Development of a real time PCR assay to distinguish between *Coxiella burnetii* and *Coxiella*-like endosymbionts

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DECLARATION

I hereby declare that this dissertation which I submit for the degree Magister Scientiae in Environmental Monitoring in the Unit for Environmental Sciences and Management, Faculty of Natural and Agricultural Sciences, North West University, Potchefstroom Campus, South Africa is my original research work and has not been submitted by me to any institution or University for the conferment of an award.

_____ _13 February 2023_____

Carina W Lourens Date

DEDICATION

This thesis is dedicated to my mother, Bets Visagé, who was and still is there for me always, no matter what. You shaped me into the woman I am today. Thank you for your never-ending support, sacrifices, dedication and faith in me, but most of all your LOVE! You are not only a mother to me, but one of my best friends. Thank you for always believing in me and teaching me that no dream is ever too big.

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Abstract

Development of a real-time PCR assay to distinguish between *Coxiella burnetii* and *Coxiella*-like Endosymbionts

by

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Q fever is classified as notifiable by the World Organisation of Animal Health (OIE, now known as the World Organisation for Animal Health, WOAH). It is a zoonotic bacterial disease caused by *Coxiella burnetii* that occurs globally, affecting humans and other vertebrates. The causative agent is a Gram-negative obligate intracellular coccobacillus, causing symptoms in humans that can vary from acute disease to chronic disease and occasional deaths.

Bacterial endosymbionts known as *Coxiella*-like Endosymbionts (CLEs) are very closely associated to *C. burnetii* and are found in various hard and soft ticks. *Coxiella* strains from soft ticks (*Ornithodoros* and *Argas* species) showed to be the closest relatives of *C. burnetii,* indicating that *the species* evolved from a soft tick endosymbiont. Although *C. burnetii* is considered the only pathogen within the genus *Coxiella,* other species have also been identified, such as *C. cheraxi*, a pathogen of crayfish, *C. avium* a pathogen of birds and *C. massiliensis* a recently identified pathogen of humans. This raised the question of the possible role that CLEs might play in Q fever infections.

There are various bacteriological, serological and molecular diagnostic tests available to test for *C. burnetii* and CLEs. In bacteriology, culturing of *C. burnetii* is often challenging, while in serology the development of antibodies takes approximately two to three weeks from start of infection, and although there is various molecular test to detect *C. burnetii* and CLEs, to date and to our knowledge, no real-time PCR-based assay has been developed to detect and differentiate between *C. burnetii* and CLE DNA simultaneously.

This study aimed to develop and validate a sensitive and specific real-time PCR test to the differentiate between pathogenic *C. burnetii* and the CLEs sharing the same host that can ultimately be used to rapidly screen for and detect possible Q fever infections in humans and animals. We targeted the conserved region of the 16S rRNA gene by designing genus-specific primers and TaqMan® minor groove binder probes specific to each of *C. burnetii* and CLEs. The primer concentration was optimized to 400 nM and probe concentrations to 100 nM for *C. burnetii* and 150 nM for CLE. The efficiency of the assay was 100.00% for *C. burnetii* and 99.03% for the CLEs. Sensitivity of the assay yielded 41 bacteria/µl for *C. burnetii* and 133 bacteria/µl for CLE. The 95% limit of detection was determined to be 139.0 and 260.0 bacteria/µl for *C. burnetii* and CLEs, respectively. A cut-off Ct-value of 32.00 was selected to distinguish positive samples from negative samples (samples below the detection limit of the assay). Consistent repeatability was observed, where the inter-run standard deviation (SD) ranged between 1.50 - 2.00 for *C. burnetii* and 0.16 - 0.34 for CLE. The intra-run SD was 0.76 - 1.98 for *C. burnetii* and 0.81 - 0.96 for CLE. The coefficient of variation (CV) ranged from 4.56 - 7.40% for *C. burnetii* and 2.17 - 2.54% for the CLEs, indicating minimal variation between replicates.

The designed probe-based real-time PCR assay was specific for *C. burnetii* when tested against other closely related pathogens (*Legionella pneumophila, Mycobacterium tuberculosis, Brucella abortus, Listeria monocytogenes, Salmonella typhimurium, Pasteurella multocida, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus, Rickettsia africae, Anaplasma marginale, Anaplasma centrale, Ehrlichia chaffeensis, Ehrlichia canis, Ehrlichia ruminantium)*, but some cross-reactivity was observed between the CLE probe and some of these pathogens. The results of the TaqMan® MGB real-time PCR assay was compared with the VetMAX™ *C. burnetii* Absolute Quant Kit and a 100% result agreement was obtained for all 90 compared samples. Out of the 121 tick samples that tested positive with the TaqMan® MGB real-time PCR assay, 20 samples were sent for Sanger sequencing, where 14 were confirmed to be CLEs and the other six failed to amplify, thus no sequence results were obtained. Thirty human samples were also compared to the serology results obtained from the National Institute for Communicable Diseases (NICD) where some samples tested positive for immunoglobulin M and G (IgM and IgG). No positive PCR results were obtained upon testing of the human samples with our test. A negative PCR result does not rule out previous contact, nor the possibility that the contact is in the incubation period. And positive antibody result only indicates previous contact with the bacterial organism, but does not rule out nor confirms an active infection or the presence of the bacterial organism.

In conclusion, the developed real-time PCR in this study was efficient, sensitive but not that specific. It is advisable that another assay be developed with a newly designed probe in order to increase the specificity of the PCR.

Key words: Q fever, *Coxiella burnetii, Coxiella*-like Endosymbionts, Tick-borne disease, qPCR, TaqMan®

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List of Abbreviations used

AFI	Acute febrile illness
ANCA	Anti-neutrophil cytoplasmic antibodies
A-se	Analytical sensitivity
A-sp	Analytical specificity
BLAST	Basic local alignment search tool
bp	Base pairs
CDC	Centre for Disease Control and Prevention
CFT	Complement fixation test
CI	Confidence interval
CLE	*Coxiella*-like endosymbionts
CLEAA	*Coxiella*-like endosymbionts of *Amblyomma americanum*
Cq	Quantification cycle
CSF	Cerebrospinal fluid
Ct	Cycle threshold
CV	Coefficient of variation
R^2	Correlation coefficient
DNA	Deoxyribonucleic acid
°C	Degree Celsius
DAFF (DALRRD)	Department of Agriculture, Forestry and Fisheries (now Department of Agriculture, Land Reform and Rural Development (DALRRD)
DAMBE	Data analysis in molecular biology and evolution
DVTD	Department of Veterinary Tropical Diseases
ELISA	Enzyme-linked immunosorbent assay
FAM	6-carboxy-fluorescein
FDG-PET/CT	Fluorodeoxyglucose positron emission tomography combined with computed tomography
G+C	Guanosine-plus-cytosine
IDT	Integrated DNA technologies

IFA	Immunofluorescence assay
IPC	Internal positive control
IS	Insertion sequence
LCV	Large-cell variant
LOD	Limit of detection
MAFFT	Multiple sequence alignment program
Mb	Megabases
MGB	Minor groove binder
µM	Micromolar
nM	Nanomolar
NICD	National Institute for Communicable Diseases
ng	Nanogram
nt	Nucleotides
OIE-WAHIS	World Animal Health Information System
PCR	Polymerase chain reaction
PPE	Personal protective equipment
%	Percentage
qPCR	Quantitative polymerase chain reaction
Q fever	Query fever
rRNA	Ribosomal ribonucleic acid
SCV	Small-cell variant
SD	Standard deviation
TEE	Trans-oesophageal echocardiography
Tm	Melting temperature
UP	University of Pretoria
USA	United States of America
µl	Microliters

Chapter 1

Introduction

Ticks are widely distributed around the world, with an estimate of 900 species reported (Barker and Murrell, 2004). They are ecto-parasites, and are second to mosquitoes the most significant vectors that are responsible for human and animal disease causing pathogens (de la Fuente, 2008). They transmit a wide spectrum of zoonotic viral (tick-borne encephalitis, Crimean-Congo haemorrhagic fever), bacterial (Lyme disease, rickettsiosis, ehrlichiosis, Q fever) and protozoan (babesiosis, anaplasmosis) pathogens. These ticks feed solely on blood and have a broad spectrum of hosts, such as mammals, birds and sometimes reptiles and amphibians. They can be co-infected with various pathogens, transmitting them to their hosts (Clay et al., 2008; Moreno et al., 2006)

Ticks of domestic animals cause considerable harm to human and livestock by transmission of many species of zoonotic pathogens. One such zoonotic disease transmitted is Query fever or otherwise shortened to Q fever. Q fever is caused by the bacteria *Coxiella burnetii,* an obligate intracellular coccobacillus that is classified as a potential bioterrorism agent by the Centre of Disease Control and Prevention (CDC). It has a very low infectivity dose and has the capability to survive extreme environmental conditions (Madariaga et al., 2003).

Although *C. burnetii* is classified as the only pathogenic *Coxiella* in the genus, some published articles found other *Coxiella*-like bacteria to be the cause of deaths in birds, with a more recent report by Angelakis et al. (2016) of *Coxiella*-like organisms in human patients who developed eschars after tick bites (Angelakis et al., 2016). This raised the question of the possible role of *Coxiella*-like endosymbionts (CLEs) in Q fever infections.

In the rural area of the Mnisi community, that borders on the Kruger National park, Mpumalanga Province of South Africa, cases of acute febrile illness (AFI) are reported constantly by the National Institute for Communicable Diseases (NICD). This community is located in the northeast part of the Bushbuckridge municipal area in Mpumalanga Province, South Africa. It is an area that forms the middle ground where humans, domesticated livestock and wildlife lives in harmony with each other (Berrian et al., 2016). Communal land totals approximately 29,500 ha of this community and shares >75% of its boundary with provincial game reserves (Manyeleti and Andover reserves) and private game reserves (Sabi Sands and Timbavati reserves). The community has an estimated population size

of 40,000 – 50,000 people that lives in ~8,500 households, spread over 12 villages and where ownership of domestic animals is very common (Simpson et al., 2018).

Although malaria has a high occurrence in this area, a high number of non-malarial acute febrile illness (AFI) cases are also presented at the community clinics. The people of the area live in close proximity with wildlife, livestock and other domestic animal such as dogs. The tick *Rhipicephalus sanguineus* is an exclusive parasite of dogs, but it has been found to bite humans as well when living in close proximity (Horak et al., 2002). A recent study (R. Ackermann, MSc dissertation 2019, UP) indicated the presence of *Coxiella* DNA in the salivary gland and midgut microbiomes of these ticks collected from dogs in the Mnisi community. With a relatively high seroprevalence rate (dip-tankers = 60.9% and febrile patients = 37.8%) (Simpson et al., 2018) of Q fever in the rural area of the Mnisi community, and suspected cross-reactivity of *C. burnetii* and *Coxiella*-like bacteria in serological detection methods, there is the need to study the involvement of CLEs with regards to Q fever infections, especially in non-malarial AFI cases.

There are various diagnostic tests available to test for *C. burnetii* and CLEs. They include bacteriological, serological and molecular detection tests. In bacteriology, culturing of *C. burnetii* is often challenging and poses a high risk to staff, thus culturing the organism require a BLS 3+ laboratory. Serological tests are considered to be the first-line test to be performed when *C. burnetii* infection is suspected (Eldin et al., 2017).

Tests such as the enzyme-linked immunosorbent assay (ELISA), the indirect immunofluorescence assay (IFA) and the complement fixation test (CFT) are the most routinely used serological tests. Unfortunately, these tests require the development of antibodies which takes approximately two to three weeks from start of infection (Maurin and Raoult; 1999, Hechemy, 2012), making it difficult for a quick diagnosis to be made.

The IFA is considered to be the gold standard serological test to be used, but reproducibility between analysts are challenging and a disadvantage of the testing method (Maurin and Raoult, 1999; Hechemy, 2012, World Organization for Animal Health, 2018). The ELISA mostly used for surveillance purposes, is a test considered to be more sensitive than the IFA and the CFT and are able to distinguish between phase I and phase II antigens. However, it can be challenging to interpret by inexperienced analysts and thus are not as routinely used as the IFA test. The CFT test, although specific, is the most

time consuming and least sensitive of all three tests. A prozone effect can be responsible for reporting of false negatives in chronic Q fever (Maurin and Raoult, 1999).

Through recent years, various polymerase chain reaction (PCR) assays have been developed for the detection of *C. burnetii* in different samples such as tissue, blood and urine samples (Madariaga et al., 2003). Various molecular tests have also been used to detect *Coxiella*-like bacteria in ticks. Such as real-time qPCR, 16S rRNA gene cloning and sequencing, direct 16S rRNA sequencing and molecular probing (Zhong, 2012). Various genes have also been targeted, such as the 16S rRNA gene, 23S rRNA gene, *groEL* gene, *rpoB* gene and the *dnaK* gene (Zhong, 2012). To date and to our knowledge, no PCR-based assay for the detection and differentiation of *C. burnetii* and CLE DNA simultaneously has been described.

In this study we developed and validated a real-time PCR test for the differentiation between pathogenic *Coxiella burnetii* and the *Coxiella*-like endosymbionts sharing the same host that can ultimately be used to rapidly screen and detect possible Q fever infections in humans and animals.

Research aim

The aim of this research project was to develop and validate a real time PCR assay, to identify and distinguish *C. burnetii* and CLE DNA simultaneously in samples.

Objectives

- Identify the most conserved region of the 16S rRNA gene of *C. burnetii* and CLEs.
- Design specific primers and probes for the detection and differentiation of *C. burnetii* and CLE DNA.
- Establishment of a real-time PCR assay.
- Validate the assay by determining analytical sensitivity, specificity as well as repeatability.
- Screen AFI patient samples to verify that results obtained correspond with serological results obtained by the NICD.

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Chapter 2

Literature review

2.1. Q fever

Q fever is a zoonotic bacterial disease with global occurrence, affecting humans and other vertebrates. Symptoms in humans can vary from acute disease (which includes hepatitis, pneumonia, headaches, influenza-like symptoms and fever), to chronic disease (most frequently endocarditis) (Angelakis and Raoult, 2010, Wielders et al., 2015). Symptoms may dissolve on their own, or lead to chronic infection and occasional deaths. About 60% of infections in humans lead to asymptomatic seroconversions (Hechemy, 2012). It is categorized as notifiable by the World Organisation of Animal Health (OIE).
Q fever is usually obtained through inhalation of the pathogen as well as through direct contact with animal infected products or reproductive tissues of animals (Angelakis and Raoult, 2010).

2.1.1. An historical background

The disease was initially reported in 1937 in Queensland, Brisbane, Australia by Edward Holbrook Derick, a medical practitioner and Director of the Pathology and Microbiology laboratory of the Health Department in Queensland (Eldin et al., 2017). In autumn of 1935, abattoir workers showed signs of febrile illness. Diseases such as typhus, undulant fever, paratyphoid fever, leptospirosis as well as the everyday animal diseases were excluded as possible causes for the fever-like disease (Derrick, 1937). The thought arose of a possible new disease as the cause of the fever-like illness and the disease was named Q fever, shortened for Query fever until more insight of the disease were available to properly name it (Derrick, 1937). In some cases, it is also referred to as Queensland fever, from where it originated.

By experimenting on guinea pigs, Derrick endeavored to isolate the causative agent of the disease, but without success (Eldin et al., 2017). Macfarlane Burnet and Mavis Freeman (Walter and Eliza Hall Institute, Melbourne) considered the causative agent to be of rickettsial origin after examining samples sent to them by Derrick and reproducing the disease in various animals. Through examinations of hematoxylin-eosin stained spleen sections, intracellular vacuoles filled with grainy substances were noticed. Giemsa staining revealed various small rods resembling rickettsial species. With this new knowledge Derrick and colleagues studied the epidemiology of the disease and concluded that the disease might be transmitted by ticks or other arthropods and that wild animals were the primary hosts, with domestic animals as secondary hosts (Derrick, 1937; Eldin et al., 2017).

In a total separate incident, at the Rocky Mountain Laboratory in Hamilton Montana, Gordon Davis observed in 1935 symptoms such as the lack of marked testicular swelling in guinea pigs after tick feedings. These ticks were collected in the Nine Mile area in Montana and the symptoms did not resemble that of the so-called Rocky Mountain spotted fever. The disease could be transmitted from infected guinea pigs to non-infected guinea pigs through injection of blood and the causative agent were unable to be grown in sterilized medium. With the intent of assisting Davis to characterize the Nine Mile agent, Harold Rea Cox joined Davis in 1936. They proved that the agent showed characteristics of both viruses and rickettsiae, just as Burnet and Freeman did in Brisbane. In 1938 Cox made a breakthrough when he was able to multiply the agent in embryonated eggs (Eldin et al., 2017).

Similarity between the Brisbane and Montana infections were noted in 1938, when the Director of the National Institutes of Health, Rolla Eugene Dreyer went to Hamilton in order to authenticate the multiplication of the Nine Mile agent in eggs. Dreyer got infected with the agent the laboratory was working on. Guinea pigs inoculated with Dreyer's blood showed symptoms of febrile illness and spleen samples harvested from the guinea pigs revealed rickettsiae species. Serological cross-protection was also established between the Nine Mile agent and microorganisms isolated from Dreyer's blood, indicating an unambiguous association between the Nine Mile agent and the Australian Q fever agent. Guinea pigs was then inoculated with Q fever obtained from Q fever infected mice spleen samples sent to Dreyer by Burnet. Dreyer was then able to prove that the isolated strain from his blood protected the animals from new infections. This cross-protection very much suggests that the Q fever agent, the Nine Mile agent and the organism isolated from Dreyer's blood were isolates of the same single microorganism. This causative agent of Q fever was then named *Rickettsia burnetii*, however, in 1948 Cornelius B. Philip suggested the creation/establishment of a new genus namely *Coxiella* and the Q fever agent was renamed as *Coxiella burnetii*, honouring both Cox and Burnet who identified the new rickettsial species (Eldin et al., 2017).

But in more recent years, sequencing of the 16S rRNA gene showed more resemblance to the order Legionellales, members of the gamma subdivision of the proteobacteria in contrast with the order Rickettsiae, members of the alpha subdivision of proteobacteria (Parker et al., 2006; Weisburg et al., 1989).

2.2. *Coxiella* classification

Coxiella burnetti was previously known as *Rickettsia burnetii* (Kingdom Bacteria, Phylum Proteobacteria, Class Alphaproteobacteria, Order Rickettsiales, Family Rickettsiaceae, Tribe I, Genus III *Coxiella*, species *burnetii*) due to the fact it shares some characteristics with rickettsiae. This classification changed when molecular phylogenetic tools became available and sequencing of the 16S rRNA gene showed more resemblance to the order Legionellales [*Legionella pneumophila* to be exact] (Figure 2.1), members of the gamma subdivision of the proteobacteria in contrast with the order Rickettsiae, members of the alpha subdivision of proteobacteria (Parker et al., 2006; Weisburg et al., 1989). Currently the classification for *Coxiella* is: Kingdom Bacteria, Phylum Proteobacteria, Class Gammaproteobacteria, Order Legionellales, Family Coxiellaceae, Genus *Coxiella*, Species *burnetii*.

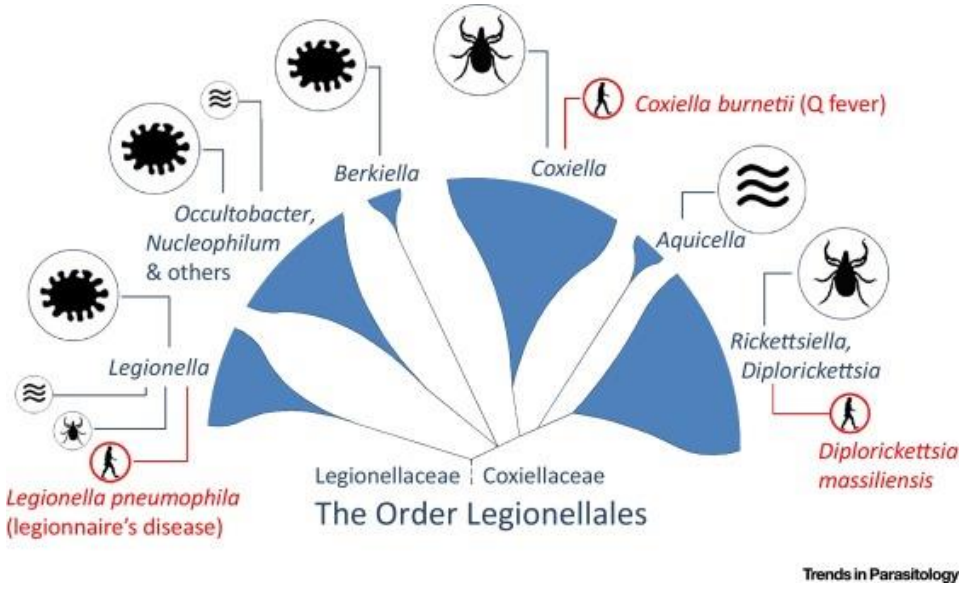

Figure 2.1. A phylogeny overview of the order Legionellales, including the families Legionellaceae and Coxiellaceae. Red is indicative of species causing human disease. For each genus, a variety of hosts (amoeba, arthropods and vertebrates including humans) is shown in circles (Duron et al., 2018).

However, using the 16S rRNA gene phylogenetic tree in other research studies, *C. burnetii* is more closely related to *Rickettsiella grylli* that is also a gamma proteobacterium and a pathogen of crickets (Roux et al., 1997; Cordaux et al., 2007) as shown in Figure 2.2. This close relatedness has also been proved with the sequencing of other genes such as *dnaB* (DNA helicase), *mucZ* (DnaJ-like membrane chaperone) and the *GroEL* (chaperonin) (Raghavan et al., 2008; Leclerque et al., 2008).

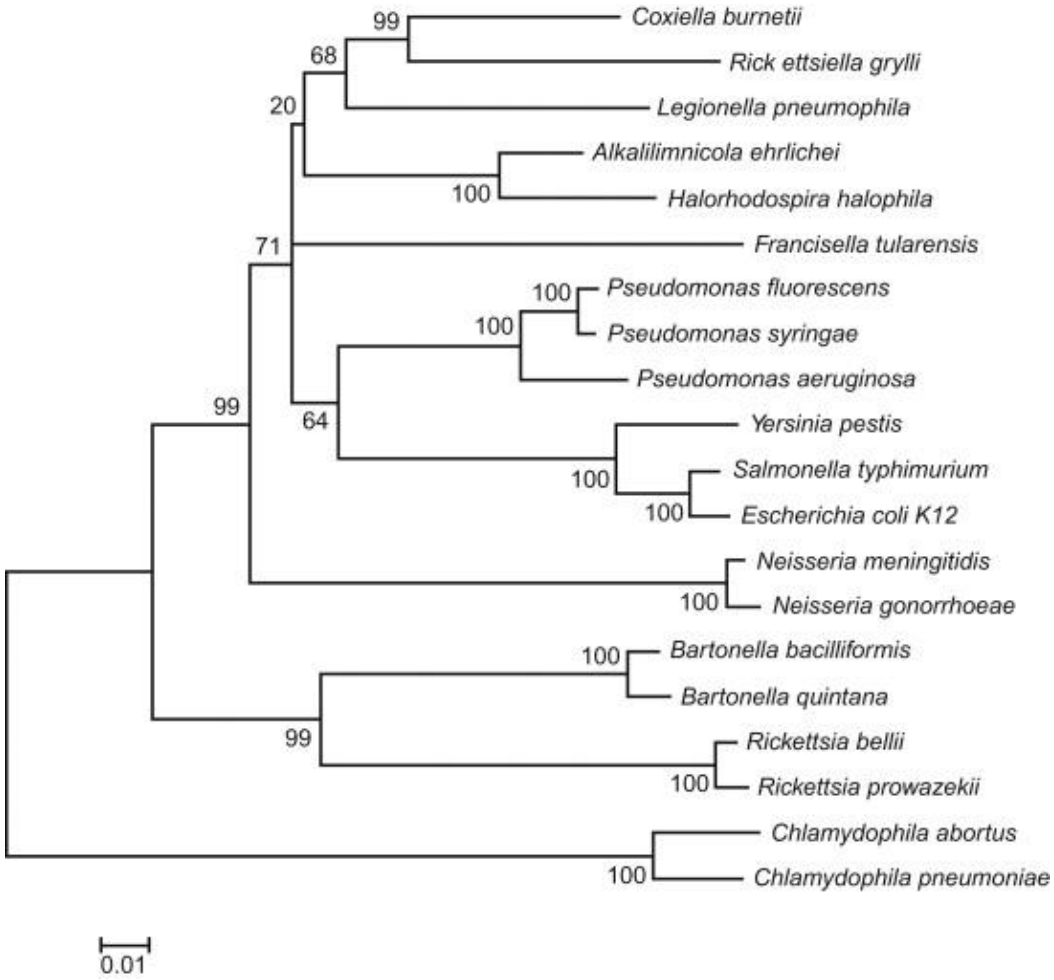

Figure 2.2. 16S rDNA sequences used to showcase phylogenetic analysis (1351 positions). The neighbour-joining tree was constructed by a maximum composite likelihood method using MEGA4. Bootstrap values (1000) replicates are shown at the nodes (Marrie et al., 2015).

Apart from the close relatedness shown by molecular phylogeny, other characteristics like the natural history of *C. burnetii* and *L. pneumophila*, also support this descent of classification into the Legionellales order (Marrie et al., 2015). Both of these pathogens corrupt the host cell because of evolved intracellular parasitic actions. Both cause infection mainly by inhalation of the pathogen from an environmental source. Pneumonitis is most often the end result of infection, since the alveolar macrophage cell in the lung is targeted by these pathogens and both can infect a variety of hosts, from aquatic to protist hosts for *Legionella,* to vertebrate and arthropod hosts for *Coxiella* (Marrie et al., 2015). Another distinctive feature, is that the *C. burnetii* genome is rich in guanosine-plus-cytocine (G+C) content (42.2%), placing it closer to the *Legionellales* order than that of *Rickettsiales* that have a G+C content of approximately 29% (Hechemy, 2012).

Although *C. burnetii* is considered the only pathogen within the genus *Coxiella,* other pathogens have also been identified, such as *C. cheraxi*, a pathogen of crayfish, *C. avium* a pathogen of birds and *C. massiliensis* a recently identified pathogen of humans (Duron et al., 2015, OIE, 2017 Iowa University). These pathogens as well as various other related tick symbionts also have their sequences recorded in GenBank (Marrie et al., 2015).

Bacterial endosymbionts in various hard and soft ticks related to *Coxiella burnetii*, are known as *Coxiella*-like endosymbionts (CLE) (Zhong, 2012). These CLEs are not identical to *C. burnetii*, but very closely related. Like *C. burnetii* all CLEs belong to the gamma-subdivision of the proteobacteria. Duron et al (2015) demonstrated through phylogenetic analysis that the *Coxiella* genus can be divided into four main clades (A to D), with *C. burnetii* isolates grouped into an exclusive subclade within the A clade (Figure 2.3). *Coxiella* strains from soft ticks (*Ornithodoros* and *Argas* species) showed to be the closest relatives of *C. burnetii,* indicating that *C. burnetii* commenced from a soft tick endosymbiont (Duron et al., 2015).

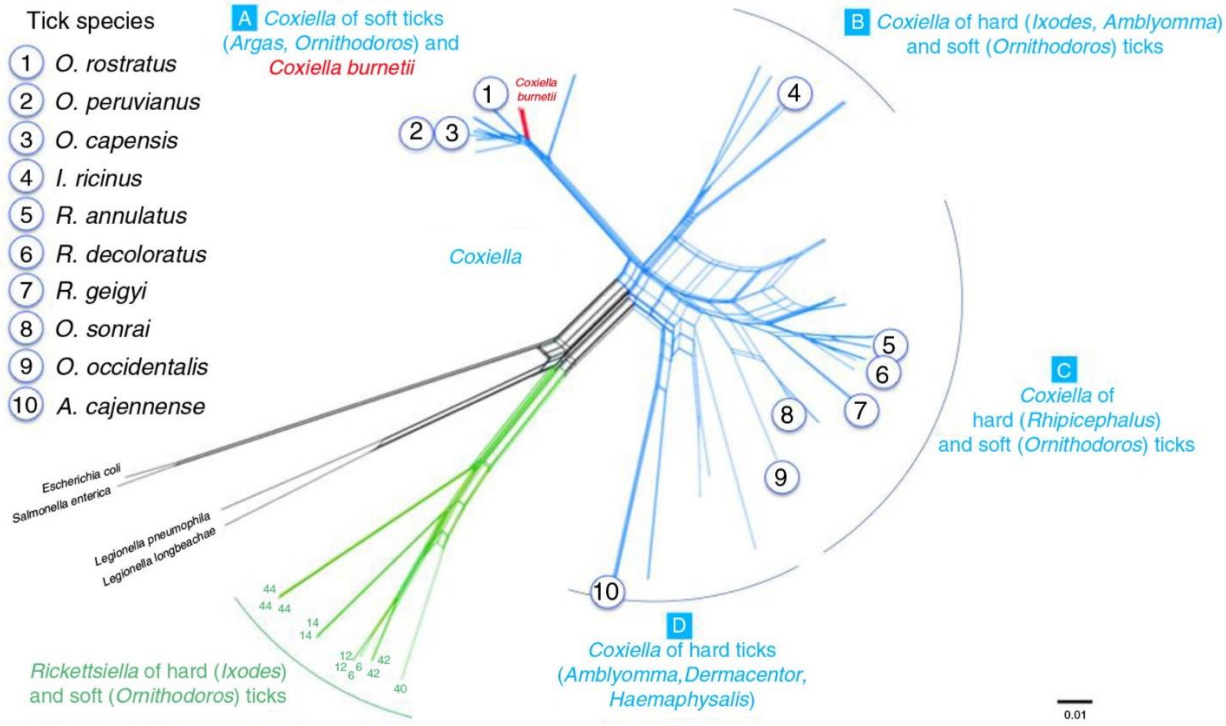

Figure 2.3. Phylogenetic analysis of concatenated 16S rRNA, 23S rRNA, *GroEL, rpoB* and *dnaK* gene sequences (3009 unambiguously aligned bp), which include 71 *Coxiella*-like strains of ticks, 15 *C. burnetii* reference strains and bacterial outgroups. The four *Coxiella* clades are labelled A to D. Each number corresponds to one tick species. Blue = *Coxiella*-like organisms; red = *C. burnetii,* green = *Rickettsiella* and black = other bacteria (Duron et al., 2015).

