

Prevalence and risk factors of *Coxiella burnetii* infection in cattle on farms in Limpopo province, South Africa

By

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Submitted in fulfilment of the requirements for the degree

Master of Science (Production Animal Studies)

in the Faculty of Veterinary Science, University of Pretoria

2022

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DECLARATION

I, Vhahangwele Sadiki, declare that this dissertation is the original report of my research and that any person's work used in this report has been properly acknowledged and referenced. In this report, I did not use or copy work completed by another student.

Signature..... Date.....

ACKNOWLEDGEMENTS

This research project would not have been a success without:

- My supervisor, Professor Adesiyun for his guidance and patient mentoring throughout this study, and for signing off on all necessary paperwork.
- My co-supervisor, Dr. Gcebe for helping me get funding for this project, for her added support, guidance, patient mentoring, and for approving all necessary paperwork.
- My gratitude goes to the Red Meat Research and Development in South Africa (RMRDSA) and the Department of Trade, Industry and Competition (DTI)-THRIP for their financial support.
- Thanks to Maruping Mangena for his advice and assistance with troubleshooting, Lesego Mashiane for driving long distances and assisting me with sampling, and also Zuki Miya for traveling with us to Limpopo to assist with sampling.
- Many thanks to the Limpopo Department of Agriculture and Rural Development permitting me to sample, as well as the state veterinarians and animal health technicians who assisted me with sampling and sample storage.
- The farmers and farm workers for allowing me to sample their cattle, for assisting with sampling, and for participating in the questionnaire.
- The entire Bacterial PCR Laboratory team at the Agricultural Research Council-Onderstepoort Veterinary Research (ARC-OVR) campus for cooperating with me and allowing me to use some of their supplies.
- My gratitude goes to Yusuf Ngoshe for his assistance with data analysis.
- My family, especially my mother for her continuous prayers, support, and encouragement throughout this project and my cousin, Tshifhiwa Malange, for his counsel and encouragement.

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ABBREVIATIONS

>	Greater than
°C	Degrees Celsius
ACCM2	Acidified cysteine citrate medium 2
AIC	Akaike's second-order information criterion analysis
AP	Apparent prevalence
ARC-OVR	Agricultural research council - Onderstepoort Veterinary Research
BTM	Bulk tank milk
CDC	Centers for Disease Control and Prevention
CFT	Complement fixation test
CGH	Comparative genomic hybridization
CI	Confidence interval
DAFF	Department of Agriculture, Forestry and Fisheries
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FMD	Foot and mouth disease
HEL	Human embryonic lung cells
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ITS	Internal transcribed spacer
LCV	Large cell variant
LPS	Lipopolysaccharide
MLVA	Multiple-locus variable-number tandem repeat
MST	Multispacer sequence typing
OR	Odds ratio
PCR	Polymerase chain reaction
QPCR	Quantitative polymerase chain reaction
<i>rpoB</i>	RNA polymerase β -subunit

RpoS	Stationary-phase sigma factor
SCV	Small cell variant
ScvA	Small-cell-variant protein A
Se	Sensitivity
SNP	Single nucleotide polymorphism
<i>sodB</i>	Superoxide dismutase
Sp	Specificity
UPGMA	Unweighted pair group method using arithmetic averages
VNTR	Variable number of tandem repeats
WHO	World Health Organization

SUMMARY

Title: PREVALENCE AND RISK FACTORS OF *COXIELLA BURNETII* INFECTION IN CATTLE ON FARMS IN LIMPOPO PROVINCE, SOUTH AFRICA

By

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Coxiella burnetii is a worldwide zoonotic bacterial pathogen and the causative agent for the zoonosis known as Q fever. The appearance of Q fever has been reported internationally and it is considered indigenous in most places in the world, but, in South Africa there are few studies of Q fever in livestock. This study aimed to determine the occurrence and prevalence of *C. burnetii* in farmed animals in Limpopo province, South Africa, and assess the risk of transmission to humans and animals.

This cross-sectional study was conducted in the Capricorn, Sekhukhune, and Waterberg district municipalities of Limpopo. Blood samples were collected from the selected cattle (male and female) from the coccygeal veins into tubes; vaginal swabs, as well as sheath wash samples, were also collected. All of the farmers and farm workers were unaware of Q fever during the sampling process. A structured questionnaire survey was administered to the farmers to collect demographic data and potential risk factors for exposure of animals and humans to *C. burnetii*. The IDEXX Q-FEVER 2/strip ELISA kit was used to detect IgG antibodies to *C. burnetii* infection. Conventional PCR targeting the *IS1111* gene fragment was used to determine the PCR prevalence. Data for ELISA and PCR were analysed separately using univariate logistic regression followed by multivariate logistic regression. To verify *Coxiella* *IS1111* gene fragment amplification, seven of the PCR products were sent to Inqaba Biotechnologies for sequencing of both the forward and reverse strands using an ABI sequencer.

Out of 383 cattle tested for antibodies against *C. burnetii*, the overall seroprevalence was 24.28% (93/383). Farms with a herd size > 150 had the highest seroprevalence of 70.37%, and a lower seroprevalence of 18.85% was observed in the herd size category of 1-50. Q fever prevalence by PCR in females was found to be 16.72% and 8.33% in males. Farms with a herd size greater than 150 had the highest PCR positivity of 44.44% and a PCR prevalence of 18.87% was observed in farms that fall under the category of 51-100 herd size. Commercial farms exhibited a prevalence of 18.14% of cattle testing positive by PCR and also a higher seroprevalence of 28.84% was observed in commercial farms. In the final multivariable logistic regression, local municipality (OR 1.09; 95% CI 1.00 - 1.20; P = 0.043) and herd size (OR 2.24; 95% CI 1.21 - 4.15; P = 0.010) remained associated with *C. burnetii* seropositivity in cattle. Only abortion history (OR 0.31; 95% CI 0.11 – 0.89; P = 0.030) remained associated with *C. burnetii* positivity by PCR. Molecular detection of *C. burnetii* in sheath scrapings and vaginal swabs by PCR targeting the IS1111 gene revealed that 15.67% were positive and the amplicons were 146 bp in size. Of the seven sequences analysed on NCBI BLAST for sequence identity, all had similarities to *C. burnetii* transposase gene fragment, confirming molecular detection of the bacterium. In conclusion, we investigated the seroprevalence, PCR prevalence, and risk factors correlated with *C. burnetii* in cattle on farms in Limpopo province, South Africa. This study discovered that *C. burnetii* is widespread in the study areas (Capricorn, Waterberg, and Sekhukhune) and should be regarded as a possible source of human Q fever.

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Coxiella burnetii is an intracellular pathogen that replicates in eukaryotic cells. The bacterium displays phagolysosome-like characteristics (acidic pH, cation peptide, and acid hydrolysates) which are acquired by its vacuole, and shows several adaptation skills to this stressful environment. The environment of the phagolysosome-like vacuole is acidic, and the proteins encoded by this bacterial pathogen buffer this environment (Eldin et al., 2017). The microorganism can occur in two forms, small cell variant (SCV) and large cell variant (LCV) (Angelakis & Raoult, 2010). The SCV form of the bacterium is stable and non-replicating, and it has condensed chromatin, a thick envelope, and appears as small rods, whereas the LCV form is a highly replicating form characterized by less dense chromatin and the envelope is similar to that of a gram-negative bacterium (Eldin et al., 2017). The bacterium can survive in dairy, meat products, aborted foetuses, manure, clothes, and animal feed for a long time. Small cell variants survive on wool for about 7-10 months, in fresh meat for a month and in milk for more than 40 months (Pexara et al., 2018).

Coxiella burnetii can exist in two antigenic phases, the virulent phase I and the avirulent phase II and are differentiated by a transformation in the lipopolysaccharide (LPS) from smooth to coarse (Stoker and Fiset, 1956; Baca and Paretsky 1983; Hackstadt et al., 1985). In phase I, the smooth LPS of *C. burnetii* interferes with the immune system's response which gives the bacterium the space and time to multiply and survive, thus making phase I a more infectious phase. The avirulent phase of *C. burnetii* remains questionable (Van den Brom et al., 2015). The less virulent form, phase II, has yet to be isolated from the host (Babudieri, 1959), phase I bacteria can be isolated from animals and humans who have been infected naturally or that have been infected in a lab (Krt, 2003; Setiyono et al., 2005), whereas the avirulent phase II is isolated during serial passages of *C. burnetii* in immunocompromised hosts such as embryonated hen eggs or cell culture systems (Krt, 2003; Thompson et al., 2003; Setiyono et al., 2005).

Domestic ruminants (sheep, cattle, and goats) and wild animals (rabbits, squirrels, deer, and buffaloes) are some of *Coxiella burnetii* hosts (Marrie et al., 1986). The hosts for *C. burnetii* can either be vertebrates or invertebrates. The reservoirs for the bacterium are mostly cattle,

sheep and goats and they are responsible for transmission to humans (Eldin et al., 2017), but the natural reservoirs for *C. burnetii* are ticks and rodents (Galiero et al., 2016). Ticks are infected naturally and *C. burnetii* multiplies in their gut and eventually shed in tick faeces. *C. burnetii* remains alive and active for a long period but its ability to infect decreases as time passes (Fard et al., 2011). *Coxiella burnetii* is shed in milk and vaginal secretions by goats and cows and in a large quantity in the faeces of sheep (Pexara et al., 2018). The main source of infection in humans is the inhalation of aerosols from birth fluids or placental materials (Marrie, 1990). Dairy cows have a higher infection rate than sheep and goats, which are the main sources of human illness, according to epidemiological data reported by Gwida (2012).

Coxiella burnetii infection is generally asymptomatic in domestic ruminants and can result in reproductive disorders which vary among ruminant species, for example in sheep, a fever can cause abortions, delivery, or weak/dead offspring, in goat herds it leads to an outbreak of abortions and in cattle it can be of no affliction, can cause infertility, abortion, metritis, and mastitis (Pexara et al., 2018). Infected ruminants can either be symptomatic/or asymptomatic (Roest et al., 2012) and they both release *C. burnetii* into the environment, and the shedding might last for a long period (Pexara et al., 2018). *Coxiella burnetii* is shed in large quantities during an abortion or parturition of infected ruminants (Babudieri, 1959; Maurin & Raoult, 1999; Pexara et al., 2018). In sheep, *C. burnetii* is shed in higher numbers in faeces whereas goats and cattle shed *C. burnetii* mostly in milk and vaginal mucus (Rodolakis et al., 2007; Pexara et al., 2018). Before and after kidding, goats excrete *C. burnetii* in their faeces, and the average excretion period is 20 days (Angelakis & Raoult, 2010).

Q fever in humans may manifest as an acute or chronic disease (Menadi et al., 2022). Acute Q fever is accompanied by flu-like symptoms, hepatitis and neurological involvement and it is usually underdiagnosed because of indefinite symptoms (Tissot-Dupont et al., 2007). Q fever acute disease is usually mild to moderately severe but can at times progress to acute respiratory distress syndrome and respiratory failure (Frean & Blumberg, 2002). Acute Q fever has a two-to-four-week incubation period (Sawyer et al., 1987). Chronic Q fever is associated with endocarditis, osteomyelitis, vascular/aneurismal infections, prolonged fever, and a post-chronic fatigue syndrome (Frean & Blumberg, 2002). The death rate for chronic Q fever has been observed to be higher than that of acute Q fever (Sawyer et al., 1987, Terhegge & Leggat, 2007). Pregnant women experience abortion as a result of placentitis and pathogenesis similar to that seen in sheep and goats. Q fever placentitis causes abortions, which are usually late-stage and can occur again in subsequent pregnancies (Norlander, 2000;

Woldehiwet, 2004). Transmission from one person to person is not common (Angelakis & Raoult, 2010). The zoonotic disease caused by this intracellular bacterial pathogen is known as Q fever (Cutler et al., 2007). Several studies have suggested that wind is also accountable the transmission of bacteria from ruminants to people (Tissot-Dupont et al., 2004; Schimmer et al., 2010).

The first cases of Q fever in South Africa were documented in 1950, in a small boy with fever and unusual pneumonia (Gear & Wolstenholme, 1950), and in an adult male with fever and atypical pneumonia (Saner & Fehler, 1950) as well as in an adult male with an intermittent fever of unclear aetiology and infection-related serological evidence (Ranking, 1950). Q fever disease in a herd can continue for a long term causing financial losses to the owners because the herd's reproduction performance will be negatively impacted (Pexara et al., 2018). Q fever disease in livestock is often unnoticed (Honarmand, 2012). Q fever infections in domestic animals have been recorded in numerous countries (Knobel et al., 2013, Haider et al., 2015, Pexara et al., 2018) and in South Africa *C. burnetii* infections in cattle have been reported in Gauteng province in 1987 (Gummow et al., 1987) and there also has been a report on *C. burnetii* infections where 38.9% cattle in Bushbuckridge municipality, Mpumalanga province were positive (Adesiyun et al., 2020). A recent study was conducted in the five districts of the Gauteng province and at a farm in the Mangaung district in Free State reported that cattle had the highest seroprevalence of 6.9% *C. burnetii* compared to sheep and pigs (Mangena et al., 2021). Infections with *C. burnetii* in cattle have not been reported in the Limpopo province of South Africa. There is also no information about the strains circulating in the Limpopo province or regarding the risk factors linked with exposure to Q fever.

Q fever outbreaks that are linked with aerosolized transmission routes are common and frequently linked to certain environmental conditions that favor dissemination (Boschini et al., 1999; Hatchette et al., 2001; Carrieri et al., 2002; O'connor et al., 2015), including windy days (Hawker et al., 1998; Boschini et al., 1999; Tissot-Dupont et al 2004; O'connor et al., 2015) and landscape (van Leuken et al., 2015).

Close contact between cows is an inherent feature of dairy herd management systems and herds with many cattle provide more opportunity for contact and transmission. Farms with a high population density are more likely to experience pathogen transmission within the herd after the introduction of *C. burnetii* (Rabaza et al., 2021). Further more high animal density

increases bacterial load and also environmental contamination (Suman et al., 2016) which may pose an increase risk of transmission to either cattle or human (Rabaza et al., 2021). Several global studies have found that the higher the prevalence of *C. burnetii* in a farm the larger the herd size (Psaroulaki et al., 2006; Anastacio et al., 2013). The higher the number of calving or lambing females during parturition, the greater the danger of *C. burnetii* transmission in farms with large herd size (Woldehiwet, 2004) as well as other management factors such as increased animal supplies such as feed and a greater number of people working on farms (Schimmer et al., 2011). As a result a large herd size is more vulnerable to *C. burnetii* infection and therefore herd size must be regarded as a risk factor for *C. burnetii* spread (Obaidat and Kersh, 2017).

Geographical local factors such as climate, vegetation, land use, and soil humidity are influence transmission of Q fever (Mori and Roest, 2018). During the Dutch outbreak, vegetation that provides dust particles shelter and reduces wind speed had an impact (Mori and Roest, 2018). It was more likely that infected particles from farms in the vicinity of sparsely vegetated areas would spread and infect the local people with Q fever. As a result, farms near contaminated sources that had wetter average soil moisture levels had a decreased chance of spreading Q fever to humans (van der Hoek et al., 2011; Mori and Roest, 2018). Most likely, the impact of moist soils on dust generation and deposition was what led to this association. Moist soils are less susceptible to be eroded by wind and have greater plant densities, providing some protection against airborne transmitted particles (van der Hoek et al., 2011; Mori and Roest, 2018). It has been reported that Q fever cases increase in winter and early spring (Porter et al., 2011).

2. LITERATURE REVIEW

2.1 *Coxiella burnetii*

Coxiella burnetii is a small, pleomorphic (0.2 - 0.4 mm wide, 0.4 - 1.0 mm long) organism (Philip, 1948).

2.2 Taxonomy/classification

Coxiella burnetii belongs to the order *Legionellales* and the family *Rickettsiaceae*. It also forms part of the gamma proteobacteria group. *Coxiella burnetii* is categorized as a Gram-negative bacterium because it possesses a similar cell wall (Angelakis & Raoult, 2010) since it cannot be stained by the Gram technique (Maurin & Raoult, 1999). It is also an intracellular pathogen that replicates in eukaryotic cells (Voth & Heinzen, 2007).

