Molecular characterization of putative virulence-associated genes in *Brucella melitensis* and *Brucella abortus* isolates from livestock specimens

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By

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GENERAL ABSTRACT

In many regions of the world, brucellosis is an important zoonotic condition, In South Africa, it is a controlled animal disease, however in human it is only a reported condition. *Brucella abortus* is the principal pathogen, whereas *Brucella melitensis* has fewer predominant infections reported in animals and humans. Majority of South Africans still depend on livestock by-products such as milk for food. Multidrug resistance of *Brucella* which could be transmitted to humans through the food chain and close contact remains a big problem. The objectives of this study were to isolate and identify *Brucella* species recovered from raw milk, blood and lymph nodes of cattle, sheep and goats using culture and molecular profiling using PCR, to detect virulence genes of *Brucella* species and also determine the antibiogram of the isolates. Standard microbiological tests were used to identify *Brucella* spp. and all strains confirmed as belonging to the genus by genus-specific PCR primers. To determine the occurrence of *Brucella* virulence-associated genes, all identified isolates were further screened for the presence of the *VirB5, VirB2, BtpA, BtpB, VceC, BetB, BPE275, BSPB* and *PrpA* virulence-associated genes. Detection of the *Bru* gene was observed in 120 isolates; 74 (62 %) from cattle; 16 (13.3%) in sheep and 33 (27.5%) from goats, lymph nodes had showed the highest isolates in cattle, while the blood samples were observed with highest isolates in sheep and goat. *Brucella abortus* isolates from cattle were susceptible to moxifloxacin (5 µg), gentamicin (10 µg), levofloxacin (5 µg), ofloxacin (5 µg) and cefixime (5 µg) (100%), while high-level of resistance (100%) to penicillin G (10 units), erythromycin (15 µg), ampicillin (10 µg), amoxicillin (10 µg), trimethoprim-sulfamethoxazole (2.5 µg) and rifampicin (5 µg) were observed. Intermediate resistance of deoxycycline (30 µg) (12%) was observed for *Brucella melitensis* in goats isolates and tetracycline (5 µg) (21%) and 44% for both goats and sheep respectively. A significant number of *Brucella* isolates had a MAR (Multiple Antibiotic Resistance) index > 0.2 indicating their source to be from high-risk sources, being previously exposed to antibiotics. Approximately 100% was observed for genes *VecC* and *BetB* from *B. arbotus*. While the lowest gene observed was *PrpA* at 4.6% from *B. arbotus*. *BetB* was detected in 34.7% while *virB2* and *prpA* (0%) were not detected in *B. melitensis*. Results of the present study showed that *Brucella* spp has a lot of different virulence genes isolated from the Eastern Cape region and that cattle are still the main reservoir for *Brucella*. Continuous monitoring and surveillance in the livestock industry must be ensured to mitigate the problem for public safety.

Keywords: Brucellosis, Virulence-associated, *Brucella*, Multiple antibiotic resistance (MAR), *Brucella melitensis, Brucella abortus,* Zoonotic, Prevalence, Pathogenicity, Polymerase Chain Reaction, Putative**.**

DECLARATION

I **RUDZANI PATRICK MANAFE** student number: 802045601 hereby declare that the dissertation titled ‘‘Molecular characterization of putative virulence-associated genes in *Brucella melitensis* and *Brucella abortus* isolates from livestock specimens*’’* represents my original work and has not been previously submitted, by me to the University of Johannesburg or any other institution in application for a degree or any other qualification.

RUDZANI PATRICK MANAFE

DEDICATION

This work is dedicated to my lovely wife Thabelo Manafe for her support, love and sacrifices she has made and also My parents (Ishmael and Mashudu Manafe) for the support they showed me.

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- ✓ First, I would like to thank the Almighty God for providing me with strength to carry out my work effectively and complete this project.

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RESEARCH OUTPUTS

Manuscript in preparation for submission

- ✓ **Manafe, R.P**. and Green, E. Detection and dissemination of putative virulence genes from Brucella species isolated from livestock in Eastern Cape Province of South Africa. To be submitted to International Journal of Environmental Research and Public Health.

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LIST OF ABBREVIATIONS AND ACRONYMS

CDC…… Centers for Disease Control and Prevention

CLSI…… Clinical and Laboratory Standards Institute

DAFF…….. Department of Agriculture, Forestry and Fisheries

EFSA…….…….. European Food Safety Authority

FAO…….…… Food and Agriculture Organization

JFAO…….……..... Joint Food and Agriculture Organization

MAR…….…….. Multiple Antibiotic Resistance

OIE……. World Organisation for Animal Health, formerly International des Epizooties

WHO…….……. World Health Organization

PCR…… Polymerase Chain Reaction

LIST OF UNITS

%	Percentage
°C	Degree Celsius
µl	Microlitre
min	Minute(s)
s	Second(s)
bp	Base pair(s)
l	Litre

ORGANIZATION OF THE DISSERTATION

- **Chapter 1: General introduction**

This chapter focuses on the background of the study, problem statement and aim and objectives of the study.

- **Chapter 2: Literature review**

This chapter presents a comprehensive review on *Brucella* species, brucellosis, pathogenicity of *Brucella*, virulence factors of *Brucella*, and identification, prevention and treatment of brucellosis are discussed.

- **Chapter 3: *Brucella* discerning and antibiogram profiling associated with *Brucella* species isolated from livestock in Eastern Cape Province, South Africa**

This chapter deals with Identification of *Brucella* spp, DNA extraction and molecular characterization of *Brucella* and also antibiotic susceptibility.

- **Chapter 4: Detection and dissemination of putative virulence genes from Brucella species isolated from livestock in Eastern Cape Province of South Africa**

This chapter deals with identification of different putative virulence genes in *Brucella* isolates, isolated from sheep, goat and cattle in Eastern Cape, South Africa. These genes are involved in pathogenicity of *Brucella* species.

- **Chapter 5: General discussion, conclusions and recommendations**

This chapter presents the general discussion and conclusion based on chapters three to chapter four. The chapter also states recommendations for future studies.

- **Appendices**

This includes Appendix I for chapter 3 and Appendix II for chapter 4

CHAPTER ONE

*General Introduction

*This chapter introduces the background of the research project, problem statement as well as aim and objectives.

1.1. INTRODUCTION

Worldwide, *Brucella* spp. are responsible for one of the most important zoonotic and contagious bacterial disease known as brucellosis, an ancient disease with worldwide prevalence, which has a large impact on human morbidity. Infection by this bacterium results in reproductive failure in livestock thus leading to a huge loss economically, impacting the international trade (Gopinath, 2016). In human, infection is caused by the three species *B. abortus*, *B. melitensis* and *B. suis* (De Miguel *et al*., 2011). Additional economic impact as a results of *Brucella* infections include, loss in milk production, or no milk production, preventative programmes for humans as well as absenteeism from work can be costly and increase production costs (Shoukat *et al*., 2017).

According to Department of Agriculture, Forestry and Fishery (DAFF), (2015) terrestrial wildlife species together with mammals have been a great source for documentation of *Brucella* spp worldwide. A number of wild animals in South Africa, namely; the hippopotamus, zebra, sable, eland, waterbuck and impala, have tested positive for brucellosis. Due to the infrequent contact with domestic animals like cattle, this species was not that important, but there has been an increase in contact between the two thus increasing the spread of bovine brucellosis in Southern Africa.

Genus *Brucella* is a Gram negative, a member of the α-2 proteobacteria which causes brucellosis, which is a group of zoonosis infections that infect both animals and humans (Ducrotoy *et al*., 2017). Brucellosis affects humans by infecting the mucous membranes such as eyes and mouth, or by handling tissues that are infected. *Brucella* can lead to long term health problems for example, Malta fever in humans which can be chronic, and meningoencephalitis in livestock to mention a few, if not treated, may lead to acute or chronic forms, and the latter can lead to long-term health impacts (Mazyck *et al*., 2006, Franco *et al*, 2007).

Furthermore, ingesting contaminated food samples such as unpasteurized milk, milk products like cheese, and not ensuring that preventative measures are taken during slaughtering can also lead to the risk of contamination in humans (DAFF, 2015). Over the years *Brucella* species that were initially recognized, include *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis* and *B*, n*eotomae* (Azam *et al*., 2016). All members of the genus *brucella,* genetically and immunologically are closely affiliated to each other thus, they vary at molecular level, as a

result they have a lot of virulence factors that cause undesirable pathogenicity (Razzaq, Alsaadi, & Al-yassari, 2014).

Brucella melitensis is the main cause of human brucellosis and is usually associated with abortion of the foetus. This pathogen serves as the main contaminant in sheep and goats, whereas the reservoir for *B*. *abortus* is cattle (Wojno *et al.*, 2016). It was reported that known bacterial pathogenic factors that directly harm eukaryotic cells, such as cytolisins, exotoxins, exoproteases and exoenzymes, and the expression of pathogenic determinants, like fimbriae, capsules, antigenic variation and plasmids was not found in *brucella* (Baldi & Giambartolomei, 2013; He, 2012). However, we cannot rule out genes that were identified as virulence genes in other organisms if they are detected in *Brucella*. Because brucellosis is currently underreported in Africa and South Africa, and no complete treatment so far is available, only certain vaccines are administered, knowing more about the virulence genes for brucellosis, can help in finding a more complete treatment at a molecular level.

1.2.PROBLEM STATEMENT

Africa is growing in population, hence milk and meat, which are animal based protein sources vital in the food industry, because they are abundant, safe and cheaper to produce (DAFF, 2015). In South Africa, millions of people depend on this high-quality protein source for their wellbeing, making livestock production that is safe from contamination of brucellosis, is vital.

There is a widespread contamination of milk and beef by *Brucella* species making brucellosis in South African livestock a problem to the economy mainly affecting the production system of cattle, impacting on the dairy as well as the beef industries. Even with only 10% of dairy herds infected, it can cause a chain reaction of millions of rands lost annually, that's why it's a controlled livestock disease in South Africa in terms of Animal Disease Act 35 of 1984 due to its impact in the economy (Du Preez and Malan, 2015). The organism also causes brucellosis in humans, making it a healthcare problem.

From 1996 to 2004, between 291 and 457 bovine brucellosis outbreaks were reported yearly to OIE14 (OIE, 2008), showing that it has been long known in South Africa. High prevalence of brucellosis was reported in intensively farmed areas in Southern Africa because of the large number of livestock available. According to Godfroid (2004), farmers in Southern Africa were reported to lose an estimated 300 million rand in 1990 due to the 14.7% of their herds being

infected. Bakunzi *et al.*, (1993), reported lower sero-prevalance detected in remote rural village cattle as compared to higher sero-prevalence in peri-urban areas.

Areas of poorly established domestic animals programme and improper public health programmes are the most prevalent areas (Wojno *et al*., 2016). The United States of America's Center for Disease Control and Prevention (CDC) identified Africa as among the most commonly affected areas. Human brucellosis in South Africa is considerably under diagnosed, hence under reported as in many parts of the world (Wojno *et al*., 2016).

Because of the potential threat of the use of *Brucella* as a biological weapon, the interest has grown with regard to production of brucellosis. Growth of tourism and migration internationally, resulted in increase in infection of different wild and domesticated animals (Al-Nassir, 2018). And also knowing the prevalence of virulence factors, and comparing them to other different virulence factors, we can determine which genes are found around which areas, or if different genes are responsible for virulence according to regions, this information can then be used to help with the control of brucellosis, specific methods can be developed to target specific genes.

1.1. AIM AND OBJECTIVES

AIM

The aim of the study is to determine the prevalence of putative virulence genes associated with *Brucella abortus and Brucella melitensis* isolates from livestock specimens using molecular methods.

OBJECTIVES

The objectives of the study are:

- To test for antimicrobial susceptibility of different *Brucella* isolates obtained from cow raw milk.
- To extract genomic DNA from isolates, determine the concentration and quality of DNA using a nano drop and gel electrophoresis.
- To perform PCR amplification of the putative virulence genes of interest using primers specific to the target genes.

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CHAPTER TWO

*LITERATURE REVIEW

*This chapter presents a comprehensive review on *Brucella* species, Brucellosis, Pathogenicity of *Brucella*, virulence factors of *Brucella*, and identification, Prevention and treatment of Brucellosis are discussed.

2.1 *Brucella*

The genus *Brucella* belongs to phylum Proteobacteria, Class Alphaproteobacteria, Order Rhizobiales, and Family Brucellaceae. Becouse of the occurrence of pleomorphic forms, the structure of all *Brucella* spp. are justly constant, apart from old cultures (Scholz & Vergnaud, 2013).This gram negative genus does not sporulate, require oxygen to grow, nonmotile and are coccobacilli (0.5- 1.5µm) (Garin-bastuji *et al*., 1998). Different *Brucella* spp have different hosts, with *Brucella melitensis*, *Brucella abortus*, *Brucella canis* and *Brucella suis* being isolated or hosted mainly in sheep, goats, cattle, dogs and pigs. Sometimes a single host can be infected by two different species, for example, both *B. abortus* and *B. melitensis* can infect the cattle at the same time (Shoukat *et al.*, 2017).

Other species include *B. Ovis* which was also isolated from sheep (Buddle, 1965); *B.neotomae* from desert wood rat (Stoenner & Lackman, 1957). Recently other species of *Brucella* have been identified from cetaceans, pinnipeds (Foster *et al*, 2007) and voles (Scholz *et al*., 2008), which are *B. ceti*, *B. pinnipediae* and *B. microti* respectively. All members of the genus *Brucella* are closely related to each other but vay at molecular level, as a result, they have a lot of virulence factors that cause undesirable pathogenicity (Razzaq *et al,* 2014).

2.2 *Brucella* species

2.2.1 *Brucella abortus*

Under normal conditions *B. abortus*, which is a facultative intracellular pathogen, contaminate amongst others, man, dog, horse, goats and sheep, but the main contaminate being cattle (Kudi *et al*, 1997). The difference is rather small between the nine *B. abortus* biovars (1-9), that so far have been reported [Centers of Disease Control and Prevention (CDC, 2005)]. Milk production decreases as a result of brucellosis infections in milk producing animals because somatic cell count in milk is multiplied, and abortion increased, as well as postpartum metritis (Meador & Deyoe, 1989). Mainly bovine brucellosis is an infection in cattle, isolation in affected livestock usually happens in the uterus, udder, and lymphoid organs (Poester *et al*., 2006; Xavier *et al*, 2010).

According to Schelling *et al*. (2003) and Muma *et al*. (2007), because cattle are the dominant distributor of bovine brucellosis in farmyards, they undergo termination of pregnancy than non-contaminated ones. Oral entry remains the pivotal route of infection by *Brucella*, such as taking in food or water that is infected as well as licking aborted foetuses or new-borns from contaminated cows (Díaz Aparicio, 2013).

Brucella is passed on to the calves by their mothers through the milk that they consume (Díaz Aparicio, 2013). Because sometimes the same teat cups can be used during milking, this increases the risk of cow to cow infection in milk producing farms (Díaz Aparicio, 2013). As a result milking of healthy cows before contaminated cows can reduce the spread of *brucellosis* (OIE, 2008; Samartino, 2003; Samartino & Enright, 1993).

