Development and analytical validation of a genus-specific *Brucella* real-time PCR assay targeting the 16S-23S rDNA internal transcribed spacer

Rejoice E. Nyarku

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Declaration

I, Rejoice Esenam Nyarku, hereby declare that all the work that has been carried out in this study was done by me as a student under the supervision and support of Professor Melvyn Quan and Dr Ayesha Hassim at the Department of Veterinary Tropical Diseases and that there is no falsification of results. All sources of information used have been acknowledged.

Signed.....

Place.....

Date.....

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Glossary of terms and abbreviations

ARC-OVR	Agricultural Research Council- Onderstepoort Veterinary Research
ATCC	American Type Culture Collection
AMOS	*Brucella abortus*, *B. melitensis*, *B. ovis* and *B. suis*
BCSP	*Brucella* cell surface protein
BCSP qPCR	*Brucella* cell surface protein real-time polymerase chain reaction
BCVs	*Brucella*-containing vacuoles
BLAST	Basic Local Alignment Search Tool
bp	base pairs
bv.	biovar
CFT	Complement Fixation Test
CFU	colony-forming unit
CI	confidence interval
C_T	Cycle threshold
CV	coefficient of variation
DALY	Disability-Adjusted Life Years
DNA	deoxyribonucleic acid
rDNA	ribosomal deoxyribonucleic acid
°C	degree Celsius
DAFF	Department of Agriculture, Forestry and Fisheries
DAMBE	data analysis in molecular biology and evolution
DVTD	Department of Veterinary Tropical Diseases
ELISA	Enzyme Linked Immunosorbent Assay
cELISA	competitive Enzyme Linked Immunosorbent Assay
iELISA	indirect Enzyme Linked Immunosorbent Assay
fg	femtogram
FAM	6-carboxy-fluorescein
FM	Farrell's media
FRET	fluorescence resonance energy transfer
g	grams
hrs	hours
h	hour
IDT	Integrated DNA Technologies
IPC	Internal positive control
IS711	insertion sequence 711
ITS	internal transcribed spacer
ITS qPCR	16S-23S rDNA internal transcribed spacer real-time polymerase chain reaction
LOD	Limit of detection
LPS	Lipopolysaccharide
MAFFT	multiple sequence alignment program
Mb	megabases
mg	Milligrams
ml	Millilitres
MGB	Minor groove binder
µm	Micrometre
µM	Micromolar
min	minutes
mTM	modified Thayer-Martin's selective medium
nM	Nanomolar
NDA	National Department of Agriculture

NHLS	National Health Laboratory Service
NICD	national institute for communicable diseases
OIE	World Organisation for Animal Health
PBS	Phosphate-buffered saline
PCR	polymerase chain reaction
PK	Protein kinase
qPCR	quantitative polymerase chain reaction
RBT	Rose Bengal Test
rRNA	ribosomal ribonucleic acid
SARs	shared anomalous regions
SAT	Serum Agglutination Test
SD	standard deviation
sec	seconds
Tm	melting temperature
UP	University of Pretoria
USA	United States of America
µl	Microliters

Abstract

Title: Development and analytical validation of a genus-specific *Brucella* real-time PCR assay targeting the 16S-23S rDNA internal transcribed spacer

Student: Rejoice E. Nyarku

Study leader: Melvyn Quan

Co-study leader: Ayesha Hassim

Department: Veterinary Tropical Diseases

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Brucellosis is an economically important bacterial disease of both animals and humans. In sub-Saharan Africa, the diagnosis of the disease remains a challenge. Brucellosis is underreported in South Africa, due to inconsistency in reports of bacteriological and serological tests, which lack adequate sensitivity and specificity in the diagnosis of the disease. They also are ineffective in confirming brucellosis during early stages of the disease.

The aim of this study was to develop a 16S-23S ribosomal deoxyribonucleic acid (rDNA) internal transcribed spacer (ITS) quantitative polymerase chain reaction (qPCR) assay for early diagnosis of brucellosis and as a rapid screening tool. To achieve this, blood, milk and tissue samples were spiked with *B*. *abortus* biovar (bv.) 1 (B01988-18 strain) to determine the analytical sensitivity and specificity of the assay. The efficiency was 105% in tissue, 99% in blood, and 93% in milk. The 95% limit of detection (LOD) of the ITS qPCR assay was highest in tissue, followed by blood, then milk; thus (1.45, 13.30 and 45.54 bacterial genome copies/PCR reaction).

Furthermore, the diagnostic performance of the assay was compared to the *Brucella* cell surface protein real time polymerase chain reaction (BCSP31 qPCR) assay. Out of 56 aborted foetal tissue samples from bovine, ovine and caprine, 33% (19/56) were positive for *Brucella* spp. The sensitivity and specificity of the ITS qPCR assay were 87% and 95% respectively, compared to the 92% and 89% for the BCSP31 qPCR assay and 47% and 55% for bacterial culture, respectively. The ITS qPCR gave earlier C_T's with a difference in C_T (ΔC_T) between ITS and BCSP31 ranging between 7.1 and 3.24.

The assay was efficient, sensitive and specific. It detected as little as 1.45 bacterial genome copies/PCR reaction in tissue, making this assay a valuable tool in early detection of the presence of the *Brucella* pathogen. It is sensitive and specific in the diagnosis of brucellosis.

Key words: diagnosis, qPCR, brucellosis, blood, milk, abomasal fluid

CHAPTER
1. INTRODUCTION

1.1. General overview

Brucellosis is a zoonotic disease that is distributed worldwide and endemic in developing countries. It is an economically important disease, which causes substantial loss to the animal industry, small-scale livestock holders and a wide range of wild animals (Franc et al., 2018, Ducrotoy et al., 2017, Franco et al., 2007, Pappas et al., 2006). Annually, about 500,000 cases are reported for human brucellosis. However, true incidence is estimated to be 5,000,000 to 12,500,000 cases per year (Hull and Schumaker, 2018). In South Africa, human brucellosis (*B. melitensis*) was reported in the Western Cape. A detailed history revealed that, the patient often fed his dog with waste from cattle, sheep and goat abattoir that was disposed of at a local open-access municipal waste site. There was no history of consumption of unpasteurised milk (Wojno et al., 2016, Centre-for-Emerging-Zoonotic-Parasitic-Diseases., 2019).

The smooth (S) *Brucella* species which includes *Brucella abortus*, *B. melitensis* and *B. suis* are the zoonotic species of economic importance which cause abortions in animals and are infectious (Whatmore, 2009). The rough (R) strain species are *B. ovis* and *B. canis* (Mancilla, 2016). In South Africa, *B. abortus* is considered the most predominant species reported and to a lesser extent, *B. melitensis* in both animals and humans (Frean et al., 2019). It is a controlled and a notifiable disease in humans and animals across sub-Saharan Africa (Ducrotoy et al., 2017). It is a “herd disease” which means that when one animal is infected, the whole herd is considered infected. This causes limitations to animal movements and international trade (Frean et al., 2019).

1.2. Problem statement

The diagnosis/detection of brucellosis is by routine serological tests and bacterial culture isolation, which is the “gold standard” in brucellosis diagnosis (Godfroid et al., 2010). However, these tests have inadequate sensitivity and specificity, are time-consuming and have the potential of causing infection to laboratory personnel. Immunological cross-reactions of *Brucella* species with *Ochrobactrum anthropi* has also been reported (Velasco et al., 1997). To avert these drawbacks, several molecular diagnostics on the basis of conventional and qPCR assays are now employed (Bricker, 2002).

Although the detection of *Brucella* either at the genus or species level by PCR has been performed in blood, milk and tissue samples with several gene targets (O'Leary et al., 2006), the performance of the 16S-23S rDNA internal transcribed spacer real- time polymerase chain reaction (ITS qPCR) in determining the sensitivity of this assay in blood, milk and tissue has not been reported. The performance of the ITS qPCR assay was analysed in the detection of *Brucella canis* in various samples, including canine semen, vaginal swabs and blood by conventional PCR (Keid et al., 2010, Keid et al., 2007a). The ITS qPCR has also been used in diagnosis of brucellosis in humans (Kattar et al., 2007). It has been proven to be rapid, specific and more sensitive for the diagnosis of brucellosis in both animals and humans because the rDNA exists in multiple copies (Keid et al., 2007b). There is therefore a constant need to develop an accurate and rapid diagnostic assay for high throughput detection of *Brucella* in order to implement appropriate control measures and subsequently contribute to the eradication of the disease in the country.

1.3. Rationale

Brucellosis is under-reported in South Africa. Several human brucellosis cases have been reported in the country. This leads to economic losses and financial implications to the livestock industry and a threat to human population. Despite the application of culture isolation, which is the 'gold standard' in the detection of *Brucella* in both animals and humans, the disease remains under-detected. This is because available serological and bacteriological tests lack adequate sensitivity and specificity, resulting in false-positives and false-negative results. Also, these tests only detect the pathogen at late stages of infection, where the disease might have been transmitted to other animal populations posing a risk to public health when products from these infected animals are consumed. It could be in part as a result of nonspecific and insidious nature of the disease. Therefore, early detection and accurate diagnosis of the disease allows the application of prompt preventive and control measures. Ultimately reducing disease burden and subsequently eradicating brucellosis. Hence, there is the need to develop and validate an accurate diagnostic tool for early detection of *Brucella*.

1.4. Justification

In South Africa, Brucellosis is of major concern as a result of the high prevalence and the substantial economic losses it creates in the livestock industry (Hesterberg et al., 2008). Diagnosis of brucellosis is based on bacterial isolation and/or serological tests. These tests can be time consuming, lack specificity, hazardous and subject to variable interpretation (Nielsen et al., 2004). In order to overcome these challenges, several conventional and real-

time PCR assays utilizing different primers derived from different polymorphic regions in the *Brucella* genome have been developed (Probert et al., 2004, Navarro et al., 2004). While these assays generally work well, cross-reactions with other closely related species have been reported, resulting in reduced assay sensitivity (Vizcaíno et al., 2000). Some of these assays are only able to differentiate a limited number of species (Hinić et al., 2008). Cross- reactions with *Ochrobactrum intermedium* and *O. anthropi* have been reported in real- time PCR assay development (Romero et al., 1995). The ITS region is highly conserved, with 100% sequence homology among *Brucella* spp. (Bricker, 2000). It has been proven to be rapid, specific and more sensitive for the diagnosis of brucellosis in both animals and humans because the rDNA exists in multiple copies (Keid et al., 2007b).

1.5. Research question

Does the development of a 16S-23S rDNA ITS qPCR assay improve the detection of the genus *Brucella* and curbing the issue of under-reporting and under-detection of the disease?

1.6. Hypothesis

Ho: There is no difference in sensitivity and specificity between 16S-23S rDNA ITS qPCR assay and BCSP31 qPCR assay in detecting *Brucella* species in milk, blood and tissues.

H1: The 16S-23S rDNA ITS qPCR assay has a higher sensitivity and specificity than BCSP31 qPCR assay in detecting *Brucella* species in milk, blood and tissues.

1.7. Aim

To develop a rapid genus-specific qPCR assay to detect *Brucella* species targeting the 16S-23S rDNA ITS region.

1.8. Objectives

- Compile a 16S-23S rDNA ITS sequence database of all *Brucella* species and closely related bacterial species.
- Identify a conserved region specific to *Brucella* species and design primers and TaqMan Minor groove binder (MGB) probes for qPCR.
- Perform an analytical validation of the assay.
- Test the assay against a small subset of diagnostic field samples.

CHAPTER
2. LITERATURE REVIEW

2.1. Aetiology

Brucellosis is caused by the genus *Brucella* (Alton and Forsyth, 1996). It belongs to the order alpha-Proteobacteria; which consists of mostly intra-cellular bacteria that are recognised as pathogenic in a number of mammalian hosts (Whatmore, 2009). *Brucella* is an intracellular, facultative, Gram-negative coccobacillus bacteria (Hinić et al., 2008), which causes abortion as well as infertility in animals and undulant fever in humans (Corbel, 1997). *Brucella* is a non-spore-forming and non-motile bacterium that measures from 0.6 to 1.5 micrometre (µm) in length and 0.5 to 0.7µm in width (Alton and Forsyth, 1996, Meyer and Shaw, 1920).

The outer cell membrane of *Brucella* intently looks like that of other Gram-negative bacilli with a prevailing lipopolysaccharide (LPS)(Alton and Forsyth, 1996). Other abortion causing pathogens including *Chlamydia abortus* and *Coxiella burnetti* share some morphological characteristics with *Brucella* on cultures, and this makes the Stamps staining method lack specificity (Alton et al., 1988, OIE Terrestrial Manual., 2018, Porter et al., 2011).

Twelve *Brucella* species have been characterised: six classical species, which includes *Brucella suis, B. melitensis, B. abortus, B. ovis, B. neotomae* and *B. canis*; an additional six novel species, which includes *B. ceti from cetaceans* and *B. pinnipedialis* from seals (Foster et al., 2007), *B. microti*, from wild rodents, *B. inopinata*, from breast implant infection in humans (Scholz et al., 2010), *B. vulpis* and *B. papionis,* from baboons (Whatmore et al., 2014). These are classified based on distinction between host preference and pathogenicity (Moreno et al., 2002). The smooth strains (*B. suis*, *B. melitensis* and *B. abortus*) are the zoonotic strains. Of the rough strains (*B. ovis* and *B. canis*), *B. ovis* is not zoonotic (Mancilla, 2016).

2.2. Clinical signs

Clinical signs vary in animals depending on the host species. Sheep and goat (*B. melitensis*), cattle (*B. abortus*) and swine (*B. suis*) brucellosis and rough strain *B. canis* are zoonotic (Moreno, 2014). The smooth strains present with spontaneous abortion, weak offspring, pyrexia, hygromas and mastitis (Megid et al., 2010). Infected pregnant cows abort with their first calving. Subsequent pregnancies can be carried for the full term, but calves that are born are weak; this phenomenon is as a result of acquired immunity after the first abortion (Megid

et al., 2010). Infected bulls show signs with orchitis, epididymitis, ampullitis, and seminal vesiculitis (Plant et al., 1976).

In infected swine, abortion, orchitis, epididymitis, infertility, arthritis, lameness and birth of weak piglets occur. Boars show signs of infection in the genital tract, with unilateral testicular enlargement. Canids present with mild pyrexia, late-term abortion and weak-litters (Megid et al., 2010).

Calves from infected cows may have latent infections. Since these animals usually test negative on serological tests, they pose an important problem in the implementation of control and elimination schemes (ter Huurne et al., 1993). Albeit, heifers with latent asymptomatic infection that can last for longer periods, can abort or deliver calves infected with the pathogen, which are critical in maintenance of brucellosis in a herd (Lapraik and Moffat, 1982). When these animals are introduced into a naïve herd, they cause abortion storms. The placenta, foetal fluid and aborted foetus serve as major sources of infection to other animals (Samartino and Enright, 1996).

In endemic areas where wild animals and small ruminants are kept in close contact, *B. melitensis* infection could occur in wild ruminants (Godfroid, 2018). These wild animals present with clinical signs of brucellosis in resemblance to that of cattle, sheep and goats. These include: orchitis, purulent/calcified arthritis, uveitis and neurological problems are observed (OIE Terrestrial Manual., 2018).

Areas where cows, sheep and goats are housed together, promotes the possibility of cross-infections with different species of *Brucell*a. For example, *B. abortus* infection in small ruminants, where there has not been any report of *B. melitensis* in that area (Allsup, 1969). It is unknown if this rare phenomenon is as a result of different management practices of small ruminants and cattle or as a result of host preference in *B. abortus* or both (Shaw, 1976). A study reported a case of persistence of *B. abortus* in sheep without the presence of a reservoir host (cow) (Shaw, 1976). An indirect evidence propose that sheep infected with *B. abortus* may serve as reservoir hosts for brucellosis in cattle (Allsup, 1969). With regards to *B. abortus* infection in horses, it was previously suggested it is a result of spill over infection from cattle to horses and are not reservoirs (Cohen et al., 1992).

2.3. Transmission

Brucella is transmitted by contact with infected animal tissues (Ferrero et al., 2014, Godfroid et al., 2005). A study demonstrated vertical transmission from infected cow to newborn calf

(Catlin and Sheehan, 1986). The low infectious dose of *Brucella* makes the infection an occupational hazard to those who are occupationally exposed, including: veterinarians, farmers, and butchers (Smits and Cutler, 2004). In humans, brucellosis is transmitted via drinking of unpasteurized milk, consumption of partially cooked meat or by-products from infected animals (Tadesse, 2016, Poester et al., 2013) or by inhalation of contaminated aerosols (López-Santiago et al., 2019).

The presence of *Brucella* species in wild animals as reservoirs is regarded as a risk (Godfroid et al., 2013, Alexander et al., 2012). This can result in a "spillback" infection from wildlife to livestock, leading to disruption of the brucellosis-free status in livestock (Godfroid, 2018). This can be observed in the interaction of bovine and camels that have contact with small ruminants infected with *B. melitensis*. This proposes that cattle and camels may not serve as a maintenance host of *B. melitensis* within their species without external source of infection such as a reservoir host (Godfroid, 2018). Although in sub-Saharan Africa, there is no evidence of direct transmission of *Brucella* spp. from wildlife to humans, infection is possible by the preparation and consumption of buffalo (*Syncerus caffer*) bushmeat (Alexander et al., 2012). Buffalo is the wildlife species most reported with brucellosis (Alonso et al., 2016). Therefore, it is important to identify and characterise *Brucella* strains from buffalo and humans to establish direct transmission of brucellosis from wildlife to humans (Godfroid, 2018).

2.4. Pathogenesis

A major characteristic of brucellosis is its extended incubation period. This period enhances an ''immunological window'' which allows the spread of *Brucella* in the host especially in phagocytes as a result of a delayed activation of the host innate immunity (Martirosyan et al., 2011).

The pathology of brucellosis is classified into three phases: the incubation phase, acute phase and the chronic phase. Chronic infection is due to the persistence of *Brucella* in host cells distributed by the reticuloendothelial system leading to hepatic, cardiovascular, neurologic, lymphoreticular, and osteoarticular disease (de Figueiredo et al., 2015). The life cycle of *Brucella* as described by Ke et al. (2015) is shown below (Figure 2.1).

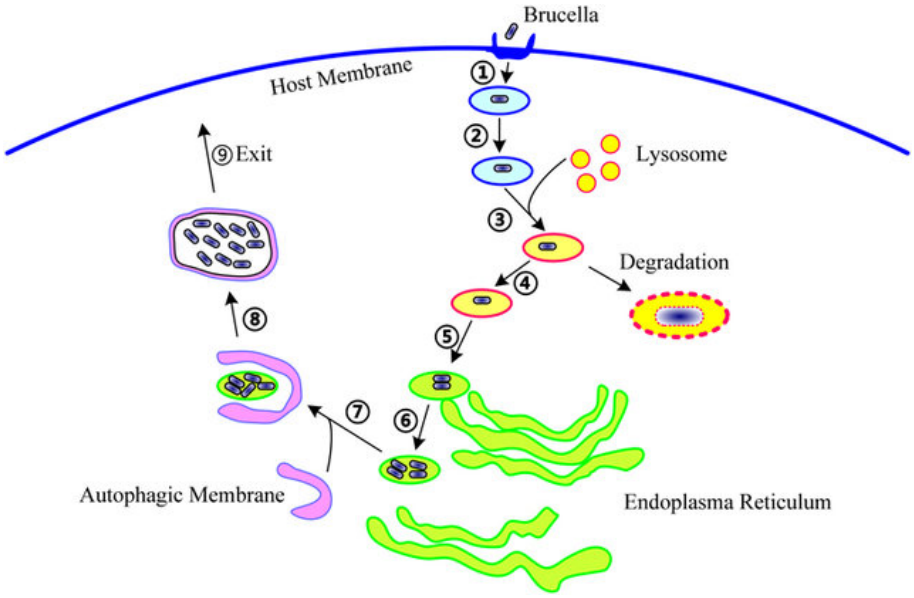

Figure 2.1. Life cycle of *Brucella* in host cells (Ke et al., 2015).