2.3. Disease Q fever in animals and humans

It is difficult to identify infected animals since they can be asymptomatic and pregnant animals show no symptoms (Eldin et al., 2017). However, although mostly asymptomatic in animals, the infection may cause reproductive disorders such as stillbirth, abortions, repeated bleeding, and premature births (Pexara et al., 2018). *Coxiella burnetii* has been found in the blood, spleen, liver, and lungs of animals during the acute phase of their infection but when they start shedding *C. burnetii* in the urine and faeces it is considered the chronic phase (Arricau-Bouvery & Rodolakis, 2005). The prevalence of Q fever has increased worldwide, primarily in livestock, putting the general public at risk (Arricau-Bouvery & Rodolakis, 2005; Cruz et al., 2018).

Infections in humans can be acute or chronic and can also be asymptomatic. Acute Q fever is identified as a flu-like disease or abnormal pneumonia or hepatitis while endocarditis, chronic fatigue syndrome, and repeated abortions are all symptoms of chronic Q fever (Galiero et al., 2016). Acute and chronic Q fever is caused by different strains of *C. burnetii* (Long et al., 2019). Asymptomatic acute Q fever develops in about 40% of infected patients, with the remainder of cases presenting as a vague, self-limiting sickness (Derrick, 1937; Maurin & Raoult, 1999). Approximately 5% of human *C. burnetii* infections who develop chronic Q fever only manifest symptoms after years of infection (Raoult et al., 2005; Wegdam-Blans et al., 2012). If a pregnant woman becomes infected, the bacterium localises in the uterus and

mammary glands, exposing the mother to the risk of chronic Q fever and future abortions. If infection occurred in the first trimester of the pregnancy, there is a high chance of abortion or premature birth occurring (Angelakis and Raoult, 2010). The effects and impacts of Q fever in African countries have been underestimated (Eldin et al., 2017). Clinical manifestations of Q fever often show in people who are over 15 years of age (Angelakis & Raoult, 2010).

2.4 Transmission *C. burnetii* infection

The transmission of Q fever to humans can occur through aerosols, the oral route and by infected ticks. The aerosol route was reported to be the main route of transmission of Q fever to humans (Maurin & Raoult, 1999). Person-to-person transmission through sexual activities is rare (Angelakis & Raoult, 2010). Transmission from one human to another human may occur through blood transfusion, bone marrow transplantation and infected placenta (Eldin et al., 2017). The bacteria in semen, milk, urine, and faeces transmit the infection to humans if there has been direct contact (Vanderburg et al., 2014). Although it is thought to be insignificant, oral transmission of the bacterium in humans (through contaminated dairy products) has been described in the literature. (Benson et al., 1963; Fishbein and Raoult, 1992). Humans inhale contaminated aerosols during slaughtering, cleaning, parturition, and all other farm activities (Keshavamurthy et al., 2019). Inhalation of polluted aerosols created by shedder animals and the contaminated environment are the primary routes of infection for animals and humans. Based on trials in which unpasteurized tainted milk was consumed, it is unclear whether Q fever can be spread through ingestion. In volunteers who swallowed tainted milk, *C. burnetii* did not produce febrile illness (Marrie et al., 1996; Kazar 2005). The infection routes of *C. burnetii* are shown in Figure 2.1.

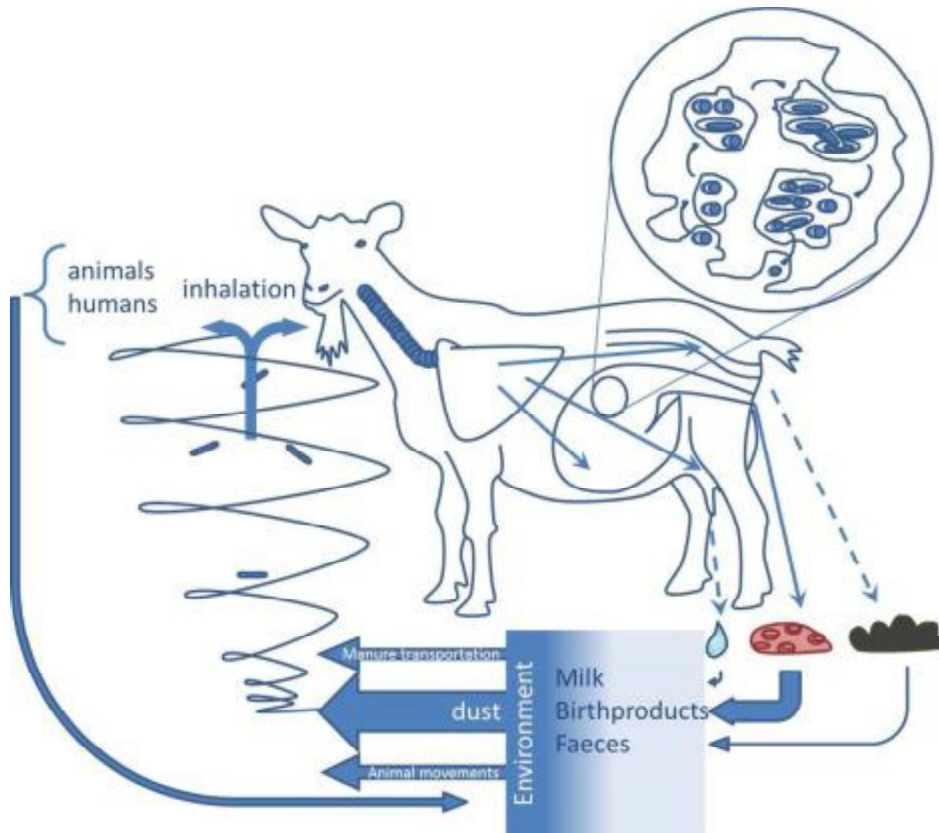


Figure 2.1: *Coxiella burnetii* infection pathways (Van den Brom et al., 2015)

2.5 Q fever sero-positivity

In 1950 the third World Health Assembly sent out a call for the investigation of the prevalence of Q fever around the world and the World Health Organization (WHO) supported the epidemiology, research and survey in 33 countries (Kaplan & Bertagna, 1955). The sudden appearance of Q fever has been reported worldwide and it is considered indigenous in most places in the world including South Africa (Woldeyohannes et al., 2018). A survey was undertaken in areas around Cairo in Egypt to investigate the seroprevalence of Q fever in human and animal populations and reported seropositivity for *C. burnetii* to be 13% in cattle, 23% in goats, 33% in sheep, and 16% in humans who were in contact with infected animals (Nahed & Khaled, 2012). In another survey conducted in Chad, the seroprevalence of *C. burnetii* was 80% in camels, 4% in cattle, 13% in goats, 11% in sheep, and 1% in humans with contact with infected animals (Schelling et al., 2003). In Zimbabwe, 39% of cattle and 10% of goats were seropositive for *C. burnetii* (Cutler et al., 2007). Q fever cases have been reported in many tourists coming back from African countries (Potasman et al., 2000). *C. burnetii* antibodies were found in 8.3% of 796 Gambia children

aged 1 to 15 years old in a serological investigation (Klaasen et al., 2013). Significant inter-species and geographical heterogeneity was discovered in a recent serological investigation of 2,699 animals across Egypt (Klemmer et al., 2018). Although the prevalence of Q fever in Africa has been studied for more than 60 years (Kaplan & Bertagna, 1955), throughout the continent, Q fever is still an essentially neglected zoonosis, and our understanding of the disease is based primarily on serology and seroepidemiological investigations (Salifu et al., 2019).

2.6 Infections and outbreaks of Q fever in humans

The first-ever outbreak of Q fever with the highest seroprevalence was reported in the Netherlands which resulted from intensive production of dairy from goats and sheep. From the outbreak (2007 to 2009), approximately 3,523 human cases which included many hospitalizations after showing clinical symptoms such as pneumonia were documented (Schimmer et al., 2008). Q fever is a threat to public health as it has demonstrated resistance to drying, heating, and disinfectants. It has a high survival rate in extreme environmental conditions. About 200-500 cases of Q fever are reported per year in Germany (Dalton et al., 2014). In the rural areas of western Kenya, Q fever was reported to be the cause of acute lower respiratory illness (Knobel et al., 2013).

Q fever outbreaks mostly originate from rural areas and are directly/indirectly associated with farms/farm animals (Parker et al., 2006). Q fever urban outbreaks have been reported following exposure to the slaughterhouses (Armengaud et al., 2004), animal research laboratories (Simor et al., 1984), parturient cats (Marrie et al., 1988), contaminated straws (Van Woerden et al., 2004) and after *C. burnetii* has spread by the wind from farmlands (Hawker et al., 1998). In recent decades, small ruminants are liable for several large and small-scale human Q fever epidemics across Europe (Georgiev et al., 2013; Vellema et al., 2014; Bauer et al., 2020; Bauer et al., 2021), as opposed to cattle, which have only caused a small number of Q fever infections (Georgiev et al., 2013; Bauer et al., 2020; Bauer et al., 2021).

Q fever is mostly regarded as a workplace hazard in humans (Porter et al., 2011). Investigations around the world reported that young children are more resistant to infection by *C. burnetii* (Angelakis & Raoult, 2010). Q fever serological evidence has been shown in adults and children, but, increased rates of *C. burnetii* seropositivity have been observed in

young children (Kobbe et al., 2008). Children over the age of 15 had a five-fold higher chance of developing symptoms in a significant Q fever epidemic in Switzerland than did children under the age of 15 (Dupuis et al., 1985). During the Dutch epidemic in 2009, a blood donor recipient tracking research revealed that a transfusion recipient had antibodies against *C. burnetii* following blood donation from a Q fever patient donor (van Kraaij et al., 2013). *Coxiella burnetii* can remain stable in blood samples at 1-6°C for 6 weeks (Kersh et al., 2013b). Q fever outbreaks in ruminants occur at the same time as infection in humans as ruminants are considered the main source of infection in humans (Jodelko et al., 2015).

Persons who are occupationally at risk, such as farmworkers, farmers, veterinarians, and para-veterinary workers, abattoir workers, and people who work in meat processing factories, account for the bulk of infections (Kazar 2005; Waag 2007). In an Australian Q fever outbreak, 58% of the 72 cases with available findings exhibited positive Q fever serology (Bond et al., 2016). In another outbreak in the United States, 60% (81/135) of people tested positive for Q fever (Biggs et al., 2014, 2016). Out of 109 human pneumonia cases, it was reported that 5% were caused by *C. burnetii* in Tanzania (Angelakis & Raoult, 2010). Since 1999, cases have been reported to the CDC (Centers for Disease Control and Prevention) in the United States. Between 2016 and 2018, the number of cases recorded each year fluctuated between 164 and 215 per year. Between 2003 and 2017, 6,591 cases were documented in Australia, averaging 400 to 600 per year (Tozer et al., 2020). Between 2016 and 2018, between 172 and 251 confirmed cases were recorded in France. During this time, the European Union recorded 922 to 1,058 Q fever cases yearly, and the highest frequencies were observed in France and Spain. A study by Whitney et al. (2009) found a prevalence rate of 22.2% among 113/508 American veterinarians, which is far higher than the prevalence rate in the general adult US population. Seropositivity was substantially more prevalent (OR 5.15) among workers in a mixed or large animal practice than those working in a small animal practice. In a study by Bosnjak et al. (2010) in Denmark *C. burnetii* antibodies were found in 31 of 87 veterinarians, in two of 15 inseminators, in five of 163 farmers, and one of 14 hoof trimmers. The lack of protective clothing or a mask and regular contact with water have all been identified to be significant risk factors for infection (Whitney et al., 2009).

2.7 Q fever in cattle

Q fever in cattle results in great losses of calves as it causes abortions, premature delivery, and weak offspring. Symptoms such as infertility, metritis, and mastitis are manifested in infected cows, but infection is mostly asymptomatic (Angelakis & Raoult, 2010). Q fever-induced abortions are typically late-stage, with no earlier signs of infection, and have a similar presentation to chlamydiosis and brucellosis (Arricau-Bouvery & Rodolakis 2005). The route of transmission from cows to calves and other species can be through milk, and through environmental contamination which results from vaginal and faecal discharge. *Coxiella burnetii* has been discovered in the sperm of naturally infected bulls, implying that sexual transmission of Q fever could occur in various mammalian species (Kruszewska and Tylewska-Wierzbanska, 1997).

Aljafar et al. (2020) reported that the results of identifying risk factors in each animal species revealed that female cows and male sheep had a higher risk of infection with *C. burnetii* (Aljafar et al. 2020). In Cameroon, Cetinkaya et al. (2000) reported that female cows had a higher seroprevalence of *C. burnetii*. *Coxiella burnetii* has a strong preference for the mammary glands and uterus, so large quantities of *C. burnetii* are found in these tissues (Ho et al., 1995; Aljafar et al. 2020), which may explain why females have higher seroprevalence than males (Cetinkaya et al., 2000).

A study by To et al. (1998) discovered a high frequency of *C. burnetii* infection in dairy cattle with reproductive problems in their investigation. Rady et al. (1985) found a high seropositivity rate among cattle with reproductive issues in Hungary. Krauss et al. (1987) found *C. burnetii* antibodies in 40% of 1193 randomly selected serum samples from 84 infertile herds in west Germany. The high incidence of *C. burnetii* positive dairy cattle with reproductive disorders indicated that *C. burnetii* positive cattle play an important role in perpetuating the infection and disseminating *C. burnetii* into the environment. (To et al., 1996; Yasumoto et al., 1997; To et al., 1998).

Two cohort studies reported that cattle with no history of abortions had no difference in *C. burnetii* seroprevalence compared to those with previous abortion cases (Maurice et al., 1968; Domenech et al., 1985). According to reports, *C. burnetii* induced abortions are more common in goats and sheep and less common in cattle. The placentas of infected livestock are reported to carry large quantities of *C. burnetii* (Lang et al., 1991; Berri et al., 2001;

Rousset et al., 2009). It has also been reported that older animals are more routinely infected by Q fever than heifers even in infected herds (Guatteo et al., 2008; Taurel et al., 2011).

According to reported studies, the highest shedding of *C. burnetii* in cows occurs around parturition and lasts for up to 2 weeks; however, low-level shedding in cattle might last for several months after parturition (Woldehiwet, 2004). A study by Guatteo et al (2006) reported that 65.4% of examined cows only shed bacteria through one route (milk, faeces, or vaginal mucus), while 6.4% shed bacteria in vaginal mucous, faeces, and milk at the same time. In another study conducted by Beaudeau et al. (2006) only 2% of infected cows excreted *C. burnetii* in vaginal mucus, faeces, and milk simultaneously. *Coxiella burnetii* is usually excreted in milk by goats and cows (Guatteo et al., 2006, Rodolakis et al., 2007). *Coxiella burnetii* is primarily shed into the surroundings throughout parturition or abortion, but *C. burnetii* positive animals can also excrete *C. burnetii* in milk, urine, feces, and vaginal mucus (Lucchese et al., 2015). It is well known that cattle herd commerce occurs often and over great distances (Widgren & Frössling, 2010).

2.8 Control methods and vaccinations

Environmental contamination can be reduced by practising good hygiene. Infected foetal fluids and membranes, as well as aborted foetuses and contaminated bedding, should be burned or buried (Angelakis & Raoult, 2010). Furthermore, manure must be treated with 0.4% lime or calcium cyanide before being applied to fields; this treatment must be carried out in the absence of wind to prevent the microorganism from scattering further away. To decrease abortions cases and the amount of *C. burnetii* shed during parturition, antibiotic therapy may be used (Angelakis & Raoult, 2010). Despite the high cost, infected animals should be kept separate from herds or given separate confinement infrastructure in which to give birth. Animal sector workers must be well educated about the risk factors for Q fever, and laboratories should have sufficient safety facilities and equipment (Angelakis & Raoult, 2010).