2.2.2 *Brucella melitensis*

Amongst all the species under the genus *Brucella,* this is the most virulent one, and within the three biovars it contains, 1 and 3 are the ones identified regularly in areas such as the Middle East, Latin America and also the Mediterranean which are sheep rearing locations (Banai, 2002; Lucero, 2008; McDermot *et al,* 2013). Because of the negative economic impact brucellosis has on the trade industry of both animals and their by-products, it has been classified as a very strong zoonosis (Banai, 2002; Benkirane, 2006; Seleem *et al*, 2010). Sheep and goat are the most favoured hosts for *B. melitensis*, but goats remain *B. melitensis*'s classic and natural hosts, cattle and other ruminants can also be affected (Verger *et al*, 1995).

Even though *B. melitensis and B. abortus* contamination is the same for ruminants and cattle respectively, the main difference in ruminant's infection from brucellosis is, stillbirths and abortion. This usually occurs in the latter stages of pregnancy and its only once in a lifetime (Blasco & Molina-Flores, 2011; Elzer *et al.,* 2002). Contamination usually happens by means of close contact of infected animals's secretions from genital tract of females (Alton, 1990) , whereas calves are contaminated by *B. melitensis* via consumption of tainted milk from their mothers (Álvarez *et al*., 2011; Kahler, 2000; Verger *et al*., 1995). Infections of the udder and contamination of milk by the bacteria results from two third contamination of goats by *B. melintesis* during pregnancy (Díaz Aparicio, 2013).

2.2.3 *Brucella ovis*

Countries in Europe known for sheep-raising are mostly contaminated by *B. ovis* because it infect sheep*,* other countries include but not least, Australia, North and South America, New Zealand, and South Africa (Burguess, 1982; DAFF, 2013). Infections such as epididymitis and sporadic loss of pregnancy in mature rams and ewes respectively is caused by *B. ovis* (Assadullah *et al*., 2010). Keeping rams in the same vicinity can also increase the risk of infection through direct contact, also ewes mating with contaminated ram in one season

(Machado *et al.,* 2015; Carrera-Chávez, 2016). Starting at 30 days of gestation, *B. ovis* unusually results in loss of pregnancy related to placentitis in ewes (Xavier *et al*., 2010), resulting in weak lambs being born (Menzies, 2007).

2.2.4 *Brucella canis*

B. canis infects mostly tamed dogs, wild carnivores and sometimes other tamed animals, the growing pet industries will only result in an increase in *Brucella* infections. (Carmichael, 1990; Scholz & Vergnaud, 2013). The geographical location of the *B. canis* has been found to be around America, especially South and Central (Xavier *et al*., 2010). Usually transmission of *B. canis* can happen in a way of direct contact with contaminated foetus, placenta or foetus fluids, resulting in infertility, abortions, testicular atrophy (Greene & Carmichael, 2006). Nevertheless contamination by this specie is very unlikely and is not serious, even though it's a zoonotic agent (Xavier *et al*., 2010). Accidental laboratory contamination and close contact with affected dogs or animals has been the only way humans got contaminated with *B. canis* (Wanke, 2004).

2.2.5 *Brucella suis*

In areas where brucellosis is a regular occurrence in cattle, tamed pigs are less likely to be infected by *B. abortus* and *B. melitensis,* but most likely to be contaminated by *B. suis* (Díaz Aparicio, 2013). Worldwide, only 3 (1, 2 and 3) biovars are the primary cause of porcine brucellosis out of the total of five, according to the European Food Safety Authority (2009). *B. suis* can also infect humans, espercially biovars 1 and 3, in regions such as America (North, South and central), Europe, Southern Asia and the Pacific (Frye, 1991; Scholz & Vergnaud, 2013).

Clinical diagnosis of *B. suis* infection in pigs can be rather difficult because of the lack of clinical signs, but in other cases it can be categorized by a genital infection with abortions (Díaz Aparicio, 2013). According to Olsen and Tatum (2016), other organs such as bones and joints can also be affected. Artificial insemination with infected boar's semen, bringing into contact between infected and non-infected animals and wildlife reservoirs, remains the biggest way of infection of pigs by porcine brucellosis in farms (Díaz Aparicio, 2013).

2.2.6 *Brucella maris* (*B. ceti* and *B. pinnipedialis*)

Seal, whale, dolphins and other sea animals have always been the main hosts for sea *Brucella* strains, since the 1990's. (Clavareau *et al.*, 1998; Ewalt, Payeur *et al*., 1994; Foster *et al.*, 1996;

Ross *et al.*, 1994; Wyatt, 1999). All marine *Brucella* were originally grouped under one specie, but recently these specie have been sub-divided into two species, namely *B.ceti* (cetaceans) and *B pinnipedialis* (seals), from originally being called *B. maris* (Foster *et al*., 2007).

These *Brucella* sea isolates don't only infect sea animals only, but also humans as well through direct contact or eating contaminated marine animals (Xavier *et al*., 2010), sometimes there has been cases of human brucellosis were there was no evidence of direct contact between patient and marine animals (Hernandez-Mora *et al*, 2008; McDonald *et al*., 2006; Sohn, 2003) , with having a connection with neurological disorder in humans for some cases (Hernandez-Mora *et al*., 2008; Sohn, 2003).

2.2.7 *Brucella neotomae*

Not a lot has been reported of this specific *Brucella* spp, being discovered in 1957, *B. neotomae*, has been known to contaminate under normal situations the dessert wood rat in the United States of America (Stoenner & Lackman, 1957).

2.2.8 *Brucella microti*

The fact that *B. microti* has been isolated in South Moravia and Czech Republic (Scholz *et al*., 2008), in the late 2000's, from Voles (*Microtus arvalis)* and in Austria at a later stage from mandibular lymph nodes of wild red foxes (*Vulpes vulpes*) (Scholz *et al,* 2009) shows that there might still be a lot of information that is unknown about this specie and the genus as a whole that is yet to be discovered world wide. Due to its location *B. microti*, is very important, because since its found in soil, all other information pertaining to environmental impact, distribution, ecology, genomic organization, zoonotic potential and how it interacts with other species requires immediate attention (Anderson *et al*, 1986).

2.3 Diseases caused by *Brucella* species

2.3.1 Brucellosis in livestock

Birth of weak offspring's or late term abortion can result from *B. abortus* infection in cattle (Olsen & Tatum, 2016). Infection during initial birthing usually results in abortion, but after that birth becomes normal, with occasional bacterial shedding. Uterine secretions were found to contain brucellae during the second birthing subsequent to infection with 20 percent of cows, 88 percent of cows had given off brucellae in their colostrum, among the negative uterine fluid cultures (Manthei & Carter, 1950). Temporal or development of concealed infection may result

in calves that are contaminated at birth, these calves can show no signs of infection and maybe serologically negative until they first give birth which can then result in abortion and a change from seronegative to seropositive is also observed this point (Wilesmith, 1978, Nicoletti, 1984).

Absentia of seen particles and low leukocyte count result in high milk quality (Emminger & Schlam, 1943), and shedding of infection into milk is usually an expected outcome resulting from *Brucella* infection, even when signs of mastitis are not typically present (Morgan, 1960). It has been reported that different geographical locations highly influence difference in frequency of disease presentation, due to *B.abortus* biotypes or even the difference in cattle, for example, in Sudan's western region, ostreoarticular lesions have been linked to infection by *Brucella*, with 92 percent of Zebu cattle with hygromas and 62 percent with arthritis were seropositive for *brucella* (Musa *et al*., 1990).

Seminal vesiculitis and epididymitis are usually connected to orchitis, and it will remain concealed in bulls and sometimes infertility is also not observed (Eaglesome & Garcia, 1992, Neta *et al*., 2010). There is also *Brucella abortus* infection in other wildlife's, such as buffalo, elk and Yak amongst others, which the infection closely relates to infection in cattle (Olsen & Johnson, 2011, Kreeger *et al*., 2000, Jackson *et al*., 2014, Nicoletti, 1980).

Goats can also be infected by *Brucella melitensis* which strongly resembles *B. abortus* infection in cattle goats. Amongst all the animals infected by br*ucella* Sheep are the most invulnerable to infection and also this is likely to be influenced by difference amongst breeds (Alton, 1990), and also unlikely to experience abortion. Even though shedding in milk over succeeding pregnancies has been seen to occur, shedding is less likely to reoccur in uterine and is very short in the following pregnancies (Tittarelli *et al*., 2005).

Epididymitis and weakened fertility is caused by *Brucella ovis* in male sheep (Menzeis, 2007), in goats infection has not yet been reported to occur naturally, but experimental infections are most likely to occur (Burgess *et al*., 1985, Ridler *et al*., 2000). About 30-50 % serologically positive rams have lesions that are perceptible (Poester *et al*., 2006, Van Metre *et al*., 2012). In rams that don't show any signs of infection, shedding of brucellae in semen still occurs, and can be spread across the herd, as a result reduction in fertility can be observed. Birth of weak lambs or even abortion has been seen to occur in ewes even though infection is less likely (Poester *et al*., 2006, Menzies, 2007).

Swine infection by *Brucella suis* is very organized and regularly observed, and lasting for a long time. Osteoarticular lesions are very common in swine, making it very distinct from other

animals, even if sometimes they don't show any signs of infection. Abortion, weak piglets, orchitis, and epididymitis are the usual reproductive signs for *Brucella suis* infection. (Poester *et al*., 2006).

Dogs also go through reproductive lesions as a result of *Brucella* infection, where late term abortion is the most likely expression of this infection in bitches. There can also be large number of brucellae in the vaginal discharge, weeks after abortion. Infertility can be caused by orchitis and epididymitis in males. Bacteremia seen in swine can also be observed in dogs. Infection by *B. canis* in dogs can also result in discospondylitis, meningoencephalitis, and uveitis sometimes (Hollett, 2006, Wanke, 2004).

2.3.2 Human brucellosis

In human brucellosis, detection is complicated due to being characterized as a 'protean manifestations' (Pappas *et al*., 2005). Because human brucellosis has flu like symptoms, it has been called Malta fever or undulant fever with an approximate of 53-100% patients infected with it (Franco *et al*., 2007). This disease can infect any organ in the human body, and can be chronic as well, but it's less likely to be fatal. The stage of disease, the species involved in infection and the organ system affected can influence clinical features of brucellosis in humans (Pappas *et al*., 2005), with 3 months incubation time and symptoms manifesting with just only 2-6 weeks after exposure (Sauret & Vilissova, 2002, Reguera *et al*., 2003).

The most usual symptoms include fever, joint pain, night sweats, and more ratified symptoms such as anorexia, weakness and weight loss, with only 10-30% patients experiencing complications and skeleton infection being the most common (Araj, 1999, Reguera *et al*., 2003). According to Pappas *et al*., (2005), peripheral arthritis, sacroiliitis, and spondylitis are the three unmistakable forms of osteoarticular disease resulting from brucellosis infection in patients (Pappas *et al*., 2005), and of the three forms, spondylitis is the most complicated to treat and can be linked to other complications such as neurological problems.

Spontaneous abortion and epididymo-orchitis have been seen as a result of *Brucella* infection in the reproductive system, which is the most likely area to be infected. Only 2% of patients undergo cardiac complications, even though endocarditis has been identified as the leading cause of death from *Brucella* infection. Surgical valve replacement sometimes is required for the aortic valve, as it's the most commonly infected part, together with an extended antibiotic therapy to remedy the infection (Reguera *et al*., 2003).

Severe diseases in humans are most often caused by *B. melitensis* and *B. suis* infection than *B. abortus,* as a result of such clear causative factors for severe disease in humans, very few studies have done interspecies comparison for disease manifestation (Troy *et al*., 2005). Infection by *B. melitensis* which is a more toxic disease than the other *Brucella* strains to humans, is distinguished by 'fever, abdominal tenderness, hepatosplenomegaly, thrombocytopenia, and liver enzyme abnormalities. And also, infection by *B. abortus* is distinguished by focal complications such as, 'osteomyelitis, arthritis, or complications with pregnancy'.

Inadequate length of antibiotic treatment can result in about 10% relapse in patients infected with brucellosis, but this usually occurs in a period of 12 months after initial infection (Pappas *et al*., 2005). Relapse after longer periods after initial infection has been reported, such as 28 years after initial presentation as reported by Ogredici *et al.* (2010). Even years after diagnosis, having completed the antibiotic treatment and a successful clinical cure for brucellosis, recent reports suggest that brucellae can still exist intracellular, sometimes even in asymptomatic patients and also patients with either chronic focal disease or complaints of chronic vague non-focal illness (Vrioni *et al*., 2008; Castano & Solera, 2009). Further studies are needed for complete eradication of brucellae, but also other studies suggest that clinical cure is very much possible to do so in many cases.

2.4 *Brucella* virulence factors

The harmfulness of *Brucella* species relies mainly on being able to persist inside cells of the host, long enough for sexual contact to occur, since the hosts always removes bacteria in reproductive secretions, as a result this allows passing of bacteria to the subsequent host. As a result of this persistence, they remain in the immune system of domesticated animals, such as cattle, goats, sheep, and swine, for a long time, because they only breed once or twice a year (Seleem *et al.*, 2008).

Brucella infect the host in a hidden way, that's why they always don't openly cause toxicity as compared to other bacteria, and also they lack classical bacterial virulence factors such as 'exotoxins, endotoxic LPS, cytolysins, a capsule, functional flagella, fimbriae, plasmids, and inducers of apoptosis (Seleem *et al*., 2008), as such their virulence factors; lipopolysaccharide, a two component system, type 43 secretion system, and a cyclic β 1, 2 glucans (CβG) has a

three way function: hiding of *Brucella* from detection in immune system, protection from host and also evasion of the immune system of the host (Seleem *et al*., 2008).

2.4.1 Lipopolysaccharide (LPS)

Brucella contain lipopolysaccharide (LPS), which is important for the integrity of the outer membrane (Cardoso *et al*, 2006). *Brucella* LPS demonstrates characteristics different from other LPSs (Lapaque, Moriyon, Moreno, & Gorvel, 2005). The LPS phenotype of *Brucella* species is either smooth or rough if they possess or lack the surface exposed O-polysaccharides (O-PS) chain respectively (Xavier *et al*., 2010). The O-PS is linked to virulence related with smooth LPS (S-LPS), in that, mutant smooth strains fail to survive in macrophages (Franco *et al*, 2007; Xavier *et al*., 2010).

The LPS is smooth in *B. melitensis, B. abortus, and B. suis* and rough in *B. canis* (Lapaque *et al.*, 2005). *Brucella* LPS has been shown to weaken antimicrobial host reactions by preventing complement activity, antibacterial- peptide attacks, and impeding the immune mediators formation (Forestier *et al* , 1999; Lapaque *et al*., 2005; Moreno *et al,* 1981). Bactericidal cationic peptides such as defensin NP-2, lactoferrin, cecropines, lysozyme, bactenecin-derived peptides, the defensin-like antibiotic polymyxin B, and the crude lysosomal extracts from polymorphonuclear leukocytes are prevented from acting against *Brucella* by LPS (Freer *et al*., 1996; Lapaque *et al*., 2005; Martinez de Tejada *et al,* 1995; Riley & Robertson, 1984). These assist *Brucella* in surviving and replicating inside the cells.

