Zoonotic diseases in high-risk populations in the Free State province, South Africa

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by

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Declaration

"I, Cornelius G. van der Westhuizen, declare that the Master's Degree research dissertation that I herewith submit for the Master's Degree qualification in Medical Microbiology at the University of the Free State is my independent work, and that I have not previously submitted it for a qualification at another institution of higher education."

Signature: Cornelius Date: 18-05-2021

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"Science knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world". - Louis Pasteur

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Abbreviation	Meaning
ANDV	Andes virus
BB	Bovine brucellosis
BSL	Biosafety level
bTB	Bovine tuberculosis
CFT	Complement fixation test
CIST	Comparative intradermal skin test
DAFF	Department of Agriculture Forestry and Fisheries
DOBV	Dobrava–Belgrade virus
EID	Emerging infectious diseases
ELISA	Enzyme-linked immunosorbent assay
G	Glycoprotein
HFRS	Haemorrhagic fever with renal syndrome
HIV	Human immunodeficiency virus
HPS	Hantavirus pulmonary syndrome
HTNV	Hantaan virus
IFA	Immunofluorescence assay
IFN-γ	Interferon-gamma
IU	International Units
KNP	Kruger National Park
LPS	Lipopolysaccharide
MAC	*Mycobacterium avium* complex
MAT	Microscopic agglutination test
MGIT	Mycobacteria Growth Indicator Tube
MIRU	Mycobacterial interspersed repetitive units
MOTT	*Mycobacterium* other than tuberculosis
MRT	Milk-ring test
MTBC	*Mycobacterium tuberculosis* complex
N	Nucleocapsid
NALC – NaOH	N-acetyl-l-cysteine–sodium hydroxide

NTM	Non-tuberculous mycobacteria
OD	Optical densities
PBS	Phosphate buffer solution
PI	Post infection
PCR	Polymerase chain reaction
PPD	Purified protein derivative
PUUV	Puumala virus
RBT	Rose Bengal test
SA	South Africa
SANGV	Sangassou virus
SAT	Standard agglutination test
SIST	Single intradermal skin test
SNV	Sin Nombre virus
TB	Tuberculosis
TST	Tuberculin skin test
VNTR	Variable number tandem repeats
WB	Western Blot

Abstract

Zoonotic diseases are infectious diseases transmitted from vertebrate animals to humans and are accountable for more than 60% of all recognized human diseases and 75% of all new or emerging infectious diseases (EID). In South Africa (SA), endemic zoonoses include *Mycobacterium bovis* (*M. bovis*), *Brucella* sp., and *Leptospira* sp. The prevalence and burden of other pathogens, such as hantaviruses, are unknown. Therefore, identifying high-risk occupations and other risk factors are important for control and preventative measures to decrease the disease burden of these zoonoses. Thus, this study aimed to investigate the incidence rate of *M. bovis* and *Brucella* sp. in cattle and farm workers in two different farming communities (communal and commercial), as well as their associated risk factors. This study aimed to document occupational exposure to *Brucella* sp., *Leptospira* sp. and hantaviruses across the Free State province, South Africa. Four commercial farms and a rural cattle farming community within the Moqhaka and Ngwathe municipal regions were selected for the purpose of this study. From these farms, sputum and blood specimens were collected from 13 commercial farm workers and 13 communal farm workers. Sputum samples were screened for *M. bovis* through Mycobacteria Growth Indicator Tube (MGIT) culture. Blood specimens from these 26 farm workers, in addition to 301 archived sera, were screened for *Brucella* sp., hantaviruses, and *Leptospira* sp. antibodies using commercially available enzyme-linked immunosorbent assays (ELISA). From the 26 farm workers, no *M. bovis* was isolated. Out of the 327 sera screened, 35/327 (10.7%) were *Brucella* sp. IgG positive, 17/327 (5.2%) *Leptospira* sp. IgM positive, and 38/327 (11.6%) hantavirus IgG positive. A combined total of 321 cattle were screened for *M. bovis* through tuberculin skin testing (TST); 71 cattle were from communal farms and 250 from commercial farms. Additionally, blood samples collected from 69 and 1793 cattle within the communal and commercial farms, respectively, were screened for *Brucella* sp. using a Rose Bengal test (RBT) and complement fixation test (CFT). A total of 8/321 (2.5%) cattle reacted positive to the TST, and two were positive using the interferon-gamma release assay. Initial RBT screening resulted in 52/1859 (2.8%) positive results, further testing using a CFT identified 19/1859 (1%) brucellosis-positive cattle. A higher percentage of brucellosis-positive animals were from communal cattle (6/69; 8.7%) compared to commercial cattle (13/1859; 1.1%). Statistical analysis and probability values were calculated using a chi-squared or Fisher's exact test in the case of sparse data. Analysis

identified higher *Brucella* sp. occupational exposure in veterinarians (p-value = 0.0006) and laboratory workers (p-value = 0.031). Further analysis showed a statistically significant correlation between people who reported illness post-exposure to animal blood/tissue (p-value = 0.029); and older age (p-value = 0.0008) with *Brucella* sp. seropositivity. Working at the abattoir (p-value = 0.024) was identified as a high-risk occupation for contracting *Leptospira* sp. In conclusion, the low incidence rate of *M. bovis* in cattle suggests limited contact with known reservoirs (i.e. buffalo or other wildlife). However, the higher incidence rate of brucellosis in communal cattle highlights the importance of implementing mass herd vaccination campaigns, particularly in communal settings. This report documents the seroprevalence of *Leptospira* sp., *Brucella* sp. and hantaviruses in various high-risk occupations in the Free State province and can be used as a basis for the development and establishment of adequate preventive or control measures.

Keywords: zoonotic diseases, brucellosis, tuberculosis, leptospirosis, hantavirus, occupation, risk factors, seroprevalence, cattle.

Chapter 1: Literature review

1.1 Introduction

Zoonotic diseases are defined as diseases that can be transmitted from a vertebrate animal to humans and are some of the oldest known infectious diseases (Herrero and Mahalingam, 2013). The prevalence of zoonotic diseases has increased over the past two decades and are accountable for more than 60% of all recognized human diseases. Furthermore, 75% of all emerging infectious diseases (EID) are of zoonotic origin and are globally responsible for 15.8% of all deaths associated with disease (Salyer et al., 2017). However, in developing countries- including South Africa (SA), the mortality rate is significantly higher at 47.3% (Wang et al., 2016). It is estimated that zoonotic diseases are globally responsible for 2.5 billion human cases accounting for approximately 2.7 million deaths each year (Gebreyes et al., 2014). EID episodes are dominated by zoonoses, where the majority (71.8%) originate from wildlife and are increasing over time (Jones et al., 2008).

Emerging or re-emerging infectious diseases are generally associated with unprecedented and unpredictable outbreaks due to their highly infectious nature (Joffe, 2011). Therefore, considerable attention is provided to these diseases as they contain substantial pandemic potential, as seen with the recent severe acute respiratory syndrome coronavirus 2 (SARS-CoV 2) outbreak (Konda et al., 2020). However, an argument can be made that endemic zoonoses, such as brucellosis and rabies, pose a similar, more chronic and insidious threat to human and animal health. These zoonoses clinically present with symptoms generally shared with a range of other common infectious diseases (i.e. malaria, typhoid fever) leading to difficulties in diagnosis and underestimating of the true burden of these diseases (Salyer et al., 2017).

Surveillance studies of zoonotic pathogens in animal-human interfaces, particularly occupations with direct animal exposure, are crucial towards preventing and controlling zoonoses. The One Health concept that follows an interdisciplinary approach between human, animal and environmental health are essential to combat human and animal disease and environmental degradation (Rock et al., 2009). During 2014-2016, the Centers for Disease Control and Prevention (CDC) implemented a One Health Zoonotic Disease Prioritisation

workshop in seven low-income countries, which included: SA, Ethiopia, Kenya, Cameroon, the Democratic Republic of the Congo, Thailand and Azerbaijan.

A total of 18 zoonotic diseases were selected as priority zoonoses amongst the seven countries which included the endemic bacterial zoonoses brucellosis, zoonotic tuberculosis (TB), leptospirosis, and emerging haemorrhagic hantaviruses (Salyer et al., 2017). These diseases were selected based on several criteria, the most important being economic, environmental, social impact, availability of interventions, epidemic or pandemic potential, severity of disease in humans, and the presence of the disease in a country or region (O'Brien et al., 2016).

In SA, zoonotic diseases are responsible for directly affecting human health and wellbeing by being a common cause of disease and indirectly by impacting food security as a result of livestock production losses (Halliday et al., 2015). Moreover, they contribute to a great disease burden, where they pose a significant public health concern. Limited information exists regarding the prevalence and incidence of zoonotic diseases in SA. Therefore, further studies are necessary to determine the true burden these diseases pose to assist in future prevention and control measures.

1.2 Mycobacterium bovis

1.2.1 Characteristics and taxonomy

Mycobacterium bovis is the etiological agent for bovine tuberculosis (bTB) in cattle and zoonotic tuberculosis in humans. These are Gram-positive acid-fast, rod-shaped, aerobic bacteria, and members of the *Mycobacterium tuberculosis* complex (MTBC). *M. bovis* is recognised worldwide as a significant health risk to the general human population, as well as to domestic cattle and wildlife. Currently, the MTBC consists of twelve members within the same genus, characterised by ≥99.9% similarity at the nucleotide level (Huard et al., 2006), which include: *M. africanum*, *M. bovis* and the *Bacillus Calmette–Guérin strain* (BCG), *M. canetti*, *M. caprae*, *M. microti*, *M. mungi*, *M. pinnipedii*, *M. suricattae, M. orygis*, *Dassie bacillus* and *M. tuberculosis* (Alexander et al., 2010). *M. tuberculosis* and *M. bovis* are the most frequently reported complex species capable of causing TB in animals and humans (Brosch et al., 2002.

Mycobacteria that do not cause TB but a disease similar to TB are collectively referred to as non-tuberculous mycobacteria (NTM) or mycobacterium other than tuberculosis (MOTT) (Wallace et al., 1990). In SA, *Mycobacterium avium* complex (MAC), *M. scrofulaceum* and *M. kansasii* are the most prevalent and responsible for 30.2%, 5.5% and 4.7% of all pulmonary NTM cases in the country, respectively (Okoi et al., 2017). *M. intracellulare* is also the most frequently isolated member of the MAC and accounts for 76.4% of all MAC infections (Okoi et al., 2017). Unlike members of the MTBC that are obligatory pathogens, NTMs are readily characterised as opportunistic environmental pathogens (Cook, 2010) that do not cause TB, but may cause TB like symptoms under certain conditions (Grubek-Jaworska et al., 2009).

1.2.2 *Mycobacterium bovis* in animals

Mycobacterium bovis was first isolated from tubercules in domestic cattle, which were later established as one of the maintenance hosts for *M. bovis* (Ayele et al., 2004). Until 1920, *M bovis* was a significant cause of disease in cattle in large parts of Europe, with herd prevalence of up to 40% reported in the United Kingdom (Brooks-Pollock et al., 2014). As a result, countries experienced substantial economic loss due to trade restrictions and reduced animal productivity (De la Rua-Domenech, 2006). Many developed countries, such as Australia and Switzerland, have successfully eradicated the disease or limited the spread of the disease through a 'test and slaughter' approach (Carneiro and Kaneene, 2018).

In SA, the first reported reference of bTB in cattle was made by Hutcheon in 1880. Suggesting that the disease was introduced during the 18th and 19th century when European cattle were imported into the country. A potential link between tuberculosis in livestock and wildlife was first suggested by Paine and Martinaglia (1929) when *M. bovis* was reported in small ungulates and greater kudu (*Tragelaphus strepsiceros*) in the Eastern Cape province.

Retrospective bTB outbreak investigations in buffalo in the Kruger National Park (KNP) suggested that the disease was transmitted from infected cattle to buffalo herds at the park's southeast corner between 1950 and 1960. However, the presence of the disease in KNP was only first discovered in 1990 in African buffalo (*Syncerus caffer*). Two years thereafter, the prevalence of bTB in KNP was estimated at 0% in the northern zone, 4.4% in the central zone and 27. 1% in the southern zone. The spread of the disease to other wildlife became evident

in 1995, when the disease was found in several wildlife species, including leopards (*Panthera pardus*), lions (*Panthera leo*), cheetahs *(Acinonyx jubatus*), olive baboons (*Papio Anubis*) and greater kudu (*Tragelaphus strepsiceros*) (Michel et al., 2006). By 1998 the prevalence of bTB in KNP had increased significantly to 16% in the central zone and 38.2% in the southern zone, mainly due to an increase in average herd prevalence, as well as an increase in the total number of infected buffalo herds (Rodwell et al., 2001).

Furthermore, a study conducted by Hlokwe et al. (2014) provided strong evidence of intra and inter-species transmission of *M. bovis* in SA. In the previously mentioned study, 491 *M. bovis* isolates were obtained from several livestock and wildlife species between 2002 and 2013 across the country and characterised using spoligotyping and variable number of tandem repeat (VNTR). Bovine TB infection was confirmed in cattle and 16 different animal species (one domestic porcine and 15 wildlife species), supporting evidence of intra and inter-species transmission. Following this, in 2014, the first evidence of *M. bovis* spillback from a wildlife reservoir host located in KNP back into an adjacent subsistence cattle farming community was established (Musoke et al., 2015).

Although *M. bovis* is the predominant member of the MTBC associated with disease in bovids, other members of the MTBC have also been isolated from cattle (Adesokan et al., 2019), most notably *M. tuberculosis*. Numerous studies reported incidences of *M. tuberculosis* infection in cattle, dating as far back as 1923 when human tuberculosis bacilli were isolated from a calf (Giltner, 1923). Since then, it was hypothesised that *M. tuberculosis* infection in cattle resulted from human-to-cattle transmission. However, this was first proven in 2005 when Ocepek *et al*. epidemiologically linked human-to-cattle transmission of *M. tuberculosis*. A study conducted by Ameni *et al*. (2011) in Ethiopian grazing cattle reported human-to-cattle transmission of *M. tuberculosis* through the traditional husbandry act of discharging chewed tobacco directly into the oral cavity of cattle to treat for internal parasites. In SA, the first isolated cases of *M. tuberculosis* in cattle were reported by Hlokwe *et al*. (2017) in the Eastern Cape province.

Bovine TB in animals is considered a chronic infection, meaning that animals may only show clinical signs months to years after contracting the infection. Infected individuals can shed *M.*

bovis bacteria for long periods without concurrent evidence of disease, resulting in transmission through direct contact or through contamination of the environment (Miller et al., 2015). Transmission most frequently occurs through inhalation of expelled droplets as a result of coughing. Husbandry acts such as indoor housing or poorly ventilated housing provide an environment of ideal transmission, with reports of *M. bovis* bacilli remaining viable for 74 days when shielded from sunlight (Phillips et al., 2003). Other modes of transmission include ingestion or inhalation of *M. bovis* when animals share the same grazing pasture or food –and water troughs with infected animals. Calves can also become infected when ingesting milk from infected cows (Zanardi et al., 2013).

Animals with advanced infection of *M. bovis* often demonstrate difficulties in breathing, coughing, decreased milk production and fertility, and other respiratory signs, which may ultimately progress to death (Cousins et al., 2004).

1.2.3 Diagnosis of *Mycobacterium bovis* in cattle

Several techniques exist that can be used to diagnose bTB in cattle, which can be divided into field tests, laboratory diagnosis and postmortem.

1.2.3.1 Tuberculin skin test (TST)

The TST, a field test, is currently used as the definitive screening test and prescribed by the World Organisation of Animal Health (OIE) for international trade (DAFF, 2016).

The TST principle relies on the delayed-type hypersensitivity to mycobacterial tuberculo-protein, which is a purified protein derivative (PPD). The PPD is injected intradermally into the neck (cervical) of the animal and generates a cell-mediated immune response to the antigen, visualised through dermal swelling, measured after 72h post-injection.

Primarily, only two variations of the TST are used depending on the available resources, history and known TB status of the herd, and exposure to other environmental *Mycobacteria* sp. These include the single intradermal skin test (SIST) and comparative intradermal skin test (CIST). The SIST is used when the negative TB status of the herd is known or if animals are tested for the first time and includes a single injection of bovine

tuberculin. After 72h, all animals are measured at the injection site, where an increase in skin thickness of ≥6 mm regards a herd as suspect (Figure 1). If no measurements above 6 mm are obtained, the herd is considered negative. However, SIST suspect animals require confirmatory testing using a CIST. The latter is used when the TB status or history of the herd is unknown or when difficulties are experienced with the interpretation of results, most likely due to non-specific reactions occurring. If multiple SIST skin reaction increases of >20mm are observed, the herd is considered positive. Animals are then subsequently slaughtered, or in some cases, positive reactive animals are treated as suspect reactors and undergo confirmatory testing (DAFF, 2016).

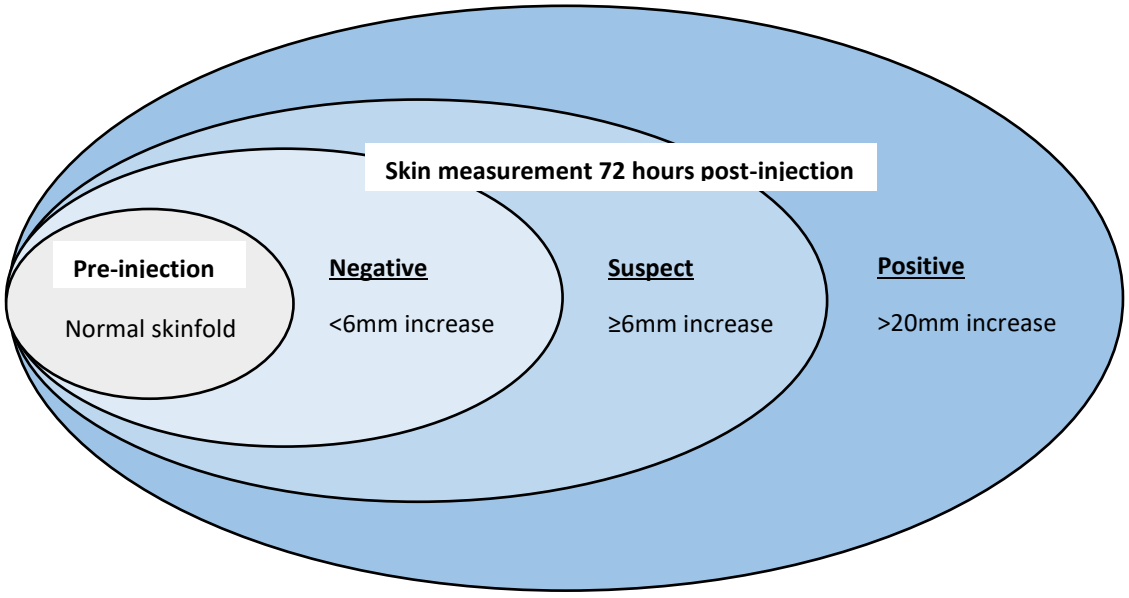

Figure 1: Interpretation of results (skin thickness increase) obtained from the single intradermal tuberculin skin (SIST) test after 72 hours post bovine tuberculin injection.

The CIST is the preferred method and more commonly used as it is able to differentiate between infections of *M. bovis* from other sensitising environmental *Mycobacteria* sp. The overall sensitivity and specificity of the CIST range from 55.1-93.5% and 88.8-100%, respectively (Fentahun and Luke, 2012). Results are determined by measuring the increase in skin thickness difference at the site injected with bovine tuberculin and the site injected with avian tuberculin. Cattle are considered positive reactors when the increase in skin thickness is equal to or greater than 4 mm at the bovine tuberculin injection site. Suspect animals are

defined as having a reaction increase of 3-4 mm, and animals with a reading difference of <2 mm are considered negative.

Advantages of TST are that they are cost-effective, convenient and readily available. However, some limitations include a low sensitivity and specificity in regions with a low TB prevalence, interpretation of results, and variations in test results due to the stage and severity of the disease.

Therefore, SISTs are incapable of differentiating between a *M. bovis* or avian reaction; for this reason, CISTs are the preferred method for diagnosis due to the increased specificity. In the case of incoherent results, a gamma-interferon release assay can be used as an ancillary test.

1.2.3.2 The gamma-interferon (IFN-γ) release assay

The gamma-interferon (IFN-γ) release assay is a laboratory-based, in vitro assay analogous to the TST. The technique is used to verify the existence of a cell-mediated immune response generated by an animal in response to being infected with *Mycobacteria* sp. (Ramos et al., 2015). In the assay, IFN-γ produced by T-lymphocytes of an infected animal is detected through monoclonal anti- IFN-γ using a sandwich enzyme-linked immunosorbent assay (ELISA). To conduct the assay, whole blood in heparin tubes are collected from animals and stimulated with antigens (i.e. bovine PPD, avian PPD, fortuitum PPD and mitogen pokeweed-positive control) to induce the release of IFN-γ before being incubated for 20-24h. Afterwards, plasma is harvested to quantify the release of IFN-γ (Lalvani and Pareek, 2010).

The overall sensitivity and specificity of the IFN-γ release assay range from 73-100% and 85-99.6%, respectively (De la Rua-Domenech, 2006). Advantages of the assay include detection during the early stages of infection and increased sensitivity (DAFF, 2016). Furthermore, animals are only required to be handled once with no waiting period between testing. However, limitations include the time sensitivity of the assay as blood needs to be processed within 6-8h after collection. Also, the assay is relatively expensive and requires a greater logistical demand than the TST. Therefore, the IFN-γ release assay is often used as an ancillary test to the TST when a quick and sensitive diagnosis is required (De la Rua-Domenech, 2006).

1.2.3.3 Other methods

Other diagnostic methods include the TB Guinea pig inoculation test, ELISA, fluorescence polarization test and bacteriological examination (Ziehl- Neelsen staining), however, these tests are not routinely used or readily available (DAFF, 2016).

1.2.4 Control

In western European countries, such as France, Denmark and the Netherlands, bTB have been successfully eradicated through strenuous testing, slaughter and other control measures to restrict animal movement (Allen et al., 2018). In SA, the bTB eradication and control scheme was implemented in 1969, following the 'test and slaughter' approach, due to the increase in economic importance of the disease (Michel et al., 2006). The approach was met with great success and led to a significant decrease of bTB in cattle within the commercial sector from a prevalence of 11.8% in 1971 to 0.39% in 1995 (Arnot and Michel, 2020). Since then, the prevalence of bTB has slowly started to increase as a result of infrequent testing and budget constraints (Michel et al., 2008).

The control scheme involves the routine screening of cattle herds using a TST. Positive reactors are branded and culled, and the herd is screened at three-month intervals until no positive reactors are observed. During this stage, farms are placed under quarantine (DAFF, 2016).

According to Arnot and Michel (2020), five requirements are to be met for a successful control scheme, which include: stringent test interpretation, compensation for animal loss, a uniform commercial farming system, government support and the absence of a wildlife *M. bovis* reservoir. However, in SA, many challenges are faced. These include a large communal farming community that are not frequently included in annual bTB screenings or surveillance studies, a lack of government financial support and the presence of wildlife maintenance hosts (African buffalo). All these are contributing factors to the persistence of *M. bovis* in both animal and potentially, in human populations.

1.2.5 *Mycobacterium bovis* in humans

Tuberculosis in humans caused by *M. bovis* is clinically referred to as zoonotic TB (Cosivi et al., 1998). Although the disease caused by *M. tuberculosis* is more frequent, it is clinically, pathologically, and radiologically indistinguishable from that caused by *M. bovis*; the latter is typically associated with extra-pulmonary TB due to the route of transmission (Scott et al., 2016).

