			
Animal demography	**ELISA positives %**	**OR (95%CI)**	**Chi(x^2)**	**P-value**
Age				
Young	4.92% (3/61)	0.3(0.08-1.36)	2.5	0.114
Adult	13.11% (8/61)			
Sex				
Female	10.81% (8/74)	0.6(0.14-2.19)	0.7	0.39
Male	6.25% (3/48)			

Animal demography	RBT Confirmed iELISA positives %	OR (95%CI)	Chi(x^2)	P-value
Age				
Young	3.28% (2/61)	0.4 (0.07-2.04)	1.4	0.24
Adult	8.20% (5/61)			
Sex				
Female	6.76% (5/74)	0.6 (0.11-3.23)	0.4	0.55
Male	4.17% (2/48)			

The distribution of RBT positive confirmed iELISA according to breed were as follows: Afrikaner 13.33% (2/13), Angus 20.00% (1/5), Bonsmara 0.00% (0/46), Brahman 0.00% (0/18), Friesian 33.33% (2/6), Nguni 8.33% (2/24) and mixed breed 0.00% (0/8).

The DNA detection rate of *Brucella* spp. from RBT and/or iELISA seropositive animal tissue was 29.03% (9/31). Thus, the prevalence of *Brucella* DNA detection using the ITS-PCR was 29.03% (9/31, 95%CI: 14.22-48.04) and 7.38% (9/122, 95%CI: 3.43-16.54), from seropositive animals and the total animals tested respectively. Of the 31 RBT and/or iELISA positive animals, 20 were female and 11 were male. The *Brucella* DNA detection was highest in seropositive females with a frequency of 19.40% (6/31) compared to the 9.70% (3/31) detection rate in seropositive males. This translates into a detection rate of 25.00% (6/20) amongst the females and 27.27% amongst the male group. From the 9 ITS-PCR positive animals, the DNA detection rate was 55.55% (5/9) higher in adult than in younger animals. However, there was no statistical difference amongst the age and sex group (P>0.05) (Table 3.3). The detection rate of *Brucella* DNA from the seropositive breeds were as follows: Afrikaner 6.67% (1/15), Angus 20.00% (1/5), Bonsmara 8.70% (4/46), mixed breed 25.00% (2/8) and Nguni 4.20% (1/24).

Table 3. 2: The 16-23S ribosomal RNA internal transcribed spacer (ITS)-PCR results of the Rose Bengal and/or iELISA seropositive animals in this study.

Animal demography	**ITS-PCR Herd positives %**	**OR (95%CI)**	**Chi(x^2)**	**P-value**
Age				
Adult	8.20% (5/61)	0.8(0.20-3.07)	0.12	0.729
Young	6.56% (4/61)			
Sex				
Female	8.11% (6/74)	0.8(0.18-3.18)	0.15	0.70
Male	6.25% (3/48)			
Animal demography	**ITS PCR_Seropositive %**	**OR (95%CI)**	**Chi(x^2)**	**P-value**
Age				
Adult	25.00% (5/20)	1.7(0.35-8.42)	0.44	0.50
Young	36.36 (4/11)			
Sex				
Female	30.00% (6/20)	0.9(0.17- 4.49)	0.03	0.87
Male	27.27% (3/11)			

From the 9 ITS-PCR positive cattle (Table 3.2), 44.4% (4/9) *Brucella* cultures which is the gold standard were isolated and confirmed with modified Ziehl-Nielsen stain, AMOS-PCR and Bruce-ladder PCR assays for speciation. The isolation rate of *Brucella* spp. from all the animals sampled and seropositive animals was 3.28% (4/122, 95%CI: 0.90-8.18) and 12.90% 4/31, respectively. From the 4 confirmed *Brucella* spp. isolates, 50.00% (2/4) were from the confirmed iELISA (RBT and iELISA) positives and the remaining 50.00% (2/4) was from the RBT positive but iELISA negative animal. Three quarters of the isolates were cultured from adult animals and a 50:50 distribution of the sex was noted from the cultured animals. The breeds that were positive on isolates were, 1 Afrikaner (adult female), 1 Angus (young bull), 1 Bonsmara (adult bull) and 1 Friesian (adult female).