During contact with host epithelial cells, binding molecules are activated. This binding activates small GTPases that generate a signalling cascade (mitogen-activated protein kinase signalling pathway). This reorganizes the cytoskeleton and re-arrange the host cell membrane to enhance invasion (de Figueiredo et al., 2015). *Brucella* initially invade the host cells (①), interact with endosomes and acquire several markers to form *Brucella*-containing vacuoles (BCVs) (②). The BCVs then fuse with the lysosome (③). At this stage, about 90% of the *Brucella* get degraded by the action of hydrolysing enzymes, and 10% survive (④). The BCV migrate to the endoplasmic reticulum (⑤), develop the replicative niche and multiply in large numbers (⑥). *Brucella* then migrate to reach the autophagy-like vacuoles (aBCVs) (⑦ and survive in the vacuoles (⑧). They exit the host cells and spread throughout the organism (⑨) (Ke et al., 2015, Celli et al., 2003).

The presence of erythritol, a carbohydrate produced by the placentae of goats, cows and pigs, plays a key role in causing abortion in animal species (Petersen et al., 2013, Samartino and Enright, 1996). Erythritol is used by *Brucella* as a growth stimulatory factor. When released from the placenta into the circulation, it translocates *Brucella* from the lymph nodes to the reproductive tissues, where the pathogen invades the chorionic villi and reaches the cotyledons on the foetal boundary of the placenta (Santos et al., 1996, Anderson et al., 1986). The bacteria replicates to high levels of about 10^{13} bacteria/gram of tissue and activates inflammatory cells, leading to vasculitis (Alexander et al., 1981, Carvalho Neta et al., 2010). This eventually causes foetal-maternal metabolic changes, resulting in foetal loss (Anderson et al., 1986).

2.5. Economic impact of brucellosis

Brucellosis is considered a neglected zoonotic disease which significantly affects countries where there are limited resources. As a result of this, there exist only a few reports that measure the economic impact of brucellosis in small ruminants (Rossetti et al., 2017). A study carried out by Singh et al. (2015) in India revealed that brucellosis is responsible for a median loss of USD 3.4 billion in livestock. Sulima and Venkataraman (2010) calculated the average annual economic losses per animal to be Rs. 1180 for sheep and Rs. 2121.82 for goats, based on calculations on reduction in meat production and yield due to disease. Brisibe et al. (1996) estimated an annual loss of USD 3.2 million in sheep and goats in Borno and Yobe States, Nigeria. Using complement fixation test, Bamaiyi et al. (2015) estimated an annual economic impact due to caprine brucellosis to be RM7,974263.8 (USD 2,572343.1) in four states of Malaysia. Comparison of results from different publications is however difficult, since every publication utilizes different criteria (Rossetti et al., 2017).

McDermott et al. (2013) estimated the economic impact of brucellosis in Africa and south/southeast Asia and reported that brucellosis was endemic in these regions, with high prevalence in small ruminants (0 – 88.8%), followed by cattle (0 – 68.8%) and 0.4 to 20% in camels. A prevalence of 11% was estimated in livestock handlers, veterinarians, and abattoir workers. Abortions caused substantial loss to producers and the state. In addition, the cost involved in replacing culled animals is high (Campero et al., 2003).

Annually, about 500,000 cases are reported for human brucellosis (Hull and Schumaker, 2018). In humans, losses associate with brucellosis arises from different factors which includes cost involved in hospital treatment, drugs, patient out-of-pocket treatment expenses, and loss of income and work-days due to illness (Rossetti et al., 2017). In a study carried out in Málaga, Spain, the total money loss due to human brucellosis was 84.307.488 pesetas with a mean of 787.920 pesetas per patient (Colmenero Castillo et al., 1989). In Africa, the cost of treating a patient ranges from 9 EUR in Tanzania to 200 euros in Morocco, and 650 euros in Algeria (Akakpo et al., 2009).

Disability-Adjusted Life Years (DALY) is used as a measure of health outcome (Roth et al., 2003). Based on the pain and impaired productivity known to result from brucellosis, Roth et al. (2003) estimated a DALY lost due to brucellosis with the assumption that the disease is a class II (0.2) disability weight. An estimated disability weight of 0.150 is proposed as the first informed estimate for chronic, localised brucellosis and 0.190 for acute brucellosis based on disability weights from the 2004 Global Burden of Disease Study (Dean et al., 2012).

2.6. Vaccination and control measures in South Africa

The broadly used brucellosis vaccines worldwide are: *B. abortus* RB51 and *B. abortus* S19, which are used to control bovine brucellosis and *B. melitensis* Rev 1 to control small ruminants brucellosis (Frean et al., 2019). S19 vaccination interferes with serological diagnosis, whiles RB51 does not. Moreover, RB51 vaccination induces antibodies reacting in indirect and competitive Enzyme Linked Immunosorbent Assay (iELISA and cELISA) respectively. Also, using RB51 in animals leads to the development of anti-S-LPS antibodies when exposed to virulent strains (Moriyón et al., 2004). This infers that infected animals cannot be differentiated from vaccinated animals during diagnosis and therefore impedes the implementation of control programmes (Sousa et al., 2017).

South African law requires the vaccination of heifers between four and eight months of age (Department of Agriculture, Forestry and Fisheries, 2017). Currently, most cattle farmers do not comply with the brucellosis vaccination strategies. Therefore they have the potential of acquiring positive *Brucella* cases in their cattle herds (Department of Agriculture, Forestry and Fisheries, 2017). Additionally, practices such as communal grazing, lack of proper fencing and mixing of animals are a risk factor of spread of the disease among livestock (Cloete et al., 2019). With regards to zoonosis, human cases of brucellosis are considered under-diagnosed and under-reported in the country, as is the case in many resourced-limited countries where brucellosis is endemic in cattle (Wojno et al., 2016).

2.7. Diagnosis

Since clinical presentations of brucellosis are non-specific, its clinical diagnosis is difficult. Hygromas are a positive indicator of the presence of brucellosis in African flocks and herds (Akakpo, 1987, McDermott and Arimi, 2002). Perhaps since infected animals are held for longer periods, paving way for the development of this pathology. Although hygromas relate more than abortions in positive brucellosis serological test, they are not pathognomonic and requires confirmation by laboratory tests (Sanogo et al., 2013). Among these tests, only bacteriological isolation and serological tests are currently valid (OIE Terrestrial Manual., 2018). This is because serological tests have higher analytical sensitivity. Whilst the diagnostic performance (sensitivity and specificity) of other deoxyribonucleic acid (DNA) detection methods are undetermined (Yu and Nielsen, 2010). Therefore, additional tests are required. The dominating and overlapping characteristic of smooth *Brucellae* C-epitope tends to complicate the detection of the infecting *Brucella* species when using serological tests, regardless of the antigen or host species tested (Ariza, 1999, Spink, 1956). Therefore,

bacteriological isolation is required to ascertain which *Brucella* species are involved and to properly comprehend the epidemiology where diverse host species are reared together (Ducrotoy et al., 2017).

The milk ring test (MRT) is an agglutination and screening test performed on milk to detect *Brucella*-infected flocks (Farrell and Robertson, 1968). It uses haematoxylin stained *Brucella* cells added to whole milk and incubated (McCaughey, 1972). Immunoglobulins in the milk are attached to fat globules. Agglutination reaction is observed in the presence of antibodies producing a purple band on top of the milk. In the absence of an antibody, the purple antigen will be dispersed in the milk sample (Poester et al., 2010). The MRT is not sensitive. However, the advantage of this test is that it can be repeated monthly, as it is inexpensive. The disadvantage is that it cannot be performed on individual animals but only on bulk milk tanks (Godfroid et al., 2010).

2.7.1. Bacteriological diagnosis

Bacterial isolation is regarded the “gold standard” for the diagnosis of brucellosis. Although this process is usually reliable and definitive, it is limited to the availability of suitable samples. It also requires specialized media, is time consuming and to prevent exposure to laboratory workers, strict biosafety rules needs to be observed (Bricker, 2002).

Different culture media have been applied in the isolation of *Brucella* species. *Brucella ovis* could be isolated on non-selective media, for example blood agar base enriched with five to ten percent sterile ovine serum/blood. Nevertheless, it promotes the growth of other commensals, fungi and bacteria on the agar plate, since approximately four to seven days of incubation is required. This minimises the diagnostic sensitivity (OIE Terrestrial Manual., 2018). Selective media, example: the modified Farrell’s media (FM) is used mostly for the isolation of the smooth *Brucella*. This media inhibits the growth of *B. ovis* and is not recommended. The modified Thayer-Martin’s (mTM) selective medium has also been described characteristically for isolating *B. ovis* (Marin et al., 1996). However, due to the haemoglobin included as a basal component, it is not translucent, thus inappropriate for the direct visualisation of colony morphology (Alton et al., 1988).

Basal media has been used in isolating *Brucella*. Although it gives clear observation of colonies on plates, it limits the growth of rough strains and development of contaminants (Alton et al., 1988). Commercial dehydrated basal media such as trypticase soy agar is available (Alton et al., 1988). Castañeda’s medium, a non-selective, biphasic medium is recommended for isolating *Brucella* from body fluids or milk in addition to an enrichment medium. This

medium is preferred, as *Brucella* tends to dissociate in broth medium, which hinders biotyping by conventional bacteriological methods (OIE Terrestrial Manual., 2018). The CITA medium, a selective culture medium has been recently formulated (De Miguel et al., 2011). This medium is translucent and suitable and the medium of choice because, it inhibits most contaminants and permits simultaneous growth of all *Brucella* species. In addition, it is more sensitive than both mTM and FM in isolating all smooth *Brucella* strains from field samples. However, the greatest diagnostic sensitivity is attained using both FM and CITA concurrently (De Miguel et al., 2011).

2.7.2. Serological diagnosis

Serological methods for brucellosis diagnosis using blood sera include the complement fixation test (CFT), rose bengal test (RBT) or the card test, the iELISA and cELISA, serum agglutination test (SAT) (not an OIE recommended test for brucellosis for the purposes of international trade), fluorescence polarisation assay and the lateral flow immunochromatography test. These methods are easy and rapid, nevertheless false-positive reactions sometimes do arise due to similarity in the O-chain structure in smooth lipopolysaccharide portion of certain bacteria like *Yersinia enterocolitica*. Cross-reactions also do occur (Bounaadja et al., 2009). It is generally known that a sero-negative animal can still be a carrier (Alton et al., 1975). Serological results need to be interpreted carefully due to ambiguity in test implementation and validation (Ducrotoy et al., 2017).

Despite the general use of RBT as screening tool and CFT as a “confirmatory” test, this method shows that RBT is low in specificity and is only a “presumptive test” (Ducrotoy et al., 2014). Enzyme Linked Immunosorbent Assay (ELISA) requires validation in the target populations. Also, cut-offs recommended by the producers in Europe are unlikely to be sufficient in sub-Saharan Africa (OIE Manual of Diagnostic Tests for Aquatic Animals., 2019). The MRT is used indiscriminately to detect antibodies in milk. It is therefore worth noting that the test can only be applied to cattle and neither small ruminants nor camels or wildlife (Alton et al., 1988).

2.7.3. Molecular identification

Molecular tools have traditionally been used to identify *Brucella* spp. from isolates obtained from bacteriologic diagnostics and are therefore part of downstream identification protocols. Molecular protocols include polymerase chain reaction (PCR) and multilocus analysis of genome regions with variable number of tandem repeats (Bricker et al., 2003, Le Fleche et al., 2006). It is recorded that qPCR is more sensitive, rapid and less labour intensive than

conventional PCR (Alarcon et al., 2006) and has recently become the preferred choice for the identification of *Brucella* due to the high sensitivity and specificity of the assays (Redkar et al., 2001, Yu and Nielsen, 2010).

AMOS PCR assay is a multiplex molecular assay that uses a cocktail of five primers to detect *B. abortus*, *B. melitensis*, *B. ovis* and *B. suis*. An initial drawback of the AMOS assay was its inability to discriminate between *Brucella* vaccine strains: S19, RB51 and field strains from cattle with naturally occurring infections. It also could only identify biovars 1, 2, and 4 of *B. abortus* , *B*. *melitensis* biovars 1, 2, and 3, *B*. *suis* (biovar 1) and *B*. *ovis* (Bricker and Halling, 1994). Bruce-ladder PCR was then used to differentiate vaccine strains (Bricker and Halling, 1995). Thereafter AMOS-ery, developed by Ocampo-Sosa et al. (2005) to identify B. abortus bv. 3, 5, 6 and 9. This test helps in determining the *Brucella* species involved, for the purposes of public health (Ocampo-Sosa et al., 2005).

Bruce-ladder multiplex assay can detect almost all the *Brucella* spp. (Lopez-Goni et al., 2011, Garcia-Yoldi et al., 2006). The newly developed Bruce-ladder multiplex PCR assay can now discriminate between all species of Brucella and discriminates the vaccine strain *B. abortus* RB51 and S19, and *B. melitensis* Rev. 1 from the *Brucella* field strains (Lopez-Goni et al., 2011, Weiner et al., 2011).

2.7.4. Molecular diagnosis

Various PCR-based assays for either the genus or species identification of *Brucella* spp. have been published but none have been validated as a definitive diagnostic test according to the OIE Brucella Terrestrial Manual (Table 2.1).

Table 2.1. PCR-based assays used in genus and species identification of *Brucella* spp. FAM- 6-carboxy-fluorescein, FRET- fluorescence resonance energy transfer, IS711- insertion sequence, rRNA- ribosomal ribonucleic acid.

Reference	Assay type	Gene target	Species	Primers/Probes
Redkar et al., 2001	qPCR	IS711	*B. abortus*	[1]F: CAT GCG CTA TGT CTG GTT AC [2]R (*B. abortus*): GGC TTT TCT ATC ACG GTA TTC [3]P 1 (*B. abortus*): GCC CTA GAA CGC CTT TCG CAA GG P 2 (*B. abortus*) CAG ATT AAG CCG AAA CGG CCC C

[1] F- Forward primer
[2] R- Reverse primer
[3] P- Probe

			B. melitensis	R (*B. melitensis)*: AGT GTT TCG GCT CAG AAT AAT C P 1 (*B. melitensis*): GGT AAG CTA TTC CAA TCT CGC TAT TG P 2 (*B. melitensis*): TAA TGG CGT CTA TTG GAT ATT ACT GCT
			B. suis	R (*B. suis*): ACC GGA ACA TGC AAA TGA C P 1 (*B. suis*): CCC AAG CGA TAA TGC ATT CAC C P 2 (*B. suis*): CCG CAT AAG TAG GGT CTA AGC CG
Navarro et al., 2002	Conventional PCR	31-kDa 16S rRNA omp-2	*B. abortus*	F (B4): TGG CTC GGT TGC CAA TAT CAA R (B5): CGC GCT TGC CTT TCA GGT CTG primers F4/R2 F (JPF): GCG CTA AGG CTG CCG ACG CAA R (JPR): ACC AGC CAT TGC GGT CGG TA
Probert et al., 2004	Multiplex qPCR (hydrolysis probes)	bcsp31	*B. spp.* *B. melitensis*	F: GCT CGG TTG CCA ATA TCA ATG C R: GGG TAA AGC GTC GCC AGA AG P: AAA TCT TCC ACC TTG CCC TTG CCA TCA
		IS711	*B. abortus*	F: GCG GCTTTTCTATCACGGTATTC R: CAT GCG CTA TGA TCT GGT TAC G P: CGC TCA TGC TCG CCA GAC TTC AAT G
		IS711	*B. melitensis*	F: AAC AAG CGG CAC CCC TAA AA R: CAT GCG CTA TGA TCT GGT TAC G P: CAG GAG TGT TTC GGC TCA GAA TAA TCC ACA
Al Dahouk et al., 2007	qPCR (hybridization probes)	31-kDa outer membrane protein	*B. spp.*	F (B4): TGG CTC GGT TGC CAA TAT CAA R (B5): CGC GCT TGC CTT TCA GGT CTG P1 (BruFL): AGG CAA CGT CTG ACT GCG TAA AGC C P2 (BruLC): ACT CCA GAG CGC CCG ACT TG AT CG
Keid et al., 2007b	Conventional PCR	16S–23S ITS	6 classical *B. spp.*	F (ITS66): ACA TAG ATC GCA GGC CAG TCA R (ITS279): AGA TAC CGA CGC AAA CGC TAC
Kattar et al., 2007	qPCR (hybridization probes)	16S–23S ITS omp25 omp31	*B. spp.*	F (Bru ITS-S): TGC CTG TTC TGT ATG AAA TCG T R (Bru ITS-A): GCA GAA AGA CCA GCT TCT CGA P1 (ITS_FL): CTT GCT CAA GCC TTG CAT AAT GAT TGA-F P2 (ITS_LC): TGT TTA ACC GCC ATC ACC GAT TGT A-p F (Bru 25-S): GGT TAT TCC TGG GCC AAG AA R (Bru 25-A): AGC CGT GAG GTA CGG CAT A

				P1 (Melit FL+): AGG GCT TTG AAG GCT CGC TGC GT-F P2 (Melit-LC+): CCC GCG TTG GCT ACG ACC TG-p F (Bru-31-F): TGG TAA GGT CAA GTC TGC GTT R (Bru-31-R): CTT CTT CAT TCC GTG TTC GTG P1 (Bru31-FL): TGA GAG CAA GGT CAA TTT CCA CAC TG-F P2 (Bru31-LC): CGC GTC GGT CTG AAC TAC AAG TTC-p
Hinić et al., 2008	qPCR (hydrolysis probes) Conventional PCR	IS711	*B. canis* *B. suis* *B. ovis* *B. abortus* *B. neotomae*	F: GCT TGA AGC TTG CGG ACA GT R: GGC CTA CCG CTG CGA AT P: FAM-AAG CCA ACA CCC GGC CAT TAT GGT-TAMRA
Bounaadja et al., 2009	qPCR (hydrolysis probes)	IS711	*Brucella spp.*	F (IS421): CGC TCG CGC GGT GGA T R (IS511): CTT GAA GCT TGC GGA CAG TCA CC P (ISTq): FAM-ACG ACC AAG CTG CAT GCT GTT GTC GAT G-TAMRA
		bcsp31		F (BCSP1163): TCT TTG TGG GCG GCT ATC C R (BCSP1199: CCG TTC GAG ATG GCC AGTT P (BCSPTq): FAM-ACG GGC GCA ATC T MGB-NFQ
	qPCR	*per*		F (Per525): GTT TAG TTT CTT TGG GAA CAA GAC AA R (Per575): GAG GAT TGC GCG CTA GCA P: FAM-TAC GAC CGG TGA AGG CGG GAT G-MGB-NFQ
	Nested PCR	*per*		F (Per51): GTG CGA CTG GCG ATT ACA GA R (Per261): GCC TTC ACC GGT CGT AAT TGT No probe
Sidor et al., 2013	Multiplex qPCR	bcsp31 16SrRNA	*Brucella spp.* in marine species	F: GCT CGG TTG CCA ATA TCA ATG C R: GGG TAA AGC GTC GCC AGA AG P: FAM-AAA TCT TCC ACC TTG CCC TTG CCA TCA-BHQ1

2.8. Biology of *Brucella*

2.8.1. Genome

The genome of *Brucella* is encoded on 2 chromosomes, about 2.05 and 1.15 Mb in size, with chromosome 1 being larger (Michaux-Charachon et al., 1997). The closest relatives to *Brucella* are *Ochrobactrum anthropi* and *Ochrobactrum intermedium* 5.22 Mb and 4.6 Mb in size respectively. Both *Brucella* chromosomes possess comparable GC content, with an average of 57.1% and 57.3% for chromosome 1 and 2 respectively. Wattam et al. (2009) analysed nine *Brucella* genomes and reported that there was similarity in the total gene number per genome of approximately 3,460. In both *Brucella* chromosomes, there are certain distinct regions which are not shared with *Ochrobactrum*. These regions are called the shared anomalous regions (SARs). The genes in the SARs including those responsible in the synthesis of the O-polysaccharide and type IV secretion, are responsible for the ability of *Brucella* to persist and survive in its intracellular niche in the host (Wattam et al., 2009, Celli et al., 2003). A further study indicates that the IncP island, which is also a SAR contains the Tra proteins, established to be part of the type IV secretion system. It is thought to have entered *Brucella* after its divergence from *Ochrobactrum* (Lawley et al., 2003).