Rodokalis et al. (2007) proposed using bulk tank milk (BTM) analysis to monitor bovine and caprine herds. Pools of ten individual milk samples from herds with a PCR-positive BTM result should be tested by PCR to identify the shedding animals. The authors suggested that if the shedders are insignificant, they can be eliminated, and the other animals should be vaccinated (Rodolakis et al., 2007). Rodokalis et al. (2007) also recommended that herds near infected herds be vaccinated. In animals, the most efficient vaccinations are made up of

inactivated entire phase 1 bacteria (Balndin, 2007). The effectiveness of vaccination, on the other hand, was higher in nulliparous animals than in parous animals. Vaccination also did not eradicate infection in previously infected goats or cattle in two different studies conducted in France (Arricau-Bouvery et al., 2005; Guatteo et al., 2008; Hogerwerf et al., 2011).

In order to protect humans from Q fever, three vaccine formulations have been proposed: attenuated live vaccine, chloroform-methanol residue extracted vaccine and Q-Vax (whole-cell formalin-inactivated vaccine), which is considered to be acceptable for human use (Chiu and Durrheim, 2007). Considering that human Q fever is frequently a work hazard, populations at risk should be prioritized during vaccinations (Maurin and Raoult, 1999). Furthermore, vaccination should most likely be considered in people at risk of developing chronic Q fever and also patients with heart valve defects, vascular disease and immunocompromised patients (Angelakis & Raoult, 2010).

2.9 Methods for isolation of *C. burnetii*

The shell vial technique is the most used for isolation of *C. burnetii* even in samples kept at -80°C for a long period before they are cultivated. The HEL cell monolayers in shell vials are inoculated with 1 ml of the sample followed by centrifugation at 70 x g at 20°C for 1 h. This step enables the attachment and invasion of *C. burnetii* into the cells. Once the cells have taken up *C. burnetii*, they are incubated at 37°C in 5% CO₂ for about 7 days. To detect the presence of the bacteria inside the cells, the cells are stained by Gimenez or immunofluorescence stain (Gikas et al., 1998; Gouriet et al., 2005).

2.10 Molecular detection of *C. burnetii*

A polymerase chain reaction (PCR) can detect *C. burnetii* DNA in a variety of samples, including placenta tissues, faeces, vaginal mucus and milk (OIE, 2012). *Coxiella burnetii* DNA can be detected from milk samples using conventional, real-time, multiplex and nested PCR assays. The PCR assays can detect both forms (SCVs and LCVs) of *C. burnetii* (Gale et al., 2015). In a study, LCVs were generated by inoculating ACCM2 with ten days old ACCM2-cultured bacteria following a 72 h incubation and isolation by centrifugation (Coleman et al., 2004). Small Cell Variants were generated similarly except that *C. burnetii* was grown for seven days (Raghavan et al., 2008).

Presently, lyophilization is used to concentrate DNA from clinical samples to increase qPCR sensitivity (Eldin et al., 2017). For the detection of *C. burnetii* infection in milk, vaginal

mucus, faeces, aborted foetuses, and placentas, PCR is used (Muskens et al., 2012). The 16S-23S RNA gene, the superoxide dismutase (*sodB*) gene, the *com1* gene, or the *IS1111* repetitive elements are sequences mostly targeted by PCR in human/animal samples (Mediannikov et al., 2010). Real-time PCR/quantitative PCR is not tedious and is used commonly as it also measures the number of bacteria (Eldin et al., 2013). For the detection of both acute and chronic Q fever, the light-cycler nested PCR which utilizes serum as a specimen and targets a multicopy element sequence has also been used for the early detection of chronic Q fever (Angelakis & Raoult, 2010). Quantitative PCR can detect *C. burnetii* in the first week of infection when sera are not yet seropositive. *Coxiella burnetii* DNA can also be isolated from blood and serum (Eldin et al., 2013).

IS1111 is a repeat element that appears in approximately 20 copies in the *C. burnetii* Nine Mile genome (Denison et al., 2007; Klee et al., 2006; Eldin et al., 2013). Previous research detailing the insertion sequence showed that the transposase's coding domain is bordered at each end (Hoover et al., 1992). This chromosomal target sequence is found more than 50 times in the Nine Mile genome (Partridge and Hall, 2003). *IS1111A*, *IS1111B*, and *IS1111C* were formed as a result of sequence differences in the various copies of *IS1111* (Hoover et al., 1992). All *C. burnetii* genomes have multiple *IS1111* copies scattered throughout the bacterial chromosome (Hoover et al., 1992; Denison et al., 2007; Beare et al., 2009; Sidi-Boumedine et al., 2014). The *IS1111* target permits very sensitive detection of *C. burnetii* DNA among the PCR-based techniques established for *C. burnetii* detection in clinical samples (Klee et al., 2006; Eldin et al., 2013) in comparison to a single copy of the *com1* gene (Kersh et al., 2012).

Conventional PCR is the most commonly used technique in research and diagnostics; however, due to the time between amplification and DNA revelation, this technique cannot be completely automated (Alsaleh et al., 2011). Second, it makes no distinction between animals that shed a large number of bacteria and those that shed only a few bacteria (Alsaleh et al., 2011). Conventional PCR which was employed in this study has been used in several other studies. In one study conducted in France, 22 new *C. burnetii* isolates were discovered in human patients suffering from chronic Q fever (Stein and Raoult, 1992). In another study, Ibrahim et al. (1997) used conventional PCR to confirm the presence of DNA in all bacterial samples by amplifying a 350-bp fragment of the 16S rDNA using two universal primers. In recent years, conventional PCR's high sensitivity and specificity have proven useful in detecting *C. burnetii* in a variety of sample types (Eldin et al., 2017).

To better understand the epidemiology of an infectious disease such as Q fever, genotyping of the bacteria must be performed. Molecular epidemiological markers that were used at first were the 16S rRNA gene sequencing, 16S-23S rRNA gene sequencing, and RNA polymerase β -subunit (*rpoB*) sequencing but were insufficient for distinguishing *C. burnetii* genotypes (D'Amato et al., 2016). The availability of whole-genome sequences (WGS) led to the discovery of comparative genomic hybridization (CGH). For genotyping, multiple-locus variable-number tandem repeat (VNTR) analysis (MLVA), multi-spacer sequence typing (MST) and single nucleotide polymorphism (SNP) methods are currently used (Eldin et al., 2017). For inconsistent repetitive DNA, MLVA is used, and its advantage is that it can employ more than one panel of markers to achieve good discriminatory power. MST genotyping aids in tracking the spread of *C. burnetii* from one region to the next, as well as from animal reservoirs to humans and it also allows inter-laboratory comparison, as the method is standardized and has been reported to be highly discriminant (Eldin et al., 2017).

2.11 Serologic diagnosis of Q fever

Serology is the first-line diagnostic tool to use in the presence of symptoms or signs of *C. burnetii* infection (Anderson et al., 2013). Anti-phase I and phase II antibodies are produced by the immune system in response to infection (Anderson et al., 2013). Increased phase II IgG antibodies and a considerable low level of phase I IgG can last for over a year after the main infection, although both phase I and II IgM often decline to a low level after a few months (Dupuis et al, 1985; Dupont et al., 1994). Phase II IgM antibodies are the first to be detected in acute Q fever, followed by phase II IgG antibodies (Angelakis and Raoult, 2010). Chronic Q fever infections are distinguished by the existence of low phase I IgG antibodies, whereas previous infections are distinguished by the existence of high phase II IgG and low phase I IgG antibodies (Boden et al., 2012).

The detection of phase II antibodies occurs between 7 to 15 days following infection and decreases in 3 to 6 months. It is recommended that two serum samples should be collected about 3 to 6 weeks apart to detect phase II antibodies (IgM and IgG) (Murphy and Field, 1970; Eldin et al., 2017). The enzyme-linked immunosorbent assay (ELISA) has been used to measure the production of *C. burnetii*-specific gamma interferon (IFN- γ). ELISA, indirect immunofluorescence assay (IFA), complement fixation test (CFT), western blotting, dot immunoblotting, microagglutination, and indirect haemolysis test can be used for diagnostic

purposes. The reference method is the IFA. For the detection of IgM antibodies at an early stage of Q fever IFA is mostly used because of its high sensitivity (Anderson et al., 2013).

For large-scale screening of livestock infection status, the ELISA is preferred (OIE, 2015). ELISA is preferred even though it only allows detection of exposure to *C. burnetii* and not the identification of the pathogen and animals shedding the bacteria (Berri et al., 2003). The CFT has low sensitivity and usually fails to identify antibodies against *C. burnetii* in sheep and goats (Kovacova et al., 1998).

The use of ELISA is advantageous because it is simple to perform, and there is less subjectivity in the interpretation of results than that of IFA and CFT and automation is possible (Anderson et al., 2013; Herremans et al., 2013; Eldin et al., 2017). The complement fixation test and IFA have been regarded as less sensitive than ELISA (Field et al., 1983; Peter et al., 1987; Cowley et al., 1992; Maurin & Raoult, 1999). ELISA is not extensively used to diagnose Q fever. ELISA has been presented as a valuable technique for seroepidemiology (Peter et al., 1987; Maurin & Raoult, 1999). In a study by Peter et al. (1987) where serum samples from 213 Q fever patients and 301 blood donors from six Swiss cities were analyzed using ELISA, as well as indirect fluorescent-antibody (IFA) and complement fixation (CF) tests. They reported that the ELISA, IFA, and CF tests all identified antibodies to *C. burnetii*. The ELISA test produced a greater percentage of positive sera from blood donor serum samples than the IFA and CF tests. Rousset et al. (2007) found a high level of agreement between IFA and ELISA results in a goat study. Commercially available ELISA tests, on the other hand, appear to lack IFA's ability to identify patients at risk of developing persistent Q fever (Herremans et al., 2013).

In this current study indirect ELISA was employed to detect IgG antibodies against *C. burnetii*. The antigen used in ELISA is typically a whole cell preparation of phase I or phase II *C. burnetii* (Peter et al., 1988; Uhaa et al., 1994; Chen et al., 2014). After incubating the antigens with serially diluted sera in phosphate-buffered saline, antibodies are detected using alkaline phosphatase-conjugated rabbit anti-human IgG, IgM, and IgA. Anti-phase I and anti-phase II antibodies can both be detected (Fournier et al., 1998). The assay in this current study was validated following manufacturer's instructions: the average optical density value of the two negative controls (NCx) at 450 nanometers (A450) must be equal to or not be more than ($\leq$) 0.500 for the assay to be valid. The two positive controls (PCx) should have an average value of equal to or not be more than ($\leq$) 2.500 at 450 nanometers (A450). Therefore,

PCx-NCx (A450) must be above or equal to ($\geq$) 0.300. In terms of results interpretation, sample to positive (S/P) % < 30% indicate a negative result, $30\% \leq \text{S/P}\% < 40\%$ indicate a suspect, while $\text{S/P}\% \geq 40\%$ indicate a positive result.

2.12 Virulence factors of *C. burnetii*

Little is known about *C. burnetii* virulence factors due to difficulty in genetic manipulation and growth of this pathogen in culture broth media. The surface area of *C. burnetii* is protected from the innate immunity recognition by LPS. Lipid A which is the centre region and clustered genes that form part of O-antigen generation are the genes that code the structural characteristics of LPS. The exportation of LPS components to the outside of the host membrane has a critical role in the virulence of *C. burnetii* (Kuley et al., 2015). Pathogens can modify the host cell proteins, and this is a major virulent process. The host response mechanism is regulated by a eukaryotic-type protein kinase and an acid phosphate (Baca et al., 1993). *Coxiella burnetii* acid phosphatase phosphorylates the host cell proteins and this leads to a reduced oxidative burst following phagocytosis (Baca et al., 1993). Type IV secretion system (T4SS) is involved in the delivery of effector proteins into the host cytosol and in the pathogenesis of the bacteria. In *in vivo* assay, ankyrin proteins encoded by *ankC* and *ankG* which are effector proteins were elevated and it was demonstrated that they disturb the apoptosis pathway of the host cell (Kuley et al., 2015).

2.13 Situation in South Africa

Coxiella burnetii infections in cattle have been reported in South Africa's former Transvaal province (now Gauteng) where 7.8% of cattle in the study were seropositive (Gummow et al., 1987) and there also has been a report on *C. burnetii* infections where 38.9% cattle in Bushbuckridge Municipality, Mpumalanga province were positive (Adesiyun et al., 2020). In addition, findings of a study by Donnelly et al. (2021) revealed that white rhinoceros in South Africa had high seropositivity (53.9%) to *C. burnetii*.

Mangena et al. (2021) conducted a study on the seroprevalence of *C. burnetii* in slaughter cattle, sheep, and pigs from abattoirs located in Gauteng province using ELISA and further reported *C. burnetii* shedding detected by PCR, they also included a report on abortion cases caused by *C. burnetii* from sheep originating in a farm in Free State province. The study reported that, among animals studied, cattle had the highest seroprevalence. Mtshali et al. (2018) concentrated on *Coxiella* PCR detection from ticks. Mangena et al., (2021) further

reported two novel *C. burnetii* MLVA genotypes that were not found in the *Coxiella* MLVA databases (*C. burnetii* 2014 Nijmegen database).

In a study conducted in Mpumalanga, South Africa, *Coxiella burnetii* IgG was found in 28/73 (38%) of non-malarial febrile patients and 39/64 (61%) of farmers, herders, and veterinary workers (Simpson et al., 2018). These are some of South Africa's few studies on *C. burnetii* animal infections. Kelly et al. (1993) discovered *C. burnetii* antibodies in 37% of humans, 39% of cattle, and 10% of goats in Zimbabwe. Seroprevalences of 6.5% and 32% were reported in cattle in Malawi and Cameroon's Adamawa region by Staley et al. (1989) and Scolamacchia et al. (2010), respectively. Mwololo et al. (2022) reported a seroprevalence of 3.00% in cattle in Kenya's Tana River and Garissa counties. A study by Prabhu et al. (2011) conducted in northern Tanzania reported a seroprevalence of 5% (24) of the 55.5% (483) febrile patients tested for acute Q fever. A seroprevalence of 19.1% was reported in febrile patients in northeastern Kenya by Njeru et al. (2016). There is currently no information on the seroprevalence of *C. burnetii* in cattle, risk factors linked to Q fever, and PCR prevalence in the Limpopo province. This research will contribute to what is previously known about Q fever in South Africa.

2.13.1 Aim of the study

- To determine the prevalence of *C. burnetii* in farmed cattle and assess the risk of transmission to humans and animals across the farms in Limpopo Province.

2.13.2 Hypotheses

- The prevalence of *C. burnetii* is high in infertile cattle than in fertile cattle.
- The prevalence of *C. burnetii* infection is higher in female than male cattle.
- Q fever infections are prevalent in cattle across the Limpopo province.
- The main drivers of transmission of *C. burnetii* in cattle on farms is lack of hygienic practices during farm activities.

2.13.3 Objectives for the study

- To investigate the prevalence of Q fever in cattle on farms across Limpopo using serological and molecular methods.

- To determine the risk factors for exposure of cattle and humans to *C. burnetii*, using a structured questionnaire administered to farmers.
- To use the data generated in the study to propose ways to prevent and control Q fever in humans and animals in Limpopo province.

CHAPTER 3. MATERIALS AND METHOD

3.1 Ethical Approval

The protocol for the study was approved by the Animal Ethics Committee (Appendix 3.1) and the Research Committee (Appendix 3.2) of the Faculty of Veterinary Science. In addition, the Section 20 Ethical Approval Committee Certificate (Appendix 3.3) was granted for the study by the Department of Agriculture, Forestry, and Fisheries (DAFF).

3.2 Description of study areas

3.2.1 Location

This cross-sectional study was conducted in Limpopo, which is South Africa's northernmost province with a total area of 125 755 km² and five district municipalities. Limpopo is one of South Africa's warmest regions, with an average daily high temperature of 26°C. For several months of the year, temperatures are consistently above 25°C, sometimes reaching 29°C. The sampled district municipalities are Capricorn which is a stopover between Gauteng and Limpopo north and between the north-western areas and the Kruger National Park and has a total area of 21 705 km². Sekhukhune which lies in the south-eastern part of the province with a total area of 13 528 km². Lastly, Waterberg which is bordered to the north by Capricorn district municipality and to the south by Sekhukhune district municipality in the east and has a total area of 44 913 km² (<https://municipalities.co.za>). These district municipalities represent 50% of the districts in the province as other areas are foot and mouth disease (FMD) restricted areas. The study was conducted in all the local municipalities that fall under each district municipality namely Blouberg, Molemole, Polokwane, Lepelle Nkumpi (Capricorn), Lephalale, Bela-Bela, Mokgalakwena and Modimolle (Waterberg) and Greater Tubatse, Ephraim Mogale, Elias Motsoaledi (Sekhukhune) (<https://municipalities.co.za/>) (Figure 3.1).