The global prevalence of human TB due to *M. bovis* remains relatively unknown, primarily due to routine diagnostics unable to differentiate between *M. bovis* and *M. tuberculosis*. Between 1954 and 1970, the global prevalence of zoonotic TB was estimated at approximately 3.1% (Cosivi et al., 1998). A crude estimated rate of 7 cases of zoonotic TB per 100 000 population/year was made more recently in 2013 by Müller *et al*. In this comprehensive review, a further estimation was made that the overall median proportions of zoonotic TB incidents were ≤1.4% in regions outside Africa and increased to 2.8% for African countries (Müller et al., 2013).

The transmission of *M. bovis* from animals to humans consists of three main routes (Grange, 2001). Ingestion of contaminated milk (Kahla et al., 2011) or other dairy products, such as soft cheeses (Harris et al., 2007), has been identified as the most frequent mode of *M. bovis* transmission. During the 1940s, an increase of *M. bovis* transmission occurred in Great Britain as a result of milk from many animals originating from different herds being pooled together to ease transport (Francis, 1947). Inhalation of aerosolised bacilli has also been recorded as a route of *M. bovis* transmission from animals to humans, especially animal handlers such as farm workers. This transmission route was supported by evidence of acid-fast bacilli witnessed in dried bovine sputum on windows and walls of a cowshed holding infected animals (Jensen, 1952).

Additionally, airborne transmission has also been identified in abattoirs when aerosols are generated during the handling of tuberculous infected carcases. This was demonstrated in South Australasia in 1988 when four abattoir workers developed pulmonary TB and another renal TB over a two-year period (Robinson et al., 1988). A third, far less common transmission mode is through traumatic inoculation of *M. bovis* into the skin. Commonly referred to as

butcher's wart, due to the occupational hazard- typically in abattoirs, when infected meat is handled (du Buf-Vereijken et al., 1999; Grange, 2001).

People who contract zoonotic TB usually develop different forms of the disease based on the route of transmission. Infection via the respiratory tract, gastrointestinal tract or localised inoculation typically manifests as pulmonary TB, extra-pulmonary TB and localised lesions, respectively (De la Rua-Domenech, 2006). Pulmonary TB is characterised by the involvement of the lungs and considered more transmissible due to coughing. In contrast, extra-pulmonary TB is less transmissible and can occur in the lymph nodes, pleura, brain, kidneys, bones and joints (CDC, 2016).

1.2.6 Diagnosis of *Mycobacterium bovis* in humans

Routine diagnostics of TB are incapable of differentiating between *M. bovis* and other *Mycobacterium* sp. and generally requires additional methods such as polymerase chain reaction (PCR) or typing to distinguish between other members of the MTBC (Raffo et al., 2017).

Sputum smear microscopy is still the most widely used diagnostic technique in developing, low-income countries (Weyer et al., 2011). However, microscopy limitations are well known, with a low sensitivity of 40% - 60% compared to culturing techniques. Furthermore, the human immunodeficiency virus (HIV) puts added pressure on smear microscopy by significantly increasing the amount of smear-negative cases and those with extrapulmonary disease (Weyer et al., 2011).

In countries such as SA, TB culture is considered the gold standard method for a confirmatory diagnosis due to the high prevalence and endemicity of the disease that creates difficulties for serology-based methods. A BD BACTEC MGIT 960 culturing system with a specialised liquid media to enhance MTBC growth and decrease the turnaround time has been developed and used extensively in TB diagnostics. This system requires a single decontaminated specimen, preferably sputum-phlegm, and incubates the specimen at 37°C for a maximum duration of six weeks (Pfyffer and Wittwer, 2012). During the incubation period, the system flags positive if any growth is detected. After six weeks, if no growth is detected, the system flags negative.

A positive culture result is subject to further antibiotic susceptibility testing to first-line drugs, including rifampicin and isoniazid, to determine patient treatment.

However, no further routine analysis is performed on TB positive results to determine the MTBC isolate responsible for the infection. Therefore, the true burden of *M. bovis* remains vastly underreported. *M. bovis* is also inherently resistant to the first-line antibiotic - pyrazinamide, resulting in complications when administering treatment.

Subsequent steps to differentiate between members of the MTBC includes the line probe assay (LPA) or RD4 conventional PCR (Warren et al., 2006). However, these methods are generally used for surveillance-based studies or complex clinical cases, often involving multidrug-resistant or extreme-drug resistant TB. Methods used to differentiate between different strains of *Mycobacteri*um *tuberculosis* complex, include spacer oligonucleotide typing (spoligotyping) and mycobacterial interspersed repetitive units (MIRU), which occur in variable number tandem repeats (VNTR), collectively known as the MIRU- VNTR PCR-based system. These methods are often used in outbreak investigations and epidemiological surveillance and not routinely performed in diagnostics.

1.3 *Brucella* sp.

1.3.1 Characteristics and taxonomy

Brucella sp. are the etiological agents for brucellosis, commonly referred to as undulant fever or Malta fever. These bacteria are Gram-negative, rod-shaped coccobacilli, intracellular pathogens approximately 0.5 microns in diameter. *Brucella* sp. are considered not true acid-fast bacteria as decolourisation by weak acids can be resisted, therefore staining red when stained using the modified Ziehl Neelsen method (Corbel and Banai, 2015).

There are currently nine *Brucella* sp. Recognised to be predominantly affecting different animal species (Seleem et al., 2010). These include *B. abortus* in cattle, *B. melitensis* and *B. ovis* in sheep and goats*, B. suis* in pigs, reindeer and small rodents, *B. canis* in dogs, *B. neotomae* in wood rats and *B. microti* in common voles. Two *Brucella* sp. have also been found in marine mammals, including *B. pinnipedialis* in pinnipeds (seals and walruses) and *B. ceti* in cetaceans (Pappas, 2010). Five out of the nine known species can infect humans, with

the most pathogenic being *B. melitensis*, followed by *B. suis*, *B. abortus* and B*. canis* (Seleem et al., 2010).

1.3.2 *Brucella* sp. in animals

As described previously, several species of *Brucella* sp. are hosted in different animal species and can cause animal brucellosis that usually involves reproductive failure (i.e. abortions, stillbirths, infertility), or in rare cases, arthritis. In animals, persistent infection and shedding of cocci in mammary and reproductive excretions are common (Garin-Bastuji, 2011). *B. abortus* and *B. melitensis*, from an epidemiological perspective incorporating both human and animal health, are considered more significant due to the higher virulence (*B. melitensis*) and widespread distribution (*B. abortus*). *B. abortus* is one of the leading causes of production losses in cattle worldwide and have also been implicated with abortions in sheep and goats (Ocholi et al., 2005). Transmission to buffaloes, camels, dogs, horses, and pigs have also been established (Garin-Bastuji, 2011).

B. melitensis typically causes abortions and infertility in sheep and goats but have also been implicated with abortions in cattle (Kolo et al., 2018). Other *Brucella* sp. capable of infecting cattle are *B. suis* (Ewalt et al., 1997) and *B. canis* (Baek et al., 2012); however, cases are rare.

Transmission of *Brucella* sp. between cattle occur most frequently through direct contact (Coetzer et al., 1994). Mainly through consumption of contaminated food and water sources and by licking infected genitalia, foetuses and other birthing products. Furthermore, studies have documented infant animals becoming infected through ingestion of milk from infected females (DAFF, 2016). Additionally, the venereal transmission of *Brucella* sp. has also been reported but are considered epidemiologically unimportant, except for regions where artificial insemination is heavily practised (Khamesipour et al., 2013).

In SA, bovine brucellosis (BB) is controlled in cattle as part of the Animal Diseases Act, 1984, due to the public health hazard and economic impact the disease causes (DAFF, 2016). BB control measures in cattle, as with bTB, uses a ‘test and slaughter’ approach. Two vaccines, namely *Brucella abortus* strain RB51 and S19, have also been developed to vaccinate heifers four to eight months of age as preventative measures (Simpson et al., 2018a). The age of the

animal is crucial when considering vaccine administration as heifers vaccinated at three months or younger develop poor immunity. Heifers older than eight months are not vaccinated to avoid an increase in false-positive reactors when herd testing is conducted (DAFF, 2016). Bulls are not vaccinated as it may cause infertility and orchitis (Simpson et al., 2018a). Although cattle vaccination in South Arica is legislated as compulsory, the responsibility is given to livestock owners who often do not comply (Tempia et al., 2019).

Further difficulties in controlling the disease include the persistence of *Brucella* sp. in wildlife that may serve as a potential source of infection, especially African buffalo which is considered a reservoir for *B. abortus*. Further implications in controlling *Brucella* sp. include its multifaceted epidemiology, socioeconomic implications, and also not being readily identified due to the variable picture associated with the disease (Ducrotoy et al., 2017).

1.3.3 Diagnosis of *Brucella* sp. in animals

Due to animal brucellosis depicting various clinical features, heavy reliance is placed on laboratory diagnosis, which include direct and indirect methods. Direct methods include culture and microscopic examination to identify the bacterium, whereas indirect methods include serology-based methods such as the Rose Bengal test (RBT), complement fixation test (CFT) and milk-ring test (MRT).

The preferred specimen for culture includes foetal specimens when abortion occurs, including the lungs, spleen, liver, and foetal stomach fluid. A selective medium, e.g. Farrell's medium, is typically used for *Brucella* sp. culture and requires an incubation period of one week before being declared as negative (Godfroid et al., 2010). In addition, milk samples can also be cultured; however, it is estimated that roughly 80% of infected female animals experience localisation of *Brucella* sp. in the supra-mammary lymph nodes and mammary glands (Hamdy and Amin, 2002).

The RBT is a well-known, highly sensitive, commonly used *Brucella* sp. antigen test for initial screenings. These tests are inexpensive, rapid agglutination tests done on a glass plate lasting four minutes with the addition of an acidic-buffered antigen (Godfroid et al., 2010). Although rapid, a positive RBT result is subject to further confirmatory testing, usually through the use

of a CFT- a requirement for international trade according to OIE guidelines (Chisi et al., 2017). A CFT allows for detecting anti-*Brucella* antibodies, IgM and IgG, activating complement (Godfroid et al., 2010). Therefore, The RBT is used for its higher sensitivity and CFT for its higher specificity (Chisi et al., 2017).

This was demonstrated by Chisi *et al.* (2017) when a sensitivity of 95.8% and a specificity of 100% for the RBT and CFT were obtained, respectively, by screening 186 cattle from *Brucella* sp. – negative herds and 46 naturally infected animals within the KwaZulu – Natal province, SA.

Animals that react positively to both the RBT and CFT are handled according to the guidelines provided by the Animal Diseases Act 35 of 1984 to control and eradicate BB. These measures include quarantine, branding and subsequent slaughtering. If a herd tests negative in both the RBT and CFT, all animals will be declared brucellosis-free, and the State Veterinarian issues a certificate to dairy herds (Chisi et al., 2017).

Other screening tests, more specifically for dairy herds, include the MRT. The test consists of mixing fresh cow milk with coloured *Brucella* sp. whole-cell antigen. The presence of *Brucella* sp. antibodies will then induce an antibody-antigen complex that migrates to the cream layer, forming a purple ring on the surface. A negative result can be determined if no colour change is observed (Cadmus et al., 2008). However, an MRT cannot be used as a diagnostic method but rather a screening method to provide early warning of the disease.

1.3.4 *Brucella* sp. in human

Brucellosis occurring in humans is known as undulant fever, Malta fever, Bang's disease or Mediterranean fever (Galinska and Zagórski, 2013). The disease is mainly transmitted to humans through direct or indirect contact with infected animals and their products (Corbel, 2006), which include foetuses, placentae, other birthing products, vaginal discharge or by-products (i.e. soft cheeses, milk, meat) from infected animals (Khan and Zahoor, 2018). This explains why infection typically occurs through ingestion or mucous membranes, skin abrasions, and in rare cases, intact skin (Tadesse, 2016). Infection through direct contact with contaminated manure has also been reported but remains rare (Makala et al., 2020).

Additionally, other rare cases of *Brucella* sp. transmission includes person-to-person transmission, in utero transmission and tissue transplantations. Aerosolised bacteria continue to be a threat of infection through inhalation or conjunctival route (Khan and Zahoor, 2018). Urbanisation, expansion of animal industries, coupled with the lack of hygienic practices in food industries and animal husbandry, have all contributed to the persistence of brucellosis as a public health hazard (Corbel, 2006).

In humans, exposure to the pathogen are observed in occupations and activities associated with handling animals that are considered reservoirs of the disease; these include veterinarians and veterinary technicians, farmers- especially those working on multi-herd farms (both commercial and subsistence), abattoir workers or informal slaughterers, zookeepers and zoo technicians, hunters, and insemination service employees (Galinska and Zagórski, 2013; Pereira et al., 2020).

Brucellosis is annually responsible for approximately 500 000 cases worldwide, with most cases reported in regions where the disease has reached levels of endemicity (Lai et al., 2017). Limited studies on the prevalence of brucellosis in SA have been conducted. The last formally published study focusing on the incidence rate of *Brucella* sp. in the SA human population reported a rate of >0.2 per 100 000 population based on a survey from 1956 to 1959 (Schrire, 1962).

Brucellosis in humans is associated with acute or subacute febrile illness with intermitted or remitted fever accompanied by malaise, anorexia and depression. Prostration may also occur and can persist for weeks or months if no treatment is administered (Ulu Kilic et al., 2013). The acute phase of the disease may develop into a chronic phase with relapse or the development of persistent, localised infection. Severe forms of the disease include neurobrucellosis, endocarditis, peripheral arthritis and optic neuropathy (Corbel, 2006). Treatment often requires diverse therapeutic strategies with more than one antibiotic, as monotherapy often leads to relapse. The World Health Organisation (WHO) recommends a treatment course of six weeks with doxycycline and rifampicin, afterwards replaced by tetracycline and streptomycin (Glowacka et al., 2018).

1.3.5 Diagnoses of *Brucella* sp. in humans

Culturing of *Brucella* sp. is still considered the gold standard for the diagnosis of brucellosis. For culture, *Brucella* sp. is isolated from tissues or body fluids (blood, urine, cerebrospinal fluid, milk and others) and then cultured on selective media such as Farrell's medium or a modified Thayer Martin media (Ledwaba et al., 2020). Identification is based on colony morphology, conventional staining (Gram-negative), and metabolic capabilities (such as oxidase-positive, catalase-positive and urease-positive) (Al Dahouk and Nöckler, 2011). However, the diagnosis of brucellosis is strictly limited due to the bacterium's fastidious nature, resulting in a time-consuming, labour-intensive procedure. *Brucella* sp. are also highly pathogenic, and cultivation of the bacterium requires a specially equipped biosafety level 3 laboratory (Smirnova et al., 2013).

Therefore, brucellosis diagnostics, especially in low-income countries, rely on serological assays. The most common antigens used for serological testing are lipopolysaccharide (smooth-S-LPS) and internal cytosolic proteins (Ducrotoy et al., 2017). *Brucella* sp. LPS is considered a strong immunogen; however, the epitopes of the LPS are the foremost aetiology of cross-reactions with other Gram-negative bacteria, such as *Vibrio cholerae* and *Yersinia enterocolitica* (Ko et al., 2012). Therefore, clinical symptoms, patient history and epidemiological information should always be taken under consideration when evaluating test results.

In endemic brucellosis areas, such as sub-Saharan Africa, detecting antibodies at low levels in healthy individuals is possible. Therefore, considerable attention is required to determine seropositivity cut-off values. For example, in patients showcasing relapses or chronicity, the cut-off values must be adjusted accordingly, whereas, in endemic regions, the limit should be higher than in non-endemic regions (Purwar et al., 2016).

Agglutination tests, such as the standard agglutination test (SAT) and Rose Bengal plate agglutination test (RBPT), have been used for more than a century to diagnose human cases of brucellosis worldwide. However, these tests are labour-intensive and incapable of differentiating between active or chronic cases of brucellosis (Mantur et al., 2010). For this reason, ELISAs are the preferred diagnostic assay, especially in endemic regions, as IgM and

IgG antibodies to *B. abortus* surface antigen can be individually identified and monitored to determine disease stage progression (Ducrotoy et al., 2018).

In a study conducted by Mantur *et al.* (2010), the IgM and IgG ELISA diagnostic accuracy compared to the SAT was evaluated in 92 patients with suspected brucellosis in an endemic region in India. Out of the 91 patients, 31 were confirmed culture-positive, and analysis showed the combined IgM, and IgG ELISA accurately diagnosed all 31 patients compared to the SAT, who accurately diagnosed 12/31 (38.8%) of the culture-positive patients. In this study, the combined IgM and IgG ELISA sensitivity and specificity were calculated at 100% and 71.31%, respectively. Furthermore, the authors found that the ELISA was 28% and 55% more sensitive in the acute and chronic phase of the disease, respectively, compared to the conventional methods (Mantur et al., 2010).

1.4 *Leptospira* sp.

1.4.1 Characteristics and taxonomy

Leptospirosis, caused by the *Leptospira* sp. are Gram-negative bacteria, highly motile, obligate aerobic spirochaetes that belong to the family *Leptospiracae* (Tilahun and Simenew, 2013). The *Leptospira* genus consists of 22 serogroups subdivided into more than 300 serovars (Boey et al., 2019). A newer classification system using comparative metagenomics analysis have divided *Leptospira* sp., previously classified as 'pathogenic' and 'intermediate', into four subclades named P1 and P2 (pathogens) and S1 and S2 (saprophytes) (Kallel et al., 2020). Within the P1 subclade, further analysis divided species into four groups: Group I (*L. interrogans*, *L. noguchii*, and *L. kirschneri*), Group II (L*. borgpetersenii*, *L. weilii*, *L. alexanderi*, *L. mayottensis*, and *L. santarosai*), Group III (*L. astonii*) and Group IV (*L. kmetyi*) (Bierque et al., 2020).

To date, only *Leptospira* sp. belonging to Groups I and II within subclade P1 have been successfully isolated from mammals or humans and considered virulent (Bierque et al., 2020). *L. interrogans* (Group I) have been the most documented and described pathogenic species and often used as a model species to describe the epidemiology of the disease. Serovars within *L. interrogans* are maintained in several animal hosts, which include: *L. interrogans* serovar Hardio in cattle, *L. interrogans* serovar Canicola in dogs, *L.*

interrogans serovar Copenhageni in rats and *L. interrogans* serovar Pomona in pigs (Monahan et al., 2009).

The bacteria are widespread in nature and maintained within the renal system of wild and domesticated animals that serve as reservoir hosts (Haake and Levett, 2015). Small mammals from the family Muridae, especially the brown rat (*Rattus norvegicus*), are considered more important reservoirs, with larger herbivores serving as additional sources of infection.

1.4.2 *Leptospira* sp. in humans

Leptospirosis, also known as Weil's disease, is a widespread and potentially fatal zoonotic bacterial disease (Haake and Levett, 2015). Globally, between 300 000 and 500 000 cases of leptospirosis are reported, with case fatality rates of up to 30% (Tilahun and Simenew, 2013). In countries where high levels of endemicity are maintained, infections are usually mild or asymptomatic (Haake and Levett, 2015). However, studies have reported more severe forms of the disease in HIV positive people within endemic countries (Biggs et al., 2013), an increasing concern due to the large burden of HIV in Sub-Saharan countries. Studies have estimated that 20-40% of all febrile illnesses of unknown origin are caused by leptospirosis (Abela-Ridder et al., 2010). Furthermore, the severe outcome of the disease is also largely dependent on host susceptibility, epidemiological conditions, and pathogen virulence (Haake and Levett, 2015).

People can become infected with *Leptospira* sp. through various portals. These can include cuts or abrasions and mucosal surfaces (i.e. oral, conjunctival and genital surfaces). Exposure can occur due to direct contact with an infected animal or through indirect contact with soil or water contaminated with the excrement of infected animals (Budihal and Perwez, 2014). For this reason, veterinarians, hunters, abattoir workers, farm workers (especially dairy) and scientists are at a higher risk of infection through mainly direct exposure.

Like many other endemic zoonoses, leptospirosis typically exhibits non-specific febrile illness during the early stages of infection, where difficulties arise in distinguishing the disease from other aetiologies (Haake and Levett, 2015). The clinical course of the disease can range from mild to lethal with a broad clinical spectrum, often causing acute episodes that are not

disease-specific. Therefore, the disease is often misdiagnosed as malaria (Crump et al., 2013) or dengue fever (Conroy et al., 2014) in areas where the latter are both endemic. Due to the wide geographical distribution and overall abundance of *Leptospira* sp.

A clinical case of leptospirosis often presents with abrupt onset of fever, followed by headaches, myalgia and rigours (75-100% of patients). The second phase of the disease, also known as the "immune" phase, is characterised by renewed fever, where more serious clinical manifestations might develop (Day, 2019). Jaundice and renal failure (classical signs of Weil's disease) may develop, as well as pulmonary haemorrhage, with case fatalities of 70% being reported (Bharti et al., 2003). Other manifestations might include acute respiratory distress syndrome (ARDS), optic neuritis, rhabdomyolysis and myocarditis (Day, 2019).

1.4.3 Diagnoses of *Leptospira* sp. in humans

The serological reference test for diagnosing leptospirosis in humans is the microscopic agglutination test (MAT) (Niloofa et al., 2015). For this test, live antigens from different *Leptospira* sp. serogroups are reacted with serum samples. Darkfield microscopy is used to examine agglutination. Live leptospirosis from different serogroups must therefore be maintained in the laboratory. However, as a minimum, the laboratory should include all *Leptospira* sp. serovars circulating locally (Musso and La Scola, 2013). MAT can be positive from day 10-12 after symptom onset, with reports depicting a sensitivity of 41% during the first week, 82% during the second to the fourth week, and 96% beyond the fourth week of illness (Musso and La Scola, 2013).

The MAT cut-off value on a single sera specimen depends mainly on the circulating serovar's prevalence. According to the CDC, a probable leptospirosis case is defined as having a titre >200 with an association to compatible illness. However, in highly endemic countries, a single titre of 800 is generally indicative of infection, but a 1600 titre has been recommended (CDC, 1997). A fourfold rise in paired sera titres is required to confirm leptospirosis, as MAT can detect both IgM and IgG antibodies. However, MAT requires high levels of expertise and the maintenance of a large panel of *Leptospira* sp. cultures (Niloofa et al., 2015).

Furthermore, live *Leptospira* sp. also creates a health risk as it requires biosafety level (BSL) 3 containment (Musso and La Scola, 2013). MAT also gives false-positive results during the early stages of infection, as IgM antibodies are only detectable after day eight of illness (Agampodi et al., 2010). Therefore, much attention is given to the use of IgM ELISA in diagnosing acute phases of leptospirosis (Niloofa et al., 2015).

Anti-leptospires IgM can be detected, with an ELISA, at 4 to 5 days post-onset of symptoms, and can persist for five months, with some studies reporting the presence of *Leptospira* IgM antibodies 11 years after initial infection (Cumberland et al., 2001). Moreover, ELISAs require minimal training with an approximate turnaround time of 2-4 hours and are therefore recurrently used in the diagnosis (Rosa et al., 2017).

1.5 Hantaviruses

1.5.1 Characteristics and taxonomy

Hantaviruses are enveloped RNA viruses belonging to the Hantaviridae family. These viruses are negative-sense with a tri-segmented genome (Khaiboullina et al., 2005). The segments consist of a large (L), medium (M) and small (S) segment that code for RNA-dependant-RNA polymerase, as well as a glycoprotein precursor (GPC), processed into the two glycoproteins (Gn and Gc) and the nucleocapsid (N) (Khaiboullina et al., 2005). These viruses are globally emerging pathogens, with novel pathogenic serotypes being frequently reported (Kruger et al., 2015).