2.4.2 Cyclic β 1, 2 glucans (CβG)

The cyclic β 1, 2 glucans (CβG) in *Brucella* are produced by cyclic β 1-2 glucan synthase and encoded by *cgs* gene (Ko & Splitter, 2003). Glucans are components of the bacterial periplasm with osmoregulatory and cholesterol-sequestering activity needed for existence of the organism in phagocytic and non-phagocytic cells (Ko & Splitter, 2003). *Brucella* glycans prevent phagosome development and modifies protein expression in vacuolar membrane, excluding proteins from lysosomes in *Brucella*-containing vacuole (Arellano-Reynoso *et al*., 2005). These pathogens evade merging of *Brucella*-containing vacuole with macrophages lysozomes using cyclic β 1, 2 glucans (Arellano-Reynoso *et al*., 2005). A Study by Arellano-Reynoso *et*

al. (2005) indicated the inability to prevent phagosome-lysosome fusion among the CβG deficient mutants and replication was abrogated.

2.4.3 Two- component system

For successful establishment of infection, *Brucella* must gain entry into the host cell (Lopez-Goni *et al*, 2003). *Brucella* has a two-component *BvrR/BvrS* gene regulation structure acting through a cascade of phosphorylation to control gene expression (Franco *et al*., 2007). The *Brucella BvrR/BvrS* two-component controlling structure is highly comparable to the controlling and sensory proteins of *Sinorhizobium* and *Agrobacterium* necessary for endosymbiosis and pathogenicity in plants, and very similar to a putative system present in the animal pathogen *Bartonella* (Lopez-Goni *et al.*, 2003). Mutations in the *BvrR/BvrS* genes hinder the entrance of *B. abortus* in non-phagocytic cells and weaken intracellular trafficking and virulence (Lopez-Goni *et al*., 2003). *BvrR* is a gene that codes for a reaction regulator protein whereas *BvrS* codes for a sensor protein with histidine kinase activity (Xavier *et al*., 2010).

This regulatory structure is necessary for recruitment of GTPase and actin filaments, and for sustaining bacterial outer membrane (Lopez-Goni *et al*., 2003; Xavier *et al*., 2010). It is postulated that this system modulates the 12 outer membrane, which is necessary for binding, cell incursion, and resistance to lethal cationic peptides (Vassalos *et al*, 2009). This system has a significant influence on the appearance of the surface proteins, coded for by *Omp25* and *Omp31 genes*. It is believed that expression of such external proteins allows *Brucella* to attach, and enter the host cells while evading lysosomal pathway. Mutants that are flawed in this structure have weakened cellular permeation and improved obliteration by phagolysosomes (Vassalos *et al.*, 2009).

2.4.4 Type 4- secretion system

Type 4 secretion systems (T4SSs) belong to family of multiprotein complexes responsible for excretion of bacterial macromolecules and proteins throughout the bacterial cell envelope (Cascales & Christie, 2003). The *Brucella* type 4 secretion system (T4SS) is encoded by the *VirB1-VirB12* genes, which are located on chromosome II (Delrue *et al*, 2004; Hong *et al*, 2000; O'Cellaghan *et al*., 1999). It transports bacterial effector proteins into the host cells; the

effector molecules play a role in trafficking of the *Brucella*-containing vacuoles headed for the replication site (Franco *et al.*, 2007).

Characterization of mechanisms and virulence factors in *Brucella*, that can be involved in the mediation and intracellular persistence have been poor (Azam *et al*., 2016).The promoter that is upstream the *virB1* gene manages the transcription of the *virB* operon (O'Callaghan *et al.*, 2002; Sieira *et al*., 2000). *Brucella suis* was the first *Brucella spp* in which the *virB* operon was initially discovered, there after it was discovered in all other *Brucella spp* and the presence of genomic sequences further proved the potential importance of this operon (O'Callaghan *et al*., 2002).

T4SS can be subdivided into 12 subunits, which are further subdivided into five parts: the stretching needle complex (composed of *VirB2*), the core/outer membrane complex (composed of *VirB7*, *VirB9*, and *VirB10*), the linking stalk (probably composed of fragments from *VirB5* or *VirB10*), the inner membrane complex (composed of *VirB3*, *VirB4*, *VirB6*, *VirB8*, and the N-terminus of *VirB10*), and the ATPases/energy center (consisting of *VirB4* and *VirB11*) (Fronzes *et al*., 2009; Low et al., 2014; Trokter *et al*., 2014).

Amongst all the subunits of *Brucella*, *VirB1*, *VirB7*, and *VirB12* don't play any role in virulence, but the others do (Comerci *et al*., 2001; den Hartigh *et al*., 2004, 2008; Sun et al., 2005). During screening of genes co-regulated by virB operon regulator, the two effectors, virB-co-regulated effectors such as *Vec A* and *Vec C* were only recently identified, even when the T4SS had already been discovered some fifteen years ago in *Brucella spp* (de Jong *et al*., 2008).

According to Myeni *et al*. (2013), during bioinformatics other genes such as *BspA*, *BspB*, and *BspF*, and *BspF*, together with *BspC* and *BspE*, were also discovered. Myeni *et al*. (2013) further states that *BspA*, *BspB*, and *BspF* are composed of 191, 187, and 428 amino acids, and contain the DUF2062 domain (Domain of Unknown Function 2062, Pfam database). The structural classification of proteins (SCOP) structural domain (flanked by two transmembrane domains), and the Gcn5-related N-acetyltransferases (GNAT)- family acetyltransferase domain, respectively (Myeni *et al*., 2013). The *BspA* and *BspB* which are ectopically expressed appear to localize at the endoplasmic reticulum (ER), whereas the *BspF* is found in and around the cytosol and plasman membrane (Myeni *et al*., 2013). When overexpressed, these three effecors inhibit the host cells' protein secretory pathway in transfected cells, and also block cellular secretion during infection (Myeni *et al*., 2013).

2.5 Prevalent *Brucella* virulence genes isolated from domestic livestock in South Africa

In a study conducted by Green *et al*., (2018) in Eastern Cape, South Africa, *Brucella* virulence-associated genes were isolated from cattle, sheep, and goats in 4 municipalities. According to this study, two *Brucella* spp. were confirmed from a total of 130 isolates, which were *B. abortus* (62.3%) and *B. melitensis* (37.7%) and then they were screened for the presence of the *ManA*, *ManB*, *Omp25*, *Omp31* and *ZnuA* virulence-associated genes. The results were as follows *ManA* (100%), *Omp25* (100%) and *Omp31* (34.6-76.9%) to be the highest genes detected while *ManB* (11.5-38.5%) and *ZnuA* (10.3-19.2%) were the lowest from all isolates in the 4 municipalities.

2.6. Distribution and outbreak of Brucellosis in South Africa and Africa in humans and livestock

There are an estimated 500 000 new cases reported every year around the world for brucellosis, which is one the most likely zoonosis (Azam *et al*., 2016). In South Africa, even if the exact occurrence is not known, brucellosis is still considered a priority zoonotic disease, with the last recorded incidence rate of >0.2 per 100 000 population, which was based on a survey done in 1956 to 1959 (Frean *et al*., 2018). But the department of health indicated an increase rate of between <0.1 and 0.3 per 100 000 population yearly (Frean, 2018). The uncontrolled movement of cattle and also the shortage of vaccination of susceptible animals has contributed to this increase in incidence rate across the country (van Helden, 2016).

Bovine brucellosis can be seen across all the nine provinces in South Africa, and is very concentrated around the Highveld regions and central arrears as seen in figure 2.1 below, which shows *B. abortus* outbreaks, which is a primarily cattle disease, from January 2015 to May 2018 as reported by DAFF, (2018). Because of poor compliance with regard to vaccination and testing of brucellosis many cattle farmers in South Africa currently are at risk of having *Brucella* positive cattle within their herds (Frean, 2018).

For an example, there was an estimated prevalence of 0% - 1.5% in KwaZulu-Natal province in South Africa, (Hesterberg *et al*., 2008), Whereas due to larger herd and extensive animal movement together with sharing common grazing sites amongst animals around Africa, has led to an increase in prevalence rate (McDermott & Arimi, 2002). With the implementation of

the heifer vaccination in South Africa, this has led to the discovery of cattle being seen as the greatest source of outbreak (Hesterberg *et al*., 2008).

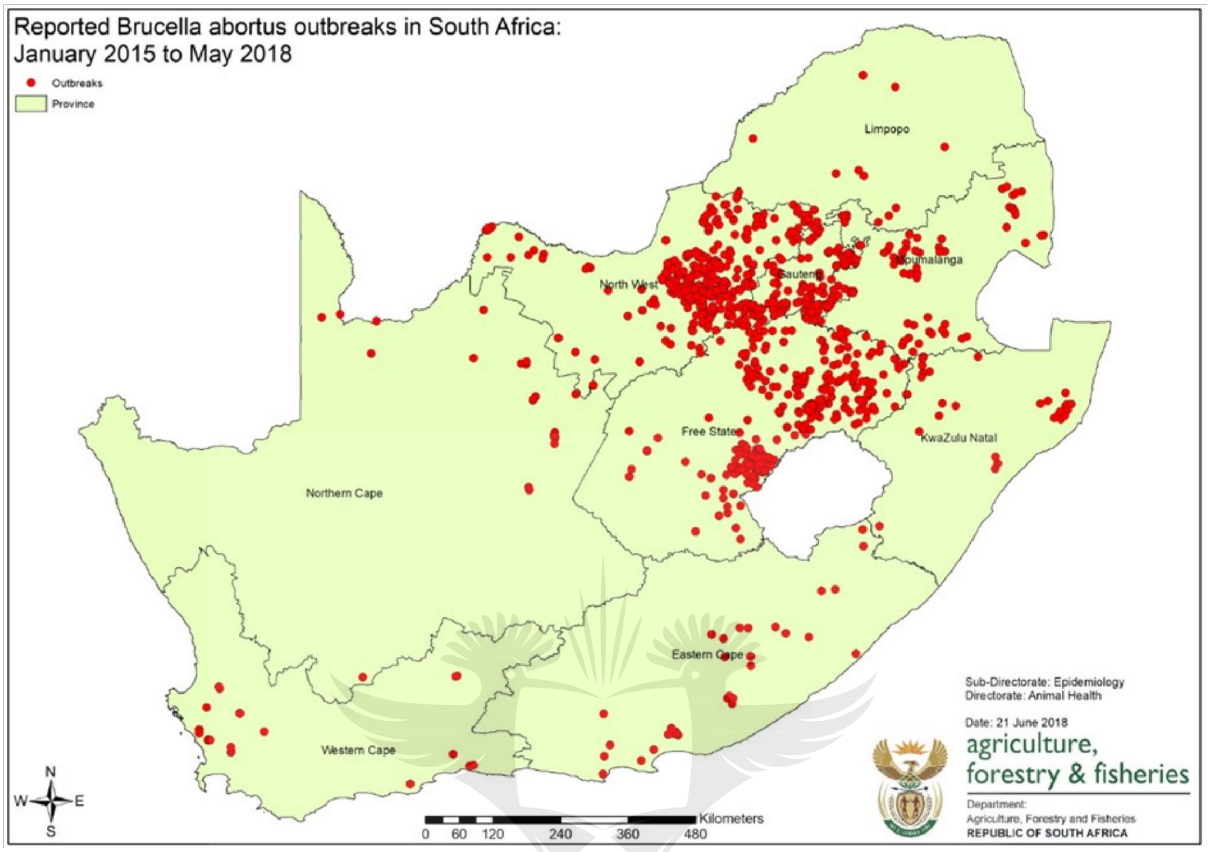

Figure. 2.1. Reported *Brucella abortus* outbreaks in animals from January 2015 to May 2018 across all nine provinces of South Africa. Image courtesy of the Sub-Directorate: Epidemiology of the Directorate Animal Health, Department of Agriculture, Forestry and Fisheries (DAFF, 2019).

Apart from the Nile delta and some Coastal strips, potential brucellosis hosts such as, cattle are found in the following countries in Africa, the Sahel and Ethiopian highlands and in Kenya, Tanzania, Uganda and other Eastern African countries of the Great Lakes area and in Southern African countries, namely Botswana, Madagascar, South Africa, South West of Angola, Zambia and Zimbabwe (Ducrotoy *et al*., 2017). In the South and North of Sahara and the tip of Africa, small ruminants are found in large quantities than cattle, even though sometimes there is overlapping, as shown in figure 2.2 a and b below.

Production systems for livestock across Sub-Sahara Africa can be categorized according to five different groups, as shown in figure 2.2c and many cattle are evenly spread on mixed rain-fed systems, but some cattle can be found where grazing is the dominant production system (McDermott & Arimi, 2002). In other words the lack of intensive livestock production

systems and dominant patoralist where the cattle and small ruminants are kept together is the main driving factor (Ducrotoy *et al*., 2017). Human brucellosis in Africa due to its neglection is still a major problem, even with lack of epidemiological valid data (Ducrotoy *et al*., 2014).

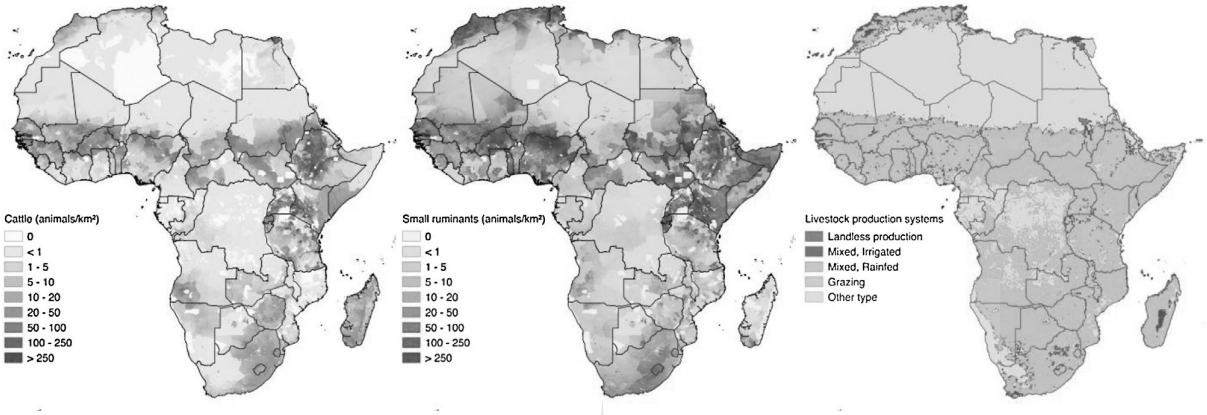

Figure 2.2. Brucella potential hosts and livestock production systems in Africa. Livestock density is expressed as the number of cattle (panel A) or small ruminants (B) per square kilometer according to categories of different sizes in order to clearly differentiate between zones of low and high livestock density (data derived from Gridded Livestock of the World; http://livestock.geo-wiki.org). Panel C shows the livestock production systems. Landless production systems are characterized by (i) less than 10% of the income that comes from non-livestock agriculture, (ii) less than 10% of the feed dry matter that is farm produced, (iii) stocking rates of more than 10 livestock units per ha of agriculture land and (iv) a higher ruminant enterprise value than that of pig or poultry. In mixed farming systems the non-livestock agriculture is responsible for a considerable part of the income. A distinction is made between rain-fed crops and land that is irrigated. In the remaining non-mixed farming systems, livestock depends on grazing activities whether extensively mobile as pastoralists or not. Land inaccessible or unsuitable for livestock is mainly categorized under "Other type"

According to McDermott *et al*. (2013), observations were made from 500 000 animals, 30 000 people and 600 food samples to determine the economic impact of brucellosis in about 259 studies across Africa and Asia, which showed that sheep and cattle have a much higher prevalence range than other animals such as pigs and goats while veterinarians, livestock handlers and abattoir workers have a higher prevalence rate than patients who are in hospital (Zinsstag *et al*., 2013).