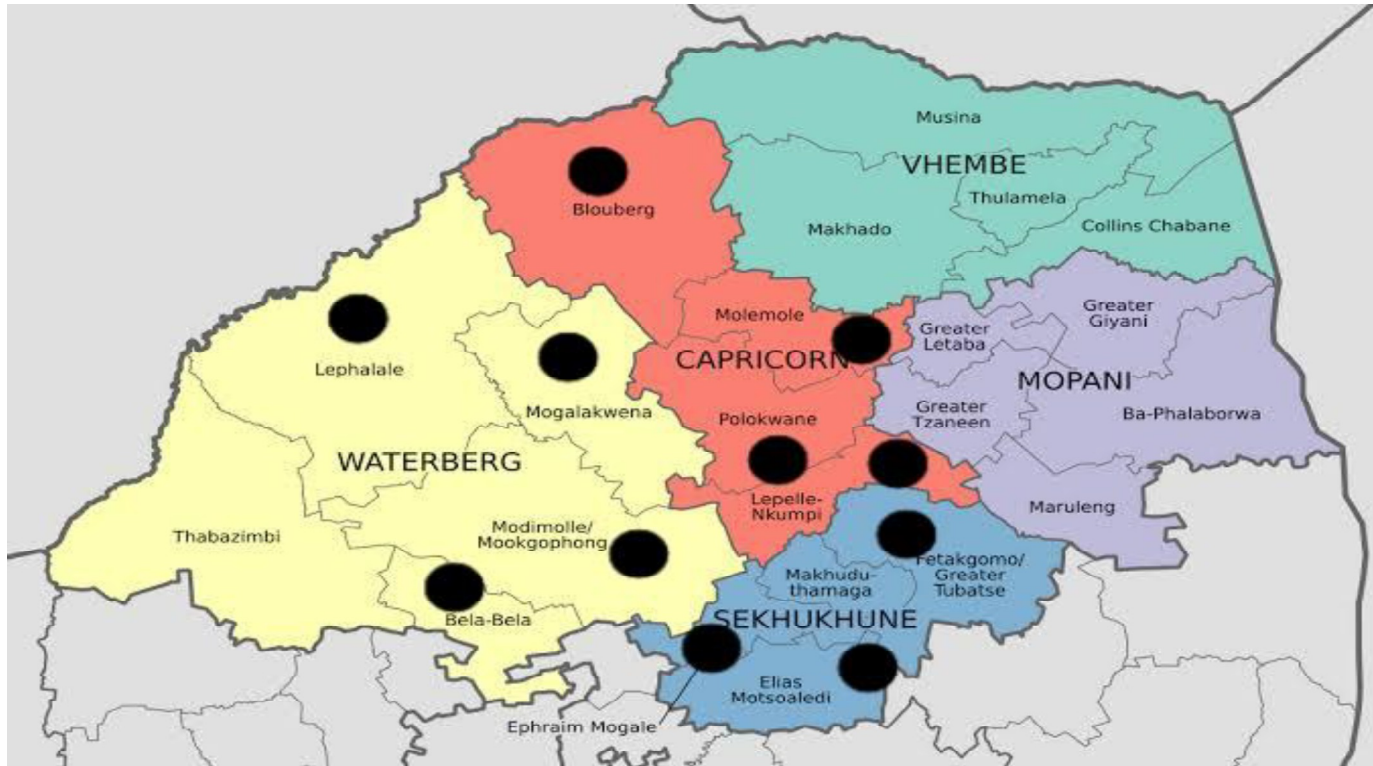


Figure 3.1: Map of Limpopo province showing districts and municipalities where samples were collected (<https://maps.google.com/maps>). The black dots represent the sampled areas.

3.2.2 Study design and sample size

The sample size for the study was calculated using the formula by Thrusfield (2007) using an expected prevalence (P_{exp}) of 50%. Because no information on the prevalence of *Coxiella burnetii* in the study population was available, a prevalence of 50% was used. The desired precision (d) was set to 5%.

$$n_0 = \frac{\{1.96^2 \times P_{exp} \times (1 - P_{exp})\}}{d^2}$$

$$n_0 = \frac{3.84 \times 0.5 \times 0.5}{0.0025} = \frac{0.96}{0.0025} = 384$$

$n_0 = 384$ cattle, however, 383 cattle were sampled.

3.2.2.1 Selection of farms

In this study convenience sampling was used because only those farmers willing to participate in the research were included. A minimum of 128 cattle were sampled from each

of the three districts in Limpopo province. It was anticipated that a vast majority of the farms will be communal in operation but commercial farms were available and included in the study. A list of cattle farms, approximate cattle populations and locations (GPS coordinates)/districts and contact numbers of the owners were obtained from the Veterinary Division in the Limpopo province. The 128 cattle collected from each district were distributed using the following criteria:

- a. Farms with less than 5 cattle: all animals were sampled
- b. Farms with 5 - 20 cattle: a maximum of 8 were sampled
- c. Farms with 21 – 30 cattle: a maximum of 12 were sampled
- d. Farms with 31 – 50 cattle: a maximum of 18 were sampled
- e. Farms with >51 cattle: maximum of 20 were sampled

Inclusion and Exclusion criteria:

Inclusion criteria: Adult cattle (bulls and cows), cattle that belonged to farms at the selected municipalities, apparently healthy cattle.

Exclusion criteria: Calves, and sick cattle.

3.2.3 Questionnaire survey

On each farm sampled, a consent form was first administered to the farm owner/attendant after explaining the study's objectives and background on Q fever in Sepedi/English to solicit their participation (Appendix 3.4). All of the farmers and farm workers were unaware of Q fever as we explained it to them during the sampling process. A structured questionnaire survey (Appendix 3.5) was administered to the farmers in their language English/Sepedi to collect demographic data and potential risk factors for exposure of animals and humans to *C. burnetii*. The information obtained from the questionnaire included the location of the farm in the province using GPS coordinates, type of farm, farm size (cattle population) and practices, herd size, sex and age of animals to be sampled, breed, disposal of aborted fetuses/placentas, abortion cases, manure uses, water supply for the animals and drainage of effluents.

3.3 Sample collection

Samples were collected only from adult cattle since no consent was given to sample calves. Blood samples were collected from the selected cattle male/female from the coccygeal vein

into tubes. Serum samples (10 ml) were collected using BD-Vacutainer® SST™ II Advance 10 ml serum collection tubes (figure 3.2). The samples were stored at 4°C until they were transported to the lab for testing. Serum samples were then harvested by centrifuging the clotted blood in collection tubes at 1000 x g for 10 min. The harvested sera were stored at -20°C until analysis. Vaginal swabs were collected from all female animals including heifers using dry swabs that were dipped in phosphate-buffered saline (PBS) (0.01 M, pH 7.4) after swabbing and stored in sterile plastic bags. Sheath scraping samples were collected from all male cattle within the farm. Scraping and simultaneous aspiration were performed with Uterine Infusion Pipettes (Kyron Laboratories) connected to a sterile disposable 10 ml hypodermic syringe with a silicon-rubber tube. During sheath collection, bulls were held in a sturdy crush with a neck clamp. Additional restraint included tying one back leg or using a Rau animal Immobilizer placed in the rectum to deliver low-level electrical stimulation. The sample was then transferred to a plastic tube containing 10 ml of PBS (0.01 M, pH 7.4).



Figure 3.2: Blood collection from the coccygeal vein of cattle in a crush pen at a farm.

3.4. Serology for antibodies against Q fever

The indirect ELISA was used to screen serum samples for Q fever because of its high sensitivity and specificity (Peter et al., 1988). The IDEXX Q-FEVER 2/strip ELISA kit (IDDEX Laboratories, Liebefeld-Bern, Switzerland) was used to detect IgG antibodies to *C. burnetii* infection following the manufacturer's instructions. The manufacturer provided the microtiter plates precoated with an inactivated antigen. The IDEXX Q-FEVER 2/strip ELISA

kit has a diagnostic sensitivity of 100% and specificity of 100%. Before use, all reagents (IDEXX Q Fever 2/strip antibody test kit reagents, as well as the frozen serum samples) were allowed to reach room temperature. Prior to use, the wash concentrate (10X) was diluted 1/10 with distilled water. All the samples including the positive and negative control were prediluted (1/400) using the wash solution. Following the predilution, 100 µl of the positive and negative controls and samples were pipetted into antigen-coated wells. The plates were then tightly sealed and incubated at 37°C for 1 h. The plates were washed three times with the wash solution and then 100 µl of the conjugate was added to each well and incubated for 1 h at 37°C. Subsequently, 100 µl of TMB substrate N.12 was added to each well after washing three times with the wash solution. The plates were then incubated at 26°C for 15 min. After adding 100 µl of the stop solution N.3 into each well, the wavelength was read at 450 nm using a Thermo Lab systems Multiskan MS Original microplate reader (Thermo Fischer Scientific, Waltham, MA, USA). To validate the assay, the following procedures were used: The average optical density value of the two negative controls (NCx) at 450 nanometers (A450) must be equal to and not ($\leq$) 0.500 for the assay to be valid. At 450 nanometers (A450), the two positive controls' (PCx) average value should be less than or equal to ($\leq$) 2.500. PCx-NCx (A450) must thus be greater than or equal to ($\geq$) 0.300. The sample to positive control (S/P) ratio was calculated according to the formula:

$$S/P \% = 100 \times \frac{\text{Sample (A450)} - \text{NCx (A450)}}{\text{PCx (A450)} - \text{NCx (A450)}}$$

In terms of results interpretation, $S/P \% < 30\%$ denoted a negative result, $30\% \leq S/P\% < 40\%$ denoted a suspect, while $S/P\% \geq 40\%$ denoted a positive result.

3.5 DNA extraction

For extraction of DNA from sheath scrapings and vaginal swabs, the High Pure PCR template preparation kit (Roche Diagnostics GmbH, Mannheim, Germany) was used. The High Pure PCR template preparation kit was used following the manufacturer's instructions. Briefly, 40 µl of proteinase K and 200 µl of the binding buffer were added to 200 µl of the sample. Following incubation at 70°C for 10 minutes, 100 µl of isopropanol was added and the mixture was then centrifuged at 8000 x g for 1 minute and the flow-through and collection tube were discarded. Following the addition of 500 µl of the inhibitor removal buffer, the mixture was centrifuged at 8000 x g for 1 minute and the flow-through and collection tube were discarded. This was followed by the addition of 500 µl of the wash

buffer and centrifugation at 8000 x g for 1 minute and the flow-through and collection tube were discarded. Following the addition of 500 µl of wash buffer, the mixture was centrifuged at 8000 x g for 1 minute and the flow-through was discarded. After centrifuging for 10 seconds at 13000 x g, the collection tube was discarded and a new tube was added, along with 200 µl of the elution buffer, before centrifuging for 1 minute at 8000 x g and storing at -20°C. The extracted DNA was amplified using PCR.

3.6 Detection of *C. burnetii* by PCR

The PCR experiments were carried out according to the procedure detailed by Mangena et al. (2021). The PCR for *C. burnetii* detection in seropositive and seronegative samples was performed in a 50 µl reaction targeting the multi-copy transposase gene in insertion element; IS1111 (de Bruin et al., 2012) with primers IS1111F 5'CGCAGCACGTCAAACCG3' and IS1111R 5'TATCTTTAACAGCGCTTGAACGTC3'. The positive control used was an in-house sample from Mangena et al. (2021) which was sequenced and the negative control used was distilled water. The reaction mixture contained 2 µl of each 10 µM primer (IS1111F and IS1111R), 25 µl of the Amplicon 2X Taq DNA polymerase Master Mix Red (Amplicon A/S, Odense, Denmark), and 10 µl of the extracted DNA. For *C. burnetii* DNA amplification, a 146 bp sequence of the IS1111 gene was amplified using BIO-RAD T100™ thermal cycler (BIO-RAD, Singapore) in which the first denaturation was at 95°C for 15 min, 35 cycles were carried out with denaturation at 95°C for the 30s, primer annealing for 30s at 60°C, followed by 72°C for 60 s and the final extension step at 72°C for 10 min.

3.6.1. Agarose gel electrophoresis

The amplicons from all the PCR assays were electrophoresed on a 1.5% ethidium bromide-stained agarose gel at 100 V for 1:30 h or until the separation of the DNA bands was observed. The size of the amplicons was determined using a 100 bp molecular weight marker. Gels were visualized under ultraviolet light using a gel documentation system (Chemi XRQ, Vacutec, UK).

3.6.2 IS1111 sequence verification of *Coxiella burnetii* positive samples

Seven of the PCR products that had brighter bands were chosen and sent to Inqaba Biotechnologies, South Africa for sequencing of both the forward and reverse strands of the

IS1111 gene using an ABI sequencer. Sequences were edited manually and pairwise alignments of the forward of reverse sequences were undertaken using the BioEdit Sequence alignment editor (version 7.1.9). The consensus sequences were analysed on the NCBI BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) platform for species identification by megablast.

3.8 Statistical analysis

3.8.1 The percentage of positive animals (% positive) for the presence of *C. burnetii* antibodies was calculated as described in the equation (a).

$$(a) \text{ Apparent prevalence} = \frac{\text{Number of positive animals}}{\text{Number of animals tested}} \times 100$$

The calculation of the 95% confidence interval for the true prevalence was performed using the following equation:

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

Where: $Z\alpha$ at a 95% confidence level is 1.96.

3.8.2 Univariate analysis

A univariable logistic regression model was employed to filter the factors against *C. burnetii* infection exposure at the individual level. In the initial analysis, the Chi-square test (P-value ≤ 0.05) was used to assess each factor's unconditional relationship with the outcome. During the univariable logistic regression analysis, the factor with the highest P-value ≥ 0.05 was eliminated. In the univariable logistic regression analysis factors with a P-value ≤ 0.05 were used to create a multilevel logistic regression model for each exposure.

3.8.3 Multivariable analysis

Factors that were found significantly associated with the outcome on the univariable analysis with a P-value ≤ 0.05 were included in the multivariate logistic regression model to examine the amount of effect on the outcome variable. The logistic model was reduced by stepwise elimination removing variables with $P > 0.05$. This procedure was performed until the model with the lowest Akaike's second-order information criterion (AIC) was identified. The odds ratios with 95% confidence interval results were noted.

CHAPTER 4. RESULTS

4.1 Serology

Out of 383 cattle tested for antibodies against *C. burnetii*, the overall seroprevalence was 24.28% (93/383). The number of cattle testing positive in municipalities was highest in Elias Motsoaledi local municipality with a seroprevalence of 70.37% (Table 4.1.1). followed by Mkgalakwena local municipality with a seroprevalence of 45% and the lowest seroprevalence of 6.90% was observed in Ephraim Mogale local municipality and the difference was statistically significant ($P < 0.001$). For the breeds of animals, the seroprevalence ranged from 9.09% (Angus) to 31.58% (Brahman). The difference was not statistically significant ($P = 0.149$). The seroprevalence in dip-tanks ranged from 6.90% in Marble Hall to 70.37% in farm 32 ($P < 0.001$). The seroprevalence of Q fever was 24.18% and 25.0% for female and male cattle respectively. The difference was not statistically significant ($P = 0.901$).