2.8.2. Factors affecting PCR assay performance

The challenges related to cross-reactivity and all the factors affecting PCR assay performance are dealt with in the sections below.

2.8.2.1 Gene targets

A number of gene markers have been investigated in the development of species-specific, biotype-specific, and genus-specific PCR assays, which includes the genes for 16S rRNA (F4/R2), 16S-23S internal transcribed spacer region (ITS), Omp 2b, Omp 2a and Omp 31, 31-kDa (BCSP31K) gene, 16S-23S rDNA interspace (ITS66/ITS279), IS711 (IS313/IS 639), per (bruc1/bruc5) (Probert et al., 2004, Yu and Nielsen, 2010).

False positive results have been observed with 16S rRNA and BCSP31 based PCR assays (Romero et al., 1995). While the IS711 is more sensitive and appears to be present in all *Brucella* examined to date, minor sequence variation between elements within this region in different *Brucella* species can cause very major changes in assay sensitivity (Gopaul et al., 2008). Another broadly used target, the Omp31 gene is absent in *B. abortus* (Gopaul et al.,

2008). The gene that encodes a 31-kDa protein has proven to be a good target due to the presence of species-specific signature regions in the gene (Matar et al., 1996).

2.8.2.2 DNA extraction method

The performance of an assay varies depending on the extraction method being used. Commercialized DNA extraction kits that use silica columns to adsorb DNA have proven to be effective in different sample matrices (Lusk et al., 2013), however, a proteinase K/phenol/chloroform method of extraction may be more sensitive compared to other extraction methods (Keid et al., 2007a). Commercial assays may give different results under different experimental conditions. The selection of an appropriate extraction method for a particular sample matrix is essential for a successful downstream analysis (Cankar et al., 2006). The efficiency of an assay also depends on the reagents being used in the PCR amplification, and the thermal cycler used in running the assays (Raggi et al., 2005, Buzard et al., 2012).

2.8.2.3 Probe chemistry

The use of Fluorescence resonance energy transfer and hydrolysis (TaqMan®) probes increases the specificity of the assay (Probert et al., 2004, Al Dahouk et al., 2007). The TaqMan MGB probe, an oligonucleotide which contain a donor fluorescent component and an acceptor at the 5′-end and at the 3′-end respectively (Figure 2.2). The fluorescence produced by the donor is quenched by the receptor as they remain in close proximity. In the extension phase, the bound hydrolysis probe is degraded by the 5'-3'-exonuclease activity of the *Taq* DNA polymerase, which generates fluorescence from the reporter (Navarro et al., 2015). TaqMan MGB probes have the advantage of producing accurate result, high sensitivity, specificity and reproducibility and the design of shorter probes (Kutyavin et al., 2000).

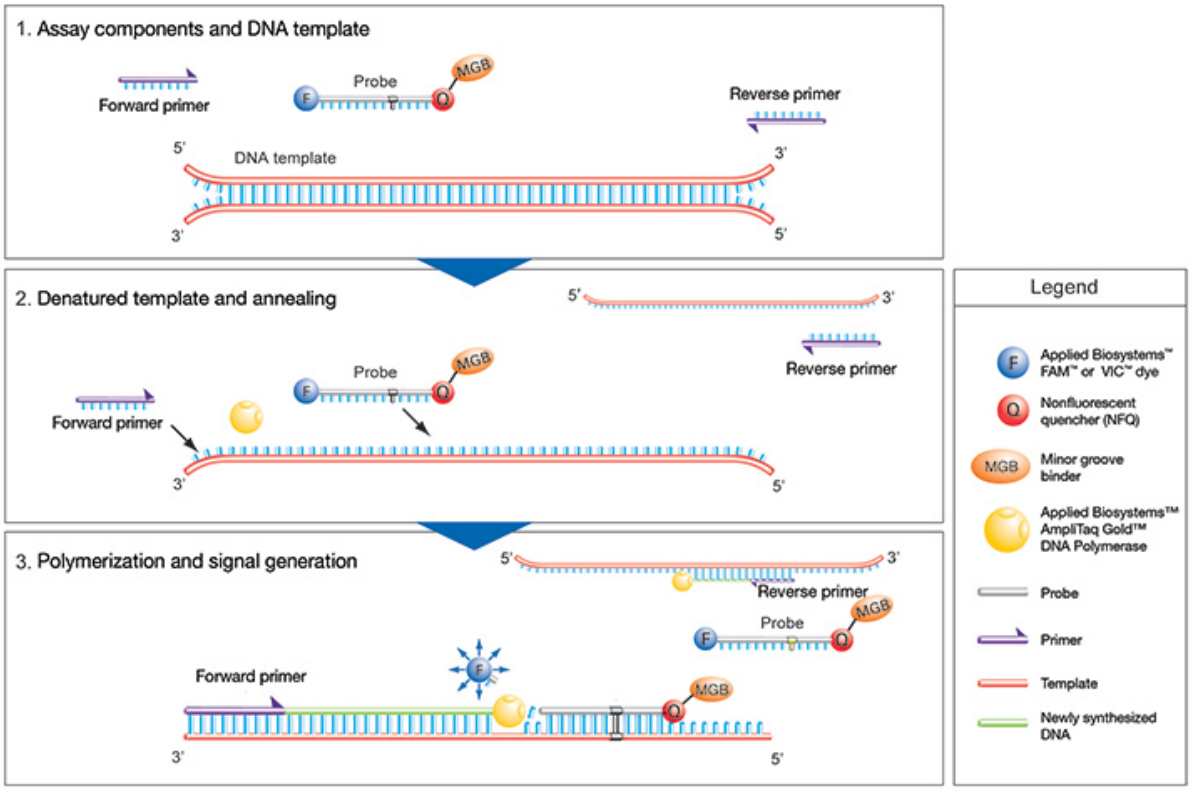

Figure 2.2. TaqMan® MGB probe-based assay chemistry (ThermoFisher, 2019).

2.8.2.4 Sample type

To ensure high diagnostic sensitivity, preparation of samples is a crucial stage recommended for a successful PCR that provides high DNA yield and purity. The ideal samples for isolation of *Brucella* species are from bovine foetuses, specifically abomasal fluid, rectal swabs, bronchial lymph nodes, lung, liver and spleen (Lusk et al., 2013). Blood and milk samples are easily obtained from animals for DNA extraction. Using blood for diagnosing brucellosis has an advantage over tissues because *Brucella* can be detected in blood during the early stages of infection. Whereas tissues can only be used when the animal is already dead.

Although tissue samples have shown valuable potential as a target for PCR diagnostics, it can only serve as a confirmatory tool after slaughter or post-mortem, rather than a screen for an active infection (López-Goñi and O'Callaghan, 2012).

Milk serves as a good sample for brucellosis diagnosis because of the persistence, ease and non-invasive nature of sample collection. Studies carried out revealed that *B. melitensis* could be repeatedly isolated from sheep (Tittarelli et al., 2005). Cattle could shed *B. abortus* in its milk nine years after primary infection (Lapraik and Moffat, 1982).

Blood, like milk, is a very accessible sample type, with the advantage that it is not limited to a subset of animals (i.e. milk can only be taken from lactating animals) and could readily be used in a screening situation. The disadvantage of using blood for screening is the uncertainties surrounding the bacteraemic phase, which varies between species and individuals within species (López-Goñi and O'Callaghan, 2012). In the case of dogs, literature suggests the period of *Brucella* bacteraemia is relatively long (Wanke, 2004), whereas a period of eight weeks has been reported for *Brucella* bacteraemia in pigs; although it can persist for longer periods in certain individuals (Dunne, 1958).

2.9. Epidemiology of *Brucella*

Despite paucity of valid epidemiological data and underreporting of brucellosis, evidence acquired over the years demonstrates that in Africa, the disease is an extensive issue (Akakpo, 1987, Ducrotoy et al., 2014, McDermott et al., 2013).

Brucellosis exhibits a wide host range and a multifaceted epidemiology (Ducrotoy et al., 2017). In addition to its socioeconomic impact, the disease is not easily identified due to the variable presentation at individual/population level (Cunningham, 1977). Although the most dominant clinical signs in small ruminants and bovines are abortions and infertility, these signs are neither disease-specific nor present in all infected animals (Cunningham, 1977). Exposure of animals to bacteria excreted could be controlled by good animal management practices. On the other hand, congenital transmission as well as the existence of animals previously asymptomatic and seronegative that later become infectious pose a serious problem (Ray et al., 1988).

In areas where brucellosis is endemic, individual overall prevalence and abortion rates are usually moderate. However, the percentage of infected herds/farms normally remains high and this signifies a possibility for the disease to escalate when transmission conditions are favourable. For instance in sub-Saharan Africa where livestock production is dominated by extensive farming systems and cattle/small ruminants are reared together (Ducrotoy et al., 2017).

The detection of antibodies against *Brucella* spp. has been identified in wildlife, example impala (*Aepyceros melampus*), zebra (*Equus burchelli*), African buffalo (*Syncerus caffer*) and blue wildbeest (*Connochaetus taurinus*) (Alexander et al., 2012, Herr and Marshall, 1981). Clinical signs associated with brucellosis in wildlife is similar to that in livestock, which includes: carpal hygroma, orchitis and abortions (Gradwell et al., 1977). Co-existence of

livestock with wild animals in the same area is a high risk factor for spread of brucellosis in wild animals (Bell et al., 1977).

The existence of *Brucella* spp. in both livestock and wild animals exacerbates the human risk of brucellosis, particularly in resource-poor areas, since they depend on animal and animal products for their livelihood. To achieve One Health in these areas, it is imperative to interrupt the transmission of the disease by implementing control measures and subsequently eradicating brucellosis (Ducrotoy et al., 2017).

2.10. Prevalence of *Brucella* spp. in sub-Saharan Africa

Prevalence of brucellosis varies worldwide. This is related to either the presence or absence of control programmes available or the vaccination status of animals (Coelho et al., 2015). Farm management practices also influences the prevalence of the disease in a herd. Normally greater in production systems where cattle are mixed together and to a lower extent, in small confined herds (McDermott and Arimi, 2002).

The true prevalence of brucellosis is difficult to determine in South Africa because of the inconsistencies of diagnostic tests and asymptomatic nature of individuals within a herd. This was detailed in the deficiencies of the available diagnostic tests, but it can be readily observed in the discrepancies in positively identified cases based on published reports (Table 2.2) and (Table 2.3) in countries across the globe.

Table 2.2. Prevalence of *Brucella* spp. as a cause of bovine and ovine abortions.

Reference	Prevalence	Assay	Target gene	Species	Sample type	Host	Extraction method
Kolo et al., 2019	12.5% (25/200) 5.5% (11/200)	Conventional PCR Culture	Genus-specific 16S-23S rRNA ITS	*Brucella* spp.	Tissues (slaughtered cattle)	Cattle	Isolate II Genomic DNA kit, Bioline (South Africa)
Huan et al., 2018	28.09% (34/120)	Conventional PCR	IS711	*B. melitensis*	Aborted foetuses	Cattle, sheep	TIANamp Genomic DNA Kit (Tiangen Biotech Co., Ltd)
Mittal et al., 2017	27.27% (24/88) 4.54%	Conventional PCR Culture	BCSP31	*B. abortus*	Foetal blood, placental tissues and foetal stomach contents	Cattle	DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) DNeasy® Stool Kit (Qiagen, Hilden, Germany)
Mahajan et al., 2017	27.1% (28/103) 6.67%	Conventional PCR Culture	omp2, IS711	*Brucella* spp. *B. abortus*	Stomach contents, aborted foetal tissue, vaginal mucus/uterine discharge	Cattle	HiPura mammalian genomic miniprep purification spin kit Himedia
Aslan et al., 2016	14%, (10/70)	qPCR		*Brucella* spp.	Aborted foetuses	Cattle	Commercial *Brucella* genus detection kit

Table 2.3. Sensitivity and specificity of published PCR assays.

Reference	Sensitivity (%)	Specificity (%)	Assay	Target gene	Species	Sample type	Host	Extraction method
Keid et al., 2007b	100.0	100.0	Conventional PCR	16S-23S rDNA interspacer (ITS)	*B. canis*	Whole blood	Dogs	Proteinase-K, sodium dodecyl sulphate and cetyl trimethyl ammonium bromide followed by phenol-chloroform purification
Kattar et al., 2007	66.7	99.7	qPCR (hybridization probe)	16S-23S rDNA interspacer (ITS)	*Brucella* spp.	Whole blood and paraffin-embedded tissues	Humans	Qiagen QIAamp DNA Mini kit (Qiagen, Hilden, Germany)
Leyla et al., 2003	97.4	100.0	Conventional PCR	Insertion sequence (IS711)	*B. melitensis*	Aborted foetuses (stomach contents)	Sheep	Simple lysis method (sonication and treatment with both non-ionic detergents and proteinase K)
Richtzenhain et al., 2002	100.0	93.0	Multiplex PCR	31 kDa outer membrane	*Brucella* spp.	Aborted foetuses	Cattle	Conventional proteinase K/ sodium dodecyl sulfate or a boiling-based extraction protocol

2.11. Status of brucellosis in South Africa

In South Africa, the existence of brucellosis was first reliably reported by Gray in 1906, although it is believed that brucellosis had been prevalent for several years before then. Gray's evidence was based on a serious outbreak in a cattle herd near Johannesburg, where the source of infection was traced to an infected cow introduced into the herd. The disease was later confirmed in 1913 by Hall, who isolated *B. abortus* from the stomach contents of an aborted foetus (Henning, 1956).

The map (Figure 2.3) shows the distribution of bovine brucellosis outbreaks in South Africa. Outbreaks of bovine brucellosis are reported in all nine provinces, but mostly in central and Highveld regions.

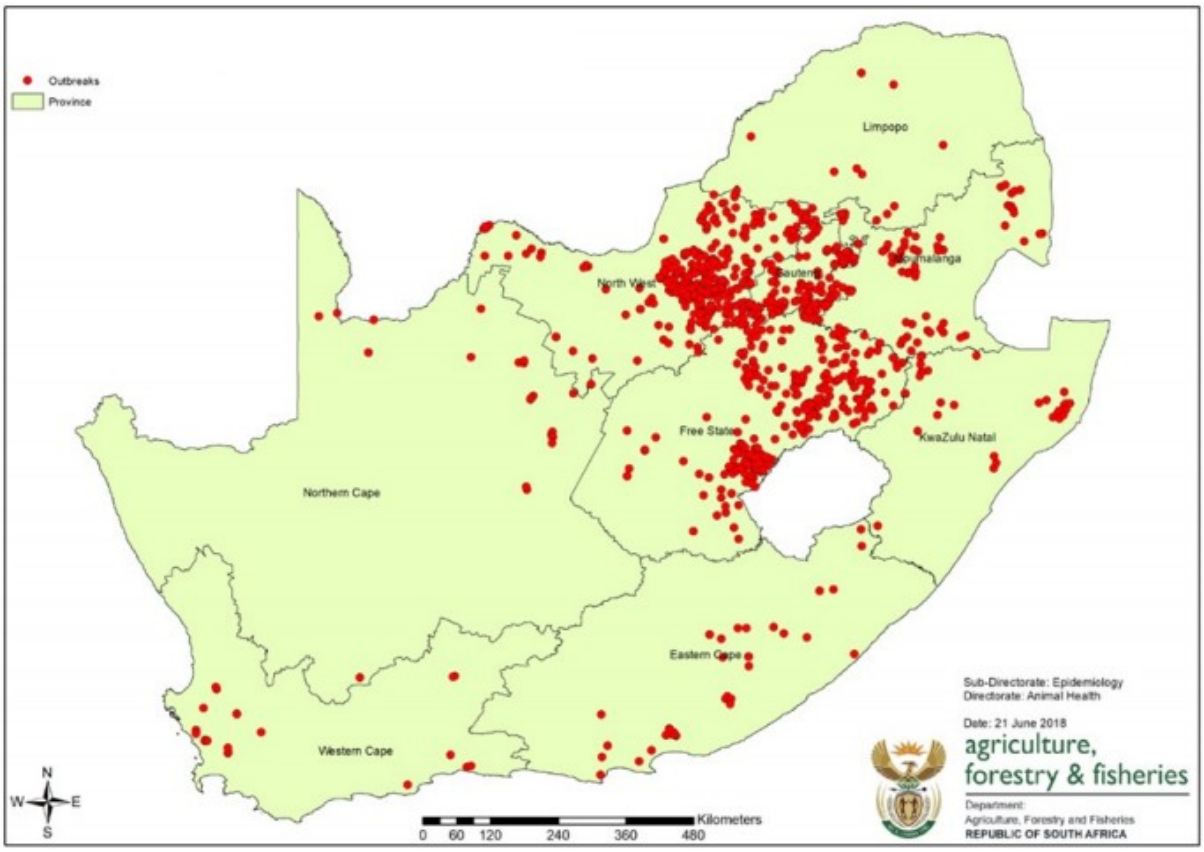

Figure 2.3. Reported *Brucella abortus* outbreaks in animals from January 2015 to May 2018 across all nine provinces of South Africa (Frean et al., 2019).

In low and middle-income countries that includes South Africa, several informal slaughter houses exist. Meat inspection do not take place in these facilities, increasing the risk of exposure to consumers (Mauff, 1980). An acute form of brucellosis in humans was reported in Johannesburg, which was implicated to be from local abattoirs (Mauff, 1980). They noted that positive blood cultures were obtained only after 7-12 days of incubation. Many laboratories

discard blood cultures after 7-10 days of incubation for practical reasons. Therefore, caution should be exercised in this regard, as there could be a possibility of positive *Brucella* cultures when kept for enough period. Inadequate information could result in false negative report on culture (Mauff, 1980).

Although some published reports on the detection of brucellosis in livestock and wild animals are available, the prevalence of the disease is unknown (Caine et al., 2017, Emslie and Nel, 2002). The S19 vaccine interferes with some serological tests (Simpson et al., 2018), which could lead to underreporting of the disease. Human brucellosis in under-diagnosed and under-reported in South Africa (Wojno et al., 2016). The disease in humans was reported in the Western Cape. A detailed history revealed that, the patient often fed his dog with waste from cattle, sheep and goat abattoir, which was disposed of at a local open-access municipal waste site. There was no history of consumption of unpasteurised milk (Wojno et al., 2016, Centre-for-Emerging-Zoonotic-Parasitic-Diseases., 2019).

2.12. Diagnostics

2.12.1. Methods for development and validation of diagnostic assays

Validation is a process that seeks to determine the fitness of an assay that has been accurately developed, optimised and standardised for an intended purpose (OIE Manual of Diagnostic Tests for Aquatic Animals., 2019).

Development and validation of an assay is a progressive process that consists of five stages (Jacobson, 1998). These include: 1. Feasibility studies on the method for a particular use (Jacobson, 1998) and the intended purpose of the assay (OIE Manual of Diagnostic Tests for Aquatic Animals., 2019), 2. Choice, optimisation, and standardisation of reagents and protocols, 3. Determination of the assay's performance characteristics, 4. Continuous monitoring of assay performance, and 5. Maintenance and enhancement of validation criteria during routine use of the assay (Jacobson, 1998).

2.12.1.1 Definition of assay purpose and feasibility studies

Defining the purpose of an assay is imperative because it serves as the basis for all subsequent steps in the validation process. Feasibility studies are carried out in the first stage of assay validation to analyse the capacity of the selected protocol and reagents to distinguish between a range of analyte concentrations (depending on the test) to an infectious agent while providing minimal background activity (Jacobson, 1998). The assay must also be defined in

terms of target animal species, target pathogen(s) or condition, and sampling matrix (OIE Manual of Diagnostic Tests for Aquatic Animals., 2019).

2.12.2. Assay development - experimental studies

2.12.2.1 Selection of optimal reagent concentrations and protocol parameters

Optimisation of the reagents and protocol involves an evaluation of accuracy, in each run of the assay, one or more sample standards of a known level of activity for the test analyte (Jacobson, 1998). It is also the process where the most significant parameters (physical, chemical and biological) of an assay are assessed and adjusted to ensure that the performance characteristics of the assay are appropriate to the intended use (OIE Manual of Diagnostic Tests for Aquatic Animals., 2019).