2.3. Aetiology

Coxiella burnetii, the pathogen that is responsible for Q fever is a Gram-negative obligate intracellular coccobacillus, measuring 0.2 to 0.4 µm in width and 0.4 to 1 µm in length (Parker et al., 2006; Maurin and Raoult, 1999). Although it has a membrane similar to gram-negative bacteria as well as classifying it as a gram-negative, it doesn't stain easily with Gram staining techniques, but rather with Gimenez staining techniques (van Schaik and Sameul, 2012). It contains growth cell variants (small-cell variant and large-cell variant) that differ in ultra-structure, antigenicity, metabolic capability, physical resistance and protein composition and because of these variants, it produces a thick, extremely resistant spore-like form (Minnick and Raghavan, 2012). It is found in the phagolysosome of the host cell phagocyte where it can remain without affecting the host cell's activity. Another distinctive feature of *C. burnetii* is its two antigenic forms, the acute/pathogenic phase I and the chronic/attenuated phase II. Phase I is isolated from humans and infected animals whereas phase II is obtained by passage in *in-vitro* cultures (O'Neill et al., 2014; World Organization for Animal Health, 2018). Both are easily distinguishable through serological antibody tests.

Coxiella burnetii has a slow replication rate (8–12 hours doubling time) and alternate between small-cell variants (SCV), the infectious form and large-cell variants (LCV), the replicative form (Figure 2.4) (Heinzen et al., 1999). Both cell variants replicate by binary fusion (Minnick and Raghavan, 2012). The SCV is 0.2 µm – 0,5 µm in length, metabolically inactive/passive and have a comprehensive intracellular membrane system which is absent in LCVs. Within the phagocyte the passiveness of the SCV is broken in response to an acidic environment (pH 4.8), that is necessary to activate metabolism (Heinzen et al., 1999, Parker et al., 2006, World Organization for Animal Health, 2018). Activation of metabolism see's the SCV develop into a LCV (Hechemy, 2012). Without activation of metabolism, the SCVs remain heat and pressure resistant and survive harsh environmental conditions which is an exceptional advantage to the bacterium (Heinzen et al., 1999; Minnick and Raghavan, 2012).

LCVs exceed 1.0 µm in size, they are the only replicating cell form and have been found to reproduce in the host monocyte (Heinzen et al., 1999; Parker et al., 2006; World Organization for Animal Health, 2018; Minnick and Raghavan, 2012). These cell variants are generally pleomorphic with a less electron dense nucleoid than that of SCVs. An endospore-like structure (ELS) with a diameter of 130 – 170 nm enclosed with a membrane of four layers of which its purpose and infectivity is not yet determined, is generated by LCVs (Minnick and Raghavan, 2012).

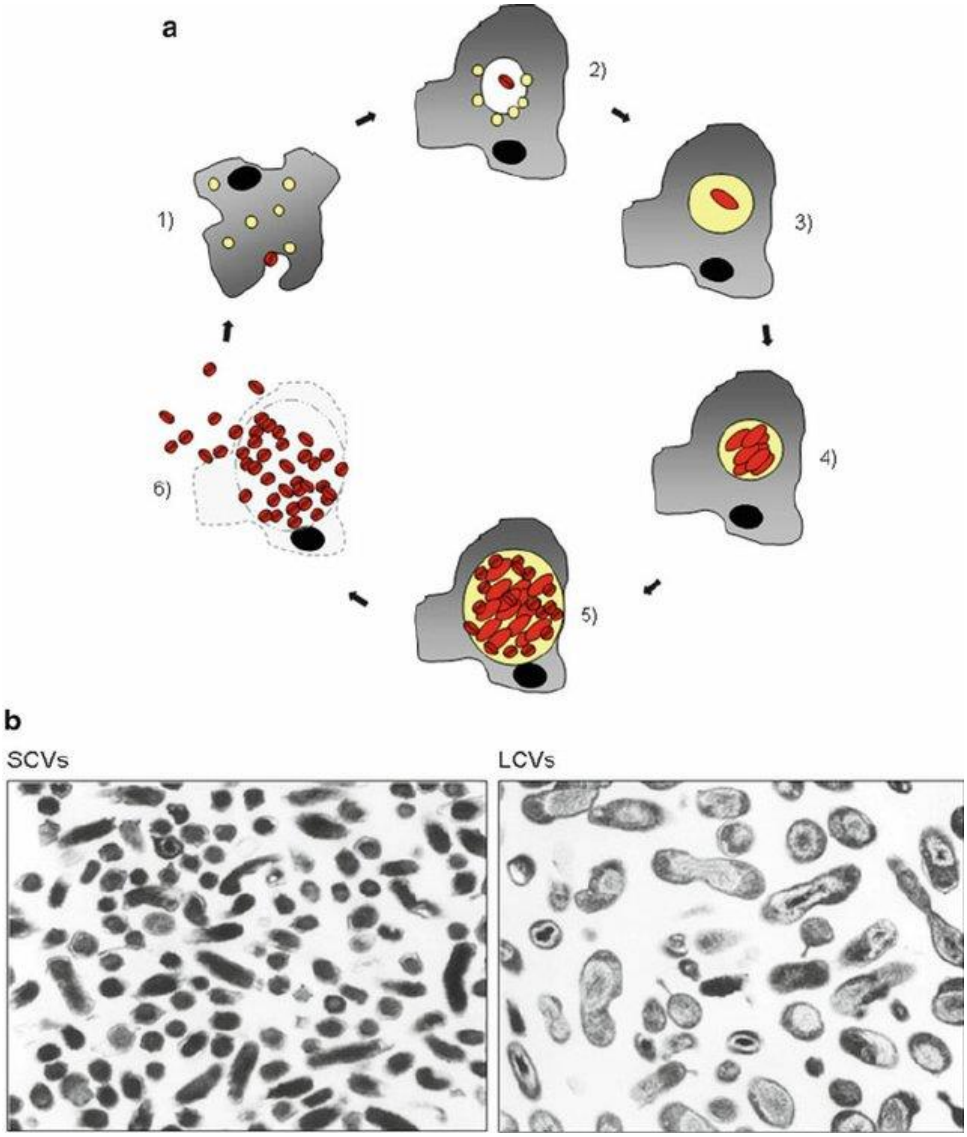

Figure 2.4. Life cycle of C. burnetii. (a) Developmental cycle of C. burnetii. (1) Passive internalization of an SCV into host cell. (2) SCV within the confines of a phogosome, with lysosomes (small yellow granules) approaching the periphery of the vacuole. (3) Phagolysosome following acidification (yellow) and subsequent morphogenesis of SCV to LCV. (4) LCV replication within the PV. (5) Metamorphosis of LCV and SCV at late log/early-stationary phase. (6) Lysis of host cell with release of SCVs to repeat cycle. (b) Purified SCVs and LCVs of C. burnetii (courtesy of R.Heinzen).

Another distinctive feature of *C. burnetii* is its two antigenic forms, the acute/pathogenic phase I and the chronic/attenuated phase II. Phase I produce a full-length virulent lipopolysaccharide (LPS) molecule and is isolated from humans and infected animals. Whereas phase II, obtained by passage in *in-vitro* cultures produces LPS molecules with lower molecular weights and noticeable basic sugar configuration, resulting in avirulent LPS molecules (O'Neill et al., 2014; World Organization for Animal Health, 2018; Hechemy , 2012). During the early stages of infection, antibodies to phase II antigens are generated, while antibodies to phase I antigens prevail if the pathogen continues longer (OIE, 2007 Iowa University).

Some *Coxiella* bacteria are considered symbionts of ticks. They interact in a positive manner towards their hosts, forming a long term relationship (Clay et al., 2008). Because the diet of ticks comprises solely of blood, lacking crucial nutrients and substances such as certain vitamins, amino-acids and cofactors, the ticks associates itself with these endosymbiotic bacteria that are able to provide these essential nutrients to them, ensuring their survival and reproduction (Tsementzi et al., 2018; Zientz et al., 2004).

2.4. Genome

Previously the genome of *C. burnetii* was estimated to be approximately 1.5 to 2.4 Mb in length, but genome sequencing indicated it to be rather in the range of 2.0 to 2.2 Mb (Hechemy, 2012). The first *C. burnetii* strain to be sequenced was isolated from a tick in 1935, the RSA493 strain (Eldin et al., 2017). Sequencing of the genome revealed a plasmid (QpH1) of 37 393 base pairs (bp) and a chromosome of 1 995 275 bp in length (Eldin et al., 2017). It encodes for 2 134 protein coding genes, with 719 (33.7%) that are hypothetical, meaning they have no significant matches to other sequenced genes (Seshadri et al., 2003). Forty-three tRNAs and 3 rRNAs, with an average ORF size of 1 585 bp long (Seshadri et al., 2003). The genome that is organized in circular chromosomes are affiliated with a single autonomous plasmid or integrated plasmid-related sequences (van Schaik and Samuel, 2012). To date there are four labelled plasmid types QpH1, QpRS, QpDG and QpDV as well as a plasmid not labelled from a Chinese *C. burnetii* isolate (Jager et al., 2002). All plasmid types contain a core region of ~16 kb, that is a conserved sequence found throughout all analysed strains of *C. burnetii*, suggesting this plasmid sequence to be a crucial part of the genome (van Schaik and Samuel, 2012).

A common feature in obligate intracellular bacteria is reductive evolution, as indicated by the high number (83) of pseudogenes found in the *C. burnetii* genome, suggesting that genome reduction is still on going. Also present in the genome is a relative high number (n = 29) of insertion sequences (IS) (Eldin et al., 2017; van Schaik and Samuel, 2012). This high number of IS elements is normally not found in obligate intracellular bacteria, thus in assuming minimal opportunity for gene transfer, it suggests that the obligate intracellular lifestyle of *Coxiella* is recently adapted (Raghavan et al., 2008; Eldin et al, 2017). D'Amato et al (2015) compared 7 sequenced strains of *C. burnetii* genomes through pangenomic analysis and found a ratio of 96% for core genome/pangenomes, with 13 542 core genes shared among all strains, 498 accessory genes, shared among certain strains and 88 exclusive genes, only found in a single strain. Seventy-four exclusive strains were found in the Dugway strain, 13 in the Q212 strain and one exclusive gene in the RSA331 strain (D'Amato et al., 2015). Thus, *C. burnetii*

genomes from various strains have a strong genomic resemblance, with a closed pangenome (Eldin et al., 2017)

The CLE genome in contrast is much smaller than that of *C. burnetii* and exhibits characteristics such as low G+C content, high substitution rates and genomic reduction (Gottlieb et al., 2015). When a free-living bacterium becomes host-associated, genome reduction is usually proposed because of a loss of selection on multiple gene functions (Gottlieb et al., 2015). When looking at the *Coxiella*-like endosymbiont of *Amblyomma americanum* (CLEAA), there is a remarkable difference in the size of the genome compared to that of *C. burnetii* as shown in figure 2.5. It consists of a circular chromosome of 656 901 bp long, encoding for 537 protein-coding genes, 39 tRNA's with a single rRNA operon (Smith et al., 2015). An estimate of 30% of *Coxiella* gene content is retained in the CLEAA genome. The existence of 23 pseudogenes, low G+C content (34.6%), low gene density (83%) and the small genome size, implies that the genome encountered reductive evolution because of the CLEAA's host dependent life style (Smith et al., 2015). Also, of importance is that no virulent genes were found within the CLEAA genome.

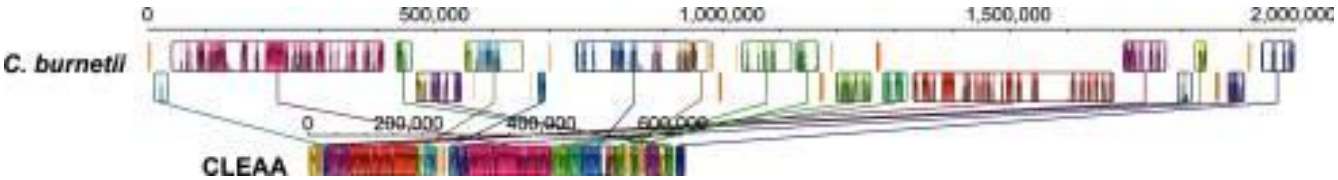

Figure 2.5. Alignment of *C. burnetii* RSA 493 and CLEAA genomes. Each contiguously coloured locally Collinear block (LCB) represents a region without rearrangement of the homologous backbone sequence. Orthologous LCBs are shown as lines between genomes (Smith et al., 2015).

The genome lengths between various CLEs also differ from each other. Tsementzi et al (2017) nearly fully sequenced the CLE bacterial genome of the tick *Rhipicephalus sanguineus* (here after referred to as CRs) and compared it to other available CLE genomes. These included the CLE genome of *Rhipicephalus microplus* (hereafter referred to as CERM), the CLE genome of *Rhipicephalus turanicus* (named Candidatus *Coxiella* mudrowiae, hereafter referred to as CRt), the CLEAA genome (as referred to above) as well as a *C. burnetii* representative strain. The differences in genomes are summarized in Table 2.1 (Tsementzi et al., 2017).

Table 2.1. Genome comparisons of the CRs genome with various available CLE genomes and a representative strain of *Coxiella burnetii*. Numbers within brackets show the counts when the duplicated areas are excluded. (Tsementzi et al., 2017).

	CRs	CRt	CERM	CLEAA	*C. burnetii*
Total length	1 717 748	1 733 840	1 194 772	656 901	1 995 281
GC%	38.1	38.2	32.7	34.6	42.7
# ORFs	1582 (1434)	1639 (1456)	836	605	2, 059
# Protein coding genes	839 (764)	912 (825)	796	554	1 823
# Pseudogenes	691 (626)	675 (579)		8	189
# rRNA	3 (3)	3 (3)	3	3	3
# tRNAs	48 (40)	48 (40)	37	39	42
# Other RNAs	1 (1)	1 (1)		1	2

CRs = CLE of *Rhipicephalus sanguineus*; CRt = CLE of *Rhipicephalus turanicus*; CERM = CLE of *Rhipicephalus microplus*; CLEAA = CLE of *Amblyomma americanum*

2.5. Epidemiology

Infection with the *C. burnetii* organism takes place globally, with the exclusion of New Zealand, Norway, Iceland and French Polynesia (OIE, 2007 Iowa University). It affects humans, wildlife, birds, reptiles and domestic animals, with ruminants featuring as the main reservoirs (Das et al., 2013; World Organization for Animal Health, 2018, Hechemy, 2012). In contrast to human infection which primarily occur in the respiratory system, Q fever infection in goats and sheep occur mainly in the reproductive organs, leading to abortions and reproductive disorder, whereas cattle is usually found to be asymptomatic (Seo et al., 2017, Hechemy, 2012).

Q fever is usually obtained through inhalation of the organism as well as through direct contact with animal contaminated products or reproductive tissues of animals (Angelakis and Raoult, 2010). The pathogen is shed in milk, urine, faeces, semen, vaginal fluids, placental and birth fluids (Rad et al., 2014). People most likely exposed on a continual basis are the ones that comes in contact with waste and birth products, such as veterinarians, farmers, zoo keepers and even abattoir workers (Hechemy, 2012). Treatment of infection does not limit or prevent shedding from taking place, as demonstrated by Astobiza et al (2010). The study showed that the pathogen is still excreted in faeces for up to 5 months after giving birth, for up to 3 months in vaginal discharges and for 4 months in milk (Astobiza et al., 2010; Hechemy, 2012).

Limited cases of human-to-human transmissions have also been documented, either through sexual transmission, blood transfusions, childbirth exposure and hospital-acquired transmissions (Eldin et al., 2017; World Organization for Animal Health, 2018). Although both are equally exposed, men are more susceptible to Q fever infections than women and this might be due to hormonal differences that

affects susceptibility to infection (Eldin et al., 2017). Prevalence for men is approximately 3.8% compared to woman with a prevalence rate of approximately 2.5%. Also, with age antibody positivity have been found to increase (Hechemy, 2012).

Because of the pathogens ability to withstand very harsh conditions, *C. burnetii* can stay "dormant" for long periods and reappear when those conditions change to more favourable (Hechemy, 2012). This and the fact that it has a very low infectivity dose (a single organism is all that is needed to cause infection), is why it is considered a bioterrorism agent, classified as category B by the Centre of Disease Control and Prevention (CDC) (Madariaga et al., 2003).

The first report of Q fever in Africa was in 1947 (Angelakis and Raoult, 2010) and in 1955 Kaplan and Bertagna reported the disease in nine other countries including South Africa (Eldin et al, 2017). In Tanzania, *C. burnetii* have been demonstrated to be a common cause of febrile illness and pneumonia, more so than malaria (Vanderburg et al., 2014). The pathogen can have a major impact on livestock production and thus impact socioeconomic and health of humans, specifically with regards to poor/rural communities (Vanderburg et al., 2014). In South Africa, in the Mnisi area that is situated in the north-eastern corner of the Bushbuckridge local Municipality, Mpumalanga Province, which is adjacent to the Kruger National Park, between 3-400 patients with acute febrile illness (AFI) are presented yearly. These cases not only share general symptoms with a wide range of infectious diseases, including mosquito-borne diseases, but also shares geographic distribution. With ticks considered to be second to mosquitoes as vectors of humans (Troughton and Levin, 2007, Dantas-Torres et al., 2011), it is possible that diseases, especially AFI can be misdiagnosed as malaria. Berrian et al (2016) suggested that dogs, cattle and cats, in that order, are the domestic animal species of most concern with regard to disease transmission (Berrian et al., 2016).

The tick *Rhipicephalus sanguineus* is an exclusive parasite of dogs, but it has been found to bite humans as well when living in close proximity (Horak et al., 2002). Ackermann (in press) studied the brown dog tick, *Rhipicephalus sanguineus,* and the microbiome revealed an average of 86-94% *Coxiella* spp found in the midgut and salivary glands of these ticks. *Coxiella*-like bacteria have been associated only with tick hosts in the past (Duron et al., 2015), but the occasional tick-to-vertebrate transmission does occur.

Although CLEs shares the same geographic distribution as *C. burnetii,* their prevalence rate is remarkably different in ticks. Mediannikov et al (2010) demonstrated a relative low prevalence rate

of 0-37,6% for *C. burnetii* in ticks sampled, in contrast to Jasinskas demonstrating a 100% prevalence rate for CLE in *A. americanum* ticks sampled. (Mediannikov et al., 2010; Jasinskas et al., 2007). In saying that, there is also a definite difference in the prevalence rate of CLEs in various ticks species, possibly because of various transmission routes (Zhong, 2012).

With a relative high prevalence rate (fip-tankers = 60,9% and febrile patients = 37,8%) (Simpson et al., 2018) of Q fever in the rural Mnisi community, and suspected cross-reactivity of *C. burnetii* and *Coxiella*-like bacteria in serological detection methods, there is the need to study the involvement of CLEs with regards to Q-fever infections, especially in non-malarial AFI cases.

2.6. Reservoirs

A vast range of vertebrates and invertebrates acts as reservoirs for the *C. burnetii* pathogen (Eldin et al., 2017). These include but are not limited to mammals (domestic and wild), birds, reptiles and arthropods, with cattle, goats and sheep as the main reservoirs (Maurin and Raoult, 1999). The pathogen is shed in milk, urine, faeces, semen, vaginal fluids, placental and birth fluids (Maurin and Raoult, 1999; Rad et al., 2014). In cattle, Q fever infection is usually asymptomatic, but goats and sheep are more prone to abortions and reproductive disorder (Seo et al., 2017). Even dogs and cats may act as reservoirs, obtaining the pathogen either through tick bites, the consumption of birth products or from milk obtained from infected ruminants and even through inhalation (Maurin and Raoult, 1999).

Ticks can be co-infected with various pathogens, transmitting them to their hosts (Clay et al., 2008; Moreno et al., 2006). While *Coxiella*-like endosymbionts (CLEs) differ from *C. burnetii*, in that it is only found in tick reservoirs (Duron et al., 2015), both organisms have been isolated from tick species, from over 40 hard tick species (including the brown dog tick *Rhipicephalus sanguineus*) to at least 14 soft tick species. Other tick species include *Amblyomma* spp*., Haemaphysalis* spp*., Ixodes* spp*., Argas* spp. *and Ornithodoros* spp. (Eldin et al., 2017; Duron et al., 2015; Zhong, 2012). During feeding time, these organisms is shed in the faeces of the ticks onto the skin of their animal hosts (Maurin and Raoult, 1999).

2.7. Transmission

Aerosol transmission: The most common route of transmission of *C. burnetii* is through inhalation of infected aerosols, with reports of aerosol molecules traveling a distance of up to 30 km on a windy day (Eldin et al., 2017). Hawker et al (1998) reported an attack rate of 384/100 000 for people living

within a 5 km radius of a *C. burnetii* point of outbreak and an attack rate of 48/100 000 for people living within a 10 km radius of a point outbreak (Hawker et al., 1998). Direct exposure to infected products such as the placenta, birthing products, faeces, urine, wool and hides can cause infection (Eldin et al., 2017; Maurin and Raoult, 1999).

Oral transmission: Although considered a minor route of infection, consumption of unpasteurised milk, cheese and even tobacco have led to infections (Marrie et al., 2015; Maurin and Raoult, 1999).

Person-to-person transmission: This transmission route is very unusual perhaps due to the intracellular character of the pathogen, but there have been reports where it did occur. Two cases after autopsies were performed, pneumonia infection of an obstetrician after delivering a baby, infections after blood transfusions, and a case where infection was obtained after a bone marrow transplantation (Eldin et al., 2107; Marrie et al., 2015).

Sexual transmission: An unreliable case has been reported of a farmer infecting his wife (Eldin et al., 2017). However, the pathogen has been detected in semen from different species, such as humans, cattle, dorcas gazelle and mice and sexual transmission was in fact demonstrated through mice (OIE, 2007 Iowa University).

Tick transmission: Although the *C. burnetii* pathogen is found in ticks, transmission through tick bites has not yet been proved (Eldin et al., 2017). The pathogen is found mainly in the gut epithelial cells of the tick, in contrast to CLEs that are found in various organs of ticks, with the ovaries, midgut and malpighian tubules the most common organs (Zhong, 2012). Because CLEs are found only in tick hosts, transmission of these bacteria relies on tick bites. The bacteria are also transmitted transovarially to their offspring, maintaining the bacterial population, whereas transovarial transmission of *C. burnetii* rarely occur in nature (Zhong, 2012).

2.8. Pathogenesis

Human infection usually follows inhalation of aerosols containing *C. burnetii*. Depending on the infectivity dose, the incubation period may range form 1-3 weeks (Maurin and Raoult, 1999; Eldin et al., 2017). Small amounts of bacteria (<10 bacteria) can cause infection. Entrance of *C. burnetii* into the body through other routes like mucous membranes, abrasions, and the gastrointestinal tract through consumption of milk from infected animals have also been reported. As previously stated, *C. burnetii* exists in two antigenic forms called phase I and phase II. The virulent and infectious form

(phase I) is found in humans with Q fever and infected vertebrate animals, whereas phase II is the non-virulent form. The alveolar macrophages get infected when the pathogen enters the lungs. This happens when the pathogen secures itself to the host cell through special proteins (spectrin-binding proteins) that arbitrate cooperation of the membrane skeleton with the plasma membrane (Madariaga et al., 2013). The bacteria are then incorporated into the host cell through phagocytosis. *Coxiella burnetii* evade intracellular death in macrophages by: (i) inhibiting the final phagosome maturation step (cathepsin fusion) and (ii) by providing resistance to the acidic surroundings of the phagolysosome by producing superoxide dismutase (Madariaga et al., 2013).

The typical development after phagocytosis of almost all organisms is amalgamation of the phagosome with a series of endosomes (intracellular vesicles), resulting in a drop in intracellular pH, followed by amalgamation with lysosomes containing hydrolytic enzymes. This results in elimination of the chronic phase II bacteria, where the T-lymphocyte-mediated response is flawed. Acute/phase I bacteria, however, can resist elimination because of efficient T-lymphocyte-mediated responses (Madariaga et al., 2013).

Secondly, to be safeguarded from being eliminated by most antibiotics, the organisms depend on an acidic pH environment for metabolic activities. Cell destruction is postponed due to the fact that *Coxiella* is capable to coordinate the cell signaling pathways. Because *C. burnetii* can survive intracellularly, it causes either acute or chronic disease. Bacterial death takes place when phagosome-lysosome fusion occurs, due to the presence of interferon-γ, however, in chronic infections interleukin-10 is overproduced by the host cell, which impedes fusion and permits intracellular survival of *C. burnetii*. Infection with *C. bumetii,* especially in cardiac and smooth muscles, produces auto-antibodies, that assist in clearing of the bacteraemic stage in acute infection, but they are not helpful in the chronic stage of infection (Madariaga et al., 2013). In addition, in the chronic phase, high titers of the antibodies lead to a rise in circulating immune complexes affecting various organs and produces a pathological condition namely glomerulonephritis and leucocytoclastic lesions (Madariaga et al., 2013).

Although *C. burnetii* is considered the only pathogen within the genus *Coxiella,* another pathogen of crayfishes has also been identified, namely *Coxiella cheraxi* (Duron et al., 2015). More recent studies also suggest that CLEs can be pathogenic to some extent. Shivaprasad et al (2008) was the first to report CLE infection in vertebrate hosts, the hawk-headed parrot and in golden mantle rosella (toucan) to be exact. The study amplified the 16S rRNA gene of CLE from the livers of these birds (Shivaprasad

et al., 2008). Also in 2008, Woc-Colburn et al reported of fatal coxiellosis in lorikeets, where the first bird died within 24 hours after clinical signs were noted. Lymphohistocytic perivascular encephalitis, cephalic vasculitis as well as microgranulomas were found in the liver, spleen and brain by histopathological scrutiny (Woc-Colburn et al., 2008). Electron microscopy of the brain revealed a prokaryotic bacterium, spherical to rod shaped, containing a trilaminar cell wall, identified as *Coxiella* species, through molecular testing (Woc-Colburn et al., 2008). The death of a female eclectus parrot (*Eclectus roratus*) due to a *Coxiella*-like bacteria were reported again in 2012 by Vapniarsky et al, showing similar symptoms as was reported by Woc-Colburn et al (2008) (Vapniarsky et al., 2012). Although the above findings were only reported in birds, Angelakis et al (2016) finally reported the detection of *Coxiella*-like organisms in human patients who developed eschars after tick bites. The organism was designated *Candidatus* Coxiella massiliensis, which is the proposed name of the bacteria that is an endosymbiont of tick vectors *R. sanguineus, R. turanicus,* and *H. pusillus* (Angelakis et al., 2016). These findings raise the question then: "Can CLEs possibly contribute to Q fever infection as well, especially in South Africa?".

2.9. Clinical signs

Symptomatology is determined by host characteristics, the route of infection and the strain involved (OIE, 2007 Iowa University, Eldin et al., 2017). In Cayenne, French Guiana, the MST 17 clone that is the unique genotype responsible for *C. burnetii* infections in Cayenne, has been found to produce increased rates of symptomatic infections, more so than clones from metropolitan France (Eldin et al., 2017). As illustrated in figure 2.6, symptoms in humans can vary from acute disease (which includes hepatitis, pneumonia, headaches, influenza-like symptoms and fever), to chronic disease (most frequently endocarditis, but also arterial aneurysm infection, osteomyelitis and Q fever during pregnancy) (Angelakis and Raoult, 2010; Wielders et al., 2015; Marrie et al., 2015), those are the two types of infections produced.

2.9.1. Acute Q fever

Self-Limited Febrile Infection

Most people (approximately 60-64%) exposed to *C. burnetii* lack symptoms of infection (Maurin and Raoult, 1999; Bacci et al., 2012). Blood donors have been found to harbor a seroconversion rate of 12.2% and 23.1% for the *C. burnetii* pathogen, in the Netherlands and Spain respectively, that is far higher than the number of symptomatic infections diagnosed per year (Eldin et al., 2017; Marrie et al, 2015). Most symptomatic infections are mild, presenting with non-specific flu-like symptoms with a sudden onset of high fever (± 40°C, that can last for up to 15 days), fatigue, headache, nausea and

myalgia (also known as muscle aches) (Maurin and Raoult, 1999; Eldin et al., 2017; Madariaga et al., 2003).

Pneumonia

Pneumonia is the second most commonly presentation of the disease, affecting middle-aged men more frequently than other human beings (Eldin et al., 2017). Although most pneumonia cases are mild, acute pneumonia symptoms include the following: fever, chills, dyspnea, cough (38% of patients report a non-productive cough and 33% a productive cough), pleuritic chest pain, vomiting, abdominal pain, sore throat, diarrhoea or constipation, myalgia, arthralgia, bradycardia and in some cases confusion. (Maurin and Raoult, 1999; Marrie et al., 2015; Eldin et al, 2017). Headaches are reported as quite severe with some cases reporting as "this is the most severe headache I have ever had in my life" (Marrie et al, 2015). Severe cases presented necrotizing pneumonia and pleural effusion and in exceptional cases, lung pseudotumor (Eldin et al., 2017). Clearing of the pneumonia symptoms is usually within 30 days, how-ever, death has been reported as a result of respiratory distress syndrome (Eldin et al., 2017).