Farms that disposed of aborted fetuses\placentas by throwing them into the bush had the highest seroprevalence of 45.45%, while farms that disposed of aborted fetuses\placentas by burning had the lowest seroprevalence of 9.52% and the difference was not statistically significant ($P = 0.126$). Cattle with no infertility history exhibited a seroprevalence of 25.07% and those with infertility history had a seroprevalence of 10.0% (Table 4.1.1). The difference was not statistically significant ($P = 0.126$). Cattle from farms where the manure was used as fertilizer showed the highest seroprevalence of 27.78% and farms that do not use their manure had a seroprevalence of 17.56% and the difference was significant ($P = 0.027$). The seroprevalence according to herd size ranged from 18.85% in the herd size category of 1 – 50 to 70.37% in the herd size category of greater than 150 and the difference was statistically significant ($P < 0.001$). A total of 215 cattle were sampled from commercial farms with 28.84% seropositive and from communal farms, 168 cattle were sampled with 18.45% seropositive. The difference was statistically significant ($P = 0.019$).

Dip-tank, local municipality, manure uses, herd size and farm type all had significant univariate associations with Q fever seropositivity (Table 4.1.1) and were included in the multivariable logistic regression. District municipality, gender, abortion history, infertility history, disposal of aborted fetuses\placentas, breed and weak offspring history were excluded in the multivariable logistic regression because they were not significant. In the final multivariable logistic regression, local municipality (OR 1.09; 95% CI 1.00 - 1.20; $P =$

0.043) and herd size (OR 2.24; 95% CI 1.21 -4.15; P= 0.010) remained linked to *C. burnetii* seropositivity in cattle (Table 4.1.2).

Table 4.1.1 Seroprevalence of *C. burnetii* (Q fever) in cattle on farms in Limpopo province, South Africa, and univariable analysis of possible risk variables in cattle for Q fever seropositivity.

Risk factor	Number of animals sampled	Number testing positive	% seropositive	P-value
District municipality				
• Capricorn	127	27	21.26	0.347
• Sekhukhune	123	28	22.76	
• Waterberg	133	38	28.57	
Local municipality				
• Bela-bela	26	7	26.92	0.000
• Blouberg	48	5	10.42	
• Burgersfort	67	7	10.45	
• Elias Motsoaledi	27	19	70.37	
• Ephraim mogale	29	2	6.90	
• Lepelle nkumpi	29	7	24.14	
• Lephalale	31	8	25.81	
• Modimolle	42	11	26.19	
• Mokgalakwena	20	9	45.00	
• Molemole	15	6	40.00	
• Moretele	9	2	22.22	
• Polokwane	35	9	25.71	
• Vaalwater	5	1	20.00	
Dip-tank				
• Alice's farm	11	5	45.45	0.000
• Blackhill	14	0	0.00	
• Earlydawn	34	5	14.71	

• Elandsbosch	5	1	20.00	
• Eqcebo	3	1	33.33	
• Farm 32	27	19	70.37	
• Ga- machikiri	6	2	33.33	
• Ga-maepa	32	4	12.50	
• Ga-maseven	35	3	8.57	
• Ga-molekane	14	7	50.00	
• Goba	15	6	40.00	
• Klipspruit	12	2	16.67	
• Marble hall	29	2	6.90	
• Mora farm	10	2	20.00	
• Rooibosveld	29	7	24.14	
• Skuinhoek	23	7	30.43	
• Sterkfontein	9	0	0.00	
• Sterkstrom	19	5	26.32	
• Tabana	13	4	30.77	
• Vlaaglaakte portion 8	9	2	22.22	
• Weldeverden	23	6	26.09	
• Zwarthoek	11	3	27.27	
Breed				
• Angus	11	1	9.09	0.149
• Beef master	1	1	100	
• Bonsmara	140	39	27.86	
• Brahman	38	12	31.58	
• Jersey	1	0	0.00	
• Nguni	192	40	20.83	
Gender				
• Female	335	81	24.18	0.901
• Male	48	12	25.00	
Abortion history				

• No	158	33	20.89	0.194
• Yes	225	60	26.67	
Disposal of aborted fetuses\placentas				
• Buried	172	45	26.16	0.126
• Burnt	21	2	9.52	
• None	179	41	22.91	
• Thrown into the bushes	11	5	45.45	
Infertility history				
• No	363	91	25.07	0.126
• Yes	20	2	10.00	
Weak offspring history				
• No	335	81	24.18	0.901
• Yes	48	12	25.00	
Manure uses				
• Fertilizer	252	70	27.78	0.027
• Not used	131	23	17.56	
ticks infestation\ presence of rodents in the farms				
• No	54	14	25.93	0.761
• Yes	329	79	24.01	
Herd size				
• 1-50	191	36	18.85	0.000
• 51-100	53	11	20.75	
• 101-150	112	27	24.11	
• >150	27	19	70.37	
Farm type				
• Commercial	215	62	28.84	0.019
• Communal	168	31	18.45	

Table 4.1.2 Multivariable analysis of risk factors linked with seropositivity to *C. burnetii* in cattle on farms in Limpopo province, South Africa.

Risk factor	OR*	95% CI**	Chi-square <i>P</i> -value
Dip tank	0.98	0.92 – 1.05	0.589
Local municipality	1.09	1.00 – 1.20	0.043
Manure uses	0.75	0.48 – 11.62	0.347
Herd size	2.24	1.21 – 4.15	0.010
Farm type	3.06	0.65 – 14.36	0.155

*OR: Odds ratio

**CI: Confidence interval

4.2 Molecular prevalence of *C. burnetii* using PCR

Of the 383 samples tested to determine *C. burnetii* prevalence by PCR, 60 (15.67%) cattle were positive. *C. burnetii* prevalence by PCR in females was 16.72% and 8.33% in males and the difference was not statistically significant ($P = 0.135$) (Table 4.2.1). Elias Motsoaledi local municipality had the highest number of 44.44% of cattle testing positive by PCR followed by Vaalwater local municipality with 40.00% and the lowest PCR prevalence of 8.33% was observed in Blouberg local municipality and the difference was statistically significant ($P < 0.001$). The PCR prevalence in breed ranged from 20.71% in the Bonsmara breed to 13.16% in the Brahman breed and the difference was not statistically significant ($P = 0.068$).

The prevalence of *Coxiella burnetii* by PCR in dip-tanks ranged from 44.44% in farm 32 to 7.14% in Ga-Molekane dip-tank. The difference was statistically significant ($P = 0.001$) (Table 4.2.1). Cattle that had an abortion history exhibited a prevalence of 20.44% by PCR and cattle with no abortion history had a PCR prevalence of 8.86% and the difference was statistically significant ($P = 0.002$). Farms that disposed of aborted fetuses/placentas by burning had the lowest PCR positivity of 9.52% and a PCR prevalence of 18.18% was

observed in farms that had the aborted fetuses\placentas thrown into the bush and the difference was statistically significant ($P = 0.016$). Cattle that had ticks infestation\ presence of rodents in the farms exhibited a PCR prevalence of 14.29% and those that had no ticks infestation\ presence of s rodents in the farms had a prevalence of 24.07% and the difference was statistically significant ($P = 0.0067$). The prevalence by PCR according to herd size ranged from 12.57% in the herd size category of 1 – 50 to 44.44% in the herd size category of greater than 150 and the difference was statistically significant ($P < 0.001$). Commercial and communal farms exhibited a prevalence of 18.14% and 12.50% by PCR, respectively but the difference was not statistically significant ($P=0.132$) The local municipality, dip-tank, abortion history, disposal of aborted fetuses\placentas, ticks infestation\ presence of s rodents in the farms and herd size were included in the final logistic regression (Table 4.2.2), however only abortion history (OR 0.31; 95% CI 0.11 – 0.89; $P = 0.030$) remained associated with *C. burnetii* positivity by PCR.

Table 4.2.1 Molecular prevalence of Q fever and univariable analysis of risk factors linked with PCR positivity to *C. burnetii* in cattle on farms in Limpopo province, South Africa.

Risk factor	Number of animals sampled	Number testing positive	% PCR positive	<i>P</i> - <i>value</i>
District municipality				
• Capricorn	127	17	13.39	0.671
• Sekhukhune	123	20	16.26	
• Waterberg	133	23	17.29	
Local municipality				
• Bela-bela	26	3	11.54	0.000
• Blouberg	48	4	8.33	
• Burgersfort	67	8	11.94	
• Elias Motsoaledi	27	12	44.44	
• Ephraim mogale	29	0	0.00	
• Lepelle nkumpi	29	5	17.24	
• Lephalale	31	4	12.90	
• Modimolle	42	10	23.81	

• Mokgalakwena	20	2	10.00	
• Molemole	15	0	0.00	
• Moretele	9	2	22.22	
• Polokwane	35	8	22.86	
• Vaalwater	5	2	40.00	
Dip-tank				
• Alice's farm	11	2	18.18	0.001
• Blackhill	14	0	0.00	
• Earlydawn	34	4	11.76	
• Elandsbosch	5	2	40.00	
• Eqcebo	3	0	0.00	
Farm 32	27	12	44.44	
Ga- machikiri	6	1	16.67	
Ga-maepa	32	5	15.63	
Ga-maseven	35	3	8.57	
Ga-molekane	14	1	7.14	
Goba	15	0	0.00	
Klipspruit	12	0	0.00	
Marble hall	29	0	0.00	
Mora farm	10	2	20.00	
Rooibosveld	29	5	17.24	
Skuinhoek	23	8	34.78	
Sterkfontein	9	0	0.00	
Sterkstrom	19	4	21.05	
Tabana	13	1	7.69	
Vlaaglaakte portion 8	9	2	22.22	
Weldeverden	23	6	26.09	
Zwarthoek	11	2	18.18	
Breed				
Angus	11	1	9.09	0.068
Beef master	1	1	100.00	

Bonsmara	140	29	20.71	
Brahman	38	5	13.16	
Jersey	1	0	0.00	
Nguni	192	24	12.50	
Gender				
Female	335	56	16.72	0.135
Male	48	4	8.33	
Abortion history				
No	158	14	8.86	0.002
Yes	225	46	20.44	
Disposal of aborted foetuses\placentas				
• Buried	172	38	22.09	0.016
• Burnt	21	2	9.52	
• None	179	18	10.06	
• Thrown into the bushes	11	2	18.18	
Infertility history				
• No	363	58	15.98	0.474
• Yes	20	2	10.00	
Weak offspring history				
• No	335	51	15.22	0.530
• Yes	48	9	18.75	
Manure uses				
• Fertilizer	252	44	17.46	0.180
• Not used	131	16	12.21	
ticks infestation\ presence of rodents in the farms				
• No	54	13	24.07	0.067
• Yes	329	47	14.29	
Herd size				
• 1-50	191	24	12.57	0.000
• 51-100	53	10	18.87	
• 101-150	112	14	12.50	

• >150	27	12	44.44	
Farm type				
• Commercial	215	39	18.14	0.132
• Communal	168	21	12.50	

Table 4.2.2 Multivariable analysis of risk factors associated with PCR positivity to *C. burnetii* in cattle on farms in Limpopo province, South Africa.

Risk factor	OR*	95% CI**	Chi-square <i>P</i> -value
Dip tank	0.98	0.89 - 1.07	0.636
Local municipality	1.06	0.93 - 1.19	0.364
Disposal of aborted foetuses\placentas	0.99	0.62 – 1.56	0.961
Abortion history	0.31	0.11 – 0.89	0.030
Herd size	1.16	0.53 – 2.48	0.711

*OR: Odds ratio

**CI: Confidence interval

4.3 *Coxiella burnetii* molecular detection and MLVA typing

Molecular detection of *C. burnetii* from sheath scrapings and vaginal swabs by PCR targeting the IS1111 gene revealed that 15.67% were positive and the amplicons were approximately 146 bp in size (Figures 4.3.1 and 4.3.2). Of the seven sequences analysed for verification on the NCBI BLAST, all had similarities to *Coxiella burnetii* transposase gene fragment (Table 4.3.1).

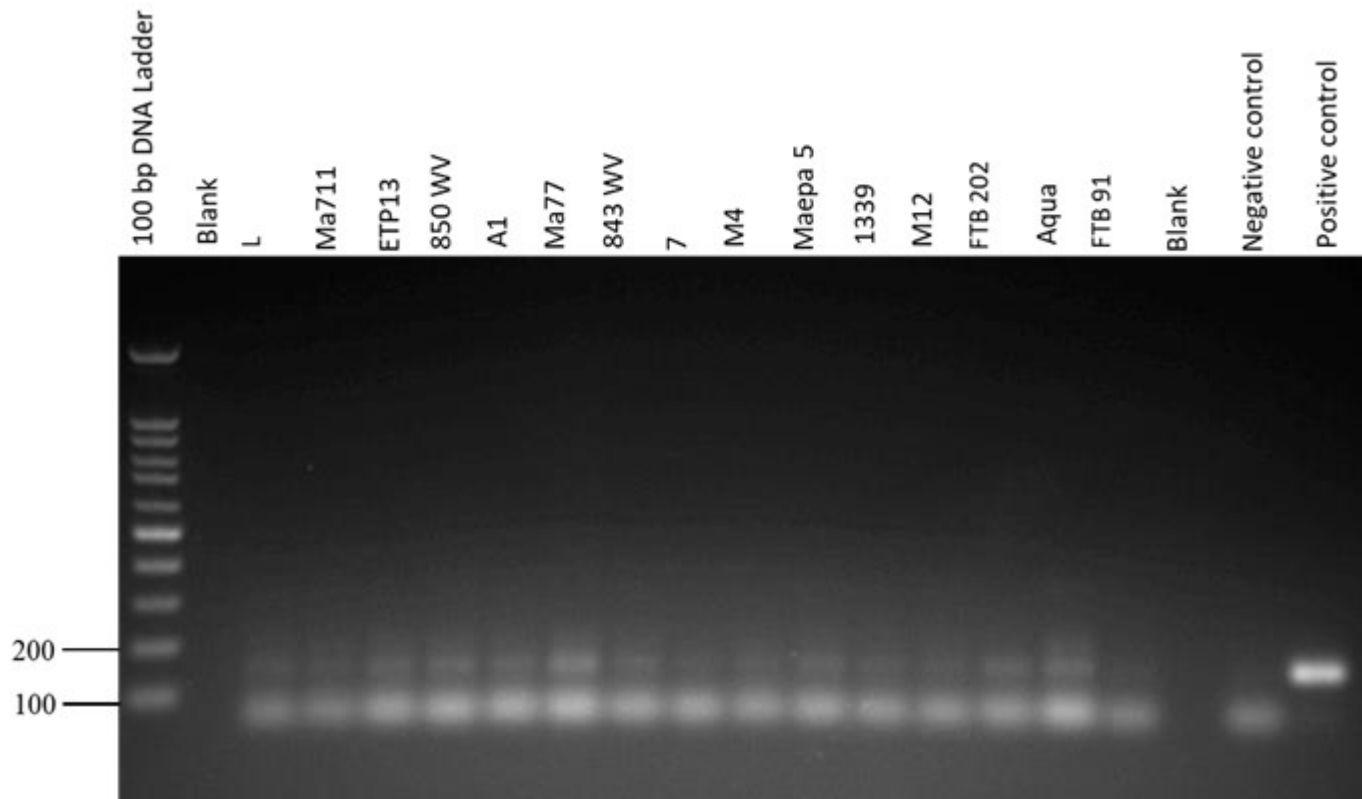


Figure 4.3.1: *IS1111* gel electrophoresis of PCR amplicons. Quick-Load® 100 bp DNA ladder was used in the first lane (New England Biolabs, Ipswich, MA, USA). The positive control is an in-house positive control from Mangena et al. (2021), distilled water was used as the negative control, and the empty lane is blank. L, Ma711, ETP 13, 850 WV, A1, Ma77, 843 wv, 7, m4, Maepa 5, 1339, M12, FTB 202, Aqua and FTB 91 are cattle vaginal swab samples.