An optimised assay repeatedly producing the same results for a sample standard and sample controls may be designated as a standardised assay (Jacobson, 1998).

2.12.2.2 Repeatability - preliminary estimates

Repeatability is the agreement between replicates within and between runs of the assay. This is achieved by evaluating results from replicates of all samples in each plate. Thus, using the same samples run in different plates within and between runs of the assay (intra-plate and inter-plate variation). This is essential to permit further development of the assay (Jacobson, 1998).

2.12.2.3 Determination of analytical sensitivity and specificity

The analytical sensitivity of an assay is the least detectable amount of the test analyte (Jacobson, 1998). It is also referred to as the LOD. Characteristically, an estimate of the LOD is carried out by spiking the analyte into the target matrix. These experiments may be intended for precise and accurate estimation of the probability point (e.g. 50% or 100%). However, in some instances, a conservative estimate of the LOD (e.g. 100%) could be acceptable. For instance, in a tenfold dilution experiment, all replicates at all dilutions might show either 100% or 0% response. The last dilution which shows 100% response may be accepted as a conservative estimate of the lower LOD. For more accurate estimate, a second stage experiment is further carried out using narrower intervals in the dilution series with focus on the region between 100% and 0% (OIE Manual of Diagnostic Tests for Aquatic Animals., 2019).

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

2.12.3. Determining assay performance characteristics

2.12.3.1 Diagnostic sensitivity and specificity

Diagnostic sensitivity is the proportion of known infected reference animals that test positive in the assay, which means that, known infected animals that test negative are regarded as false negative results. Whereas diagnostic specificity is the proportion of uninfected animals that test negative in the assay, meaning that uninfected animals that test positive on the assay are regarded as false positive results (Jacobson, 1998). These parameters are derived from testing a series of samples from reference animals, where the history and disease status is known (Jacobson, 1998).

To estimate the diagnostic sensitivity and specificity of the developed assay, the test results must be categorised into positive or negative. This is achieved by including a cut-off point/ threshold on the continuous scale of test results (Jacobson, 1998).

2.12.3.2 Repeatability and reproducibility

Repeatability and reproducibility are estimates of precision in the assay. Precision is the degree of dispersion of results for a sample tested repeatedly. This implies, a minor amount of dispersion indicates a precise assay (Jacobson, 1998).

Reproducibility is the degree of agreement between results of samples tested in different laboratories (Jacobson, 1998).

2.12.4. Monitoring validity of assay performance

The ability of a positive or negative test result to predict infection status in the target population depends on the prevalence of infection (Jacobson, 1991).

Prevalence estimates are necessary for calculating the predictive values of positive and negative test results. It is therefore not possible to predict the infection status from test results when test values are reported without estimates of the diagnostic sensitivity and specificity of the assay (Jacobson, 1991).

2.12.5. Maintenance and enhancement of validation criteria

Constant monitoring and maintenance of a validated assay is required to retain its designation. When the assay is routinely used, it is consistently monitored to assess repeatability and accuracy in order to achieve internal quality control (Cembrowski et al., 1992). In addition, reproducibility between laboratories must be assessed at least twice each year (Jacobson, 1998).

CHAPTER
3. MATERIALS AND METHODS

3.1. *In-silico* analysis

For this study, an *in-silico* analysis was done on all available *Brucella* and closely related species. This included 132 *Brucella* species and 126 closely related species (*O. anthropi*, *O. intermedium*, *O. oryzae*, *O. otitici*, *O. ogrignonense*, *O. spp*, *S. virus*, *O. pituitosum*, *C. tokpelaia*). A list of all accession numbers is shown in Appendix E.

3.1.1. Assay design

Using GenBank accession number of *B. abortus* X95889, nucleic acid sequences of the 16S-23S rDNA ITS region of *Brucella* spp. and *Ochrobactrum* spp. were retrieved from GenBank (https://www.ncbi.nlm.nih.gov, 2019), using a nucleotide Basic Local Alignment Search Tool (BLAST®) (Altschul et al., 1990), (National Center for Biotechnology Information). The highly similar sequences (megablast) program was selected. The resulting sequences were downloaded in FASTA format. Next, the sequences of *O. intermedium* AJ867325 were downloaded using a discontiguous megablast. This was done to obtain more sequences of other bacteria with close homology to *Brucella*.

The retrieved genetic sequences were aligned online using a multiple sequence alignment programme MAFFT version 7 (Katoh et al., 2002). Data analysis in molecular biology and evolution software **(**DAMBE) (Xia and Xie, 2001) was used to identify identical sequences. Sequences were edited with BioEdit (Alzohairy, 2011). A TaqMan® MGB assay was designed using Primer Express v3.0 (ThermoFisher Scientific, USA).

The default primer/probe design parameters were as follows: melting temperature (Tm) of 58-60 degree Celsius (°C) and 68 – 70°C respectively; a percentage GC content of 30 - 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 (a ‘G’ residue has a quenching effect).

The probe was labelled with the reporter fluorescent dye FAM (Table 3.1) (Applied Biosystems, USA).

Table 3.1. Primers and TaqMan® minor groove binder (MGB) probe targeting the 16S-23S internal transcribed spacer (ITS) region of *Brucella* spp. and labelled with caboxyfluorescein (FAM) dye.

Name	Sequence (5′→3′)	Length	T_m	%GC
BrucellaITS_F	GTCGGCCTTGCGAAGCT	17	59	65
BrucellaITS_R	GCCCAGATACCGACGCAAA	19	61	58
BrucellaITS_P	FAM-ATCTGTGGATCGCGTAGTA-MGB	19	69	47

3.2. Bacterial reference strains and growth conditions

Reference isolates of *Brucella* spp. obtained from the Department of Veterinary Tropical Disease (DVTD) diagnostics laboratory, University of Pretoria and closely related non-*Brucella* species, such as *O. anthropi* were used for this study (Table 3.2). All bacterial isolates were sub-cultured on blood agar at 37˚C for 72 hours (hrs) for *Brucella* spp. and 24 hrs for other bacteria (Keid et al., 2007b).

Table 3.2. Bacterial strains used in this study.

Organism	Strain
Brucella abortus bv. 1	B01988-18
Brucella abortus bv. 1	B01897-18
Brucella abortus bv. 2	B01872-18
Brucella abortus bv. 1	B01853-18
Brucella abortus bv. 1	B01060-18
Brucella abortus bv. 1	B01100-18
Brucella melitensis	BMR 1 8001
Ochrobactrum anthropi	[4]ATCC 49687
Ochrobactrum intermedium	DNA (Genbank accession number AJ867325)*
Escherichia coli	ATCC 25922
Streptococcus agalactiae	ATCC 27956
Salmonella typhimurium	ATCC 13311
Pasteurella multocida	ATCC 12945/43137
Enterococcus faecalis	ATTC 29212
Pseudomonas aeruginosa	ATCC 27853
Chlamydia abortus	DVTD, UP

Cultures were obtained from the DVTD diagnostic laboratory. Colonies were harvested and stored in 10% glycerol and stored at -80°C for further use. These were then thawed and sub-cultures were prepared. These were streaked and inoculated on Selecta-MEDIA Blood Tryptose agar + 5% sheep blood agar (ThermoFisher Scientific, USA). The *Brucella* strains were cultured in an HF151UV CO_2 incubator (Heal Force, China) for 72 hrs. Bacterial colonies

[4] ATCC- American Type Culture Collection, bv. – biovar, DVTD, UP – Department of Veterinary Tropical Diseases, University of Pretoria. * DNA synthesised by Integrated DNA Technologies (USA).

on the plates were scraped off using 10 µl sterile inoculating loops and transferred to 1.5 millilitres (ml) Eppendorf tubes containing 200 µl phosphate-buffered saline (PBS) (Sigma-Aldrich, USA). Bacteria were quantified using a TC20™ automated cell counter (Bio-Rad Laboratories, Singapore).

Brucella can sometimes present in two or more clumps (Godfroid et al., 2013). Following Stamp staining without bipolar staining, *Brucella* shows as red in colour on a blue background. When using the peroxidase-labelled antibody conjugate or fluorochrome technique, *Brucella* stains purple in colour. However, the modified acid-fast staining works since *Brucella* can withstand weak acid treatment and together with the fluorochrome methods, they are good presumptive or primary tests for brucellosis (Godfroid et al., 2013, Roop et al., 1987).

3.3. Sample handling

3.3.1. Preparation of control samples

Blood was collected from a cow with no history of brucellosis at the Agricultural Research Council- Onderstepoort Veterinary Research (ARC-OVR) laboratory, South Africa. This animal is kept as a donor for experimental purposes. Prior to spiking, the blood was sent to the Pathology department of the Onderstepoort Veterinary Teaching Hospital to be tested (complete blood count and parasitology), carrying out a complete blood count (Appendix I). It also tested *Brucella* negative on qPCR using the developed and optimised ITS qPCR assay. Fresh milk was collected from a farm with no history of brucellosis, located at Irene, Gauteng, South Africa. Negative tissue samples were also collected. Both milk and tissue samples tested negative on the ITS qPCR. This was used as negative control. Afterwards, 180µl of negative blood and milk samples were spiked with 20 µl of the stock solution (*Brucella* colonies harvested in PBS) and then further tested to ensure it tested *Brucella* positive. Next, 20-30 milligrams (mg) of known *Brucella* positive foetal aborted tissue by culture isolation, was also tested by ITS qPCR and used as a positive control for tissues. Therefore, all three sample matrices had both positive and negative controls and used in the PCR reaction for all runs.

3.3.2. Spiking of samples

B. *abortus* bv. 1 (B01988-18 strain) with a bacterial cell count of 3.19×10^7 cells/ml was diluted with PBS in a ten-fold dilution series from 10^0 to 10^{-8}. Blood, milk or tissue sample matrix (180 µl) samples were spiked with 20 µl of the *B. abortus* dilution. Dilution calculations are shown in (Appendix G).

3.3.3. Sample size

This was a preliminary testing of field samples for diagnostic performance of the assay. The number of available samples (56) were used for the study.

3.4. DNA purification

For each sample type, the preferred appropriate method of extraction was used and run on the KingFisher™ Duo Prime purification system (ThermoFisher Scientific, USA). Tissue samples used to test diagnostic performance of the assay were prepared in triplicates. All samples were extracted using the MagMAX CORE™ Nucleic Acid Purification Kit (ThermoFisher Scientific, USA) according to the manufacturer's manual. VetMAX™ Xeno™ Internal Positive Control (IPC) DNA was added in all workflows.

3.4.1. Blood and milk

DNA from blood and milk samples was purified using the simple workflow of the MagMAX CORE™ Nucleic Acid Purification Kit. Samples were spiked as described in (3.3.2). A total of 200 µl of ach sample (180 µl milk/blood + 20 µl *B. abortus* stock solution) were extracted. The method per sample was as follows: the bead/protein kinase (PK) mix was prepared by combining 20 µl magnetic beads with 10 µl PK. Lysis/binding mixture was prepared by adding equal volumes (350 µl) of lysis solution and binding solution. Next, 500 µl 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). Row A contained 30 µl bead/PK mix, 200 µl sample and 700 µl lysis/binding buffer per well. The prepared plate was loaded onto the KingFisher™ Duo Prime Purification System (ThermoFisher Scientific, USA) and run for 27 minutes (min) using the MagMAX_CORE_DUO.bdz programme. DNA was eluted in 90 µl of elusion buffer. Purified DNA was transferred into 1.5 ml Eppendorf tubes and stored at -20°C.

3.4.2. Aborted foetal tissues

To determine which extraction method was more efficient in purifying DNA from tissues, the simple and digestion methods the MagMAX CORE™ Nucleic Acid Purification Kit of extraction were compared. The digestion method was determined to be more efficient than the simple method, as this method produced lower C_T's with high fluorescence on qPCR than using the simple method.

PK solution was prepared by adding 90 µl of PK buffer to 10 µl of PK per sample. Approximately 20–30 mg of tissue was transferred to the PK solution and incubated for 2 h at 55°C. The samples were centrifuged briefly to collect the contents at the bottom of the tube and the supernatant added to a well containing 20 µl magnetic beads. The sample was mixed for 2 min at room temperature by pipetting up and down several times. The lysis/binding solution was prepared as mentioned in (3.4.1) and 700 µl of lysis/binding solution added to each sample Two µl of VetMAX™ Xeno™ IPC DNA was added to the lysis/binding solution. Samples were processed on the KingFisher™ KingFisher™ Duo Prime Purification System.

3.4.3. Foetal abomasal fluid

The complex workflow of the MagMAX CORE™ Nucleic Acid Purification Kit was used to purify DNA from foetal abomasal fluid (300 µl of each sample), by following the manufacturer's protocol. Bead/PK mix was prepared, as described in (3.4.1) and 30 µl of the mix added to wells in row A of the deep-well plate. Lysis solution was prepared by adding 2 µl VetMAX™ Xeno™ IPC DNA to 450 µl of MagMAX™ CORE Lysis Solution per sample. The abomasal fluid samples were briefly vortexed and 300 µl of each sample added to the lysis solution. The samples were vortexed for 3 min and centrifuged at 15,000 *g* for 2 min. Six hundred µl of the supernatant was transferred to the well with the bead/PK mix and mixed for 2 min at room temperature by pipetting the solution up and down several times. MagMAX™ CORE Binding Solution (350 µl) was added to each well and samples processed on the KingFisher™ Duo Prime Purification System.

3.5. Real-time PCR

The VetMAX™-Plus qPCR Master Mix (ThermoFisher Scientific, USA) was used for DNA amplification. PCR amplification mixture comprised of 12.5 µl 2× qPCR Master Mix, 400 nanomolar (nM) (final concentration) forward/reverse primers, 150 nM (final concentration) probe, 1 µl VetMAX™ Xeno™ Internal Positive Control - VIC™ Assay (ThermoFisher Scientific, USA), 2 µl template DNA, and nuclease-free water to make up a total volume of 25 µl per reaction. The negative control contained all PCR reagents except the DNA template.

Detection and amplification of purified DNA by qPCR was performed on the StepOnePlus™ Real-Time PCR System, running StepOne Software v2.3 system (ThermoFisher Scientific, USA), using the thermal cycling conditions described in (Table 3.3).

Table 3.3. Thermal cycling conditions for the VetMAX™-Plus qPCR Master Mix (ThermoFisher Scientific, USA).

Thermal cycling step	Stage	Number of cycles	Temperature	Time
Enzyme activation	1	1	95°C	10 min
Amplification	2	40	95°C	15 sec
			55°C	45 sec

3.5.1. Assay optimisation

The primer and probe concentration of the genus-specific qPCR assay was optimised by testing diverse primer/probe concentrations in triplicates for *B. melitensis* (BMR 1 8001) and *B. abortus* (B01988-18). For primer optimization, a combination of four primer concentrations in the PCR reaction (800, 400, 200 and 100 nM) of both forward and reverse primers were tested, with a constant probe concentration in the PCR reaction kept at 250 nM. The optimised primer concentration determined above was used for probe optimisation by varying probe concentrations in the PCR reaction (250, 200, 150, 100 and 50 nM). The combination of primer and TaqMan probe concentration that yielded optimal assay performance (low cycle threshold (C_T), efficient (steep) amplification slope and low primer/probe concentration) was chosen for further experiments.

3.6. Analytical validation

The intended purpose of the assay is to i) contribute to the eradication or elimination of brucellosis from defined populations, ii) confirm diagnosis of suspect or clinical cases and iii) estimate prevalence of infection or exposure to facilitate risk analysis (surveys, herd health status, disease control measures) in samples submitted for veterinary diagnostics. The analytical performance of the assay was determined by following the Stage 1 validation pathway in Chapter 1.1.2 of the OIE Manual of Diagnostic Tests and Vaccines for Aquatic Animals of the World Organization for Animal Health (OIE Manual of Diagnostic Tests for Aquatic Animals., 2019).

3.6.1. Efficiency

B. *abortus* bv. 1 (B01988-18 strain) with a bacterial cell count of 3.19×10^7 cells/ml was diluted with PBS in a ten-fold dilution series from 10^0 to 10^{-8}. Blood was collected from a cow with no history of brucellosis at the ARC-OVR laboratory, South Africa. This animal is kept as a donor for experimental purposes. Fresh milk was collected from a farm with no history of brucellosis,

located at Irene, Gauteng, South Africa. Samples were prepared as previously described (3.3.1).

Blood, milk or tissue sample matrix (180 µl) samples were spiked with 20 µl of the *B. abortus* dilution. Samples were spiked as illustrated previously (3.3.2). DNA was purified from each dilution and the PCR performed in triplicate. The average C_T values for each dilution was calculated using Microsoft Excel. The calculation for the log bacteria per reaction is given in (Appendix G).

Standard curves for each run and dilution series were plotted showing C_T value versus log genome copies/reaction. Linear regressions of the qPCR amplification data were performed using Microsoft Excel. The slope of the regression line of the standard curve was used to calculate the efficiency of the PCR assay as: PCR efficiency (%) = $(10^{-1/\text{slope}} - 1) \times 100$.

3.6.2. Sensitivity

To determine the sensitivity of the assay, the last dilution which produced a positive result in all replicates, obtained from the efficiency analysis (70.89 bacteria/PCR reaction) was used to prepare a two-fold dilution series of *B*. *abortus* bv. 1 (B01988-18 strain) with PBS to cover the non-linear range of the assay. The two-fold dilution was carried out in order to obtain a more accurate estimate (OIE Manual of Diagnostic Tests for Aquatic Animals., 2019). Dilutions ranged from 10^0 to 10^{-9}. Dilution calculations are described in (Appendix G). Blood, milk and tissue sample matrix samples spiked with *B. abortus*. DNA from each dilution was purified five times and each purified DNA sample tested by PCR five times (each dilution was tested 25 times in total).

There are three genome copies of the 16S-23S ITS region per *Brucella* sp. bacterium (Kattar et al., 2007). Results are expressed in bacterial copies, where one bacterium is equivalent to three genome copies.

SPSS Statistics 25 software (IBM, USA) was used for probit analysis to determine the LOD.

3.6.3. Specificity

Bacterial strains as listed in table (Table 3.2) were cultured as described in section (3.2). A loop fool of colonies was harvested and mixed well in 1 ml of PBS for all the strains in Eppendorf tubes. Then these samples were each diluted 1:10 in PBS, the DNA purified using the MagMAX CORE™ Nucleic Acid Purification Kit as per section (3.4.1), and tested by PCR as described in (3.5). The final concentration of the DNA was not determined. However, DNA

was eluted in 90 µl of MagMAX CORE™ elusion buffer and 2 µl of DNA was used in the PCR reaction for all the strains. The rest of the colonies were scraped off the plate in PBS for storage. The MagMAX CORE™ Nucleic Acid Purification Kit includes an optimised lysis solution with an adjusted extraction process which improves the recovery of nucleic acid from all bacteria, including Gram-positive bacteria (ThermoFisher Scientific, 2020). Hence, no further test was carried out to check if DNA extraction worked for all.

The *O. intermedium* bacterium was not available in our laboratory as a culture and therefore the DNA sequence of *O. intermedium* that encompassed the target region of the PCR assay was synthesized by IDT (Integrated DNA Technologies) (Appendix D).

The primers were tested against a panel of other Gram-positive and Gram-negative bacteria as listed (Table 3.2).

3.6.4. Repeatability

Data obtained from the sensitivity results were used to determine the inter-run and intra-run repeatability of the assay, using Microsoft Excel. Inter-run variation was determined by using the same samples in multiple runs, which were done on multiple days. However, it involved one operator.

3.7. Diagnostic performance on field samples

3.7.1. Sample preparation

In order to ensure that there are no cross reactions in samples submitted for diagnostics from the field, a blind study of random positive and negative samples was obtained from the Bacteriology laboratory of the Department of Veterinary Tropical Diseases. A total of 56 aborted foetal tissue samples were used to evaluate the diagnostic performance of the assay. The samples comprised 24 foetal abomasal fluid samples and 32 tissue samples (which included aborted foetal liver, lung and placenta from bovine, caprine and ovine). DNA from these samples were extracted as laid out in section (3.4) and tested using ITS qPCR and the BCSP31 qPCR (Probert et al., 2004) assays in parallel.