Hepatitis

Hepatitis is associated with elevated liver enzymes (two to three times the normal) in nearly all infections, accompanied by fever, chills and headache (Maurin and Raoult, 1999; Marrie et al., 2015; Eldin et al., 2017). Other symptoms reported are anorexia, vomiting, occasional diarrhoea and agonizing hepatomegaly, with jaundice in rare cases (Eldin et al., 2017). Fever lasts for about 10 days if the patient has been started on antibiotic treatment. Liver biopsy often reveal granulomas often described as "doughnut" granulomas. More recent studies reported epithelloid granulomas with eosinophilic infiltration, comprehensive belched fibrin, lacking the ring granuloma as well as acute cholangitis without granuloma (Eldin et al., 2017). Kaech et al (2009) suggested that autoimmune antibodies in Q fever patients may cause autoimmune liver disease such as primary biliary cirrhosis (Kaech et al., 2009; Marrie et al., 2015). A relatively good prognosis is usually found with acute hepatitis (Eldin et al., 2017).

Cardiac involvement

Pericarditis, myocarditis and acute endocarditis are all forms associated with Q fever infections. The myocarditis form is rare, but very serious (Eldin et al., 2017). Abnormalities on the electrocardiogram are often the only indication of myocarditis (Maurin and Raoult, 1999). Endocarditis is regarded as the "chronic" form of Q fever, but a recent case study revealed this new clinical entity namely acute

endocarditis, where the infection caused a major secretion of auto-antibodies, including IgG aCL, causing auto-immune valvular lesions (Million et al., 2016; Eldin et al., 2017).

Neurological signs

This type of infection is rare and can be witnessed alone or with the involvement of other organs (Eldin et al., 2017). Symptoms include headaches, meningitis and meningoencephalitis, with meningoencephalitis as the most common symptom of the disease, followed by meningitis and peripheral myelitis (Eldin et al., 2017). Lymphocytic meningitis has also been reported through investigations of the cerebrospinal fluid (CSF). Due to immunological disorders, post-infectious neurological signs have been reported such as peripheral sensory neuropathy or Guillian-Barré syndrome (Skiba and Barner, 2009; Rustcheff, 2005; Eldin et al., 2017). These have been shown to improve with steroid therapy (Eldin et al., 2017).

Rare clinical infections

Dermatological signs: Skin infections consisting of maculopapular or vesicular exanthema and occasional purpuric lacerations have been observed, as well as granulomatous panniculitis and erythema nodosum (Eldin et al., 2017).

Bone marrow involvement: Pathology reveals a classic “doughnut” or “fibrin ring” granuloma amid *C. burnetii* primary infections. (Eldin et al., 2017).

Acute lymphadenitis: Lymph nodes associated with fever, headaches, pneumonia and hepatitis are the cervical, axillary, mediastinal, abdominal and inguinal lymph nodes (Eldin et al., 2017).

Cholecystitis: Infection of the gallbladder have been reported with patients reporting of right upper quadrant pain and fever. A diffuse symmetrical thickened and hypodense gallbladder is usually observed through abdominal CT scanning (Eldin et al., 2017).

Auto-immunity: During acute Q fever, anti-smooth antibodies, anti-neutrophil cytoplasmic antibodies (ANCA) and anti-nuclear and anti-phospholipid antibodies have been observed. This observation usually leads to misdiagnosis of Q fever with Good-pasture’s syndrome, Crohn’s disease, Still’s disease, polymyalgia rheumatic, polyarteritis nodosa, essential type II cryoglobulinemia and giant-cell arteritis (Eldin et al., 2017).

Fatigue syndrome: Fatigue may be experienced for a long period after Q fever infections. Symptoms include fatigue, headaches, sweats, arthralgia, myalgia, swollen lymph nodes, blurred vision and muscle fasciculations (Marrie et al., 2015).

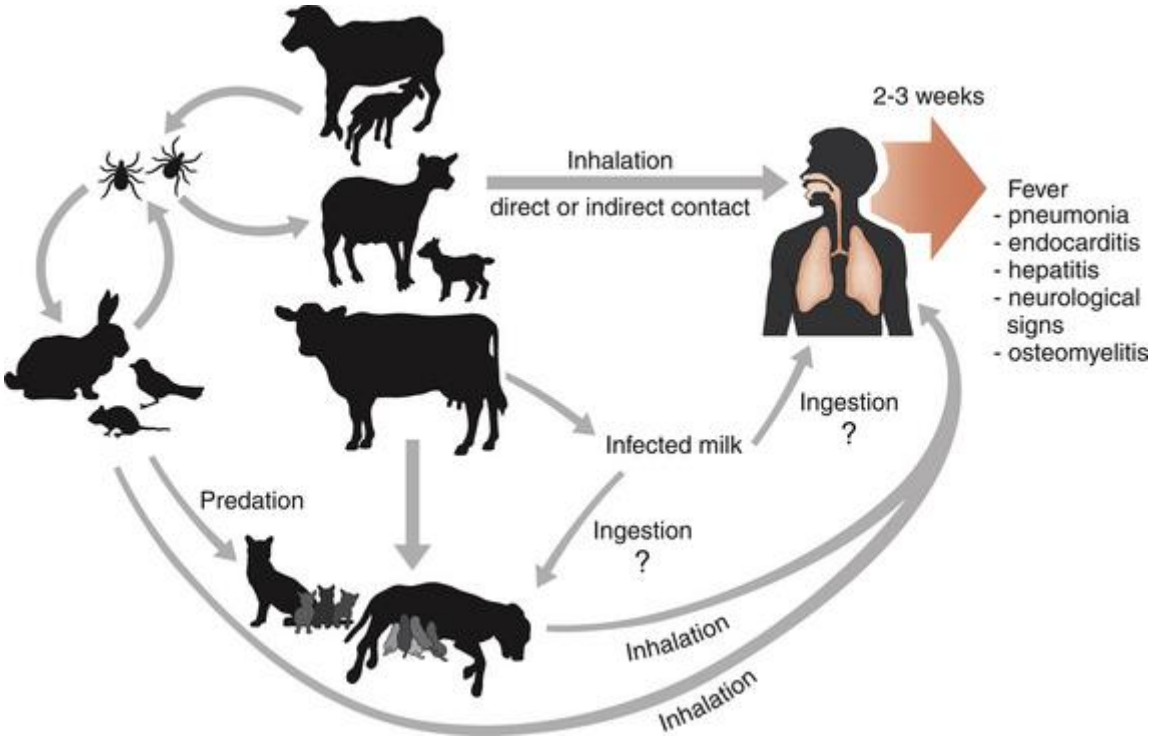

Figure 2.6. Transmission and clinical signs of *C. burnetii* infection **(Q fever.com au)**.

2.9.2. Chronic Q fever

Endocarditis

With chronic *C. burnetii* infection, endocarditis has been reported to be the most common manifestation seen. A time period of 12 months is usually how long it takes to diagnose endocarditis from onset of symptoms (Marrie et al., 2015). Factors such as male sex, age above 40 years and the most important risk factor, underlying valvular heart disease aid in advancement of endocarditis after primary infection, with underlying valvular heart disease estimated at 39% of cases (Eldin et al., 2017). Patients with formerly known valvular heart disease, presenting with unusual native or a bioprosthetic valve and a baffling infection/inflammatory syndrome is the primary indication symptoms of endocarditis (Marrie et al., 2015). Chills, isolated relapsing fever, night sweats, weight loss, fatigue, hepatosplenomegaly, stroke and sudden cardiac insufficiency are all non-specific symptoms of *C. burnetii* endocarditis infections (Marrie et al., 2015; Eldin et al., 2017). Mycotic aneurysms can also present as a symptom and this must be differentiated from isolated vascular infection of a pre-existing aneurysm (Eldin et al., 2017).

Underlying *C. burnetii* infections can last for years causing permanent damage by destruction of the heart valves (Eldin et al., 2017). Million et al (2013) reported that increased levels of IgG aCL (specifically levels higher than 90 IUs) are linked with valvulopathy and is a predictive indicator of advancement to chronic endocarditis (Eldin et al., 2017). Fibrosis, calcification, minor inflammation, vascularization and minimal/absent vegetation is usually revealed during pathological examinations, with histochemical analysis aiding to confirm diagnosis as the mentioned signs can be confused with non-infectious cardiac valve damage (Eldin et al., 2017). Transoesophageal echocardiography (TEE) is a tool used to assist clinicians when investigating patients with traditional bacterial endocarditis (Marrie et al., 2017). Fluorodeoxyglucose positron emission tomography combined with computed tomography (FDG-PET/CT) scanning is a more recent tool that are not only able to identify valvular infections, but also intravascular infections elsewhere as well as osteomyelitis (Marrie et al., 2015; Eldin et al., 2017).

Vascular infections

Infections of vascular grafts or aneurysms can occur after a primary *C. burnetii* infection, with the abdominal and thoracic aortas the most frequent manifestation areas. These infections are mainly diagnosed when complications occur because of the fact that *C. burnetii* vascular infections are supressed and usually present with a wide range of symptoms such as unidentified fever or weight loss (Eldin et al., 2017). Complications of importance include, but are not limited to aortoduodenal fistulas leading to haemorrhage, spondylodiscitis usually linked to psoas ulcerations, graft or aneurysm rupture as well as embolic complications. With vascular infections, the fatality rates are between 18% and 26% (Eldin et al., 2017).

Osteomyelitis infections

Up to 2015, only 20 cases of these type of bone and joint infections has been described. (Marrie et al., 2015), but the number of infections has increase over recent years (Eldin et al., 2017). Cases of osteomyelitis infections found in literature include 11 cases of multifocal osteomyelitis in children, 6 cases of isolated spondylodiscitis and 8 cases of prosthetic joint arthritis (7 of the hip and 1 of the knee) (Eldin et al., 2017).

Infections during Pregnancy

Although the majority of cases are usually asymptomatic, infection during pregnancy can lead to miscarriage, neonatal death, premature births, malformations, oligohydramnios and growth

retardation (Marrie et al., 2015; Eldin et al., 2017). First trimester infections usually end with miscarriages and second trimester infections with prematurity (OIE, 2007 Iowa University).

Shivaprasad et al (2008) was the first to report *Coxiella*-like endosymbiont (CLE) infection in vertebrate hosts, the hawk-headed parrot and in golden mantle rosella (toucan) to be exact. Clinical signs of these birds include anorexia, lethargy, weight loss and respiratory distress (Shivaprasad et al., 2008). Also in 2008, Woc-Colburn et al reported of lorikeets infected with a CLE, showing clinical signs that included head pressing, hemiparesis, seizures, obtunded mentation, weakness, lethargy and weight loss (Woc-Colburn et al., 2008). Infection of CLE in an *Eclectus* parrot that showed clinical signs ranging from inapparent illness to severe lethargy, altered mental status, seizures and sudden death was reported in 2012 by Vapniarsky et al. Angelakis et al (2016) finally reported the detection of CLEs in human patients who developed scalp eschars and neck lymphadenopathy after tick bites (SENLAT) (Angelakis et al., 2016).

2.10. Treatment

Australia produces a vaccine called Q-Vax that is considered to be 98% effective, providing protection for up to 5 years. The vaccine consists of the Henzerling phase I strain of whole-cell *C. burnetii* cells, inactivated in formalin (Chiu and Durrheim, 2007). A side/negative effect of the vaccine can be shivering and induration and because of this, the immune status of the patient needs to be determined either through serology or skin testing before the vaccine can be administered (Waag, 2007).

Various treatment studies have been conducted. For acute Q fever, doxycycline, erythromycin and azithromycin have been tested. All patients were cured, with doxycycline (100 mg twice daily for 14 days) showing the best results, with a mean of 2-3 days for fever duration and recovery of pneumonia, followed by azithromycin and then erythromycin (Parker et al., 2006; Marrie et al., 2015). Fluoroquinolones have excellent cerebrospinal infiltration abilities, making these group of antibiotics suitable for treatment of *C. burnetii* meningitis infections (Drancourt et al., 1991; Eldin et al., 2017; Marrie et al., 2015). Anti-inflammatories are also recommended when symptoms show no response to antibiotics (Parker et al., 2006).

Treatment for chronic Q fever, especially predisposing valvular lesions is usually done with doxycycline (100 mg, twice daily) and hydroxychloroquine (200 mg, three times a day) (Raoult et al., 1999; Marrie et al., 2015; Eldin et al., 2017). Treatment with doxycycline and hydroxychloroquine versus treatment with doxycycline and ofloxacin, showed that the doxycycline and hydroxychloroquine administration

has a compelling lower relapse time (Marrie et al., 2015). Chloroquines are responsible for raising the efficacy of doxycycline, by raising the pH in the phagolysosome (Parker et al., 2006). How-ever a negative effect of this combined antibiotic administration is photosensitivity in patients, requiring patients to have routine heart and eye examinations (Parker et al., 2006). A fundamental part of treatment is the screening of antibody titres on a 3-monthly basis, how-ever no consensus has yet been reached on the exact antibody titre at which treatment can be stopped (Marrie et al., 2015). A titre of below 1:200 is recommended for IgA and IgG antibodies to phase I antigen (Parker et al., 2006). Rifampicin have also been suggested to be effective in treatment of *C. burnetii* (Maurin and Raoult, 1999).

Co-trimoxazole have been found to be very effective in the treatment of children under the age of 8 years and pregnant women till delivery (Parker et al., 2006). Whereas a large percentage (81%) of pregnant women not treated with co-trimoxazole during a case study presented with obstetrical complications (Carcopino et al., 2007; Marrie et al., 2015). Mothers should be made aware that antibiotics as well as the pathogen, is excreted in breast milk (Parker et al., 2006).

Treatment with tetracyclines are recommended for animal herds infected with *C. burnetii* (OIE, 2007 Iowa University).

Zhong et al (2007) reported on successful treatment of CLEs in the *Amblyomma americanum* tick with tetracycline or rifampicin, how-ever treatment of the CLE negatively impacted the reproductive fitness of the tick (longer oviposition and producing fewer eggs) (Zhong et al., 2007). All the patients in the study of Angelakis et al (2016), that developed eschars after tick bite, due to CLE infections were also treated with doxycycline, seeing that it is the preferred treatment for many tick-transmitted infections (Angelakis et al., 2016).

2.11. Prevention

Regarding humans

Personal protective equipment (PPE) should be worn by anybody handling suspected samples containing *C. burnetii*. These include masks, surgical gloves, protective gowns or overalls (Madariaga et al., 2003). Obstetricians should take special care to either wear a face shield or by wearing a N95 protection mask and protective eye glasses when assisting an infected woman with birth. This should also be applicable for veterinarians and farmers with pregnant animals (Eldin et al., 2017; OIE, 2007 Iowa University). Contaminated laundry should not be shaken after a delivery, to minimize

aerosolization and a respirator can also be used during medical procedures (OIE, 2007 Iowa University). Although not recommended, a biosafety level II cabinet can be used when staining smears or tissue samples as well as during serological procedures. A biosafety level III laboratory is the only laboratory to be used when culturing the *Coxiella* organism (Madariaga et al., 2003; Eldin et al., 2017). By using a 0.05% hypochlorite, 5% peroxide, or phenol-based-solutions, any spills of potential infectious material should be cleaned, and biohazardous waste should be disinfected and then autoclaved. Used equipment and instruments can be disinfected with authorized disinfectants containing dual quaternary ammonium-detergent composites that inactivate the organisms after 30 minutes of contact time, followed by autoclaving (Madariaga et al., 2003; Eldin et al., 2017). If the suitable disinfectants and an autoclave is not available instruments can also be boiled for 10 minutes. One should just be aware that the spore-like form of *C. burnetii* may be resistant to the disinfectants used, to ultraviolet radiation, to heat and desiccation procedures used. Aerosolization procedures should be avoided at all cost during post-mortem examinations and personnel should wear a N95 respiratory protection mask (Madariaga et al., 2003).

Regarding animals

Spread of the pathogen can be prevented or controlled through isolation of animals that aborted for up to 14 days. Preventing contamination of feed through excretions can also be done by lifting the feed cages from the ground (Marrie et al., 2015). Burning or burying of aborted materials and foetuses are recommended, while making use of PPE. Through culling and vaccination programmes of sheep and goats, the pathogen can also be kept at bay (Marrie et al., 2015, OIE; 2017 Iowa University). Uninfected farms can be safeguarded to some extent by reducing the introduction of new stock and environmental controls can be practised by cleaning and disinfecting risk areas often (OIE, 2007 Iowa University). Good manure management can also be exercised, such as avoiding the spread of manure during windy days, thus minimizing aerosolization of the pathogen. Practising regular dipping procedures for tick control and invoking movement restrictions when an outbreak occur, can also be helpful in minimizing the spread of the disease (OIE, 2007 Iowa University). And lastly, ingestion of any raw milk should be avoided (OIE, 2007 Iowa University).

2.12. Laboratory identifications

Culturing of *C. burnetii* is very difficult and poses a high risk to staff, thus culturing the organism require a BLS 3+ laboratory. The bacteria are a slow-grower that can be isolated *in vitro* from cell cultures, including Vero cells, mouse macrophage-like cells and fibroblast cells. It can also be isolated from embryonated eggs or laboratory animals (OIE, 2007 Iowa University). How-ever embryonated eggs

are less efficient than cells and isolation in laboratory animals like mice and guinea pigs shows a high degree of cross-contamination between infected and uninfected animals (Fournier et al., 1998; Maurin and Raoult, 1999; World Organization for Animal Health, 2018; OIE, 2017 Iowa University).

In the laboratory, because of *C. burnetii*'s size and shape, one needs to be careful not to confuse it with other pathogens like *Chlamydia abortus* and *Brucella* (World Organization for Animal Health, 2019). Although the *Coxiella* bacteria harbour a membrane similar to a gram-negative bacterium, it is not stainable by gram-stain. Staining is usually done thorough methods such as the Gimenez, Stamp, Macchiavello, Giemsa and modified Koster staining methods, with best results obtained through the first three staining methods (Maurin and Raoult, 1999; World Organization for Animal Health, 2019).

Serological tests are considered to be the first-line tests to be performed when *C. burnetii* infection is suspected (Eldin et al., 2017). These tests require phase I (acute infection) and phase II (chronic infection) antibodies present in serum samples as well as laboratory production of phase I and phase II antigens. Laboratory animals are used to obtain phase I antigens and continues passage in cell culture or eggs are used to obtain phase II antigens (Eldin et al., 2017). Tests such as the Enzyme-linked immunosorbent assay (ELISA), the indirect immunofluorescence assay (IFA) and the complement fixation test (CFT) are the most routinely used serological tests (Table 2.2). Unfortunately, these tests require the development of antibodies which takes approximately two to three weeks from start of infection (Maurin and Raoult, 1999; Hechemy, 2012), which hampers early diagnosis. A distinguishing factor between acute Q fever and chronic Q fever serological wise, is that with acute Q fever, antibodies against phase II antigen is higher than antibodies against phase I antigen and the reverse is true for chronic Q fever, with antibodies higher against phase I antigen than phase II antigen (Marrie et al., 2015).

The IFA is considered to be the gold standard serological test to be used, but reproducibility between analysts poses a problem / disadvantage of the test (Maurin and Raoult, 1999; Hechemy, 2012; World Organization for Animal Health, 2018). Although small percentage wise, another disadvantage of the test is the reporting of cross-reactions with *Legionella micdadei* and *Bartonella* (Eldin et al., 2017). Angelakis et al (2016) also reported of cross-reactivity between *C. burnetii* and *Coxiella*-like bacteria referred to as *Candidatus* Coxiella massiliensis during their IFA screening of samples. The *C. burnetii* IFA test resulted in positive detection for *Candidatus* Coxiella massiliensis, while all patients were in fact negative for *C. burnetii* (Angelakis et al., 2016).

Small volumes of phase I as well as phase II antigens of the *C. burnetii* Nine Mile strain is used in the IFA test. Seroconversion of sera taken 7 to 21 days apart (acute phase and convalescent phase) are regarded as positive if a fourfold increase in phase II IgG or IgM antibodies are obtained between the two serum samples. Cut-off titre values for acute phase II Q fever is considered to be ≥ 1:200 for IgG and ≥ 1:50 for IgM. In the past chronic Q fever were identified if an IgG antibody titre of ≥ 1:800 were obtained against phase I antigen (Fournier et al., 1996; Maurin and Raoult, 1999; Eldin et al., 2017), but this has been revised more recently to be ≥ 1:1600 (Frankel et al., 2011; Marrie et al., 2015).

The ELISA mostly used for surveillance purposes, is a test considered to be more sensitive than the IFA and the CFT and detect both phase I and phase II antigens. However, it can be challenging to interpret by inexperienced analysts and thus are not as routinely used as the IFA test. Acute Q fever are identified if an IgG antibody titre of ≥ 1:1 024 and an IgM antibody titre of ≥ 1:512 are obtained against phase II antigen, and chronic Q fever if IgG as well as IgM antibody titres of ≥ 1:128 are obtained against phase I antigen (Maurin and Raoult, 1999).

The CFT test, although specific, is the most time consuming and least sensitive of all three tests. A prozone effect can be responsible for reporting of false negatives in chronic Q fever. A diagnosis is made for acute Q fever when and antibody titre of ≥ 1:40 is obtained against phase II antigen and for chronic Q fever when an antibody titre of > 1:200 is obtained against phase I antigen (Maurin and Raoult, 1999).

Molecular detection methods do not rely on culturing of organisms nor the production of antibodies (Zhong, 2012). Because some animals shed the organism before developing antibodies and other animals does not seroconvert at all, PCR testing is considered to be another huge advantage in confirming *C. burnetii* presence (OIE, 2007 Iowa University). Various conventional and real-time PCR assays have been developed and successfully implemented to detect *C. burnetii* in samples. The test is specific, sensitive, less hazardous and have a fast turnaround time (Madariaga et al., 2003). Through recent years, various PCR primers and probes have been developed for the detection of *C. burnetii* in different samples such as tissue samples, blood and urine (Madariaga et al., 2003). Multiplex PCR assays are able to detect more than one pathogen simultaneously, cutting the diagnostic time by 75% (Madaraga et al., 2003).

Target genes used varied from the 16-23S RNA, the superoxide dismutase gene, the *icd* gene, the *com1* gene, the IS*30* gene and the IS*1111* gene in animal as well as human samples (Eldin et al., 2017; World Organization for Animal Health, 2018). Nested PCR lacks specificity. Real-time PCR or otherwise referred to as quantitative PCR (qPCR), is a fast turnaround test that quantifies the bacteria in samples through the amplification of a specific sequence (Eldin et al., 2017; World Organization for Animal Health, 2018).

About 20 copies of the I*S1111* gene is present in the *C. burnetii* Nine Mile reference genome. Depending on the isolate, the insertion sequence (IS*1111*) can vary greatly (from 7 and 110 bp of the genome). By targeting the *IS1111* gene, quantification may be affected with different strains, however it is the most sensitive (Eldin et al., 2017, World Organization for Animal Health, 2018). By targeting this gene, the qPCR is able to pick up the bacterium in serum samples within the first 2 weeks of infection, when antibodies are not yet fully formed for serological testing as was reported by Schneeberger et al (Schneeberger et al., 2010). Other studies targeting the *ompA* gene and the IS*30A* gene, showed lower sensitivity than the IS*1111* gene. The *ompA* gene, showed an 88% sensitivity for valvular samples, a 69% sensitivity for blood samples and a 50% sensitivity for urine samples (Eldin et al., 2017). Through lyophilisation extracted DNA can be concentrated, resulting in a much more sensitive qPCR test when targeting the IS*1111* gene (Eldin et al., 2017).

Various molecular tests have also been used to detect *Coxiella*-like bacteria in ticks. Such as real-time qPCR, 16S rRNA gene cloning and sequencing, direct 16S rRNA sequencing and molecular probing (Zhong, 2012). Various genes have also been targeted, such as the 16S rRNA gene, 23S rRNA gene, *groEL* gene, *rpoB* gene and the *dnaK* gene. To date and to our knowledge, no PCR-based assay for the detection and differentiation of *C. burnetii* and CLE DNA simultaneously has been described.

Another identification tool, that is relatively new is the Immuno-PCR that combines amplification of PCR with specificity and versatility of ELISA. It has greater sensitivity (90%) than the ELISA (35%) and the IFA (25%) in sera tested during the first two weeks of infection and a specificity of 92% (Eldin et al., 2017).

Table 2.2. Summary of test methods available for the diagnosis of Q fever and their purpose. Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limit its application; - = not appropriate for this purpose; n/a = not applicable.

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection - surveillance	Immune status in individual animals or populations post-vaccination
Agent identification						
PCR	+++	n/a	+++	+++	++	+[1]
Culture	+	n/a	+	-	+	-
Staining	+	n/a	+	+	+	-
Genotyping	n/a	n/a	n/a	n/a	++	n/a
Detection of immune response						
ELISA	+++	n/a	+++	++	+++	+++
IFA	++	n/a	++	++	++	++
CFT	-	n/a	-	++	+	+

2.13. Laboratory developed and validated assays

In development of a PCR assay, it is imperative to know that efficiency and specificity of a PCR are dependent on the correct selection of the primers (Hyndman and Mitsuhashi, 2003). Different aspects should be taken into consideration when designing a primer, such as the location (if a particular gene is targeted, the primer sequence is required to contain that region of interest), the amplicon size (to maximize binding specificity, the ideal length should be 18-30 nucleotides (nt)), guanine + cytocine (G+C) content (the ideal G+C content that a good primer should contain is around 20 - 80% and every primer should contain one, but no more than two G or C nt's within the last 5 bases of the 3' end in order to reduce nonspecific primer bindings), the melting temperature (primer pairs shouldn't have a difference of more than 2 - 3°C in melting temperatures (Tm) and the Tm should ideally fall within the 58 - 60°C range), and secondary structures (inverted repeat sequences can form stable hairpins which can cause inefficient primer bindings and therefore should not be found within primers) (Hyndman and Mitsuhashi, 2003; Green and Sambrook, 2018). It is important to design the primers as close to the probe as possible, without overlapping the probe.

The TaqMan® MGB probes allow for shorter probe design, which is very useful for shorter conserved region identification in a variable region and also has the advantage of increased assay sensitivity because the 3'-end has a non-fluorescent quencher-MGB attached, responsible for lower background signals (Kutyavin et al., 2000). Specificity of the assay is increased by the use of a fluorescence resonance energy transfer and hydrolysis (TaqMan®) probe. The TaqMan MGB probe, is an oligonucleotide which contain a donor fluorescent component and an acceptor at the 5'-end and at

the 3'-end respectively (Figure 5.1). They are designed to bind downstream to one of the primers during the PCR reaction and to give off a fluorescent signal during the reaction. The receptor quenches the fluorescence produced by the donor, as they remain in close proximity. During the extension phase, deterioration of the hydrolysis probe takes place via the 5'-3'-exonuclase activity of the Taq DNA polymerase, which bring forth fluorescence from the reporter (Navarro et al., 2015). TaqMan MGB probes have the ability to produce precise results, high sensitivity, specificity and reproducibility and the advantage of designing shorter probes (Kutyavin et al., 2000).

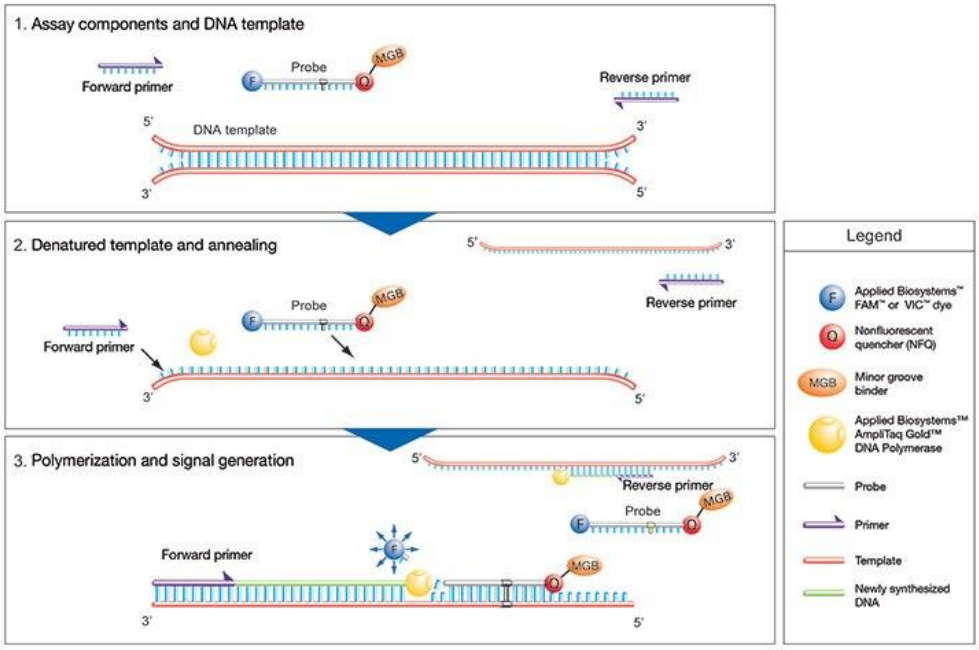

Figure 2.7. TaqMan ® MGB probe-based assay chemistry (ThermoFisher, 2020).