The AMOS PCR assay characterized the four *Brucella* isolates as *B. abortus* with one mixed culture consisting of *B. abortus* and *B. melitensis* (Fig 3.2). Purification attempts to isolate both *B. abortus* and *B. melitensis* from mixed culture failed as only *B. abortus* was isolated from mixed culture. *B. abortus* specific primers amplify a 498 bp amplicon, while *B. melitensis* specific primers amplify 731 bp using AMOS multiplex PCR assay. Bruce-ladder PCR assay identified 3 cultures as *B. abortus* with banding pattern of 1682, 794, 587, 450, 152 bp and the mixed culture as *B. melitensis* with banding pattern of 1682, 1071, 794, 587, 450 and 1562 bp (results not shown; Garcia- Yoldi et al, 2006).

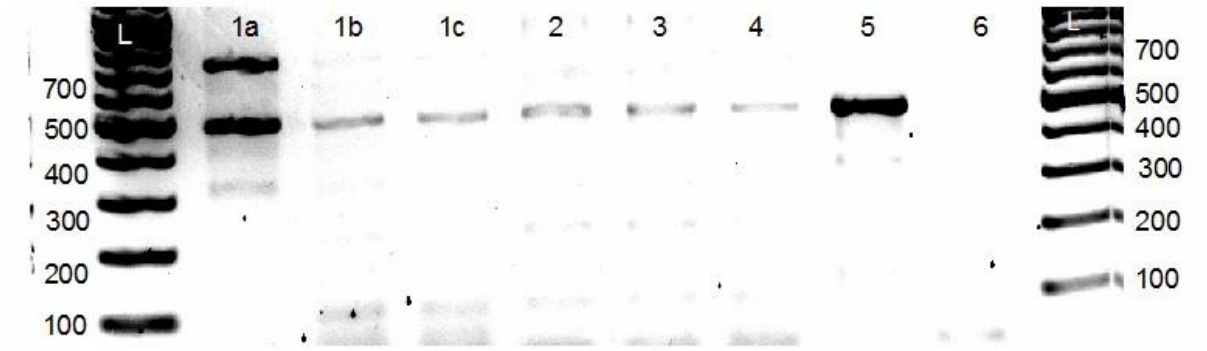

Figure 3. 2 AMOS–PCR gel electrophoresis products using species specific primers from *Brucella* cultures established from tissue samples from slaughter cattle at the Hammanskraal abattoir.

Lanes 1a, 1b, 1c are AMOS-PCR products amplified *B. abortus* and *B. melitensis* DNA from the same animal's spleen, liver and lymph, respectively while lanes 2 - 4 are AMOS-PCR amplicons from cultures from three animals with *B. abortus* DNA, while lane 5 is a *B. abortus* bv 1 S19 strain positive control and lane 6 is a negative control. The 498 bp amplicon identifies *B. abortus* bv 1, 2 and 4 while 731 bp amplicon identifies *B. melitensis* bv 1, 2 and 3.

Chapter 4

4.1 Discussion

This study provided evidence of seropositivity and the isolation of *Brucella* in slaughter cattle at a local abattoir in Hammanskraal, South Africa. The RBT and iELISA serological tests were used for this analysis to evaluate cattle seropositivity to brucellosis using serum samples. Overall, the study estimated a seroprevalence of 5.74% (7/122) in the cattle using RBT corroborated with iELISA (RBT and iELISA). A similar serological study carried out in Gauteng abattoirs reported an estimated seroprevalence of 5.5% (11.0% RBT, 5% iELISA) in slaughtered cattle (Kolo et al., 2019). Other countries showed varying proportions of seroprevalence in slaughtered cattle such as 7.8% (RBT and cELISA) in south-western Nigeria (Ayoola et al., 2017) and 3.40% for RBT and 5.93% for iELISA in Cameroon (Awah-Ndukum et al., 2018). Similar serological findings were recorded in native cattle farming systems in Zimbabwe (5.6%) and Ivory Coast (4.6%) (Matope et al., 2010, Sanogo et al., 2013). The different prevalence observed in this study comparable to other studies, may be due to the varying sample size, study frame, type and number of diagnostic tests used, as well as, the control measures and farming practises in each country.