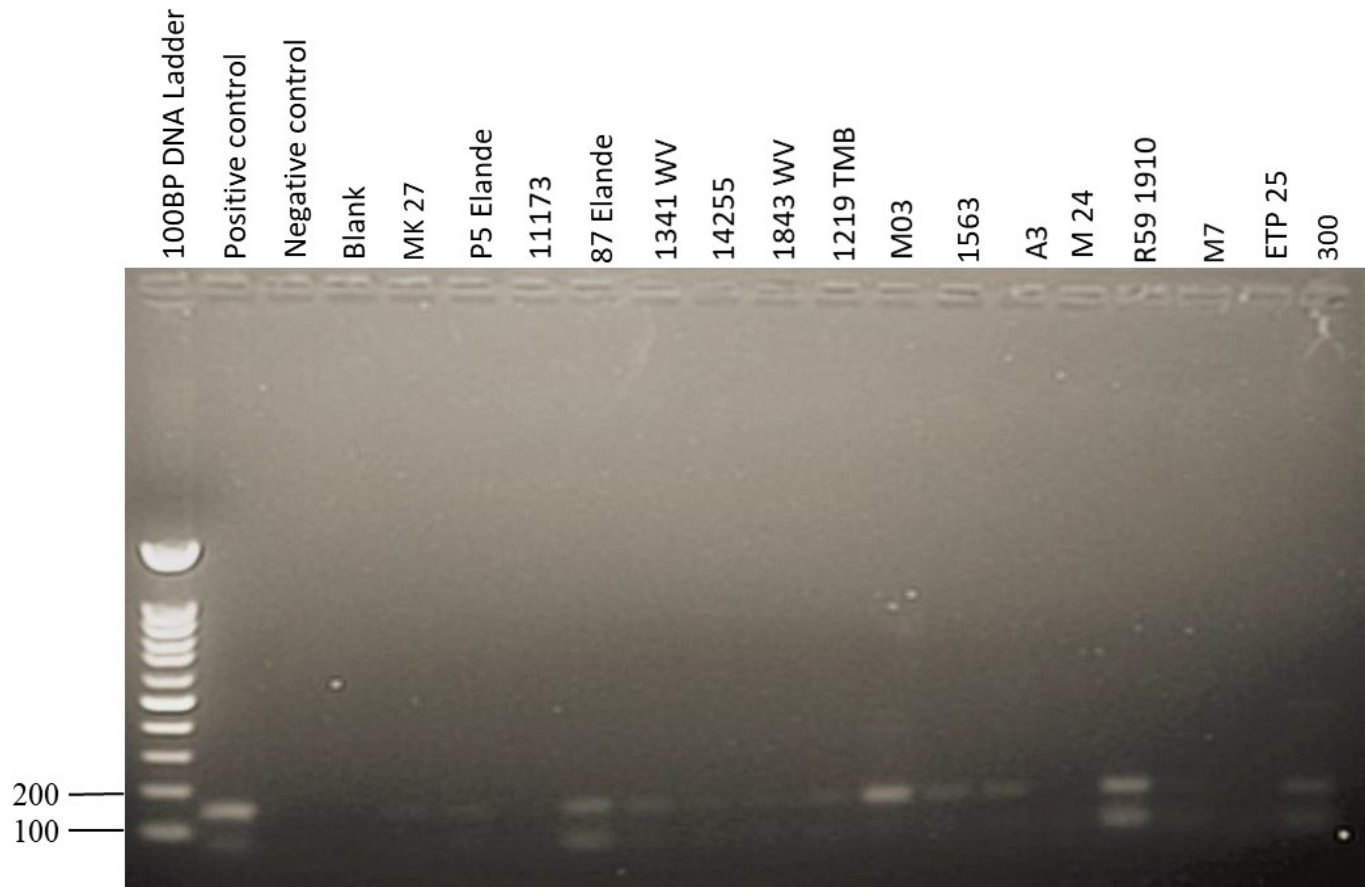


Figure 4.3.2: IS1111 gel electrophoresis of PCR amplicons. Quick-Load® 100 bp DNA ladder was used in the first lane (New England Biolabs, Ipswich, MA, USA). The positive control is an in-house positive control from Mangena et al. (2021), distilled water was used as the negative control, and the empty lane is blank. Mk 27, P5 Elande, 11173, 87 Elande, 1341 WV, 14255, 1843 WV, 1219 TMB, M03, 1563, A3, R59 1910, M7 ETP 25 AND 300 are cattle vaginal swab samples.

Table 4.3.1: The sample ID, sequence identity, percent identity , query coverage, and accession number of the seven sequences analysed using NCBI BLAST.

Sample ID	Sequence identity	Percent identity	Query coverage	E values	Base pairs matching	Genbank accession number
L	<i>Coxiella burnetii</i> strain 54T1 transposase gene, partial cds	99%	98%	1e-66	547	MT268532.1
A1	<i>Coxiella burnetii</i> strain 54T1 transposase gene, partial cds	94%	91%	3e-31	566	MT462981.1
FST059	<i>Coxiella burnetii</i> strain 54T1 transposase gene, partial cds	95%	84%	2e-34	547	MT268532.1
M4	<i>Coxiella burnetii</i> strain 54T1 transposase gene, partial cds	99%	85%	4e-40	547	MT268532.1
Maepa 18	<i>Coxiella burnetii</i> insertion sequence IS1111A transposase gene, partial cds	90%	46%	4e-23	566	MT462981.1
Maepa 24	<i>Coxiella burnetii</i> insertion sequence IS1111A transposase gene, partial cds	96%	59%	<u>2e-36</u>	<u>429</u>	<u>MT462981.1</u>
ETP 19	<i>Coxiella burnetii</i> strain YL4 insertion sequence IS1111, partial sequence	92%	57%	<u>4e-27</u>	<u>247</u>	<u>KY484070.1</u>

4.4. Comparison of *C. burnetii* prevalence using ELISA and PCR

There was agreement in the results for 153 (34.95%) cattle out of 383 samples tested for antibodies against *C. burnetii* using indirect ELISA and for *C. burnetii* DNA using IS1111

conventional PCR. Relatively fewer cattle (15.67%) were positive by PCR (60) than by ELISA (93) (Table 4.4.1). Of the total ELISA positive cattle, 55 (59.14%) were also positive by PCR. Out of all the samples that were not positive by PCR (323), 55 (17.03%) of those were also negative by ELISA. Of the overall PCR positive cattle, 38 (63.33%) of those also tested positive by ELISA.

Table 4.4.1: Relationship between ELISA and PCR prevalence in cattle on farms in Limpopo province, South Africa.

	Frequency (n)	Percent
ELISA		
Negative	290	75.72
Positive	93	24.28
PCR		
Negative	323	84.33
Positive	60	15.67
ELISA Negative and PCR Positive		
ELISA negative	268	92.41
PCR positive	22	7.59
Total	290	
ELISA Positive PCR Positive		
ELISA negative	55	59.14
PCR positive	38	40.86
Total	93	
PCR Negative ELISA Positive		
ELISA negative	268	82.97
ELISA positive	55	17.03
Total	323	

PCR Positive		
ELISA Positive		
PCR positive	22	36.67
ELISA positive	38	63.33
Total	60	
ELISA Negative		
PCR Negative		
PCR negative	268	100
Total	268	

CHAPTER 5. DISCUSSION AND CONCLUSIONS

To the best of our knowledge, the seroprevalence of 24.28% observed in this study using indirect ELISA is the first evidence of *Coxiella burnetii* presence in cattle on farms in Limpopo province, South Africa. This seroprevalence is comparable to and falls within the range noted in other countries. A study conducted in Egypt revealed a seroprevalence of 19.3% in cattle using an indirect ELISA (Klemmer et al., 2018) which is slightly lower than the findings in the current study. The seroprevalence observed in this study is higher than that of a study conducted in the abattoirs of the five districts of the Gauteng province by Mangena et al. (2021) who reported a seroprevalence of 9.40% in cattle using an indirect ELISA. The seroprevalence noted in this study is lower than the seroprevalence in a study by Adesiyun et al. (2020) which reported a significantly higher prevalence of 38.9% in cattle in Bushbuckridge Municipality, Mpumalanga province using an indirect ELISA.

Gummow et al. (1987) reported a seroprevalence of 7.8% using CFT in the Transvaal (Gauteng) province of South Africa, which is much lower than what was obtained in this study. Kelly et al. (1993) reported a seroprevalence of 39% in cattle in Zimbabwe using IFA which is higher than that obtained in this study. A much higher seroprevalence of 59.8% was reported by Adesiyun et al. (1984) in Nigerian cattle using capillary agglutination test (CAT). The differences in the seroprevalence obtained in all of these studies can be ascribed to factors such as study locations (South Africa (Limpopo, Mpumalanga, Gauteng); Zimbabwe; Nigeria and Egypt), the years in which samples were collected (October 1983 to February

1984; March 1985 to July 1986; April to September 2013; October 2015 to March 2016; November 2020 to September 2021) and serological test used (ELISA, CFT, IFA and CAT). Furthermore, IgG antibodies can be detected for up to a year after exposure (Conan et al., 2020). These variables have the potential to influence exposure and detection of *C. burnetii* infections (Muleme et al., 2016, Plummer et al., 2018, Larson et al., 2019).

In terms of risk factors for *C. burnetii* exposure in cattle, significant correlations are mostly associated with management methods (Carbonero et al., 2015; Nokhodian et al., 2016; Boroduske et al., 2017). Since we only sampled cattle in our study, we were unable to determine prevalence variation by animal species and we also did not document any cattle housing system however the seroprevalence data in this study shows that the cattle in these district municipalities are exposed to *Coxiella burnetii*. Other studies, however, have shown that prevalence in animals vary according to species, herd size, housing systems, and geographic region (Wittenbrink et al., 1994; Hilbert et al., 2012; Georgiev et al., 2013). In terms of year-to-year variations and geographic region, our research supports the suggestions made by Adesiyun et al. (2020) who reported a seroprevalence of 38.90% in the Mpumalanga province in samples collected between April to September 2013. Mpumalanga has a subtropical climate with hot summers and mild to cool winters that transition to cold and frosty conditions in the Highveld (Municipality, 2012)

Gummow et al. (1987) that reported a seroprevalence of 7.80% in the Transvaal (Gauteng) province in samples collected between March 1985 to July 1986. Only the months of July and August experience the coldest weather. The samples of this current study were collected between November 2020 to September 2021. As mentioned in the materials and method of this thesis, Limpopo has an average daily high temperature of 26°C. The majority of Limpopo is warm all year with little cold experienced during winter. Cases of Q fever have been reported to rise in the winter and early spring (Porter et al., 2011). It has also been documented that seasonal and year-to-year variations in *C. burnetii* infections are possible (Hilbert et al., 2014). The difference in the seroprevalence noted in all these studies could be due to year-to-year variations and geographic regions.

In our study, local municipality had significant associations with Q fever seropositivity in cattle ($P < 0.001$). There was a higher seroprevalence in Elias Motsoaledi (70.37%) compared to other municipalities. Mangena et al. (2021) reported a seroprevalence of 12.20% in Tshwane district of Gauteng province which was the highest in their study but lower than the

findings observed in this study. *Coxiella burnetii* positive cattle were found in all local municipalities and as a result, Q fever is most likely an existing disease in Limpopo province.

In the current study, statistically significant ($P = 0.027$) associations were observed between *C. burnetii* infection and manure use. This study revealed that cattle from farms that used manure as fertilizer had a higher seroprevalence (27.78%) than those that did not (17.56%). Manure spreading has been identified as a risk factor for human *C. burnetii* infections (Porter et al., 2011). The main source of Q fever infections in humans is the inhalation of contaminated aerosols which can occur during the spreading of manure, therefore there is a possibility of human Q fever cases in Limpopo. Wind can also carry contaminated manure and dust, exposing more animals and workers on the farm to infection. The wind has been reported to play a critical role in the transmission of *C. burnetii* between ruminant farms and from ruminants to humans (Tissot-Dupont et al., 2004; Schimmer et al., 2010; O'Connor et al., 2015).

Farms with herd sizes greater than 150 had a significantly ($P < 0.001$) higher seroprevalence (70.37%), while farms with herd sizes between 1 and 50 had a statistically significantly ($P < 0.001$) lower seroprevalence of 18.85%. The findings agree with the report of Cadmus et al. (2020) who reported a higher seroprevalence (20.51%) in the herd size category of above 50 but a lower seroprevalence (9.52%) in smaller herds (10-30). Adamu et al. (2018) demonstrated that the larger the herd size, the greater the risk of disease transmission through interaction with other infected animals or herds while grazing on contaminated pasture or sharing of watering stations.

In this study commercial farms exhibited a statistically significantly ($P = 0.019$) higher seroprevalence (28.84%) compared to communal farms. This difference could be because commercial farms have more cattle, as well as the fact that infection can occur during the movement of animals to and from auctions and during the auction itself. It has been reported that herd size is related to antibody positivity (Ryan et al., 2011; Anastacio et al., 2013).

A seroprevalence of 25.07% was observed in cattle with no infertility history in this study and the difference was statistically not significant ($P = 0.126$). Infection with *Coxiella burnetii* has frequently been associated to infertility (Krauss et al., 1987; Aitken 1989; To et al., 1998). However, Lo'pez-Gatius et al. (2012) reported in a study of 781 parous cows with 50% seroprevalence against *C. burnetii*, that they discovered early fertile cows (cows that were pregnant before 90 days of lactation) had a greater chance of being seropositive than the

rest of the cattle. In a review article, Garcia-Ispuerto et al. (2014) revealed that there are several factors that can influence the findings of studies aimed at correlating *C. burnetii* infection with the rate of conception: the spread of various *C. burnetii* genotypes, what consequence does the *C. burnetii* strain have on the cow?, the monitoring or non-monitoring of other traditionally influencing factors such as past postpartum diseases, the inseminating semen, the inseminator, or the season. There hasn't been a study providing evidence that *C. burnetii* is linked to low cattle conception rates (Agerholm, 2013; Garcia-Ispuerto et al., 2014).

In the current study, a prevalence of 15.67% for *C. burnetii* was determined by IS1111 PCR which is considerably lower than the seroprevalence of 24.28% determined by the indirect PCR. In this current study, ELISA positivity (17.03%) and PCR negativity (82.97%) indicated a previous or past *C. burnetii* infection with no bacterial shedding through vaginal mucus in cows or preputial discharge in bulls. In contrast, the presence of PCR positive samples (7.59%) and ELISA negative samples (92.41%) can be attributed to a recent *C. burnetii* infection in cattle in which antibodies against *C. burnetii* have not developed, or the level of these antibodies is not yet detectable (Muskens et al., 2011; Anastácio et al., 2016; Menadi et al., 2022). *Coxiella burnetii* antibodies have been observed to emerge 15 days after the beginning of the disease (Maurin & Raoult, 1999). The presence of the pathogen in cattle and the evidence of exposure as shown by using both PCR (36.67%) and ELISA (63.33%) positivity in the current study suggests an active circulation of the pathogen, and possibly past exposure reflected by the ELISA results, as earlier reported (Freick et al., 2017; Barkallah et al., 2018).

Coxiella burnetii DNA was detected in all except two local municipalities in which antibodies were present and *C. burnetii* DNA was detected in all breeds except the Jersey breed which had antibodies against *C. burnetii* indicative of past exposure to the pathogen in these municipalities and in the Jersey breed. The Bonsmara breed had a high prevalence of shedders (20.71%), whereas the Brahman breed had a high seroprevalence (31.58%) suggestive that the infection was recent in some of the Bonsmara cattle and past infections in most of the Brahman cattle, as *C. burnetii* DNA was detected in only 13.16% of the Brahman cattle.

In this study, we detected *C. burnettii* DNA in 20.44% of cattle from farms with a history of abortions. The seroprevalence (26.67%) noted in this study is also higher in farms with

abortion history cases. It has been documented in the literature that infections with *C. burnetii* were responsible for 22% and 37% of abortion cases in studies in Italy (Parisi et al., 2006) and in Cyprus (Cantas et al., 2011), respectively.

There is a strong association ($P = 0.002$) between *C. burnetii* infection and abortion history in this current study. *Coxiella burnetii* infections have been linked to significant economic losses in cattle and other animals due to late abortions, early births, and low birth weights (Honarmand, 2012). *Coxiella burnetii*-associated abortion in cattle is rarely identified in larger surveys on the causes of abortion in endemic areas (Anderson et al., 1990; Kirkbride, 1993). Poor hygiene practices, according to Cantas et al. (2011), may explain the relatively high occurrence of *C. burnetii* abortions in most rural regions. The current study was conducted in mostly rural areas such as Ga-Maepa, Vlaagglaakte portion 8, Zwarthoek, Goba, Earlydawn, Blackhill, Farm 32, Rooibosveld, Ga-Molekane, Ga-Maseven and Ga-Machikiri, with all having a history of abortions in cattle. Vaccination with a phase 1 vaccine has been shown to reduce the occurrence of abortions and other reproductive problems in livestock (Arricau-Bouvery et al., 2005; Hogerwerf et al., 2011).