An optimised assay is where controls and standards produce the same results repeatedly (Jacobson, 1998). Thus, optimization conditions are important to develop an accurate and robust assay. Indications of inadequate optimization are a lack of reproducibility between replicates as well as inefficient and insensitive assays (Jacobson, 1998). Good primer combinations that generate the most sensitive and reproducible results are those that produce the lowest Ct-value (the cycle number where the fluorescence signal crosses the threshold) and the highest ΔRn (a measure of sensitivity and an indicator of the significance of the fluorescence signal produced by the PCR) (Green and Sambrook, 2018). Optimization is also the process where the physical, chemical and biological parameters are determined and altered, ensuring appropriate performance characteristics for the intended use (World Health Organisation, 2018). The two primary approaches are optimization of primer and probe concentration and/or annealing temperatures.

To optimize primer concentrations is to construct a matrix of reactions where the forward and reverse primers are separately varied and tested in combination (Green and Sambrook, 2018). A primer matrix is tested against a constant probe concentration of 250 nM (the final concentration in the reaction volume), ensuring that the probe is in ample abundance to avoid probe limitations and to guarantee maximum sensitivity. The lowest of the primer concentrations that amplifies the most efficiently is then used to test different probe concentrations typically in the range of 50 – 250 nM. And as with the primers, the probe concentration that produces the lowest Ct-value and highest ΔRn, are identified and used for further testing (Green and Sambrook, 2018).

Ruijter et al. (2009) described the PCR efficiency as the increase in the number of DNA copies per cycle. In theory, the number of DNA copies doubles after each cycle, but in practice, the DNA increases by a factor of (1+n) where n is the cycle efficiency. This means that doubling of the DNA concentration is implied when the efficiency of n = 1.

The lowest quantity of analyte that can be detected in a sample is known as analytical sensitivity or the limit of detection (Jacobson, 1998). It portrays the capability of the test to diagnose disease and determine treatment endpoints (Burd, 2010).

Analytical specificity is the degree to which the assay does not cross-react with other organisms (Jacobson, 1998). It is the capability of the assay to differentiate the target organism, including antibody or genomic sequence, from non-target organisms including matrix components (OIE Manual of Diagnostic Tests for Aquatic Animals., 2019).

Repeatability represents estimates of precision within an assay (within the same run and between runs). It implies that a minor amount of distribution indicates an accurate assay (Jacobson, 1998). This is achieved by using the same samples, run in different plates within and between runs of the assay (intra-plate and inter-plate variation).

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Chapter 3

Materials and Methods

3.1. Sampling area

Sampling of *Rhipicephalus sanguineus ticks* was conducted in the Clare A region of the rural Mnisi community (Figure 3.1) during a recent study (R. Ackermann, MSc dissertation 2019, UP).

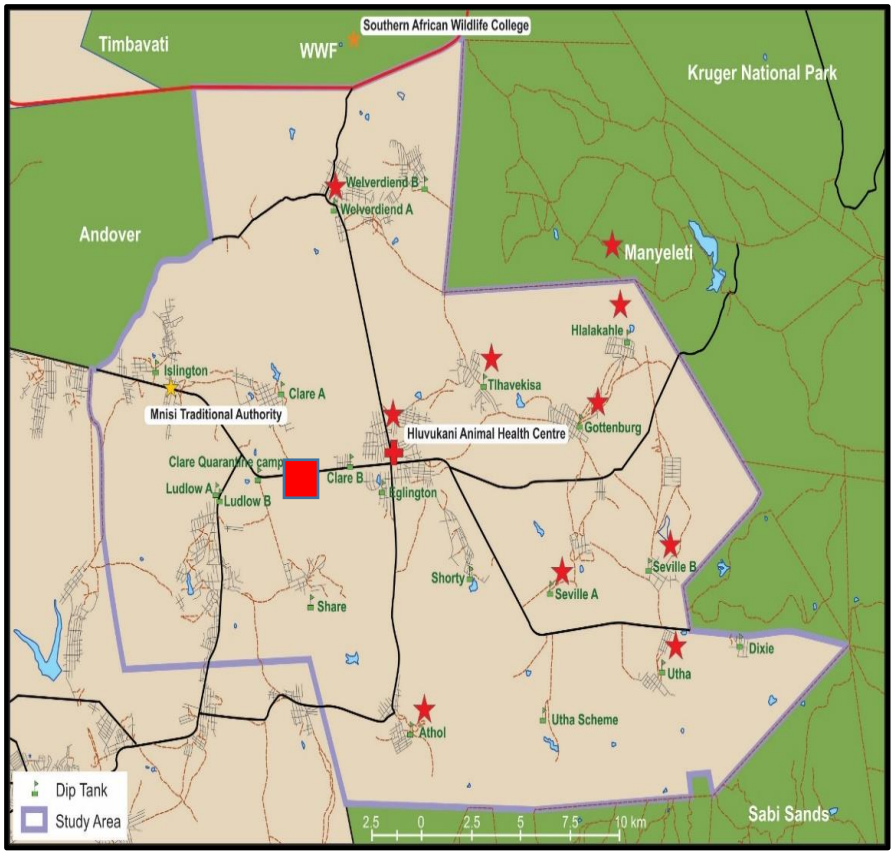

Figure 3.1. The red square on the map is indicative of the sample area (Clare A) in the Mnisi Community. Surrounding areas is the Kruger National Park, Manyeleti Game reserve, Sabi Sands and Andover. (map produced by Estelle Mayhew).

3.2. Sample size

A sample size of 149 was the minimum number of samples required, to detect at least one positive *Coxiella* species, with a prevalence rate of 2% and a probability level of 0.95 in an infinite population size (http://epitools.ausvet.com.au/). The samples were obtained from a previous study (R. Ackerman, MSc dissertation, 2019, UP) and consisted of extracted DNA samples from midgut (MG) and salivary gland (SG) pools of *R. sanguineus* ticks collected from dogs as well as extracted human

DNA samples, positive by serology for *Coxiella* species obtained from the National Institute for Communicable Diseases (NICD).

3.3. Purification of tick and human nucleic acid

Purified DNA was obtained from a previous study (R. Ackermann, MSc dissertation 2019, UP) where the DNA was purified from MG and SG pools of *R. sanguineus* ticks (each tick pool = 3-10 ticks/dog) using a modified PureGene Ethanol Extraction (MPEE) method (Scoles et al. 2005), eluted in 50 µl DNA hydration solution (Qiagen, Hilden, Germany) and stored at -20°C until further processing. A further 200 µl mixture of six pools containing midgut and salivary glands (each tick pool = 10 ticks/dog) were extracted using a DNeasy Blood and Tissue kit (DBTK) method (Qiagen, Hilden, Germany), eluted in 200 µl of AE buffer, and stored at -20°C until further processing.

Tick MG and SG were dissected and pooled in groups of 30 with three biological replicates. Tissues were stored in Cell Lysis Solution (Qiagen, Valencia, CA, USA) and Proteinase K (100 µl proteinase K in 10 ml Cell Lysis buffer). Genomic DNA was isolated using the PureGene Extraction kit (Qiagen), following the manufacturer's instructions. Pellets were re-suspended by the addition of 50 µl DNA Hydration solution and stored at -20°C until further processing.

Some already extracted human DNA obtained from the NICD of patients with clinical signs of non-malarial acute febrile illness (AFI) was used. The NICD extracted the DNA either from 200 µl blood, using the QIAamp DNA mini kit ® (Qiagen, Hilden, Germany) following the manufacturer's instructions, eluted in 200 µl of AE buffer, and stored at -20°C until further processing or through their robotics system.

3.4. TaqMan® MGB real-time PCR assay design

3.4.1. Experimental assay design

Twenty four near-full length 16S rDNA CLE sequence data reads that were generated from *R. sanguineus* ticks from a previous study (R. Ackermann, MSc dissertation, UP) (hereafter referred to as CLE DVTD 1-4) was used together with published *C. burnetii* sequences obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank) (Altschul et al., 1990). The sequences were used to design primers targeting nucleotide differences between *C. burnetii* and CLEs in the 16S rRNA genes. Primers and probes were optimised and the efficiency of the assay calculated from the slope of a standard curve regression line. A positive nucleic acid control strain of *C. burnetii* was obtained through the French National Reference Laboratory (ANSES) and cloned for use as a positive control. The PCR was

validated by determining the sensitivity, specificity as well as repeatability in human blood spiked with *C. burnetii* and CLE plasmid DNA, respectively. After optimisation of the PCR, serological positive *Coxiella* samples from febrile patients obtained from the NICD were tested. Twenty samples that tested positive on the PCR were sent for Sanger sequencing for confirmation.

3.4.2. *In silico* PCR primer and probe design

Variable sequences of the 16S rRNA gene of *C. burnetii* and CLEs, that fell within the 100 - 1600 bp range were downloaded in FASTA format. GenBank accession numbers of sequences used, is shown in tabular form (Table 3.1). The sequences were then aligned with MAFFT online version (https://mafft.cbrc.jp/alignment/server), using the default settings (Katoh et al., 2017). The *C. burnetii* strain ATCC VR-615 sequence with accession number NR_104916 was used as the reference sequence. Poorly aligned sequences were manually deleted. The Data Analysis in Molecular Biology and Evolution software (DAMBE) was used to identify and collapse identical/duplicate sequences (Xia, 2018). Sequence alignments were edited with BioEdit Sequence Alignment Editor (Hall, 1999, Alzohairy, 2011).

Table 3.1. Genbank accession numbers of Coxiella burnetii and CLEs used for identification of differential/conserved areas. The accession number followed by _ and the number at the end, represents the sequences collapsed due to it being identical to the first sequence.

GenBank Accession numbers					
Coxiella burnetii		***Coxiella*-like Endosymbionts**			
CB_NR104916_2	CB_KY498541	CLE_KU324478_10	CLE_KT371073	CLE_KP994838_4	CLE_KR820005
CB_JQ740886	CB_KX825917	CLE_KU324471_3	CLE_DVTD_1	CLE_KP994836_2	CLE_KR820012
CB_CP000890	CB_MG722701_2	CLE_KT835661	CLE_DVTD_3	CLE_KP994834_2	CLE_KR820007
CB_LC464975_3	CB_MG722702_2	CLE_KT835660	CLE_DVTD_4	CLE_KP994840_2	CLE_KP994816_2
CB_GU797243	CB_MG722703	CLE_MH645182	CLE_DVTD_2	CLE_KP994830_2	CLE_KF913928_3
CB_D89799	CB_MG640364	CLE_KP994799	CLE_MG050149	CLE_KP994832_2	CLE_KF913922
CB_D89797	CB_MG640363_2	CLE_KP994790_2	CLE_MG050151	CLE_KR820006	CLE_KF913924
CB_D89795	CB_JX154095	CLE_KP994771_2	CLE_KP994850_2	CLE_KR820011	CLE_KF913926
CB_D89798	CB_JX154094	CLE_KP994776_3	CLE_KP994846_2	CLE_KR820015	CLE_KF913925
CB_AY342037	CB_MN900581	CLE_KP994784_2	CLE_KP994844_2	CLE_KP994826_2	CLE_KF913921
CB_MN880312	CB_MN900572	CLE_KP994782_2	CLE_MG050150	CLE_KP994824_2	CLE_KR820010
CB_D89792	CB_MN900571_5	CLE_KP994769_2	CLE_MG050148	CLE_KP994818_2	CLE_KR820013
CB_D89800	CB_MN121701	CLE_KP994794_4	CLE_MH645186	CLE_KP994780_2	CLE_KR820014
CB_D89791	CB_MN121700	CLE_KP994773_2	CLE_MH645188	CLE_KP994822_2	CLE_KR820016
CB_D89796	CB_MN900580	CLE_KF913920_2	CLE_MH645192	CLE_KP994820	CLE_KP994806_2
CB_FJ787329	CB_MN900570	CLE_KF913917	CLE_MH645194	CLE_KP994819	CLE_KP994804_2
CB_KT835663_2	CB_MN473203	CLE_KF913919	CLE_NZ_MH645193_4	CLE_KP994796_2	CLE_KR820009
CB_M21291	CB_MN121702	CLE_KF913910_8	CLE_MH645189	CLE_MK671680_11	CLE_MN612066_2
CB_MT158656	CB_MN900579	CLE_KF913918_7	CLE_MH645190	CLE_KP994814_2	CLE_KR820008
CB_MG564251	CB_MN473206	CLE_KF913916_2	CLE_MH645187	CLE_KP994812_2	CLE_KP994786_2
CB_MG564253		CLE_KF913906	CLE_KP994842_2	CLE_MH645183	CLE_MN612068
CB_MG564250		CLE_KF913914	CLE_KP994802_2	CLE_MH645184	
CB_MG564252		CLE_KF913893	CLE_KP994798_2	CLE_MH645185	
CB_KT945016_3		CLE_KF913900	CLE_KP994788_2	CLE_KP994808_2	
CB_KU291433_6		CLE_KF913905_2	CLE_KP994778_2	CLE_KP994810_2	
CB_KU215908		CLE_KF913912	CLE_KP994848_2	CLE_KR820017	

Conserved regions were identified and a set of group-specific primers were designed (one primer set for both *C. burnetii* and CLEs within a 100% conserved region) along with species-specific TaqMan® minor groove binder (MGB) probes (Table 3.2) using Primer Express® version 3.0.1 (Applied Biosystems, USA). A region specific to the *C. burnetii* and CLE group was selected for probe placement which was flanked by conserved regions which the primers targeted. A nucleotide Basic Local Alignment Search Tool (BLASTn) screening was performed for both the primers and probe on NCBI web site (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to test for non-specific binding *in silico*.

The default primer/probe design parameters were as follows: melting temperature (Tm) of 58 - 60°C and 68 – 70°C, respectively; a percentage GC content of 20 - 80%; the last five nucleotides of the 3' end of the primer do not contain more than two 'G' and 'C' residues; the 5' end of probe does not contain a 'G' residue (because a 'G' residue has a quenching effect). The C*. burnetii* probe was labelled with a fluorescent reporter dye FAM (6-carboxyfluorescein) and the CLE probe with a VIC fluorescent reporter dye at the 5'-end. A non-fluorescent quencher-minor groove binder (NFQ-MGB) was attached to the 3'-end.

3.4.3. Real-time PCR optimization

The lyophilized stock of primers (Integrated DNA Technologies, USA) was resuspended to 100 µM stock concentration with Tris-EDTA (TE buffer). Dilutions to 20 µM working stocks aliquots were made with ultrapure 18.2 MΩ.cm water (Elix® Essential 5 and Synergy® water purification systems, Merck). The probe (ThermoFisher Scientific, USA) was resuspended with TE buffer to 100 µM and further dilutions to 20 µM working stock aliquots were prepared with the same diluent. All stocks prepared were stored at -20°C until use. Primer and probe concentrations were tested in triplicate using the StepOnePlus™ Real-time PCR System (Applied Biosystems) with StepOne Software v2.3 system (ThermoFisher Scientific, USA) and a TaqMan ™ Fast Advanced Master Mix (catalog number 4444557, LTC Technologies, SA). The primer concentration was optimized by testing both forward and reverse primers with a 100 nM, 200 nM, 400 nM and 800 nM concentration in the PCR reaction with a constant probe concentration of 250 nM in the PCR reaction. A negative control consisting of PCR mix and 2µl water was included. The optimised primer concentration determined above (400 nM) was used for probe optimization using a probe concentration range of 50 nM, 100 nM, 150 nM, 200 nM and 250 nM. The standard amplification curve was visually analyzed and the primer/probe combinations that yielded optimal assay performance (low cycle threshold (CT), efficient (steep) amplification slope and low primer/probe concentration) was chosen for further experiments.

3.5. Construction, purification and cloning of a *C. burnetii* and CLE PCR amplicon for use as a positive control

A DNA sample of *C. burnetii* was obtained from the National Reference Laboratory for Q fever, ANSES, France. This DNA sample was used to amplify the near full-length sequence of *C. burnetii* 16S rRNA gene (~ 1321-1429 bp) in order to construct a plasmid *C. burnetii* positive control. For the CLE positive control, a positive tick DNA sample (# 1102M), collected during a previous study (R. Ackermann, MSc dissertation 2019, UP) and confirmed via sequencing, was used. One set of primers for both organisms was designed by using Whitehead Scientific's Integrated DNA Technologies, PrimerQuest Tool (https://sg.idtdna.com/pages/tools/primerquest) shown in Table 3.3.

Table 3.2. Primers designed for cloning of plasmid positive controls.

Name	Primer sequence (5'-3')	Length	Tm (°C)	% GC
CoxClone_F-primer	GTA GCG GTG AAA TGC GTA GA	20	62	50
CoxClone_R-primer	GTA AGG GCC ATG ATG ACT TGA	21	62	47,6

The PCR master mix consisted of 12.5 µl 2x Phusion Flash High-Fidelity PCR master mix (Phusion DNA polymerase, deoxynucleotides, Phusion HF Buffer and Phusion GC Buffer containing $MgCl_2$, dimethyl sulfoxide) (ThermoFisher Scientific, USA), 0,25 µl of a 10 µM forward primer, 0,25 µl of a 10 µM reverse primer, 9.5 µl of PCR grade water and 2,5 µl template to make up a 25 µl total reaction. Template amplification was performed on the Veriti GeneAmp® PCR systems 2700 (Applied Biosystems). The optimum annealing temperature was determined by testing the reaction against a range of temperatures (55°C, 56°C, 57°C, 58°C, 59°C, 60°C, 61°C, 62°C, 63°C, 65°C and 66°C) to see which temperature gave the clearest band when ran on a 2% agarose gel. The reaction set-up was repeated, but this time it was ran at the determined optimum temperature as shown in Table 3.4.

Table 3.3. PCR conditions for the amplification of the cloned C. burnetii and CLE 16S rRNA gene.

PCR steps	Number of cycles	Temperature (°C)	Duration
Initial denaturation	1	98°C	10 seconds
Denaturation	35	98°C	1 second
Annealing		58°C	5 seconds
Extension		72°C	15 seconds
Final extension	1	72°C	1 second
Holding step		4°C	∞

The PCR amplicons from the above PCR assay was run on a gel for confirmation of DNA product/successful amplification. A 2% agarose gel was prepared in 1x tris-acetate-ethylenediamine

tetra-acetic acid buffer (TAE) with ethidium bromide. From the PCR product, 5 µl was mixed with 2 µl of loading dye (ThermoFisher Scientific, USA) and run for 30 min at 120 volts, on a Labnet GI XL Enduro machine. The size of the band was determined with the aid of a Quick-Load 100 bp + DNA ladder with a concentration of 50 µg/ml (BioLabs,USA). The PCR product was visualized with a ChemiDoc™ XRS+ System with Image Lab™ Software version 3.0 (Bio-Rad) Gel Doc imaging system.

To prevent interference with the sequence analysis, all primers, nucleotides, enzymes and any impurities were removed. This was done using the PureLink® PCR Purification Kit (Invitrogen by Life Technologies, ThermoFisher, USA), according to the manufacturer's instructions. The DNA was eluted in 50 µl elution buffer.

Cloning was done using the Clone Jet PCR cloning Kit (ThemoFisher Scientific, USA). The purified PCR amplicons was cloned into a pJET1.2/blunt-end cloning vector (ThermoFisher Scientific, USA). The purified DNA fragments which were ligated into the vector by means of the T4 DNA ligase were transformed into competent high efficiency *Escherichia coli* NEB 5-alpha $F^1/_q$ (Biolabs, USA) cells and the culture was incubated in a shaking incubator (225-250 rpm) at 37°C for 1 hour in imMedia™ Amp Blue S.O.C liquid broth (# 15544-034, Invitrogen,USA). The culture was then plated onto two imMedia™ Amp Blue (Invitrogen,USA) agar plates, by using standard techniques and incubated overnight at 37°C.

To verify the presence of *C. burnetii* and CLE inserts into the cloning vector, colony PCR was performed on three to five colonies. The PCR reaction mixture consisted of 12.5 µl 2x Phusion Flash High-Fidelity PCR master mix (ThermoFisher Scientific, USA), 0,25 µl of a 10 µM forward primer, 0,25 µl of a 10 µM reverse primer, 9.5 µl of PCR grade water and 2,5 µl template to make up a 25 µl total reaction, following the same PCR conditions listed previously in Table 3.5, but with 30 cycles performed instead of 35 cycles. By using a 2% agarose gel, the PCR product was analysed.

From the overnight cultures, plasmid extractions were performed using the PureLink ™ Quick Plasmid Miniprep Kit (Invitrogen, ThermoFisher Scientific, USA), following the manufacturer's instructions. The plasmid DNA was eluted in 75 µl TE buffer, of which 15 µl was sent to Inqaba Biotec™ (Pretoria, RSA) for Sanger sequencing in order to confirm the *C. burnetii* as well as CLE sequences. The remaining plasmid DNA was stored at -20°C for use as positive control DNA in the real-time PCR.

The concentration of the eluted plasmid DNA was determined by taking an average of three readings from the Qubit ® 2.0 Fluorometer (Invitrogen by Life Technologies, USA) as well as the Trinean Xpose (Anatech Instruments, South Africa) spectrophotometer. The copy number of the plasmid was calculated by taking the obtained spectrophotometer result in ng/µl and dividing it with the weight of the plasmid in nanograms.

3.6. Validation

3.6.1. Analytical efficiency

A total blood cell count was performed (Veterinary Diagnostic Laboratory, Onderstepoort, SA) on a human blood sample (Appendix 1) that was used as matrix in the determination of the assay efficiency. This was done by separately preparing a 10-fold dilution series (10^{-1} to 10^{-10}) of *C. burnetii* plasmid DNA (starting concentration of 5.85 ng/µl) and CLE plasmid DNA (starting concentration of 23.90 ng/µl) in TE buffer. Ten microlitres of each plasmid DNA dilution was added to 190 µl of the human blood to make a dilution series of plasmid spiked blood. DNA from each dilution was purified and run in triplicate with the real-time PCR within a single run. Generated results were used to draw a standard curve showing Ct value versus log genome copies/reaction. The slope of the regression line of the standard curve was used to calculate the efficiency of the PCR assay using the following formula:

$$PCR\ efficiency\ (\%) = 100 \times (10^{-1/slope} - 1)$$

3.6.2. Analytical sensitivity

The limit of detection (LOD) is a measure of the analytical sensitivity (A-se) of an assay. Analytical sensitivity is usually expressed as the minimum detectable concentration of the analyte. A plasmid construct, containing the target sequence was tested in a dilution series in a human blood matrix, where after the number of genome copies detected by real-time PCR was then estimated.

In order to cover the non-linear range of the assay, the last dilution which produced a positive result in all replicates obtained from the efficiency analysis (10^{-6}) was used to prepare a two-fold dilution series for both *C. burnetii* plasmid DNA as well as CLE plasmid DNA (separately) in ultra-pure water. According to the OIE Manual (2018), a two-fold dilution range provides a more accurate estimate (OIE Terrestrial Manual, 2018). Ten two-fold dilutions were prepared ranging from 1:2 to 1:1024. Five separate nucleic acid purifications were performed from each dilution and each purified dilution tested five times in separate PCR runs. Thus, each dilution was tested 25 times in total. The data

generated were used to calculate the 95% LOD (input concentration giving a positive result in 95% of the replicates) by probit analysis (SPSS Statistics v25 software, IBM Analytics, USA).

3.6.3. Analytical specificity

Specificity of the real-time PCR was determined using extracted DNA from other bacteria (obtained from the NICD) closely related to *C. burnetii* as well as in-house constructed plasmids of other tick-borne pathogens. (Table 3.5).

Table 3.4. Organisms used to test specificity of the designed TaqMan ® MGB real-time PCR.

Organism	**Strain**
Legionella pneumophila	NICD – ATCC 33152
Mycobacterium tuberculosis	NICD
Brucella abortus	NICD – CEZPD – SBPRL
Listeria monocytogenes	NICD
Salmonella typhimurium	ATCC 13311
Pasteurella multocida	ATCC 12945
Escherichia coli	ATCC 25922
Klebsiella pneumoniae	ATCC 700603
Pseudomonas aeruginosa	ATCC 27853
Staphylococcus aureus	ATCC 25923
Rickettsia africae	RE 18/030
Anaplasma marginale	UP Proefplaas Farm (C14) – 9678/29
Anaplasma centrale	Onderstepoort Biological Products, South Africa
Ehrlichia chaffeensis	RE 18/053
Ehrlichia canis	Onderstepoort Biological Products, South Africa – Constructed plasmid
Ehrlichia ruminantium	Onderstepoort Biological Products, South Africa
Babesia microti	DNA obtained from the University of Utrecht (Zivkovic *et al.,* 1984) – Constructed plasmid

The ATCC reference bacterial strains obtained from the Department of Veterinary Tropical Diseases (DVTD) diagnostic bacteriology laboratory, University of Pretoria, were cultured on blood agar plates and incubated at 37°C for 24 hours (hrs). A single colony from each of the reference organisms was harvested and thoroughly mixed in 1 ml PBS in a 1.5 ml microcentrifuge tube. A further 1:10 dilution

in PBS was made from which the DNA was purified using the MagMAX CORE™ Nucleic Acid Purification Kit.

3.6.3.1. Nucleic acid purification

A total of 200 µl of the 1:10 dilution of each organism was extracted. The lysis/binding mixture was prepared by adding equal volumes (350 µl) of lysis solution and binding solution and the bead/protein kinase (PK) mixture was prepared by combining 20 µl magnetic beads with 10 µl PK. Five hundred microlitres each of wash solution 1 and 2 was added to rows B and C respectively of a KingFisher deep-well 96 plate (ThermoFisher Scientific, USA). In row A, 30 µl bead/PK mix, 200 µl sample and 700 µl lysis/binding buffer per well was added. In a separate elution strip, 90 µl of elution buffer was added to each well. The prepared plate as well as the elution strip was loaded onto the KingFisher™ Duo Prime Purification System (ThermoFisher Scientific, USA) and run using the MagMAX_CORE_DUO.bdz programme. After purification, the DNA was eluted in 90 µl elusion buffer for all the strains. From this, 2 µl of DNA was added to 18 µl of the prepared Master Mix (described below at the real-time PCR) and the PCR was run on the StepOnePlus™ Real-time PCR System (Applied Biosystems) with cycling conditions listed in Table 3.6. The elution strip with the rest of the DNA was sealed with an elution strip cap and stored at -20°C.

3.6.4. Repeatability

The inter-run and intra-run repeatability of the assay was determined from data obtained from the sensitivity results, using Microsoft Excel. Inter-run variation was determined over multiple days, by using the same samples in multiple runs. However, it involved one operator. The total coefficient of variation (CV) was calculated by the formula:

CV = total SD / (mean CT value of all replicates).

3.6.5. PCR assay conditions

All qPCR assays were run in a total reaction volume of 20 µl comprising of 10 µl TaqMan™ Fast Advanced Master Mix (catalog number 4444557, LTC Technologies, SA), 400 nM (final concentration) of each forward and reverse primers, 100 nM (final concentration) of the *C. burnetii* probe, 150 nM (final concentration) of the CLE probe, 2 µl template DNA and 6,95 µl nuclease free water. The positive control comprised of PCR mix with 2 µl *C. burnetii* plasmid DNA and 2 µl CLE plasmid DNA respectively. The negative control contained all the above-mentioned PCR reagents except the DNA template. The

DNA template was replaced with 2 µl nuclease free water. The qPCR cycling conditions are described in Table 3.6.

Table 3.5. Thermal cycling conditions for the TaqMan ™ Fast Advanced qPCR Master Mix (ThermoFisher Scientific, USA).

PCR steps	Stage	Number of cycles	Temperature (°C)	Duration
UNG incubation	1	1	50°C	2 minutes
Polymerase Enzyme activation	2	1	95°C	20 seconds
Denaturation	3	40	95°C	1 second
Annealing / Extension	4	40	57°C	20 seconds

3.7. Screening of field-derived samples with newly developed Real-time PCR assays

Real-time quantitative PCR was performed on a total of 130 tick samples and 78 human samples in MicroAmp optical 96-well reaction strips using the StepOnePlus™ Real-time PCR System (Applied Biosystems), with StepOne Software v2.3 system (ThermoFisher Scientific, USA).