Currently, around 40 hantaviruses are known, of which 22 are pathogenic and hosted by small mammals (Munrir et al., 2021). These viruses are also categorised based on their geographical distribution and the respective syndromes they cause. Old World hantaviruses are found in Europe and Asia and can cause haemorrhagic fever with renal syndrome (HFRS) in humans. Hantaan virus (HTNV), Puumala virus (PUUV) and Dobrava–Belgrade virus (DOBV) are some of the more common hantaviruses of the Old World (Munrir et al., 2021). However, the Seoul virus (SEOV) - an Old-World hantavirus, has a more widespread distribution due to the widespread distribution of its rodent reservoir- the Norway rat (*Rattus norvegicus*) (Kim et al., 2018).

New world hantaviruses have a geographical distribution in North and South America and includes multiple viruses. The Sin Nombre virus (SNV) was the first hantavirus identified in the United States in 1993 (Eliott et al., 1994). These viruses are responsible for the more severe hantavirus pulmonary syndrome (HPS), with mortality rates of >40% (Jonsson et al., 2008; Munrir et al., 2021).

Each hantavirus species is hosted by a unique small mammal, where its geographical distribution reflects that of its host. For example, the striped field mouse (*Apodemus agrarius*) is geographically distributed throughout East Asia. It serves as the host for the Old-World hantavirus- HTNV, responsible for causing Korean hemorrhagic fever in humans who share the same geographical region (Tian et al., 2017).

All pathogenic hantaviruses are hosted by rodents, whereas the pathogenicity of viruses hosted in shrews, moles and bats remains not fully understood (Vaheri et al., 2013). Host animals get chronically infected with the virus (Kallio et al., 2007), and despite the presence of neutralising antibodies, there is active replication of the virus (Schönrich et al., 2008). Studies have shown that after infection, the animal host does not show signs of illness and can shed the virus throughout their lifespan (Avšič-Županc et al., 2019).

1.5.2 Hantaviruses in Africa

Early serological studies performed in Africa on rodents and humans suggest hantavirus presence in several African countries. However, no indigenous African hantavirus species were identified or isolated until 2006 (Klempa et al., 2006) due to difficulties in hantavirus isolation from culture and the usage of primers specific for known European and Asian hantaviruses (Witkowski et al., 2014). However, the development of a broadly reactive reverse transcriptase PCR (RT-PCR) targeting a highly conserved L segment made it possible to identify African hantaviruses molecularly (Klempa et al., 2006). The method was called the Pan-Hanta- PCR and was utilised for the first hantavirus surveillance study in small mammals in Guinea, West Africa. This led to identifying the first two African hantaviruses (Klempa et al., 2006; Klempa et al., 2007).

The first virus, named the Sangassou virus (SANGV), was identified in the African wood mouse (*Hylomyscus simus*) (Klempa et al., 2006). Phylogenetic analysis indicated that SANGV belongs

to the Murinae-associated hantaviruses. The second African hantavirus, named Tanganya virus (TGNV), was identified in a Therese's shrew (*Crocidura theresae*) in Guinea (Klempa et al., 2007). The only other shrew-borne hantavirus species identified was the Thottapalayam virus, found in India (Carey et al., 1971).

Since then, several shrew-borne hantaviruses were discovered in West Africa. Initial findings suggested that hantaviruses were only endemic in western Africa, with the Guinea forest established as a biodiversity hotspot (Witkowski et al., 2014). However, the discovery of the first hantavirus, named Tigray virus (TIGV), in Ethiopia (sub-Saharan Africa) disproved the endemicity assumption. The TIGV was identified (Meheretu et al., 2012) from an Ethiopian white-footed mouse (*Stenocephalemys albipes*) and represented the second African Murinae hantavirus. Further investigations on Hantaviruses in Africa led to the discovery of the first bat-borne hantavirus (Weiss et al., 2012). This discovery changed the view on hantaviruses as being predominantly rodent-borne viruses. The Magboi virus (MGBV) was discovered in a slit-faced bat (*Nycteris hispida*) in Sierra Leone and paved the way for future discoveries.

However, all hantavirus discoveries in Africa, except for SANGV, were based on molecular methods, more specifically broadly reactive RT-PCRs. The SANGV is, to date, the only African hantavirus that has successfully been isolated from cell culture and available for future studies, including pathogenicity (Witkowski et al., 2014).

Though hantaviruses have been identified in sub-Saharan East and West Africa, in SA, no information exists on these viruses' occurrence and health impacts (Witkowski et al., 2014). Since 2010, research on hantavirus identification and detection has been conducted in SA. To date, in an ongoing study conducted by Witkowski et al. (2014), more than 2000 lung samples from various small mammals have been obtained and screened for hantavirus infections. Capture sites were distributed throughout several biomes, with most samples originating from the Western Cape (fynbos biome). Both rodent and insectivore (shrews) samples screened negative for hantaviruses using the Pan-Hanta-PCR.

However, the moderately low seroprevalence rate from patients in SA does not exclude the possibility of hantavirus disease occurring in the county. For example, seroprevalence studies

on hantaviruses in Germany showed a similar low seroprevalence of 1%, but registered roughly 300 patients, per population of 80 million, in the year 2012 (Krüger et al., 2013).

1.5.3 Hantaviruses in humans

Infections caused by hantaviruses can manifest as a severe clinical disease that often relates to respiratory disorders, kidney failure, muscular disorders and cardiac disease (Munrir et al., 2021). As mentioned previously, HFRS and HPS are the two most serious disorders caused by the old world and new world Hantaviruses, respectively. Both disorders are recognised initially with fever, respiratory distress, lethargy, nausea and vomiting.

It is estimated that hantaviruses were the cause of 300 000 to 400 000 human infections in Europe and Asia between 2000 and 2009 (Guterres and Lemos, 2018), and more than 4000 cases of HPS have been reported in South America up until 2013 (Figueiredo et al., 2014). These numbers are considered significantly high when case fatality rates of HFRS have been reported at 5-15% and up to 50% for HPS (Krautkrämer and Zeier, 2014). The most common mode of virus transmission is the inhalation of aerosolised virus when the virus is shed into the environment through excreta and saliva from infected rodents (Forbes et al., 2018). However, person-to-person transmission of the Andes virus has also been documented (Martinez-Valdebenito et al., 2014).

Hantavirus infections in SA have not been reported, and limited information exists regarding the prevalence of these viruses in the country. In another study conducted by Witkowski *et al*. (2014), 1442 patient samples were screened for evidence of hantavirus infection in the SA Cape region from August 2010-April 2012. Patient sample screening was serology based, with the first screening showing an ELISA reactive percentage of 14.5% (210 out of 1442 samples): 111 samples against Puumala virus-recombinant nucleocapsid protein (PUUV-rN), 152 samples against Dobrava-Belgrade virus-specific recombinant nucleocapsid protein (DOBV-rN), and 53 samples against both antigens. All samples were then subjected to confirmatory testing using a Western Blot (WB) and immunofluorescence assay (IFA), and a seroprevalence of 1% (14 out of 1442) was determined (Witkowski et al., 2014).

Furthermore, no hantavirus diagnostics are routinely carried out in laboratory settings that could contribute to the underreporting of these viruses. The possibility of non-pathogenic hantaviruses circulating in SA does exist. However, more investigations are required.

1.5.4 Diagnoses of hantaviruses in humans

Hantavirus diagnostics rely on clinical findings and serological methods, as clinical signs alone are not enough to diagnose an infection. During the onset of symptoms, patients have detectable levels of anti-hantavirus IgM and IgG (Bi et al., 2008). IFA was the first diagnostic method to diagnose hantavirus infection; the test was performed by fixing infected hantavirus cells on glass slides as antigens. However, IFA assays are limited due to the low yield of viruses in cell lines; furthermore, the use of virus-infected cells can only be done in BSL 3 or -4 laboratories (Chandy and Mathai, 2017).

Today, serological assays make use of hantavirus recombinant antigens. IgM and IgG indirect-ELISA (iELISA) and IgG capture ELISA are the most commonly used methods for laboratory diagnosis. High levels of anti-N IgM are usually detected during the onset of symptoms (Manigold and Vial, 2014). The advantages of using an ELISA for diagnostics is the ability to track disease phase through IgM and IgG detection, also valuable for surveillance studies through IgG detection. ELISAs using the more conserved hantavirus N protein antigen is also able to detect multiple hantaviruses due to cross-reactivity (Kruger et al., 2015). Limitations include not being able to detect the hantavirus serotype causing disease. However, the only serological method able to differentiate serotypes is the neutralisation assay, which is not routinely used due to the duration (one to two weeks), specialised techniques and virus containment requirements needed to conduct the assay (Yoshimatsu and Arikawa, 2014).

Antibody response against Gn and Gc proteins are not targeted for diagnostic purposes as they arise significantly later and are not typically conserved across different hantaviruses (Chandy and Mathai, 2017).

1.6 Problem statement and justification

In SA, bTB and BB infection in cattle are controlled diseases associated with extensive morbidity that consequently lead to livestock production losses. Furthermore, human disease

caused by these bacteria is a notifiable condition due to their associated mortality and morbidity, and therefore, a significant public health concern with substantial economic impact.

Commercial farms that produce dairy and beef for a larger market are subject to more strenuous, routine screening for bTB and BB in cattle herds than communal farm workers who practice subsistence farming. This frequently leads to underestimating of the true burden of these diseases, not only amongst animals but also humans who are more dependent on their animals for their livelihood.

For this reason, investigations should aim to include human and animal health in both commercial and communal farms to determine a more accurate incidence rate. If the herd status is known, risk factor information can then be obtained to determine interspecies transmission.

However, these investigations are not frequently performed in SA, and limited studies exist on the incidence rate and transmission of *M. bovis* and *Brucella* sp. in SA, especially in the Ngwathe and Moqhaka municipal regions, Free State province.

Moreover, studies on occupational exposure to zoonotic diseases in the Free State province are neglected. These populations are at an increased risk of contracting a zoonotic infection due to their direct or indirect contact with animals. Diseases such as brucellosis and leptospirosis are known to circulate and continue to burden the healthcare system due to their highly infectious nature. Research on hantaviruses in SA is a relatively new field with limited information regarding seroprevalence, transmission, pathogenicity and geographical distribution.

Identifying occupations at higher risk of contracting a certain zoonotic infection is therefore important in future control strategies and creating awareness amongst health care workers of populations at an increased risk of infection.

1.7 Aim and Objectives

1.7.1 Aim

This study aimed to investigate the incidence rate of *Mycobacterium bovis* and *Brucella* sp. in cattle and farm workers in two different farming communities (communal and commercial), and their associated risk factors. This study aimed to document occupational exposure to *Brucella* sp., *Leptospira* sp. and hantaviruses across the Free State province, SA.

1.7.2 Objectives

1. To screen sputum samples collected from communal and commercial farm workers for *M. bovis* infection through culture.
2. To screen blood samples collected from communal and commercial farm workers for the detection of anti-*Brucella* sp. IgG antibodies through the use of an ELISA.
3. To screen cattle for bTB using TST, IFN-γ release assay and milk culture, and BB using a RBT and CFT.
4. To acquire demographic- and risk factor information from human participants through the use of a questionnaire.
5. To determine occupational exposure to zoonotic diseases by detecting anti-*Leptospira* sp. IgM, anti-*Brucella* sp. IgG and anti-hantavirus IgG antibodies using an ELISA.
6. To perform statistical analysis to determine the probability value between demographical data, exposure and infection.

Chapter 2: Incidence and risk of transmission of zoonotic tuberculosis and brucellosis in communal and commercial farms.

2.1 Introduction

Tuberculosis and brucellosis are primarily considered to be two of the most widespread and important bacterial zoonoses worldwide (Cantas and Suer, 2014). These diseases are associated with large-scale livestock production losses, especially in cattle, which contribute to the economic importance of these diseases (Marcotty et al., 2009). Especially in developing countries where subsistence farming communities are greatly dependant on their livestock for their livelihood. Infected animals are also able to transmit the disease to humans (Cowie et al., 2014), creating a public health concern due to the severe clinical manifestations of these diseases that may develop.

According to the WHO Global Tuberculosis report published in 2020, a total of 360 000 people fell ill with TB in South Africa in 2019 (WHO, 2020). However, the proportion of cases caused by zoonotic TB are unknown due to routine diagnostics not differentiating between the different aetiological agents. Furthermore, in South Africa, limited information exists on the incidence rate of brucellosis in the human population. The last formally published study estimates the incidence rate at >0.2 per 100 000 population between 1956 and 1959 (Schrire, 1962). However, in other sub-Saharan countries, seroprevalence rates as high as 10% have been reported in smaller communities (Nabukenya et al., 2013).

M. bovis is the causative agent of bTB in cattle and belongs to the MTBC. Other members of the MTBC have also been implicated in causing disease in livestock and wildlife, such as *M. tuberculosis*, however it is reported less frequently. *M. bovis* can be transmitted from cattle to humans through the consumption of contaminated, unpasteurised dairy products and raw meat. Direct contact with a wound of an infected animal is also an established transmission route, mainly through hunting and slaughtering (Brooks-Pollock et al., 2014). In some cases, *M. bovis* can also be transmitted through direct contact with aerosolised droplets released when an animal is sneezing or coughing. However, these cases are considered rare (Pollock et al., 2006). People infected with *M. bovis* typically exhibit symptoms associated with 'normal' TB disease, caused by *M. tuberculosis*. However, clinical representation is

associated with the locality of the disease in the infected patient's body. Due to the most common route of infection, *M. bovis* are proportionately more likely to cause extra-pulmonary TB in humans (Cruz and Starke, 2007).

B. abortus is the aetiological agent for BB in cattle, however *B. melitensis* and *B. suis* have also been implicated (Díaz, 2013). In humans, the predominant pathogen responsible for causing disease is *B. abortus*, with *B. melitensis* being reported less frequently (Frean et al., 2018). *B. abortus* infection in cattle are associated with abortion and morbidity (Rich and Perry, 2012). The bacterium is transmitted to humans through the consumption of contaminated, unpasteurised dairy as well as raw meat (Frean et al., 2018). Direct contact with infected animals can also act as a transmission route, especially when direct contact is made with aborted –or birthing material when assistance in parturition is required. In humans, the disease is highly variable in its clinical manifestation ranging from fever, weakness, fatigue to more long-term severe signs that can include endocarditis, arthritis, and recurrent fevers (Celli, 2019).

Bovine TB and BB are controlled diseases in South African livestock where control measures are in place but not without challenges, more notably in smaller farming communities where subsistence farming is practised (Arnot and Michel, 2020). Both diseases are controlled following a 'test and slaughter' approach, and although effective, is not entirely feasible in smaller communal settings. In these communities, non-compliance remains an obstacle when animals are to be tested and with limited to no government compensation for the slaughter of reactive animals, communities are hesitant towards annual testing (Amanfu, 2006; Arnot and Michel, 2020). Two BB vaccines for cattle have also been developed, and although cattle vaccination in South Arica is legislated as compulsory, the responsibility is given to livestock owners who often do not comply (Tempia et al., 2019).

In the Moqhaka and Ngwathe municipal regions, limited data is available regarding the prevalence of bTB and BB in cattle and farm workers. Determining the prevalence and associated risk factors would contribute to identifying possible problem areas and aid in the control of bTB and BB in these locations.

2.2 Materials and methods

2.2.1 Ethical considerations

Ethical approval was obtained from the Health Sciences Research Ethics Committee (UFS-HSD2019/1075/270801), Animal Research Ethics (UFS-AED2019/0111) and Environmental & Biosafety Research Ethics Committee (UFS-ESD2019/0086). Furthermore, permission was obtained from DALRRD, before any animal testing was conducted (Appendix D-H).

2.2.2 Study area

This study was conducted on two separate farming populations, a rural cattle farming community and four commercial farms (two beef and two dairy farms). The rural cattle farming community is located in Maokeng, Kroonstad rural, Free State province, SA, and the commercial farms are located within the Moqhaka and Ngwathe municipal regions in the Free State province (Figure 2). All screening locations are under the responsibility of the Kroonstad State Veterinary Services.

Figure 2: Study sites in the different municipal regions within the Fezile Dabi district, Free State province, South Africa. Available at https://municipalities.co.za/map/107

2.2.3 Study design

A prospective, cross-sectional study was conducted on two different populations, which included livestock farm workers and cattle. All farms selected for this study had cattle scheduled for routine bTB and/or BB screening as part of an ongoing surveillance program. Subsequently, farm workers on the chosen sites were also screened for MTBC and *Brucella* IgG antibodies and asked to complete a questionnaire.

2.2.4 Study population

2.2.4.1 Livestock

Two commercial dairy farms, designated as farm A and farm B and two commercial beef farms, designated as farm C and D were identified. A total of 33, 117, 439 and 1157 cattle were screened for BB from farms A, B, C and D, respectively. For bTB, 33, 126 and 91 cattle were screened from farms A, B and C, respectively. Farm D was only scheduled for BB screening and not bTB. In the Maokeng community kraal, a total of 71 cattle were screened for bTB and 69 were screened for BB.

The screening was conducted on all animals present on the farm above the age of six months for bTB and 18 months for BB. However, on farm C only a single herd (n=91) were screened for bTB as the herd consisted mainly of new introductions and heifers of which the bTB status was unknown. The remaining animals (n=348) were declared bTB negative based on previous screenings performed by the Kroonstad State Veterinary Services.

2.2.4.2 Farm workers

All farm workers in the Maokeng community kraal (n=13), commercial dairy farm B (n=7) and commercial beef farm C (n=6), above the age of 18, were approached, and agreed to participate in the study. Unfortunately, specimen collection could not be achieved for farm A and farm D, due to COVID-19 travel restrictions. A convenience sampling method was used and all workers present on each location agreed to participate.

2.2.5 Informed consent

All study participants were given an information document available in English, Afrikaans and Sesotho, which included the study outline in layman's terms and personal contact information (Appendix A-C). The contents of the information document were also verbally explained to each participant in their preferred language. After being informed, participants were asked to sign a consent form. Specimen collection was only performed after obtaining informed consent.

2.2.6 Specimen collection and screening

2.2.6.1 Livestock

Single intradermal skin test (SIST)

All cattle herds were initially screened for bTB using a SIST to determine current status. The protocol was carried out according to the Bovine Tuberculosis Manual, as approved by the Department of Agriculture, Land Reform and Rural Development (DALRRD), formerly known as the Department of Agriculture Forestry and Fisheries (DAFF) in September 2016. Briefly, all animals were herded into a kraal, where female animals were separated from their calves to limit injury. Small groups of animals were herded into a crush with a neck clamp to ensure convenient and safe testing. An injection site, halfway between the juncture of the neck and head and the fold in front of the shoulder and halfway between the bottom and top of the neck was selected and inspected for any existing lumps or skin damage before being shaved.

An area of approximately 50 mm x 50 mm was shaven with a double-sided razor and disinfected using a 70% ethyl alcohol solution. The skin thickness of the animal was measured, pre-injection, using a Hauptner pistol grip. The animal was then injected intradermally with 0.1 mL of 5 000 International Units (IU) of bovine tuberculin PPD (OBP, Pretoria) using a McClintock syringe. Testing was carried out by the Kroonstad State Veterinary Services.

After 72 hours, all animals were brought back for the reading of results. Each reaction site was observed for evidence of swelling or a colour change and examined for reaction consistency (hard or soft swelling), presence of oedema as well as heat. All reaction characteristics were noted before any measurements were conducted. A calliper was placed

over the thickest part of the reaction and measured in tenths of millimetres. The difference in skin thickness, pre-and post-injection, was determined for each animal by subtracting the measurement obtained after 72 hours from the initial skin thickness measurement, as seen below (Equation 1).

Equation 1:

Pre-injection, normal skin measurement (mm) – 72h post-injection skin measurement (mm) = increase in skin thickness (mm)

Herds were regarded as negative when no animal had an increase in skin thickness of <6 mm, including non – specific reactions. Suspect herds were defined as having a single animal with an increase in skin thickness of >6mm, combined with evidence of positive skin reactions. Herds with animals showing large typical inflammatory reactions with an increase of skin thickness of ≥20 mm were regarded as positive.

All suspect and positive herds were re-tested after three months using a CIST.

Comparative intradermal skin test (CIST)

The CIST follows the same protocol as the SIST, however, instead of solely injecting 0.1 mL of 5000 UI bovine tuberculin PPD, 0.1 mL of 2500 UI avian tuberculin PPD (OBP, Pretoria) is also injected. Both the bovine and avian tuberculin PPD are injected intradermally on the same side of the animal's neck, with the bovine tuberculin PPD being injected closer to the shoulder and avian tuberculin closer to the head. The distance of the two site injections was approximately 15 cm apart. After 72 hours, an increase in skin thickness was measured as follows:

The bovine reaction increase was determined by subtracting the 72h post-injection skin measurement (bovine PPD) from the pre-injection normal skin measurement. Using the same formula, the increase in skin thickness for the avian reaction was calculated (Equation 2a and 2b)

Equation 2a:

Bovine reaction:

Pre-injection, normal skin measurement (mm) – 72h post-injection (bovine PPD) skin measurement (mm) = ***increase in skin thickness, bovine reaction (mm)***

Equation 2b:

Avian reaction:

Pre-injection, normal skin measurement (mm) – 72h post-injection (avian PPD) skin measurement (mm) ***= increase in skin thickness, avian reaction (mm)***

Increase in skin thickness, bovine reaction (mm) *–* ***increase in skin thickness, avian reaction (mm) = difference between bovine and avian reaction increase***

A positive difference of <2 mm was regarded as a negative reactor, 3 – 4 mm suspect, and an increase of >5 mm was regarded as a positive reactor.

All suspect and positive reactors were subjected to further testing using a gamma-interferon test and milk bTB culture.

Interferon-gamma assay (Bovigam™)

The IFN-ɣ release assay was performed on all CIST-suspect and positive animals as per the standard operating procedure. Briefly, two whole blood samples in 6 mL heparin tubes were collected from the tail vein of each animal by the Kroonstad State Veterinary Services. Tubes were inverted several times to ensure thorough mixing. All blood samples were kept at an ambient room temperature and transported to the Tuberculosis Laboratory at Onderstepoort Veterinary Institute (OVI) within 6 hours after collection.

In a class 2 biosafety cabinet, 1.5 mL of heparinised blood from each animal was dispensed into five different 2 mL sterile plastic tubes and stimulated with bovine PPD, avian PPD, fortuitum PPD (Tuberculosis laboratory at OVR, Pretoria) and pokeweed mitogen (Thermo Fisher Scientific, Waltham, USA) positive control. All tubes were inverted several times to ensure thorough mixing and incubated at 37°C for 24 hours.

After incubation, samples were centrifuged for 5 minutes at maximum speed to ensure sufficient plasma separation. All plasma was harvested and transferred into sterile tubes. Samples were kept at 4°C for a maximum of three days before being used for the Bovigam™ (Thermo Fisher Scientific, USA) assay.

The release of interferon-gamma from stimulated blood was detected by using a Bovigam™ test kit as per the manufacturer's instructions with minor modifications as described by Michel et al (2011). Steps requiring plate washing was done using a 96-well plate washer (BioTek ELx50, USA), and the optical densities (OD) of the stimulated blood were measured at 450 nm using plate reader (BioTek Elx800, USA). Whole blood stimulated with pokeweed mitogen was used as a positive control and unstimulated blood was used as a negative control. The OD values for the plasma stimulated with bovine PPD, avian PPD, fortuitum PPD and pokeweed mitogen were recorded as OD_{bov}, OD_{av}, OD_{fort} and OD_{pwh}, respectively. Unstimulated blood was recorded as OD_{neg}. Animals were regarded as bTB positive when ($OD_{bov} - OD_{av} > 2$ and $OD_{fort} - OD_{neg} \leq 0.15$). Animals were classified as avian reactors when $OD_{av} > (OD_{bov} + 0.1 \times OD_{bov})$. Animals depicting an immune response to bovine PPD and fortuitum PPD were classified as multiple reactors if ($OD_{bov} - OD_{av} < 0.2$ and $OD_{fort} - OD_{neg} > 0.15$). Animals depicting an equal immune response to both bovine PPD and avian PPD were classified as equal reactors $(OD_{bov} + 0.1 \times OD_{bov}) > OD_{av} > (OD_{bov} - 0.1 \times OD_{bov})$. To validate the results, the OD value of the blood stimulated with pokeweed mitogen (OD_{pwh}) should be > 0.5.