3.7.2. Real-time PCR

The BCSP31 qPCR assay was used to compare to the developed ITS qPCR assay for the diagnostic validation because, the BCSP31 gene target is one of the most commonly used target in the detection of *Brucella* spp. in animals (Gupta et al., 2014). Also, expert information

sought from experienced laboratory personnel reveals that BCSP31 assay is routinely used by OIE reference laboratories in the detection of *Brucella* spp. in animals.

The BCSP31 qPCR can be used in a real-time PCR format. The assay described by (Probert et al., 2004) was used in a multiplex qPCR format. However, the primers and probe for the *Brucella* spp. as described by the authors was used for our assay (Table 3.4). This assay was used on a different platform (StepOne real-time PCR machine), the same used for validation of the ITS qPCR.

Table 3.4. Sequences and primers used for targeting *Brucella* spp. using the BCSP31 PCR assay (Probert et al., 2004).

PCR identification	Forward primer	Reverse primer	Probe
Brucella spp.	GCTCGGTTGCC AATATCCAATGC	GGGTAAAGCG TCGCCAGAAG	6-FAM-AAATCTTCCACCTTGCCCTTGCCATCA-BHQ1

The BCSP31 qPCR was performed using the VetMAX™-Plus qPCR Master Mix as described in (3.5), but with 300 nM forward and reverse primer concentrations and 100 nM probe concentration in the final reaction.

3.7.3. AMOS PCR

The AMOS PCR which discriminates the four species of *Brucella* was performed according to (Bricker and Halling, 1994, Weiner et al., 2011). Non-template control and *B. abortus* was used as negative and positive controls respectively. Species-specific forward primers (Table 3.5) at a 0.1 micromolar (µM) final concentration were used, 0.2 µM concentration of *IS711* reverse primer, 1x MyTaq™ Red PCR Mix (Bioline, South Africa) and 2 µl DNA template in 25 µl PCR reaction. PCR was run on Veriti® 96-Well Thermal Cycler (Applied Biosystems®). PCR conditions comprised of: a denaturation step at 95°C for 5 min, then 35 cycles at 95°C for 1 min, 55°C for 2 min and an extension step at 72°C for 10 min. PCR products were then analysed by gel electrophoresis using a 2% agarose gel (2 g of agarose in 100 ml of Tris-acetate-ethylenediamine tetraacetic acid) (Sigma-Aldrich) stained with 0.5 µg/ml ethidium bromide, viewed under ultraviolet light. The size of the PCR product amplicon was determined by using 100 base pair (bp) DNA ladder (Invitrogen, ThermoFisher® scientific, South Africa).

Table 3.5. Sequences and primers used for different *Brucella* species in the AMOS PCR assay targeting the IS711 gene (Bricker and Halling, 1994).

Primers	Sequence (5′→3′)	Amplicon size in base pair (bp)
B. abortus	GAC GAA CGG AAT TTT TCC AAT CCC	498
B. melitensis	AAA TCG CGT CCT TGC TGG TCT GA	731
B. ovis	CGG GTT CTG GCA CCA TCG TCG GG	976
B. suis	GCG CGG TTT TCT GAA GGT GGT TCA	285
IS711	TGC CGA TCA CTT AAG GGC CTT CAT	

3.7.4. Diagnostic sensitivity and specificity statistical analysis

Diagnostic sensitivity and specificity of the PCR assay were estimated in the absence of a gold standard assay, by using a three-test one-population Bayesian latent class model that allowed for conditional dependence between two of the tests and independence of the third test (Branscum et al., 2005) (Appendix F).

Modes were obtained from references (Table 2.3). For multiple references, the average of the modes were used (Table 3.6). For the sensitivity of the ITS qPCR assay, the references reported on the diagnostic sensitivity in blood samples. For the diagnostic sensitivity in aborted material, the prior for this parameter was adjusted upwards. No reference could be obtained for “prevalence”, the proportion of tested aborted material positive for *Brucella* spp. submitted to a diagnostic laboratory in South Africa and expert opinion (Dr A. Jonker, DVTD) was obtained.

Table 3.6. Priors used in a three-test one-population Bayesian latent class model for the diagnosis of *Brucella* spp. in field samples by bacterial culture, *Brucella* cell surface protein polymerase chain reaction- BCSP31 qPCR assay and ITS qPCR assay. Prevalence = proportion of tested aborted material positive for *Brucella* spp.

Parameter	Mode	5/95th percentile	α-value	β-value
Sensitivity_Culture	0.440	0.10	1.6376	1.8114
Specificity_Culture	0.590	0.15	1.9571	1.6651
Sensitivity_BCSP	0.990	0.70	8.7838	1.0786
Specificity_BCSP	0.930	0.70	12.1696	1.8407
Sensitivity_ITS	0.900	0.70	15.0342	2.5594
Specificity_ITS	0.998	0.80	13.6318	1.0253
Prevalence	0.125	0.80	1.1386	1.9702

The model was run in OpenBUGS, version 3.2.3 rev 1012, a programme for Bayesian analysis of complex statistical models using Markov chain Monte Carlo techniques (Gelfand and Smith, 1990, Lunn et al., 2009). Two chains were used and initial values were generated by forward sampling from the prior distribution for each parameter. The first 10,000 iterations were discarded, and the next 50,000 iterations used for posterior inferences. Model convergence was assessed by visual inspection of the trace plots.

CHAPTER
4. RESULTS

4.1. Assay design

When the sequence of the 16S-23S rDNA ITS region of our selected reference was BLAST searched, only 391 sequences appeared, while 500 sequences were needed. Sequences that were less than 15 bases were deleted. Identical sequences were collapsed and the region selected to design primer and probes was trimmed using DAMBE. Numerous duplicate sequences were obtained, therefore a discontiguous megablast was used resulting to 258 sequences, which were downloaded from GenBank (Appendix E) and aligned.

Using the default parameters of the Primer Express software, no acceptable primer pairs were found. However, when the maximum melting temperature (Tm) was increased from 60 to 61, suitable primers and probe were found (Table 3.1). Using *Brucella abortus* X9889 for numbering, primers and a hydrolysis probe were designed to specifically amplify a region from nucleotides 200-290 of all *Brucella* spp. (Figure 4.1).

A BLAST analysis of the primer and probe sequences on on GenBank indicated specificity for *Brucella* spp. sequences *in silico*. This implies no other sequences apart from *Brucella* spp. sequences were retrieved.


220
230
240
250
260
270
280
X95889_Babortus
CP024716_Bmelitensis (4)
CP029757_Bmelitensis (40)
CP030752_Babortus (41)
CP000708_Bovis (1)
CP027643_Bcanis (11)
CP007717_Bsuis (23)
CP006899_Bceti (2)
CP007743_Bpinnipedialis (2)
CP016980_Bspp_Rattus_spp (1)
LT599048_Bspp_Pedulis (1)
LT605586_Bspp_Tlymma (1)
CP019347_Bspp_Pedulis (1)
CP019391_Bspp_Pedulis (1)
LN998034_Bvulpis (2)
AB841199_Ooryzae (5)
AB841201_Ooryzae
AB841179_Ointermedium
AJ867331_Otritici
X95892_Oanthrois
AB841147_Oanthropi (26)
AB841163_Oanthropi
AB841144_Oanthropi
AJ242581_Ogrignonense
CP022603_Ospp (2)
CP015775_Opgrignonense (2)
EF059910_Ocytisi (2)
FJ842650_Ospp
AJ867336_Otritici (3)
AJ867335_Otritici
AJ867329_Otritici
AJ867328_Ointermedium
AJ867333_Ointermedium
AJ867325_Ointermedium (44)
AB841183_Ointermedium (3)
AB841166_Ointermedium (2)
AJ867345_Ointermedium
AJ867338_Ointermedium
AB841182_Ointermedium
AJ867344_Ointermedium

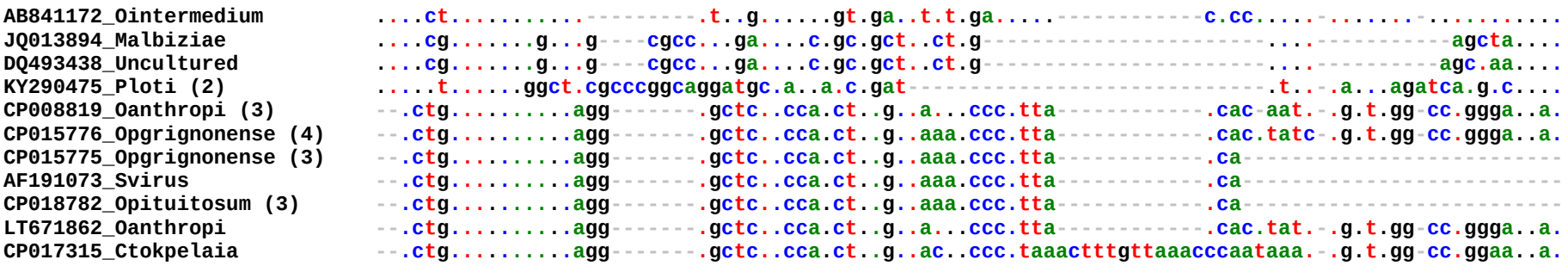

Figure 4.1. Multiple sequence alignment of the 16S-23S rDNA internal transcribed spacer (ITS) region of *Brucella* spp. and closely related *Ochrobactrum* spp. available on GenBank. N= the number of Identical sequences which have been grouped and labelled with a randomly selected sequence in the group. The number of identical sequences in a group is indicated in brackets after the name of the group. Primers and probe have been indicated by arrows and rectangle respectively. X95889 was used for numbering of sequences.

4.2. DNA purification

The digestion and simple extraction methods of the MagMAX™ CORE Nucleic Acid Purification Kit were compared and lower C_T's were obtained with the digestion method compared to the simple method, with a mean difference of 5.78. This comparison was only done for the tissue samples because either of these two extraction methods (simple or digestion) could be used for DNA extraction from tissues, as outlined by the manufacturer. We therefore wanted to find out which of the extraction technique was more efficient. For blood and milk samples, the recommended extraction method is the simple method.

The digestion method was therefore more efficient than the simple method (The average difference in C_T was 5.78 C_T lower using the digestion method, which equates to a 55-fold increase (using an efficiency of 100%) in sensitivity.

Table 4.1). The difference between simple and digestion method (ΔC_T) ranged between 0.11 to 9.45. The average difference in C_T was 5.78 C_T lower using the digestion method, which equates to a 55-fold increase (using an efficiency of 100%) in sensitivity.

Table 4.1. Comparison of cycle threshold (C_T) values, using the simple and digestion method of the MagMAX™ CORE Nucleic Acid Purification Kit (ThermoFisher Scientific), for purification of nucleic acid from foetal tissues. ΔC_T = C_T (simple method) – C_T (digestion method).

Sample	Simple method	Digestion method	ΔCT
B1569/19_RB	37.12	35.82	1.30
B1569/19_LN	37.31	37.20	0.11
B706/19	29.73	22.03	7.70
B705/19/591b/19	31.35	23.74	7.61
S0713/19	24.92	16.43	8.49
B1644/19/S2151/19	29.74	20.29	9.45

4.3. Assay optimisation

The primer and probe concentrations were optimised by performing PCR with varying primer-probe combinations (Figure 4.2). The combination of 400 nM forward and reverse primer and 150 nM probe combination resulted in an amplification plot with a low C_T, efficient (steep) amplification slope and low primer/probe concentration.

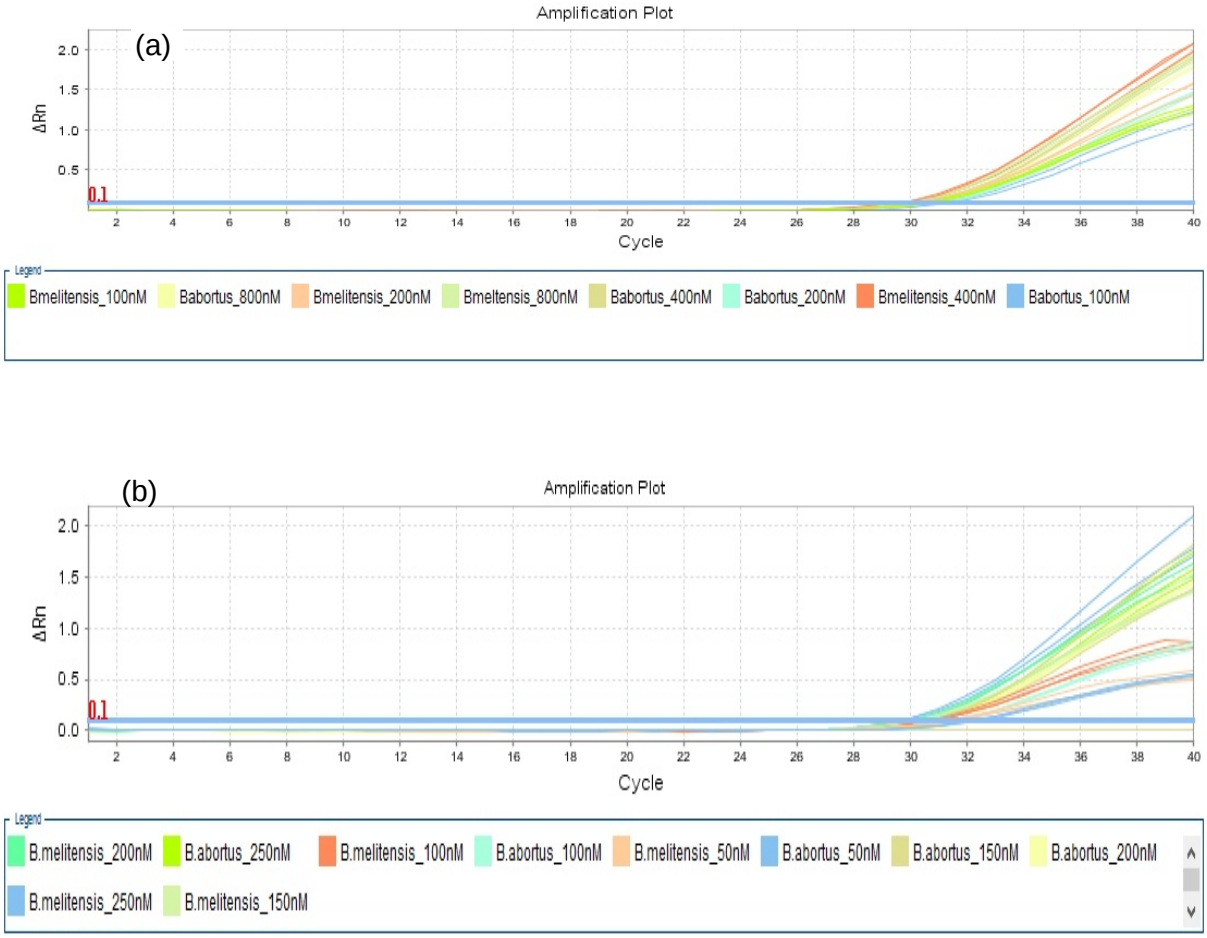

Figure 4.2. Primer (a) and probe (b) optimization of a genus-specific 16S-23S rDNA internal transcribed spacer *Brucella* spp. PCR assay. The blue line indicates a change in normalised fluorescence (ΔRn) of 0.1, the value used to determine the cycle threshold.

4.4. Analytical validation

Analytical sensitivity of an assay is the least detectable amount of the test analyte (Jacobson, 1998). It is also referred to as the LOD. The LOD is important because it aids in determining the concentration to be used as a low positive control that will be monitored to ensure consistency of performance between runs at levels near the cut-off and to ensure that the LOD does remains constant when new reagents are used. It describes the ability of the test to diagnose disease and determine treatment endpoints (Burd, 2010).

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

The PCR efficiency is the increase in amplicon per cycle (Ruijter et al., 2009). In theory, the number of templates doubles after each cycle. However, practically, the DNA increases by a factor of (1+n) where n is the cycle efficiency. Thus, an efficiency of n=1 implies a doubling of the DNA concentration. The efficiency is calculated according to the formula: $(1 + X)^n = Y$, where X is the mean efficiency per cycle, n is the number of PCR cycles, and Y is the degree of amplification (yield) after n cycles (Saiki et al., 1985).

Diagnostic sensitivity is the proportion of known infected reference animals that test positive in the assay, which means that, known infected animals that test negative are regarded as false negative results. Whereas diagnostic specificity is the proportion of uninfected animals that test negative in the assay, meaning that uninfected animals that test positive on the assay are regarded as false positive results (Jacobson, 1998). These parameters are derived from testing a series of samples from reference animals, where the history and disease status is known (Jacobson, 1998).

4.4.1. Efficiency

A ten-fold serial dilution of *B*. *abortus* (10^0-10^{-8}) was prepared in PBS. Blood, milk and tissue sample matrices were spiked with various dilutions of *B. abortus*.

Amplification of the DNA was linear for positive results within the range tested, i.e. $10^{-0.15}$ to $10^{4.85}$ bacteria/reaction (blood and milk) and $10^{-1.15}$ to $10^{4.85}$ bacteria/reaction (tissue) (Figure 4.3). This was equivalent to $10^{2.20}$ to $10^{7.20}$ bacteria/ml blood or milk, and $10^{1.20}$ to $10^{7.20}$ bacteria/ml digested tissue. Calculation of the log bacteria/PCR reaction is shown in (Appendix G).

The efficiency of the PCR assay was 105% in tissues, 99% in blood and 93% in milk. The PCR is efficient in the blood and milk samples. The acceptable efficiency ranges between 90-100 % (ThermoFisher-Scientific, 2020). In a 10- fold dilution series, a slope with -3.33 gives an efficiency of 100 %. This gives an assumption that the number of DNA template molecules in each PCR step doubles perfectly (Svec et al., 2015).

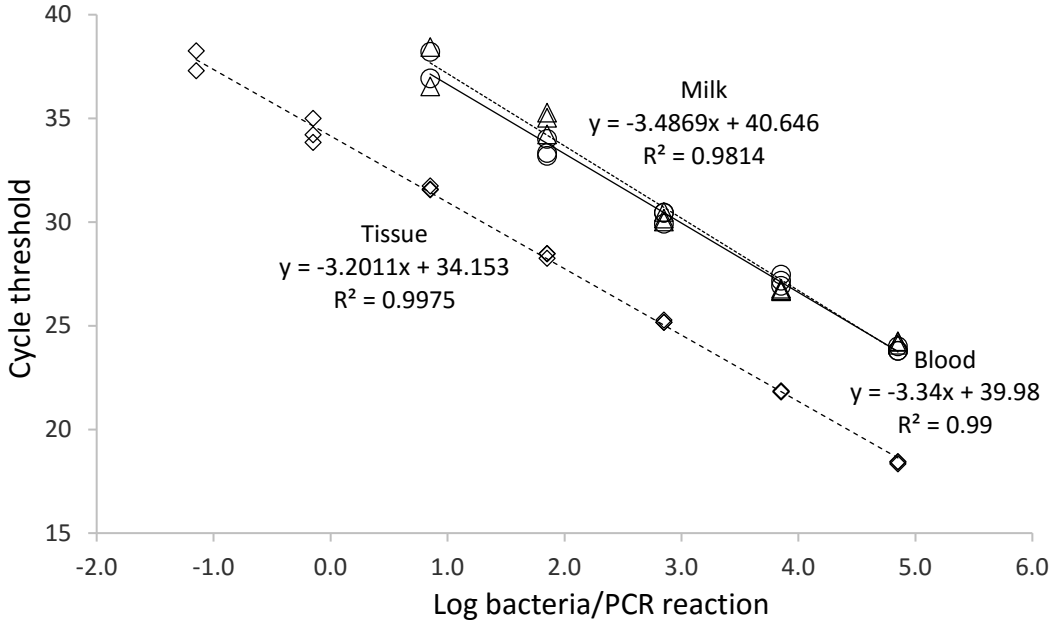

Figure 4.3. Standard curves of a genus-specific 16S-23S rDNA internal transcribed spacer *Brucella* spp. PCR assay using different matrices (i.e. blood, milk and tissue). Each dilution was run in triplicate. The equation of the regression line is indicated, as well as the coefficient of determination (R^2).