Burying aborted fetuses/placentas may also spread the infection because the aborted fetus/placentas are buried in the farm, where dogs and other animals can dig up the grave to feed, exposing them to the infection and transmitting Q fever to other animals and farm workers. Throwing away the aborted fetuses/placentas into the bushes spreads *C. burnetii* infection even further. In farms where the aborted materials were burned, there was a comparable prevalence (9.52%) and seroprevalence (9.52%). Keshavamurthy et al. (2019) reported that throwing away aborted material or parturition products or feeding them to dogs was an unsanitary management practice that increased the risk of *C. burnetii* infection in cattle. A similar study conducted in France concluded that when the placenta and/or fetus of aborted cows were not properly removed, the risk of having at least one seropositive nulliparous female increased (Taurel et al., 2011). During parturition, large quantities of *C. burnetii* are secreted through birth products (Guatteo et al., 2007; OIE, 2015). The high prevalence observed in farms that threw away/buried their aborted materials in this study can be attributed to a lack of Q fever awareness. As mentioned previously that during the sampling process, all the farmers and farm workers did not know of Q fever. A Q fever awareness programme should be implemented in South Africa so that farmers and farm workers can practice proper hygiene measures.

The association between herd size, animal density, and an increased risk of *C. burnetii* infection has been well established (Agger and Paul, 2014; Nusinovici et al., 2015). In this study a high frequency (44.44%) of animals shedding *C. burnetii* was observed in herd size category of > 150 and the difference was statistically significant ($P < 0.001$). This is similar to the reports by others (McCaughey et al., 2010; Ryan et al., 2011) who observed a significant association between increasing herd size and test positivity in bulk tank milk (BTM) samples. This association can be explained, in part, by a larger population at risk, an increased risk of pathogen introduction and transmission within and between herds, such as increased feed and animal supply, and more professionals working at or visiting the farm (Schimmer et al., 2011). Farms with many animals have a higher chance of contracting and developing *C. burnetii* infection, thus herd size must be considered a risk factor for *C. burnetii* spread (Anastácio et al., 2016). A frequency of 18.87% of PCR positivity, reflective of carriage and shedding of *C. burnetii*, was observed in farms with herd sizes ranging from 51 to 100 animals, which could be attributed to fewer animals, less cattle activity on the farms, and a better management system.

Although the difference was not significant in this study, the frequency of cows shedding *C. burnetii* as detected in vaginal swab samples was 16.72%, while the corresponding frequency of shedding in bulls detected in the preputial materials was 8.33%, both of which were lower than the seroprevalence observed for both sexes. Vaccination may prevent shedders from spreading the infection to other cattle and people on farms. However, there is currently no policy plan on a cattle vaccination programme in South Africa.

Limitations

The present study had limitations which include the number of samples collected being lower than that calculated prior to the study. Failure to sample two local municipalities, due to financial constraints, therefore resulting in a smaller sample size. The recent discovery of *Coxiella*-like bacteria (CLB), which are closely related but genetically distinct from *C. burnetii*, has created a diagnostic quandary due to cross-reactivity for many *C. burnetii* specific genes, including the *IS1111*. In this study, however, we used conventional PCR, which is regarded as one of the most sensitive for epidemiological screening. The influence of confounding factors on the analysis cannot be ignored.

Recommendations

Conduct large-scale studies in Limpopo province in animals and people to determine the prevalence of *C. burnetii*, as well as the risk factors of *C. burnetii* infection in humans. Based on the identified risk factors, management measures should be developed to control or prevent Q fever. These studies should investigate the seroprevalence and PCR prevalence of *C. burnetii* infection to differentiate between past and current exposure, as well as conduct a molecular characterization of the pathogen to determine the strains circulating in the country. Other studies should be conducted on ticks and Coxiella-like bacteria. A long-term plan for animal vaccination should be developed to reduce the number of infections in animals and to also reduce animal-to-human transmission. Awareness campaigns should also be launched to educate farmers about the risks of Q fever and proper hygiene practices. It is recommended that a surveillance system for Q fever control and prevention be developed in South Africa.

Conclusions

Finally, the study examined the seroprevalence, PCR prevalence, and risk factors for *C. burnetii* in cattle on farms in South Africa's Limpopo province. This study determined that *C. burnetii* is present in the study areas (Capricorn, Waterberg and Sekhukhune). Local municipality found to be significantly associated with Q fever seroprevalence, while abortion history was found to be significantly associated with the prevalence of Q fever determined by PCR. In the study areas, *C. burnetii* should also be regarded as a possible source of human Q fever.

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APPENDICES

Appendix 3.1 Animal ethics committee

Appendix 3.2 Research ethics committee

Appendix 3.3 Section 20 Ethical Approval Committee Certificate

Appendix 3.4 Consent form

Appendix 3.5 Questionnaire

Appendix 3.6 ELISA supplementary table

Appendix 3.7 Sequences supplementary table

Appendix 3.1 Animal ethics committee



Faculty of Veterinary Science Animal Ethics Committee

7 May 2021

Approval Certificate Annual Renewal (EXT1)

AEC Reference No.: REC172-19
Title: PREVALENCE, RISK FACTORS AND CHARACTERISTICS OF COXIELLA BURNETII IN CATTLE ON FARMS IN LIMPOPO PROVINCE, SOUTH AFRICA
Researcher: Ms V Sadiki
Student's Supervisor: Prof AA Adesiyun

Dear Ms V Sadiki,

The **Annual Renewal** as supported by documents received between 2021-04-12 and 2021-05-07 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2021-05-07.

Please note the following about your ethics approval:

1. The use of species is approved:

Species	Number Available
Cattle	196
Samples	
Sheath washes/ scrapings	98
Vaginal swabs	98
Whole blood	196 (10ml per animal)
Opportunistic sampling of aborted fetuses	To be reported on

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2022-05-07.
3. Please remember to use your protocol number (REC172-19) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
5. **All incidents** must be reported by the PI by email to Ms Marieze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
6. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.
Yours sincerely



Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8434
Fax +27 12 529 8321
Email: marleze.rheeder@up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

Appendix 3.2 Research ethics committee



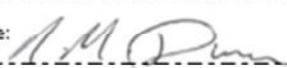
Faculty of Veterinary Science

Research Ethics Committee

Project Title	PREVALENCE, RISK FACTORS AND CHARACTERISTICS OF COXIELLA BURNETII IN CATTLE ON FARMS IN LIMPOPO PROVINCE, SOUTH AFRICA
Project Number	REC172-19
Researcher / Principal Investigator	Ms V Sadiki

Dissertation / Thesis submitted for	Masters
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Supervisor	Prof AA Adesiyun
------------	------------------

APPROVED	Date: 2019-11-04
CHAIRMAN: UP Research Ethics Committee	Signature: 

Office of the Chairman: Research Ethics Committee
Room 2-24, Pathology Building, Onderstepoort
University of Pretoria, Private Bag X04
Onderstepoort 0110, South Africa
Tel +27 (0)12 529 8062
Email
www.up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

Appendix 3.3 Section 20 Ethical Approval Committee Certificate



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: HerryG@daff.gov.za
Reference: 12/11/1/1

Dr Nomakorinte Gcebe
Onderstepoort Veterinary Institute
100 Old Soutpan Road
Onderstepoort
0110
Email: GcebeN@arc.agric.za

Dear Dr Gcebe,

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

Your application sent per email on 17 May 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/her by any other act of the Republic of South Africa;
2. The study is approved as per the application form dated 17/05/2019 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. All potentially infectious material utilised, collected or generated during the study are to be destroyed at the completion of the study. Records must be kept for five years for auditing purposes;

4. Samples may only be collected from animals where the state veterinary official has confirmed that the area is not under any restriction due to disease which the species is susceptible to;
5. Samples from abattoirs may only be removed subject to obtaining permission from the owner and the provincial veterinary official providing oversight for that specific abattoir;
6. All samples must be packaged and transported in accordance with International Air Transport Association (IATA) requirements and the National Road Traffic Act, 1996 (Act No. 93 of 1996);
7. Isolates of *Coxiella burnetii* and *Toxoplasma gondi* as well as extracted DNA and purified protein derivatives from this study may be stored at the OVI Bacteriology laboratories and any further use or distribution is subject to obtaining a separate Section 20 approval;
8. 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: Prevalence of Q-fever and Toxoplasmosis in slaughtered and farmed animals in Free State, North West and Limpopo Provinces of South Africa and development of cell mediated immunity biomarkers.

Researcher: Dr Nomakorinte Gcebe

Institution: Onderstepoort Veterinary Institute

Our ref Number: 12/11/1/1

Your ref: n/a

Expiry date: 2022-04

Kind regards,



DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH

Date: 2019-07-30

- 2 -

SUBJECT: S20 PERMISSION FOR: PREVALENCE OF Q-FEVER AND TOXOPLASMOSIS IN SLAUGHTERED AND FARMED ANIMALS IN FREE STATE, NORTH WEST AND LIMPOPO PROVINCES OF SOUTH AFRICA AND DEVELOPMENT OF CELL MEDIATED IMMUNITY BIOMARKERS.- LJVR

Appendix 3.4 Consent form



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**PARTICIPANT'S INFORMATION & INFORMED CONSENT DOCUMENT
FOR A PARTICIPANT ADMINISTERED QUESTIONNAIRE**

STUDY TITLE: prevalence, risk factors and characteristics of *Coxiella burnetii* in cattle on farms in Limpopo province, South Africa

Principal Investigator: Vhahangwele Sadiki

Supervisor: Professor A.A. Adesiyun

Co-supervisor: Dr N Gcebe

Institution: University of Pretoria, faculty of veterinary science

Daytime telephone number: 0125299138

DATE AND TIME OF INFORMED CONSENT DISCUSSION:

		20
Dd	Month	Year

:	
Time	



Dear Prospective Research Participant

Dear Mr / Ms / Mrs

1) INTRODUCTION

You are invited to volunteer to participate in a research study. I am doing this research for a Master of Science degree purposes at the University of Pretoria. The information in this document is provided to help you to decide if you would like to participate. Before you agree to take part in this study, you should fully understand what is involved. If you have any questions, on any issues which are not fully explained in this document, do not hesitate to ask the researcher. You should not agree to take part unless you are completely happy with the kind of questions that will be asked.

2) THE NATURE AND PURPOSE OF THIS STUDY

The aim of this study is to determine the occurrence of *C. burnetii* in farmed cattle as well as characterizing circulating strains and assessing the risk of transmission to humans and animals across the farms in Limpopo Province, by doing so we wish to learn more about the disease Q-fever in cattle.

3) EXPLANATION OF PROCEDURES AND WHAT WILL BE EXPECTED FROM PARTICIPANTS

3.1 This study involves answering some questions regarding the cattle in your farm. We would like you to complete a questionnaire. It will take approximately 10 minutes. We will collect the questionnaire from you before you leave the farm. We will be available to help you with the questionnaire, if required. The researcher will keep the completed questionnaires in a safe place to make sure that only people working on the study will have access to it. Please do not write your name on the questionnaire. This will ensure that your answers are kept confidential (so nobody will know what you have answered).



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The questionnaire consists of two parts:

Part 1: Socio-demographic information which involves answering some questions about the location of the farm, farm size, herd size, type of farm, etc.

Part 2: Health Questionnaire which involves answering some questions about the medical history, any symptoms you may have noticed from the cattle such as abortion, infertility or weak offspring that could indicate Q-fever.

If there are questions that are too sensitive for you to answer, you do not need to answer them.

3.2 Sample collection

Do you grant full consent for the collection of data on your farm and cattle? Yes/No

Do you grant full consent for the collection of samples from your cattle? Yes/No

4) RISK AND DISCOMFORT INVOLVED

There is no foreseeable physical discomfort or risk involved, but if any of the animals should get injured during sampling, the state veterinarian will take care of the injury and the farm owners will be given the telephone number of the veterinarian and researcher to call if the injury worsens.

5) POSSIBLE BENEFITS OF THIS STUDY

This study may help provide up-to-date data on the prevalence of Q-fever in cattle farms, it may also provide recent data on the strains of *C. burnetii* currently circulating in the province and recommend ways to reduce contamination of the environment by aborted fetuses and manure and the information collected about transmission to humans and animals can be used to educate farmers, veterinarians and the public at large to improve their knowledge.

6) ETHICS APPROVAL



This Protocol was submitted to the Faculty of Veterinary Science Research Ethics Committee, University of Pretoria, Veterinary Science Campus, Telephone numbers 012 356 3084 / 012 356 3085 and written approval has been granted by that committee.

7) REUSE AND STORAGE OF DATA

No material of any kind, including data and research findings, obtained or resulting from the procedure would be passed on to any third party or used for any purpose other than that specified in this form, except with the written consent of the undersigned owner of the animal. Data will be stored electronically on a South African database.

If you have any questions concerning this study, you may contact: Dr. Gcebe on 0125299138.

8) CONFIDENTIALITY

All records from this study will be regarded as confidential. All results will be published or presented in such a way that it is not possible to identify the participants.

9) COMPENSATION

You will not be paid to take part in the study. There are no costs involved for you to be part of the study.

10) CONSENT TO PARTICIPATE IN THIS STUDY

- I confirm that the person requesting my consent to take part in this study has told me about the nature and process, any risks or discomforts, and the benefits of the study.
- I have read and the above written information about the study was explained to me using the local vernacular for my understanding.



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- I have had adequate time to ask questions and I have no objections to participate in this study.
- I am aware that the information obtained in the study, including personal details, will be anonymously processed and presented in the reporting of results.
- I understand that I will not be penalised in any way should I wish to discontinue with the study and my withdrawal will not affect my employment or student status.
- I am participating willingly.
- I have received a signed copy of this informed consent agreement.

Participant's name (Please print)

Date

Participant's signature

Date

Researcher's name (Please print)

Date

Researcher's signature

Date

AFFIRMATION OF INFORMED CONSENT BY AN ILLITERATE PARTICIPANT

(if suitable)

I, the undersigned, _____ have read and have explained fully to the participant, named _____, the participant informed consent document, which describes the nature and purpose of the study in which I have asked the participant to participate. The explanation I have given has mentioned both the possible risks and benefits of the study. The participant indicated that he/she understands that he/she will be free to withdraw from the study at any time for any reason and without jeopardizing his/her standard care.

I hereby certify that the participant has agreed to participate in this study.



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Participant's name (Please print)

Date

Participant's Signature or Mark

Date

Investigator's Name (Please print)

Date

Investigator's Signature

Date

Name of the person who witnessed the
Informed consent (Please print)

Date

Signature of the Witness

Date



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Questionnaire

You will have to fill the following questionnaire if you decide to join the study. Please do not complete the section if you are unsure of the required information. The project PI is available to explain all sections.

Essential information (required)

Answering the questions is completely voluntary and participants may leave out any questions that make them feel uncomfortable. This data will be used anonymously in our network and your privacy will be protected to the best of our ability.

CATTLE FARM QUESTIONNAIRE IN LIMPOPO PROVINCE

Date _____

Farm / Locality Details

Survey ID

Metropolitan / District		Locality name		Farm name		GPS coordinates	
Type of farm— Also: semi-intensive? Extensive? Intensive?	Feedlot	Cow-calf	Communal	Farm size acres		Tick infestation/presence of rodents	

Details and History of Animals Sampled

Breed/type	pure	crosses	Type	steers	bulls	Adult	Calves
Sex category	M	F	Herd size				
Age							
Disposal of aborted fetuses/placentas				Abortion cases			
Manure uses							
Water supply				Drainage of effluents			
No. and types of samples taken							

Appendix 3.6 ELISA supplementary table

Table 4.1: Sample to positive ratio in percentage, mean of the controls, positive and negative of the samples tested for antibodies against *C. burnetii* by indirect ELISA.