It should be noted that all samples stimulated with bovine PPD were initially screened to determine any positive reactors. Any sample with an optical density of ≥ 0.38 was regarded positive as previously described by Michel (2008). All positive reactors were subject for re-testing with the inclusion of avian PPD, fortuitum PPD and controls.

Milk culture

Milk was collected from all suspected and positive female animals, based on the results of the CIST. A single 50 mL milk sample was collected directly from the udder in a screw cap container. Samples were obtained aseptically in the early morning during routine milking. All obtained samples were transported to the Tuberculosis Laboratory at Onderstepoort

Veterinary Institute for culture. Methods were carried out according to the Tuberculosis laboratory's standard operating procedure.

Briefly, individual milk samples were transferred to a 250 mL bottle and decontaminated using 1% cetylpyridinium chloride (CPC) to achieve an equal volume of 100 mL. Samples were mixed thoroughly and incubated at 20°C ± 2°C for one week in the dark. Afterwards, each sample was transferred, in equal amounts (50 mL), to two 50 mL centrifuge tubes and centrifuged at 3500 x g for 30 minutes. The supernatant was discarded, and sterile distilled water was added to achieve a final volume of 25 mL. Samples were mixed well and centrifuged at 3500 x g for 10 minutes. The supernatant was poured off until approximately 3 mL was left in the tube. Caution was taken not to discard the cream. The pellet was mixed well and inoculated onto 4X Lowenstein Jensen (LJ) - pyruvate and 2X LJ- glycerol media using an inoculation loop. Samples were incubated at 37°C ± 2°C for 10 weeks.

2.2.5.2 Sputum collection from farm workers

Participants were instructed by a qualified nurse on how to produce an adequate sputum phlegm specimen. This was achieved by instructing participants to take a deep breath and then slowly breathe out, to take another deep breath and then cough until sputum accumulates in the mouth. All specimens were collected into individually marked 40 ml screw-cap containers and stored at 2 - 8°C for a maximum of five days before analysis. Subsequently, two blood samples were also collected from each participant. Blood was collected in BD Vacutainer® (Serum Separator Tubes) SST™ II Advance 5.0 ml collection tubes. Samples were inverted five to six times before allowing to clot for <30 minutes at an ambient room temperature. All blood specimens were centrifuged at 1300 x g for 15 minutes (20°C to 25°C) in a fixed angle unit, within five hours after collection.

Serum was harvested and transferred to 2 ml cryovials (Thermo Fisher Scientific) and stored at -80°C until further downstream analysis.

2.2.7 TB Diagnostics

2.2.7.1 Sputum decontamination

A total of 26 sputum samples were collected. All samples were decontaminated using a BD BBL™ MycoPrep™ kit. Firstly, a 0.1 ml phosphate buffer with a pH of 6.8 was prepared and autoclaved at 121°C for 15 minutes. Afterwards, an N-acetyl-l-cysteine–sodium hydroxide (NALC – NaOH) solution was prepared according to the kit specifications.

In a class 2 safety cabinet, equal amounts (approximately 10 mL) of specimen and NALC – NaOH solution were added into a sterile 50 mL centrifuge screw cap container. The mixture was then vortexed until the specimen was in a complete liquid state and allowed to sit at room temperature for 15 minutes, with occasional shaking in 5 - minute intervals. Afterwards, phosphate buffer was added to the mixture up to the 50 mL mark, mixed through vortexing, and then centrifuged at 3000 x g for 15 minutes. All supernatant fluid was then decanted, and the pellet was re-suspended in a small amount of phosphate buffer. The pellet was used immediately for culture.

2.2.7.2 Sputum culture

All TB culturing was carried out at the Tuberculosis Laboratory, Department of Medical Microbiology, UFS. A BD BACTEC™ MGIT™ 960 Supplement Kit was used according to manufacturer's instructions, containing both a growth supplement and antibiotic mixture, required for selective growth. Aseptically, 0.8 mL of the antibiotic – growth supplement mixture was added into a 7 mL MGIT tube labelled with the corresponding specimen number. Lastly, 0.5 mL of the specimen pellet in a small amount of phosphate buffer was added into the tube.

All samples were cultured within a BD BACTEC™ MGIT™ 960 Mycobacteria Culture System at ± 37°C for up to 42 days. The incubation process was monitored every other day. If growth was detected, the system flagged positive, and the sample was removed and stored at 4°C. After 42 days, the system flagged negative and all samples were discarded appropriately.

2.2.7.3 Modified Ziehl Neelsen staining

A modified Ziehl Neelsen stain was performed on all MIGIT cultures with any evidence of growth (flagged positive). Briefly, a smear on 1 mL fixative from each positive culture was prepared in a biological level 2 safety cabinet. Smears were air-dried and heat fixated. Prepared slides were flooded with Kinyoun carbol fuchsin for 5 minutes and gently rinsed with water. Decolourisation was performed by flooding the slides with a 3% acid alcohol solution (3% HCl in ethanol) for 2 minutes and then rinsed with water. Lastly, methylene blue was used as a counterstain by flooding the slides for 2 minutes and then rinsing off with water (Ziehl, 1883; Neelsen, 1883).

Smears were air-dried and viewed under a microscope using the 100X oil immersion objective. Positive cultures were identified as a slide with acid-fast bacilli (red) clumped together to form serpentines. Dispersed acid-fast bacilli (red) were identified as MOTTs, and all negative acid-fast (blue) stains were identified as other bacteria.

2.2.7.4 Line probe assay (LPA)

<u>DNA extraction</u>

DNA extraction was performed on all culture-positive samples using a GenoLyse® DNA Extraction kit from Hain Lifescience. Briefly, in a biological level 2 safety cabinet, 1 mL of liquid MIGIT culture was transferred into a sterile Eppendorf tube and centrifuged at 10 000 x g for 15 minutes to form a pellet. The supernatant was discarded, and the pellet re-suspended in 100 µl lysis buffer through vortexing. Samples were incubated for 5 minutes at 95°C. Neutralization buffer (100 µl) was added into each sample and centrifuged at maximum speed (14 800 x g) for 5 minutes. Extracted DNA was stored at -20°C until used.

<u>PCR amplification and hybridization</u>

In a pre-PCR laboratory, a master mix was prepared using a GenoType *Mycobacterium* CM kit as per manufacturer's instructions. The contents for a single 50 µl reaction are described below in Table 1.

Table 1: Reagents for a single PCR reaction

Reagents	Volume (µl)
Primers Nucleotide Mix (PNM)	35
10x PCR buffer [15 mM $MgCl_2$]	5
$MgCl_2$ [25 mM $MgCl_2$]	2
PCR grade H_2O	2.8
Taq polymerase [1.25 U]	0.2
DNA	5
Total	**50**

PCR conditions for a 50 µl reaction were followed according to the manufacturer's instructions as tabulated in Table 2.

Table 2: PCR conditions for a 50 µl reaction

Time	Temperature (°C)	Number of cycles
15 minutes	95°C	1
30 seconds 2 minutes	95°C 65°C	10
25 seconds 40 seconds 40 seconds	95°C 50°C 70°C	20
8 minutes	70°C	1

Following amplification, hybridization was performed using a GenoType *Mycobacterium* CM VER 2.0 kit (Hain Lifescience) in an automated GT – Blot 48 (Hain Lifescience) hybridization apparatus. Visible hybridization bands on the DNA strips were compared to a reference key to differentiate between *M. tuberculosis* complex and 27 clinically relevant NTMs.

2.2.8 Serology

2.2.8.1 Farm workers

All serum samples (n=26) were screened for the presence of *Brucella* IgG antibodies using an ELISA kit (Vircell), according to the manufacturer's instructions. Briefly, an initial 1:10 sample dilution was performed for each participant sera by adding 20 µl sera to 180 µl of phosphate buffer saline (PBS). All samples were then further diluted with kit-specific sample diluent to a 1:20 dilution. The optical densities (OD) of each well were measured at 450 nm, against a reference wavelength of 630 nm. The mean OD cut-off values were determined and the results were interpreted as instructed by the manufacturer.

2.2.8.2 Livestock

Whole blood collected from the tail vein (Figure 4) of cattle (n=1862) was sent to either the Onderstepoort Veterinary Institute or Grahamstown Veterinary Laboratory for Brucellosis screening where a rose Bengal test (RBT) was performed on all samples. All animals with a positive RBT result were subjected to further testing using a CFT.

Figure 3: Whole blood collected from the tail vein of an animal for brucellosis screening.

2.2.9 Risk factors

Upon obtaining consent, a confidential interview was conducted with each participant. Each participant was given an information document and questionnaire (Appendix 1) available in

their preferred language (English, Sesotho or Afrikaans). The contents of the information document were explained verbally to each participant in their language preference. Afterwards, each participant was asked a series of questions to gather demographical data and possible risk factor information.

2.2.10 Statistical analysis

Database establishment and the necessary manipulation of data were done in Excel® 2016. Descriptive statistics were calculated: namely, frequencies and percentages for categorical variables and medians and quartiles for numerical variables due to skewed distributions. Associations between categorical variables and laboratory outcomes were assessed using chi-squared or Fisher's exact test in the case of sparse data. Differences between laboratory outcome groups regarding numerical variables were assessed using Mann-Whitney tests. All statistical analyses were performed by the Department of Biostatistics using SAS Version 9.4.

2.3 Results

2.3.1 Intradermal tuberculin skin test

A combined total of 321 cattle were screened for bTB in the Moqhaka and Ngwathe municipal regions within the Fezile Dabi district. Out of the 321 cattle screened, 71/321 (22.1%) were from the Maokeng community kraal, 33/321 (10.3%) from farm A, 126/321 (39.3%) from farm B and 91/321 (28.3%) from farm C. Farm D was not scheduled for bTB screening. Only 301/321 (93.8%) cattle returned after 72h for the result readings (Table 3), the remaining animals (n=20) broke out of the kraal and were not available. Based on the results of the SIST, 3/51 (5.9%) cattle were suspect reactors in the Maokeng community and 4/33 (12.1%) were suspect reactors in farm A (Appendix I). No SIST was performed on farm C, instead, a CIST was used due to the farms' previous history with positive reactors (Appendix J).

According to the CIST results, no suspect or positive reactors were identified in farm A. Out of the 126 cattle screened on farm B, 8/126 (6.3%) were positive reactors and 8/122 (6.3%) were suspect reactors. No CIST was performed in the Maokeng community due to the animals not being available for a three-month follow-up. Furthermore, based on the previous negative SICT results, no CIST was performed on farm C.

Table 3: Results of the intradermal tuberculin skin test in cattle from the Moqhaka and Nqwathe municipality regions.

		Community	Commercial Dairy		Commercial Beef	
		Maokeng	Farm A	Farm B	Farm C	Farm D*
No. of cattle screened with SIST		71	33	-	91	-
No. of cattle returned after 72h for result readings		51	33	-	91	-
SIST screening results	Positive	0	0	-	0	-
	Suspect	3	4	-	0	-
	Negative	48	29	-	91	-
No. of cattle screened with CIST		-	33	126	-	-
No. of cattle returned after 72h for result readings		-	33	126	-	-
CIST screening results	Positive	-	0	8	-	-
	Negative	-	33	110	-	-
	Suspect	-	0	8	-	-

Farm D was not scheduled for bTB screening, SIST: Single intradermal skin test, CIST: Comparative intradermal skin test

2.3.2 Interferon-gamma assay (IFN-ɣ) and milk culture

The IFN-ɣ assay was performed on all CIST-suspect and positive animals which all originated from Farm B (excluding the three animals from the Maokeng community kraal that were unavailable). Out of the 16 positive/suspect animals, whole blood was collected from 13/16 (81%). Two of the animals passed away during the three-month waiting period, and the third had hypocalcaemia when sampling was conducted (no sample available). The IFN-ɣ assay was therefore conducted on 7/8 CIST-positive animals and 6/8 CIST-suspect animals. Three out of the 13 cattle had a positive IFN-ɣ result (two positive and one avian reactor). The remaining 10 samples were negative (Table 4).

Subsequently, milk samples collected from the seven positive -and six suspect reactors were all culture-negative (Table 4).

Table 4: Interferon-gamma release assay and milk culture results of cattle with positive and suspect CIST reactions.

Animal no.	Animal status based on CIST result	CIST skin reaction increase (mm)	External characteristics of injection site	IFN-γ results	Milk culture results
1	Positive	4.6	C	Negative	Negative
2	Positive	4.7	C	Negative	Negative
3	Suspect	3.3	C	Negative	Negative
4	Suspect	3.7	F	Negative	Negative
5	Positive	5.4	C	Negative	Negative
6	Positive	10.4	A, skin condition	Positive	Negative
7	Suspect	3.6	C	Negative	Negative
8	Positive	5.6	C	Negative	Negative
9	Suspect	3.9	C	Negative	Negative
10	Positive	6.7	C	Negative	Negative
11	Positive	4.8	C, mild D	Positive	Negative
12	Suspect	3.9	C	AV	Negative
13	Suspect	3.8	C	Negative	Negative
C: Circumscribed, A: Adhesions, D: Diffuse, F: Flat, AV: Avian reactor					

2.3.3 Farm worker sputum culture results

A total of 26 sputum samples were collected from farm workers for culture (Table 5). From these samples, 13/26 (50%) were from the Maokeng community kraal cattle owners, 7/26 (27%) from commercial dairy farm B and 6/26 (23%) were from commercial beef farm C. Samples were collected from all farm workers/owners with direct exposure to cattle. No sample collection occurred at farms A and D.

Table 5: All sputum culture-positive results collected from farm workers where the herd status is known.

Sample no.	Sex	Age	Herd status	Species
Maokeng community kraal				
1	Male	52	Suspect	*Nocardia* sp.
2	Male	23	Suspect	*Mycobacterium intracellulare*
3	Male	74	Suspect	*Mycobacterium intracellulare*
5	Female	57	Suspect	*Mycobacterium* sp.
Commercial dairy farm B				
23	Female	30	Positive	*Mycobacterium* sp.
Commercial beef farm C				
21	Male	27	Negative	*Mycobacterium* sp.
24	Male	25	Negative	*Nocardia* sp.

The results displayed in Table 5, show no *M. bovis* or MTBC species were detected in all samples. Seven out of the 26 samples (27%) flagged as culture positive. *Nocardia* sp. was detected in 2/7 (28.6%) samples, *Mycobacterium intracellulare* in 2/7 (28.6%) samples and 3/7 (42.8%) samples could only be identified as *Mycobacterium* sp.

2.3.4 Risk factors

P-value scores were calculated for any statistical significance regarding a culture-positive result (Table 6). No variables were considered to be of any statistical significance ($p \leq 0.05$).

Table 6: The proportions of individuals in each category of each variable investigated during the study.

Variable	Culture positive (n=7)	Culture negative (n=19)	P-value
Age (years)			
Max	74	69	0.544
Min	23	21	
Mean	41.1	45.5	

Variable	Culture positive (n=7)	Culture negative (n=19)	P-value
Experience (years)			
Max	28	40	0.977
Min	4	0.17	
Mean	13.1	12.5	
Sex			
Male (%)	5 (71.4)	14 (73.7)	1.0
Female (%)	2 (28.6)	5 (26.3)	
Residence			
Rural (%)	7 (100)	17 (89.5)	1.0
Urban (%)	0	2 (10.5)	
Livestock exposure: Cattle			
Yes (%)	7 (100)	19 (100)	1.0
No (%)	0	0	
Pigs			
Yes (%)	1 (14.3)	0	0.269
No (%)	6 (85.7)	19 (100)	
Sheep			
Yes (%)	3 (42.9)	10 (52.6)	1.0
No (%)	4 (57.1)	9 (47.4)	
Goats			
Yes (%)	0	1 (5.3)	1.0
No (%)	7 (100)	18 (94.7)	
Assisted in parturition?			
Yes (%)	4 (57.1)	11 (57.9)	0.665
No (%)	3 (42.9)	8 (42.1)	
Regular consumption of raw milk?			
Yes (%)	4 (57.1)	15 (78.9)	0.340
No (%)	3 (42.9)	4 (21.1)	
Serious illness post animal tissue exposure?			
Yes (%)	0	1 (5.3)	1.0
No (%)	7 (100)	18 (94.7)	
Additional animal exposure (e.g. hunting)?			
Yes (%)	5 (71.4)	12 (63.2)	1.0
No (%)	2 (28.6)	7 (36.8)	
Diagnosed with TB?			
Yes (%)	0	2 (10.5) – underwent treatment	1.0
No (%)	7 (100)	17 (89.5)	

2.3.5 Rose Bengal -and complement fixation test

A total of 1862 cattle were tested for *Brucella* sp. using the RBT (Table 7). Three samples were haemolysed and not adequate for testing, resulting in 1859 cattle samples that were used for testing. A total of 52/1859 (2.8%) cattle had a positive RBT result, the remaining samples 1807/1859 (97.7%) all tested RBT negative.

Table 7: RBT results of cattle from the Maokeng community kraal and four commercial farms.

	No. of cattle tested	**No. of conclusive results (%)**	**No. of RBT positive reactions (%)**	**No. of RBT negative reactions (%)**
Community				
Maokeng Com Kraals	69	69 (100)	8 (11.6)	61 (88.4)
Commercial (Dairy)				
Farm A	33	33 (100)	0	33 (100)
Farm B	117	117 (100)	0	117 (100)
Commercial (Beef)				
Farm C	449	448 (99.8)	9 (2)	439 (97.8)
Farm D	1194	1192 (99.8)	35 (2.9)	1157 (97.1)
Total (%)	**1 862**	**1 859(99.8)**	**52 (2.8)**	**1807 (97.2)**

All 52 cattle with a positive RBT result were confirmed using the CFT. From these results 19/52 (36.5 %), 9/52 (17.3%) and 27/52 (51.9%) were confirmed CFT positive, suspect and negative, respectively (Table 8).

Table 8: CFT results of cattle from Maokeng community kraal and four commercial farms with a positive RBT result.

	No. of cattle tested	No. of conclusive results	No. of RBT positive reactions (%)	No. of CFT positive reactions (%)	No. of CFT suspect reactions (%)	No. of CFT negative reactions (%)
Community						
Maokeng Com Kraals	69	69 (100)	8 (11.6)	6 (8.7)	0	2 (2.9)
Commercial (Dairy)						
Farm A	33	33 (100)	0	-	-	-
Farm B	117	117 (100)	0	-	-	-
Commercial (Beef)						
Farm C	449	448 (99.8)	9 (2)	0	4 (0.9)	5 (1.1)
Farm D	1194	1192 (99.8)	35 (2.9)	13 (1.1)	2 (0.2)	20 (1.7)
n = (%)	**1 862**	**1 859(99.8)**	**52 (2.8)**	**19 (1.0)**	**6 (0.3)**	**27 (1.5)**

2.3.6 Farm worker *Brucella* sp. IgG ELISA results

Whole blood samples were collected from all 26 farm workers, from which 4/26 (15.4%) were IgG-positive and 22/26 (84.6%) were IgG-negative. From the four IgG-positive results, 3/4 (75%) were from the Maokeng community kraal where the herd status is confirmed as BB positive. The remaining IgG positive sample, 1/4 (25%), were from commercial beef farm C with a BB suspect herd (Table 9).

Table 9: Information on the four farm workers with a *Brucella* IgG positive result.

Sample no.	Sex	Age	Herd status
Maokeng community kraal			
1	Male	52	Positive
3	Male	74	Positive
7	Male	78	Positive
Commercial beat farm C			
17	Male	52	Suspect

Statistical analysis was performed on all results and a p-value for each variable was calculated to determine statistical significance (Table 10). According to these results, 'age' was considered as statistically significant (p-value = 0.023), where an increase in age had a strong correlation with having a positive *Brucella* IgG result (Table 10).

Table 10: The proportions of individuals in each category of each variable investigated during the study.

Variable	*Brucella* IgG seropositive result (n=4)	*Brucella* IgG seronegative result (n=22)	P-value
Age (years)			
Max	78	70	**0.023**
Min	52	21	
Median	64	40.7	
Experience (years)			
Max	28	40	0.373
Min	7	0.17	
Median	15.5	12.6	
Sex			
Male (%)	4 (100)	15 (68.2)	0.546
Female (%)	0	7 (31.8)	
Residence			
Rural (%)	4 (100)	20 (90.9)	1.0
Urban (%)	0	2 (9.1)	
Livestock exposure: Cattle			
Yes (%)	4 (100)	22 (100)	1.0
No (%)	0	0	
Pigs			
Yes (%)	1 (25)	0	0.154
No (%)	3 (75)	22 (100)	
Sheep			
Yes (%)	1 (25)	12 (54.5)	0.593
No (%)	3 (75)	10 (45.5)	
Goats			
Yes (%)	0	1 (4.5)	1.0
No (%)	4 (100)	21(95.5)	
Assisted in parturition?			
Yes (%)	3 (75)	9 (40.9)	0.306
No (%)	1 (25)	13 (59.1)	
Regular consumption of raw milk?			
Yes (%)	2 (50)	17 (77.3)	0.287
No (%)	2 (50)	5 (22.7)	
Serious illness post animal tissue exposure?			
Yes (%)	0	1 (4.5)	1.0
No (%)	4 (100)	21 (95.5)	
Additional animal exposure (e.g. hunting)?			
Yes (%)	2 (50)	15 (68.2)	0.591
No (%)	2 (50)	7 (31.8)	

Variable	*Brucella* IgG seropositive result (n=4)	*Brucella* IgG seronegative result (n=22)	P-value
Diagnosed with TB?			
Yes (%)	1 (25)	1 (4.5)	0.289
No (%)	3 (75)	21 (95.5)	

2.4 Discussion

In this study, based on the results of the intradermal tuberculin skin test, 8/301 (2.7%) cattle tested bTB positive (all originating from farm B), and 15/301 (5%) were suspect cases. Further analysis using the IFN-ɣ assay on seven CIST-positive animals determined two animals as true bTB positive (2/301; 0.7%) and one as an avian reactor, highlighting the importance of combining both the CIST and IFN-ɣ test for an accurate diagnosis. Previous studies evaluating the use of the CIST as a confirmatory test found an increase in false-positive reactions when animals are exposed to environmental *Mycobacteria* sp. such as those commonly found in herbage, soil and water (Monaghan et al., 1994).

The reason for the increased specificity with the IFN-γ assay is the inclusion of *M. fortuitum* (fortuitum PPD) to detect non-specific sensitisation in positive skin reactors (Michel et al., 2011), emphasising the importance of combining the IFN-γ assay as an ancillary test with the CIST to differentiate between avian, bovine and other NTM reactors. Unfortunately, neither positive animals were slaughtered to inspect for visible lesions and culture. Therefore, no differentiation could be made between *M. bovis* and *M. tuberculosis* or any other member of the MTBC. However, on the same farm (farm B) in 2018, an animal had a positive CIST result and was subsequently slaughtered. No visible lesions were detected (personal communication from veterinarian). Nonetheless, lymph node tissue was sent for culture, and *M. tuberculosis* was confirmed. Therefore, the possibility arises that both animals may be infected with *M. tuberculosis* based on the farms' history. Both animals were also first-time reactors, having tested negative with the CIST eight months prior to the positive result. Investigations to identify a possible zoonotic transmission source to humans were not found as all sputum culture results were MTBC negative, except for one positive NTM.