2.7 Laboratory identification of *Brucella*

2.7.1. Staining

Chlamydophila abortus and *Coxiella burnetii* can be used as reference point because they are abortive agents and they stain red can be used to check for aborted material, hence the use of stamp staining method is used even though it's not specific but it gives a good indication (Alton *et al.*, 1988).

Brucella spp. which are gram negative and resist weak acid treatment, usually occur singly and are observed in clusters of two or more and also they are coccobacillus measuring 0.6-1.5 µm long and 0.5-0.7 µm wide in size stain red after stamp staining (Godfroid *et al.*, 2010).

2.7.2. Culture

In biotyping of strains, isolation of bacteria is always performed. This is also the case with brucellosis diagnosis, where the choice of sample is very critical as it depends on clinical signs observed. Hence valid samples are needed for diagnosis, samples such as aborted foetuses (stomach, spleen, and lung), foetal membranes, vaginal secretions, colostrum, milk, sperm, and fluid collected from the knee or hygroma (Alton *et al*., 1988).

Farrell medium is the medium of choice when isolating *Brucella* spp., because it contains antibiotics that prevent other bacteria from growing and only allows *Brucella* spp., To grow (Alton *et al*., 1988). Different *Brucella* spp. have different CO_2 requirements based on their different biovars, biovars between 1-4 need CO_2 (*B. abortus* wild type), while biovars 5,6,9 don't need CO_2 for growth others, such as *B. abortus* wild type (biovars 5, 6, 9), *B. abortus* S19 vaccine strain, *B. melitensis*, and *B. suis,* (Chisi *et al*., 2017).

2.7.3. Serological tests

There are lots of serological tests that can be used in the characterization of *Brucella* spp., but Rose Bengal Test (RBT), Serum Agglutination Test (SAT), and Complement Fixation Test (CFT) are widely used. Buffered Brucella antigen test also known as RBT relies heavily on reduction of the ability of immunoglobin antibodies to bind to antigen under low pH conditions (Corbel, 2006b). According to Serra and Vinas (2004), the principle of RBT is that when

stained antigen and serum mix together, any agglutination that results shows a positive reaction (Serra and Vinas, 2004).

The SAT has always been used as an alternative to the RBT, because of its lack of sensitivity and specificity even though its cheap (Agasthya *et al.*, 2012; Clavijo *et al.,* 2003*;* Vancelik *et al.*, 2008). In contrast to the SAT, the CFT has good sensitivity and specificity, but difficult to perform and is very costly as well in terms of facilities and staff needed (Corbel, 2006a).

2.7.4. Molecular characterization

For the identification and characterization of *Brucella* spp. Many different polymerase chain reaction (PCR) techniques have been developed, because such methods often rely on detection of specific genes or sequences such as the *Bru* gene, 16s-23s ribosomal ribonucleic acid (rRNA) coding for *Brucella* spp. and also the *bcsp*31 gene encoding a 31-kDa protein (Baddour & Alkhalifa, 2008; Khamesipour, Rahimi *et al.,* 2014; Ouahrani-Bettache *et al.*, 1996).

Because of its sensitivity, PCR can detect very small amounts, even only after 24 hours incubation, this helps with early identification and treatment. Other various target genes such as., *Omp25*, *Omp31*, *ManA*, etc. within the *Brucella* spp, have also been identified by PCR to detect *Brucella* virulence-associated (Gee *et al*., 2004).

2.8 Antibiotic resistance

Brucellosis is a very important zoonotic disease for both animals and human respectively. There has been a lot of attention directed towards development of antibiotic therapy for humans with various combination of drugs tried sometimes alone or in combination, drugs such as doxycycline, rifampicin, streptomycin, and corticosteroids have been used to treat simple infection or treat chronic infection of brucellosis with successful outcome; but setback have been reported in other cases. Currently there is no definite treatment of brucellosis in livestock (Ghodasara *et a.l,* 2011), this is because of the evasive nature of *Brucella* infection.

Both phagocytic and non-phagocytic cells can be infected by *Brucella,* (Miraglia *et al*., 2018). By inhibiting the phagosome-lysosome fusion, which result in survival, intracellular replication, and chronic infection of the host cells, small number of *Brucella* are able to escape the killing mechanisms of the host (Bellaire *et al*., 2005; Starr *et al*., 2008 and Celli *et al*., 2003). As a result of this *Brucella* have many unique mechanisms that they use to evade

detection by the innate immune system and are able to evade antibiotics used as well, this is also called adaptive immunity (Baldwin et al., 2006).

However, there are many different antibiotics that have been found to be effective in routine treatment of *Brucella* species, antibiotics such as rifampin, tetracycline, trimethoprim/sulfamethoxazole, deoxycycline, quinolones, streptomycin and ceftriaxone gave good results during treatment. But a combination of deoxycycline together with streptomycin or rifampin has been put forward by World Health Organization (WHO) in their guidelines for *Brucella* treatment [Food and Agricultural Organization (FAO)/(WHO) 2000; Young 2009].

2.9 Prevention and treatment measure for Brucellosis

2.9.1 Livestock prevention and treatment

2.9.1.1. Control Brucellosis and control of unrestricted animal movements

Veterinary services for animals must be improved and new and improved diagnostic methodologies must be developed to control the spread of brucellosis, this can be achieved by normalizing all diagnostic kits and vaccines (Pappas *et al.*, 2006). Monitoring of infected animals and reporting of new cases is very important in developing risk analysis, this should also include both domesticated and also wild animals (Rao, 2010).

Checking the movements of animals is also very important, because if not checked, infected animals can be introduced to uninfected herds, if the status of those animals is not known, hence serosurveilance is very important. Maybe quarantine and serosurveilance of new animals before they are introduced into the herd to ensure that these animals are free from brucellosis (Alton, 1990; Kabagambe *et al*., 2001; Refai, 2002).

2.9.1.2. Vaccination

Vaccination is one of the most economical methods to control any disease. Because of lack of or very limited vaccination for animals infected with brucellosis it can also lead to an increase in resistibility of the disease if it's not administered continuously or consistently (Omer *et al*., 2000). If done properly vaccination can help strengthen the host against *brucella* infections, hence reduce the risk of abortion and spread of brucellosis (Omer *et al*., 2000). *B. melitensis* strain Rev1 vaccine and *B. abortus* strain 19, strain RB51 are used in sheep or goat, and cattle respectively (DAFF, 2015).

Ruminants around the ages of three to four months can have immunity against brucellosis for three to five years when vaccinated with the Rev 1 vaccine (Blasco & Molina-Flores, 2011; Olsen & Stoffregen, 2005; Zinsstag *et al*., 2005). Only heifers between 4 to 8 months, which are sexually immature can be vaccinated by strain 19 of *B. abortus* against Brucellosis only once as required by law should (Vemulapalli *et al*, 2002). Calves between 4 and 12 months are vaccinated by Strain RB51 (Vemulapalli *et al*., 2002).

2.9.2 Human control, prevention and treatment

2.9.2.1. Public health education, safety and hygiene

Infection of brucellosis amongst humans can be increased by touching infected materials without proper protection and consumption of raw infected milk (Corbel, 1997), as such public education is very important towards brucellosis in order to minimize or reduce infections. Hence teaching individuals about risk factors, transmission, prevention, and dangers of brucellosis is of vital importance (Rahman *et al*., 2012).

Pasteurization has been found to be very effective in killing *Brucella* spp. But this is not available across South Africa. *Brucella* strains can be killed by boiling milk for several minutes at 80–85 ºC (Corbel, 2006a). During handling of animals, overalls, rubber gloves and rubber boots should be used for protection. But sometimes washing of hands with soap remains one of the most simple and effective ways to prevent infections, if gloves are not available is highly recommended (Samartino, 2003).

2.9.2.2. Diagnostic

Underreporting of Brucellosis in humans is of serious concern globally (Corbel, 2006a), as well as locally in South Africa. Consequently, treatment is not always available because of lack of diagnostic and medical tools. Hence the human health care system must contain appropriate test facilities for quicker and more precise diagnosis of brucellosis and also include a strong antimicrobial treatment regime prescription.

A perfect test for diagnosis of brucellosis is yet to be advanced, which is related to vaccine development. Polymerase chain reaction (PCR) remains one of the most effective ways of diagnosing brucellosis in humans and livestock, together with detection of *Brucella* organisms or DNA through culture. Anti- *Brucella* antibodies in serum has been relied upon to properly

diagnose Brucellosis, because culture carries a lot of risk, such as contamination of staff and requires use of highly skilled staff in very high security laboratories and also it has poor sensitivity which can depend on host species and length of contamination (World Organization for Animal Health, 2009a,b and c).

2.9.2.3. Treatment of human brucellosis

The distinctive interchange with the human immune system makes *Brucella* difficult to cure, because brucellosis is most likely to become a chronic infection, hence this can only be solved by introducing timely diagnosis and treatment, which will prevent chronic infections (Ke *et al.,* 2015). With high treatment failure and relapse occurring at a very high rate (Ariza *et al*., 1995), brucellosis has become a very expensive disease to treat and this result in patients suffering when they can't afford treatment (Franco *et al.,* 2007), even with several treatments regimes currently available (Skalsky *et al*., 2008).

For antibiotics to be effective, they need an appropriate length of time to be effective against all appearance of human brucellosis. Activity *in vitro* against *Brucella* spp has been observed for many different antimicrobial drugs (Corbel, 2006a; Seleem *et al*., 2010). According to Seleem *et al*., 2010, doxycycline amongst the tetracycline's group are the drug of choice, because it can be administered twice per day for six weeks orally, whereas tetracycline is given every six hours or six weeks also, but because of the gastrointestinal side effects its rarely used (Seleem *et al*., 2010).

An addition of aminoglycoside in treatment of human brucellosis is needed, because tetracyclines have between 10 and 20% relapse rate (Seleem *et al*., 2010). Streptomycin and gentamicin are two of the most favoured aminoglycosides, with streptomycin (1g/day intramuscularly) being the most preferred choice when used in combination with the tetracyclines, given for two to three weeks to patients (Corbel, 2006a). Single dose of Gentamicin on a daily basis is more active in vitro against *Brucella* spp. than streptomycin, but it is associated with unfavourable side effects (Corbel, 2006a).

According to Abdel-Maksoud *et al*., 2012, there has been other combination of therapies, which are an alternative to the tetracyclines, which are doxycline-floroquinolone and trimethoprim-sulphamethoxazole combinations (Abdel-Maksoud *et al*., 2012). Rifampicin, gentamicin and co-trimoxazole have been reported to offer alternatives for treatment of pregnant woman infected with brucellosis (Cekovska *et al.*, 2010; FAO, 2006; WHO, 2005). All the treatment alternatives currently are not safe or reliable to be able to effectively and completely immunize

humans against brucellosis (Surendan *et al*., 2011), even with vaccines being developed worldwide. As a result of vaccination not being recommended, in the former Soviet Union and China have resorted to using live *B. abortus* strain 19-BA and 104M respectively (Seleem *et al*., 2010).

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CHAPTER THREE

Discerning antibiogram affiliated with *Brucella* species isolated from livestock in Eastern Cape Province, South Africa

*This chapter deals with Identification of *Brucella* spp, DNA extraction and molecular characterization of *Brucella* and also antibiotic susceptibility.

Abstract

Brucellosis is a widespread zoonotic disease which causes major public health and economic concerns. The objective of the study was to detect *Brucella* spp., determine antimicrobial susceptibility and identification of virulence-associated genes isolated from raw milk, blood, and lymph nodes samples of cattle, sheep, and goats. Samples were inoculated into *Brucella* selective media to recover *Brucella* isolates. Detection of the *Bru* gene was observed in 120 isolates including 74 (62 %) from cattle; 16 (13.3%) in sheep and 33 (27.5%) from goats. Lymph nodes showed the highest isolates in cattle, and blood samples with highest isolates in sheep and goat. *Brucella* isolates were susceptible to moxifloxacin, gentamicin, levofloxacin, ofloxacin and cefixime (100%), while high-level of resistance (100%) to streptomycin, penicillin G, erythromycin, ampicillin, amoxicillin, trimethoprim-sulfamethoxazole and rifampicin were observed. Intermediate resistance was observed doxycycline (12%) in goats isolates and tetracycline (21%) and 44% for both goats and sheep respectively. A significant number of *Brucella* isolate had a MAR index > 0.2 indicating their source to be from high-risk sources, being previously exposed to antibiotics. The results of this study specifically indicate that cattle, goats and sheep in the East Cape are reservoirs of *Brucella* spp that are resistant to antibiotics and that they are the potential pool of antibiotic genes that may be spread to other pathogens in the population posing a risk to public health.

3.1 Introduction

Brucellosis in animals, both wild and tamed is caused by *brucella* spp. and their main reservoirs being sheep, cattle, swine, dogs, camels, and desert woodrats (Martirosyan *et al*., 2011; Bargen *et al*., 2012). Animals are not the only hosts for this bacterium, humans with direct contact with contaminated animals or products can contract brucellosis (Atluri *et al*., 2011; Bargen *et al*., 2012). Body organs such as the liver, lymph nodes and reproductive tract can be more effected by brucellosis and also can lead to chronic lasting for several weeks (Atluri *et al.,* 2011). The mucous membrane can be easily penetrated in natural hosts by *brucella*, leading to infection in the reproductive and respiratory tract (Ke *et al*.,2015).

The two main *brucella* spp. *B. melitensis* and *B. abortus,* infect cattle and small ruminants resulting in infertility and abortions respectively, consequently causing enormous economic losses for the community and the country (Corbel, 2006). Because of the poor reporting and misdiagnosis of brucellosis in other countries, the number of cases appear low. Even though few countries have never reported brucellosis infections, it is still a serious concern world-wide (Musallam *et al*., 2015). South Africa is one of the countries in Africa where information is still not distributed enough, considering that a lot of rural arears in the different provinces depend on livestock for sustenance and hence proper diagnosis of the causative agents of brucellosis is very important for the development of any control program, such as feeding the animals with antibiotics.