RBT is a cheap and rapid preferred serology test in South Africa used as a screening test for brucellosis, because of its highly sensitivity for immunoglobulin detection of IgG1 and IgM (Nielsen et al., 2005a). Due to RBT's high sensitivity, it is more likely to obtain false positive results than to miss brucellosis hence the need for the recommended complementary tests, such as complement fixation test (CFT), cELISA or iELISA that are more specific (Nielsen, 2002). The confirmation iELISA test used in this study, solely detects IgG in cattle sera against *Brucella* organisms (Nielsen et al., 2005a). In this study, the seroprevalence in cattle with RBT and iELISA was 22.1% and 9.02% respectively. These results are suggestive of false positive or present or past exposure to *Brucella* spp. including vaccine in the slaughter cattle. The vaccination status was unknown. The results showed no statistical significance between the sex and seropositivity to bovine brucellosis. However, it must be noted that highest seroprevalence detected by RBT and iELISA was found in females (6.76%) than in males (4.17%), most likely attributed by the fact that the females are kept longer than males and are rarely sold for slaughter by herdsmen unless they have poor reproductive performance (Mangen et al., 2002). In addition, most genetically superior bulls are kept for a shorter period than the females in a breeding herd, resulting in decreased exposure in males especially none breeding males compared to females (Kebede et al., 2008). Furthermore, female cattle can remain as a source of infection that spreads from one animal to another either through lactation or mating (Jajere et al., 2016). Another factor for female predominance can be due to lowered immunity of female animals induced by stress that is associated with pregnancy

and calving (Mai et al., 2012). The difference in seroprevalence can also be because of the predilection sites of *Brucella* spp., which are predominantly the reproductive tract and a gravid uterus (Ayoola et al., 2017).

In this study, the *Brucella* antibody seropositivity was found to be higher in older animals than younger animals. According to (Kebede et al., 2008) and (Matope et al., 2010) cattle susceptibility to *Brucella* spp. infection is influenced by the age of the animal. This result may be due to the increased risk of exposure with age of cattle and possible proliferation of the brucellosis organism that can produce a latent or apparent clinical manifestation. Although age and sex were both not statistically significant, 71.43% of RBT positives confirmed with iELISA were adults and female. Therefore, this study can confirm that brucellosis is a disease of sexual mature animals in accordance to with other research studies (Ndazigaruye et al., 2018, Al-Majali et al., 2009).

Serology remains the most widely used method for brucellosis diagnosis and detection of antibody is considered the most practical and economic diagnostic tool (Corbel, 2006). However, the major disadvantages of serological tests are that no serological test is 100% accurate therefore series serological method is used, and serological test except cELISA cannot differentiate vaccine strains from field strains and are liable to false-positive results due to other cross -reacting bacteria (Godfroid et al., 2010, Gall and Nielsen, 2004).

Godfroid et al. (2010) indicated that isolation and PCR are the only methods that allow for 100% specificity. This study made use of *Brucella* genus specific ITS-PCR which confirmed the presence of *Brucella* DNA in the tissue samples. This molecular method was able to detect *Brucella* DNA in 29.03% (n=9) of the total seropositive animal isolates. The DNA detection was highest in females and adults with a frequency of 19.4% and 55.55% respectively. Keid et al. (2007) validated the ITS-PCR specificity. The sensitivity of the ITS-PCR for brucellosis in cattle tissue has not been determined but *Brucella* spp. were cultured from ITS-PCR positive tissue, which was also found by Kolo et al. (2019). Kolo et al. (2019) were able to establish a herd prevalence of 75% (10/14) in cattle using PCR for prevalence estimation. These authors further established a detection rate of 92% in lymph nodes tissues using ITS-PCR (Kolo et al., 2019). Furthermore, AMOS-PCR was able to demonstrate and differentiate infection of both *B. abortus* and *B. melitenis* from culture colonies in this study. These results are comparable to other studies that also demonstrated mixed infections in cattle (Kolo et al., 2019, Thenamutha et al., 2017).