Conversations with the farm owner and workers revealed that during 2018 (when *M. tuberculosis* was cultured from an animal on the farm), a farm worker diagnosed with TB was present on the farm. However, the worker passed away at the end of 2018. Also, throughout this study, no cattle were introduced into the herd. Therefore, another possible explanation could be the reactivation of latent bTB.

An additional explanation for the positive result with no evidence of an external infection source is the reactivation of a latent state of bTB. Previous reports have shown that cavitation of caseous lesions can occur in cattle herds infected with bTB and are required for the bacteria to go into a state of dormancy (Van Rhijn et al., 2008). This phenomenon of reactivation is documented more frequently in humans than in cattle. However, the Australian TB eradication programme supports some evidence of latent bTB reactivation in cattle whereby a number of infected animals were detected, culled, and then years after, more infected animals were detected with no external infection source (Cassidy, 2006).

Upon further investigations, Cape ground squirrels (*Xerus inauris*) were identified as the only source of wildlife interaction. Currently, there is no data on the susceptibility of *M. bovis* in *X. inauris* colonies. However, *M. bovis* has been isolated from California ground squirrels (*Citellus Beecheyi*) in the US (McCoy and Chapin, 1911) and eastern grey squirrels (*Sciurus carolinensis*) in the UK (Delahay et al., 2007). Therefore, *X. inauris* might be a potential source of *M. bovis* infection in cattle, though further investigations are required.

M. bovis was not detected in any human participants in both commercial and community-based settings. *Nocardia* sp. was isolated from two farm workers (2/7), both of which were confirmed to consume unpasteurised milk regularly. Previous studies have confirmed the transmission of *Nocardia* sp. from cattle to humans by consuming dairy products from cattle infected with the bacteria (Wahba et al., 2011). Furthermore, the thermostability of *Nocardia* sp. at high temperatures means that the organism can survive the industrial pasteurisation process, with reports showing viability at 64°C for 30 minutes or 74°C for 15 seconds (Dhanashekar et al., 2012).

Nocardia sp. infection in cattle is generally associated with poor hygienic conditions when animals are milked. Udder washing and intramammary infusions with contaminated soil are considered significant risk factors for bovine mastitis. A condition that occurs when *Nocardia* sp. metastasises to the udder tissue of an animal, causing an inflammatory response (Ribeiro et al., 2008). Cattle were not inspected for bovine mastitis during this study. However, the isolation of *Nocardia* sp. from two workers indicates the bacteria's presence in the environment. One of the *Nocardia* sp. positive cases were from a farmer in the Maokeng community with a bTB suspect herd, based on the results of the SIST.

Nocardia sp. have been known to cause difficulties in the differential diagnosis of bTB in animals (DAFF, 2016). Lesions caused by *Nocardia* sp. closely resembles that of other members of the MTBC, and cross-reactivity between *Nocardia* sp. and mammalian and avian tuberculin has been demonstrated under experimental conditions. However, under the same experimental conditions, cross-reactivity with the tuberculin disappears after 72 hours. Therefore, a three-month follow-up CIST would have ruled out *Nocardia* sp. as the possible source of sensitivity (DAFF, 2016). Unfortunately, the three suspect animals were unavailable for the CIST, and the herd remains suspect.

The remaining culture isolates from the farm workers were *M. intracellulare* (2/7) and other *Mycobacterium* sp. (3/7) that could not be differentiated to species level by the GenoType *Mycobacterium* CM VER 2.0 kit. *M. intracellulare*, part of the MAC, was isolated from two farmers based in the Maokeng community, who consumed unpasteurised milk daily. Although contaminated water and soil consumption are considered more significant risk factors in contracting *M. intracellulare* (Maekawa et al., 2011), previous studies have successfully isolated *M. intracellulare* from bovine milk samples (Mdegela et al., 2004; Franco et al., 2013) and could, therefore, possibly act as transmission route in both cases.

Both *M. intracellulare*-positive farm workers had cattle that reacted positively to the SIST and were considered suspect cases. It remains possible that the animals may have been infected with avian TB (*M. intracellulare*) that cross-reacted with the bovine PPD, consequently leading to a positive skin result as no avian PPD was injected for comparative analysis. The inclusion of a CIST or IFN-γ would have made it possible to differentiate between a bovine or avian

reaction. However, as mentioned previously, all three animals were unavailable for subsequent testing.

The isolation of *M. intracellulare* and *Nocardia* sp. from farm workers, especially in this limited sample pool, is significant. Both organisms are opportunistic pathogens and a common cause of pulmonary disease in immunocompromised individuals (Corti and Fioti, 2003; Manosuthi et al., 2007). *M. intracellulare*, commonly referred to as MAC disease, is a known cause of great morbidity and mortality in patients with HIV. In a review published by Heidary et al. (2019) on the frequency of MAC infections in HIV-positive patients revealed an infection frequency of 10.6% in 18,463 HIV-positive patients. However, the review only included one article from SA, which only included 100 of the 18463 (0.5%) patients. Therefore, the frequency of MAC infections may be significantly higher in SA due to the country having the highest number of people living with HIV globally, which accounting for 20.4% of the adult population (Bulled and Singer, 2020).

Out of the 1859 cattle screened for BB, 19/1859 (1%) were positive based on the CFT results. Limited studies have reported on the prevalence or incidence rate of BB in SA cattle populations. Previous findings have reported on the seroprevalence of BB in Gauteng, Mpumalanga (Mnisi area) and KwaZulu Natal, and determined a seroprevalence of 2.33%, 0.88% and 1.3%, respectively, with the latter two focusing on communal cattle in municipal dip tanks (Govindasamy et al., 2016; Matekwe, 2011; Chisi et al., 2014). Moreover, the introduction of compulsory calf vaccinations in SA has significantly decreased the overall prevalence of BB from approximately 10.5% in 1976 to 1.4% in 1988 (Godfroid et al., 2004). These results are in accordance with the results obtained in this study, where an incidence rate of 1% was determined. Furthermore, in the Maokeng community, 6/69 (8.7%) cattle were CFT positive. These results were also expected due to the seroprevalence of BB in subsistence farming communities, almost always exceeding 5% in sub-Saharan Africa (Chisi et al., 2014).

Bovine brucellosis' higher incidence rate in communal settings is likely attributed to how animals are managed and a lack of disease awareness amongst farmers (Cloete et al., 2019). In commercial settings, animals are raised on enclosed land where movement is restricted

and controlled. Also, in these settings, BB control measures such as mass herd vaccinations and annual testing are implemented more stringently to adhere to certain standards. However, in communal farming settings, grazing land is shared amongst farmers where cattle herds recurrently interact with other herds, increasing the risk of transmission (Madzingira et al., 2020).

The remaining BB positive animals (13/1192;1.1%) were from a commercial beef farm D. The higher incidence rate is likely due to less frequent testing of animals compared to dairy farms, where milk has to be routinely screened for brucellosis through the use of an MRT. On this farm, significantly more animals were screened for BB, with different herds spread out over a larger surface area, increasing the likelihood of these animals coming in contact with potentially infectious sources as well as maintaining it.

Brucella sp. IgG positive antibody titres were detected in 4/26 (15.4%) farm workers. From the 13 Maokeng communal farm workers screened, 3/13 (23%) were IgG positive with a confirmed BB positive herd. Two out of the three farmers consumed unpasteurised milk regularly from their herd, and the third was confirmed to have assisted his animals in parturition several times over past years. The other positive reactor was from a commercial beef farm with a BB suspect herd, as determined by the results of the CFT. The incidence rate of *Brucella* sp. seropositivity in the community could result from limited knowledge regarding the prevention and transmission of the disease (Cloete et al., 2019). Also, at the time of specimen collection, all participants appeared to be healthy. However, *Brucella* sp. can cause persistent chronic infections in humans, and a clinical form of disease may develop due to immune compromisation in the future (Ulu Kilic et al., 2013).

According to the determining risk factors for *Brucella* IgG, age was considered statistically significant (p-value = 0.023). These results are similar to other reports, which found an increase in age to be strongly associated with seropositivity (Al Sekait, 1999; Centinkaya et al., 2005). The decrease in lower seropositivity in younger age, compared to the older age group, is likely due to the increased exposure older individuals have with livestock. Also, in younger age groups (<40 years), morbidity is dependent mainly on the pathogenicity of

the *Brucella* sp., whereas older individuals are typically more susceptible to infection as a result of being immunocompromised (Al Sekait, 1999).

In conclusion, the IFN-γ assay significantly increases the specificity of the CIST and should be considered when several positive reactors are identified in a herd. The overall low incidence rate of TB in cattle (0.7%) in all study sites, coupled with the negative farm worker MTBC culture results, indicates an effective control scheme being practised in the area or the absence of a wildlife reservoir host. However, the high incidence rate of *Brucella* sp. in both cattle and farmers in the Maokeng community, compared to the commercial farms, is concerning and emphasises the importance of implementing mass herd vaccination campaigns in communal settings.

Chapter 3: Seroprevalence and occupational exposure to selected zoonotic diseases in the Free State province.

3.1 Introduction

Zoonotic diseases frequently occur at unprecedented rates in human and animal populations and cause major concern in public health and veterinary communities (Brown, 2004). The emergence or re-emergence of these diseases almost always involve dynamic or constant interactions amongst different human, livestock and wildlife populations within dynamically changing environments (Allen et al., 2017). Therefore, continuous surveillance and seroepidemiological investigations of zoonotic diseases are crucial in identifying high–risk groups and estimating the actual burden of the disease (Asante et al., 2019). Occupational groups or recreational activities involving frequent exposure to animals are at an increased risk of contracting zoonotic infections, highlighting the importance of focusing on these groups to determine the disease burden.

In SA, zoonoses such as leptospirosis and brucellosis are considered to be endemic in the country, meaning that the conditions for their spread and maintenance exist (Maudlin et al., 2009). Furthermore, the occurrence or epidemiology of other diseases, such as those caused by hantaviruses, are not yet known. A concern of endemic zoonoses is the possibility of disease re-emergence due to a rapid increase in incidence or geographical range (Maudlin et al., 2009). Therefore, populations with continuous exposure to animals are frequently the subject of seroepidemiological investigations. However, in developing countries which includes SA, seroprevalence and incidence data of zoonotic diseases are exceedingly scarce and underreported (WHO, 2006).

Occupational exposure to zoonotic pathogens includes direct and indirect modes of transmission (Batelli, 2008). For diseases such as brucellosis, direct transmission is the most common infection route, primarily through contact with infected aborted material or other birthing products (Corbel, 2006). Other recurrent transmission modes include consuming dairy from infected animals and direct contact with fluids and tissue of infected animals. Therefore, occupations such as veterinarians, abattoir workers and farmers, with repeated

direct exposure to livestock or other animals are at an increased risk of infection (Pereira et al., 2020).

Leptospirosis and hantaviruses are rodent-borne pathogens, primarily transmitted through indirect contact with the environment contaminated with the excreta of infected rodents (Blasdell et al., 2019; Dearing and Dizney, 2010). Therefore, occupations that share the same environment as the rodent reservoir hosts are at a greater risk of infection. *Leptospira* sp. and hantaviruses are also known to infect livestock and wildlife, and for *Leptospira* sp., direct contact with infected livestock is a well-documented mode of transmission (Avšič-Županc et al., 2019; Ellis, 2015). However, the epidemiology and transmissibility of hantaviruses in non-rodent animals are still moderately under-investigated (Avšič-Županc et al., 2019). Therefore, direct occupational exposure to these animals (e.g. farmers and abattoir workers) may potentially be at a higher risk of infection.

Serology-based methods, focusing mainly on detecting anti-IgG antibodies specific to a desired aetiological agent, have been extensively used in determining the seroprevalence of disease in diverse populations. IgG antibodies are targeted as their presence are indicative of past infection, and detectable levels of IgG antibody titres can persist in human sera years after exposure (Lévesque et al., 2007). In the case of brucellosis, infection with the bacteria will see a predominance of IgM antibodies within the first week, followed by detectable IgG levels within the second week of infection (Al Dahouk and Nöckler, 2011). Both IgM and IgG antibodies will then continue to rise in titre and peak within the first four weeks, after which IgM levels would start to decline to undetectable levels. *Brucella* sp. IgG antibody titres after four weeks post-infection would also gradually decline but will continue to persist over extended periods, even after a person has undergone successful antibiotic treatment (Al Dahouk and Nöckler, 2011).

Similarly, hantavirus infection in humans would present a gradual increase of IgM antibodies detectable at five days post-infection (PI) during the virus's incubation phase (Engdahl and Crowe, 2020). Hantavirus IgM antibodies reach peak titres at the end of the febrile phase and continue to decline in titre, reaching very low levels at the start of the convalescent phase at 40 days PI. Hantavirus IgG antibodies also gradually increase at five days PI and continue to

do so until a plateau is reached at the start of the convalescent phase 40 PI (Engdahl and Crowe, 2020). Neutralising antibodies titres of 1:400 and <1:100 for ANDV and SNV, respectively, have been detected in patients 11 years PI through the use of a focus-reduction neutralisation assay (Valdivieso et al., 2006).

For leptospirosis, seroconversion may occur as early as day 5-7 after disease onset but may take up to 10 days or longer. *Leptospira* IgM antibodies reach peak titres at 10-14 days after symptom onset, whereas IgG antibodies reach peak titres 28 days after the initial onset of symptoms (Picardeau et al., 2014). However, the detection of *Leptospira* IgG antibodies is variable and often not detected or may only be detectable for short periods (WHO, 2003). Therefore, *Leptospira* IgM antibodies are frequently targeted for seroprevalence studies as IgM levels remain detectable for several years after exposure (Cumberland et al., 2001).

In SA, limited information exists regarding zoonoses in high-risk populations due to their occupation or recreational activities. These populations are at higher risk of contracting a zoonotic disease due to prolonged exposure and frequent direct or indirect contact with animals. Determining the seroprevalence of *Brucella* sp., *Leptospira* sp. and hantaviruses can indicate which populations are at a higher risk, subsequently aiding in preventative or control schemes.

3.2 Material and Methods

3.2.1 Ethical considerations

Ethical approval was obtained from the University of the Free State Health Sciences Research Ethics Committee (UFS-HSD2019/1075/270801) and Environmental & Biosafety Research Ethics Committee (UFS-ESD2019/0086) before any specimen collection or analysis were performed.

3.2.2 Study design and specimen collection

In this cross-sectional study, 327 serum samples were available for this study. From these, 299 were archived serum samples collected from healthy individuals between April 2016 – February 2017 as part of an unrelated study (HSREC34/2016 and ETOVS152/06). All samples were stored at -80°C at the Division of Virology at the UFS. In addition, sera collected from 28

healthy farm workers during November 2019 and March 2020 were also included, as indicated in chapter two, section 2.2.4.2.

3.2.3 Study area and sample composition

All 327 samples used in this study were collected within the Free State province and included the following high-risk populations: farm workers (n=28), abattoir workers (n=207), veterinarians (n=12), stable grooms (n=32), recreational hunters (n=46) and laboratory workers (n=2). Demographical and occupational information were obtained through a questionnaire. Additional information included livestock exposure and any reports of illness after a participant was directly exposed to animal tissue/fluids.

3.2.4 Serology

3.2.4.1 Sample preparation

All serum samples were retrieved from the -80°C freezer and left to thaw at room temperature. An initial 1:10 dilution series was performed on all samples in a 96 well Sterilin™ U-bottom plate by adding 20 µl of serum to 180 µl PBS.

3.2.4.2 *Brucella* IgG antibody detection

All serum samples were screened for *Brucella* IgG specific antibodies, using a commercially available indirect ELISA (Vircell; Granada, Spain), and steps were carried out per the manufacturer's instructions. Briefly, serum samples were screened at a dilution of 1:20. For each sample, 100 µl of diluted serum were added to the wells of a 96-well plate coated with LPS antigen of *B. abortus*, strain S-99 and left to incubate (covered) at 37±1°C for 45 minutes. Wells were then aspirated and washed five times with a 20X phosphate buffer containing Tween®-20 and Proclin™ 300, using a Tecan HydroSpeed™ automated plate washer (Männedorf, Switzerland).

Immediately afterwards, 100 µl of anti-human IgG peroxidase conjugate in a Neolone and Bronidox buffer solution were added into each well, covered, and incubated at 37±1°C for 30 minutes. Wells were then aspirated and washed five times, before adding 100 µl of a substrate solution containing tetramethylbenzidine. The plate was then left to incubate at room temperature for 20 minutes in a dark room. Immediately afterwards, 50 µl aliquots of

a stopping solution containing 0.5 M sulphuric acid were added into each well and measurements were taken within one hour.

Optical densities (OD) were measured at a wavelength of 450 nm (with a reference read at 630 nm) using a BioTek® 800TS™ Absorbance Reader (Winooski, USA). The mean OD value was calculated for the cut-off serum, and the antibody index was calculated by dividing the sample OD with the cut-off serum mean OD, multiplied by 10. An index of <9 was considered negative, 9 – 11 equivocal and >11 positive. All results that returned as equivocal were retested.

3.2.4.3 *Leptospira* IgM antibody detection

The detection of *Leptospira* specific antibodies was carried out using a commercially available Panbio IgM ELISA (Windsor, Australia), according to the manufacturer's instructions. Briefly, serum samples were screened at a dilution of 1:100. For each sample, 100 µl of diluted serum were added to a 96-well plate coated with *Leptospira* antigen. The plate was then covered and incubated at 37±1°C for 30 minutes. Wells were washed six times with a 20X phosphate buffer containing Tween®-20 and 0.1% Proclin™. Afterwards, 100 µl of horseradish peroxidase conjugated goat anti-human IgM solution was added into each well and the plate incubated (covered) at 37±1°C for 30 minutes.

Wells were washed six times before adding 100 µl of 3, 3', 5, 5'; -tetramethylbenzidine (TMB) chromogen substrate into each well. Following this, the plate was covered and incubated for 10 minutes in a dark room before immediately adding 100 µl of 1M phosphoric acid stopping solution.

Optical density values were measured, within 30 minutes after adding the stopping solution, at a wavelength of 450 nm with a reference filter at 630 nm. The cut-off value was determined by calculating the average absorbance of the calibrators tested in triplicate, multiplied by the calibrator factor (batch specific). Results were calculated as "Panbio units" by dividing the sample absorbance read with the cut-off value. A result of <0.9, 0.9 to 1.1 and >1.1 was defined as negative, equivocal, or positive, respectively. All samples with an equivocal result were retested.

3.2.4.4 Hantavirus IgG antibody detection

To detect hantavirus specific IgG antibodies, a commercially available EUROIMMUN Anti-Hanta Virus Pool 1 "Eurasia" ELISA (Lübeck, Germany) was used. This *in vitro* assay is able to detect human IgG antibodies against old-world hantavirus strains (Hantaan, Dobrava and Puumala), and the procedure was carried out according to the manufacturer's instructions. Briefly, serum samples were screened at a dilution of 1:100. For each sample, 100 µl of diluted serum were added into the wells of a 96-well plate coated with a mixture of hantavirus recombinant nucleocapsid protein (N-protein) antigens. Three calibrators at 200 relative units per millilitre (RU/mL), 20 RU/mL and 2 RU/mL were also tested. Plates were covered and samples were incubated at 37±1°C for 60 minutes. The plates were then washed three times with a 1x concentrate wash buffer.

Thereafter, 100 µl of peroxidase-labelled anti-human IgG (rabbit) enzyme conjugate was loaded into each well, covered with a protective foil, and left to incubate for 30 minutes at room temperature. As mentioned above, plate washing was carried out and 100 µl of TMB/H_2O_2 chromogen substrate solution was added into each well. The plate was sealed and samples were incubated at room temperature for 15 minutes in a dark room. Directly afterwards, 100 µl of stopping solution containing 0.5 M sulphuric acid were added to each well, and the absorbance of each sample was read at 450 nm with a reference wavelength of 630 nm within 30 minutes.

Results were determined semi-quantitatively. The ratios of test sample to calibrator was determined by dividing the absorbance of the serum sample with the absorbance of calibrator two (20 RU/mL), as seen below in equation three. A ratio of <0.8 was negative, ≥ 0.8 to <1.1 equivocal and ≥ 1.1 positive. Equivocal samples were subjected to retesting.

Equation 3:

$$\frac{Serum\ absorbance\ at\ 450\ nm}{Calibrator\ absorbance\ (20\ RU/mL)\ at\ 450\ nm} = Ratio$$

3.2.5 Statistical analysis

Database establishment and the necessary manipulation of data were done using Excel® 2016. Descriptive statistics were calculated, namely frequencies and percentages for categorical variables and medians and quartiles for numerical variables due to skewed distributions. Associations between categorical variables and laboratory outcomes were assessed using chi-squared or Fisher's exact test in the case of sparse data. Differences between laboratory outcome groups regarding numerical variables were assessed using Mann-Whitney tests. All statistical analyses were performed by the Department of Biostatistics, University of the Free State, using SAS Version 9.4.

3.3 Results

3.3.1 Demographics

A total of 327 serum samples were analysed from participants at a higher risk of contracting a zoonotic infection due to their occupation and frequent outdoor exposure. The maximum, minimum and mean age for participants was 78, 18 and 36 years, respectively (Table 11). Most samples were from male participants, 281 (85.9 %), compared to females, 46 (14.1%). Furthermore, 208 (63.6%) participants were from more urbanised settings, as compared to living in a rural area, 119 (36.4%) (Table 11).

The majority of participants (63.3%) were abattoir workers or reported exposure to animals through informal slaughtering, followed by farm workers (40.4%), stable grooms (13.1%), veterinarians (3.7%) and laboratory workers (0.9%). It should be noted that several participants reported exposure to more than one occupation/activity. For example, abattoir workers frequently reported subsistence farming as secondary exposure.

Table 11: Socio-demographical and occupational/recreational exposure data of all participants.

Variable		Frequency (n=327, %)
Age (years)	Maximum	78
	Minimum	18
	Mean	36
Sex	Male	281 (85.9)
	Female	46 (14.1)
Residence	Rural	119 (36.4)
	Urban	208 (63.6)
Type of exposure reported by each participant*	Farming	132 (40.4)
	Recreational hunting	86 (26.3)
	Abattoir worker (slaughtering)	207 (63.3)
	Stable groom	43 (13.1)
	Veterinary work	12 (3.7)
	Laboratory work	3 (0.9)
	Livestock	158 (48.3)
	Illness post-exposure to animal tissue/fluids	12 (3.7)
Some participants reported more than one type of exposure		

The proportion of exposure reported by all participants, including exposure to more than one occupation or activity, can be seen in figure five. Abattoir work and informal slaughtering were most frequently reported (43%), followed by farming (27%), hunting (18%), stable groom (9%), veterinary- (2%) and laboratory (1%) work.