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Chapter 4

Results

4.1. TaqMan® MGB real-time PCR assay design

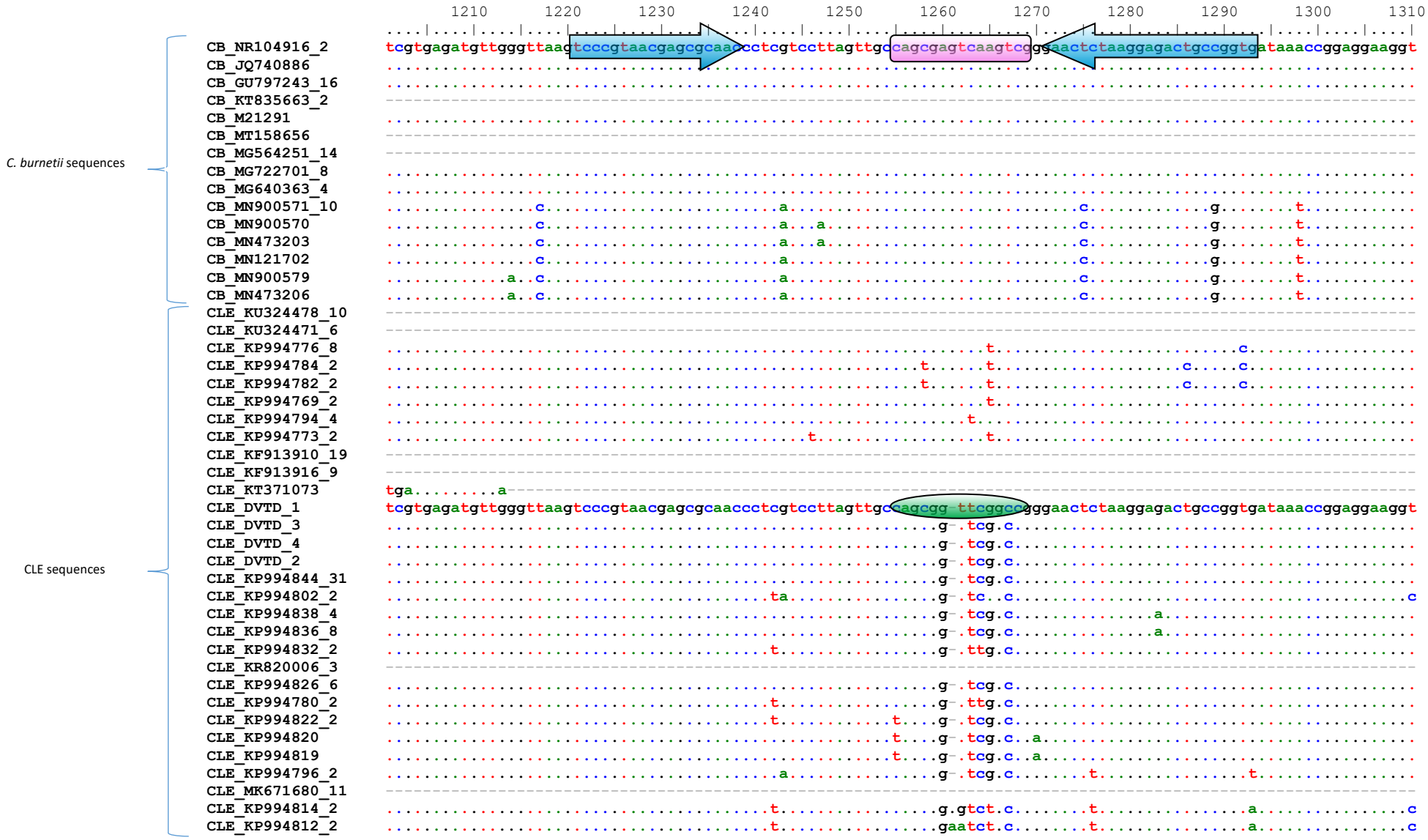
A total of 767 sequences that fell within the 100 - 2000 bp range of the 16S rRNA genes of both *Coxiella burnetii* and *Coxiella*-like endosymbionts (CLE) were downloaded and aligned. The reference sequence used in our search was GenBank sequence NR_104916 (*Coxiella burnetii* strain ATCC VR-615). All non-*Coxiella* sequences were manually deleted which left 707 sequences. These sequences were grouped into *C. burnetii* and CLE groups. Included in the CLE group was 24 near-full length CLE 16S rRNA gene sequences obtained from *R. sanguineus* ticks. Sequences that aligned poorly were deleted, leaving 62 sequences of *C. burnetii* and 135 sequences of CLEs. Identical sequences were then removed with DAMBE software, leaving a total of 44 *C. burnetii* sequences and 63 CLE sequences (Figure 4.1).

By using the default primer/probe parameters, one set of primers were designed for both *C. burnetii* and CLEs within a conserved region flanking species-specific region using Applied Biosystems Primer Express® 3.0 software (Table 4.1). A region specific to the *C. burnetii* and CLE groups was identified and probes designed for each group within that region. Both probes have a melting temperature of 69°C. The lengths of the probes were respectively 15 bp for the *C. burnetii* probe and 13 bp for the CLE probe and the percentage GC content was 60% and 77% respectively.

Table 4.1. Sequences and characteristics of the forward / reverse primers (F/R) and specific probes (P) targeting the 16S rRNA gene of Coxiella burnetii and CLEs.

Name	Primer sequence (5'-3')	Length	Tm (°C)	% GC
F-primer	TCC CGT AAC GAG CGC AAC	18	59,4	61
R-primer	CAC CGG CAG TCT CCT TAG AGT T	22	58,5	55
*Coxiella burnetii*_P (CBP)	CAG CGA GTC AAG TCG	15	69	60
CLE_P (CLEP)	CAG CGG TTC GGC C	13	69	77

A BLAST screening of the designed primers and probes in GenBank® showed no non-specific binding, therefore indicating *in silico* specificity of the TaqMan® MGB real-time PCR assay.


1210
1220
1230
1240
1250
1260
1270
1280
1290
1300
1310
C. burnetii sequences
CLE sequences
CB_NR104916_2
CB_JQ740886
CB_GU797243_16
CB_KT835663_2
CB_M21291
CB_MT158656
CB_MG564251_14
CB_MG722701_8
CB_MG640363_4
CB_MN900571_10
CB_MN900570
CB_MN473203
CB_MN121702
CB_MN900579
CB_MN473206
CLE_KU324478_10
CLE_KU324471_6
CLE_KP994776_8
CLE_KP994784_2
CLE_KP994782_2
CLE_KP994769_2
CLE_KP994794_4
CLE_KP994773_2
CLE_KF913910_19
CLE_KF913916_9
CLE_KT371073
CLE_DVTD_1
CLE_DVTD_3
CLE_DVTD_4
CLE_DVTD_2
CLE_KP994844_31
CLE_KP994802_2
CLE_KP994838_4
CLE_KP994836_8
CLE_KP994832_2
CLE_KR820006_3
CLE_KP994826_6
CLE_KP994780_2
CLE_KP994822_2
CLE_KP994820
CLE_KP994819
CLE_KP994796_2
CLE_MK671680_11
CLE_KP994814_2
CLE_KP994812_2

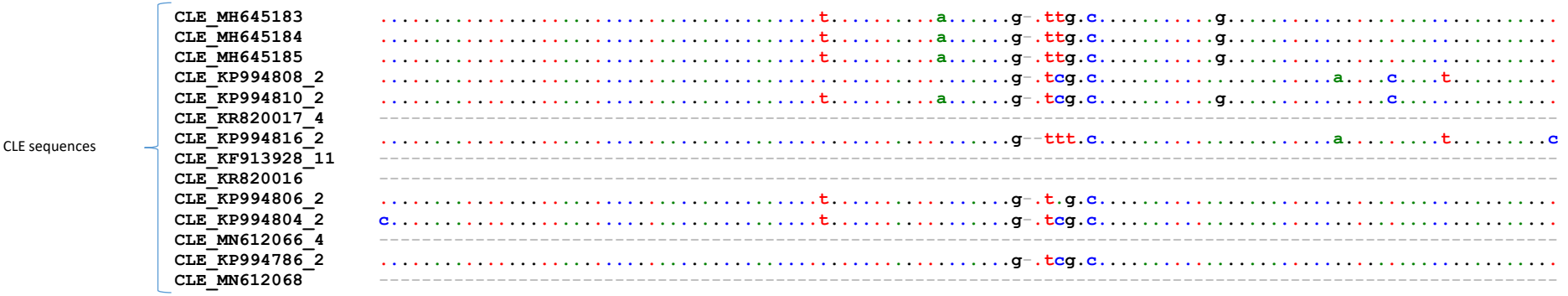
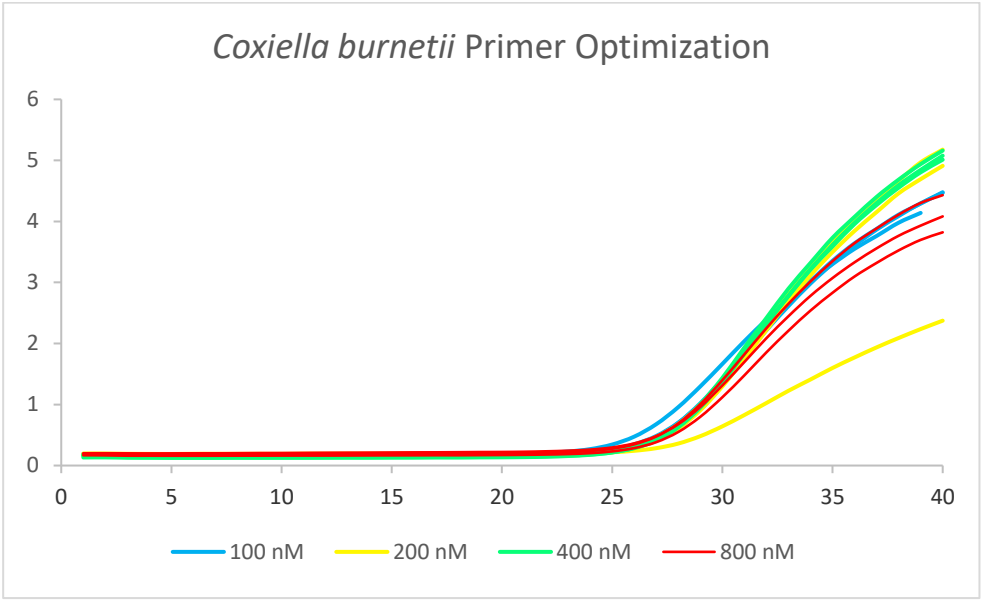


Figure 4.1. Variation of the 145 aligned C. burnetii and CLE sequences, the primers (indicated by blue arrows) and probe region for C. burnetii *(indicated by pink rectangle) and probe region for CLE (indicated by green circle) of a TaqMan® MGB assay to detect* C. burnetii *and CLEs. Sequences are identified by the accession number followed by the number of sequences collapsed due to being identical to the first sequence. The dots represent identical nucleotide to the reference sequence and the dashed represent gaps.*

4.1.1. Primer and probe optimization

With the *C. burnetii* probe all four primer concentrations showed little difference in Ct-values (Ct values ranged from 26.91 for the 100 nM, 27.76 for the 200 nM, 27.10 for the 400 nM and 27.25 for the 800 nM), but the concentration with the lowest Ct value was determined to be 100 nM. With the CLE probe, the optimum primer concentration was determined to be 400 nM. Because one set of primers was designed for both *C. burnetii* and CLEs, the primer concentrations needed to be the same for both *C. burnetii* and CLE. Thus because the *C. burnetii* concentration values showed little difference, the optimum primer concentration decided on was 400 nM (Figure 4.2).

a)



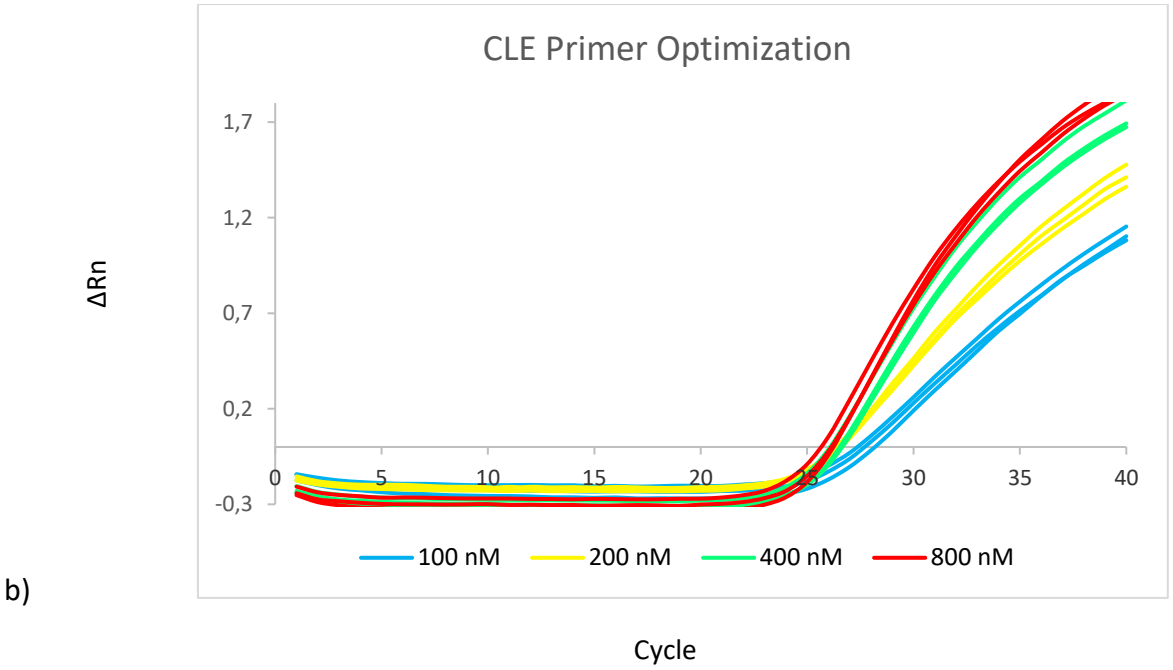
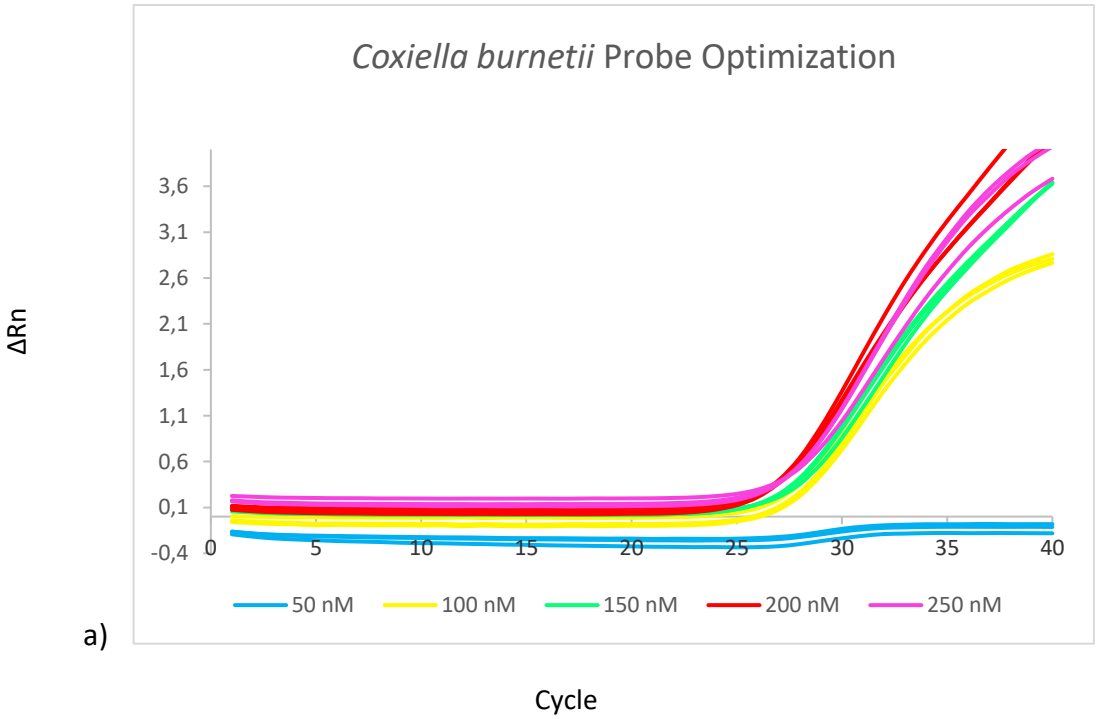


Figure 4.2. Primer concentration optimization curve of a TaqMan ® MGB assay for a) Coxiella burnetii and b) CLEs.

Five probe concentrations were tested for each of the *C. burnetii* and CLE probes against the 400 nM primer concentration. The optimum concentration for the *C. burnetii* probe was determined to be 100 nM and for the CLE probe was determined to be 150 nM as these concentrations yielded low Ct values and a steep efficient amplification curve reading (Figure 4.3).



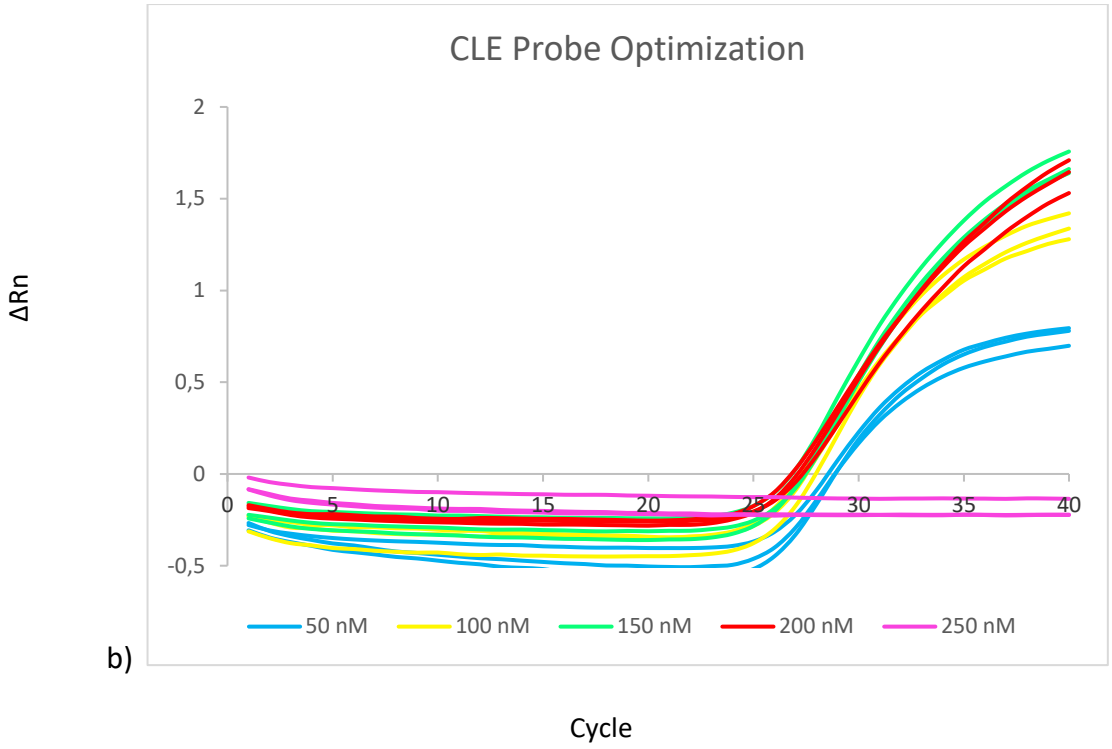


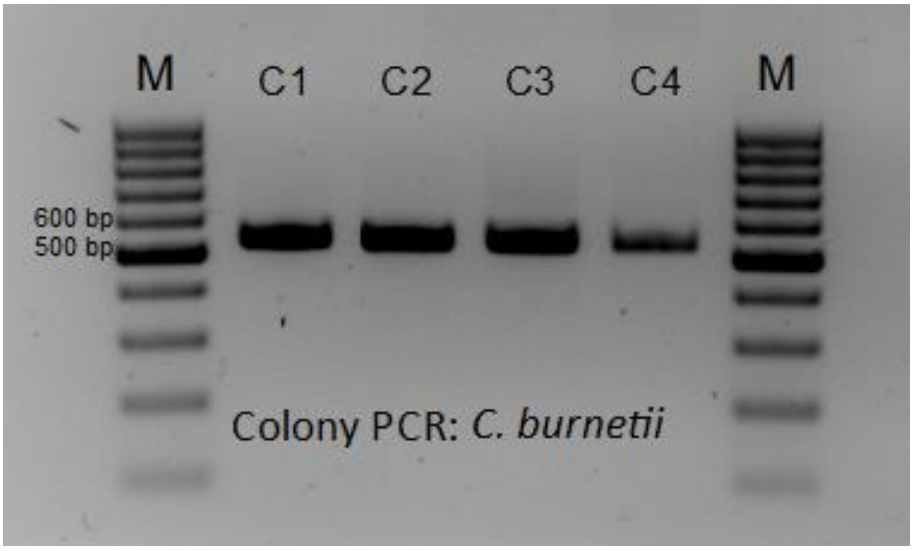
Figure 4.3. Probe concentration optimization curve of a TaqMan ® MGB assay for a) Coxiella burnetii and b) CLEs.

4.1.2. *Coxiella burnetii* and CLE plasmid positive control

A 529 bp region for both *C. burnetii* and CLE (inclusive of the TaqMan® MGB real-time PCR assay region of the 16S rRNA gene) was successfully amplified by the primers designed for the cloning of the plasmid positive controls (Table 3.4). The amplified product was cloned into a pJET 1.2 cloning vector (ThermoFisher Scientific, USA) and four to five colonies were randomly picked, analysed and visualized on a 2% agarose gel to check for the presence of the inserts. Both *C. burnetii* and CLE colony inserts were visualised at around 500 - 600 bp (Figure 4.4). The sequence results obtained from the Sanger sequencing performed by Inqaba Biotec™ (Pretoria, RSA) were analysed using the Staden software and confirmed to be 100% identical with both *C. burnetii* and CLE sequences respectively.

The DNA concentration of the *C. burnetii* plasmid DNA was 5.85 ng/µl and for the CLE plasmid DNA was 23.9 ng/µl. According to Afseth & Mallavia, (1997) the *C. burnetii* gene only contains one copy of the rRNA operon and that one plasmid is equivalent to one bacterium. Thus, the copy number was calculated to be 1.93 x 10^9 bacteria copies/µl for *C. burnetii* and 6.89 x 10^9 bacteria copies/µl for the CLE respectively.

(a)



(b)

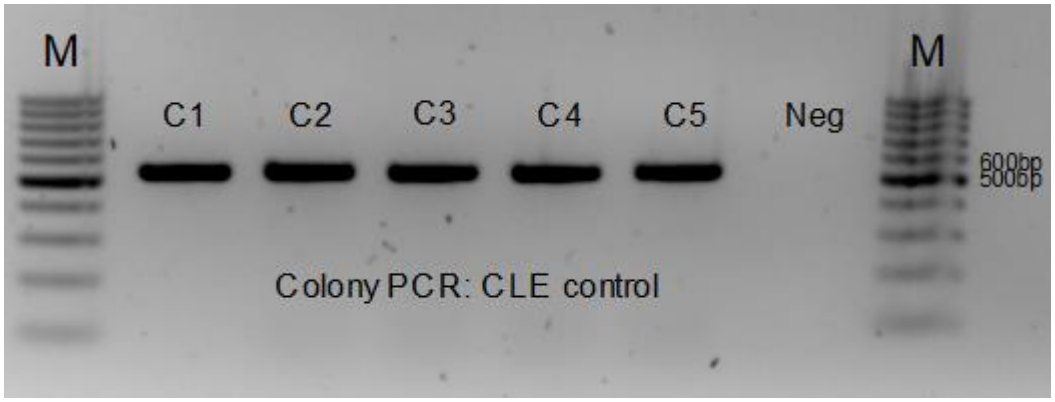
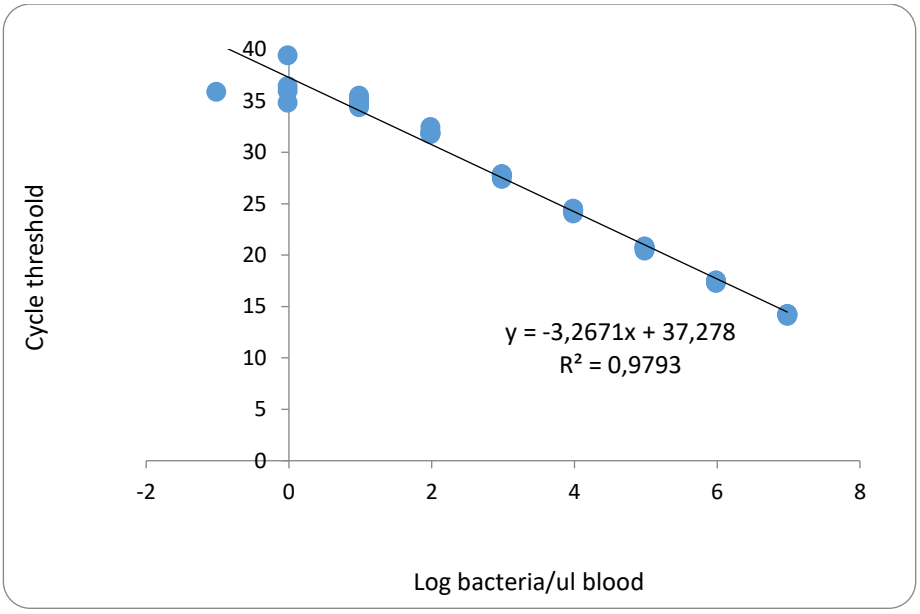


Figure 4.4. (a) Colony PCR on electrophoresis for C. burnetii and (b) CLE. C1 = colony 1, C2 = colony 2, C3 = colony 3, C4 = colony 4, C5 = colony

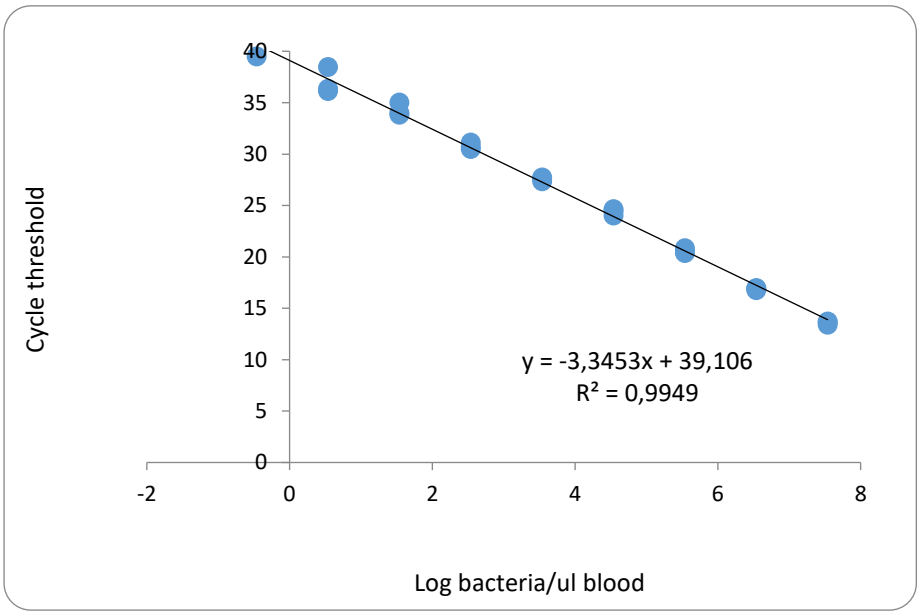
4.2. Validation

4.2.1. Analytical efficiency

The efficiency of each assay was determined from the regression equations. The assay was linear between $10^{-0.02}$ to $10^{6.98}$ bacteria copies/µl blood for *C. burnetii* and $10^{0.54}$ to $10^{7.54}$ bacteria copies/µl for CLE. The correlation coefficient (R^2) is a measure of how well the data fit the model and demonstrates the linearity of the standard curve. Linear regression analysis indicated the R^2 to be 0.9793 for *C. burnetii* and 0.9949 for CLE, and based on the slope the efficiency of the assay was determined to be 100.00% for *C. burnetii* and 99.03% for CLE (Figure 4.5).



(a)



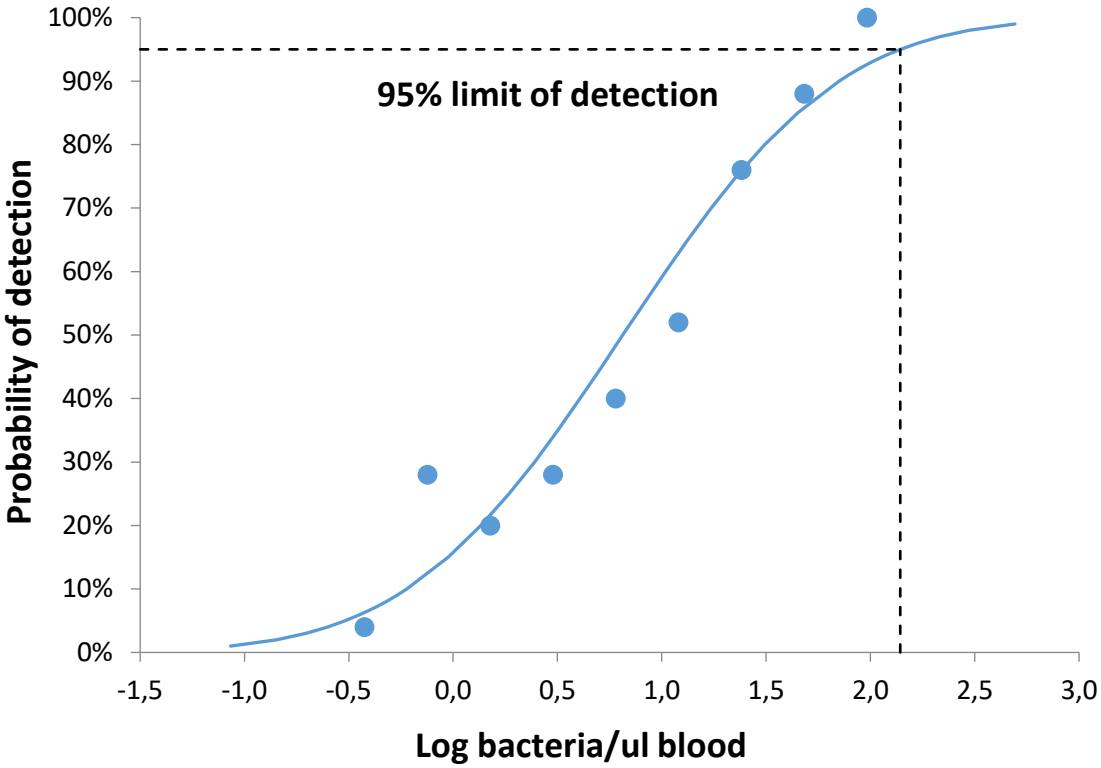
(b)

Figure 4.5. Standard curves displaying linearity for (a) C. burnetii, and (b) CLE.