The quality of the diagnostic test is determined by its sensitivity and specificity, each relating to the test ability to reveal the actual/true status of the disease (Gall and Nielsen, 2004). The recommended diagnostic ‘gold standard’ of bovine brucellosis is culture. *Brucella* spp.

isolation is “absolute” proof that the animal is infected although not all infected animals give a positive culture (Godfroid et al., 2010, Hosein et al., 2017). In the absence of bacterial growth, false negative results should always be considered since the sensitivity of culture is low (Padilla et al., 2010). This may arise from suboptimal collection or selecting samples from uninfected tissues, transport and storage of specimens, and/or deficiencies in culture media and incubation conditions or because *Brucella* spp. are fastidious organisms. The gold standard has a low sensitivity as reported by Gall and Nielsen (2004) and Ndazigaruye et al. (2018) with 46.0% sensitivity and 100.0% specificity of isolation of *Brucella* spp. from animal tissue samples. O’Grady et al. (2014) isolated *Brucella* spp. from 86.8% lymph nodes from an infected brucellosis dairy herd. From the retropharyngeal (RP), internal iliac (IL), supramammary (SP) lymph nodes 90.5% of the *Brucella* cultures were isolated from RP. Kolo et al. (2019) isolated from 92.0 % (23/25) of lymph nodes from ITS-PCR positive cattle in Gauteng abattoirs. In this study, only a 50.0% (2/4) isolation rate from lymph nodes positive by AMOS PCR was obtained, in comparison to the aforementioned. The interpretation of the isolation rate of this study should be done with caution and could have been influenced by the absence of bacterial growth and the sample size. Nonetheless, the isolation rate of the bacteria from lymph nodes suggests that pooling of lymph nodes can be used to assay *Brucella* DNA (Kolo et al., 2019). This is significant and valid for the definitive diagnosis of *Brucella*. The use of abattoirs to collect animal tissues during routine inspection for screening can reinforce the test and slaughter policy and eliminate leaving “suspect’’ animal in a herd indefinitely until they test positive, as per the current practice (DAFF, 2017)**.**

Tissue collected from seropositive slaughtered cattle were used to obtain isolates and identified mainly *B. abortus,* as well as an animal with mixed infection of *B. abortus* and *B. melitensis*. The bacteriological characterization of *B. melitensis* from the tissue of slaughter cattle is of epidemiological importance and magnifies the risks to which humans are exposed. *B. melitensis* is the most virulent *Brucella* species reported in human cases and is associated with severe acute disease as reported by (Corbel, 2006). The zoonotic implication of this findings poses a significant public health risk, which emphasise the importance of establishing epidemiological data for the occurrence of *B. melitensis* in livestock. The most recent case of *B. melitensis* bv 1 isolated in humans in South Africa was reported in 2015 in the Western Cape Province (Wojno et al., 2016). A study carried out in Eastern Cape province reported *B. abortus* from tissues and blood samples in cattle as well as *B. abortus* and *B. melitensis* in sheep at abattoirs (Caine et al., 2017). The first cases of *B. melitensis* bv 2 and 3 isolated in cattle in the Gauteng Province was reported by Kolo et al. (2019), Kolo et al. (2018). *B. melitensis* has thus been reported in 3 different provinces in South Africa (Wojno et al., 2016, Caine et al., 2017, Kolo et al., 2018, Kolo et al., 2019). The findings of this study correlate with

the results of the previous studies (Caine et al., 2017, Kolo et al., 2019, Wojno et al., 2016) and confirms that *B. abortus* as well as *B. melitensis* is present in cattle population in South Africa.

The presence of *B. melitenisis* in both cattle and humans indicate that there is a high probability that it is present in the sheep and goats within this geographical region. The cross-species transmission from small stock to cattle has not fully been established. Literature suggest that spillover across species and farms could be due to rearing of cattle together with sheep and goats (Godfroid et al., 2013). Cattle in communal areas usually graze on shared pastures and can travel over several kilometres, which could lead to contamination of large areas. Generally, there are no restricted calving/lambing pens, and this can serve as a source of infection for other cattle using the same pasture. The isolation of *B. abortus* is not surprising because it has been documented as the most common species in the cattle population in South Africa (Coetzer et al., 1995). However, the significance of this finding is in the fact that it is present in slaughter cattle that were assumed or "deemed" to be healthy. This heightens the risk of exposure in humans directly involved in daily activities with cattle such as rearing, slaughter or handling (Pappas et al., 2006).