4.4.2. Analytical sensitivity

The analytical sensitivity was determined by preparing two-fold serial dilutions from $10^{-0.38}$ to $10^{2.33}$ genome copies/reaction (blood and milk) and $10^{-2.38}$ to $10^{0.33}$ genome copies/reaction (tissue). Values were obtained from the results of the probit analysis, SPSS of log values for each dilution. The analytical sensitivity also known as the LOD of an assay is the least detectable amount of the test analyte (Jacobson, 1998). The LOD is important because it aids in determining the concentration to be used as a low positive control that will be monitored to ensure consistency of performance between runs at levels near the cut-off and to ensure that the LOD does remains constant when new reagents are used. It describes the ability of the test to diagnose disease and determine treatment endpoints (Burd, 2010).

Probit regression analysis was used to calculate the 95% LOD of the assay. The assay detected 13.30 genome copies of *Brucella* DNA in blood, 45.54 in milk and 1.45 in tissues (Figure 4.4 and Table 4.2). Sensitivity in tissue was better in tissues than in milk.

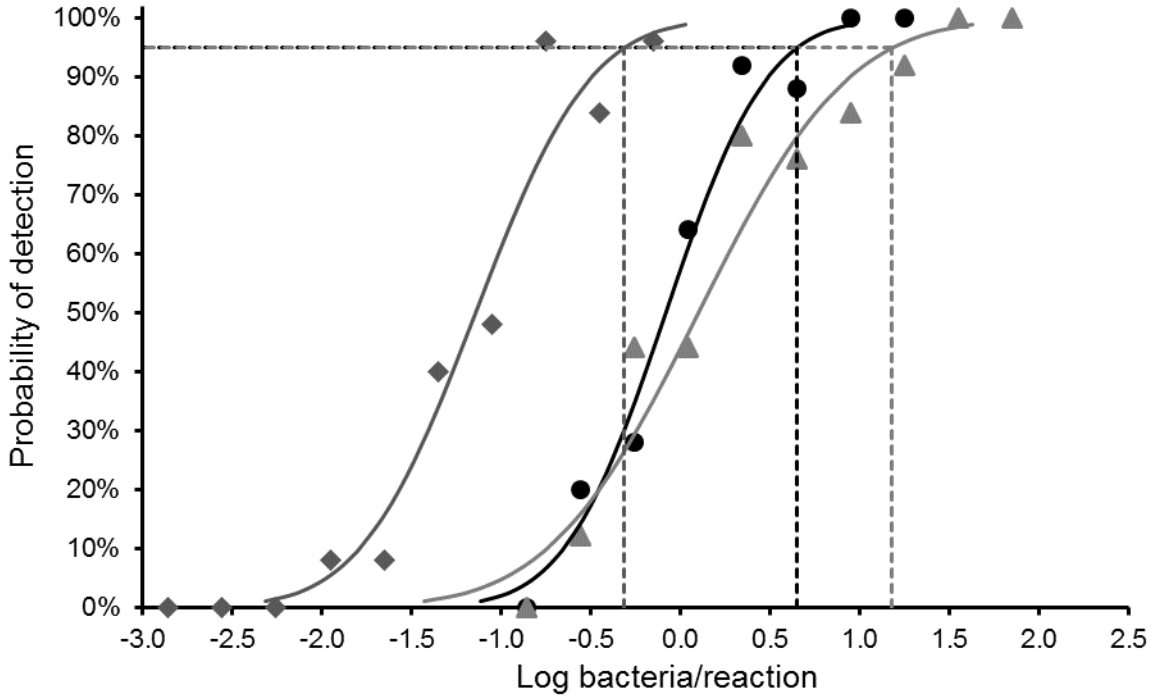

Figure 4.4. The 95% limit of detection (dotted lines) of a genus-specific 16S-23S rDNA internal transcribed spacer *Brucella* spp. PCR. The diamond shapes indicates tissue, circles indicates blood and triangles indicates milk.

Table 4.2. The 95% limit of detection of a genus-specific 16S-23S rDNA internal transcribed spacer (ITS) *Brucella* spp. PCR determined by probit analysis. CI – confidence interval.

Sample	Bacteria/PCR reaction	Genome copies/PCR reaction	Log genome copies/PCR reaction	Lower 95% CI	Upper 95%CI
Blood	4.43	13.30	1.12	0.96	1.37
Milk	15.18	45.54	1.66	1.45	1.96
Tissue	0.48	1.45	0.16	-0.02	0.42

4.4.3. Analytical specificity

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

The developed ITS-PCR assay specifically amplified DNA from all *Brucella* spp. and no amplification was recorded from other closely related bacterial spp. (Figure 4.5).

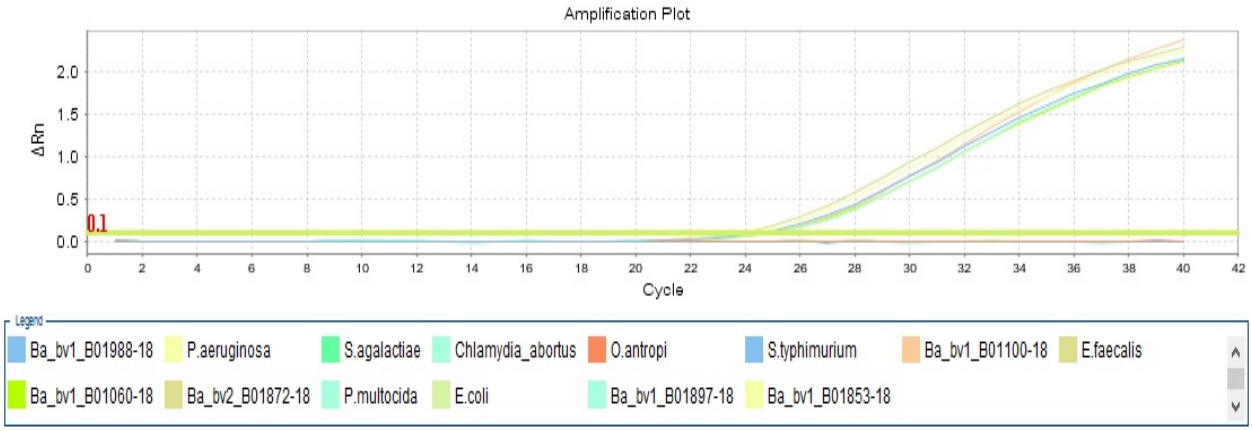

Figure 4.5. Specificity of a genus-specific 16S-23S rDNA internal transcribed spacer *Brucella* spp. PCR assay. Amplification curves are shown for all the *Brucella* spp. tested. None of the other bacterial species tested positive (baseline). The green/yellow line indicates a change in normalised fluorescence (ΔRn) of 0.1.

This figure shows the results for 14 bacterial strains tested (Table 3.2). *O. intermedium* and *B. melitensis* BMR 1 8001 were tested separately (Appendix J). All the other strains did not show any amplification, thus the baseline.

4.4.4. Repeatability

Repeatability is the agreement between replicates within and between runs of the assay. This is achieved by evaluating results from replicates of all samples in each plate. Thus, using the same samples run in different plates within and between runs of the assay (intra-plate and inter-plate variation). This is essential to permit further development of the assay (Jacobson, 1998).

The standard deviation of the inter-run and intra-run reproducibility was in the range of 0.1 to 1.7. The coefficient of variation (CV) ranged from 1.7% to 4.7% (Table 4.3). This means there is minimal variation between replicates.

Table 4.3. Inter- and intra-assay reproducibility for the detection of Brucella SD-standard deviation, CV-coefficient of variation.

Sample	Log bacteria/ PCR reaction	Inter-run SD	Intra-run SD	Total Mean	Total SD	CV (%)
Blood	1.25	0.13	0.56	31.35	0.56	1.77
Blood	0.95	0.35	0.81	32.79	0.88	2.68
Blood	0.65	0.32	0.8	33.91	0.82	2.42
Blood	0.35	0.25	0.92	35.07	0.94	2.68
Blood	0.04	0.8	0.87	36.06	1.13	3.14
Blood	-0.26	0.58	0.94	36.29	1.04	2.86
Blood	-0.56	0.41	1.19	36.71	1.17	3.18
Milk	1.85	0.32	0.81	31.00	0.82	2.65

Milk	1.55	0.31	0.82	32.74	0.80	2.45
Milk	1.25	0.43	1.22	33.73	1.37	4.07
Milk	0.95	0.46	1.01	34.23	1.14	3.34
Milk	0.65	0.32	0.90	35.41	1.06	2.98
Milk	0.35	0.40	0.84	36.03	0.91	2.51
Milk	0.04	0.29	0.60	36.76	0.54	1.46
Tissue	-0.15	0.27	1.74	34.87	1.61	4.61
Tissue	-0.45	0.52	1.32	35.78	1.30	3.62
Tissue	-0.75	0.43	1.47	34.67	1.44	4.16
Tissue	-1.05	1.05	0.00	36.16	1.51	4.17

4.5. Diagnostic performance on field samples

Fifty-six samples were tested by isolation, BSCP PCR and ITS qPCR (Table 4.4). Cut off for both BSCP31 and ITS qPCR was C_T 38 (45% LOD) and then results shown in (Table 4.5).

The ITS qPCR was more sensitive than BCSP31 qPCR because the difference in C_T was 1x increase in sensitivity. The diagnostic sensitivity of culture, ITS and BCSP31 assay are shown in (Table 4.6).

The ITS qPCR assay produced earlier C_T values than the BCSP31 assay. The difference in C_T; ΔC_T observed between ITS and BCSP31 was between 7.1 and 2.9 (Table 4.4). The C_T cut off was set at 38 using the equation: y = -3.2011x + 34.153, where x is the 95% confidence limit of a probability of 50% (0.22). The sensitivity and specificity of the ITS assay was 87% and 95% respectively, whereas the sensitivity and specificity of the BCSP31 assay was 92% and 89% respectively. That of culture was 47% and 55% respectively. The prevalence of brucellosis in aborted tissues was 33% (19/56).

Table 4.4. Comparison of bacterial culture, BCSP31 and ITS qPCR assays for the diagnosis of *Brucella* spp. from foetal abomasal fluid, foetal tissue and milk samples. Cut-off values of + < C_T = 38.00 > - were used to categorise positive (+) and negative (-) samples for both the BCSP31 and ITS (45% LOD) PCR assays. $\Delta C_T = C_T$ (ITS) - C_T (BCSP31).

Sample name	Sample type	Culture	BCSP 31 (C_T)	ITS (C_T)	ΔC_T
S0713/19	Foetal abomasal fluid	+	+ (20.02)	+ (16.78)	3.24
B1644-19_S5151-19	Foetal abomasal fluid	+	+ (20.60)	+ (17.25)	3.35
B705/19_S91b/19	Foetal abomasal fluid	+	+ (21.53)	+ (18.16)	3.37
B1988-18	Foetal abomasal fluid	+	+ (26.21)	+ (22.40)	3.81
B1631_18	Foetal abomasal fluid	-	-	+ (37.95)	
B1756/19_S2327/19	Foetal abomasal fluid	-	-	- (39.89)	
5699/18	Foetal abomasal fluid	-	-	-	
7683/18	Foetal abomasal fluid	-	-	-	
B1167_19	Foetal abomasal fluid	-	-	-	
B1377/18	Foetal abomasal fluid	-	-	-	

B1431/19	Foetal abomasal fluid	-	-	-	
B1499_18	Foetal abomasal fluid	-	-	-	
B1554/19	Foetal abomasal fluid	-	-	-	
B1568_19	Foetal abomasal fluid	-	-	-	
B1588_19	Foetal abomasal fluid	-	-	-	
B1593/19_B632/18	Foetal abomasal fluid	-	-	-	
B1800/18_S290/18	Foetal abomasal fluid	-	-	-	
B1803/19_S238/19	Foetal abomasal fluid	-	-	-	
B2063/19_S2718/19	Foetal abomasal fluid	-	-	-	
B618/18_B661/18	Foetal abomasal fluid	-	-	-	
B646/19_S865/19	Foetal abomasal fluid	-	-	-	
B647/19_S865/19	Foetal abomasal fluid	-	-	-	
B7482/19	Foetal abomasal fluid	-	-	-	
S3855_18	Foetal abomasal fluid	-	-	-	
B1644/19/S2151/19	Aborted foetal tissue	+	+ (27.34)	+ (20.24)	7.10
B1857/19_S2461/19	Aborted foetal tissue (bovine lung)	+	+ (27.83)	+ (21.65)	6.18
B706/19	Aborted foetal tissue	+	+ (28.76)	+ (21.90)	6.86
B705/19/591b/19	Aborted foetal tissue	+	+ (29.56)	+ (23.67)	5.89
B1857/19_S2461/19	Aborted foetal tissue (bovine liver)	+	+ (36.23)	+ (31.02)	5.21
B1569_19_RB	Aborted foetal tissue	+	- (39.58)	+ (35.44)	4.14
B1569_19_LN	Aborted foetal tissue	+	-	+ (37.19)	
B1144_17	Aborted foetal tissue	-	+ (36.51)	+ (31.90)	4.61
B1860/19_S2445/19	Aborted foetal tissue (bovine lung)	-	-	+ (29.26)	
B2063/19_S2718/19	Aborted foetal tissue (bovine liver)	-	-	+ (34.92)	
B215/18_S504/18	Aborted foetal tissue (ovine liver)	-	-	+ (35.68)	
B2063/19_S2718/19	Aborted foetal tissue (bovine lung)	-	-	+ (36.80)	
B1756/19_S2327/19	Aborted foetal tissue (caprine placenta)	-	-	+ (37.59)	
B2033/19_S2672/19	Aborted foetal tissue (ovine lung)	-	-	+ (37.92)	
B2033/19_S2672/19	Aborted foetal tissue (ovine placenta)	-	-	- (38.08)	
B1010/19_S1309/19	Aborted foetal tissue (ovine lung)	-	-	- (38.12)	
B1010/19_S1309/19	Aborted foetal tissue (ovine placenta)	-	-	- (38.69)	
B1488/18_B1434/18	Aborted foetal tissue (ovine placenta)	-	-	- (39.24)	
B1010/19_S1309/19	Aborted foetal tissue (ovine liver)	-	-	- (39.79)	
B1593/18_B632/18	Aborted foetal tissue (ovine lung)	-	-	-	
B1702_19_S2207_19	Aborted foetal tissue	-	-	-	

B1702_19_S2267_19	Aborted foetal tissue	-	-	-	
B1800/18_S290/18	Aborted foetal tissue (ovine lung)	-	-	-	
B1800/18_S290/18	Aborted foetal tissue (ovine placenta)	-	-	-	
B2033/19_S2672/19	Aborted foetal tissue (ovine liver)	-	-	-	
B215/18_S504/18	Aborted foetal tissue (ovine lung)	-	-	-	
B540/18_S631/19	Aborted foetal tissue (caprine liver)	-	-	-	
B540/19_S631/19	Aborted foetal tissue (caprine lung)	-	-	-	
B647/19_S8651/19	Aborted foetal tissue (ovine placenta)	-	-	-	
B771/18_S1322/18	Aborted foetal tissue (caprine placenta)	-	-	-	
B856/18_S1430/18	Aborted foetal tissue (caprine lung)	-	-	-	
B856/18_S1430/18	Aborted foetal tissue (caprine liver)	-	-	-	
S51	Milk	+	-	+ (36.42)	
1857	Milk	-	+ (22.10)	+ (19.20)	2.90
B1569/19_milk_RB_A	Milk	-	+ (36.82)	+ (31.52)	5.30
361	Milk	-	-	+ (35.30)	
LANI	Milk	-	-	+ (35.83)	
101216	Milk	-	-	+ (36.09)	
N36	Milk	-	-	+ (36.25)	
436	Milk	-	-	+ (36.39)	
437	Milk	-	-	+ (36.67)	
ROSE	Milk	-	-	+ (36.73)	
333	Milk	-	-	+ (37.08)	
A330	Milk	-	-	+ (37.24)	
V2	Milk	-	-	+ (37.56)	
566	Milk	-	-	+ (37.64)	
1228	Milk	-	-	- (38.38)	

Table 4.5. Fifty-six aborted foetal samples submitted to DVTD for *Brucella* spp. testing and classified according to test results. BSCP31 – PCR assay (Baily et al., 1992), Culture – bacterial blood agar culture ITS – internal transcribed spaced PCR (this study).

	Culture+ BSCP31+	Culture+ BSCP31-	Culture- BSCP31+	Culture- BSCP31-
ITS+	9	2	1	7
ITS-	0	0	0	37

Table 4.6. Estimates of the diagnostic sensitivity and specificity of the PCR assay.

	Median	95% probability interval
Sensitivity of the ITS-PCR	0.868	0.701 - 0.963
Specificity of the ITS-PCR	0.949	0.800 - 0.996
Sensitivity of the BCSP31-PCR	0.917	0.700 - 0.993
Specificity of the BCSP31-PCR	0.886	0.701 - 0.977
Sensitivity of bacterial isolation	0.469	0.102 - 0.868
Specificity of bacterial isolation	0.549	0.150 - 0.903

The AMOS PCR characterised *Brucella* species. Lane 3 shows amplification of a 498 bp for sample B1631_18 (*B. abortus*) (Figure 4.6), positive on ITS qPCR but negative on both BCSP31 qPCR and culture. Lane 4 shows amplification of a 731 bp for sample B1569/19_milk_RB_A (*B. melitensis*), positive on both ITS qPCR and BCSP31 qPCR, but negative on culture. *Chlamydia abortus* and *B. ovis* was isolated concurrently in sample B1144_17. Lane 5 shows amplification of a 976 bp for sample B1144_17 (*B.* ovis), which was positive on both ITS qPCR and BCSP31 qPCR. Lane 6 and 7 shows the negative and positive control respectively. Lane L shows 100 bp DNA ladder (Invitrogen, ThermoFisher® scientific, South Africa)

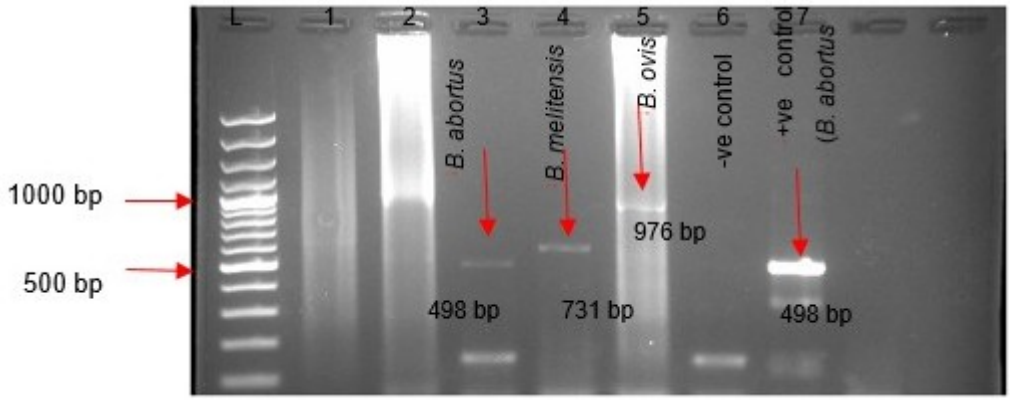

Figure 4.6. Gel electrophoresis of products from AMOS-PCR of the IS711 gene using species-specific primers.

CHAPTER
5. DISCUSSION

It is reported that brucellosis is a ‘‘difficult disease’’ in terms of its epidemiology (distribution and wide host range), economic importance and the difficulty in disease identification (Cunningham, 1977). Our study represents successful optimisation of a TaqMan MGB qPCR assay targeting the 16S-23S rDNA ITS region to detect the genus *Brucella*. The high sensitivity and the specific amplification of the required target gene sequence in the developed assay has potential for diagnostic validation.

The 16S-23S rDNA ITS qPCR assay was designed using Primer Express software. Using default parameters, no acceptable primer pairs were found. However, when the maximum melting temperature (Tm) was increased from 60°C to 61°C, an assay was developed. This may be because the melting temperature (Tm) of a PCR amplicon depends on its GC content and length (Kalendar et al., 2017). Rijpens et al. (1996) tested their primer sets against close relatives of *Brucella* spp. for specificity. With an annealing temperature of 50°C, neither of the primer pairs were specific for *Brucella* spp. There was cross-reaction with *O. anthropi*. However, when the annealing temperature was increased to 55°C, the assay was 100% specific. This confirms the fact that the melting temperature or annealing temperature should be put into consideration when designing primers and probes.