Brucellosis can be managed through the use of antibiotics such as tetracycline, rifampin, trimethoprim-sulphamethoxazole (SXT), and streptomycin, administered orally or injected (Pappas *et al.,* 2005 and Hall, 1991). Antibiotics are antimicrobial agents produced by microorganisms, they function by inhibiting or killing other microorganisms (Amabile-Cueva, 2016). Because *brucella* is an intracellular bacterial pathogen that infects host macrophage cells, a specialized agent that is able to penetrate the macrophages and function within their cytoplasm is needed for the treatment of brucellosis. However, *Brucella* isolates are becoming resistant to the antibiotics recommended by World Organization Health (WHO) (Baykam *et al.*, 2004).

Treatment of brucellosis has always been a problem world-wide, with various combination of drugs be in experimented on or used, but this can lead to general misuse resulting in great concern for brucellosis treatment, because this can lead to resilience of the bacteria. However, during regular treatment other antibiotics such as, rifampin, tetracycline and few others have

been effective against *brucella* spp., but the World Health Organization according to their guidelines has put forward a mixture of doxycycline together with streptomycin as a possible treatment therapy for brucella infections (FAO/WHO, 2000; Young 2000). The aim of this study was to investigate the prevalence of putative virulence genes associated with *Brucella abortus and Brucella melitensis* isolates from livestock specimens in Eastern Cape, South Africa using molecular methods.

3.2 Materials and methods

3.2.1 Identification of *Brucella*

3.2.1.1 Sample collection

A total of 1955 samples comprising milk, blood, and lymph nodes were collected from 880 cattle, 555 sheep, and 520 goats from the livestock production sector of the Amathole District Municipality (A), Buffalo City Metropolitan Municipality (B), OR Tambo District Municipality (C) as well as random samples collected from cattle slaughtered in Queenstown and East London abattoirs (D) in 2016. An amount of 10ml of blood samples were collected from cattle every Wednesday, from the caudal tail vein while those from sheep and goats were collected from the jugular vein. All samples were collected with individual needles and stored in 25 ml sterile EDTA vacutainer tubes (OIE, 2008). The tubes containing blood were immediately stored on ice until further analysis could be conducted. Raw milk samples, 10 ml, were collected in individual 100 ml sterile bottles from each quarter of dairy cows, sheep, and goats based on the method of Alton *et al*. (1989) and kept at 4°C for further analysis. Tissue samples of the mammary lymph nodes from cattle slaughtered in Queenstown and East London abattoirs were collected by the slaughtering staff personell and processed for bacterial isolation in accordance with the description of Alton *et al*. (1989).

3.2.1.2 Bacterial isolation

Centrifuge tubes containing 10 ml of milk samples were centrifuged at 6000 × g for 15 min at 4 ºC (Eppendorf 5430 R, Eppendorf). The cream and deposit obtained after the skimmed milk had been discarded were mixed and spread with a swab-stick on *Brucella* Agar (Merck, Johannesburg, South Africa) with *Brucella* supplement (Liofilchem, Roseto D.A., Italy). The

plates were incubated at 37ºC with 5-10% CO_2 to enhance anaerobic conditions. The presence of *Brucella* colonies was inspected after 2, 4, and 7 days. Blood from cattle, sheep and goats were inoculated into a Castaneda biphasic medium which consisted of both a solid and liquid Brucella medium (Merck, Johannesburg, South Africa) with *Brucella* supplement (Liofilchem, Roseto D.A., Italy). The Castaneda bottles were incubated for 21 days, supplemented with 5% CO_2, with periodic tipping (OIE, 2008 and Carmichael, 1990). Lymph nodes were immersed in alcohol and flamed before being cut into small pieces and spread on the surface of *Brucella* Agar (Alton *et al*., 1989)

3.2.1.3 DNA extraction and molecular characterization of *Brucella* species

DNA was extracted from presumptive isolates using the Zymo Research bacterial or fungal mini-prep kit (Zymo Research Corp, Irvine, USA) following the manufacturer's instructions. Genus-specific primers (Bru-F, Bru-R) for identification of *Brucella* sequences and species-specific primers shown in Table 3.1, were used as described by Khamesipour *et al*., (2013) and Bricker and Halling (1994, 1995).

Table 3. 1: Primer sets and amplicon sizes specific for the different *Brucella* species and vaccine stains.

Strain	Primer set	Primer sequence (5'-3')	Conditions	Amplicon size (bp)	References
Brucella species	*Bru*	CTATTATCCGATTGGTGGTCTG	5 minutes initial denaturation at 95 ºC followed by 35 cycles of denaturation at 94 ºC for 45 seconds, 64.9 ºC annealing for 1 minute and 72 ºC extension for 1 minute. A final elongation step at 72 ºC for 7 minutes.	245	(Khamesipour, 2013)
	Bru	GGTAAAGCGTCGCCAGAAGG			
B. abortus	*IS711*	TGCCGATCACTTTCAAGGGCCTTCAT	2 minutes initial denaturation at 94 ºC followed by 35 cycles of denaturation at 95 ºC for 20 seconds, 60 ºC annealing for 20 seconds and 72 ºC extension for 30 seconds. A final elongation step at 72 ºC for 7 minutes.	498	(Ewalt and Bricker, 2000)
	AB	GACGAACGGAATTTTTCCAATCCC			
B. melitensis	*IS711*	TGCCGATCACTTTCAAGGGCCTTCAT	2 minutes initial denaturation at 94 ºC followed by 35 cycles of denaturation at 95 ºC for 20 seconds, 60 ºC annealing for 20 seconds and 72 ºC extension for 30 seconds. A final elongation step at 72 ºC for 7 minutes.	731	Ewalt and Bricker, 2000)
	BM	AAATCGCGTCCTTGCTGGTCTGA			
B. abortus vaccine strain S19	*ERI1*	GCGCCGCGAAGAACTTATCAA	2 minutes initial denaturation at 94 ºC followed by 35 cycles of denaturation at 95 ºC for 20 seconds, 60 ºC annealing for 20 seconds and 72 ºC extension for 30 seconds. A final elongation step at 72 ºC for 7 minutes.	178	Ewalt and Bricker, 2000)
	ERI2	CGCCATGTTAGCGGCGGTGA			
B. abortus vaccine strain RB51	*IS711*	TGCCGATCACTTTCAAGGGCCTTCAT	2 minutes initial denaturation at 94 ºC followed by 35 cycles of denaturation at 95 ºC for 20 seconds, 60 ºC annealing for 20 seconds and 72 ºC extension for 30 seconds. A final elongation step at 72 ºC for 7 minutes.	364	Ewalt and Bricker, 2000)
	RB51	CCCCGGAAGATATGCTTCGATCC			
Rev-1 vaccine	*P1*	TGGAGGTCAGAAATGAAC	2 minutes initial denaturation at 94 ºC followed by 35 cycles of denaturation at 95 ºC for 20 seconds, 60 ºC annealing for 20 seconds and 72 ºC extension for 30 seconds. A final elongation step at 72 ºC for 7 minutes.	282	(Mullis and Faloona, 1987)
	P2	GAGTGCGAAACGAGCGC			

3.2.1.4 Gel electrophoresis

Agarose gel (1.5%) was prepared using 1 × TBE buffer (10 × TBE buffer: 1M Tris, 1 M Boric acid, 50 mM EDTA, [pH 8.3]). The gel was stained with 5 µl ethidium bromide (165, 169). A KAPA universal DNA molecular weight marker and a 100 bp ladder (Fermentas) were used as size standards. Gel electrophoresis was performed at 100 V for 45 min and amplicons were visualized under UV light and photographed using an Alliance 4.7 XD-79 System (Uvitec, Cambridge, UK).

3.2.2 Antibiotic Susceptibility

3.2.2.1 Antibiotic susceptibility testing

Susceptibility of *Brucella* isolates to 15 antibiotics – ciprofloxacin (5 µg), rifampicin (5 µg), amoxicillin (10 µg), doxycycline (30 µg), tetracycline (30 µg), trimethoprim + sulfamethoxazole (2.5 µg), ampicillin (10 µg), erythromycin (5 µg), ofloxacin (5 µg), cefixime (5 µg), moxifloxacin (5 µg), gentamicin (10 µg), penicillin G (10 units), levofloxacin (5 µg) and cefoxitin (30 µg) were determined using the Kirby Bauer disk diffusion method (Bauer *et al*., 1966), selection of this antibiotics were determined by the knowledge of the organism and its sensitivities. Briefly, Mueller-Hinton agar (Merck, Johannesburg, South Africa) plates supplemented with *Brucella* supplement (Liofilchem, Roseto D.A., Italy) were inoculated with bacterial suspensions calibrated to 0.5 McFarland standard turbidity using spread plate technique and antibiotic disks (Mast Diagnostics, Merseyside, United Kingdom) were applied.

Plates were incubated at 37 ^{o}C in 5% CO_2 for 48 hours and zones of inhibition were interpreted as resistant, sensitive, or intermediate using the interpretative chart method according to CLSI guidelines (CLSI, 2014).

3.2.2.2 Multiple antibiotic resistance

Multiple Antibiotic Resistance (MAR) phenotypes, patterns and indexing were generated for the resistant isolates (Ateba *et al*., 2008). The MAR index of individual isolates identified was calculated using the formula described by Krumperman (1983). MAR index of isolate = No. of antibiotics to which isolate was resistant / total no. of antibiotics to which isolate was exposed. A MAR index of ≥ 0.2 indicates a high-risk environment where antibiotics are often used (Osundiya *et al*., 2013).

3.3. Results

3.3.1 Confirmation of bacterial isolates

The *Bru* gene was successfully amplified from 130 isolates of the 1955 total samples collected (Figure 3.1, Table 3.2). The highest number of isolates, 81(62.3%) was detected, in cattle, while the lowest number, 16 (12.3%), was observed in the samples from sheep. The study detected highest number of isolates in the blood 31 (23.8%) and lymph node 32 (24.6%) samples of cattle.

Table 3.2: Molecular characterization of *Brucella* spp. from blood, raw milk and lymph nodes of cattle, sheep, and goats.

Municipality	Cattle					Sheep					Goats				
	No. of samples Tested	*Bru* gene	*B. abortus*	*B. melitensis*	*B. abortus* vaccine strain	No. of samples Tested	*Bru* gene	*B. abortus*	*B. Melitensis*	*Rev-1* vaccine strain	No. of samples Tested	*Bru* gene	*B. abortus*	*B. melitensis*	*Rev-1* vaccine strain
A	405	16	16	2	0	135	13	0	13	0	215	10	0	10	0
B	275	22	19	1	0	315	0	0	0	0	110	17	0	17	0
C	100	21	19	3	0	55	2	0	2	0	100	3	0	3	0
D	100	22	20	1	0	50	1	0	1	0	95	3	0	3	0
Total	880	81	74	7	0	555	16	0	16	0	520	33	0	33	0

Key*= Municipalities were named as A, B, C and to preserve confidentiality.

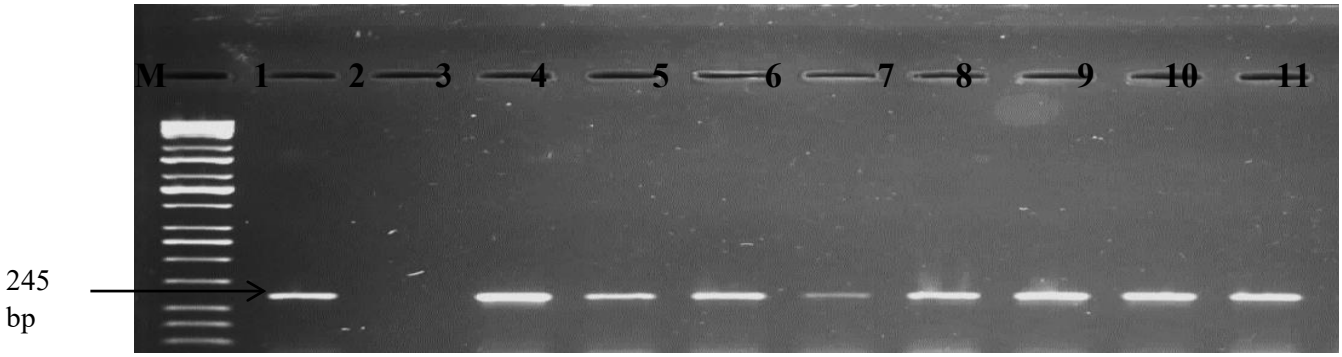

Figure 3.1: PCR products for the detection of *B. abortus* in raw milk, blood, and lymph node tissue samples. Universal DNA Ladder (Kappa); Lane 1: 13047K, Lane 2: Negative control, Lane 3: 24, Lane 4: 212, Lane 5: 129, Lane 6: 10026K Lane 7: ST13, Lane 8: EH35, Lane 9: EH 34, and Lane 10: ND12. The expected molecular size of the *Bru* gene is 245 bp.

3.3.2 Brucella species characterization

B. abortus (56.9%), *B. melitensis* (37.7%) and *B. abortus* vaccine strain S19 (5.4%) were confirmed from the isolates (Table 3.2; Figures 3.2, 3.3 and 3.4, appendix 1). *B. melitensis* Rev-1 vaccine strain and *B. abortus* vaccine strain RB51 was not amplified from any of our samples.

Figure 3.2: PCR products for the detection of *B. abortus* in raw milk, blood, and lymph node tissue samples. Lane M: Universal DNA Ladder (Kappa); Lane 1: 13047K, Lane 2: Q24, Lane 3: 24, Lane 4: 212, Lane 5: 129, lane 6: 10026K Lane 7: ST13, Lane 8: EH35, Lane 9: EH 34, and Lane 10: ND12. The expected molecular size of the *IS711+AB* fragments is 498 bp.

3.3.3 Antibiogram profile

Brucella isolates were susceptible to moxifloxacin, gentamicin, levofloxacin, ofloxacin and cefixime (100%). These antibiotics may be used in the management of brucellosis in cattle, goats and sheep. Intermediate resistance was observed doxycycline (12%) in goats isolates and tetracycline (21%) and 44% for both goats and sheep respectively (Table 3.3).

Table 3. 3: Antibiotic susceptibility testing of *Brucella* isolated from cattle, sheep and Goat.