The South African bovine brucellosis scheme is mandatory only for high risk herds suspected or confirmed of infection using RBT and CFT and is optional for all other livestock owners (DAFF, 2016). It is compulsory for heifers between the ages 4 and 8 months to be vaccinated with the *B. abortus* S19 strain against brucellosis. However, there are no strict regulations to ensure that this practice is adopted across all farms in South Africa since the responsibility has been passed to livestock owners, who do not meet their obligations. Due to the voluntary nature of this scheme, the status of herds in South Africa against bovine brucellosis is unknown and consequently, cattle with a known or unknown brucellosis status are sent for slaughter. Additionally, vaccination failure due to breaking of vaccine cold chain, incorrect application or timing of vaccinations (when using *B. abortus* S19), especially in the absence of veterinarians and para-veterinarians (Hinman et al., 1992, Wiedermann et al., 2016) are considerable contributing factors to the widespread dissemination of bovine brucellosis across the country.

The ultimate objective of abattoir facilities is to protect the health of the consumers by ensuring that the food from such establishments do not pose a risk to the public health. According to the Consumer Protection Act (Act No. 68 of 2008), it is the responsibility of the seller to ensure the quality and safety of the animal or animal product that is being supplied. This requires both owners and abattoir facilities to ensure that animals are healthy at point of sale or for consumption. The isolation and molecular methods used in this study was to demonstrate that

abattoirs are resources that can provide both serological and tissue samples necessary for the diagnosis of brucellosis. Moreover, the identification of the *Brucella* spp. in livestock can be used for epidemiological investigation and trace strains back to their farm origin (Padilla et al., 2010). The isolation of mixed *Brucella* species infection in cattle is pivotal to redirect the entire focus of the bovine brucellosis scheme away from only cattle and to consider other species such as small stock. Such invaluable information can be factored into the development of relevant control and eradication strategy of the brucellosis scheme in South Africa. Improving animal disease monitoring and eradication programs requires holistic approaches.

The discussion and review of the bovine brucellosis scheme have identified several factors that are delaying the control and eradication of brucellosis in infected herds. One important factor that has great significance to this study is the unwillingness of abattoirs in South Africa to slaughter *Brucella*-positive cattle and in turn results in lower slaughter prices to be paid to the farmer (DAFF, 2017). Furthermore, the fact that brucellosis infected animals have no value other than slaughter value regardless of their full genetic value further discourages owners to participate in the brucellosis testing (DAFF, 2017). Nonetheless, this study demonstrated the imperative value of abattoirs and their contribution to disease control and monitoring.

The current policy of repeated test and slaughter of test-positive animals in infected herds is in line with international practices (DAFF, 2017). However, the lack of incentive for farmers to present their animals for regular testing and to comply with control and movement measures neutralizes efforts to control and eliminate the disease in the country. Due to lack of participation from farmers, infected herds can go undetected unduly for prolonged periods and pose a risk to neighbouring farms aiding to the dissemination of the disease (DAFF, 2017). Therefore, routine or random inspection of slaughter cattle can help identify infected farms or herds of owners who are deliberately reluctant to cooperate in such schemes. Furthermore, the use of abattoirs will ensure that both serology and direct diagnostic methods through culture and/or PCR are achieved with 100% certainty of an animal's disease status. This in turn will mitigate or minimize the dissemination of the disease across South Africa and will give the government an advantage in the epidemiology and control of the disease.

Therefore, consideration by the Department of Agriculture Forestry and Fisheries (DAFF) would be to strategically identify local abattoirs in every province that can be used for screening/ surveillance purposes. The integration of routinely recorded data from slaughter houses can be a useful approach to quantify disease risks in production animals (Innocent et al., 2017). Slaughterhouse data can be combined with cattle tracing to monitor brucellosis over time and space if geographical information is available (Vidal et al., 2016, Innocent et al.,

2017). This will assist in the materialization of an animal Identification, recording and traceability (AIRT) system that would facilitate movement control and ensure that bovines from quarantined farms are not sold or moved, as per review discussion (DAFF, 2017). Abattoir surveillance can effectively complement epidemiological surveillance and active surveillance policies. Moreover, discussions to create incentives for farmers by considering the genetic value of test-positive cattle and availing more manpower (veterinarians, para-veterinarians) and other resources for the test of brucellosis, while collaborating with private sectors, NGOs or universities for a sustainable program is still underway and can positively project the reviewed brucellosis scheme into a successful program.