4.2.2. Analytical sensitivity

Two-fold dilution ranges of human blood spiked with plasmid control DNA from $10^{-0.02}$ to $10^{6.98}$ bacteria copies/µl blood for *C. burnetii* and $10^{0.54}$ to $10^{7.54}$ bacteria copies/µl for CLE were prepared. The 95% LOD was calculated at 139 *C. burnetii* bacteria/µl with a 95% confidence interval of 51 - 1063. For the CLE, the 95% LOD was calculated at 260 bacteria/µl with a 95% confidence interval of 203-402 (Figure 4.6). In order to categorize positive and negative samples, a cut-off Ct value of 32 was selected.

Samples was considered negative / below the detection limit of the assay if a value above this was obtained and considered positive if a value below this was obtained. This value equated to 41.25 bacteria/µl *C. burnetii* or a 80 - 85% LOD and 133.09 bacteria/µl CLE or a 55 - 60% LOD.



(a)

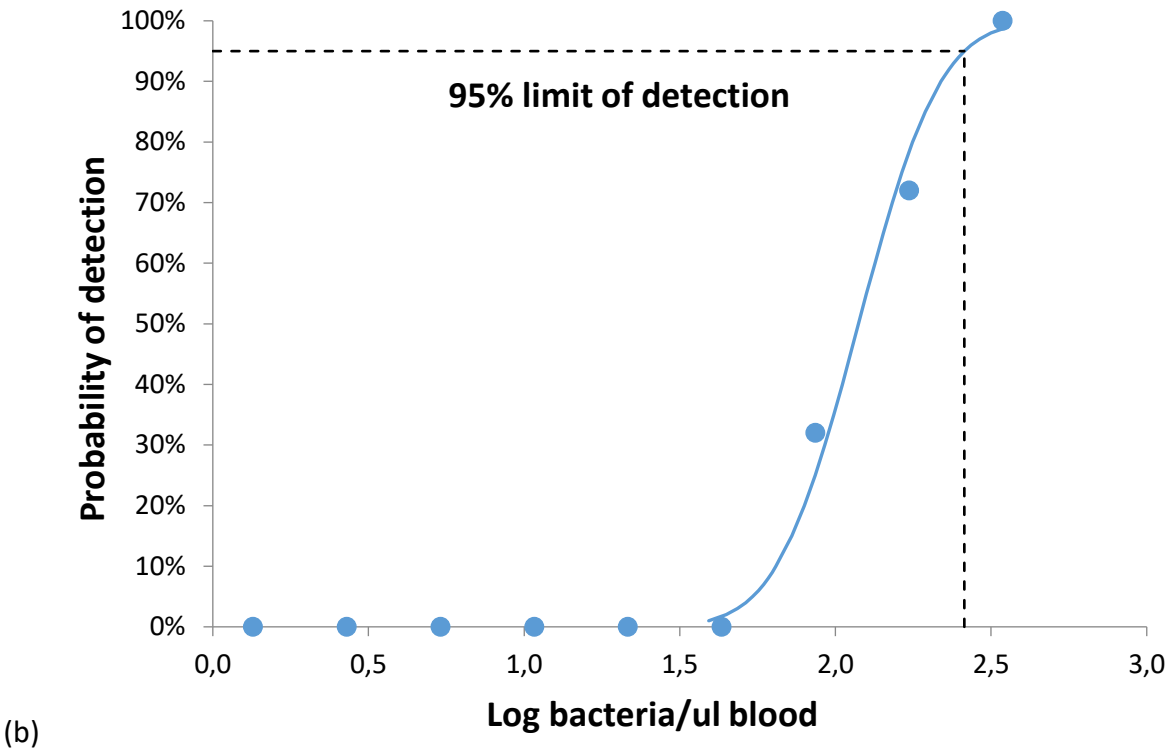


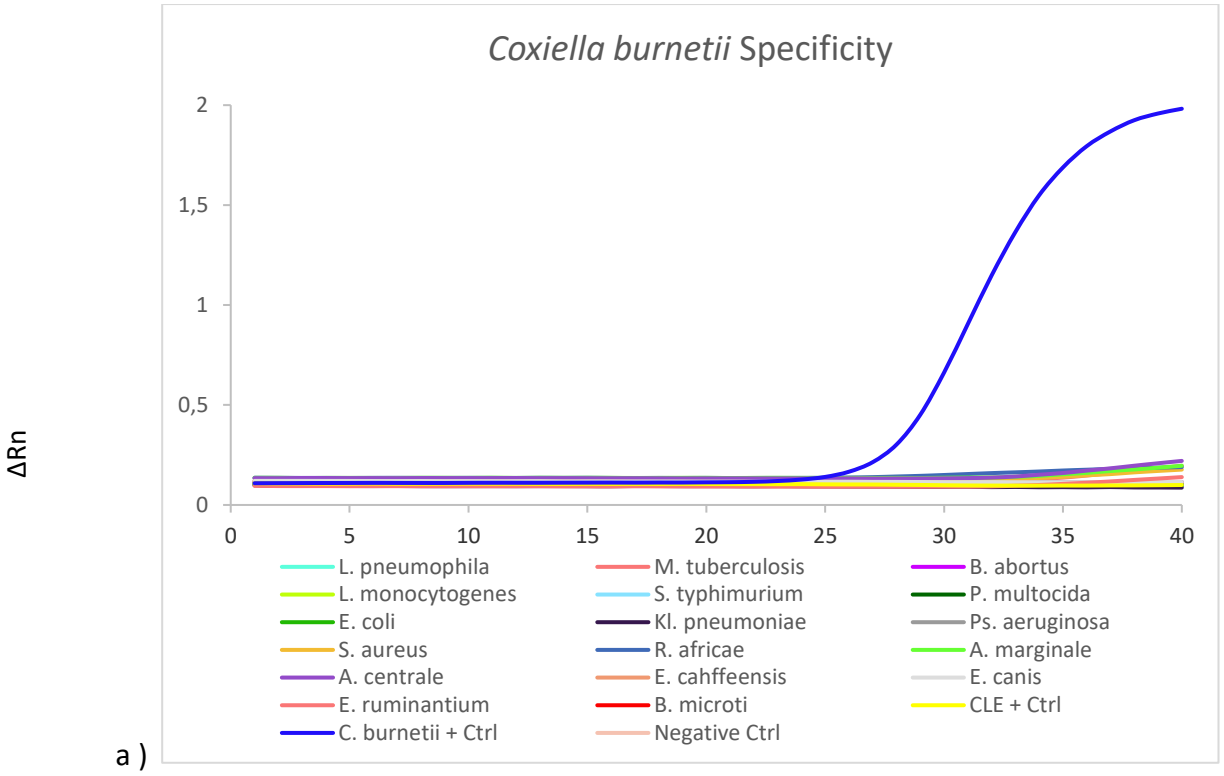
Figure 4.6. The 95% limit of detection (dotted line) of a TaqMan® MGB assay to distinguish between Coxiella burnetii and CLE. (a) Represent C. burnetii 95% LOD and (b) represent CLE 95% LOD.

Table 4.2. The 95% limit of detection of a 16S rDNA PCR determined by probit analysis. CI = Confidence Interval.

Organism	Bacteria/ul of blood	Log bacteria /µl	Lower 95% CI	Upper 95% CI
C. burnetii	41.25	1.615	51	1063
CLE	133.09	2.124	203	402

4.2.3. Analytical specificity

The analytical specificity was evaluated using DNA extracted from bacterial species expected to be transmitted by ticks. The developed qPCR amplified the DNA from *C. burnetii* and CLE. However, cross reactivity was noted with the CLE probe against six other organisms, namely *Salmonella typhimurium, Klebsiella pneumoniae, Rickettsia africae, Ehrlichia canis, Ehrlichia chaffeensis* and *Babesia microti*. (Figure 4.7). This shows that the experiment was not successful. The alignment represents few single nucleotide differences, perhaps HRM assay or other assays can differentiate this. However, 16S rRNA doesn't seem to be an ideal marker for this study.



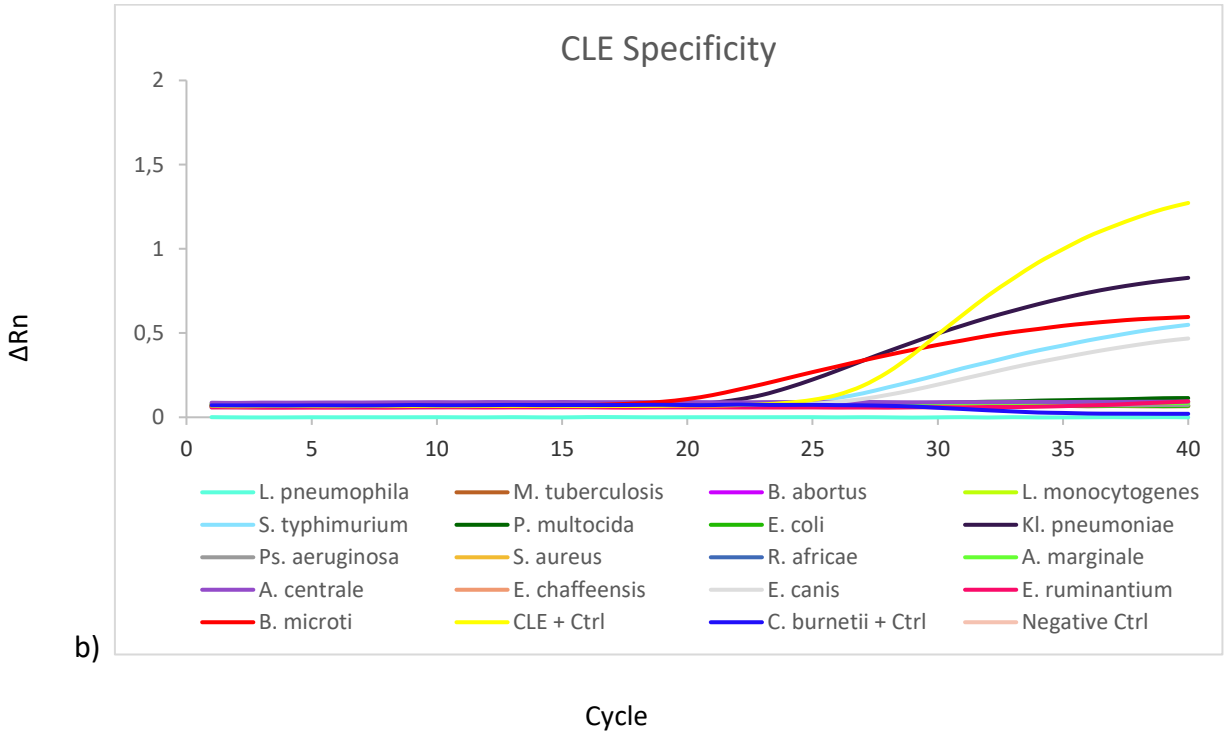


Figure 4.7. Specificity testing of a TaqMan® MGB assay to distinguish between Coxiella burnetii and CLEs, shows a) no amplifications with Coxiella burnetii probe, but b) amplification with six of the organisms tested with the CLE probe amplification with six of the organisms tested.

An alignment with all the organisms was done to see if there could be possible cross reaction with the CLE probe, but the alignment suggested that there should be no cross reactivity with the other 16S rRNA genes of the various bacteria species included (Figure 4.8). For the *Babesia* the 18S rRNA gene is usually used, thus the *Babesia* couldn't be included in the alignment. However, a BLASTn search done against all *Babesia's* in the database found that our designed probe area was identical to several *Babesia* spp., explaining why the *Babesia microti* sample tested positive during the specificity testing of the designed PCR assay.

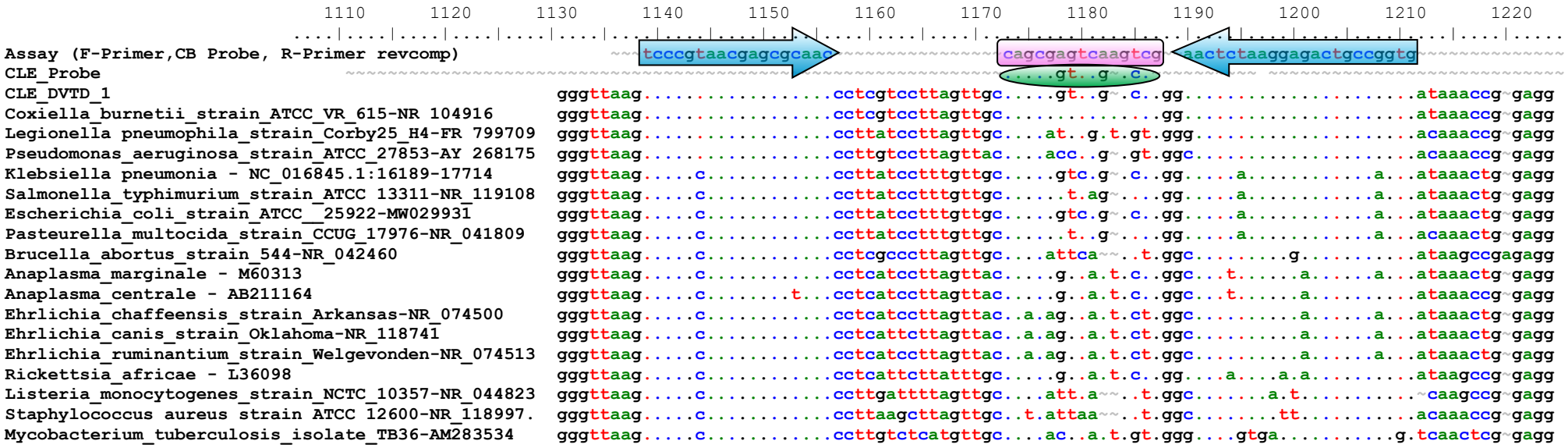


Figure 4.8. Multiple sequence alignment used to demonstrate specificity of the assay, showcasing theoretically no cross reactivity with the primers or probes.

In order to try and increase the specificity of the assay, the annealing temperature was increased by performing a gradient increase annealing temperature PCR. The annealing temperature was increased with 1°C from 60°C till 70°C (Table 4.3).

Table 4.3. Ct values for the gradient increase PCR. Undeter = undetermined.

	Ct values for										
	60°C	**61°C**	**62°C**	**63°C**	**64°C**	**65°C**	**66°C**	**67°C**	**68°C**	**69°C**	**70°C**
S. typhimurium	25.96	25.51	26.27	27.36	29.91	32.37	33.64	Undeter	Undeter	Undeter	Undeter
K. pneumonia	23.32	22.84	21.53	22.99	23.66	25.95	25.60	28.45	29.39	32.46	38.21
R. africae	24.05	24.67	22.49	22.59	22.47	22.46	Undeter	Undeter	Undeter	Undeter	Undeter
E. canis	28.45	29.02	29.13	30.98	31.83	35.39	Undeter	Undeter	Undeter	Undeter	Undeter
E. chaffeensis	27.50	27.25	28.99	34.15	36.84	Undeter	Undeter	Undeter	Undeter	Undeter	Undeter
B. microti	20.74	21.34	21.00	21.87	24.30	26.74	Undeter	Undeter	Undeter	Undeter	Undeter
CLE + Ctrl	26.09	26.09	26.32	25.99	25.83	25.57	25.98	25.98	26.67	27.55	30.73
CB + Ctrl	27.21	27.65	27.48	27.32	27.46	26.58	26.2	26.68	27.05	28.46	34.00

A real-time PCR assay was also run at 60°C with only the primers included. No probes were included as the probes contain a dye that may influence sequencing of the PCR products. These PCR products of the six organisms that showed amplification in the specificity assay were sent to Inqaba Biotec™ (Pretoria, RSA) for Sanger sequencing in order to verify whether the DNA of the organisms are pure or was accidentally contaminated with CLE plasmid during DNA loading of the PCR. The sequencing results of these PCR products produced very short sequences, too short to enable a complete assembly. Thus, contamination cannot be ruled out.

4.2.4. Repeatability

The inter-run standard deviation (SD) ranged between 1.50 - 2.00 and the intra-run SD 0.76 - 1.98 for *C. burnetii* and for the CLE the inter-run SD ranged between 0.16 - 0.34 and the intra-run SD 0.81 - 0.96. The variation between replicates and different runs is represented by the coefficient of variation (CV). This ranged from 4.56 - 7.40% for *C. burnetii* and 2.17 - 2.54% for the CLEs (Table 4.4). This indicated that there was minimal variation between replicates.

Table 4.4a. Inter- and Intra-assay reproducibility for the detection of Coxiella burnetii. SD- standard deviation and CV=Coefficient of variation.

Bacteria/µl	Inter-run SD	Intra-run SD	Total Ct Mean	Total SD	CV %
96.46	1.50	0.76	34.30	1.57	4.56
48.25	2.05	1.28	35.87	2.27	6.33
24.13	2.00	1.98	35.78	2.65	7.40
12.06	2.23	-	35.75	2.76	7.73
6.03	-	-	36.17	2.93	8.09

Table 4.4b. Inter- and Intra-assay reproducibility for the detection of Coxiella*-like Endosymbionts (CLEs). SD- standard deviation and CV=Coefficient of variation.*

Bacteria/µl	Inter-run SD	Intra-run SD	Total Ct Mean	Total SD	CV %
344.31	0.16	0.96	35.68	0.91	2.54
172.18	0.34	0.81	38.29	0.83	2.17
86.09	0.61	-	38.89	1.05	2.71

4.3. Screening of field-derived samples by real-time PCR

From the 130 tick samples tested, zero tested positive for *C. burnetii* while 121 samples tested positive for CLE. Of the human samples which showed positive IgM and IgG results, none tested positive with the designed PCR test (Table 4.6). Sixty (60) of the tick samples and thirty (30) of the human samples were also tested with the VetMAX™ *C. burnetii* Absolute Quant Kit (Applied Biosystems – ThermoFisher Scientific), following the manufacturer's instructions for detection and comparison with the designed assay. A positive control (included in the kit) as well as a negative control (consisting of PCR mix and water) gave the expected results. No positive *C. burnetii* results were found with this kit.

4.4. Real-time PCR result verification

Twenty samples that tested positive on the real-time PCR were sent for sanger sequencing in order to verify the results obtained by our developed PCR. Of the twenty samples, fourteen (14) were reported to be *Coxiella*-like Endosymbionts. The other six (6) samples failed to amplify and were not processed till the end (Table 4.5).

Table 4.5. Sanger sequencing results.

Sample ID	Gene length	Overlapping gene length	% Positive Identity
1018M	393	387	98.47
1014S	413	409	99.03
1011M	415	386	93.01
1008M	412	382	92.72
1001M	389	384	98.71
905S	414	413	99.52
503S	405	394	97.04
308S	389	386	99.23
301S	393	390	99.24
201S	389	389	99.49
106S	438	437	99.77
A6	389	381	97.94
A9	393	388	98.73
H3	380	375	98.68
H5	Amplification failed		
403S	Amplification failed		
216M	Amplification failed		
204S	Amplification failed		
117S	Amplification failed		
105S	Amplification failed		

Table 4.6. Human samples tested by NICD for Coxiella burnetii and tested with the designed real-time PCR for Coxiella burnetii as well as CLEs.

Sample ID	Real-time PCR Result	NICD Serology Results	
		IgM	IgG
Z00256	Negative	Positive	Positive
Z00257	Negative	Negative	Negative
Z00258	Negative	Negative	Negative
Z00259	Negative	Negative	Negative
Z00260	Negative	Negative	Negative
Z00261	Negative	Positive	Positive
Z00262	Negative	Negative	Negative
Z00263	Negative	Negative	Negative
Z00264	Negative	Negative	Positive
Z00265	Negative	Negative	Negative
Z00266	Negative	Negative	Negative
Z00267	Negative	Negative	Positive
Z00268	Negative	Negative	Positive
Z00269	Negative	Negative	Positive
Z00270	Negative	Negative	Positive
Z00271	Negative	Negative	Negative
Z00272	Negative	Negative	Negative
Z00273	Negative	Negative	Negative
Z00274	Negative	Negative	Negative
Z00275	Negative	Negative	Negative
Z00276	Negative	Negative	Equivocal
Z00278	Negative	Negative	Negative
Z00279	Negative	Negative	Negative
Z00280	Negative	Negative	Negative
Z00281	Negative	Negative	Positive
Z00282	Negative	Negative	Negative
Z00283	Negative	Negative	Negative
Z00284	Negative	Negative	Negative
Z00285	Negative	Negative	Negative
Z00286	Negative	Negative	Negative
Z00287	Negative	Negative	Negative
Z00288	Negative	Negative	Negative
Z00289	Negative	Negative	Negative
Z00290	Negative	Negative	Negative
Z00291	Negative	Negative	Negative
Z00292	Negative	Negative	Negative
Z00293	Negative	Negative	Positive
Z00294	Negative	Negative	Negative
Z00295	Negative	Negative	Negative

Sample ID	Real-time PCR Result	NICD Serology Results	
		IgM	IgG
Z00296	Negative	Negative	Positive
Z00297	Negative	Negative	Negative
Z00298	Negative	Negative	Negative
Z00299	Negative	Negative	Equivocal
Z00300	Negative	Negative	Positive
Z00301	Negative	Negative	Negative
Z00302	Negative	Negative	Positive
Z00303	Negative	Negative	Negative
Z00304	Negative	Negative	Positive
Z00305	Negative	Negative	Negative
Z00306	Negative	Negative	Positive
Z00307	Negative	Negative	Negative
Z00308	Negative	Negative	Negative
Z00309	Negative	Negative	Negative
Z00310	Negative	Negative	Negative
Z00311	Negative	Negative	Negative
Z00312	Negative	Negative	Negative
Z00313	Negative	Negative	Negative
Z00314	Negative	Negative	Equivocal
Z00315	Negative	Negative	Negative
Z00316	Negative	Negative	Negative
Z00317	Negative	Negative	Negative
Z00318	Negative	Negative	Negative
Z00319	Negative	Negative	Negative
Z00320	Negative	Negative	Negative
Z00321	Negative	Negative	Negative
Z00322	Negative	Negative	Negative
Z00323	Negative	Negative	Negative
Z00324	Negative	Negative	Negative
Z00325	Negative	Negative	Negative
Z00326	Negative	Negative	Negative
Z00327	Negative	Negative	Negative
Z00328	Negative	Negative	Negative
Z00329	Negative	Negative	Negative
Z00330	Negative	Equivocal	Negative
Z00331	Negative	Negative	Negative
Z00332	Negative	Negative	Negative
Z00333	Negative	Negative	Negative
Z00334	Negative	Negative	Negative

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Chapter 5

Discussion

Coxiella burnetii is responsible for causing Q fever and it is classified as a bioterrorist disease by the Centre of Disease Control (CDC). *Coxiella burnetii* has a very low infectivity dose and has the ability to withstand very harsh environmental conditions (Madariaga et al., 2003). The *C. burnetii* bacterium is difficult to culture and serological tests requires antibodies which takes time to develop and persist even after the infection has been eliminated. It is considered to be the only pathogen in the *Coxiella* group, but more recent studies also suggest that *Coxiella*-like Endosymbionts (CLEs) can be pathogenic to some extent as reported by Angelakis et al (2016). These findings raise the question then: "Can CLEs possibly contribute to Q fever infection as well, especially in South Africa?".

Because it is highly conserved, the 16S rRNA gene is often used for the classification of bacteria and for the development of group- or species-specific assays (Clarridge, 2004), hence, it was selected for use in this study. This study, developed a rapid TaqMan® MGB real-time PCR assay for distinguishing between *C. burnetii* and CLEs. This was achieved through the successful designing and optimization of a TaqMan® MGB real-time PCR assay that targeted the 16S rRNA gene with genus-specific primers and probes specific to each of *C. burnetii* and CLEs.

A forward and reverse primer combination of 400 nM together with a probe concentration of 100 nM for *C. burnetii* and 150 nM for CLE yielded the lowest Ct values with efficient amplification. This is slightly higher to the primer concentration of 300 nM and equal to the *C. burnetii* probe concentration of 100 nM used by Klee et al. (2006) where they validated a TaqMan-based assay targeting the *icd* and *IS1111* genes. Jones et al. (2010) also targeted the *IS1111* gene of *C. burnetii* and made use of Taqman assay with a primer concentration of 150 nM and a probe concentration of 100 nM. The primer and the probe placements was designed to include the nucleotide variation of the *C. burnetii* and CLE sequences.

Validation of this assay included the determination of the efficiency, sensitivity as well as the specificity in separate runs, human blood (Appendix 1) spiked with different concentrations of plasmid DNA containing the *C. burnetii* target gene and CLE target gene was used. The efficiencies of the qPCR assay were determined from linear regression equations (the slope of the semi-log regression line plot of Ct-values plotted against $\log_{10}$ of the input nucleic acid). For *C. burnetii* the efficiency was determined to be 100% and 99.03% for the CLEs. Klee et al. (2006) reported an efficiency of 90% in their development of a real-time PCR for both the *icd* and *IS1111* genes, and Howe et al. (2009) reported an efficiency of 95% for their TaqMan assay, where they also targeted the *IS1111* gene. Both of these assays have a lower efficiency than our study's efficiency.

Determination of the sensitivity of the assay yielded 41 bacteria/µl for *C. burnetii* and 133 bacteria/µl for CLE. Klee et al. (2006) reported the genome equivalent of their PCR to be $1{,}3x10^7$ GE/µl with a LOD of 100-0.75 GE/reaction for the *icd* gene and 25-0.2 GE/reaction for the *IS1111* gene, while Howe et al. (2009) reported 4.9 genome equivalents of *C. burnetii* genomic DNA for their *IS1111* gene. From the sensitivity curve, the LOD of the assay, which is defined as the concentration at which 95% of positive samples are detected (Bustin et al., 2009) or the lowest concentration of nucleic acid, giving positive results in all replicates tested (Kralik & Ricchi, 2017) was determined to be 139.00 bacteria/µl for *C. burnetii* and 260.00 bacteria/µl for CLEs. A cut-off Ct-value of 32.00 was selected to distinguish positive samples from negative samples.

Specificity of the assay was determined by testing a panel of closely related bacteria against *C. burnetii* and *Coxiella*-like endosymbionts as well as testing against other tick transmitted organisms. The CLE probe reacted with six of the organisms tested, namely *Salmonella typhimurium, Klebsiella pneumonia, Rickettsia africae, Ehrlichia canis, Ehrlichia chaffeensis* and *Babesia microti*. Because CLEs is present in almost all ticks, one can assume that during DNA extraction, the CLE DNA was also extracted resulting in the CLE probe reacting with those organisms. However, it is not the case with the *Salmonella* and *Klebsiella*, since the ATCC strains of these two organisms were used and a single bacterial colony was used for DNA extraction. With increasing the annealing temperature of the assay, the Ct-values of these organisms did decrease making the test more specific, except for the *Klebsiella pneumonia* organism. Sequencing of the real-time PCR product produced very short sequences, so short that it was not possible to do a complete assembly of these sequences. Thus, we were unable to determine whether the DNA was pure when tested or whether it was contaminated with CLE. A BLASTn search revealed that the designed probe however was identical to other *Babesia* species such as *Babesia ovata, Babesia microti* and *Babesia bigemina*, explaining why it reacted to the *Babesia microti* DNA.

Klee et al. (2006) reported negative PCR results against a panel of closely related bacteria they tested confirming specificity of their PCR for both the *icd* and *IS1111* genes. The panel included *Legionella pneumophila, Francisella tularensis* ssp. *Novicida* and ssp. *Tularensis, Bacillus subtilis, Bacillus anthracis, Bacillus cereus, Bacillus thuringiensis, Bacillus megaterium, Bacillus licheniformis, Staphylococcus aureus, Streptococcus equi, Pseudomonas putida, Pseudomonas auruginosa, Pseudomonas flurescens, Burkholderia mallei, Burkholderia pseudomallei, Burholderia stabilis, Burkholderia multivorans, Yersinia enterocolitica, Yersinia pseudotuberculosis, Yersinia pestis, Brucella melitensis* biotype 1*, Brucella abortus* biotype 1, *Brucella suis* biotype 1, *Brucella ovis* biotype 1, *Klebsiella oxytoca, Serratia marcescens, Proteus mirabilis* and *Escherichia coli.*

Jones et al. (2010) also reported that they found no cross-reactions for their *IS1111* assay, resulting in a 100% specificity. Jaton et al. (2013) also reported a 100% analytical specificity against all the pathogens they tested,

how-ever during a blind testing, an overall specificity of 98% were observed (100% specificity on their EDTA blood samples tested, 95.5% on their serum samples tested and 83.3% on their valvular biopsies tested).

Repeatability represents estimates of precision within an assay (within the same run and between runs). It implies that a minor amount of distribution indicates an accurate assay (Jacobson, 1998). This is achieved by using the same samples run in different plates within and between runs of the assay (intra-plate and inter-plate variation). The CV ranged from 4.56 - 7.40% for *C. burnetii* and 2.17 - 2.54% for the CLEs (Table 4.3). This indicated that there was minimal variation between replicates.

The type of extraction method used influences the performance of an assay. Various commercially available kits have demonstrated to be successful in extracting DNA over different sample models, however it has been suggested that a proteinase K/phenol/chloroform method of extraction may be more sensitive compared to other extraction methods (Cankar et al., 2006). Under various developmental conditions, different results may be obtained, which makes the selection of a relevant extraction method for a particular sample model crucial, in order to obtain good quality PCR results (Cankar et al., 2006). The efficiency of an assay is also determined by the quality reagents used in the PCR amplification and the thermal cycler. (Raggi et al., 2005; Buzard et al., 2012).