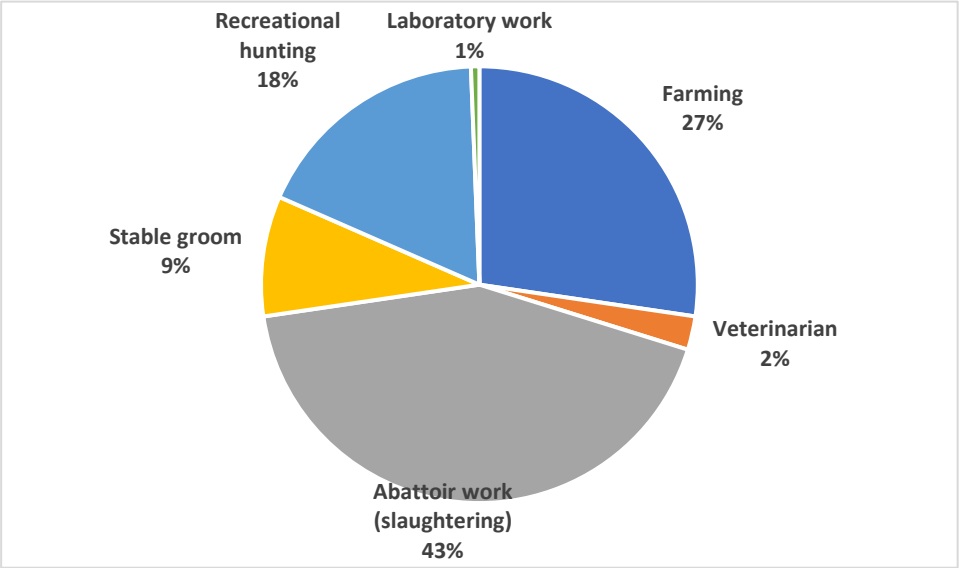

Figure 4: Pie chart depicting the various occupancies and potential sources of exposure in percentage values (%).

3.3.2 Serology

All 327 serum samples were screened for IgG antibodies against *Brucella* sp. and hantaviruses, and IgM antibodies for *Leptospira* sp. The absorbance values of all samples were normalised by calculating the ratio for each sample to that of the cut-off control. Ratio values calculated for each sample in all three assays (*Brucella* sp. *Leptospira* sp. hantavirus) were plotted and depicted in figure 5, 6 and 7. Raw absorbance values at 450 nm for each specimen are shown in Appendix K-M.

IgG antibodies against *Brucella* sp. were detected in 35 out of 327 (10.7%) participant sera, 3/327 (0.9%) were equivocal, and 289/327 (88.4%) were negative. Equivocal samples were subject to retesting, and results obtained after the retest were used (Figure 5).

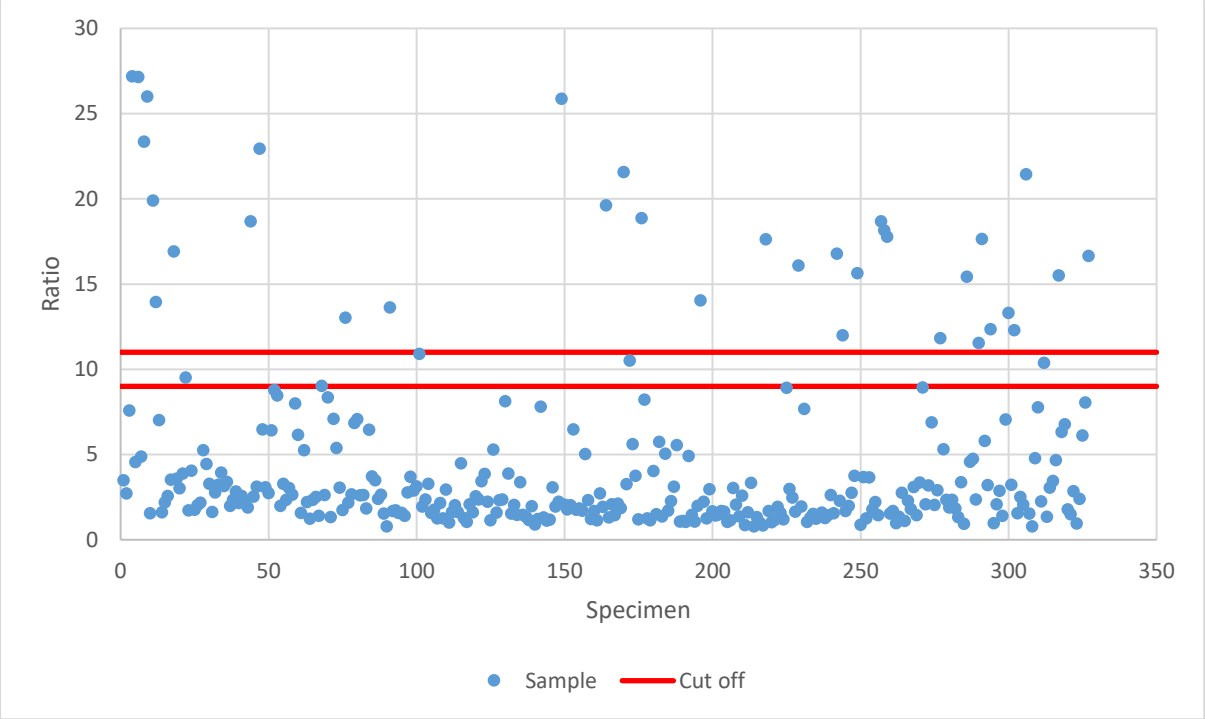

Figure 5: Ratio values calculated for each sample in the Brucella sp. IgG ELISA assay. A ratio of <9 was considered negative, 9 – 11 equivocal and >11 positive.

Results obtained from the *Brucella* sp. IgG ELISA for each occupational group is shown below in Table 12. Out of the 35 *Brucella* IgG positive reactors, 20/35 (57.1%) were abattoir workers, 6/35 (17.1%) veterinarians, 4/35 (11.4%) farm workers, 2/35 (5.7%) hunters, 2/35 (5.7%) stable grooms, and 1/35 (2.9%) were from a laboratory worker.

Table 12: Number of positive, equivocal and negative *Brucella* sp. IgG reactors per occupation group.

Occupation	Number of participants, (%)	Number of positive samples, ratio of >11 (%)	Number of equivocal samples, ratio of 9 – 11 (%)	Number of negative samples, ratio of <9 (%)
Abattoir workers	207 (63.3)	20 (9.7)	2 (1.0)	185 (89.4)
Veterinarians	12 (3.7)	6 (50.0)	1 (8.3)	5 (41.7)
Stable groom	32 (9.8)	2 (6.3)	0 (0)	30 (93.8)
Recreational hunters	46 (14.1)	2 (4.3)	0 (0)	44 (95.7)
Farm workers	28 (8.6)	4 (14.3)	0 (0)	24 (85.7)
Laboratory workers	2 (0.6)	1 (50.0)	0 (0)	1 (50.0)
Total, %	327	35 (10.7)	3 (0.9)	289 (88.4)

Out of the 327 participants screened for the presence of *Leptospira* sp. IgM antibodies, 17/327 (5.2%) were positive, 9/327 (2.8%) equivocal and 301/327 (92%) were negative. Results depicted in figure six were after subsequent retesting of equivocal samples where the results after the retest was used.

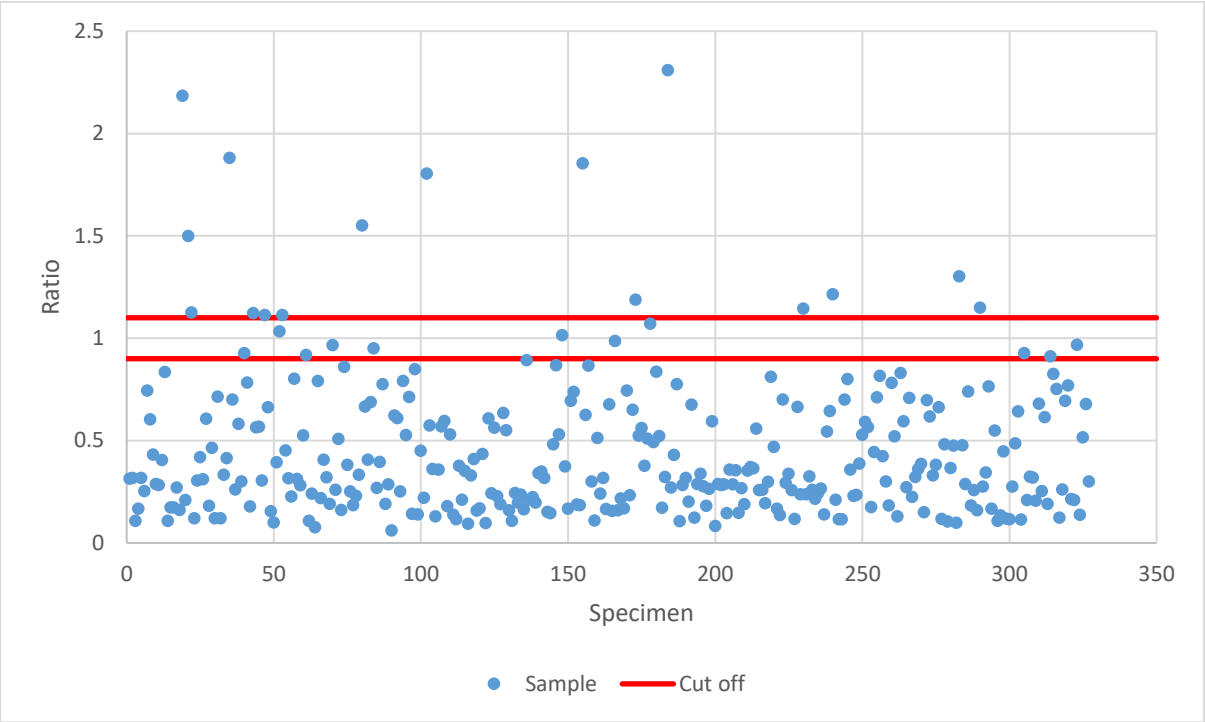

Figure 6: Ratio values calculated for each sample in the *Leptospira* sp. IgM ELISA assay. A ratio of <0.9, 0.9 to 1.1 and >1.1 was defined as negative, equivocal, or positive, respectively.

Leptospira sp. IgM antibodies were detected in three different occupation groups (Table 13). Out of the 17 positive reactors, 14/17 (82.4%) were abattoir workers, 2/17 (11.8%) stable grooms, and 1/17 (5.9%) were from a hunter.

Table 13: Number of positive, equivocal and negative *Leptospira* sp. IgM reactors per occupation

Occupation	Number of participants, (%)	Number of positive samples, ratio of >1.1 (%)	Number of equivocal samples, ratio of 0.9 - 1.1 (%)	Number of negative samples, ratio of <0.9 (%)
Abattoir workers	207 (63.3)	14 (6.7)	5 (2.4)	188 (90.8)
Veterinarians	12 (3.7)	0 (0)	0 (0)	12 (100)
Stable groom	32 (9.8)	2 (6.3)	1 (3.1)	29 (90.6)
Recreational hunters	46 (14.1)	1 (2.2)	0 (0)	45 (97.8)
Farm workers	28 (8.6)	0 (0)	3 (10.7)	25 (89.3)
Laboratory workers	2 (0.6)	0	0	2 (100)
Total, %	327	17 (5.2)	9 (2.8)	301 (92.0)

A total of 38 out of 327 (11.6%) participant sera were positive for hantavirus IgG, 21/327 (6.4%) equivocal and 268/327 (82%) were negative (Figure 7). Equivocal samples were subject to retesting, and results obtained after the retest were used.

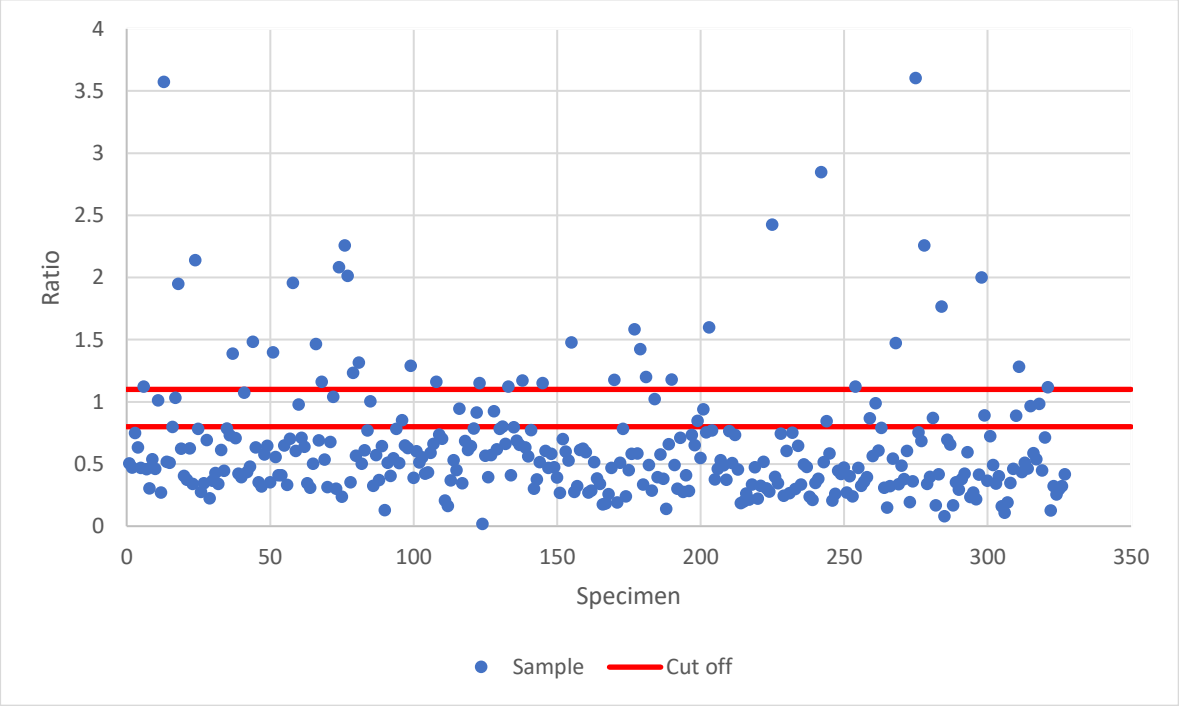

Figure 7: Ratio values calculated for each sample in the hantavirus IgG ELISA assay. A value of <0.8 was negative, ≥ 0.8 to <1.1 equivocal and ≥ 1.1 positive.

Positive, equivocal and negative results as per primary occupation are described below in Table 14. From the 38 hantavirus IgG positive reactors, 26/38 (68.4%) were from abattoir workers, 4/38 (10.5%) stable grooms, 4/38 (10.5%) hunters, 3/38 (7.9%) farm workers, and 1/38 (2.6%) from a veterinarian (Table 14).

Table 14: Number of positive, equivocal and negative hantavirus IgG reactors per occupation group.

Occupation	Number of participants, (%)	Number of positive samples, ratio of ≥ 1.1 (%)	Number of equivocal samples, ratio of ≥ 0.8 to <1.1 (%)	Number of negative samples, ratio of <0.8
Abattoir workers	207 (63.3)	26 (12.6)	10 (4.8)	171 (82.6)
Veterinarians	12 (3.7)	1 (8.3)	1 (8.3)	10 (83.3)
Stable groom	32 (9.8)	4 (12.5)	3 (9.4)	25 (78.1)
Recreational hunters	46 (14.1)	4 (8.7)	4 (8.7)	38 (82.6)
Farm workers	28 (8.6)	3 (10.7)	3 (10.7)	22 (78.6)
Laboratory workers	2 (0.6)	0 (0)	0 (0)	2 (100)
Total, %	327	38 (11.6)	21 (6.4)	268 (82.0)

3.3.3 Statistical analysis

The maximum, minimum, and mean age for all hantavirus IgG positive participants was 65, 19 and 39 years, respectively. Statistical analysis showed no significance (p-value < 0.05) to any variables (Table 15)

Table 15: The proportions of individuals in each category of each variable investigated for hantavirus IgG antibody detection during the study.

Variable	Hantavirus IgG positive (n=38)	Hantavirus IgG negative (n=289)	P-value
Age (years)			
Max	65	78	0.136
Min	19	18	
Mean	38.	36.	

Variable	Hantavirus IgG positive (38)	Hantavirus IgG negative (289)	P-value
Sex			
Male (%)	33 (86.8)	248 (85.8)	1.000
Female (%)	5 (13.2)	41 (14.2)	
Residence			
Rural (%)	14 (36.8)	105 (36.3)	1.000
Urban (%)	24 (63.2)	184 (63.7)	
Farmer			
Yes (%)	8 (21.1)	55 (19.0)	0.827
No (%)	30 (78.9)	234 (81.0)	
Farm worker			
Yes (%)	8 (21.1)	63 (21.8)	1.000
No (%)	30 (78.9)	226 (78.2)	
Livestock exposure			
Yes (%)	23 (60.5)	144 (49.8)	0.231
No (%)	15 (39.5)	145 (50.2)	
Hunter			
Yes (%)	7 (18.4)	79 (27.3)	0.327
No (%)	31 (81.6)	210 (72.7)	
Abattoir worker			
Yes (%)	24 (63.2)	157 (54.3)	0.386
No (%)	14 (36.8)	132 (45.7)	
Stable groom			
Yes (%)	5 (13.2)	38 (13.1)	1.000
No (%)	33 (86.8)	251 (86.9)	

Variable	Hantavirus IgG positive (n=38)	Hantavirus IgG negative (n=289)	P-value
Veterinarian			
Yes (%)	1 (2.6)	11 (3.8)	1.000
No (%)	37 (97.4)	278 (96.2)	
Laboratory worker			
Yes (%)	0 (0)	3 (1.0)	1.000
No (%)	38 (100)	285 (99.0)	
Illness post-exposure to animal blood/tissue			
Yes (%)	1 (2.6)	11 (3.8)	1.000
No (%)	37 (97.4)	278 (96.2)	

Statistical analysis of the *Brucella* IgG positive results showed that age (p-value = 0.0008), veterinary work (p-value = 0.0006), and laboratory work (p-value = 0.031) are all significant risk factors (Table 16). Participants who reported illness post exposure to animal tissue/blood (p-value = 0.029) also showed statistical significance to *Brucella* IgG seropositivity.

Table 16: The proportions of individuals in each category of each variable investigated for *Brucella* sp. IgG antibody detection during the study.

Variable	*Brucella* IgG positive (n=35)	*Brucella* IgG negative (n=292)	P-value
Age (years)			
Max	78	74	0.0008
Min	18	18	
Mean	43.4	35.6	
Sex			
Male (%)	32 (91.4)	249 (85.3)	0.443
Female (%)	3 (8.6)	43 (14.7)	

Variable	*Brucella* IgG positive (n=35)	*Brucella* IgG negative (n=292)	P-value
Residence			
Rural (%)	15 (42.9)	104 (35.6)	0.458
Urban (%)	20 (57.1)	188 (64.4)	
Farmer			
Yes (%)	9 (25.7)	54 (18.5)	0.363
No (%)	26 (74.3)	238 (81.5)	
Farm worker			
Yes (%)	12 (34.3)	59 (20.2)	0.08
No (%)	23 (65.7)	233 (79.8)	
Livestock exposure			
Yes (%)	16 (45.7)	144 (49.3)	0.723
No (%)	19 (54.3)	148 (50.7)	
Hunter			
Yes (%)	9 (25.7)	77 (26.4)	1.000
No (%)	26 (74.3)	215 (73.6)	
Abattoir worker			
Yes (%)	20 (57.1)	161 (55.1)	0.859
No (%)	15 (42.9)	131 (44.9)	
Stable groom			
Yes (%)	4 (11.4)	39 (13.4)	1.000
No (%)	31 (88.6)	253 (86.6)	
Veterinarian			
Yes (%)	6 (17.1)	6 (2.1)	0.0006
No (%)	29 (82.9)	286 (97.9)	
Laboratory worker			
Yes (%)	2 (5.7)	1 (0.3)	0.031
No (%)	33 (94.3)	291 (99.7)	

Variable	*Brucella* IgG positive (n=35)	*Brucella* IgG negative (n=292)	P-value
Illness post-exposure to animal blood/tissue			
Yes (%)	4 (11.4)	8 (2.7)	0.029
No (%)	31(88.6)	284 (97.3)	

Analysis conducted on the *Leptospira* seropositive IgM results depicted abattoir work or informal slaughtering (p-value = 0.024) as the only significant risk factors (Table 17).

Table 17: The proportions of individuals in each category of each variable investigated for *Leptospira* IgM antibody detection during the study.

Variable	*Leptospira* IgM positive (n=17)	*Leptospira* IgM negative (n=310)	P-value
Age (years)			
Max	65	78	0.9076
Min	23	18	
Mean	36.1	36.5	
Sex			
Male (%)	13 (76.5)	268 (86.5)	0.275
Female (%)	4 (23.5)	42 (13.5)	
Residence			
Rural (%)	8 (47.1)	111 (35.8)	0.438
Urban (%)	9 (52.9)	199 (64.2)	
Farmer			
Yes (%)	1 (5.9)	62 (20.0)	0.212
No (%)	16 (94.1)	248 (80.0)	
Farm worker			
Yes (%)	3 (17.6)	68 (21.9)	0.755
No (%)	14 (82.4)	242 (78.1)	

Variable	*Leptospira* IgM positive (n=17)	*Leptospira* IgM negative (n=310)	P-value
Livestock exposure			
Yes (%)	7 (41.1)	153 (49.4)	0.621
No (%)	10 (58.9)	157 (50.6)	
Hunter			
Yes (%)	2 (11.8)	84 (27.1)	0.257
No (%)	15 (88.2)	226 (72.9)	
Abattoir worker			
Yes (%)	14 (82.4)	167 (53.9)	0.024
No (%)	3 (17.6)	143 (46.1)	
Stable groom			
Yes (%)	2 (11.8)	41 (13.2)	1.000
No (%)	15 (88.2)	269 (86.8)	
Veterinarian			
Yes (%)	0 (0)	12 (3.9)	1.000
No (%)	17 (100)	298 (96.1)	
Laboratory worker			
Yes (%)	0 (0)	3 (1.0)	1.000
No (%)	17 (100)	307 (99.0)	
Illness post-exposure to animal blood/ tissue			
Yes (%)	0 (0)	12 (3.9)	1.000
No (%)	17 (100)	298 (96.1)	

3.4 Discussion

For *Brucella* IgG, seropositivity of 10.7% was determined (35 out of 327). Currently, limited information is available regarding the seroprevalence of *Brucella* sp. in SA human populations. However, from 1936 to 1938, two *Brucella* sp. seroprevalence studies were conducted in the country. The first study conducted by Campbell and Greenfield (1937) reported a seroprevalence of 4.8% (32 out of 661) when 661 patients with fever of unknown

origin were screened using an agglutination test in the Cape region. The second study was a seroprevalence survey study conducted by Barnetson (1939) to determine *Brucella* sp. agglutinins in 1900 blood samples from patients with suspected typhoid fever between 1936-1938, an incidence of 2.5% (40 out of 1577) was reported.

Both previously mentioned studies were conducted over 80 years ago using agglutination tests developed by Wright and Smith (1897). Though sufficient at the time, these tests are prone to false-positive reactions due to antibody cross-reactivity, typically with IgM under neutral or slightly acidic pH levels (Nielsen, 2002). These studies also placed primary focus on febrile patients. Although the results are of significance, determining the prevalence of *Brucella* sp. in healthy individuals is necessary to assist diagnostics in areas of endemicity (Govindasamy, 2020).

The last formally published study focusing on the incidence rate of *Brucella* sp. in the SA human population reported a rate of >0.2 per 100 000 population based on a survey from 1956 to 1959 (Schrire, 1962). The severe lack of information regarding the incidence or prevalence of *Brucella* sp. in the SA population could be explained by the absence of a human brucellosis surveillance program (Frean et al., 2018). In addition, even though brucellosis is considered a notifiable condition in SA, the disease is underreported due to the broad clinical presentation that frequently overlaps with more common causes of diseases, such as typhoid fever, tuberculosis and rickettsial infections (Zerfu et al., 2018).

A few studies on occupational exposure to *Brucella* sp. amongst healthy individuals in SA have been conducted. This includes a survey study of zoonotic diseases contracted by 88 SA veterinarians (Gummow, 2003). Out of the 88 veterinarians surveyed, 56 (63.6%) contracted one or more zoonotic diseases, with 7/88 (8%) reporting illness due to brucellosis. In another study conducted amongst 64 dip-tank workers (people who work at dip tanks) in Bushbuckridge, Mpumalanga, an incidence rate of 0% (0/64) was determined using a Brucellacapt® assay with a reported sensitivity and specificity of 96% and 97.5%, respectively (Simpson et al., 2018b).