4.2 Limitations

This study did not assess associated risk factors at farm, cattle herd and abattoir level which could have provided better epidemiological insights to the study. Another limitation was the unknown vaccination status of these animals, particularly cattle vaccinated with *B. abortus* S19, which can result in positive serology. The sample size of the study was small; therefore, prevalence should be interpreted with caution. AMOS only detects *B. abortus* bv 1, 2 and 4 but Bruce-ladder detects all *B. abortus* biovars and similar Bruce-ladder and AMOS-PCR results were obtained. However, Bruce-ladder does not detect mixed infections. Biotyping of the *Brucella* isolates will be conducted in future to provide important epidemiological data on the dissemination of the disease and allow traceability back to origin. Biotyping will confirm the biovars of the *Brucella* isolate.

Chapter 5

5.1 Conclusion

In conclusion, *Brucella* spp. were prevalent in cattle slaughtered in the abattoir which poses a risk to the abattoir workers. The presence of *B. abortus* and *B. melitensis* in tissues of slaughter cattle stresses the importance of using abattoirs for important public health and economic disease surveillance. In order to identify and explore the risk factors associated with the occurrence and the spread of brucellosis among cattle herds, further epidemiological studies on infected farms and cattle herds in the study area must be conducted. It is important to ascertain the prevalence of the disease both on farms (humans and cattle) and abattoir facilities.

5.2 Recommendations

This study has shown that it is possible to survey abattoirs to retrieve brucellosis status of slaughter animals. The use of bacteriological and molecular methods demonstrated valuable data output that are useful for trace-back to the farm of origin and establish geographical location of infected farms. It would be advisable to establish risk factors across all levels (from farm to slaughter). The risk factors that could influence seropositivity in animals is the possible exposure to aborted foetal material during grazing, bull sharing, introduction of new animals onto farms without quarantine and vaccination. State veterinarians can be a resource by providing locations on geographic information systems (ArcMap) of all suspected and infected farms.

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Appendix A: Animal Ethics Certificate

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**The frequency of *Brucellosis* detection in cattle slaughtered in an Abattoir in Hammanskraal and the associated risk factors**
PROJECT NUMBER	**V054-18**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. M Hausiku**

STUDENT NUMBER (where applicable)	**U_29168938**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPESIES/SAMPLES	**Bovine**
NUMBER OF ANIMALS	**200**
Approval period to use animals for research/testing purposes	**June 2018 - June 2019**
SUPERVISOR	**Prof. H van Heerden**

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 4 February 2019
CHAIRMAN: UP Animal Ethics Committee	Signature

S*4285-15*

Appendix B: Research Ethics Certificate

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science

Research Ethics Committee 13 August 2020

LETTER OF APPROVAL

Ethics Reference No	**V054-18**
Protocol Title	**Prevalence of Brucellosis in cattle slaughtered in a local abattoir in Hammanskraal, South Africa**
Principal Investigator	**Dr M Hausiku**
Supervisors	**Prof H van Heerden**

Dear Dr Hausiko,

We are pleased to inform you that your submission conforms to the requirements of the Faculty of Veterinary Sciences Research Ethics committee.

Please note the following about your ethics approval:

1. Please use your reference number (V054-18) on any documents or correspondence with the Research Ethics Committee regarding your research.
2. Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
3. Please note that ethical approval is granted for the duration of the research as stipulated in the original application (for Post graduate studies e.g. Honours studies: 1 year, Masters studies: two years, and PhD studies: three years) and should be extended when the approval period lapses.
4. The digital archiving of data is a requirement of the University of Pretoria. The data should be accessible in the event of an enquiry or further analysis of the data.

Ethics approval is subject to the following:

1. The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.
2. **Applications using Animals:** FVS ethics recommendation does not imply that AEC approval is granted. The application has been pre-screened and recommended for review by the AEC. Research may not proceed until AEC approval is granted.

We wish you the best with your research.

Yours sincerely

PROF M. OOSTHUIZEN
Chairperson: Research Ethics Committee

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