A total of 130 tick samples and 78 human samples were tested with the newly designed assay. Out of these samples, 60 tick and 30 human samples were compared with the VetMAX™ *C. burnetii* Absolute Quant Kit (Applied Biosystems – ThermoFisher Scientific). No positive *C. burnetii* samples were detected with the designed real-time PCR assay or with the VetMAX™ *C. burnetii* Absolute Quant Kit (Applied Biosystems – ThermoFisher Scientific). However, 121 tick samples tested positive for CLE DNA with the newly designed real-time PCR assay that distinguish between *C. burnetii* and CLEs. Of the samples that tested positive for CLE, twenty were randomly picked and sent to Inqaba Biotechnical Industries (Pty) Ltd for sanger sequencing in order to verify the results obtained with the designed PCR. Of the twenty samples, fourteen were reported as *Coxiella*-like Endosymbionts (CLEs), while the other six did not show any amplification, thus no results were obtained for those three. This indicates that the newly developed PCR assay is fairly accurate.

The human samples were also compared to the serology results obtained by NICD where some samples tested positive for immunoglobulin M and G (IgM and IgG) antibodies for *C. burnetii*. In response to an infection, either by bacteria, viruses or any other microorganisms, plasma cells produce proteins called immunoglobulins. (Stevens & Miller, 2017). As the first response towards a new infection, IgM antibodies are produced, providing short-term protection. They increase for a few weeks and then decrease with the beginning of IgG production (Stevens & Miller, 2017). 70-80% of the immunoglobulins in blood is represented by IgG antibodies. During the start of an infection, certain IgG antibodies are produced, rising a few weeks after it begins, then decreasing and

stabilizing. Whenever a recurring infection occur, the body preserve a series of IgG antibodies that can be swiftly reproduced. IgG antibodies form the basis of continuing protection against microorganisms (Stevens & Miller, 2017). No positive PCR results were obtained upon testing of the human samples with our test. Previous contact with a pathogen is not ruled out when a negative result is obtained, nor the possibility that the pathogen is in the incubation period. And positive antibody result only indicates previous contact with the bacterial organism, but does not rule out nor confirms an active infection or the presence of the bacterial organism.

The fact that the NICD found positive serology results against *C. burnetii* that we didn't pick up on with our newly developed PCR might be due to some cross-reactivity taking place between *C. burnetii* and CLEs, and again raising the question whether CLEs play a possible role in Q fever infections. This was also speculated on by Angelakis et al. (2006), where human patients in France developed eschars after tick bites. The organism identified by Angelakis et al. (2016) were identified through molecular techniques to be that of a *Coxiella*-like bacteria namely *Candidatus* Coxiella massiliensis.

Real-time PCR assays saves time and money because fluorogenic probes are used which detect specific amplification products and prevent non-specific amplification, thus eliminating visualization of the PCR product through electrophoresis analysis. A further advantage is that it reduces potential PCR contamination in the laboratory because amplified products are analysed and disposed of, without having to open the reaction tubes even once.

A drawback however of TaqMan® MGB real-time PCR assays are that the reagents are more costly than those of conventional PCR and that an expensive thermocycler is required for testing, which many laboratories can't afford. Multiplexing capabilities are also limited due to the number of dyes available. But despite these drawbacks, TaqMan® MGB real-time PCR assays provide dependable results within a short time.

The prevalence rate of Q fever infections in not only South Africa but the whole Africa continent ranges from low to relatively high. During 1993 a prevalence rate of 37% to *C. burnetii* antibodies in humans were reported in Zimbabwe, and with regards to animals a prevalence rate of 13% were reported in cats in Zimbabwe and 2% reported in South Africa. A rate of 15% in dogs, 10% in goats and 39% in cattle were reported in Zimbabwe, were as 8% in cattle were reported in South Africa (Frean & Blumberg, 2007). Simpson et al. (2008) also reported a relative high prevalence rate of Q fever infections (dip-tankers = 60,9% and febrile patients = 37,8%), especially in non-malarial acute febrile illness (AFI) cases presented at the Mnisi community in South Africa. Halajian et al. (2016) also reported *C. burnetii* antibody titers found in cattle in the provinces of Gauteng, North-West, Limpopo and Mpumalanga (the old Transvaal province) in South Africa as well as the existence of *C. burnetii* in ticks from domestic ruminants in Kwa Zulu Natal and the Free State provinces in South Africa.

Taking this into consideration, it is crucial to have a fast, extremely sensitive, and specific test that can distinguish between these two organisms. Secondly because *C. burnetii* is classified as a bioterrorist agent by the Centre of Disease Control (CDC) due to its very low infectivity dose and ability to withstand very harsh environmental conditions (Madariaga et al., 2003), such a test will be of great value for timely diagnosis and treatment.

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Conclusion

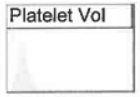
The real-time PCR assay developed in this study showcased high sensitivity toward both *Coxiella burnetii* and *Coxiella*-like Endosymbionts (CLEs). The developed assay produces rapid results; and showed that the PCR is sensitive and reproducible. However, the specificity of the assay is not what was hoped for, since the CLE probe cross reacted. It is thus advisable that another assay be developed with a newly designed probe and that more species should be included during the specificity testing of the assay that could result in higher specificity in order to characterize CLEs.

Appendix 1

Total Blood Count

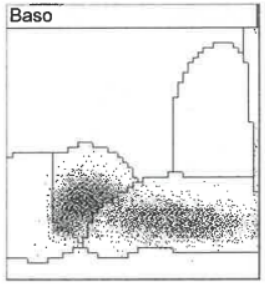
VET LAB	
Instrument Number	IR10671824
Aspiration Date/Time	08/20/2021 09:04:35 AM
Sample Selectivity	CBC\DIFF
Sample Type	PATIENT
SID	TROPICALDIS
Species	Human
Patient Name	
FOR LABORATORY USE ONLY	

Platelet Vol

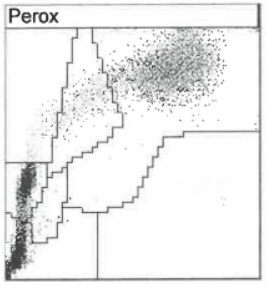


Baso X

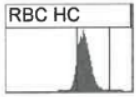
Baso



Perox



RBC HC



RBC Volume

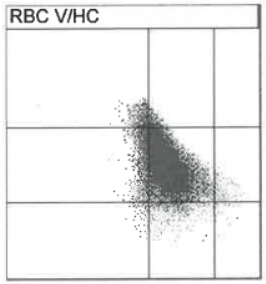
CBC			
WBC		9.14	x10³ cells/µL
RBC		5.41	x10⁶ cells/µL
HGB	H	159	g/L
Cellular HGB		155	g/L
HCT	L	0.50	L/L
MCV		92.0	fL
MCH		29.5	pg
MCHC	H	320	g/L
CHCM	H	312	g/L
CH		28.4	pg
RDW	H	16.4	%
HDW	H	24.9	g/L
PLT		281	x10³ cells/µL
MPV		8.9	fL
PDW		60.9	%
PCT		0.25	%

Routine Retic	%	#	
Retic			x10⁹ cells/L
CHr		n/a	pg
CHm		n/a	pg

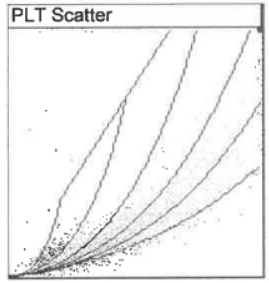
Routine WBC Differential	%	#	
WBC		9.14	x10³ cells/µL
Neut	52.2	4.77	x10³ cells/µL
Lymph	34.6	3.16	x10³ cells/µL
Mono	6.8	0.62	x10³ cells/µL
Eos	2.5	0.23	x10³ cells/µL
Baso	0.6	0.05	x10³ cells/µL
LUC	3.4	0.31	x10³ cells/µL
NRBC	0.0	0.00	x10⁹ cells/L
LI		2.24	
MPXI		-1.5	
WBCP		9.63	x10³ cells/µL

HGB Trans

RBC V/HC



PLT Scatter



RBC Rate

Perox Rate

Platelet		
PLT	281	x10³ cells/µL
MPV	8.9	fL
PDW	60.9	%
PCT	0.25	%
MPC	27.9	g/dL
PCDW	5.2	g/dL
MPM	2.30	pg
PMDW	1.04	pg
Large Plt	13	x10³ cells/µL
RBC Fragments	0.02	x10⁶ cells/µL
RBC Ghosts	0.01	x10⁶ cells/µL
PLT Clumps Count	31	

Retic Parameters	Gated	Mature	Retic	
MCV	n/a	n/a	n/a	fL
CHCM	n/a	n/a	n/a	g/L
CH	n/a	n/a	n/a	pg
CHDW	n/a	n/a	n/a	pg
RDW	n/a	n/a	n/a	%
HDW	n/a	n/a	n/a	g/L
%Micro	n/a	n/a	n/a	
%Macro	n/a	n/a	n/a	
%Hypo	n/a	n/a	n/a	
%Hyper	n/a	n/a	n/a	
%Low CH	n/a	n/a	n/a	
%High CH	n/a	n/a	n/a	
MCV Delta			n/a	
CHCM Delta			n/a	
CH Delta			n/a	
CHDW Delta			n/a	
RDW Delta			n/a	
HDW Delta			n/a	g/L

Baso Rate

Morphology Flags	
MACRO	+
HYPO	++
ANISO	+

Sample/System Flags

Retic Scatter Absorption

Appendix 2

***Rhipicephalus sanguineus* real-time PCR results with a cut-off Ct-value of 32.**

	Sample ID	Target Name	Cт	Cт Mean	Cт SD	Real-time PCR Result	*C. burnetii* Quant Kit
1	1018 S	CBP	Undetermined			CB Negative	Negative
1	1018 S	CLEP	25.58216476	25.99	0.574912667	**CLE Positive**	
2	1018 M	CBP	Undetermined			CB Negative	Negative
2	1018 M	CLEP	19.42271042	20.30	1.239295363	**CLE Positive**	
3	1017 S	CBP	Undetermined			CB Negative	Negative
3	1017 S	CLEP	23.97870255	23.99	0.013214555	**CLE Positive**	
4	1017 M	CBP	Undetermined			CB Negative	Negative
4	1017 M	CLEP	22.16919708	22.23	0.085974179	**CLE Positive**	
5	1016 S	CBP	Undetermined			CB Negative	Negative
5	1016 S	CLEP	22.49925041	22.46	0.052654564	**CLE Positive**	
6	1016 M	CBP	Undetermined			CB Negative	Negative
6	1016 M	CLEP	24.13118553	24.19	0.078805842	**CLE Positive**	
7	1015 S	CBP	Undetermined			CB Negative	Negative
7	1015 S	CLEP	24.57043266	24.61	0.056859806	**CLE Positive**	
8	1015 M	CBP	Undetermined			CB Negative	Negative
8	1015 M	CLEP	22.03205299	22.06	0.045428235	**CLE Positive**	
9	1014 S	CBP	Undetermined			CB Negative	Negative
9	1014 S	CLEP	20.59474945	20.59	0.008212229	**CLE Positive**	
10	1014 M	CBP	Undetermined			CB Negative	Negative
10	1014 M	CLEP	23.24921227	23.30	0.067194887	**CLE Positive**	
11	1012 S	CBP	Undetermined			CB Negative	Negative
11	1012 S	CLEP	27.08667564	27.09	0.001215178	**CLE Positive**	
12	1012 M	CBP	Undetermined			CB Negative	Negative
12	1012 M	CLEP	24.5822525	24.52	0.085506178	**CLE Positive**	
13	1011 S	CBP	Undetermined			CB Negative	Negative
13	1011 S	CLEP	22.9432354	22.80	0.20524773	**CLE Positive**	
14	1011 M	CBP	Undetermined			CB Negative	Negative
14	1011 M	CLEP	22.73297501	22.72	0.01259685	**CLE Positive**	
15	1009 S	CBP	Undetermined			CB Negative	Negative
15	1009 S	CLEP	23.47709274	23.46	0.02647901	**CLE Positive**	
16	1009 M	CBP	Undetermined			CB Negative	Negative
16	1009 M	CLEP	24.90244865	24.93	0.037294228	**CLE Positive**	
17	1008 S	CBP	Undetermined			CB Negative	Negative
17	1008 S	CLEP	25.12412071	25.16	0.047354177	**CLE Positive**	
18	1008 M	CBP	Undetermined			CB Negative	Negative
18	1008 M	CLEP	21.64760971	21.62	0.032803062	**CLE Positive**	
19	1007 S	CBP	Undetermined			CB Negative	Negative
19	1007 S	CLEP	21.5515976	21.58	0.035530131	**CLE Positive**	
20	1007 M	CBP	Undetermined			CB Negative	Negative
20	1007 M	CLEP	22.98818398	22.94	0.068411417	**CLE Positive**	

	Sample ID	Target Name	Cт	Cт Mean	Cт SD	Real-time PCR Result	*C. burnetii* Quant Kit
21	1003 S	CBP	Undetermined			CB Negative	Negative
21	1003 S	CLEP	26.12191391	26.11	0.01539675	**CLE Positive**	
22	1003 M	CBP	Undetermined			CB Negative	Negative
22	1003 M	CLEP	27.5861702	27.65	0.096352413	**CLE Positive**	
23	1001 S	CBP	Undetermined			CB Negative	Negative
23	1001 S	CLEP	26.64463615	26.60	0.068188883	**CLE Positive**	
24	1001 M	CBP	Undetermined			CB Negative	Negative
24	1001 M	CLEP	22.04404068	22.12	0.101992674	**CLE Positive**	
25	906 S	CBP	Undetermined			CB Negative	Negative
25	906 S	CLEP	26.93057251	26.97	0.052432027	**CLE Positive**	
26	906 M	CBP	Undetermined			CB Negative	Negative
26	906 M	CLEP	26.96995354	26.90	0.09775506	**CLE Positive**	
27	905 S	CBP	Undetermined			CB Negative	-
27	905 S	CLEP	21.29762459	21.28	0.01927291	**CLE Positive**	
28	905 M	CBP	Undetermined			CB Negative	-
28	905 M	CLEP	22.32877922	22.34	0.018744221	**CLE Positive**	
29	902 S	CBP	Undetermined			CB Negative	-
29	902 S	CLEP	26.16737938	26.41	0.345459849	**CLE Positive**	
30	902 M	CBP	Undetermined			CB Negative	-
30	902 M	CLEP	24.7645359	24.92	0.223503724	**CLE Positive**	
31	806 S	CBP	Undetermined			CB Negative	-
31	806 S	CLEP	20.66014099	26.59	8.384703636	**CLE Positive**	
32	806 M	CBP	Undetermined			CB Negative	-
32	806 M	CLEP	25.50641823	25.49	0.019102974	**CLE Positive**	
33	805 S	CBP	Undetermined			CB Negative	-
33	805 S	CLEP	21.97861671	22.58	0.857306004	**CLE Positive**	
34	805 M	CBP	Undetermined			CB Negative	-
34	805 M	CLEP	25.21686363	25.26	0.058274593	**CLE Positive**	
35	803 S	CBP	Undetermined			CB Negative	-
35	803 S	CLEP	23.38983917	23.35	0.058741242	**CLE Positive**	
36	803 M	CBP	Undetermined			CB Negative	-
36	803 M	CLEP	28.35805893	28.27	0.122256882	**CLE Positive**	
37	801 S	CBP	Undetermined			CB Negative	-
37	801 S	CLEP	24.27874947	24.29	0.009018751	**CLE Positive**	
38	801 M	CBP	Undetermined			CB Negative	-
38	801 M	CLEP	24.87914085	24.81	0.103561215	**CLE Positive**	
39	605 S	CBP	Undetermined			CB Negative	Negative
39	605 S	CLEP	27.99188805	27.99	0.006321353	**CLE Positive**	
40	605 M	CBP	Undetermined			CB Negative	Negative
40	605 M	CLEP	30.53278542	30.71	0.256058633	**CLE Positive**	
41	603 S	CBP	Undetermined			CB Negative	Negative
41	603 S	CLEP	25.2864418	25.33	0.057632614	**CLE Positive**	

	Sample ID	Target Name	Cт	Cт Mean	Cт SD	Real-time PCR Result	*C. burnetii* Quant Kit
42	603 M	CBP	Undetermined			CB Negative	Negative
42	603 M	CLEP	30.90935707	31.03	0.175059795	**CLE Positive**	
43	601 S	CBP	Undetermined			CB Negative	Negative
43	601 S	CLEP	26.94318008	26.95	0.014300257	**CLE Positive**	
44	601 M	CBP	Undetermined			CB Negative	Negative
44	601 M	CLEP	27.05044174	27.03	0.034936704	**CLE Positive**	
45	503 S	CBP	Undetermined			CB Negative	Negative
45	503 S	CLEP	24.79677773	24.85	0.073640324	**CLE Positive**	
46	503 M	CBP	Undetermined			CB Negative	Negative
46	503 M	CLEP	29.13100243	29.04	0.135475487	**CLE Positive**	
47	502 S	CBP	Undetermined			CB Negative	Negative
47	502 S	CLEP	27.90770721	28.03	0.169585437	**CLE Positive**	
48	502 M	CBP	Undetermined			CB Negative	Negative
48	502 M	CLEP	27.36351776	27.44	0.110884652	**CLE Positive**	
49	501 S	CBP	Undetermined			CB Negative	Negative
49	501 S	CLEP	26.96670723	27.09	0.176424682	**CLE Positive**	
50	501 M	CBP	Undetermined			CB Negative	Negative
50	501 M	CLEP	26.47962379	26.55	0.10044302	**CLE Positive**	
51	408 S	CBP	Undetermined			CB Negative	Negative
51	408 S	CLEP	27.39684296	27.60	0.289696544	**CLE Positive**	
52	408 M	CBP	Undetermined			CB Negative	Negative
52	408 M	CLEP	28.38254166	29.02	0.896348178	**CLE Positive**	
53	407 S	CBP	Undetermined			CB Negative	Negative
53	407 S	CLEP	31.5963707	31.75	0.218005076	**CLE Positive**	
54	407 M	CBP	Undetermined			CB Negative	Negative
54	407 M	CLEP	31.30037308	31.27	0.042179216	**CLE Positive**	
55	406 S	CBP	Undetermined			CB Negative	Negative
55	406 S	CLEP	26.67590714	26.80	0.171870127	**CLE Positive**	
56	406 M	CBP	Undetermined			CB Negative	Negative
56	406 M	CLEP	29.83804512	30.04	0.283986151	**CLE Positive**	
57	405 S	CBP	Undetermined			CB Negative	Negative
57	405 S	CLEP	23.53994751	23.85	0.433958799	**CLE Positive**	
58	405 M	CBP	Undetermined			CB Negative	Negative
58	405 M	CLEP	24.36094475	24.34	0.024659615	**CLE Positive**	
59	404 S	CBP	Undetermined			CB Negative	Negative
59	404 S	CLEP	28.39547348	28.58	0.264881819	**CLE Positive**	
60	404 M	CBP	Undetermined			CB Negative	Negative
60	404 M	CLEP	26.43386078	26.70	0.382959068	**CLE Positive**	
61	403 S	CBP	Undetermined			CB Negative	Negative
61	403 S	CLEP	23.92926216	24.19	0.37476033	**CLE Positive**	
62	403 M	CBP	Undetermined			CB Negative	Negative
62	403 M	CLEP	27.0246067	27.13	0.151141971	**CLE Positive**	

	Sample ID	Target Name	Cт	Cт Mean	Cт SD	Real-time PCR Result	*C. burnetii* Quant Kit
63	309 S	CBP	Undetermined			CB Negative	Negative
63	309 S	CLEP	26.01482201	26.03	0.017270092	**CLE Positive**	
64	309 M	CBP	Undetermined			CB Negative	Negative
64	309 M	CLEP	26.86133385	26.99	0.186252654	**CLE Positive**	
65	308 S	CBP	Undetermined			CB Negative	Negative
65	308 S	CLEP	21.62199783	21.61	0.009918334	**CLE Positive**	
66	308 M	CBP	Undetermined			CB Negative	Negative
66	308 M	CLEP	24.29865837	24.32	0.036058821	**CLE Positive**	
67	305 S	CBP	Undetermined			CB Negative	Negative
67	305 S	CLEP	31.66254997	31.68	0.025420282	**CLE Positive**	
68	305 M	CBP	Undetermined			CB Negative	Negative
68	305 M	CLEP	31.42549324	31.55	0.178088978	**CLE Positive**	
69	301 S	CBP	Undetermined			CB Negative	Negative
69	301 S	CLEP	21.99509621	22.14	0.208201379	**CLE Positive**	
70	301 M	CBP	Undetermined			CB Negative	Negative
70	301 M	CLEP	26.49225807	26.72	0.323291272	**CLE Positive**	
71	216 S	CBP	Undetermined			CB Negative	-
71	216 S	CLEP	28.66767883	28.76	0.137243629	**CLE Positive**	
72	216 M	CBP	Undetermined			CB Negative	-
72	216 M	CLEP	23.26203918	23.30	0.055640582	**CLE Positive**	
73	215 S	CBP	Undetermined			CB Negative	-
73	215 S	CLEP	Undetermined			CLE Negative	
74	215 M	CBP	Undetermined			CB Negative	-
74	215 M	CLEP	Undetermined			CLE Negative	
75	214 S	CBP	Undetermined			CB Negative	-
75	214 S	CLEP	27.60107422	28.19	0.82590425	**CLE Positive**	
76	214 M	CBP	Undetermined			CB Negative	-
76	214 M	CLEP	25.58882904	25.68	0.135247558	**CLE Positive**	
77	213 S	CBP	Undetermined			CB Negative	-
77	213 S	CLEP	26.04420662	26.14	0.132608145	**CLE Positive**	
78	213 M	CBP	Undetermined			CB Negative	-
78	213 M	CLEP	28.09529686	28.26	0.236386508	**CLE Positive**	
79	209 S	CBP	Undetermined			CB Negative	-
79	209 S	CLEP	27.60525894	27.62	0.025270576	**CLE Positive**	
80	209 M	CBP	Undetermined			CB Negative	-
80	209 M	CLEP	31.46601105	31.57	0.152839988	**CLE Positive**	
81	208 S	CBP	Undetermined			CB Negative	-
81	208 S	CLEP	27.1434288	27.12	0.038062986	**CLE Positive**	
82	208 M	CBP	Undetermined			CB Negative	-
82	208 M	CLEP	26.31924629	26.43	0.151836544	**CLE Positive**	
83	204 S	CBP	Undetermined			CB Negative	-
83	204 S	CLEP	21.28888893	22.35	1.496810555	**CLE Positive**	

	Sample ID	Target Name	Cт	Cт Mean	Cт SD	Real-time PCR Result	*C. burnetii* Quant Kit
84	204 M	CBP	Undetermined			CB Negative	-
84	204 M	CLEP	22.09217644	21.89	0.283604443	**CLE Positive**	
85	201 S	CBP	Undetermined			CB Negative	-
85	201 S	CLEP	22.64339828	22.71	0.096572258	**CLE Positive**	
86	201 M	CBP	Undetermined			CB Negative	-
86	201 M	CLEP	28.01416588	28.14	0.172340825	**CLE Positive**	
87	117 S	CBP	Undetermined			CB Negative	-
87	117 S	CLEP	22.21792412	22.75	0.758838832	**CLE Positive**	
88	117 M	CBP	Undetermined			CB Negative	-
88	117 M	CLEP	27.05163574	26.96	0.124970466	**CLE Positive**	
89	116 S	CBP	Undetermined			CB Negative	-
89	116 S	CLEP	28.75936508	29.95	1.690852046	**CLE Positive**	
90	116 M	CBP	Undetermined	38.36		CB Negative	-
90	116 M	CLEP	31.14515305	31.17	0.037984762	**CLE Positive**	
91	115.2 S	CBP	Undetermined			CB Negative	-
91	115.2 S	CLEP	35.93560028	35.68	0.365176469	CLE Negative	
92	115.2 M	CBP	Undetermined			CB Negative	-
92	115.2 M	CLEP	33.80093384	33.93	0.187903464	CLE Negative	
93	114 S	CBP	Undetermined			CB Negative	-
93	114 S	CLEP	35.63134766	35.29	0.476716608	CLE Negative	
94	114 M	CBP	Undetermined	39.63		CB Negative	-
94	114 M	CLEP	32.43854523	32.44		CLE Negative	
95	112 S	CBP	Undetermined			CB Negative	-
95	112 S	CLEP	20.13814163	22.04	2.695023298	**CLE Positive**	
96	112 M	CBP	Undetermined			CB Negative	-
96	112 M	CLEP	31.03725052	31.21	0.244280443	**CLE Positive**	
97	109 S	CBP	Undetermined			CB Negative	-
97	109 S	CLEP	27.91299248	28.22	0.429118305	**CLE Positive**	
98	109 M	CBP	Undetermined			CB Negative	-
98	109 M	CLEP	27.93951416	27.93	0.009713331	**CLE Positive**	
99	108 S	CBP	Undetermined			CB Negative	-
99	108 S	CLEP	28.11605263	28.15	0.051494684	**CLE Positive**	
100	108 M	CBP	Undetermined			CB Negative	-
100	108 M	CLEP	30.70183372	30.78	0.106432594	**CLE Positive**	
101	106 S	CBP	Undetermined			CB Negative	-
101	106 S	CLEP	18.93590736	21.69	3.895650148	**CLE Positive**	
102	106 MF	CBP	Undetermined			CB Negative	-
102	106 MF	CLEP	28.88016891	28.96	0.114078373	**CLE Positive**	
103	105 S	CBP	Undetermined			CB Negative	-
103	105 S	CLEP	22.88839149	23.76	1.23890698	**CLE Positive**	
104	105 MF	CBP	Undetermined			CB Negative	-
104	105 MF	CLEP	28.61892509	28.60	0.024000101	**CLE Positive**	

	Sample ID	Target Name	Cт	Cт Mean	Cт SD	Real-time PCR Result	*C. burnetii* Quant Kit
105	103 S	CBP	Undetermined			CB Negative	-
105	103 S	CLEP	31.73790169	32.17	0.613378942	**CLE Positive**	
106	103 M	CBP	Undetermined			CB Negative	-
106	103 M	CLEP	33.35009003	33.43	0.114741929	CLE Negative	
107	102 S	CBP	Undetermined			CB Negative	-
107	102 S	CLEP	24.01666069	24.26	0.349167407	**CLE Positive**	
108	102 M	CBP	Undetermined			CB Negative	-
108	102 M	CLEP	25.32230186	25.36	0.053825233	**CLE Positive**	
109	101 S	CBP	Undetermined			CB Negative	-
109	101 S	CLEP	32.95610428	32.96	0.011358744	CLE Negative	
110	101 M	CBP	Undetermined			CB Negative	-
110	101 M	CLEP	33.83240128	34.17	0.479732275	CLE Negative	
111	A1	CBP	Undetermined			CB Negative	-
111	A1	CLEP	26.87572861	26.944	0.097083412	**CLE Positive**	
112	A2	CBP	Undetermined			CB Negative	-
112	A2	CLEP	26.56647491	26.638	0.101207733	**CLE Positive**	
113	A3	CBP	Undetermined			CB Negative	-
113	A3	CLEP	28.27923584	28.285	0.008410488	**CLE Positive**	
114	A4	CBP	Undetermined			CB Negative	-
114	A4	CLEP	28.53792763	28.521	0.024294117	**CLE Positive**	
115	A5	CBP	Undetermined			CB Negative	-
115	A5	CLEP	27.45244408	27.579	0.179014191	**CLE Positive**	
116	A6	CBP	Undetermined			CB Negative	-
116	A6	CLEP	26.14412308	26.099	0.064214267	**CLE Positive**	
117	A7	CBP	Undetermined			CB Negative	-
117	A7	CLEP	27.05367661	27.324	0.382010937	**CLE Positive**	
118	A8	CBP	Undetermined			CB Negative	-
118	A8	CLEP	26.52062225	26.384	0.192733154	**CLE Positive**	
119	A9	CBP	Undetermined			CB Negative	-
119	A9	CLEP	25.3313179	25.307	0.034316301	**CLE Positive**	
120	A10	CBP	Undetermined			CB Negative	-
120	A10	CLEP	29.39139366	29.669	0.39267242	**CLE Positive**	
121	H1	CBP	Undetermined			CB Negative	-
121	H1	CLEP	28.78933525	28.856	0.094707005	**CLE Positive**	
122	H2	CBP	Undetermined			CB Negative	-
122	H2	CLEP	26.14553833	26.048	0.138430476	**CLE Positive**	
123	H3	CBP	Undetermined			CB Negative	-
123	H3	CLEP	26.6776886	26.672	0.007891239	**CLE Positive**	
124	H4	CBP	Undetermined			CB Negative	-
124	H4	CLEP	28.05894089	28.185	0.178306118	**CLE Positive**	
125	H5	CBP	Undetermined			CB Negative	-
125	H5	CLEP	25.5405674	25.505	0.049628083	**CLE Positive**	

	Sample ID	Target Name	Cт	Cт Mean	Cт SD	Real-time PCR Result	*C. burnetii* Quant Kit
126	H6	CBP	Undetermined			CB Negative	-
126	H6	CLEP	28.10753441	28.129	0.030507576	**CLE Positive**	
127	H7	CBP	Undetermined			CB Negative	-
127	H7	CLEP	27.82615662	27.895	0.097257391	**CLE Positive**	
128	H8	CBP	Undetermined			CB Negative	-
128	H8	CLEP	28.1282692	28.419	0.411833376	**CLE Positive**	
129	H9	CBP	Undetermined			CB Negative	-
129	H9	CLEP	25.84353828	25.847	0.004596367	**CLE Positive**	
130	H10	CBP	Undetermined			CB Negative	-
130	H10	CLEP	29.76959419	29.830	0.085062459	**CLE Positive**	

Human real-time PCR results with a cut-off Ct-value of 32.