A forward and reverse primer combination of 400nM with a 150nM probe concentration displayed a plot with both the lowest C_T value and efficient amplification with minute variation between replicates. This corresponds to the optimal concentrations required for PCR experiments with TaqMan MGB probes.

A BLAST analysis of the primers and probe sequences on GenBank showed specificity for *Brucella* spp. sequences. We determined the specificity of the assay by testing *Brucella* species against a panel of closely related bacteria. The developed ITS qPCR assay gave a good specificity by amplifying DNA from all *Brucella* species and no amplification for other closely related bacterial species. *Ochrobactrum anthropi* and *O. intermedium,* which are the closest to the genus *Brucella*, were also tested. There was no amplification of these pathogens. Our result is congruent with Keid et al. (2007b), their ITS-PCR was specific since there was no amplification of *O*. *anthropi* DNA. Same result was obtained by Kattar et al. (2007) who used hybridization probes specific to the ribosomal 16S–23S ITS region, omp31 and omp25. All 3 assays exhibited an analytical sensitivity and specificity of 100%.

The developed ITS assay efficiency was 99% in blood, 93% in milk and 105% in tissues. An efficiency of more than 100 % means more than two copies of the target sequence are generated in each qPCR cycle. An efficiency exceeding 100% was recorded in tissues. This might be because the digestion method was used in extracting DNA from tissues. The type of extraction method can affect PCR results. *Brucella* is a difficult to lyse cell. Therefore, digestion of tissue and the use of PK solution enhances lysing of the bacteria (Romero and Lopez-Goñi, 1999). This might have caused the high value in tissues as compared to blood and milk. To be confident with this result, 6 tissue samples were extracted by both the simple and digestion method according to the manufacturer's protocol. It clearly showed that the digestion method gave earlier C_T values with higher amplification curves. The difference in C_T; ΔC_T between the simple and digestion method ranged between 0.11 to 9.45. This implies that the method of DNA extraction could influence PCR results. Romero and Lopez-Goñi (1999) used different extraction method for DNA extraction from milk. They observed an increase in efficiency of *Brucella* DNA extraction when high concentrations of Sodium dodecyl sulfate was added to lysis buffer and proteinase K at high incubation temperatures. They reported a LOD of 5-50 *Brucella* colony-forming units (CFU/ml) of milk. They achieved greater reproducibility when the samples were incubated with 75 ug of RNase per ml at 50°C for 2 hrs. Thus, Digestion with RNase and subsequently incubating with proteinase K. Moriyon and Berman (1982) have shown that *Brucella* cell envelops are more resistant to non-ionic detergents such as Ethylenediaminetetraacetic acid and Tris-HCl as compared to *Escherichia coli*.

The LOD of the developed ITS-PCR assay at a 95% confidence interval was 13.30, 45.54 and 1.45 genome copies/reaction in blood, milk and tissues respectively of *B. abortus* bv. 1 (B01988-18 strain). On the contrary, Kattar et al. (2007) reported a LOD of 2 genome equivalents/reaction on ITS-PCR in blood. They also recorded 3.8 femtogram (fg) of *B*. *canis* DNA (Keid et al., 2007b). It is deduced that 3.8 fg of *Brucella* DNA is equivalent to the DNA of less than two bacterial cells, putting into consideration the molecular mass of the *Brucella* genome (Baily et al., 1992).

Since *Brucella* exhibit high affinity for the fat phase of the milk, we did not manipulate the milk sample by spinning. Rijpens et al. (1996) described a PCR procedure targeting the 16S-23S rRNA spacer region. They used an enzymatic method to extract the milk components. A sensitivity of 2.8×10^4 *Brucella* CFU/ml of milk for a single PCR and reverse hybridization and 2.8×10^2 CFU/ml for a nested PCR was recorded. They again evaluated the specificity of the PCR. There were no false-positive reactions with 56 non-*Brucella* strains, except for probe BRU-ICG1, which cross-reacted with all the *O. anthropi* strains tested and one Rhizobium strain.

Fekete et al. (1992) carried out a study in 105 bovine tissue samples. *Brucella* DNA was extracted using the phenol-chloroform extraction method. As few as 5 *Brucella* cells/100 mg tissue was detected. In our assay, we used 20–30 mg of tissue for extraction. This confirms that, even with little tissue sample available, the developed ITS qPCR could still detect as little as 1.45 genome copies/reaction of *Brucella* in 20–30 mg tissue sample. Our assay shows that tissue is more sensitive. However, it can still detect *Brucella* in either milk, blood or tissue.

A study by Rijpens et al. (1996) to detect *Brucella* spp. in raw milk by nested PCR and Reverse Hybridisation with 16S-23S rRNA spacer probes gave a sensitivity of 2.8 x 10^2 CFU/ml. A study was carried out by (Mukherjee et al., 2015) to optimise a BCSP 31 gene for qPCR. Their analytical sensitivity was 30 fg and detected up to one copy number of the plasmid and 1x10^4 *Brucella* cells/reaction from spiked bovine tissue matrices.

The variation in the analytical sensitivity could again be as a result of the different extraction method being used as mentioned previously. MagMAX CORE™ Nucleic acid Purification Kit (Applied Biosystems™) was used in DNA extraction. The kit allows purification of RNA and DNA from viruses, parasites and bacteria using magnetic particle technology. It reduces false-negative results by effectively removing PCR inhibitors, making the resulting nucleic acid ideal for qPCR applications. The use of paramagnetic beads has a wide binding surface area which can disperse in solution. This allows thorough nucleic acid binding, washing, and elution (Applied Biosystems™). The use of an IPC, example the VetMAX™ Xeno™ IPC DNA aids in interpretation of results by identifying opposing factors such as contamination, amplification inhibition, or difficulties in nucleic acid extraction. It also confirms that a negative result is truly negative.

The standard deviation of the inter-run and intra-run reproducibility was in the range of 0.1 to 1.7. The CV ranged from 1.7% to 4.7%. This indicates minimal variation in results.

To evaluate how the assay performs on field samples, a blind test was carried out on 56 samples that were obtained from the Bacteriology diagnostic laboratory, DVTD. The aim of carrying out the test on field samples was also to determine this assay's potential as a screening tool in an abortion panel. Therefore, DNA was extracted and amplified on qPCR machine for both ITS and BCSP31 qPCR assays.

Test results obtained were then sent to the Bacteriology diagnostic laboratory to compare with their culture results. Thereafter, samples that came out positive on ITS but negative on both BCSP31 and culture, were further tested on AMOS PCR to determine the species involved. It is interesting to note that when these positive samples were re-cultured by the Bacteriology

diagnostic laboratory, there was growth of *Brucella* colonies, which were initially negative on culture. Therefore, the colonies were harvested and DNA extracted and amplified following the same protocol as described previously. Again, *Brucella* positive results were obtained for these samples. This shows that, positive *Brucella* cases are "missed" in traditional diagnostics. It also confirms that brucellosis is underreported in the country, since bacteriology is the "gold standard" of identification. Also, the negative results obtained from culture initially could be as a result of the use of selective media. Farrell's selective media is used mostly for the isolation of the smooth *Brucella*. This media inhibits the growth of *B. ovis*. This was confirmed on the AMOS PCR results. The AMOS PCR amplified a 976 bp (*B.* ovis) for sample B1144_17, which was positive on both ITS and BCSP31 assay. This sample tested positive for *Chlamydia abortus* (*C. abortus*) on culture initially, but negative for *Brucella*. But when this sample was cultured again, there was growth. The colonies were extracted and tested again on PCR and *B. ovis* was confirmed. This shows that this animal was co-infected with *B. ovis* and *C. abortus*. There could be an interaction between these organisms that has been under-reported because it has not been identified as a co-infection due to diagnostic limitations. Co-infections of *B. ovis* and *C. abortus* has been reported (Barkallah et al., 2014).

Sample B1631_18 (*B. abortus*) was positive on ITS but negative on both BCSP31 and culture. Sample B1569/19_milk_RB_A (*B. melitensis*) was positive on both ITS and BCSP31 assay, but negative on culture. This is a public health problem because *B. melitensis* is the main species that causes brucellosis in humans, and this was tested in milk which is one of the main route of transmission to humans. This not being isolated on culture is a problem.

We compared the diagnostic sensitivity and specificity of ITS- and BCSP31 qPCR assays in aborted tissue samples. To the best of our knowledge, no report has been given on the validation of ITS qPCR assay in the detection of *Brucella* in aborted tissue samples. Out of the 56 samples tested in our study, 9 were positive for all three tests, 37 were negative for all, 7 were negative for both culture and BCSP31 but positive on ITS,1 was positive for both ITS and BCSP31 but negative on culture. Two were positive for both culture and ITS but negative on BCSP31. This could be because of low number of bacteria in this sample. The C_T of the ITS-PCR for these samples were 37.19 and 36.42. Meaning that, the ITS assay could detect very minute amounts of DNA in a sample (Keid et al., 2007b) as compared to the BCSP31. One would say that the BCSP31 is more sensitive than culture, which is true. Nevertheless, it is worth noting that BCSP31 is highly conserved in all *Brucella* species and biovars, except *Brucella ovis* (Bricker et al., 1988).Therefore, we assume that this sample could be *Brucella ovis*. This supports the fact that the developed ITS assay can detect any species of *Brucella*.

Also, 4 samples were positive by ITS but negative on both BCSP31 and culture; this again could be as a result of small amounts of target DNA in these samples.

The ITS qPCR assay gave earlier C_Ts than the BCSP31 qPCR. The difference in C_T; ΔC_T observed between ITS and BCSP31 was between 7.1 in aborted foetal tissues and 3.24 in abomasal fluid. (Table 4.2). This result agrees with that of Kattar et al. (2007) who found out that the C_T values were 3 cycles lower for ITS-PCR in tissues than that of blood. This indicates a higher bacterial load in tissues and the possible use of these assays for diagnosing focal disease. The difference in C_T values observed between the ITS and BCSP31 assays could be as a result of the DNA copy number in these gene targets. The BCSP31 gene which encodes for a 31-kDa *Brucella abortus* antigen is present in a single copy, whereas the 16S-23SrDNA ITS region exists in three copies (Kattar et al., 2007). Therefore, it is reported that a gene target with multiple copy numbers is more sensitive.

The diagnostic sensitivity and specificity of the ITS and BSCP31-PCR was 87%/95% and 92%/89% respectively. In contrast, Katter et al., recorded sensitivity and specificity of an ITS qPCR assay of 66.7%, 99.7% respectively compared with culture at 77%, 100% on whole blood and paraffin-embedded tissue. Keid et al. (2007b) standardized and evaluated an ITS qPCR on whole blood of naturally infected dogs for the detection of *Brucella* species. Their assay had a sensitivity and specificity of 100% when positive and negative animals from group 1 and group 2 respectively were analysed. But when negative suspected dogs in group 3 were included as negative animals, diagnostic specificity decreased to 86.45%. This reduction in diagnostic specificity of PCR was attributed to animals in group 3, which comprised of animals positive by either RSAT or 2ME-RSAT or came from kennels where *B*. *canis* had been isolated from at least one dog. Our ITS assay shows a greater specificity of 95% than the BCSP31 assay; 89%.

The sensitivity and specificity of culture was 47% and 55% respectively. This shows that PCR is more sensitive than culture. The disparity in diagnostic sensitivity and specificity of PCR from various studies could be due to the use of different protocols for DNA extraction, amplification, different target genes and the choice of reference test (Keid et al., 2007b, Newby et al., 2003).

Interestingly, the ITS qPCR performance, when statistically compared to BCSP31 qPCR in our study, does not reflect the theory of ‘‘an increased sensitivity corresponding to a multiple rDNA copy number’’. This could be as a result of the prior values used in determining the sensitivity of the ITS assay in the Bayesian statistical model. The priors were obtained from the prevalence, sensitivity and specificity results from published literature. There is lack of

information available for studies carried out on detection of *Brucella* in aborted foetal tissues using the BCSP31 gene target by qPCR. Hence Priors for BCSP31 qPCR was obtained from Richtzenhain et al. (2002) (Table 2.3), which is a multiplex conventional PCR. This might have contributed to the difference in our result.

According to Ling et al (2014), non-identifiable latent class models are known to be greatly influenced by the subjective prior information used (Ling et al., 2014). For instance, in a study carried out in Thailand to determine the sensitivity and specificity of bovine tuberculosis screen tests, using a Bayesian method, they found out that there was no substantial effect on both the sensitivity and specificity of all three tests when non-informative distributions were used as priors for any of the parameters. There was a 2.7% change in the posterior estimate of the *M. bovis* Ab ELISA Sensitivity from 47.4-48.7%. However, they observed a larger difference in the posterior estimates of single intradermal tuberculin test from 62.4-31.2%. They attributed this to the model robustness and a stronger effect of the prior parameters in the model (Singhla et al., 2019).

The clinical signs that are most apparent in cattle, sheep and goats are abortions and infertility in an acute infection. In chronic cases, hygromas are mostly presented (Godfroid, 2018). However, these clinical signs are not pathognomonic as there are other causes of abortions in ruminants such as *Chlamydia abortus* in ovine. Therefore, proper diagnosis is required to confirm the presence of the pathogen in order to effect prompt control strategies to prevent further spread of the disease.

The prevalence of brucellosis in aborted foetal tissues was 33% (19/56). This shows that prevalence has increased from the initial diagnostic reports. Kolo et al. (2019) reported a prevalence of 12.5% (25/200) from screened cattle tissues by conventional ITS-PCR, however, this was conducted on abattoir from apparently healthy slaughters. A study in Assiut Governorate, upper Egypt to estimate the cause of late abortion in the sheep flocks found in this area. A total of 47 aborted foetuses with related placenta were examined. *Brucella* spp. DNA was detected in 25.5% (12/47)) of tissue samples by conventional PCR (Hussein et al., 2019).

According to the Department of Agriculture, Forestry and Fisheries (DAFF), veterinary services are unevenly distributed in the country and this is a major constraint especially in underserved and resource poor areas resulting in increased animal disease burdens (Department of Agriculture, Forestry and Fisheries, 2017). Farmers who own animals in these areas are not obliged to test their animals in order to know the disease status on their farms. This is compounded by the current Bovine brucellosis Scheme set out by DAFF, which states

that the testing of brucellosis is compulsory for only high-risk herds that have either been confirmed or suspected of being infected. Therefore, participating in the testing scheme is voluntary to all other livestock owners and herds.

The voluntary testing of animals contributes to the persistence of the disease in the country. This is confirmed by observing that bovine brucellosis is a problem especially in smallholder or non-commercial farms in most provinces (Western-Cape Government, 2016). This is because brucellosis control is more tailored to commercial farms with little or no veterinary services (testing of animals) rendered to smallholder farms. About 41.5% of the nation's herds were tested between 1977 and 1978. A prevalence of 6.6% was recorded. In north-eastern parts of Kwa-Zulu-Natal, Hesterberg et al. (2008) reported a prevalence of 1.45%-15.6%.

Another issue of concern is that, because the current bovine brucellosis testing scheme is compulsory only for high- risk herds and voluntary for all other herds and livestock owners (Department of Agriculture, Forestry and Fisheries, 2017), the status of numerous herds which were not classified as high- risk remains unknown (Department of Agriculture, Forestry and Fisheries, 2017). This leads to the assumption that the prevalence of the disease in the country is higher than previously reported (Western-Cape Government, 2016). There is minimal information on brucellosis in small ruminants, pigs and wildlife (Ducrotoy et al., 2017).

The presence of *Brucella* spp. in livestock is a major risk to public health, because livestock and animal products are the only source of infections in humans (Marcotty et al., 2009). In humans, the isolation rate of *B. melitensis* is higher than *B. abortus*. This affirms that the higher virulence of *B. melitensis* in humans is a great public health issue. Human brucellosis was reported in the Western Cape, South Africa. History revealed that, the patient often fed his dog with waste from cattle, sheep and goat abattoir which was disposed of at a local open-access municipal waste site (Wojno et al., 2016, Centre-for-Emerging-Zoonotic-Parasitic-Diseases., 2019).

Diagnosis of brucellosis mainly relies on serological and bacteriological techniques. These methods reach only suitable levels of sensitivity at a late phase of the disease when clinical signs are already apparent. It is difficult to type *Brucella* species by conventional techniques, making molecular methods the most preferred choice for typing strains after they are isolated (OIE Manual of Diagnostic Tests for Aquatic Animals., 2019). Serological results need to interpreted carefully due to ambiguity in test implementation and validation (Ducrotoy et al., 2017).

All the above mentioned including: economic importance of brucellosis, voluntary testing of animals in the country, little information on prevalence of the disease in the country, the public health cases recorded and difficulty in diagnosing the disease shows that brucellosis is under-detected and underreported in South Africa.

In view of this, our study aimed to develop and validate a 16S-23S rDNA ITS qPCR assay using a TaqMan MGB probe to detect any *Brucella* species in blood, milk and tissues.

5.1. Benefits of study

Our study represents successful optimisation of a TaqMan MGB qPCR assay targeting the 16S-23S rDNA ITS region for the detection of the genus *Brucella*. The high sensitivity and the specific amplification of the target gene sequence in the developed assay is excellent. This prevents the issue of misdiagnosis of the presence of this pathogen and allows farmers to take prompt measures to prevent the spread of brucellosis between farms and the reduction of disease risk to humans. The control of brucellosis in humans begins with the control in animal populations. Therefore, early and correct diagnosis of the disease by our assay aids in the control of the disease in South Africa.

Also, the assay could detect *Brucella* DNA in tissues with lower number of bacteria in tissues. This means that even with a lower bacterial load in tissues, the ITS assay can still detect *Brucella*. Positive results on ITS and negative on culture is an indication that brucellosis is underreported in South Africa, since bacterial isolation is the “gold standard” of diagnosing the disease. Our assay will help to detect positive herds that were initially missed out and erroneously marked as negative. This then assists in culling out positive animals and eventually will enable proper control measures on farms.

5.2. Recommendations

With persistence in exposure to abortions or foetal fluids, immunity offered by the best vaccines could be broken, therefore, regular testing is recommended. It is not feasible to apply the test and slaughter method in the private sector since farmers keep replacement animals all through the year. Therefore, regular field visits are necessary in order to be certain of full vaccine coverage. This problem is compounded by the difficulties of including small herds with very few animals and localizing all animals in nomadic pastoralist setting (Frean et al., 2019). This assay should be used in the development of multiplex assays that can detect co-infections and causative organisms during an abortion storm. This will aid our understanding in the management of this “difficult disease.”

Since the current brucellosis scheme focuses more on the testing of cattle, we recommend that small ruminants and other livestock herds should be included in the test scheme. This is because *B. melitensis* could be missed out in small ruminants that are kept in contact with cattle herds. We recommend that access to veterinary services should be available to smallholder farmers also since little attention is paid to this sector. This could be a contributing factor to the persistence of the disease in the country.

Until the ITS assay is extensively validated, it should be used in conjunction with culture isolation which is the “gold standard”.

5.3. Limitations

Vaccinated animals will come up positive in our test because, the developed ITS qPCR is a genus specific assay. Therefore, cannot differentiate between vaccinated animals from *Brucella* infected animals. Hence, downstream test such as Bruce-ladder, an assay which can differentiate *Brucella* species and vaccine strains (*B. abortus* RB51, *B. abortus* strain 19 and *B. melitensis* REV1) could be used in addition to the ITS qPCR assay. Proper field information is still important.