Sample ID	S/P%	Mean	positive/negative
A1	-20.38	-	negative
A2	9.51	-	negative
A3	16.62	-	negative
A4	30.98	-	Suspect-negative
A5	28.85	-	negative
A6	-16.36	-	negative
A7	17.79	-	negative
A8	71.39	-	positive
A9	9.09	-	negative
A10	-14.90	-	negative
A11	29.05	-	negative
A12	66.33	-	positive
A13	52.76	-	positive
A14	16.53	-	negative
A15	0.97	-	negative
A16	28.07	-	negative
A17	-20.94	-	negative
A18	19.09	-	negative
A19	-17.07	-	negative
A20	-3.53	-	negative
A21	22.41	-	negative
A22	-26.60	-	negative
A23	-12.52	-	negative

A24	22.62	-	negative
A25	44.5	-	positive
A26	7.30	-	negative
A27	16.11	-	negative
A28	31.40	-	Suspect-negative
A29	-14.72	-	negative
A30	15.42	-	negative
A31	17.86	-	negative
A32	101.99	-	positive
A33	-17.76	-	negative
A34	9.96	-	negative
B1	-21.92	-	negative
B2	-5.87	-	negative
B3	-1.46	-	negative
B4	-4.33	-	negative
B5	8.04	-	negative
B6	30.34	-	suspect
B21	-7.90	-	negative
B22	6.71	-	negative
B23	-15.76	-	negative
B24	-1.72	-	negative
B31	-7.16	-	negative
B32	51.04	-	positive
B33	3.91	-	negative
B34	8.70	-	negative
K1	-17.13	-	negative
K2	20	-	negative
K3	-10.06	-	negative
K18	-13.43	-	negative

K19	13.14	-	negative
K20	10.10	-	negative
K24	10.94	-	negative
K25	50.59	-	positive
K30	33.53	-	suspect
K31	61.11	-	positive
K32	97.5	-	positive
K33	-11.78	-	negative
C2	-15.97	-	negative
C3	55.6	-	positive
C4	-9.02	-	negative
C5	29.93	-	negative
C6	23.70	-	negative
C7	-4.30	-	negative
C8	-17.13	-	negative
C9	1.22	-	negative
C10	26.82	-	negative
C11	47.93	-	positive
C12	-10.59	-	negative
C13	-1.10	-	negative
C14	59.51	-	positive
C21	80.94	-	positive
C22	-19.05	-	negative
C23	-6.61	-	negative
C24	-19.96	-	negative
C29	-25.90	-	negative
C30	-24.80	-	negative
C31	-22.02	-	negative
C32	-21.13	-	negative

C33	13.95	-	negative
C34	24.89	-	negative
M1	10.97	-	negative
M2	28.74	-	negative
M3	15.84	-	negative
M4	-8.01	-	negative
M5	13.92	-	negative
M6	30.97	-	Suspect-negative
M7	18.67	-	negative
M8	39.19	-	Suspect-positive
M9	8.32	-	negative
PCx	-	2.156	
NCx	-	0.495	
M11	140.91	-	positive
M10	-472.72	-	negative
M12	-154.5	-	negative
M13	-8.18	-	negative
M14	-430	-	negative
M15	-72.75	-	negative
M16	1.72	-	negative
M17	-5.2	-	negative
M18	3.6	-	negative
M19	4.3	-	negative
M20	7.9	-	negative
M21	-3.9	-	negative
M26	37.5	-	Suspect-positive
M27	64.6	-	positive
M28	86.6	-	positive
M29	24.1	-	negative

M30	33.56	-	Suspect-negative
M31	9.5	-	negative
M32	-36.7	-	negative
M33	19.3	-	negative
MM1	1.54	-	negative
MM2	27.9	-	negative
MM9	11.1	-	negative
MM10	6.2	-	negative
MM11	14.9	-	negative
MM12	2.7	-	negative
MM13	6.3	-	negative
MM14	30.8	-	Suspect-negative
MM15	2.1	-	negative
MM18	5.0	-	negative
MM19	3.6	-	negative
MM20	17.3	-	negative
MM21	21.0	-	negative
MM25	0.85	-	negative
MM26	3.2	-	negative
No Tag	-3.3	-	negative
6138	26.5	-	negative
6249	-3.6	-	negative
6350	2	-	negative
6461	-0.9	-	negative
6572	-1.8	-	negative
Mo1	0.1	-	negative
Mo2	-1.2	-	negative
2416	-2.9	-	negative
Mo3	-1.37	-	negative

1	3.0	-	negative
2 Bons	41.3	-	positive
3	-3.9	-	negative
4	0.87	-	negative
5	0	-	negative
6	-0.4	-	negative
7	14.2	-	negative
8	40.1	-	positive
9	-2.8	-	negative
12	-1.6	-	negative
13	15.5	-	negative
14	-2.7	-	negative
15	-2.3	-	negative
Don 1222	0.6	-	negative
Don 1471	-2.5	-	negative
AJS 13393	1.67	-	negative
Don 16144	-1.2	-	negative
Don1596	-1	-	negative
Don 16133	7	-	negative
Don 1327	-3.3	-	negative
Don 18291	7.2	-	negative
Don 284	2.3	-	negative
Don 18287	12.2	-	negative
Don 286	0.4	-	negative
Morongwa1	7.5	-	negative
Morongwa2	2.4	-	negative
Morongwa3	12.4	-	negative
Morongwa4	0.3	-	negative
Morongwa5	0.2	-	negative

Morongwa6	-2.9	-	negative
Morongwa7	9.8	-	negative
Morongwa8	-0.2	-	negative
Morongwa9	0.5	-	negative
7	53.8	-	positive
8	7.8	-	negative
9	28.0	-	negative
14	9.6	-	negative
15	10.6	-	negative
PCx	-	2.465	
NCx	-	0.132	
16	2.0	-	negative
17	6.6	-	negative
18	61	-	positive
19	14.4	-	negative
20	-1.67	-	negative
21	-3.1	-	negative
22	-2.9	-	negative
23	-2.1	-	negative
24	2.5	-	negative
R591 905	0.8	-	negative
R59 56	-3.9	-	negative
R59 12	-3.47	-	negative
11	3.7	-	negative
1910	-2.3	-	negative
56	2.4	-	negative
87	-2.9	-	negative
71129	2.4	-	negative
No tag	2.465	-	negative

ETP 1	0.132	-	negative
ETP 2	43.4	-	positive
ETP 3	0.42	-	negative
ETP 4	23	-	negative
ETP5	113.8	-	positive
ETP 6	15.5	-	negative
ETP 7	9.9	-	negative
ETP 8	33.5	-	Suspect-negative
ETP 9	8.4	-	negative
ETP 10	8.2	-	negative
ETP 11	10.7	-	negative
ETP 12	44.5	-	positive
ETP 13	7.3	-	negative
ETP 14	10.8	-	negative
ETP 19	2.8	-	negative
ETP 21	1.6	-	negative
ETP 22	8.8	-	negative
ETP 23	62.3	-	positive
ETP 24	10	-	negative
welde1	16.6	-	negative
welde2	21.2	-	negative
welde3	52.9	-	positive
1218 DSI	29.4	-	negative
87 elande	28.7	-	negative
P5 elande	1.0	-	negative
Elands 3	18.7	-	negative
Elands 4	21.9	-	negative
Elands 5	6.7	-	negative
MK 1	4.5	-	negative

MK 2	12.2	-	negative
MK 3	58.6	-	positive
MK 4	-0.6	-	negative
MK 5	9.6	-	negative
MK 6	10.7	-	negative
MK 7	8	-	negative
MK 8	12.6	-	negative
MK 9	1.2	-	negative
MK 10	-0.6	-	negative
MK 11	1.5	-	negative
MK 12	0.2	-	negative
MK 13	13.9	-	negative
MK 14	21.9	-	negative
Machikiri 1	15.9	-	negative
Machikiri 4	6	-	negative
Machikiri 5	27.5	-	negative
Machikiri 6	66.4	-	positive
Machikiri 7	-4.2	-	negative
Machikiri 8	-3.8	-	negative
EM2	22.9	-	negative
EM1	19.7	-	negative
EM3	36.0	-	suspect
EM4	61.3	-	positive
EM5	17.6	-	negative
EM6	11.1	-	negative
EM7	15.3	-	negative
EM8	13.4	-	negative
EM9	31.4	-	Suspect-negative
EM10	109.6	-	positive

EM11	4.7	-	negative
EM12	25.8	-	negative
EM13	5.7	-	negative
EM14	73.2	-	positive
EM15	1	-	negative
EM16	8.6	-	negative
EM17	11.5	-	negative
EM18	7.9	-	negative
EM19	6.3	-	negative
EM20	4.5	-	negative
EM21	28.9	-	
PCx	-	2.214	
NCx	-	0.179	
EM22	12.3	-	negative
EM23	0.8	-	negative
EM24	5.8	-	negative
EM25	27.2	-	negative
EM26	1.9	-	negative
EM27	67.1	-	positive
EM28	33.9	-	Suspect-negative
Indruk 16110	1	-	negative
Braap	49.6	-	positive
Z1766	-2.3	-	negative
Z18167	-4.6	-	negative
LT21	-1.3	-	negative
SWP269	-1.6	-	negative
FST057	47.9	-	positive
AB	29.9	-	negative
LT22	9.6	-	negative

300	16.1	-	negative
FTB137	33.3	-	Suspect-negative
FTB152	223.6	-	positive
MYM249	28.3	-	negative
BAR105116	32.6	-	Suspect-negative
FTB202	399.4	-	positive
FTB115	32.2	-	Suspect-negative
FTB35	42.1	-	positive
L	36.3	-	Suspect-positive
301	273.5	-	positive
AQUA	46.5	-	positive
FTB38	16.2	-	negative
FST0L	74.2	-	positive
FTB53	6.9	-	negative
SW37	22.2	-	negative
FTB91	104.8	-	positive
JACK	23.8	-	negative
PASCAL	24.1	-	negative
LUCAS	88.7	-	positive
WV 1	59.9	-	positive
850 WV	527.6	-	positive
WV 6	125.3	-	negative
1816	185	-	positive
WV 8	44.2	-	positive
WV 9	30.8	-	Suspect-negative
WV 10	47.6	-	positive
1843 WV	26.2	-	negative
WV 12	195	-	positive
WV 13	4.6	-	negative

1341 WV	215	-	positive
WV 19	94	-	positive
WV 22	514	-	positive
WV 23	80.1	-	positive
893 WV	-5.4	-	negative
WV 25	89.7	-	positive
14255	16.0	-	negative
WV 27	48.8	-	positive
WV 28	43.0	-	positive
Ma700	33	-	Suspect-negative
Ma71	545	-	positive
Ma72	18.5	-	negative
Ma73	77.7	-	positive
Ma74	455	-	positive
Ma75	4.8	-	negative
Ma76	240.4	-	positive
Ma77	35.7	-	Suspect-positive
Ma78	73.2	-	positive
Ma79	67.8	-	positive
Ma710	160.6	-	positive
Ma711	94.6	-	positive
Ma712	153	-	positive
Ma713	9.0	-	negative
Ma714	56	-	positive
Ma715	78.5	-	positive
Ma716	54.9	-	positive
Ma717	14.7	-	Negative
Ma718	63.4	-	positive
Ma719	174.1	-	positive

Ma720	194.5	-	positive
Ma721	1.3	-	negative
Ma722	9.7	-	negative
Ma723	18.7	-	negative
Ma724	78.66	-	positive
Ma725	220	-	positive
Ma726	117.3	-	positive
Ma727	89.3	-	positive
Ma728	21.0	-	negative
Ma729	141.3	-	positive
Ma730	86.3	-	positive
Ma731	135.1	-	positive
Ma732	51.7	-	positive
Ma733	133.8	-	positive
Ma734	25.25	-	negative
Maepa 1	13.25	-	positive
Maepa 2	33.64	-	Suspect-negative
Maepa 3	8.04	-	negative
Maepa 4	-4.49	-	negative
PCx	-	0.827	
NCx	-	0.11	
Maepa 5	12.2	-	negative
Maepa 6	24.97	-	negative
Maepa 7	12.53	-	negative
Maepa 8	132.07	-	positive
Maepa 9	2.38	-	negative
Maepa 10	19.18	-	negative
Maepa 11	0.63	-	negative
Maepa 12	41.28	-	positive

Maepa 13	51.48	-	positive
Maepa 14	33.33	-	Suspect-negative
Maepa 15	15.95	-	negative
Maepa 16	49.46	-	positive
Maepa 17	40.65	-	positive
Maepa 18	-12.39	-	negative
Maepa 19	-0.81	-	negative
Maepa 20	-0.63	-	negative
Maepa 21	-6.65	-	negative
Maepa 22	-5.39	-	negative
Maepa 23	2.34	-	negative
Maepa 24	-7.19	-	negative
Maepa 25	74.39	-	positive
Maepa 26	3.82	-	negative
Maepa 27	17.11	-	negative
Maepa 28	12.6	-	negative
Maepa 29	45.9	-	positive
Maepa 30	88	-	positive
Maepa 31	46.3	-	positive
Maepa 32	41.7	-	positive
PCx	-	1.856	
NCx	-	0.165	

*PCx - Average of the two positive controls.

* NCx - Average of the two negative controls.

* S/P% - Sample to positive ratio in percentage.

*Positive - $S/P\% \geq 40\%$ (93)

*Negative - $S/P\% < 30\%$ (290)

*Suspect - $30\% \leq S/P\% < 40\%$ (21)

* Suspect-negative/positive – Suspect negative (17)/positive (4) after second run.

Appendix 3.7 Sequences supplementary table

Table 4.3: sample ID and seven sequences blasted by Megablast on NCBI BLAST.

Sample ID	Sequence
L	5'TCGCAGCACGTCAAACCGTATGTCAAAAGTAACAAGAATGATCGTAA CGATGCGCAGGCGATAGCTGAAGCGGCTTCCCGCGCCTCGATGCGGTTT GTCGCGGGGTAAAACGGTGGAACAACAAGACGTTCAAGCGCTGTAAA GATAA3'
A1	5'CGCAGCACGTCAAACCGTATGTCAAAAGTAACAAGAATGATGGTAAC GATGCGCAGGCGATAGCTGAAGTGGCTTCCCGCGCCTTGTTCGCGTTT GTTCCCCGGGG3'
FST059	5'TTATCTTTAACAGCGCTTGAACGTCTTGTTGTTCCACCGTTATGCCCCG CACAAWCCGCAYCGAGGCGCGGGAAGCCGCTTCAGCTATCGCCTGCGG ATGAGTTTGTGCCCTCCTG3'
M4	5'CAAGGGGAATTGAAATATCCGCAGGCGATAGCTGAAGCGGCTTCCCG CGCCTCGATGCGGTTTGTGCGGGGTAAAACGGTGGAACAACAAGACGT TCAAGCGCTGTAAAGATAA3'
Maepa 18	5'GAACTTATAAACTCCTAAGACCGACCACCGCATCGAGACGCGACTAA CCGCTTCAGCTATCGAATGCCATCGTTACGATCATTCTTGTTACTTTTGA CATACGGGTTGACGTGCTGCGAGTTGTTTGGTGTCTGGAGGGGGGGCTGA GGGGGGTGGAGGGGGTTGGGCACGGTATGGCTCTTGGGGAGGGGGGGG GG3'
Maepa 24	5'CAAAGTTTGTGACAGCGGACCGCGCACAAACCGCATCGAGGCGCGCT AAGCCCTTCAGCTATCGCCTGCCATCGTTACGATCATTCTTGTTACTTTT GACATACGGTTTGTGCTGCTGCGACTATTGAAACATATGATTTTTATTA AGCTCGGGTGTACGTGCTGCGA3'
ETP 19	5'CATTTCGCAGCACGTCAACCGAGGTTTCATAAAAATCGTATGTTTCAATC GGCGCAGCGTGTCAAACCGTATGTCAAAAGTAACAAGAATGATGGTAA CGATGGGCAGCGATAGCTGTATGGGCTTCGCGCGCCTCGATGCGGTTTG TCCGCCCGCCGCATTAGTTATTT3'