Antibiotics	Cattle			Total (%)			Goats			Total (%)			Sheep			Total (%)		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
Ciprofloxacin (10 µg)	9	0	65	12	0	88	33	0	0	100	0	0	16	0	0	100	0	0
Rifampicin (5 µg)	0	0	74	0	0	100	0	0	33	0	0	100	0	0	16	0	0	100
Amoxicillin (10 µg)	0	0	74	0	0	100	0	0	33	0	0	100	0	0	16	0	0	100
Doxycycline (30 µg)	0	0	74	0	0	100	0	4	29	0	12	88	0	0	16	0	0	100
Tetracycline (5 µg)	0	0	74	0	0	100	0	7	26	0	21	79	0	7	9	0	44	56
Trimethoprim- sulfamethoxazole (2.5 µg)	0	0	74	0	0	100	33	0	0	100	0	0	16	0	0	100	0	0
Ampicillin (10 µg)	0	0	74	0	0	100	0	0	33	0	0	100	0	0	16	0	0	100
Erythromycin (15 µg)	0	0	74	0	0	100	0	0	33	0	0	100	0	0	16	0	0	100
Ofloxacin (5 µg)	74	0	0	100	0	0	33	0	0	100	0	0	16	0	0	100	0	0
Cefixime (5 µg)	61	0	13	82	0	18	33	0	0	100	0	0	16	0	0	100	0	0
Moxifloxacin (5 µg)	74	0	0	100	0	0	33	0	0	100	0	0	16	0	0	100	0	0
Gentamicin (10 µg)	74	0	0	100	0	0	33	0	0	100	0	0	16	0	0	100	0	0
Penicillin G (10 units)	0	0	74	0	0	100	0	0	33	0	0	100	0	0	16	0	0	100
Levofloxacin (5 µg)	74	0	0	100	0	0	33	0	0	0	0	100	16	0	0	100	0	0
Cefoxitin (30 µg)	0	0	74	0	0	100	33	0	0	100	0	0	16	0	0	100	0	0

S=Susceptibility, I=Intermediate, R=Resistance

3.3.3.1 MAR indices (MARI*)*

Multiple antibiotic resistance (MAR) phenotypes and MAR indices (MARI)

The characterization of *Brucella* spp. for their MAR phenotypes and MAR indices (MARI) is shown in Table 3.4. Forty-seven isolates were resistant to at least five (Rifampin, Amoxicillin, Doxycycline and Ampicilin) antibiotics. Multiple antibiotic resistances were observed against 5-11 antibiotics. The predominant MAR phenotype was A5: CIP^R E^R PG^R RP^R A^R DXT^R T^R SXT^R AP^R which occurred in 35% of the *Brucella* isolates. On the other hand, the MARI for all the isolates ranged from 0.3- 0.7, with the mean being 0.5.

Table 3.4. Antibiotypes and MARI of *Brucella* isolates.

Antibiotic code	Antibiotype	Number of antibiotics	MARI
A1	E^R PG^R RP^R A^R AP^R	5	0.3
A2	E^R PG^R RP^R A^R DXT^R AP^R	6	0.5
A3	E^R PG^R RP^R A^R DXT^R T^R AP^R	7	0.5
A4	E^R PG^R RP^R A^R DXT^R T^R SXT^R AP^R FOX^R	9	0.6
A5	CIP^R E^R PG^R RP^R A^R DXT^R T^R TS^R AP^R	9	0.6
A6	CIP^R E^R PG^R RP^R A^R DXT^R T^R SXT^R AP^R CFM^R FOX^R	11	0.7

* A, amoxicillin; CIP, ciprofloxacin; DXT, doxycycline; PG, penicillin G; CFM, cefixime; SXT, trimethoprim/sulfamethoxazole; RP, rifampicin; E, erythromycin; Fox, cefoxitin; T, tetracycline; AP, ampicillin.

3.5 Discussion

Due to the rapid urbanization and accessibility in the past 10 to 20 years, the need for meat and milk increased tremendously (NDA, 2006). Therefore, any infectious disease that can affect this industry must be analyzed thoroughly, because if not checked it can even destroy the industry as a whole. An example of such an infectious disease is brucellosis, which is caused by the *Brucella* species (Cloeckaert and Vizcaino, 2004; Araj, 2010). This disease remains a worldwide problem, but it is particularly damaging in Africa, where similar livestock systems, environmental conditions and cultural principles are observed (Refai, 2002 and Gwida *et al*., 2010). Currently cattle remain the main source for zoonosis in human infection. There is not a lot of control programs, but vaccination does exist (Islam *et al*., 2013).

Source of infection of *Brucella* can vary amongst livestocks, both domesticated and non-domesticated. Majority of total confirmed isolates for *Brucella* spp., were 62.3% from cattle, 25.4% from goats, and 12.3% from sheep. According to the results, raw milk samples detected a lower number of the *Bru* gene as compared to lymph nodes in municipalities C and D (Table 3.2). Because milk was collected from commercial dairy animals, the low incidence can be explained by the fact that farmers in those farms follow a strict vaccination and control strategy, minimizing infections on their milk and dairy products, where as in other small farms or non-commercial farms where farmers don't follow those strict vaccination protocols incidence of infection in milk might be higher (Hesterberg *et al*., 2008).

Brucella had a high detection in lymph nodes than tissue samples, according to Corbel and Brinley-Morgan (1984). In this study, this resulted in higher detection in cattle (24%), followed by goats (3.1%) and finally sheep (0.8%), showing that even with lymph nodes detection, the amount of detection differed according to animals. Detection therefore of *Brucella* depends on the time it took for the organism to spread within the lymph nodes during infection, resulting in a high number or a low number of incidence (Corbel and Brinley-Morgan, 1984; Khamesipour *et al.* 2013). This though was an outcome contrary to this study. The samples for this study were collected post-mortem, but this was indicated as being far from an ideal situation for a diagnostic test by O'Leary *et al*, (2006).

According to Khamesipour *et al.* (2014), *Bru* gene was found in higher percentage in lymph nodes as compared to blood samples, this was in contrary to this study as this gene was found in higher percentage in blood samples than lymph nodes with *Brucella* identified in 23.9% of cattle, 7.7% in sheep and 20% in goat's blood samples. Apart from a study done by O'Leary

et al. (2006), whole blood in our study attested to be a good sample for *Brucella* detection. All the *Brucella* spp. were identified as *B. abortus*, *B. melitensis,* and *B. abortus* vaccine strain S19 in this study. These results, agrees with the report of Bricker and Halling, (1995) who observed the detection of these strains in similar samples.

Within all the isolates, higher detection percentage was confirmed in *B. abortus* (56.9%) and the lower percentage in *B. melitensis* (37.7%), this was contrary to a study done in Egypt by Mohamed *et al*., (2013), who detected a lower percentage (16.4%) of *B. melitensis* from infected cattle and sheep, this then further strengthens observations made by Corbel, (2006), that *B. melintensis* and *B. abortus* infected largely small ruminants and cattle, respectively. As previously reported, infection by Brucella can have a devastating loss in economy as a result of abortions and infertility (Mohamed *et al*., 2013). This devastation can be compounded by the resistance of the organisms to commonly used antibiotics.

Common residues found in milk, such as beta-lactams, tetracyclines and aminoglycosides (e.g. streptomycin) are the type of classes of antimicrobials mainly used in treatment of diseases like mastitis in dairy cattle (Gustavsson *et al*., 2004). Antibiotic resistance in bacteria has a potential of becoming widespread, due to the misuse of antibiotics during treatment of mastitis, because of transmission of resistant zoonotic and non-zoonotic bacteria through food, resulting in increased severity if similar antibiotics are used to treat pathogens (Bywater *et al*., 2004; Mervius *et al*., 2005).

Livestock may be an effective medium for the propagation of antimicrobial resistant *Brucella spp*. in the population (Watkins *et al*., 2016). Studies on drug-resistant *Brucella* isolates in South Africa are essential. In the present study antibiotic resistance showed a high-level resistance (100%) to streptomycin, penicillin G, erythromycin, ampicillin, amoxicillin, trimethoprim-sulfamethoxazole and rifampicin which was similar to what Maves *et al*. (2011) and Abdel-Maksud *et al*. (2012) observed in their studies. These results are however conflicting with the studies by Pauletti *et al*. (2015) who observed a 100% penicillin G and erythromycin sensitivity. Trimethoprim-sulfamethoxazole (TS) is is highly recommended for brucellosis treatment, mainly in children of younger than 8 years (Osudiya *et al*., 2013). The resistance of *Brucella* to β-lactam antibiotics can be related to the popular use of penicillin in the treatment of animal diseases which results in an increase in resistance to antibiotics of β-lactam.

Brucella isolates were susceptible to moxifloxacin, gentamicin, levofloxacin, ofloxacin and cefixime (100%). These antibiotics may be used in the management of brucellosis in cattle,

goats and sheep. Intermediate resistance was observed doxycycline (12%) in goats isolates and tetracycline (21%) and 44% for both goats and sheep, respectively. Adzitey *et al.* (2012) states that organisms that show intermediate resistance tend to easily become resistant.

Multidrug resistance was observed in 39.1% of the isolates with different multiple antibiotic-resistant phenotypes (MARP). The predominant MARP was CIP^R E^R PG^R RP^R AR DXT^R T^R SXT^R AP^R CFM^R FOX^R found in 11 (9.1%%) isolates cutting across different patho-types. Other MARP showing resistance against 5–9 different antibiotics were also detected at different frequencies. One of the biggest effects of multiple antibiotic resistance is the restricted effective treatments available to combat brucellosis, which was previously considered to be curable. The reclassification of many illnesses as recurrent with relevant clinical effects such as prolongation of the illness, higher therapy costs and an elevated risk of mortality has been influenced by multi-drug resistance (Adzitey *et al*., 2012).

In order to quantify health risks, associate to the proliferation of pharmaceutical resistance in the community, multiple antibiotic resistance index (MARI) was used. A multiple antibiotic resistant index (MARI) value of 0.2 is used to discriminate between low and high risk for infection, and the MARI value above 0.2 means that a strain of the bacteria is obtained from a high-pollution setting or from high antibiotic use (Osudiya *et al.* 2013). The MARI estimates obtained for isolates from our study ranged from 0.3 to 0.7 and are greater than 0.2, suggesting that the isolates originated from environments with high use or contamination of antibiotics. The high MARI values obtained in this study may suggest the exposure of the isolates to antibiotics pressure, which might have resulted from inappropriate use of antibiotics among the population in the study area.

3.6 Conclusion

The results of this study specifically indicate that cattle, goats and sheep in the East Cape are reservoirs of *Brucella* spp that are resistant to antibiotics and that they are the potential pool of

antibiotic genes that may be spread to other pathogens in the population posing a risk to public health. There is major development of multidrug resistance overtime or acquired resistance that is shown by the *Brucella spp*, because appropriate measures are not put in place. This situation is worrisome as certain severe bacterial infections may lack therapeutic options in the near future. This problem, together with a large number of immunocompromised citizens in South Africa, demands that the spread of antibiotic resistance be monitored as a priority to ensure the health of the general population.

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CHAPTER FOUR

Detection and prevalence of putative virulence genes from *Brucella* species isolated from livestock in Eastern Cape Province of South Africa

* This chapter will be published in International Journal of Environmental Research and Public Health, It discusses identification of different putative virulence genes in *Brucella* isolates, isolated from sheep, goat and cattle in Eastern Cape, South Africa. These genes are involved in pathogenicity of *Brucella* species. It was still under preparation on submission of the dissertation. It will be written and prepared by Manafe R.P[1] and Green E.[2].

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Abstract

Brucella, has many different virulence factors that act as causative agents of brucellosis pending on the environment and other factors, some virulence factors may play a role more than others during infection and as a result play a role in becoming a causative agent for pathogenesis. *Brucella melitensis* and *Brucella abortus* are considered to be pathogenic to humans. The genetic regularity of nine potential causes of virulence of two *Brucella* species in Eastern Cape livestock was examined. A hundred and twenty isolates were used in the detection of *virB2, virB5, vceC, btpA, btpB, prpA, betB, bpe275* and *bspB* virulence factors using PCR. Approximately 100% was observed for genes *VecC* and *BetB* from *B. arbotus*. While the lowest gene observed was PrpA at 4.6% from *B. arbotus*. *BetB* was detected in 34.7% while *virB2* and *prpA* (0%) were not detected in *B. melitensis*. The results from this research suggest that most isolates of *Brucella* have virulence-related genes associated with disease pathogenesis. Our findings showed that *Brucella* strains in the Eastern Cape Province are extremely virulent as virulence characteristics exist in most strains investigated.

4.1 Introduction

Brucellosis is a condition most widely considered to have an impact on domestic animals and marine species. This disease can also infect humans as well through the intake of tainted food products or interaction with the infected animal (Hamdy & Zaki, 2018). The classification of this genus *Brucella* has always been according to the type of species they infect; with *B. melitensis* infecting goats and sheep, while *B. abortus* infecting cattle, *B. suis* infecting pigs, *B. neotomae* infecting desert woodrats, *B. ovis* infecting rams, and *B. canis* infecting dogs, (Alton *et al.*, 1988; Godfroid *et al*., 2011). Other species have also been isolated from cetacean and pinniped species, such as *B. ceti* and *B. pinnipedialis* respectively (Foster *et al.*, 2007), *B. microti* detected from vole (Al Dahouk *et al*., 2003) and ultimately *B. inopinata* from human breast implants (Scholz & Vergnaud, 2013).

The last group of *Brucella* spp. has a much lower zoonotic potential than the first three traditionally big species, because of the high zoonotic potential, these first three *Brucella* species may lead to a broad range of infections, including abortions in animals, leading to huge losses in economy locally and internationally, this has resulted in the increase in the implementation of programs to eradicate brucellosis in wide variety of animals, more especially cattle's, and pigs (OIE, 2009).

All members of the genus *Brucella* strongly resemble each other according to their genetic and immunological evidence (Gandara *et al*., 2001). Hence the expression of their virulence factors such as antigenic heterogeneity, exopolysaccharides, exotoxins, exoenzymes, fimbriae, flagella and secretion processes are responsible for their pathogenicity (Bentley *et al.,* 2005; Cossart *et al.*, 2005; He, 2012). Even so, there is a level of dissimilarities in virulence and also the level of virulence differs according to animals, with humans and guinea-pigs very much the same level (Smith and Ficht, 1990). *Brucella* infect the host in a hidden way, without causing toxicity as compared to other bacteria because classical bacterial virulence factors including exotoxins, endotoxic LPSs, cytolysins, capsules, functional flagella, fimbrias, plasmids and apoptosis inductors are missing (Seleem *et al.*, 2008), as such their virulence factors; lipopolysaccharide, a two component system, type 43 secretion system, and a cyclic β 1, 2 glucans (CβG) has a three way function: hiding of *Brucella* from detection in immune system, protection from host and also evasion of the immune system of the host (Seleem *et al*., 2008).

The type IV (T4SS) secretion mechanism responsible for, the excretion of bacterial macromolecules and proteins in the microbial cells envelope belongs to the multi-protein class (Cascales & Christie, 2003). The T4SS structure can be broken down into 12 subunits which are further partitioned into five parts: the stretching needle complex (composed of *VirB2*), the core / outer membrane complex (consisting of *VirB7*, *VirB9*, and *VirB10*), the connecting stalk (likely composed of VirB5 or VirB10 fragments), the inner membrane complex (composed of *VirB3*, *VirB4*, *VirB6*, *VirB8*, and the *VirB10* N-terminus), and the *VirB10* energy centre (consisting of virB4 and virB11). Amongst all the subunits of *Brucella*, *VirB1*, *VirB7*, and *VirB12* don't play any role in virulence, but the others do (Den Hartigh *et al*., 2004).