In this study, a higher seroprevalence of 10.7% amongst healthy, high-risk individuals was determined than the previously mentioned studies. However, both previously mentioned studies included a smaller study population and focused on a single high-risk group. Nonetheless, the higher seroprevalence obtained in this study might indicate a higher disease burden in the Free State province than the previously mentioned studies.

The higher seropositivity is likely due to the participants' occupation and recreational activities, putting them at a higher risk of contracting an infection. Based on the results of this study, two high-risk occupational groups were identified as having a higher *Brucella* IgG seropositive rate compared to the other occupations: laboratory workers (p= 0.031) and veterinarians (p= 0.0006).

Only three participants documented potential zoonotic exposure through laboratory work, from which two were *Brucella* IgG seropositive. Although statistically significant, a larger sample pool is required to support the results. However, it is well known that laboratory workers, especially those working in *Brucella* sp. endemic regions, are at an increased risk of contracting a *Brucella* sp. infection due to it being one of the most common laboratory-acquired infections (Noviello et al., 2004). *Brucella* sp. are readily aerosolised and only requires an infective dose of 10 to 100 bacilli (Pappas et al., 2006); therefore, diagnostic steps that generate aerosols such as open-cap centrifugation are of considerable risk (Maley et al., 2006). Furthermore, due to the broad non-specific clinical representation, *Brucella* sp. are not always suspected and can lead to accidental exposure during isolation or culturing of clinical specimens (Traxler et al., 2013). In this study, only three people reported laboratory work (two out of three were *Brucella* sp. IgG positive) as exposure and a more extensive laboratory worker sample pool is required for an in-depth analysis. However, given the small sample pool, it is indicative that laboratory workers are at high-risk of contracting *Brucella* sp., as a result of inadequate protocols put in place to prevent laboratory transmission, or laboratory workers not proceeding with caution when handling potential hazardous specimens.

Brucella sp. IgG antibodies were detected in 6 out of 12 veterinarians sampled. It is a well-known fact that veterinarians are at an increased risk of contracting a *Brucella* sp. infection due to frequent exposure and mode of transmission of the pathogen. *B. abortus*, *B.*

melitensis, *B. suis* and *B. canis* are common causes of spontaneous abortions and stillbirths in cattle (Hovingh, 2009), goats and sheep (Okoh, 1980), pigs (Onunkwo et al., 2011) and dogs (Carmichael, 1990), respectively. This significantly increases the risk of exposure for veterinarians due to the high concentration of shedding *Brucella* sp. bacilli reaching maximum levels on aborted foeti, placentae and other birthing products (Corbel, 2006). However, the true significance of the high incidence rate observed in the veterinarians can only be determined when more in-depth seroepidemiological investigations are performed in both the general healthy population and high-risk occupational groups extending over different geographical areas.

In this study, an increase in participant age (p= 0.0008) and illness post-exposure to animal/blood tissue (p= 0.029) were statistically significant with a positive *Brucella* sp. IgG result. The mean age of the *Brucella* sp. IgG positive group was 43.4 years compared to the negative group with a mean age of 35.6 years. As previously mentioned, in chapter two, older age (> 40 years of age) has been previously identified as a risk factor for brucellosis (Ramos et al., 2008). It is likely because of repeated exposure to the infective agent (Mukhtar, 2010). Furthermore, 11 out of the 327 participants (3.3%) reported falling ill after being exposed to animal blood or tissue. Of these 11 participants, five (45.5%) were veterinarians, three (27.3%) abattoir workers, and three (27.3%) were professional hunters. Direct contact with infected animal tissue and fluids has been documented as a common mode of transmission (Franco et al., 2007), highlighting the importance of clinicians to suspect brucellosis when patients report regular exposure to animal blood or tissue.

Leptospira IgM antibodies were detected in 5.2% (17 out of 327) of participants. Limited studies have been conducted on *Leptospira* sp. in the SA human population (De Vries et al., 2014). In a cross-sectional serosurvey study conducted in healthy individuals using a microscopic agglutination test (MAT), a high seroprevalence of 18.9% (41 out of 217) was determined in the Durban area (Taylor et al., 2008). Furthermore, two studies conducted on febrile patients using an IgM ELISA determined seroprevalence of 7.8% (26 out of 332) and 6.8% (5 out of 74) in the Johannesburg (Saif, 2013) and Bushbuckridge municipality in the Mpumalanga province (Simpson et al., 2018b), respectively. The latter study conducted in

Mpumalanga also screened 64 dip-tank workers and determined a seroprevalence of 21.9% (14 out of 64) using an IgM ELISA (Simpson et al., 2018b).

The *Leptospira* sp. seroprevalence determined in this study is than to the previously mentioned results obtained in other studies. This is likely due to multiple factors promoting increased transmission of *Leptospira* sp. in the Gauteng (GP) and KwaZulu-Natal (KZN) provinces, compared to the Free State province. These factors include warmer climate and heavier rainfall increasing the transmission of *Leptospira* sp. as described previously (Chadsuthi et al., 2012; Sumi et al., 2017; Matsushita et al., 2018). Based on South Africa's annual climate summary in 2018, the GP and KZN provinces experience on average heavier rainfall and higher temperatures compared to the Free State province (SAWS, 2019). Another contributing factor is the geographical distribution and population density of the preferred murid reservoir host. However, limited information exist regarding the *Leptospira* sp. murid host distribution and density in South Africa.

Leptospira sp. IgM antibodies were detected in 17 participants from three different risk groups, which include 15/17 (88.2%) abattoir workers, 2/17 (11.8%) stable workers and 1/17 (5.9%) professional hunters. According to the statistical analysis, abattoir workers (p= 0.024) are at a statically higher risk of contracting *Leptospira* sp. than the other at-risk groups. These results are in accordance with results obtained in other countries that have also identified abattoir workers at an increased risk (Schoonman and Swai, 2009; Mirambo et al., 2018). No studies have been conducted in SA to investigate the seroprevalence of leptospirosis in abattoir workers. However, extensive seroepidemiological investigations of *Leptospira* sp. in livestock have been conducted in SA.

A study conducted in the Free State province screened for *Leptospira* sp. in various livestock, with results showing a high seroprevalence rate of 54% and 45% in cattle and pigs, respectively (Gummow et al., 1999). More recent investigations conducted across the country have also reported high seroprevalence results amongst different livestock populations. One study on the seroprevalence of *Leptospira* sp. in slaughtered cattle in a Gauteng abattoir reported an overall rate of 27.6% (55 out of 199) using a MAT (Dogonyaro et al., 2020). Another study focusing on the seroprevalence of *Leptospira* sp. amongst 663 horses within

the Gauteng, KwaZulu – Natal and Western Cape provinces determined a prevalence of 24 – 74%, 20 – 54% and 26 – 39%, respectively (Simbizi et al., 2016). The high seroprevalence in livestock supports the findings that abattoir workers are at an increased risk of contracting a *Leptospira* sp. through direct contact with infected animal tissue or fluids.

In conclusion, the current study provided valuable information on the seroprevalence of *Leptospira* sp. and *Brucella* sp. in different high-risk populations. Occupational groups, including veterinarians and laboratory workers, were identified as high-risk groups for *Brucella* sp. infection and abattoir workers were at an increased risk of *Leptospira* sp. infection. Furthermore, older individuals and illness post-exposure to animal tissue/blood correlated significantly with *Brucella* IgG.

Out of the 327 samples screened for hantavirus IgG using an ELISA, 38/327 (11.6%) were seropositive. The ELISA used in this study detects a mixture of nucleocapsid antigens of the old-world hantavirus serotypes: HTNV, DOBV and PUUV. Due to the close phylogenetic relationship and nucleocapsid homogeneity shared between old world hantaviruses and supporting evidence that hantaviruses circulating in Africa are closely related to old world hantaviruses (Witkowski et al., 2014), a commercial old world hantavirus IgG ELISA was chosen for this study to detect cross-reactivity.

An IgG seroprevalence of 11.6% is significantly high, especially in a country where no hantaviruses have been isolated or described. The most recent hantavirus seroprevalence study conducted on the average population in the SA Cape region by Witkowski et al. (2014) determined a seroprevalence of 14.5% when residual blood after routine diagnostics were screened (210 out of 1442). In the previously mentioned study, an in-house IgG ELISA was developed using the recombinant nucleocapsid proteins from PUUV and DOBV. However, a seroprevalence of 14.5% was also considered high. Therefore, the authors performed subsequent confirmatory tests using an IFA and Western Blot (WB) analysis on all initial IgG serum positive samples determined by the ELISA. A result was only confirmed positive when all three assays were positive. Ultimately, a seroprevalence of 1% was found after subsequent testing.

The use of an IgG ELISA as an initial hantavirus screening assay, followed by WB and IFA analysis as confirmatory tests, forms part of an IgG screening algorithm first used and described in a hantavirus seroepidemiological study in Guinea (Klempa et al., 2013). In the former study, 253 sera samples were collected from two rural villages in Guinea and screened using an IgM and IgG ELISA comprising of DOBV, PUUV and SANGV recombinant nucleocapsid antigens. Thirty – two out of the 253 sera were reactive (12.2%), and after confirmatory testing using an IFA and WB, a seroprevalence of 1.2% was confirmed (Klempa et al., 2013).

In both studies conducted by Klempa et al. (2013) and Witkowski et al. (2014), specificity was preferred over sensitivity due to the subsequent confirmatory tests decreasing the overall sensitivity. However, both studies were conducted in areas where no information is available regarding true hantavirus infections, and for this reason, emphasis should be placed more on specificity than sensitivity. The initial IgG ELISA screening results obtained in this study (11.6%) are similar to the initial ELISA results obtained in the two previously mentioned studies. Therefore, using an IgG ELISA consisting of recombinant nucleocapsid antigens of DOBV, PUUV and HTNV can be used as the first screening method. However, confirmatory tests such as a neutralisation assay are required to determine a true infection rate. This poses a challenge due to the high levels of biosafety requirements needed to propagate and culture hantaviruses and was beyond the scope of this study.

Based on the statistical analysis, no significant exposure risk was observed for positive IgG participants. This may be because of the statistical analyses being performed on all seropositive participants after initial screening. Subsequent confirmatory testing would have expectedly decreased the number of positive reactors, which ultimately might contribute to some statistical significance due to working with true positive specimens. However, this would only be speculative and confirmatory testing would be required to support any evidence.

It should be noted that limited information exists on the species, distribution, transmission, reservoir host or pathogenicity of hantaviruses in SA. Only serological investigations have suggested the presence of these viruses in the country (Lee et al., 1999; Witkowski et al., 2014). The most well established and common mode of transmission of hantaviruses is

through the inhalation of aerosols contaminated with infected rodent excreta (Hardestam et al., 2008). Therefore, any outdoor activity mentioned in this study can be considered high-risk when rodents are known to occur in the environment.

The results obtained for the seroprevalence of *Leptospira* sp., *Brucella* sp. and hantaviruses in various high-risk occupations in SA can be used as a basis for justifying further studies and the development and establishment of adequate preventive or control measures.

Chapter 4: Conclusion

The control of emerging zoonotic diseases throughout the world requires an integrated approach that includes human and animal health. The rapid response to recent zoonotic outbreaks, such as the SARS-CoV 2 pandemic, Hendra virus in Australia, West Nile virus in the US, and Ebola virus in West and Central African countries, demonstrates the effectiveness of financial and political support to control emerging diseases on a global scale (Mableson et al., 2014).

However, in contrast to emerging infectious diseases, neglected zoonotic diseases are found throughout the world. They pose a chronic and insidious threat to animal and human health, responsible for the deaths of approximately half a million people each year (Christou, 2011). Zoonotic TB, brucellosis, leptospirosis and hantaviruses are common in the developing world, supported by the close proximity and reliance people have on their livestock or wildlife, residing in rural areas, and poor resilience, which all favours transmission.

This study demonstrates the effectiveness of control schemes in lowering the incidence rate of *M. bovis* in cattle, ultimately resulting in decreased transmission and human infection. Another explanation for the lower incidence rate could be an absent reservoir host (i.e. buffalo or other wildlife species). Surprisingly, in this study, the only two TB positive animals are also suspected *M. tuberculosis* infections, based on the farm's history, possibly indicating transmission from humans to animals. The first case reports of *M. tuberculosis* infection in cattle in SA were only recently reported and published by Hlokwe et al. (2017), indicating that increased transmission of *M. tuberculosis* from humans (or other possible sources) to animals may occur. However, this is only speculative, and culture results from both animals are needed to confirm *M. tuberculosis*.

The use of TST is still an effective and convenient screening method for bTB. However, as mentioned previously, the INF-y release assay provides far fewer false-positive results and should still be considered an ancillary test to TST. It could be argued that the INF-y assay should be performed more regularly, especially on farms with incoherent skin reactions as a result of environmental *Mycobacterium* sp. contamination. Unlike avian *Mycobacterium* sp.

where a differentiation could be made from *M. bovis* using a CIST, environmental *Mycobacterium* sp. are far more present and difficult to distinguish.

In contrast, although the incidence rate of BB in this study is relatively low and corresponds to results obtained in other studies, a significantly higher incidence rate was witnessed in cattle and farm workers within the communal setting compared to the commercial farms. More effort should therefore be made to include rural farming communities in control strategies.

Although compliance within these communities remains a challenge, different approaches should be considered to increase participation. For example, in this study, farmers received lumpy skin disease vaccinations when animals were brought for the initial TST. After 72h when results were gathered, branding and general inspections were given. Public health officials should also prioritize education and awareness in these communities to promote human and animal health, emphasizing zoonotic disease transmission and prevention. Several studies have shown that a lack of education and general disease awareness is directly associated with higher infection rates in humans (Tebug et al., 2015; Alemayehu et al., 2021).

In this study, occupational exposure to *Brucella* sp. was the most significant in veterinarians and laboratory workers (p-value < 0.05). Both occupations have been previously identified and described as high-risk occupations for *Brucella* sp., but have not been documented in the Free State province. However, a larger sample pool of laboratory workers is required for more in-depth analysis.

Veterinarians may inadvertently act as biological sentinels for *Brucella* sp. Although human-to-human transmission of *Brucella* sp. is rare, several instances have been reported. A review by Tuon et al. (2017) on 45 human-to-human transmission cases of *Brucella* sp. identified 61% of cases occurring in children less than one year of age (congenital and breastfeeding). Other cases included blood transfusion, sexual transmission, bone marrow transplantations and aerosols from infected patients. Therefore, veterinarians may potentially spread infections to other humans. For this reason, professional and policy measures should be implemented and regulated to decrease the risk of veterinarians becoming infected.

This report identified abattoir workers as a high-risk occupational group for *Leptospira* sp. in the Free State, indicating that *Leptospira* sp. are circulating in high concentrations in livestock within the Free State province. More frequent and thorough investigations are therefore required to determine the prevalence of *Leptospira* sp. in livestock and to identify the serovars circulating for diagnostic purposes. Abattoirs should also enforce more strenuous preventative measures to reduce infections.

Occupational exposure to hantavirus were also evaluated and although the initial hantavirus IgG seroprevalence data are the same as those obtained in other studies (Klempa et al., 2013; Witkowski et al., 2014), no occupational exposure or other risk factors were statistically significant. However, confirmatory testing would provide more information regarding hantavirus exposure, as no information currently exists on the transmission and pathogenicity of these viruses in SA.

In SA, a large proportion of the human population are still dependent on animals for their livelihood, whether as a source of food,trade, companionship, or the services they provide. Therefore, more emphasis should be placed on populations at higher risk of contracting zoonotic infections regarding epidemiological investigations. Identifying high-risk populations for different zoonotic infections across different geographical regions will ultimately aid in implementing effective preventative measures, and assisting clinicians in diagnosing patients who present undifferentiated febrile illness.

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Appendices

Appendix A: Consent document

CONSENT DOCUMENT HSREC number: UFS-HSD2019/10752708

CONSENT TO PARTICIPATE IN RESEARCH

You have been asked to participate in a research study. You have been informed about the study by ……..

You may contact Dr J. Musoke (051) 405 3462 or Mr. C.G van der Westhuizen (+27) 76 811 1810 at any time if you have questions about the research. You may contact the Secretariat of the Health Sciences research Ethics Committee of the Faculty of Health Sciences, UFS at telephone number (051) 4052812 if you have questions about your rights as a research subject.

Your participation in this research is voluntary, and you will not be penalized if you refuse to participate or decide to terminate participation. If you agree to participate, you will be given a signed copy of this document as well as the participant information sheet, which is a written summary of the research.

The research study, including the above information has been verbally described to me. I understand what my involvement in the study means and I voluntarily agree to participate. I consent that

1. I will donate blood and produce a sputum sample on 1 occasion as described in the information document
2. My donated blood may be stored and analyzed as part of future research related to zoonotic and arboviral research
3. Research data obtained from the study may be published anonymously in a scientific journal

Name of participant: Telephone no:
Email address:

Signature of Participant	Date
Signature of Witness *(Where applicable)*	Date
Signature of Translator *(Where applicable)*	Date

Appendix B: Information document

INFORMATION DOCUMENT

Study title: Zoonotic diseases in high-risk populations in the Free State province, South Africa.

Good day

We, the Department of Microbiology and Division Virology, are doing research to determine if there are cases of zoonotic diseases (*Mycobacterium bovis*, *Brucella* spp., *Leptospira* spp., *Coxiella burnetii* and hantaviruses) in people who are generally healthy but work in places that puts them at a higher risk for getting these infections that are transmitted from animals to humans. These diseases are transmitted to humans from animals by drinking unpasteurized milk, contact with blood and tissue of infected animals, inhaling dust that are contaminated and through tick-bites. In this project we want to test your blood for antibodies (proteins that fight against viruses and bacteria) against these diseases and store your blood for future tests to determine the prevalence of other diseases spread by insects and animals. We will also use the sputum sample you produce to look for a possible infection with a bacterium (*Mycobacterium bovis*) usually found in cattle that may cause tuberculosis (TB).

Invitation to participate: We are inviting you to participate in this research study

What is involved in the study: We are inviting people who work in areas that put them at a higher risk for getting zoonotic diseases, to participate in the study. We will request blood and sputum from you on 1 occasion. We require 2 x 10 ml blood tube and 1 sputum sample. Patients who survive infection with these bacteria and viruses have antibodies against them in their blood. Therefore, we want to test your blood to determine if you have been exposed to these diseases at some time in your life. Your sputum will be used to see if you have an infection with TB that you could've gotten from animals.

Risks: There are no foreseeable risks of being involved in the study:

Benefits: If we can identify which bacteria and viruses are circulating in the area and how they are most likely transmitted from animals to humans. Then we can inform public health officials

in the area. This will aid in clinical awareness amongst health practitioners.

Participation is voluntary and refusal to participate will involve no penalty; the subject may discontinue participation at any time.

Reimbursements: All procedures will be performed at no extra cost to you. You will not receive any reimbursement for participating in the study.

Cost: Participants will not pay any costs to participate in the study

Confidentiality: All information provided by you will be kept strictly confidential. Data obtained from the questionnaire will be anonymous. The consent form is the only document that will contain the participants' name. From that point onwards, a sample number will be assigned to the questionnaire form.

Contact details of researcher– Mr. C.G van der Westhuizen (+27) 76 811 1810, Dr J.M. Musoke (051) 405 3462 MusokeJ@ufs.ac.za, Department of Medical Microbiology, University of the Free State, Bloemfontein.

Contact details of REC Secretariat and Chair – For reporting complaints or problems please contact

Ms M Marais, Research Division, Faculty of Health Sciences, University of the Free State, E-mail address: EthicsFHS@ufs.ac.za ☎ (051) 4052812

Appendix C: Questionnaire

Sample number:_____ **Date**_____

Gender:_____ **Location**_____

Age:_____

1. How long have you been a farm worker? _____

2. What animals are you responsible for?

Cattle	Sheep	Goats	Pigs	Horses	Other

3. Which of the following duties are you responsible for?

Herding	Feeding	Dipping	Milking	Sheep shearing	Other

4. Do you have a problem with rodents (mice, rats) at your home?

Yes	No

5. Have you ever worked with aborted foetuses, or assisted in livestock birthing?

Yes	No

If yes, how often?

> 5 times	< 5 times

6. Have you ever consumed unpasteurised milk or milk products?

Yes	No

If yes, how often?

Daily	Weekly	Monthly	Yearly

7. Do you have any pets living with you in your household?

Yes	No

8. Have you had any previous exposure to livestock or wildlife?

Yes	No

9. Have you ever had a serious illness after you were exposed to animal tissues?

Yes	No

10. Have you ever been diagnosed with tuberculosis (TB)?

Yes	No	Don't want to answer

If yes, when were you diagnosed, within?

The past 6 months	The past 12 months	Over 1 year ago

If no, have you ever had swollen lymph nodes (*see picture below*)?

Yes	No	I Don'tknow

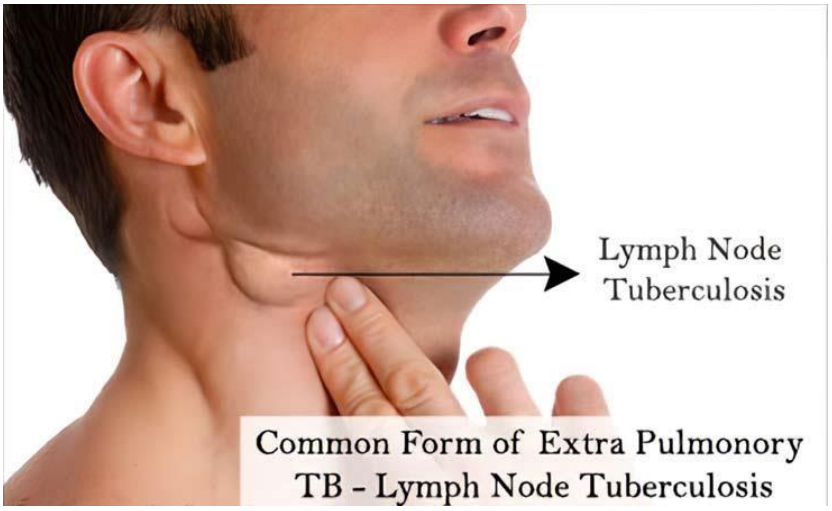

Figure 1: An example of swollen lymph nodes from a person with extra-pulmonary TB. *Available at:https://www.medindia.net/patients/patientinfo/extra-pulmonary-tuberculosis.htm*

Appendix D: Health Sciences Research Ethics Committee ethical clearance letter

Health Sciences Research Ethics Committee

15-Aug-2019

Dear **Mr Cornelius Van Der Westhuizen**

Ethics Clearance: **Prevalence of zoonotic diseases in the Free State and Limpopo Province, South Africa** Principal Investigator: **Mr Cornelius Van Der Westhuizen**
Department: **Medical Microbiology Department (Bloemfontein Campus)**

APPLICATION APPROVED

Please ensure that you read the whole document

With reference to your application for ethical clearance with the Faculty of Health Sciences, I am pleased to inform you on behalf of the Health Sciences Research Ethics Committee that you have been granted ethical clearance for your project.

Your ethical clearance number, to be used in all correspondence is:**UFS-HSD2019/1075/2708**

The ethical clearance number is valid for research conducted for one year from issuance. Should you require more time to complete this research, please apply for an extension.

We request that any changes that may take place during the course of your research project be submitted to the HSREC for approval to ensure we are kept up to date with your progress and any ethical implications that may arise. This includes any serious adverse events and/or termination of the study.

A progress report should be submitted within one year of approval, and annually for long term studies. A final report should be submitted at the completion of the study.