CHAPTER
6. REFERENCES

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7. APPENDICES

Appendix A: Research ethics approval certificate

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science

Research Ethics Committee

Project Title	Development and analytical validation of genus-specific real-time PCR and xMAP assays targeting the 16S-23S rDNA internal transcribed spacer (ITS)
Project Number	REC012-19
Researcher / Principal Investigator	Rejoice Esenam Nyarku

Dissertation / Thesis submitted for	Masters

Supervisor	Prof M Quan

APPROVED	Date: 2019-03-26
CHAIRMAN: UP Research Ethics Committee	Signature:

Office of the Chairman: Research Ethics Committee
Room 2-24, Pathology Building, Onderstepoort
University of Pretoria, Private Bag X04
Onderstepoort 0110, South Africa
Tel +27 (0)12 529 8052
Email
www.up.ac.za

Appendix B: Animal ethics approval certificate

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science

Animal Ethics Committee

Approval Certificate
New Application

AEC Reference No.: V004-19
Title: Development and analytic validation of genus-specific real-time PCR and xMAP assays targeting the 16S-23S rDNA internal transcribed spacer (ITS)
Student name: Rejoice Esenam Nyarku
Supervisor: Prof. M Quan

Dear Dr Nyarku

The **New Application** as supported by documents received 11 January 2019 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 29 January 2019.

Please note the following about your ethics approval:

1. The use of the samples obtained from project v089-16 is approved.
2. Ethics Approval is valid for 1 year and needs to be renewed annually by December 2019.
3. Please remember to use your protocol number (V004-19) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

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

We wish you the best with your research.

Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8483
Fax +27 12 529 8321
Email aec@up.ac.za
www.up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

Appendix C: Section 20 approval certificate

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/6

Dr Rejoice Esemnam Nyarku
Department of Veterinary Tropical Diseases,
Faculty of Veterinary Science,
Onderstepoort, Pretoria

Dear Dr Nyarku,

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

Your application, submitted on 14 December 2018, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following research/study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;
3. Only bacterial culture isolates from the laboratory of the Department of Veterinary Tropical Diseases obtained in the previous study "Prevalence and characterisation of selected

pathogens in slaughter animals at Gauteng province abbattoirs" by Dr. van Heerden may be used;

4. All research procedures have to be completed in the Bacteriology Laboratory of the Department of Veterinary Tropical Diseases (Biosafety level 2+);
5. Bacterial cultures and DNA extractions may be stored at the Department of Veterinary Tropical Diseases Laboratory;
6. The stored samples may only be used for further research after having obtained new Section 20 approval;
7. The stored samples may not be outsourced without prior written approval from DAFF;
8. Only a registered waste disposal company may be used for the removal of waste generated by or during the study;
9. This section 20 expires on the 31 December 2019.

Title of research/study: "Development and analytical validation of genus-specific real-time PCR and xMAP assays targeting the 16S-23S rDNA internal transcribed spacer (ITS)."
Researcher (s): Dr Rejoice Esemnam Nyarku,
Institution: Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, Onderstepoort, Pretoria
Your Ref./ Project Number:
Our ref Number: 12/11/1/1/6

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2019 -01- 08

CLASSIFICATION: CONFIDENTIAL

SUBJECT: xxxxxxxxxxxxxxxxxxxxxxxxx

Appendix D 16S-23S rDNA ITS region of *Brucella abortus* and *Ochrobactrum intermedium*.

>X95889_Brucella_abortus
taaggaagatcgagaattggaaagaggtcggatttatccggatgatccttctccatctta
ttagaacatagatcgcaggccagtcagcctgacgatcgcttgcaggcgtgccgccttcgt
ttctctttcttcattgttgattgctcacgggccgtaccgcagctgacgctgctggccctg
cgcaggcgcggcccatcagggccgacggccggtcggccttgcnaagcttcgcttcggggt
ggatctgtggatcgcgtagtagcgtttgcgtcggtatctgggcttgtagctcagttggtt
agagcacacgcttgataagcgtggggtcggaggttcaagtcctcccaggcccaccaagtt
acttgatgaggggccgtagctcagctgggagagcacctgctttgcaagcaggggtcgtc
ggttcgatcccgtccggctccaccatcatgttggtgttgagacggatattggcaatcaac
aaaagaaagaaacaagtttgcggactnttacgaaagtctgcctgttctgtatgaaatcgt
gaagagaagatgtaatcggatcaactgaagagttgatgtcgcaagaagcttgctcaagcc
ttgcataatgattgatgtgtttaaccgccatcaccgattgtatctcgagaagctggtctt
tctgctgatactgttgaaacgagcatttgcagtcgaatggcaacattcggcgtcgcataa
tgcggctttaagagctgagttttgatggatattggcaatgagagt

>AJ867325_Ochrobactrum_intermedium
taaggaagcttcggaacaggaagacgctggatttatccagatgacctttccctcgcttat
tagaacatagatcgcagagtagtcgctctgacgatcgctttgcaggcgtgccgccttcgt
ttctctttcttcattgttgattgctcacgggccgtatcgcagctgacgctgctggccctg
cgcaggcgcggcccatcggggccgacggccggtcggccttgcgaagctttgcttcggggg
tggacttctggatcgcgtagcagcgtttgcgtcggtatctgggcttgtagctcagttggt
tagagcacacgcttgataagcgtggggtcggaggttcaagtcctcccaggcccaccatgt
tacatgataaggggccgtagctcagctgggagagcacctgctttgcaagcaggggtcgt
cggttcgatcccgtccggctccaccatcgtgttttggtgtagagacggatattggcaatc
aacgaaagaaagaaacaagtttgcggacttttttgaaagtctgcctgttctgtatgaaat
cgtgaagagaagatgtaatcggatcaactatccagttgatgtcgcaatggcttgctcaag
ccttgcattatgattgatgtgtttaaccgccgtcaccgattgtatctcgagaagctggtc
tttctgctgatactgttgaaacaagcatttgcagtcgaatggcaacattcggcgtcgcat
aatgcggctttaaaagctgttttgatggatattggcaatgagagt

>AJ867325_Ochrobactrum_intermedium sequence synthesised by Integrated DNA Technologies (USA)
5′-ccg gtc ggc ctt gcg aag ctt tgc ttc ggg ggt gga ctt ctg gat cgc gta gca gcg ttt gcg tcg gta tct ggg ctt g-3′

Appendix E: 16S-23S rDNA ITS GenBank® accession numbers used in study

X95889
CP024716
CP029757
CP030752
CP000708
CP027643
CP007717
CP006899
CP007743
CP016980
LT599048
LT605586
CP019347
CP019391
LN998034
AJ867325
AJ867346
AJ867339
AJ867348
AB841189
AJ867324
AJ867347
AJ867345
AJ867337
AJ867340
AB841180
AB841177
AB841165
AB841166
AB841191
AB841194
AB841187
AB841183
AB841164
AB841176
AB841169
AJ867342
AB841178
AB841171
AB841173
AJ867338
AB841175
AB841184
AB841182
AJ867341
AB841148
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AB841167
AB841172
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AJ867333
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AJ867329
AJ867336
AJ867330
AJ867335
KF479474
AJ867320
AB841141
AB841186
AB841185
AB841150
LT671862
AB841199
AB841200
AB841198
AB841195
AB841200
EF059911
EF059911
AB841201
CP008820
AB841147
AJ867304
FJ410394
AB841156
AB841160
AB841140
AB841179
AB841154
AB841149
AB841146
AB841158
AB841159
AB841163
AB841144
CP022603
AJ242581
CP018782
JX461335
MF765457
CP018782
AJ550273
FJ356346
CP015775
EF059910
FJ842650
KY290475
FR716502
MH198744
MG976748
MF614948
MG976734
JQ013894
DQ493438
DQ659515
FJ550813
MG675070
KY290475
AJ242581
AJ550273
CP018782
FJ356346
CP015775
CP017315
LT671861
AB841151
AB841153
AB841141
AB841186
AB841185
MG675070
CP008819
LT671862
CP022604
AF191073
CP015776
CP018782
CP015775
AF191073
CP018782
CP015775
CP018782
LT671862
CP017315
MF765457
AB841193

Appendix F: Three-test one-population Bayesian latent class model

```
model{
  y[1:K, 1:K, 1:K] ~ dmulti(p[1:K, 1:K, 1:K], n)
  p[1,1,1] <- pi*SeVI*(Sefat1*Sefat2+covDp) + (1-pi)*(1-SpVI)*((1-Spfat1)*(1-Spfat2)+covDn)
  p[1,2,1] <- pi*SeVI*(Sefat1*(1-Sefat2)-covDp) + (1-pi)*(1-SpVI)*((1-Spfat1)*Spfat2-covDn)
  p[1,1,2] <- pi*(1-SeVI)*(Sefat1*Sefat2+covDp) + (1-pi)*SpVI*((1-Spfat1)*(1-Spfat2)+covDn)
  p[1,2,2] <- pi*(1-SeVI)*(Sefat1*(1-Sefat2)-covDp) + (1-pi)*SpVI*((1-Spfat1)*Spfat2-covDn)
  p[2,1,1] <- pi*SeVI*((1-Sefat1)*Sefat2-covDp) + (1-pi)*(1-SpVI)*(Spfat1*(1-Spfat2)-covDn)
  p[2,2,1] <- pi*SeVI*((1-Sefat1)*(1-Sefat2)+covDp) + (1-pi)*(1-SpVI)*(Spfat1*Spfat2+covDn)
  p[2,1,2] <- pi*(1-SeVI)*((1-Sefat1)*Sefat2-covDp) + (1-pi)*SpVI*(Spfat1*(1-Spfat2)-covDn)
  p[2,2,2] <- pi*(1-SeVI)*((1-Sefat1)*(1-Sefat2)+covDp) + (1-pi)*SpVI*(Spfat1*Spfat2+covDn)
  ls <- (Sefat1-1)*(1-Sefat2)
  us <- min(Sefat1,Sefat2) - Sefat1*Sefat2
  lc <- (Spfat1-1)*(1-Spfat2)
  uc <- min(Spfat1,Spfat2) - Spfat1*Spfat2
  rhoD <- covDp / sqrt(Sefat1*(1-Sefat1)*Sefat2*(1-Sefat2))
  rhoDc <- covDn / sqrt(Spfat1*(1-Spfat1)*Spfat2*(1-Spfat2))
  pi ~ dbeta(1.1386, 1.9702) ## Mode=0.125, 95% sure < 0.80
  Sefat1 ~ dbeta(15.0342, 2.5594) ## Mode=0.90, 95% sure > 0.70
  Spfat1 ~ dbeta(13.6318, 1.0253) ## Mode=0.998, 95% sure > 0.80
  Sefat2 ~ dbeta(8.7838, 1.0786) ## Mode=0.99, 95% sure > 0.70
  Spfat2 ~ dbeta(12.1696, 1.8407) ## Mode=0.93, 95% sure > 0.70
  SeVI ~ dbeta(1.6376, 1.8114) ## Mode=0.44, 95% sure > 0.1
  SpVI ~ dbeta(1.9571, 1.6651) ## Mode=0.59, 95% sure > 0.15
  covDn ~ dunif(lc, uc)
  covDp ~ dunif(ls, us)
}

list(n=56, K=2)
list(pi=0.125, Sefat1=0.90, Spfat1=0.998, Sefat2=0.99, Spfat2=0.93, SeVI=0.44, SpVI=0.59)
y[,1,1] y[,1,2] y[,2,1] y[,2,2]
9 1 2 7
0 0 0 37
END

#fat1 = ITS PCR, fat2 = BCSP31 PCR and VI = Bacterial isolation
```

Appendix G: Dilutions calculations

Stock (in 1 ml) = 3.19E+7 cells

Blood/milk efficiency	Dilutions in PBS	Blood/milk (200 µl)	Bacteria in PCR reaction	Bacteria/ml blood/milk
10^{0} (20ul + 180 blood)		3.19E+06	70888.89	1.60E+07
10^{-1} (20ul + 180 blood)	3.19E+06	3.19E+05	7088.89	1.60E+06
10^{-2} (20ul + 180 blood)	3.19E+05	3.19E+04	708.89	1.60E+05
10^{-3} (20ul + 180 blood)	3.19E+04	3.19E+03	70.89	1.60E+04
10^{-4} (20ul + 180 blood)	3.19E+03	3.19E+02	7.09	1.60E+03
10^{-5} (20ul + 180 blood)	3.19E+02	3.19E+01	0.71	1.60E+02
10^{-6} (20ul + 180 blood)	3.19E+01	3.19E+00	0.07	1.60E+01
10^{-7} (20ul + 180 blood)	3.19E+00	3.19E-01	0.01	1.60E+00
10^{-8} (20ul + 180 blood)	3.19E-01	3.19E-02	0.00	1.60E-01
10^{-9} (20ul + 180 blood)	3.19E-02	3.19E-03	0.00	1.60E-02
10^{-10} (20ul + 180 blood)	3.19E-03	3.19E-04	0.00	1.60E-03

Blood/milk sensitivity				
2^{0}	31900.00	3190.00	70.89	15950.00
2^{-1}	15950.00	1595.00	35.44	7975.00
2^{-2}	7975.00	797.50	17.72	3987.50
2^{-3}	3987.50	398.75	8.86	1993.75
2^{-4}	1993.75	199.38	4.43	996.88
2^{-5}	996.88	99.69	2.22	498.44
2^{-6}	498.44	49.84	1.11	249.22
2^{-7}	249.22	24.92	0.55	124.61
2^{-8}	124.61	12.46	0.28	62.30
2^{-9}	62.30	6.23	0.14	31.15

Tissue efficiency	Dilutions in PBS	PK solution (100 µl) + 25 mg tissue	Bacteria in PCR reaction
10^{0} (10 +90 PK solution)		3.19E+06	70888.89
10^{-1} (10 +90 PK solution)	3.19E+06	3.19E+05	7088.89
10^{-2} (10 +90 PK solution)	3.19E+05	3.19E+04	708.89
10^{-3} (10 +90 PK solution)	3.19E+04	3.19E+03	70.89
10^{-4} (10 +90 PK solution)	3.19E+03	3.19E+02	7.09
10^{-5} (10 +90 PK solution)	3.19E+02	3.19E+01	0.71
10^{-6} (10 +90 PK solution)	3.19E+01	3.19E+00	0.07
10^{-7} (10 +90 PK solution)	3.19E+00	3.19E-01	0.01
10^{-8} (10 +90 PK solution)	3.19E-01	3.19E-02	0.00
10^{-9} (10 +90 PK solution)	3.19E-02	3.19E-03	0.00
10^{-10} (10 +90 PK solution)	3.19E-03	3.19E-04	0.00

Tissue Sensitivity	Dilution in PBS	PK sln + tissue (100)	Bacteria in PCR reaction
2^{0}	319.00	31.90	0.71
2^{-1}	159.50	15.95	0.35
2^{-2}	79.75	7.98	0.18
2^{-3}	39.88	3.99	0.09
2^{-4}	19.94	1.99	0.04
2^{-5}	9.97	1.00	0.02
2^{-6}	4.98	0.50	0.01
2^{-7}	2.49	0.25	0.01
2^{-8}	1.25	0.12	0.00
2^{-9}	0.62	0.06	0.00

Appendix H: Sensitivity calculations

Dilution	Bacteria/reaction	Log Bacteria/reaction	Genome copies/reaction	Log genome equivalents/reaction
Blood				
2^{0}	70.89	1.851	212.667	2.328
2^{-1}	35.44	1.550	106.333	2.027
2^{-2}	17.72	1.249	53.167	1.726
2^{-3}	8.86	0.947	26.583	1.425
2^{-4}	4.43	0.646	13.292	1.124
2^{-5}	2.22	0.345	6.646	0.823s
2^{-6}	1.11	0.044	3.323	0.522
2^{-7}	0.55	-0.257	1.661	0.220
2^{-8}	0.28	-0.558	0.831	-0.081
2^{-9}	0.14	-0.859	0.415	-0.382
Milk				
2^{0}	70.89	1.851	212.667	2.328
2^{-1}	35.44	1.550	106.333	2.027
2^{-2}	17.72	1.249	53.167	1.726
2^{-3}	8.86	0.947	26.583	1.425
2^{-4}	4.43	0.646	13.292	1.124
2^{-5}	2.22	0.345	6.646	0.823
2^{-6}	1.11	0.044	3.323	0.522
2^{-7}	0.55	-0.257	1.661	0.220
2^{-8}	0.28	-0.558	0.831	-0.081
2^{-9}	0.14	-0.859	0.415	-0.382
Tissue				
2^{0}	0.71	-0.15	2.13	0.328
2^{-1}	0.35	-0.45	1.06	0.027
2^{-2}	0.18	-0.75	0.53	-0.274
2^{-3}	0.09	-1.05	0.27	-0.575
2^{-4}	0.04	-1.35	0.13	-0.876
2^{-5}	0.02	-1.65	0.07	-1.177
2^{-6}	0.01	-1.96	0.03	-1.478
2^{-7}	0.01	-2.26	0.02	-1.780
2^{-8}	0.00	-2.56	0.01	-2.081
2^{-9}	0.00	-2.86	0.00	-2.382

Appendix I: Blood analysis result

02/12/2019 04:14 PM
P04951842

VETERINARY DIAGNOSTIC LABORATORY
P/BAG X04, ONDERSTEPOORT, 0110
Request/Result Detail - CLR0001

Client

QUAN, MELVYN
DEPARTMENT OF VETERINARY TROPICAL DISEASES
FACULTY OF VETERINARY SCIENCES
PRETORIA GP 0110
W 125298142

Patient

NO 9648/5

BOVINE

Completed 21 of 21 Results

Accession #	Item	Priority	Status		Dates
D-CP10683-19	CBC	ROUTINE	COMPLETE	**Requested:**	02/12/2019 11:11am
Requesting DVM:	UNKNOWN			**Collection:**	02/12/2019 11:11am
Student:				**Received:**	02/12/2019 11:11am
Comments:				**Needed:**	02/12/2019 11:11am
				Status:	02/12/2019 12:37pm

. - HAEMATOLOGY

TEST	RESULT		UNITS	REF RANGE	RESULT DATETIME
HAEMOGLOBIN	116		g/L	80 - 140	02/12/2019 12:32pm
RED CELL COUNT	6.33		x10^12/L	5.5 - 9	02/12/2019 12:32pm
HAEMATOCRIT	0.30		L/L	.24 - .4	02/12/2019 12:32pm
MEAN CORPUSCULAR VOLUME	47.6		fL	40 - 60	02/12/2019 12:32pm
MEAN CORPUSCULAR HAEMOGLOBIN	18.4		pg	No Reference Range	02/12/2019 12:32pm
MEAN CORPUSCULAR HAEMOGLOBIN CONCENTRATION	38.6	H	g/dL	30 - 36	02/12/2019 12:32pm
RED CELL DISTRIBUTION WIDTH	18.1		%	No Reference Range	02/12/2019 12:32pm
WHITE CELL COUNT	5.33		x10^9/L	4 - 10	02/12/2019 12:32pm
SEGMENTED NEUTROPHIL	2.05		x10^9/L	.6 - 4	02/12/2019 12:34pm
BAND NEUTROPHIL	0.03		x10^9/L	0 - .12	02/12/2019 12:34pm
LYMPHOCYTE	2.29	L	x10^9/L	2.5 - 7.5	02/12/2019 12:34pm
MONOCYTE	0.40		x10^9/L	.03 - .84	02/12/2019 12:34pm
EOSINOPHIL	0.56		x10^9/L	0 - 2.4	02/12/2019 12:35pm
BASOPHIL	0.00		x10^9/L	0 - .2	02/12/2019 12:33pm
OTHER	0.00		%	No Reference Range	02/12/2019 12:33pm
PLATELET COUNT	348		x10^9/L	200 - 600	02/12/2019 12:35pm
NRBC/100 WBC	0			0 - 9	02/12/2019 12:35pm
MORPHOLOGY	1+ ANISOCYTOSIS, FEW REACTIVE LYMPHOCYTES, 1+ NEUTROPHIL TOXICITY SEVERITY, MODERATE NEUTROPHIL TOXICITY, MILD MONOCYTE ACTIVITY, 1+ SCHISTOCYTOSIS			No Reference Range	02/12/2019 12:36pm
PLATELET COMMENT	MILD PLATELET AGGREGATION			No Reference Range	02/12/2019 12:36pm
PARASITE ID	NO PARASITES SEEN ON SNEAR			No Reference Range	02/12/2019 12:37pm
SAMPLE CONDITION	CLEAR			No Reference Range	02/12/2019 12:36pm

Comments:

LYMPHOCYTE	This result has been verified
LYMPHOCYTE	This result has been verified
MORPHOLOGY	FRAGILE WBC2+

Containers

8019-27240

Appendix J: *Ochrobactrum intermedium* and *Brucella melitensis* BMR 18001