Other new proteins such as *VirB*-co-regulated effectors (*Vce*) like *VceA* and *C*, *Brucella* putative effectors (*BPE*), *Brucella* secreted proteins (*Bsps*) according to Myeni *et al*., (2013) have been discovered and also *BtpA* (*Brucella* TIR domain containing proteins), *PrpA* (proline racemase protein A) has also been discovered which triggers IL-10 secretion resulting in non-responsiveness of the immune system during infection (Spera *et al*., 2006). *BetB* is another virulence factor which is involved in the oxidation of betaine aldehyde to glysine betaine using Nicotinamide Adenine Dinucleotide (NAD).

Due to its effect on cattle, milk and other dairy related products as well as human infections, bovine brucellosis requires further investigation in order to minimize infection. The genetic predominance analysis of virulence-related genes is therefore very significant, especially in South Africa and even in Africa as a whole, for understanding and preventing the disease. The

objective of this study was to determine occurrences of nine putative virulence-associated genes from *B. arbotus* and *B. melitensis* isolated from cattle, sheep, and goats in selected municipalities of the Eastern Cape Province, South Africa.

4.2 Materials and methods

4.2.1 DNA extraction

DNA was extracted from 120 isolates including, 81 (62.3%) cattle, 33 (25.4%) goats and 16 (12.3%) sheep (Table. 4.1) in 4 municipalities using a Wizard Genomic DNA Purification kit (Promega® Corporation, Madison, USA) following the manufacturer's instructions.

4.2.2 Molecular detection of putative genes of *Brucella*

Oligonucleotide primers targeting the *VirB5* gene encoding linking stalk of the T4SS, *VirB2* gene for the stretching needle complex of the T4SS, *BtpA* and *BtpB* genes for TIR proteins, *Vcec* gene which is *VirB*-co regulator, *Bet* gene coding for betaine aldehyde dehydrogenase, *BPE275* gene, *BSPB* gene and *PrpA* virulence gene were used in the polymerase chain reaction (Table 4.1). The PCR assays were carried out in a 25 µl reaction volume as described by (Hashemifar *et al.*, 2017).

Table 4. 1: List of primers and PCR conditions for the amplification of *Brucella* virulence associated genes.

Gene	Primer set	Primer sequence (5'-3')	PCR conditions	Amplicon size (bp)
VirB5	VirB5-F VirB5-R	ATTCTCAGCTTCGCATTC TCACCGCTTCGTAGAGAT	Initial denaturation at 94 °C for 4 min followed by 30 cycles of heat denaturation at 94 °C for 60 sec, primer annealing at 56 °C for 45 sec and DNA extension at 72 °C for 1 min. Final extension at 72 °C for 10 min to complete synthesis of all strands.	274
BtpA	BtpA-F BtpA-R	CTATCAGGCTAAGCAATTC CGTAGGAAACTTTATGCC	Initial denaturation at 94 °C for 4 min followed by 30 cycles of heat denaturation at 94 °C for 60 sec, primer annealing at 56 °C for 45 sec and DNA extension at 72 °C for 1 min. Final extension at 72 °C for 10 min to complete synthesis of all strands.	458
BtpB	BtpB-F BtpB-R	TTAACCAGCACGAATACACG CTACGATCAGTTTGCAGCG	Initial denaturation at 94 °C for 4 min followed by 30 cycles of heat denaturation at 94 °C for 60 sec, primer annealing at 61 °C for 45 sec and DNA extension at 72 °C for 1 min. Final extension at 72 °C for 10 min to complete synthesis of all strands.	579
VceC	VceC-F VceC-R	CGCAAGCTGGTTCTGATC TGTGACGGGTAATTTGAAGC	Initial denaturation at 94 °C for 4 min followed by 30 cycles of heat denaturation at 94 °C for 60 sec, primer annealing at 61 °C for 45 sec and DNA extension at 72 °C for 1 min. Final extension at 72 °C for 10 min to complete synthesis of all strands.	482
BetB	BetB-F BetB-R	GCTCGAAACGCTGGATAC AGGCGATGATTGACGAGC	Initial denaturation at 94 °C for 4 min followed by 30 cycles of heat denaturation at 94 °C for 60 sec, primer annealing at 60 °C for 45 sec and DNA extension at 72 °C for 1 min. Final extension at 72 °C for 10 min to complete synthesis of all strands.	393
BPE275	BPE275-F BPE275-R	TGTCGCGGTCTATGTCTATC AATGAGGACGGGCTTGAG	Initial denaturation at 94 °C for 4 min followed by 30 cycles of heat denaturation at 94 °C for 60 sec, primer annealing at 59 °C for 45 sec and DNA extension at 72 °C for 1 min. Final extension at 72 °C for 10 min to complete synthesis of all strands.	466
VirB2	VirB2-F VirB2-R	GCTGTCGCGGATTCTACC CGGAATGCCATCTTGTAAC	Initial denaturation at 94 °C for 4 min followed by 30 cycles of heat denaturation at 94 °C for 60 sec, primer annealing at 60 °C for 45 sec and DNA extension at 72 °C for 1 min. Final extension at 72 °C for 10 min to complete synthesis of all strands.	198
BSPB	BSPB-F BSPB-R	TATCCATGGTATATGCGCC ATAAAGGCCGGGAATGAC	Initial denaturation at 94 °C for 4 min followed by 30 cycles of heat denaturation at 94 °C for 60 sec, primer annealing at 62 °C for 45 sec and DNA extension at 72 °C for 1 min. Final extension at 72 °C for 10 min to complete synthesis of all strands.	336
PrpA	PrpA-F PrpA-R	AACCTCAATGGATCGACC ACGGTCGATAGCCTTGTC	Initial denaturation at 94 °C for 4 min followed by 30 cycles of heat denaturation at 94 °C for 60 sec, primer annealing at 58 °C for 45 sec and DNA extension at 72 °C for 1 min. Final extension at 72 °C for 10 min to complete synthesis of all strands.	672

(Hashemifar *et al*, 2017).

4.2.4 Gel electrophoresis

Agarose gel (1.5%) was prepared using 1× TBE buffer (10× TBE buffer: 1M Tris, 1 M Boric acid, 50 mM EDTA, [pH 8.3]) and stained with 5 µl ethidium bromide. A Quick load 1kb DNA ladder was used as a size standard. Gel electrophoresis was performed at 100 V for 45 min and amplicons were visualized under UV light and photographed using a UV transilluminator (UVP Chem doc, Bio-Rad®, US).

4.3 Results

4.3.1 Frequency of putative genotypes in *B. melitensis* and *B. abortus* isolates

From the 120 isolates as shown below in Table 4.2, complete occurrence of *bet*B and *bsp*B were found to be 73% and 72% respectively irrespective of the strain. The highest occurrence of *bet*B, *vce*C and *bsp*B (100%) was observed from *B. abortus* while the lowest occurrence was observed in btpA (7%) and prpA (5.6%). There was no *vir*B2 and *prp*A detected (0%) in *B. melitensis* while *bet*B (34.7%) was the highest virulent-determinant observed for *B. melitensis*, as seen in Figure 4.2 below, which is a representation of many other gel picture findings.

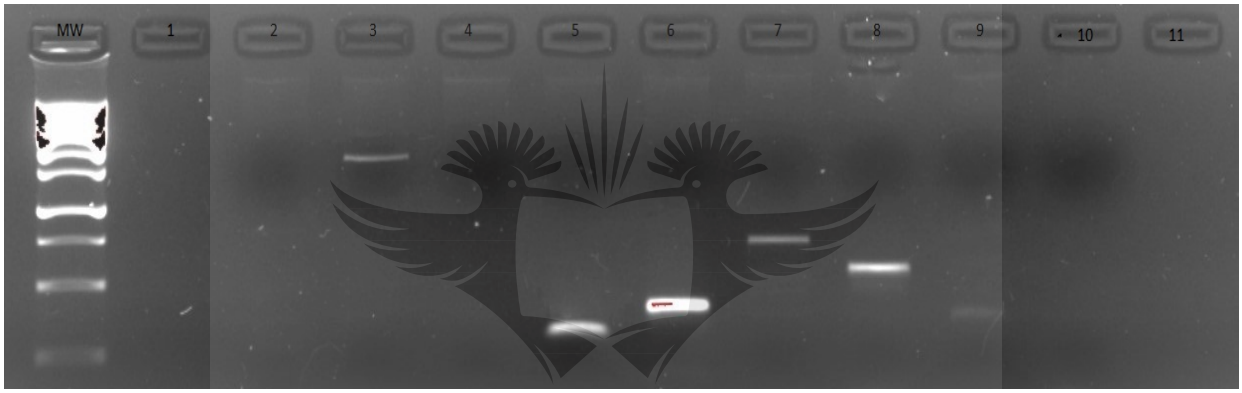

Figure 4.2: Amplification product from the primer pair of the 9 Putative virulence gene of ND 19 sample. Lane M: 1 kb DNA ladder (Inqaba); Lane 1: Negative control, Lane 2: No band, Lane 3: *PrpA* (674bp) gene, Lane 4: No band, Lane 5: *BetB* (393 bp) gene, Lane 6: (466 bp) gene, Lane 7: *BtpB* (579 bp) gene, Lane 8: *VceC* (*482* bp) gene, and Lane 9: *BtpA* (458 bp) gene.

Table 4.2: *Virulence-associated genes prevalence among the 120 Brucella isolates from animal sources*

Target strains	No. (%)	No. of putative virulence genes in studied strains								
		VirB5	*BtpA*	*BtpB*	*VceC*	*BetB*	*BPE275*	*VirB2*	*BSPB*	*PrpA*
B. melitensis	49 (41%)	3 (6.1%)	2 (4.1%)	1 (2%)	9 (18.4%)	17 (34.7%)	3 (6.1%)	0 (0%)	9 (18.4%)	0 (0%)
B. abortus	71 (63%)	10 (14.1%)	24 (33.8%)	5(7%)	71(100%)	71(100%)	70(98.6%)	65(91.5%)	71 (100 %)	4 (5.6%)
TOTAL	120(100%)	13(11%)	26(22%)	6(5%)	80(67%)	88(73%)	73(61%)	65 (54%)	86 (72%)	4 (3%)

4.4 Discussion

Brucellosis, which is caused by *Brucella* spp. infection is a disease that can be acquired from the aborted fetus or the infected animal body through direct contact with mucosa or abraded skin, fluids, and tissue. *Brucella* gently get into host cells through conjunctivae, respiratory tract and abraded skin, spreading through mononuclear phagocytes and finally into reticuloendothelial sites, inhibiting bactericidal reactions within macrophages (Saeedzadeh *et al*., 2012; Godfroid *et al.*, 2000; Moreno and Moriyon, 2002; Ugalde *et al.*, 2003; Rajashekara *et al.*, 2006; Starr and Wehrly, 2008; Xavier *et al.*, 2010; Brambila-Tapia *et al.*, 2014). To the best of our knowledge, up to now, no article has documented the molecular characterization of this specific putative virulence-associated genes isolated from cattle, sheep, and goats in South Africa.

Brucella's cell envelop protein is very important for its pathogenicity (Brambila-Tapia *et al*., 2014). The functioning of the genes is linked to the envelop protein, therefore the envelop protein must adapt to environmental stress, intracellular modulatory activity and possess the ability to survive. (Sabri *et al*, 2014). Colonization or elimination of pathogens in host cell is usually controlled by the initial interaction between the virulence genes and the envelop protein, because they institute signals that lead to the change of gene expression patterns in the cell envelope proteins (Awwad *et al*., 2015), Neta *et al*., 2010,and Starr & Wehrly, 2008).

Amongst all the virulence genes in this study, *BetB* , which encodes betaine dehydrogenase (BADH) is one of the most predominant, this gene is responsible for oxidizing betaine aldehyde to glysine betaine, which helps maintain stability of osmotic stress in all eukaryotic and prokaryotic cells (Lee, 2014), this can explain the ability for the bacteria to resist any attack by the immune systems during infection or transport into the cells, hence increasing its pathogenicity (Rossetti, 2017).

The present study also revealed that a total of 80 *Brucella* spp. comprising *B. abortus* (18.4%) and *B. melitensis* (100%) strains contained *VceC* gene. *VceC* is an essential precursor of *Brucella* T4SS. It is stated that 20 amino acids at the C-terminal of *VceC* are needed for their displacement into the host cell, even though they're not preserved in all *Brucella* species (Myeni, 2013). The interplay between *VceC* with ER chaperon, Bip, leads to the positioning of *VceC* to ER that triggers the unfolded protein reaction that induces ER stress, followed by the initiation of proinflammatory reactions throughout infection.

According to Myeni (2013), *BspB* which forms about 72% of the detected genes in our study, together with *BspA* and *BspF* was one of the three *Bsp* genes predicted by bioinformatics and later identified by TEM-1 -fusing protein method, which are composed of 191, 187 and 428 amino acids. These genes appear to be localized at the ER and their function is to inhibit or stop the host cells protein secretory pathway when overexpressed in cells that are infected and also, they are responsible for preventing cellular secretion during infection of cells (Myeni, 2013). The overexpression of this genes means that if the other two genes were to be isolated also, they would probably be expressed as well, because of the complementary functions of these genes.

Within the T-pilus in *A. tumefaciens VirB*2 was identified as a major part (Eisenbrandt *et al*.,1999). Conjugative pili of IncP plasmids and the Ti plasmid T pilus are composed of cyclic subunits (Lai *et al.*, 1998). As a result, Processed *VirB2* is the major subunit of the promiscuous pilus of *Agrobacterium tumefaciens*. Another subunit of the *VirB*, which is the *VirB5* was put forward to be a small component at the end of the pilus, which meant it mediates the unique adhesion to cells of the recipient as wells as unintended association of *VirB5* with the periplasm pilus base. Vir Proteins are responsible for stabilizing the *VirB5* and Mediate Its Association with the T Pilus of *A. tumefaciens*. The presence of *VirB2* and *VirB5*, even in small amounts, their presence alone means the survival of *Brucella* within the host since they are involved in modulation of the host immune response to infection, and adhesion and thus ensuring the survival of *Brucella* (Jong, 2008).

The low expression of the other genes can be explained by the fact that, because they are found in different stages within the infection system, their expression will always differ, and also the microenvironment with which the isolation of *Brucella* was done, hence there will always be some genes that will not be highly expressed, while some are highly expressed. However, their existence is alarming, as this indicates a lot more to be learned about the *Brucella* species.

B. abortus was identified in majority of samples collected from cattle, goat and sheep, and also with all the samples analysed for the 9 virulence genes large number of these genes were found to be expressed in isolates of *B. abortus* with *B. melitensis* accounting for a small number of genes expressed even though, according to Razzaq, *et al*., (2014) it is the more virulent spp. For this study *B. abortus* was the most isolated *Brucella* spp than *B. melitensis*. With all the samples analysed, cattle rather sheep or goat samples contained majority of these putative virulence genes.

There are genes that are associated with virulence or survival of organisms, characterization of these genes will help in development of safe and protective vaccines, like with the development of live attenuated vaccines inducing high protection levels (Gheibi, 2018). An example of such vaccine development, is the engineering of vaccines based on various deletion in *B.abortus* virulence genes that lead to significant attenuation, including purine biosynthesis pathway genes, ferro chelatase hem H mutant, lipid A fatty acid transporting gene, phosphoglycerate kinase encoding gene, the LPS biosynthesis pathway genes and the Type IV secretion *virB* genes just to mention a few (Conde-Alvarez, 2013), (Alcantra, 2004), (Trant, 2010) (Hartigh, 2004) (Ferguson, 2004), and (Almiron, 2001).