The HSREC functions in compliance with, but not limited to, the following documents and guidelines: The SA National
Health Act. No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP(2006); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 461 (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services- (HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; The International Conference on Harmonization and Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH Tripartite), Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the HSREC of the Faculty of Health Sciences.

For any questions or concerns, please feel free to contact HSREC Administration: 051-4017794/5 or email EthicsFHS@ufs.ac.za.

Thank you for submitting this proposal for ethical clearance and we wish you every success with your research.

Yours Sincerely

Dr. SM Le Grange
Chair : Health Sciences Research Ethics Committee

Health Sciences Research Ethics Committee
Office of the Dean: Health Sciences
T: +27 (0)51 401 7795/7794 | E: ethicsfhs@ufs.ac.za
IRB 00006240; REC 230408-011; IORG0005187; FWA00012784
Block D, Dean's Division, Room D104 | P.O. Box/Posbus 339 (Internal Post Box G40) | Bloemfontein 9300 | South Africa www.ufs.ac.za

Appendix E: Health Sciences Research Ethics Committee subsequent approval letter

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

Health Sciences Research Ethics Committee

16-Oct-2020

Dear **Mr Cornelius Van Der Westhuizen**

Ethics Number: UFS-HSD2019/1075/270801

Ethics Clearance: **Prevalence of zoonotic diseases in the Free State Province, South Africa**

Principal Investigator: **Mr Cornelius Van Der Westhuizen**

Department: **Medical Microbiology Department (Bloemfontein Campus)**

SUBSEQUENT SUBMISSION APPROVED

With reference to your recent submission for ethical clearance from the Health Sciences Research Ethics Committee. I am pleased to inform you on behalf of the HSREC that you have been granted ethical clearance for your request as stipulated below:

- Minor Amendment:

Title change- there will be no sample collection performed in Limpopo, therefore, "Limpopo" is excluded from the title.

Sample collection of approximately 250 abattoir workers in Limpopo will no longer take place. Instead, 381 samples previously obtained for another zoonotic study will be used.

The HSREC functions in compliance with, but not limited to, the following documents and guidelines: The SA National Health Act.

No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP(2006); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 461 (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services- (HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; The International Conference on Harmonization and Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH Tripartite), Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the HSREC of the Faculty of Health Sciences.

For any questions or concerns, please feel free to contact HSREC Administration: 051-4017794/5 or email EthicsFHS@ufs.ac.za.

Thank you for submitting this request for ethical clearance and we wish you continued success with your research.

Yours Sincerely

Dr. SM Le Grange
Chair : Health Sciences Research Ethics Committee

Health Sciences Research Ethics Committee
Office of the Dean: Health Sciences
T: +27 (0)51 401 7795/7794 | E: ethicsfhs@ufs.ac.za
IRB 00011992; REC 230408-011; IORG 0010096; FWA 00027947
Block D, Dean's Division, Room D104 | P.O. Box/Posbus 339 (Internal Post Box G40) | Bloemfontein 9300 | South Africa www.ufs.ac.za

Appendix F: Animal Research ethical clearance letter

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA
UFS UV

Animal Research

25Oc-

Dear Mr Cornelius Van Der

Student Project Number: UFS-

Project Title: Zoonotic diseases in high-risk populations in the Free State and Limpopo

Department: Medical Microbiology Department (Bloemfontein Campus)

You are hereby kindly informed that, at the meeting held on 24-Oct-2019, the Ethics Committee approved the above

Kindly take note of the

1
A progress report with regard to the above study has to be submitted Annually and on completion of the project. Reports are submitted by logging in to RIMS and completing report as described in SOP AEC007: Submission of Protocols, Modifications, Reports and Reporting of Adverse Events which is available on the UFS

2
Researchers that plan to make use of the Animal Experimentation Unit must ensure to and receive a quotation from the Head, Mr. Seb

3
Fifty (50%) of the quoted amount is payable when you receive the letter of

Yours

Mr. Gerhard Johannes van
Chair: Animal Research Ethics

Appendix G: Environmental & Biosafety ethical clearance letter

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
RESEARCH DEVELOPMENT
NAVORSINGSONTWIKKELING

Environmental & Biosafety Research Ethics Committee

17-Oct-2019

Dear **Mr Cornelius Van Der Westhuizen**

Project Title: **Zoonotic diseases in high-risk populations in the Free State and Limpopo provinces, South Africa.**

Department: **Medical Microbiology Department (Bloemfontein Campus)**

APPLICATION APPROVED

This letter confirms that this research proposal was given ethical clearance by the Biosafety & Environmental Research Ethics Committee of the University of the Free State.

Your ethical clearance number, to be used in all correspondence is: **UFS-ESD2019/0086**

Please note the following:

1. **This ethical clearance is valid for one year from the issuance of this letter.**
2. **If the research takes longer than one year to complete, please submit a Continuation Report to the Ethics Committee before ethical clearance expires.**
3. **If any changes are made during the research process (including a change in investigators), please inform the Ethics Committee by submitting an Amendment.**
4. **When the research is concluded, please submit a Final Report to the Ethics Committee.**

Thank you for your application and we wish you well in all of your research endeavours.

Yours Sincerely

Prof. RR (Robert) Bragg
Chairperson: Biosafety & Environmental Research Ethics Committee
University of the Free State

Directorate: Research Development
T: +27 (0)51 401 9398 | +27 (0)51 401 2075 | E: smitham@ufs.ac.za
Johannes Brill Building, Room 106D, First Floor
205 Nelson Mandela Drive | Park West, Bloemfontein 9301 | South Africa
P.O. Box 339 | Bloemfontein 9300 | South Africa | www.ufs.ac.za

Appendix H: Department of Agriculture, Forestry and Fisheries section 20 permit

agriculture,
forestry &
fisheries

Department:
Agriculture, Forestry and Fisheries

REPUBLIC OF SOUTH AFRICA
Directorate Animal Health, Department of Agriculture, Forestry and Fisheries Private Bag
X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 . E-mail: Herr-yG@daff.gov.za Reference:
12/11/1/4 (1256)

Dr Jolly Musoke
Faculty: Health Sciences University of the Free Sate

Tel: 051 405 3019

E-mail: MusokeJ@ufs.ac.za

Dear Dr Musoke,

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)

Your application sent with the email on 26 July 2019 requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers. I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1 . This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;

2. The study is approved as per the application form received on 26 July 2019 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this study under this Section 20 permit. Please apply in writing to HerryG@daff.gov.za;

3. The study must be conducted in compliance with the Veterinary and Para-Veterinary Professions Act 1982 (Act No. 19 of 82);

4. Sampling may only be conducted in partnership with Free State Province Veterinary Services in the Kroonstad area as indicated and may only originate from farms/ areas that are not under relevant veterinary restriction. As indicated during the

application process, only bovine samples collected through Veterinary Services will be utilised and may include blood and milk samples;

5. Samples may be sent to the ARC-OVR for Tuberculosis and Brucellosis diagnostics as indicated;
6. Samples must be packaged and transported in accordance with the National Road Traffic Act, 1996 (Act No. 93 of 1996);
7. Any incidence or suspected incidence of a controlled or notifiable disease in terms of the Animal Diseases Act 1984 (Act no 35 of 84), must be reported immediately to the responsible State Veterinarian of the area;
8. All potentially infectious material utilised, collected or generated during the study is to be destroyed at the completion of the study using the specified waste contractor. Records must be kept for five years for auditing purposes;
9. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 approval.

Title of research/study: "Zoonotic diseases in high-risk populations in the Free State and Limpopo provinces, South Africa".

Researcher: Dr Jolly Musoke

Institution: Faculty: Health Sciences, University of the Free Sate

Our ref Number: 12/11/1/4 (1256)

Your ref: UFS-HSD2019/1075

Expiry date: December 2020

Kind regards,

DR. MPHO MAJA

DIRECTOR OF ANIMAL HEALTH

DIRECTOR ANIMAL HEALTH

Date: 2019 -08- 0 8

SUBJECT: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)

Appendix I: SICT and CIST results of positive and suspect cattle in the Maokeng community and commercial dairy farm A.

Single intradermal tuberculin skin test results of suspect cattle from the Maokeng community kraal.

Tag no.	Normal skinfold baseline (mm).	Skinfold after 72h (mm).	Bovine difference (72h – baseline) in mm.	Remarks	Diagnosis
15728	10.9	17.6	6.7	C	Suspect
15743	9.3	16.4	7.1	C	Suspect
10763	9.8	16.2	6.4	C	Suspect
C: Circumscribed, mm: millimetres, h:hours					

Single intradermal tuberculin skin test results of suspect cattle from commercial dairy farm A.

Tag no.	Normal skinfold (mm).	Skinfold after 72h (mm).	Bovine difference (72h – normal) in mm.	Remarks	Diagnosis
157	5.9	12.2	6.3	C	Suspect
154	6.3	15.6	9.3	C	Suspect
141	7.8	16.4	8.6	C	Suspect
158	8.1	17.5	9.4	C	Suspect
C: Circumscribed, mm: millimetres, h:hours					

Comparative intradermal tuberculin skin test results of the four SIST suspect animals in commercial dairy farm A.

	Bovine tuberculin			Avian tuberculin					
Tag no.	**Normal skinfold (mm).**	**Skinfold after 72h (mm).**	**Bovine difference (72h – normal) in mm.**	**Normal skinfold (mm).**	**Skinfold after 72h (mm).**	**Avian difference (72h – normal) in mm.**	**Bovine Diff – Avian Diff (mm).**	**Remarks**	**Diagnosis**
157	5.7	7.2	1.5	6.3	7.1	0.8	0.7	NAD	Negative
154	5.9	9.3	3.4	6.8	8.7	1.9	1.5	NAD	Negative
141	6.1	7.6	1.5	6.8	6.8	0	1.5	NAD	Negative
158	7.7	9.6	1.9	9.3	10.4	1.1	0.8	NAD	Negative
NAD: No abnormalities detected, mm: millimetres, h:hours									

Appendix J: CIST results of positive and suspect cattle from commercial dairy farm B

Comparative intradermal tuberculin skin test results of the 16 animals with positive and suspect reactors in commercial dairy farm B.

	Bovine tuberculin			Avian tuberculin					
Tag no.	**Normal skinfold (mm).**	**Skinfold after 72h (mm).**	**Bovine difference (72h – normal) in mm.**	**Normal skinfold (mm).**	**Skinfold after 72h (mm).**	**Avian difference (72h – normal) in mm.**	**Bovine Diff – Avian Diff (mm).**	**Remarks**	**Diagnosis**
247	7.1	11.1	4.0	6.9	6.7	-0.2	4.2	C	Positive
418*	9.3	13.9	4.6	7.8	7.7	-0.1	4.7	C	Positive
420*	5.8	10.9	5.1	6.7	7.2	0.5	4.6	C	Positive
518*	5.9	15.4	9.5	7.4	10.0	2.8	6.7	C	Positive
523*	7.1	13.8	6.7	6.8	7.9	1.1	5.6	C	Positive
524*	7.5	17.3	9.8	8.4	7.8	-0.6	10.4	A, skin condition	Positive
606*	5.2	10.8	5.6	6.1	6.9	0.8	4.8	C, mild D	Positive
207*	5.4	8.6	3.2	6.2	6.1	-0.1	3.3	C	Suspect
250*	6.3	11.1	4.8	7.7	8.6	0.9	3.9	C	Suspect
253	6.1	10.2	4.1	6.7	7.7	1.0	3.1	C	Suspect
508	7.5	11.6	4.1	8.7	9.1	0.4	3.7	C	Suspect
513*	6.2	10.9	4.7	7.3	8.1	0.8	3.9	C	Suspect
521*	5.8	13.1	7.3	6.4	8.3	1.9	5.4	C	Positive
604*	8.8	13.3	4.5	8.6	9.4	0.8	3.7	F	Suspect
605*	6.6	11.6	5.0	8.2	9.6	1.4	3.6	C	Suspect
622*	6.8	11.2	4.4	9.2	9.8	0.6	3.8	C	Suspect

C: Circumscribed, A: Adhesions, D: Diffuse, F: Flat mm: millimetres, h:hours
****: animals from which whole blood was collected for IFN-y assay.***

Appendix K: Brucella IgG ELISA plate results measured at 450nm.

Key	
	Positive control
	Negative control
	Cut-off control
	Specimen

	1	2	3	4	5	6	7	8	9	10	11	12
A	3,327	0,403	0,619	0,343	0,393	0,175	0,223	0,747	0,138	0,231	0,191	0,327
B	0,059	2,399	0,141	0,826	0,29	0,207	0,274	0,175	0,463	0,738	0,234	0,308
C	0,919	0,431	0,193	0,152	0,144	0,249	1,936	0,29	0,194	0,116	0,605	0,211
D	0,849	2,064	0,226	0,357	0,244	0,19	0,571	0,207	0,109	0,627	0,625	0,223
E	0,307	2,298	0,311	0,155	0,285	0,227	0,271	0,268	0,206	0,475	0,23	
F	0,239	0,136	1,495	0,179	0,347	0,2	0,242	0,233	0,221	0,27	0,231	
G	0,669	1,759	0,316	0,191	0,277	0,166	0,566	0,706	0,124	0,154	0,161	
H	2,403	1,233	0,266	0,464	0,3	1,65	0,775	0,544	0,189	1,151	0,57	

	1	2	3	4	5	6	7	8	9	10	11	12
A	2,693	0,17	1.346	0,123	0,103	0,112	0,201	0,119	2,551	0,496	0,128	0,553
B	0,154	0,155	0,191	0,289	0,205	0,521	0,144	0,77	0,203	0,228	0,205	0,369
C	0,929	0,155	0,232	0,099	0,159	0,156	0,332	0,128	0,175	0,12	0,142	0,117
D	1,044	0,138	0,323	0,16	0,251	0,229	0,144	0,11	0,198	0,166	0,209	1,861
E	0,15	0,273	0,154	0,199	0,232	0,232	0,133	0,116	0,638	0,112	0,183	
F	0,077	0,363	0,173	0,16	0,337	0,801	0,113	0,303	0,172	0,267	2,127	
G	1.182	0,284	0,122	0,441	0,381	0,382	0,193	0,192	0,18	0,19	0,322	
H	0,165	0,309	0,211	0,125	0,219	0,15	0,087	0,219	0,168	1,934	0,911	

	1	2	3	4	5	6	7	8	9	10	11	12
A	3,214	0,166	0,118	0,247	0,115	0,372	0,129	1,792	0,177	0,188	0,406	0,188
B	0,059	0,639	0,123	0,139	0,127	0,087	0,214	0,216	0,141	0,221	0,197	0,107
C	1,125	0,151	0,115	0,33	0,337	0,148	0,175	0,854	0,16	0,306	0,246	0,149
D	1,104	0,562	0,547	0,184	0,229	0,111	0,133	0,116	0,291	0,418	0,159	0,306
E	0,915	0,189	0,162	0,157	0,151	0,092	0,992	0,144	0,172	1,741	2,08	
F	0,138	0,251	0,118	0,163	0,287	1,964	0,331	0,168	1,87	0,098	2,023	
G	0,126	0,346	0,22	0,188	0,095	0,186	0,274	0,136	0,254	0,408	1,98	
H	0,448	0,618	1,564	0,183	0,179	0,114	0,182	0,156	1.04	0,139	0,17	

	1	2	3	4	5	6	7	8	9	10	11	12
A	2,653	0,124	1,026	0,081	0,278	0,279	0,415	1,346	0,531			
B	0,039	0,291	0,46	1,339	1,071	1,067	0,673	0,549	0,698			
C	0,834	0,775	0,202	0,396	0,083	0,134	0,194	0,588	1,444			
D	0,902	0,18	0,164	0,411	0,18	0,217	0,901	0,154				
E	0,095	0,276	0,202	0,204	0,249	0,179	0,117	0,13				
F	0,197	0,597	0,158	1,001	0,121	1,861	0,265	0,246				
G	0,156	0,177	0,114	1,531	0,611	0,133	0,299	0,082				
H	0,268	0,252	0,292	0,502	1,154	0,067	0,404	0,208				

Appendix L: Leptospira IgM ELISA plate results measured at 450nm.

Key	
	Positive control
	Negative control
	Calibrator
	Specimen

	1	2	3	4	5	6	7	8	9	10	11	12
A	2,193	0,114	0,277	0,143	0,124	0,479	0,386	0,88	0,359	0,219	0,172	0,829
B	0,049	0,217	0,571	1,027	0,318	0,179	0,388	0,955	0,782	0,13	0,126	0,184
C	1,044	0,173	0,074	0.955	0,082	0,398	0,208	0,309	0,074	0,817	0,157	0,271
D	1,049	0,509	0,119	0,082	0,489	0,205	0,945	0,216	0,165	0,177	0,228	0,531
E	1,068	0,413	0,118	0,209	0,082	0.817	0,454	0,155	0,052	0,348	1,062	0,13
F	0,215	0,295	0,185	0,287	0,228	0,536	0,106	0,549	0,542	0,11	0,456	
G	0,217	0,197	0,11	0,213	0,283	0,122	0,068	0,214	0,15	0,589	0,278	
H	0,074	0,194	1,496	0,415	1,288	0,953	0,27	0,192	0,278	0,261	0,471	

	1	2	3	4	5	6	7	8	9	10	11	12
A	1,935	0,39	0,288	0,381	0,06	0,155	0,156	0,218	0,65	0,4	0,433	0,416
B	0,043	0,161	0,141	0,115	0,211	0,36	0,126	0,223	0,239	0,555	0,1	0,761
C	1,264	0,507	1,156	0,339	0,262	0,146	0,151	0,203	0,107	0,192	0,794	0,335
D	0,805	0,337	0,367	0,088	0,101	0,121	0,105	0,097	0,444	0,07	0,102	0,359
E	0,888	0,456	0,231	0,074	0,108	0,406	0,572	0,093	0,473	0,328	0,139	0,241
F	0,183	0,091	0,083	0,241	0,278	0,352	0,135	0,308	0,12	0,154	0,108	
G	0,039	0,544	0,229	0,135	0,062	0,102	0,143	0,556	0,118	0,203	0,477	
H	0,398	0,089	0,364	0,225	0,389	0,069	0,126	0,339	1,188	0,106	0,149	

	1	2	3	4	5	6	7	8	9	10	11	12
A	2,272	0,544	0,069	0,18	0,094	0,241	0,305	0,432	0,173	0,594	0,368	0,664
B	0,049	0,339	0,184	0,118	0,233	0,237	0,108	0,154	0,09	0,52	0,114	0,338
C	1,068	0,112	0,206	0,172	0,186	0,363	0,088	0,744	0,354	0,233	0,288	0,084
D	0,939	0,209	0,131	0,386	0,231	0,167	0,455	0,154	0,419	0,149	0,462	0,54
E	0,995	1,502	0,439	0,054	0,095	0,168	0,191	0,211	0,79	0,152	0,693	0,386
F	0,331	0,176	0,08	0,187	0,174	0,126	0,219	0,168	0,137	0,252	0,275	
G	0,747	0,279	0,187	0,184	0,123	0,194	0,167	0,143	0,075	0,343	0,195	
H	0,32	0,504	0,219	0,186	0,23	0,527	0,076	0,157	0,074	0,384	0,119	

	1	2	3	4	5	6	7	8	9	10	11	12
A	2,271	0,273	0,562	0,405	0,292	0,098	0,27	0,638	0,117			
B	0,052	0,306	0,099	0,244	0,649	0,788	0,175	0,105	0,438			
C	1,357	0,327	0,409	0,627	0,141	0,413	0,577	0,221	0,576			
D	1,393	0,127	0,088	0,155	0,466	0,545	0,215	0,589	0,255			
E	1,169	0,592	0,31	0,219	0,091	0,097	0,521	0,653				
F	0,231	0,524	0,403	0,135	0,114	0,787	0,162	0,181				
G	0,601	0,28	0,083	0,975	0,379	0,177	0,773	0,177				
H	0,191	0,324	1,106	0,233	0,101	0,275	0,7	0,821				

Appendix M: Hantavirus IgG ELISA plate results measured at 450nm.

Key	
	Positive control
	Negative control
	Calibrator
	Specimen

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,91	0,244	0,104	0,156	0,267	0,282	0,573	0,214	0,342	0,448	0,873	0,297
B	0,095	0,18	1,382	0,144	0,086	0,536	0,253	0,158	0,274	0,206	0,778	0,342
C	1,47	0,433	0,199	0,241	0,141	0,273	0,136	0,158	0,246	0,121	0,136	0,125
D	0,387	0,176	0,196	0,131	0,164	0,162	0,123	0,25	0,133	0,261	0,476	0,22
E	0,074	0,117	0,308	0,827	0,131	0,152	0,222	0,128	0,119	0,365	0,218	0,143
F	0,194	0,207	0,349	0,313	0,236	0,438	0,249	0,271	0,193	0,116	0,508	
G	0,181	0,177	0,753	0,106	0,171	0,167	0,136	0,756	0,566	0,805	0,193	
H	0,299	0,369	0,24	0,133	0,303	0,184	0,54	0,233	0,276	0,091	0,235	

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,979	0,171	0,165	0,494	0,373	0,006	0,281	0,238	0,247	0,116	0,162	0,216
B	0,096	0,232	0,256	0,294	0,146	0,24	0,477	0,328	0,201	0,136	0,144	0,332
C	1,533	0,332	0,217	0,298	0,291	0,167	0,173	0,127	0,166	0,26	0,074	0,101
D	0,426	0,315	0,235	0,087	0,29	0,242	0,338	0,159	0,113	0,264	0,077	0,191
E	0,072	0,373	0,179	0,068	0,273	0,348	0,275	0,218	0,297	0,252	0,109	0,247
F	0,273	0,275	0,183	0,156	0,334	0,262	0,278	0,489	0,255	0,114	0,199	
G	0,054	0,267	0,25	0,225	0,386	0,313	0,498	0,257	0,224	0,121	0,5	
H	0,216	0,548	0,281	0,191	0,489	0,34	0,269	0,199	0,629	0,218	0,08	

	1	2	3	4	5	6	7	8	9	10	11	12
A	1,008	0,157	0,064	0,132	0,361	0,345	0,103	0,349	0,233	0,335	0,188	0,264
B	0,102	0,564	0,309	0,344	0,176	0,214	0,152	0,114	0,223	0,274	0,112	0,352
C	1,544	0,23	0,554	0,306	0,216	0,086	0,243	0,284	0,11	0,096	0,527	0,285
D	0,471	0,134	0,23	0,356	0,249	0,092	0,142	0,124	0,098	0,121	0,22	0,371
E	0,077	0,405	0,141	0,257	0,229	0,122	0,13	0,353	0,162	0,209	0,152	0,145
F	0,745	0,184	0,334	0,408	0,175	0,099	1,141	0,14	0,178	0,197	0,167	
G	0,274	0,271	0,129	0,354	0,359	0,156	0,186	0,303	1,34	0,221	0,184	
H	0,669	0,178	0,192	0,752	0,238	0,222	0,161	0,156	0,242	0,126	0,327	

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,82	0,589	0,302	0,707	0,168	0,146	0,138	0,234	0,101			
B	0,053	0,134	0,274	0,031	0,238	0,289	0,184	0,215	0,118			
C	1,519	0,194	0,904	0,278	0,093	0,196	0,355	0,393	0,128			
D	0,401	0,151	0,135	0,262	0,107	0,136	0,513	0,179	0,166			
E	0,028	0,242	0,158	0,066	0,086	0,16	0,174	0,285				
F	0,059	0,077	0,348	0,14	0,165	0,063	0,203	0,447				
G	0,128	0,144	0,066	0,117	0,801	0,042	0,185	0,05				
H	0,217	1,444	0,166	0,151	0,356	0,075	0,386	0,128				