Sample ID	Target Name	Cт	Cт Mean	Real-time PCR Result	*C. burnetii* Quant Kit	NICD Serology Results	
						IgM	IgG
Z00256	CBP	Undetermined		CB Negative	Negative	Positive	Positive
Z00256	CLEP	Undetermined		CLE Negative			
Z00257	CBP	Undetermined		CB Negative	Negative	Negative	Negative
Z00257	CLEP	Undetermined		CLE Negative			
Z00258	CBP	37.26235199	37.90	CB Negative	Negative	Negative	Negative
Z00258	CLEP	Undetermined		CLE Negative			
Z00259	CBP	Undetermined		CB Negative	Negative	Negative	Negative
Z00259	CLEP	38.39077759	38.39	CLE Negative			
Z00260	CBP	38.98345184	39.11	CB Negative	Negative	Negative	Negative
Z00260	CLEP	Undetermined		CLE Negative			
Z00261	CBP	Undetermined		CB Negative	Negative	Positive	Positive
Z00261	CLEP	Undetermined		CLE Negative			
Z00262	CBP	Undetermined		CB Negative	Negative	Negative	Negative
Z00262	CLEP	Undetermined		CLE Negative			
Z00263	CBP	Undetermined		CB Negative	Negative	Negative	Negative
Z00263	CLEP	Undetermined		CLE Negative			
Z00264	CBP	Undetermined		CB Negative	Negative	Negative	Positive
Z00264	CLEP	39.35374069	39.35	CLE Negative			
Z00265	CBP	Undetermined		CB Negative	Negative	Negative	Negative
Z00265	CLEP	Undetermined		CLE Negative			
Z00266	CBP	Undetermined		CB Negative	Negative	Negative	Negative
Z00266	CLEP	Undetermined		CLE Negative			
Z00267	CBP	Undetermined		CB Negative	Negative	Negative	Positive
Z00267	CLEP	37.62314606	37.62	CLE Negative			

Sample ID	Target Name	Cт	Cт Mean	Real-time PCR Result	*C. burnetii* Quant Kit	NICD Serology Results	
						IgM	IgG
Z00268	CBP	Undetermined		CB Negative	Negative	Negative	Positive
Z00268	CLEP	Undetermined		CLE Negative			
Z00269	CBP	Undetermined		CB Negative	Negative	Negative	Positive
Z00269	CLEP	Undetermined		CLE Negative			
Z00270	CBP	39.24816132	39.25	CB Negative	Negative	Negative	Positive
Z00270	CLEP	Undetermined		CLE Negative			
Z00271	CBP	Undetermined		CB Negative	Negative	Negative	Negative
Z00271	CLEP	Undetermined		CLE Negative			
Z00272	CBP	Undetermined		CB Negative	Negative	Negative	Negative
Z00272	CLEP	Undetermined		CLE Negative			
Z00273	CBP	39.85912323	39.37	CB Negative	Negative	Negative	Negative
Z00273	CLEP	38.6348381	38.63	CLE Negative			
Z00274	CBP	39.39825821	39.40	CB Negative	Negative	Negative	Negative
Z00274	CLEP	Undetermined		CLE Negative			
Z00275	CBP	38.40999985	38.41	CB Negative	Negative	Negative	Negative
Z00275	CLEP	Undetermined		CLE Negative			
Z00276	CBP	39.35124969	39.21	CB Negative	Negative	Negative	Eqv
Z00276	CLEP	37.94195557	37.94	CLE Negative			
Z00278	CBP	Undetermined		CB Negative	Negative	Negative	Negative
Z00278	CLEP	Undetermined		CLE Negative			
Z00279	CBP	Undetermined		CB Negative	Negative	Negative	Negative
Z00279	CLEP	Undetermined		CLE Negative			
Z00280	CBP	38.06633759	38.07	CB Negative	Negative	Negative	Negative
Z00280	CLEP	Undetermined		CLE Negative			
Z00281	CBP	38.61993408	39.16	CB Negative	Negative	Negative	Positive
Z00281	CLEP	Undetermined		CLE Negative			

Sample ID	Target Name	Cт	Cт Mean	Real-time PCR Result	*C. burnetii* Quant Kit	NICD Serology Results	
						IgM	IgG
Z00282	CBP	39.64930344	39.65	CB Negative	Negative	Negative	Negative
Z00282	CLEP	37.45347977	37.45	CLE Negative			
Z00283	CBP	36.95035934	37.70	CB Negative	Negative	Negative	Negative
Z00283	CLEP	37.83246231	37.83	CLE Negative			
Z00284	CBP	39.528965	39.53	CB Negative	Negative	Negative	Negative
Z00284	CLEP	Undetermined		CLE Negative			
Z00285	CBP	39.52526093	39.53	CB Negative	Negative	Negative	Negative
Z00285	CLEP	Undetermined		CLE Negative			
Z00286	CBP	Undetermined		CB Negative	Negative	Negative	Negative
Z00286	CLEP	39.35252762	39.35	CLE Negative			
Z00287	CBP	38.94620514	38.95	CB Negative	-	Negative	Negative
Z00287	CLEP	Undetermined		CLE Negative			
Z00288	CBP	39.76354218	39.76	CB Negative	-	Negative	Negative
Z00288	CLEP	Undetermined		CLE Negative			
Z00289	CLEP	39.72231293	39.72	CB Negative	-	Negative	Negative
Z00289	CBP	38.19997406	38.20	CLE Negative			
Z00290	CBP	Undetermined		CB Negative	-	Negative	Negative
Z00290	CLEP	Undetermined		CLE Negative			
Z00291	CBP	39.96834564	39.97	CB Negative	-	Negative	Negative
Z00291	CLEP	Undetermined		CLE Negative			
Z00292	CBP	Undetermined		CB Negative	-	Negative	Negative
Z00292	CLEP	38.95496368	38.95	CLE Negative			
Z00293	CBP	38.8385582	38.50	CB Negative	-	Negative	Positive
Z00293	CLEP	39.24509811	39.25	CLE Negative			
Z00294	CBP	38.45378876	38.45	CB Negative	-	Negative	Negative
Z00294	CLEP	Undetermined		CLE Negative			

Sample ID	Target Name	Cт	Cт Mean	Real-time PCR Result	*C. burnetii* Quant Kit	NICD Serology Results	
						IgM	IgG
Z00295	CBP	37.56427383	37.56	CB Negative	-	Negative	Negative
Z00295	CLEP	Undetermined		CLE Negative			
Z00296	CBP	37.75812149	38.51	CB Negative	-	Negative	Positive
Z00296	CLEP	38.03518677	38.04	CLE Negative			
Z00297	CBP	38.92179489	38.72	CB Negative	-	Negative	Negative
Z00297	CLEP	Undetermined		CLE Negative			
Z00298	CBP	37.75593567	37.76	CB Negative	-	Negative	Negative
Z00298	CLEP	Undetermined		CLE Negative			
Z00299	CBP	39.41082382	39.41	CB Negative	-	Negative	Eqv
Z00299	CLEP	Undetermined		CLE Negative			
Z00300	CBP	38.2765007	38.28	CB Negative	-	Negative	Positive
Z00300	CLEP	32.9999733	33.17	CLE Negative			
Z00301	CBP	38.36566925	39.12	CB Negative	-	Negative	Negative
Z00301	CLEP	38.699646	38.70	CLE Negative			
Z00302	CBP	38.0725975	38.52	CB Negative	-	Negative	Positive
Z00302	CLEP	Undetermined		CLE Negative			
Z00303	CBP	39.51987839	39.52	CB Negative	-	Negative	Negative
Z00303	CLEP	37.40222549	37.40	CLE Negative			
Z00304	CBP	39.51950836	39.31	CB Negative	-	Negative	Positive
Z00304	CLEP	35.19132233	35.93	CLE Negative			
Z00305	CBP	39.08099747	39.48	CB Negative	-	Negative	Negative
Z00305	CLEP	36.85262299	37.48	CLE Negative			
Z00306	CBP	36.99150085	36.99	CB Negative	-	Negative	Positive
Z00306	CLEP	Undetermined		CLE Negative			
Z00307	CBP	39.72470474	39.72	CB Negative	-	Negative	Negative
Z00307	CLEP	Undetermined		CLE Negative			

Sample ID	Target Name	Cт	Cт Mean	Real-time PCR Result	*C. burnetii* Quant Kit	NICD Serology Results	
						IgM	IgG
Z00308	CBP	Undetermined		CB Negative	-	Negative	Negative
Z00308	CLEP	35.75631714	35.76	CLE Negative			
Z00309	CBP	36.71162033	38.29	CB Negative	-	Negative	Negative
Z00309	CLEP	Undetermined		CLE Negative			
Z00310	CBP	Undetermined		CB Negative	-	Negative	Negative
Z00310	CLEP	Undetermined		CLE Negative			
Z00311	CBP	39.01562881	39.02	CB Negative	-	Negative	Negative
Z00311	CLEP	38.35284805	36.91	CLE Negative			
Z00312	CBP	37.50035095	37.80	CB Negative	-	Negative	Negative
Z00312	CLEP	36.91605759	36.92	CLE Negative			
Z00313	CBP	37.06738663	37.82	CB Negative	-	Negative	Negative
Z00313	CLEP	Undetermined		CLE Negative			
Z00314	CBP	37.77329254	37.77	CB Negative	-	Negative	Eqv
Z00314	CLEP	Undetermined		CLE Negative			
Z00315	CBP	38.44049072	38.89	CB Negative	-	Negative	Negative
Z00315	CLEP	Undetermined		CLE Negative			
Z00316	CBP	36.93459702	37.50	CB Negative	-	Negative	Negative
Z00316	CLEP	Undetermined		CLE Negative			
Z00317	CBP	37.36182785	37.36	CB Negative	-	Negative	Negative
Z00317	CLEP	Undetermined		CLE Negative			
Z00318	CBP	Undetermined		CB Negative	-	Negative	Negative
Z00318	CLEP	39.6479187	39.65	CLE Negative			
Z00319	CLEP	35.70201874	35.70	CB Negative	-	Negative	Negative
Z00319	CBP	36.92644882	36.93	CLE Negative			
Z00320	CBP	37.79728317	38.03	CB Negative	-	Negative	Negative
Z00320	CLEP	38.05287933	38.05	CLE Negative			

Sample ID	Target Name	Cт	Cт Mean	Real-time PCR Result	*C. burnetii* Quant Kit	NICD Serology Results	
						IgM	IgG
Z00321	CBP	37.79066849	38.62	CB Negative	-	Negative	Negative
Z00321	CLEP	Undetermined		CLE Negative			
Z00322	CBP	37.71296692	38.20	CB Negative	-	Negative	Negative
Z00322	CLEP	35.76668167	36.07	CLE Negative			
Z00323	CLEP	39.84941101	39.85	CB Negative	-	Negative	Negative
Z00323	CBP	38.15871048	38.16	CLE Negative			
Z00324	CBP	Undetermined		CB Negative	-	Negative	Negative
Z00324	CLEP	36.02671432	36.03	CLE Negative			
Z00325	CBP	39.22101593	39.22	CB Negative	-	Negative	Negative
Z00325	CLEP	35.13005066	35.13	CLE Negative			
Z00326	CBP	Undetermined		CB Negative	-	Negative	Negative
Z00326	CLEP	Undetermined		CLE Negative			
Z00327	CBP	39.02412415	39.02	CB Negative	-	Negative	Negative
Z00327	CLEP	Undetermined		CLE Negative			
Z00328	CBP	36.73842239	36.76	CB Negative	-	Negative	Negative
Z00328	CLEP	39.128582	39.13	CLE Negative			
Z00329	CBP	37.31650925	37.32	CB Negative	-	Negative	Negative
Z00329	CLEP	37.4728508	37.47	CLE Negative			
Z00330	CLEP	34.55107117	35.08	CB Negative	-	Eqv	Negative
Z00330	CBP	36.00003052	36.78	CLE Negative			
Z00331	CBP	38.59870529	38.60	CB Negative	-	Negative	Negative
Z00331	CLEP	39.64345551	39.64	CLE Negative			
Z00332	CBP	39.56196213	39.53	CB Negative	-	Negative	Negative
Z00332	CLEP	35.17008972	35.18	CLE Negative			
Z00333	CBP	34.81618118	36.43	CB Negative	-	Negative	Negative
Z00333	CLEP	Undetermined		CLE Negative			
Z00334	CBP	36.83336258	36.83	CB Negative	-	Negative	Negative
Z00334	CLEP	33.68598557	33.69	CLE Negative			

Real-time PCR results and Sanger sequencing results comparison.

Sample ID		Real-time PCR result	Sanger Sequencing result
1	102S	CLE	CLE
2	106S	CLE	CLE
3	117S	CLE	No amplification
4	201S	CLE	CLE
5	204S	CLE	No amplification
6	216M	CLE	No amplification
7	301S	CLE	CLE
8	308S	CLE	CLE
9	403S	CLE	CLE
10	503S	CLE	CLE
1	905S	CLE	CLE
12	1001M	CLE	CLE
13	1008M	CLE	CLE
14	1011M	CLE	CLE
15	1014M	CLE	CLE
16	1018M	CLE	CLE
17	A6	CLE	CLE
18	A9	CLE	CLE
19	H3	CLE	CLE
20	H5	CLE	CLE

CLE = *Coxiella*-like Endosymbiont

Appendix 3

Section 20

agriculture, land reform & rural development

Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Land Reform and Rural Development
Private Bag X138, Pretoria 0001

Enquiries: Ms Marna Laing • Tel: +27 12 319 7442 • Fax: +27 12 319 7470 • E-mail: MarnaL@dalrrd.gov.za
Reference: 12/11/1/1/6 (2741ZY)

Responsible person: Mrs Carina Lourens
Institution: Department of Veterinary Tropical Diseases, Faculty of Veterinary Sciences
University of Pretoria, Onderstepoort 0110
Email: Marinda.oosthuizen@up.ac.za

Dear Mrs Carina Lourens and Prof Marinda Oosthuizen

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

Title of research project / study: "Development of a real-time PCR assay to distinguish between *Coxiella burnetii* and *Coxiella*-like endosymbionts."

Your application, received 2022-10-31, requesting permission under Section 20 of the Animal Diseases Act, 1984 (Act No 35 of 1984) to perform the research project or study stipulated above, refers.

1. Based on the information provided in your application, the Director of Animal Health has no objection to this study. The study may continue if statement 1.1 to 1.6 hereunder (as applicable) are, and remain, accurate. Should the scope of your research project change in any way you are required to inform the Section 20 Secretariat and may not proceed with any activities until written permission to do so have been granted by the National Director: Animal Health.

    1.1. No work will be done with controlled and notifiable animal diseases (list can be obtained / requested from this office), which includes any animal diseases which do not occur in South Africa;
    1.2. No imported material of animal origin or imported animal pathogens will be utilized in the study;
    1.3. No samples that originate from a biobank will be used in the study, except for DNA extracted from ticks stored at the Research and Training Laboratories in DVTD, from the study "Tick-born disease dynamics at the human/wildlife/livestock/ interface in the Mnisi community area of South Africa" (12/11/1/1/8 775MG);
    1.4. No clinical studies will be performed in the target species, either in a laboratory or in the field;
    1.5. The areas where the samples are to be collected are not under restriction for controlled or notifiable diseases to which the species of animal, from which the samples are obtained, is susceptible;
    1.6. No samples or products that have not been passed as fit for human consumption will be obtained from an abattoir.

2. In addition to the conditions mentioned in point 1, you are responsible for ensuring that your research project or study complies with all or part of the following, as applicable:

2.1. Permission to perform research under Section 20 of the Animal Diseases Act, 1984 (Act no 35 of 1984) 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, including the Veterinary and Para-Veterinary Professions Act, 1982 (Act No 19 of 1982), the Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act, 1947 (Act No 36 of 1947), the Medicines and Related Substances Control Act, 1965 (Act No 101 of 1965), the Genetically Modified Organisms Act, 1997 (Act No 15 of 1997) and the National Environmental Management: Biodiversity Act, 2004 (Act No 10 of 2004);

2.2. No part of the study may begin until valid ethical approval has been obtained in writing from the relevant South African authority;

2.3. All biological or potentially infectious material 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);

2.4. Any incidence or suspected incidence of a controlled or notifiable disease in terms of the Animal Diseases Act, 1984 (Act No 35 of 1984), must be reported immediately to the responsible state veterinarian;

2.5. Samples or material may not be outsourced or used for further/other research without prior written approval from the Director of Animal Health;

2.6. All potentially infectious material utilised or generated during or by the study is to be destroyed at completion of the study and only a registered waste disposal company may be used for the removal of waste generated during or by the study;

2.7. Records must be kept for five years for auditing purposes.

Written permission from the Director of Animal Health must be obtained prior to any deviation from the conditions. Application must be sent in writing to MamaL@dalrrd.gov.za.

Failure to obtain written permission as above may be considered a contravention of the Animal Diseases Act, 1984 (Act No 35 of 1984).

Expiry date of this permit: 30 November 2025

Kind regards,

Dr Mpho Maja
DIRECTOR: ANIMAL HEALTH
Date: 2022 -11- 02

Appendix 4

DAFF Import Permit

agriculture, land reform & rural development

Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

Directorate of Animal Health
Import-Export Policy Unit
Private Bag X138
Pretoria, 0001
Republic of South Africa
Tel: (27)-012-319 7514
Fax: (27)-012-329 8292
PERMIT NO: 13/1/1/30/2/0-202104000562
Valid from: 2021-04-08
Expiry date: 2021-07-08

DIRECTORATE ANIMAL HEALTH
08 APR 2021
1
DIRECTORATE ANIMAL HEALTH
PRIVATE BAG X138 PRETORIA 0001

IMPORTER:
UNIVERSITY OF PRETORIA
DEPT OF VETERINARY TROPICAL DISEASES
PRIVATE BAG X04
ONDERSTEPOORT
0110

VETERINARY IMPORT PERMIT FOR PATHOLOGY SPECIMENS
[Issued in terms of the Animal Diseases Act, 1984 (Act No. 35 of 1984)]

Authority is hereby granted for you to import ONE VIAL CONTAINING 50µl C. BURNETII DNA FOR USE AS A POSITIVE CONTROL into Republic of South Africa:

From: NATIONAL REFERENCE LABORATORY FOR ANIMAL Q FEVER, FRANCE
subject to the following conditions:

1. The consignment must be accompanied by this original permit and an original veterinary health certificate, complying with the conditions stipulated overleaf (IMP.PATH.CE.10/2013), duly completed and signed by an official veterinarian, authorised thereto by the Veterinary Authority of FRANCE.
2. The specimens are to be securely packed and transported in leakproof containers, sealed by an authorised official of the Veterinary Authority of the exporting country;
3. The specimens must be kept and used for purposes of testing/research at the laboratories of UNIVERSITY OF PRETORIA FACULTY OF VETERINARY SCINECES under the personal supervision of CARINA LOURENS;
4. On completion of tests/research the specimens, including all contaminated/infectious things or animal products (as defined by the Animal Diseases Act, 1984 [Act No. 35 of 1984]) derived/produced from or that came into contact with the above-mentioned specimens, must be destroyed by incineration. Records of the incinerations must be maintained for a period of 5 years, and made available for auditing to the Veterinary Authority upon request.
5. The consignment must be airfreighted through port of entry OR TAMBO. **Samples may only be imported as manifest cargo under an airwaybill number and may not be imported as personal luggage.**
6. The consignment must be accompanied by this permit and its arrival reported immediately to the inspecting veterinary official: KEMPTON PARK Tel: 011 393 7980, and may not be released without his/her written permission.
7. Upon arrival the inspecting veterinary official will inspect the consignment and release it to the importer only after he/she is satisfied that all the import conditions have been complied with in full.
8. **This permit does not absolve the importer from compliance with the provisions of any other legislation relating to this import.**
9. This permit is subject to amendment or cancellation by the Director Animal Health at any time and without prior notice being given.
10. This permit is valid for three (3) months from date of issue and FOR ONE CONSIGNMENT ONLY.

SPECIAL CONDITIONS:

DIRECTOR: ANIMAL HEALTH

NOTE:

- All imports for research purposes require Section 20 permission in compliance with the Animal Diseases Act.
- Any consignment imported into South Africa packed with either wood packaging material or dunnage, will require treatment to remove any pests present (by heat or methyl bromide fumigation). Treatment must be indicated as per IPPC prescript on wood packaging material. [Directorate: Inspection Services Tel: 012 309 8754 or Fax 086 732 4768 or www.daff.gov.za]

(IMP.PATH.CE.10/2013)

Appendix 5

NWU – Animal Ethics - N W U - 0 0 5 3 5 - 2 0 - A 5

Private Bag X1290, Potchefstroom
South Africa 2520

Tel: 086 016 9698
Web: http://www.nwu.ac.za/

North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC)

Tel: 018 299-1208
Email: Ethics-AnimCare@nwu.ac.za (for animal studies)

29 November 2020

ETHICS APPROVAL LETTER OF STUDY

Based on approval by the North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC) on 29/11/2020, the NWU-AnimCareREC hereby approves your study as indicated below. This implies that the NWU-AnimCareREC grants its permission that, provided the general conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: Development of a real time PCR assay to distinguish between *Coxiella burnetii* and *Coxiella*-like endosymbionts
Principal Investigator/Study Supervisor/Researcher: Prof M Oosthuizen
Student: CW Lourens - 33623139

Ethics number:

N	W	U	-	0	0	5	3	5	-	2	0	-	A	5
Institution				Study Number						Year			Status	

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Application Type: Single study
Commencement date: 29/11/2020
Expiry date: 30/11/2021

Risk: Category 0

Approval of the study is provided for a year, after which continuation of the study is dependent on receipt and review of an annual monitoring report and the concomitant issuing of a letter of continuation. A monitoring report is required at the end of November annually until completion.

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, the following general terms and conditions will apply:

- *The principal investigator/study supervisor/researcher must report in the prescribed format to the NWU-AnimCareREC:*
  - *annually on the monitoring of the study, whereby a letter of continuation will be provided annually, and upon completion of the study; and*
  - *without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.*
- *The approval applies strictly to the proposal as stipulated in the application form. Should any amendments to the proposal be deemed necessary during the course of the study, the principal investigator/study supervisor/researcher must apply for approval of these amendments at the NWU-AnimCareREC, prior to implementation. Should there be any deviations from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.*
- *Annually a number of studies may be randomly selected for active monitoring.*

- *The date of approval indicates the first date that the study may be started.*
- *In the interest of ethical responsibility, the NWU-AnimCareREC reserves the right to:*
  - *request access to any information or data at any time during the course or after completion of the study;*
  - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process;*
  - *withdraw or postpone approval if:*
    - *any unethical principles or practices of the study are revealed or suspected;*
    - *it becomes apparent that any relevant information was withheld from the NWU-AnimCareREC or that information has been false or misrepresented;*
    - *submission of the annual monitoring report, the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and/or*
    - *new institutional rules, national legislation or international conventions deem it necessary.*
- *NWU-AnimCareREC can be contacted for further information via Ethics-AnimCare@nwu.ac.za or 018 299 1208*

Special conditions of the research approval due to the COVID-19 pandemic:

Please note: Due to the nature of the study i.e. (laboratory work involving the development of a PCR assay to determine the presence of specific bacterial pathogens in previously collected animal blood samples), this study will be able to proceed during the current alert level, following receipt of the approval letter. No additional COVID-19 restrictions have been placed on the study except that the researcher must ensure that before proceeding with the study that all research team members have reviewed the North-West University COVID-19 Occupational Health and Safety Standard Operating Procedure.

NWU-AnimCareREC would like to remain at your service and wishes you well with your study. Please do not hesitate to contact the NWU-AnimCareREC for any further enquiries or requests for assistance.

Yours sincerely,

Digitally signed by
Prof Christiaan B Brink
Date: 2020.11.30
08:22:58 +02'00'

Chairperson: NWU-AnimCareREC

Current details (22654704) G:\My Drive\9. Research and Postgraduate Education\9.1.5.4 Templates\9.1.5.4.2_NWU-AC_EAL.docm
18 November 2020

File Reference: 9.1.5.4.2

Appendix 6

NWU – Animal Ethics - N W U - 0 0 5 3 5 - 2 0 - A 5 – Continuation letter

NWU®
NORTH-WEST UNIVERSITY
NOORDWES-UNIVERSITEIT
YUNIBESITI YA BOKONE-BOPHIRIMA

Private Bag X6001, Potchefstroom
South Africa 2520

Tel: +2718 299-1111/2222
Web: http://www.nwu.ac.za

Faculty of Health Sciences Ethics Office for Research, Training and Support

Tel: 018 299 2092
Email:Wayne.Towers@nwu.ac.za

30 November 2021

Prof O Thekisoe
Unit for Environmental Sciences and Management

Dear Prof Thekisoe

FEEDBACK ON NWU-ANIMCAREREC ANNUAL MONITORING REPORT: NWU-00535-20-A5

We would like to thank you for submitting the annual monitoring report for your project entitled, ***"Development of a real time PCR assay to distinguish between Coxiella burnetii and Coxiella-like endosymbionts"***, to the North-West University Animal Care, Health and Safety in Research Ethics Committee (NWU-AnimCareREC) in a timely manner. Please find below the decision of the NWU-AnimCareREC regarding the continuation of your project.

Classification	Mark with X	Comment	
Clarification			
Completion (Final report)			
Suspended			
Continuation	X	**Date of next monitoring report:**	30 November 2022
Termination			

Please note: Due to the nature of the study i.e. (laboratory work involving the development of a PCR assay to determine the presence of specific bacterial pathogens in previously collected animal blood samples), this study will be able to proceed during the current alert level, following receipt of the approval letter. No additional COVID-19 restrictions have been placed on the study except that the researcher must ensure that before proceeding with the study that all research team members have reviewed the North-West University COVID-19 Occupational Health and Safety Standard Operating Procedure.

Should you have any further queries, please feel free to contact Mr Buti Majola at your earliest convenience (E-mail: Ethics-AnimMonitoring@nwu.ac.za; Tel: 018 299 2197). We wish you well in your future endeavours.

Yours sincerely

Digitally signed by Christiaan B Brink (Tiaan)
Date: 2022.02.10 11:32:23 +02'00'

Chairperson: NWU-AnimCareREC

Appendix 7

NWU – Human Ethics - NWU 00176-21-A1

Private Bag X1290, Potchefstroom
South Africa 2520

Tel: 086 016 9698
Web: http://www.nwu.ac.za/

North-West University Health Research Ethics Committee (NWU-HREC)

Tel: 018 299-1206
Email: Ethics-HRECApply@nwu.ac.za (for human studies)

24 August 2021

ETHICS APPROVAL LETTER OF STUDY

Based on approval by the North-West University Health Research Ethics Committee (NWU-HREC) on 24/08/2021, the NWU-HREC hereby approves your study as indicated below. This implies that the NWU-HREC grants its permission that, provided the general conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: Development of a real-time PCR assay to distinguish between *Coxiella burnetii* and Coxiella-like endosymbionts
Principal Investigator/Study Supervisor/Researcher: Prof CM Oosthuizen
Student: CW Lourens - 33623139

Ethics number:

N	W	U	-	0	0	1	7	6	-	2	1	-	A	1
Institution				Study Number						Year			Status	

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Application Type: Single study
Commencement date: 24/08/2021
Expiry date: 31/08/2022

Risk: Minimal

Approval of the study is provided for a year, after which continuation of the study is dependent on receipt and review of an annual monitoring report and the concomitant issuing of a letter of continuation. A monitoring report is due at the end of August annually until completion of the study.

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, the following general terms and conditions will apply:

- *The principal investigator/study supervisor/researcher must report in the prescribed format to the NWU-HREC:*
  - *Annually on the monitoring of the study, whereby a letter of continuation will be provided annually, and upon completion of the study; and*
  - *without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.*
- *The approval applies strictly to the proposal as stipulated in the application form. Should any amendments to the proposal be deemed necessary during the course of the study, the principal investigator/study supervisor/researcher must apply for approval of these amendments at the NWU-HREC, prior to implementation. Should there be any deviations from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.*
- *Annually a number of studies may be randomly selected for active monitoring.*
- *The date of approval indicates the first date that the study may be started.*
- *In the interest of ethical responsibility, the NWU-HREC reserves the right to:*
  - *request access to any information or data at any time during the course or after completion of the study;*
  - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process;*

- *withdraw or postpone approval if:*
  - *any unethical principles or practices of the study are revealed or suspected;*
  - *it becomes apparent that any relevant information was withheld from the NWU-HREC or that information has been false or misrepresented;*
  - *submission of the annual monitoring report, the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and/or*
  - *new institutional rules, national legislation or international conventions deem it necessary.*
- *NWU-HREC can be contacted for further information via Ethics-HRECApply@nwu.ac.za or 018 299 1206*

Special conditions of the research approval due to the COVID-19 pandemic:

Please note: Due to the nature of the study i.e. (laboratory work involving the molecular analysis of previously collected biological samples), this study will be able to proceed during the current alert level, following receipt of the approval letter. No additional COVID-19 restrictions have been placed on the study except that the researcher must ensure that before proceeding with the study that all research team members have reviewed the North-West University COVID-19 Occupational Health and Safety Standard Operating Procedure.

The NWU-HREC would like to remain at your service and wishes you well with your study. Please do not hesitate to contact the NWU-HREC for any further enquiries or requests for assistance.

Yours sincerely,

Digitally signed by Prof Petra Bester
Date: 2021.08.25 14:23:36 +02'00'

Chairperson NWU-HREC

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