4.5 Conclusion and recommendations

The results of the present study demonstrated that *Brucella* spp. has a lot of different genes responsible for its virulence, and these genes contribute to the infection of *Brucella* in many ways, and expression depends to great degree how infected the host is when the samples were collected, and isolated, and environmental factors also plays a role as well. Cattle remain the major carrier of *Brucella* spp, with *B. abortus* the main isolated spp. Both *BetB* and *BspB* virulence genes occurred to be dominant amongst *Brucella abortus* and *Brucella melitensis* isolates, with *PrpA* virulence gene being the least dominant of the nine genes tested. Therefore, more information needs to be circulated especially to the general public about this disease, since vaccines are not readily available, especially in the poorer arears, hence Public health enlightenment should be focused on the zoonotic aspect of the disease as it relates to consumption of unpasteurized milk and other food items obtained from diseased animals.

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CHAPTER FIVE

General Discussions, Conclusions and Recommendations

*This chapter presents general discussion, conclusion and recommendations for future studies.

5.1. GENERAL DISCUSSION

Brucella is not readily identified due to its wide variety of hosts, multifaceted epidemiology, and the socio-economic implications it has, hence Brucellosis is a "difficult disease", according to Franc *et al*., (2018). Sanogo *et al.,* (2012) indicated that, livestock farming in Africa has many challenges which affect their development, these challenges include parasitic, viral and bacterial infectious diseases which are proving hard to manage for the local farmers, resulting in huge economic losses through its negative impacts on production, and also a potentially debilitating infection of man due to its zoonotic potential.

Molecular techniques are very important in the identification of *Brucella spp*. However, this has been used in combination with antibiotics, but the inappropriate use of antibiotics ultimately led to emergence of resistant microbial population further emphasizing the importance of studying virulence genes and their importance in brucella pathogenesis. According to Azam *et al.,* (2018), there is a need for more research into the molecular pathobiology and immunological properties of *Brucella* spp which will then lead into the development of better and safer vaccines for both livestock and humans.

Out of the total antibiotics used in feeds and global antibiotic consumption, tetracyclines and beta lactams effectively constitute about 50 percent each respectively (Tiwari *et al*., 2013). Due to the build-up in immunity against antibiotics, most prescribed treatment rarely works as a result of resistant bacterium. In the present study, streptomycin was highly resistant followed by cephalothin and trimethroprim-sulfamethoxazole respectively. *Brucella* isolates were also susceptible to tetracycline, doxycycline and ciprofloxacin. Misuse of antibiotics is a serious problem when dealing with brucellosis in livestock and also in humans. This misuse of antibiotics when admistering them, has resulted in several antibiotics not being effective anymore and *Brucella* being resistant. Intermediate resistance was observed on erythromycin with Adzitey *et al* (2012) stating that, organisms that shows intermediate resistance also tend to easily become resistant. However, tetracycline, doxycycline and ciprofloxacin can be used as antibiotics of choice in our setting.

The multi antibiotic resistance (MAR) index of our isolates ranges from 0.14-0.57. At least 15% of *Brucella* isolates were resistant to four antibiotics with MAR index of 0.57, 20 % were resistant to three antibiotics with MAR index of 0.43, with 60% of isolates were resistant to two antibiotics with MAR index of 0.29 and finally only 5 % of isolates were resistant to one antibiotic with MAR index of 0.14. A MAR index value greater than 0.2 indicate high risk source of contamination where antibiotics are often used, and the source has been exposed to antibiotics previously (Osundiya *et al*., 2013). Many feeds used in farms these days contains traces amounts of antibiotics, which can also result in acquire resistance. Genetic modification or mutation has alo being reported to be major factor in acquiring new resistance against first line antibiotics. For example, rifampicin -resistant mutants have been identified in patients who previously had brucellosis and recovered, this was reported after they relapsed (Maurin, 2015). As a result, this can also further provide proof that the *Brucella* species can have acquired resistance to rifampin antibiotic.

Resistance to trimethroprim-sulfamethoxazole was observed in majority of *Brucella* isolates, this antibiotic is highly recommended for brucellosis treatment, mainly in children of younger than 8 years of age (Osundiya *et al*., 2013). Streptomycin is also used to treat brucellosis, it indicated 100% resistance, which means that it cannot be used to effectively treat *Brucella* pathogen. Multidrug resistance was observed in most if not all of the isolates, this in return is a serious issue as raw milk, mainly from cattle available to workers and the community in these rural areas may act as a major vector for transmission of antibiotic resistant *Brucella* strains that can cause serious health risks to humans consuming the unpasteurized milk. The antibiotic resistant strains are exacerbated by the presence of virulence factors.

The absence of classical virulence factors such as exotoxin, endotoxin lipopolysaccharide (LPS), capsule, pilus and cytolysin does not prevent *Brucella* from surviving and replicating within the host, this is because they use smart mechanisms for invasion (Pizzaro-Cerda *et al.,* 2000), such as *BvrR* and *BvrS*. These two components of regulatory systems function by changing the host's cell cytoskeleton upon *Brucella* invasion (Lopez-Goni *et al.,* 2002). *Brucella* spp. contains a wide variety of virulent factors as seen from this study, with different functions, one of these virulent factors is Cyclic b-1,2-glucan synthase (Cgs) which is responsible for complex formation of host-cell interaction (Guidolin *et al.,* 2018 and Guidolin *et al.,* 2015).

Other systems that assist with invasion are the Type IV secretion systems (T4SSs), which assist by avoiding fusion of the phagosome with lysosome (Marchesini *et al*., 2016 and Hartigh *et al.,* 2008) ensuring survival intra-cellularly. Evasion of host immunity is very crucial for *Brucella* survival, hence the integrity of LPS on the *Brucella* surface appears to be crucial as it does not exhibit strong endotoxic activity (Lapaque *et al.,* 2005).

Virulence factors which were present in this study are *VirB2, VirB5, VceC, BtpA, BtpB, BetB*, *Bpe*275 and *BspB* which were present in all *Brucella* isolates accept *PrpA* virulence gene, which was the only absent gene amongst the studied genes. Majority of these virulence genes analysed were from isolates identified as *B. abortus*, and with *B. melitensis* accounting for a small number of genes expressed even though, according to Razzaq *et al.,* (2014) it is the more virulent spp.

The findings of the present study showed that most *Brucella* isolates from this region (Eastern Cape in South Africa) have virulence factor in their genome and *B arbotus* remains the most isolated *Brucella* spp. from wide range of livestock (sheep, cattle and goats). *B. abortus* further

demonstrated that it also contains more virulence factors than *B. melitensis* even though it is more prevalent from cattles. Therefore, *Brucella spp*. needs to be given immediate consideration, distribution of information, because a lot of farmers don't know, and sometimes this is a serious issue as all the preventative measures that are being used might be based on old information. A thorough understanding of virulence factors and the molecular properties of the different isolates of *Brucella* in this region can help to control brucellosis.

5.2. CONCLUSION

It's clear that Brucellosis prevention and treatment is a multifaced approach, not only are antibiotics important in treatment, but also knowledge of factors causing virulence has become very important over the years due to rapid rise in antibiotic resistance. This study provides evidence that commonly prescribed antibiotics may no longer be effective against *Brucella* species. *Brucella* species showed high resistance to antibiotics such as streptomycin (s), trimethroprim-sulfamethoxazole (ts) and cephalotin (cip). A significant number of *Brucella* isolate had a MAR index > 0.2 indicating their source to be from high-risk sources, being previously exposed to antibiotics. High rate and increasing trend of resistance causes the use of antibiotics to decrease in clinical practice. However, tetracycline, ciprofloxacin and doxycline antibiotics may still be of benefit in treating brucellosis because *Brucella* species are more sensitive to them.

From this study, multiple virulence genes involved in pathogenesis of *Brucella* were identified, from the same isolates. All these isolates are from one area, meaning that, there is a need to conduct more research involving virulence factors because there might be more genes that are not yet discovered. Because *Brucella* is mainly found in sheep, goats, and cattle, they pose a serious health risk to humans' exposure, as these are animals that humans come into contact the most, so there is therefore the need for adequate risk prevention strategies to protect the animals and preserve public health.

5.3. RECOMMENDATION

Existing contact sessions between government veterinary services and cattle farmers could be utilised optimally to create awareness of brucellosis and to provide relevant information on animal and human health and disease prevention. Information transfer would probably be more

efficient if provided through methods that are acceptable to the community. Non-compliance for farmers is a problem, especially for rural farmers. Because information is known on which *Brucella* species are more dominant, vaccines can be developed that can be more specific to those species, and those vaccines can be made readily available, even for rural and poor farmers. Because many farmers allow their livestock to graze normally in the field, they can be trained on how to deliver the vaccines easily and also how to minimize human transmissions as well.

Proper education, training and communication remain the main focus when combating *Brucellosis,* so awareness campaigns have to be increased to accommodate every farmer, big or small and not wait for the farmers to approach the authorities but the authorities to approach the farmers to force compliance. This in turn will help decrease misuse of antibiotics, and also resolving the multidrug resistance issue currently experienced in the livestock farming in rural arears. More research should be conducted, around the country to try and identify more virulence factors.

5.4. REFERENCES

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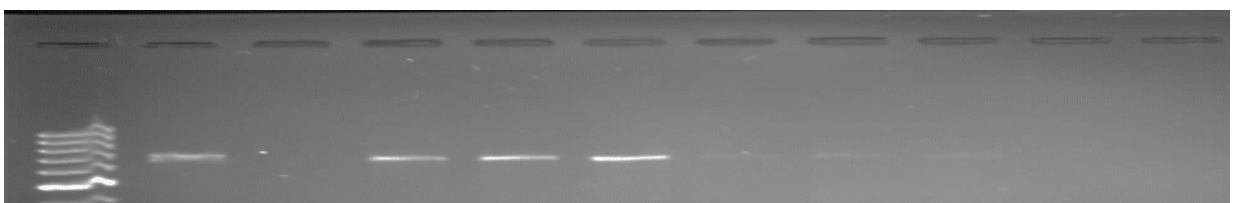
APPENDICES

Appendix I for Chapter 3



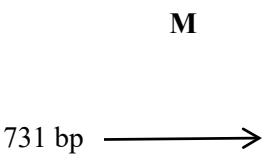


Figure 3.3: PCR products for the detection of *B. melitensis* in raw milk, blood and lymph node tissue samples. Lane M: 100 bp DNA Ladder (Fermentas); Lane 1: 13047K, Lane 2: Negative control, Lane 3: 24, Lane 4: 212, Lane 5: 129, lane 6: 10026K Lane 7: ST13, Lane 8: EH35, Lane 9: EH 34, and Lane 10: ND12. The expected molecular size of the *IS711+BM* fragments is 731 bp.

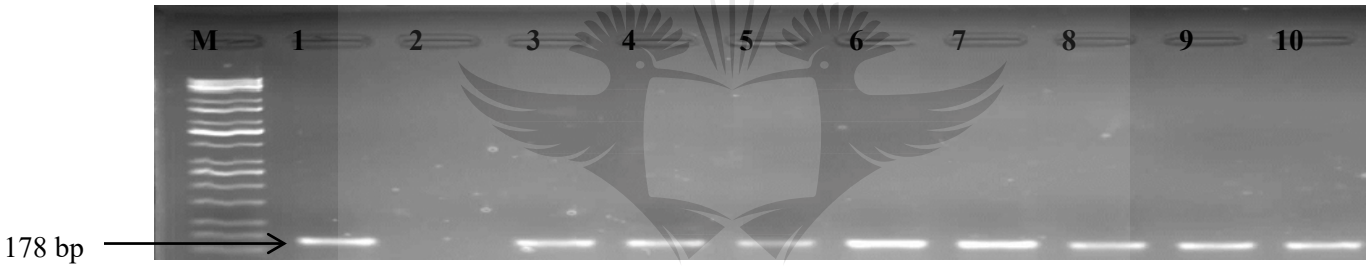


Figure 3.4: PCR products for the detection of *B. abortus* vaccine strain S19 in raw milk, blood and lymph node tissue samples. Lane M: Universal DNA ladder (Kapa); Lane 1: 13047K, Lane 2: Negative control, Lane 3: 24, Lane 4: 212, Lane 5: 129, lane 6: 10026K Lane 7: ST13, Lane 8: EH35, Lane 9: EH 34 and Lane 10: ND12. The expected molecular size of the *eri* gene is 178 bp.

Appendix II for Chapter 4

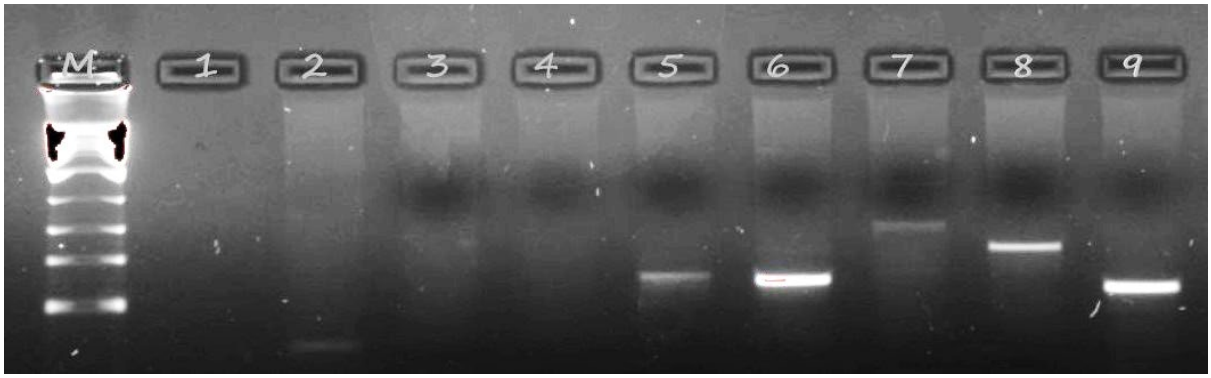


Figure 4.3: Amplification product from the primer pair of the 9 Putative virulence gene of 147 sample. Lane M: 1 kb DNA ladder (Inqaba); Lane 1: Negative control, Lane 2: *VirB5* (274bp), Lane 5*: BtpA* (458 bp) gene, lane 6*: Bet B* (393 bp) gene, Lane 7: Btp B (579) gene, Lane 8: *VceC* (482 bp) gene, Lane 9: *BSPB* (336 bp) gene.

Figure 4.4: Amplification product from the primer pair of the 9 Putative virulence gene of 12T sample. Lane M: 1 kb DNA ladder (Inqaba); Lane 1: Negative control, Lane 2: *VirB5* (274bp) gene, Lane 3: *BPE275* (466 bp) gene, Lane 5: *BtpA* (458 bp) gene, lane 6: *Bet B* (393 bp) gene, Lane 7: *Btp B* (579) gene, Lane 9: *BSPB* (336 bp) gene.