The prevalence of bacterial contamination with reference to *Brucella abortus* in slaughtered carcasses in selected abattoirs in the North West Province, South Africa

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Abstract

Bacterial, and particularly *Brucella abortus* contamination in carcasses is a public health concern. Bovine brucellosis is a chronic disease of livestock caused by Gram negative coccobacilli bacteria of the genus *Brucella* and is a major threat to public health and animal trade in the world. The disease causes serious losses in the economy of the world due to late term abortions, weak animals and stillbirth.

The aim of this study was to isolate *Brucella abortus* and other gram negative bacteria susceptible to contaminate bovine carcasses slaughtered in selected abattoirs in the North West Province, South Africa. In order to achieve this aim, abattoirs in Zeerust, Stella, Vryburg, Koster and Potchefstroom were selected in the North West Province, South Africa for the study. The following samples (Uterus, Placenta, Lymph tissues (mandibular and mammary lymph node) and Spleen were randomly collected for 5 days from each abattoir.

Polymerase chain reaction (PCR) was used to confirm genetic profile from positive preliminary results obtained during the study. The results were found negative for Brucella when using the real time PCR tests. Brucella genomes IS711 and the universal primer were used.

Results obtained revealed no positive *Brucella abortus* contamination from all samples analysed; however, other similar bacteria that could have led to confusion were isolated from some carcasses. Results of molecular identification showed that isolated strains were mostly *Enterococcus spp (35%), Clostridium histolyticum (22%), Staphylococcus aureus (10%), Streptococcus australis (8%), Macrococcus spp. (4%), Bacillus spp. (4%), Lactococcus spp. (4%) Lactobacillus spp. (4%), Vagococcus spp. (2%), Peptostretococcus russellii (2%),* and *Aneurinibacillus spp.* (2%). The presence of these bacteria in organs analysed might be due to poor hygiene in abattoir processes, possible contamination of water or other faecal material during processing.

In addition, the molecular identification of strains revealed that were not yet fully identified and full similarities were not obtained from the Gene bank. The presence of these unidentified strains was an important finding as it raises questions on mutations, and appearance of new strains due probably to the movement of animals, populations and climatic changes.

Despite the absence or non-isolation of positive *Brucella abortus* pathogens in samples analysed, the contamination of carcasses by other pathogenic gram negative bacteria constitutes a public health risk for meat consumers. There is a need of constant monitoring of animals sent to abattoirs particularly from non-tested farms. In addition there is a need to educate and train abattoir workers on basic hygiene practices to reduce the contamination of carcasses.

Prevalence of Bovine brucellosis is still high in some areas, thus regular monitoring of abattoirs remains the key to food safety. Extensive surveys on longer periods should be done in advance. However, despite the interest in Brucella strains, other pathogenic strains remain a challenge for both abattoir workers and consumers. Monitoring, implementation of Hazard analysis and critical control point (HACCP) in abattoirs and identification, antibiotic susceptibility studies need to be done routinely to reduce risks of contamination but also of outbreaks provoked by new strains.

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Declaration

I Isaac K Mosimane, North West University student declare that this document is my own work. Any Material generated through joint work has been acknowledged and the appropriate publications cited. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work. To my fully knowledge it has not been submitted previously by another person for degree at North West University or any other.

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Dedication

This study is dedicated to my family and friends; they always had hope and faith in me and taught me a lot when I thought I could not make it to the end.

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LIST OF ALL ABBREVIATIONS AND ACRONYMS

B. cains:	Brucella canis
B. ceti:	Brucella ceti
B. melitensis:	Brucella melitensis
B. neotomae:	Brucella neotaomae
B. ovis:	Brucella ovis
B. pinnipedialis:	Brucella pinnipedialis
B. suis:	Brucella suis
B.abortus:	Brucella abortus
B. cereus:	Bacillus cereus
S. aureus:	Staphylococcus aureus
CDC:	Centre for Diseases Control and Prevention
CT:	Coomb Test
Cu:	Copper
DCs:	Dendritic Cells
DNA:	Deoxyribonucleic Acid
e.g:	Example
ECP:	Exposure Control Plan
et al.:	And Others
FAO:	Food and Agriculture Organisation
FEE:	Foreign Exchange Earnings
HACCP:	Hazard Analysis and Critical Control Point

LPS : Lipopolysaccharide

PCR: Polymerase Chain Reaction

Rmp: Rounds Per Minutes

S19: Strain 19

SAT: Serum Agglutination Test

Spp.: Species

WHO: World Health Organisation

Zn: Zinc

List of units

% Percentage

/ Per

°C Degree Celsius

G Gram

mL Milli litre

mm Milli metre

µL Micro litre

CHAPTER 1

INTRODUCTION

Brucellosis is a chronic disease common among livestock. It is threat to public health and animal trade in the world in general. It has a high economic impact in animal production in different countries and humans can be infected when they come in direct contact with the bacteria from infected animals or contact with an environmentally contaminated animal discharges, consuming uncooked meat and unpasteurized milk (OIE Manual, 2004). Brucellosis is one of the most important and second most common zoonotic diseases after rabies. The disease affects the genital organs causing them to be inflamed, thus showing of sterility, abortion, drop in milk and formation of localized lesions in the lymphatic system and joints (World Health Organisation, 1971; Center for Diseases Control, 2005).

Brucellosis caused by Brucella organisms, has a variety of *Brucella spp* and affects wild and domestic animals. The causative agent of the disease has been confirmed in the past one thousand years (Capasso, 2002). In cattle, Brucellosis is caused by *Brucella abortus* which is one of the Gram-negative bacteria coccobacilli and the cells appear as short and slender with length of 0,5-0,7µm (Alton, 1988; Leslie *et al*., 1998; Corbel, 2005). These bacteria have different host preferences and have the ability to cause diseases in human beings. There are six classifications of Brucella, but they differ in their pathogenic composition of Brucella spp.: *B. abortus, B. melitensis, B. suis, B. canis, B. ovis and B. neotomae* (Bargen *et al*., 2012) *B. ceti and B. pinnipedialis* (Hernandez *et al*., 2013).

Brucella abortus differ with its pathogen from other Brucella species and contain plasmid or genomic islands that relate to pathogenicity within its genome (Edgardo *et al*., 2002). The genome structure also lacks genes that can programme common virulence aspects including capsules, resistance forms, antigenic variation, exotoxins, cytolysins, plasmids, fimbriae or lysogenic phages (Detilleux *et al*., 1990). Brucella most of the time it target dentratic cell, macrophages and trophoblasts cells (Billard *et al*., 2005). The bacteria needs the host for replication since outside the host, it cannot survive of the bacteria the cell produces endospores to be able to survive in unfavourable conditions for long periods in aerobic or anaerobic respiration it is a facultative bacterium and growth is not affected (Detilleux *et al*., 1990).

The bacteria can also survive for several months in water and aborted foetuses (McEwen and Paterson, 1939). *B. abortus* can be transmitted by fomites. Calves can be infected through trans-placental processes during the gestation phase or during nursing phases of calves with contaminated milk or the bacteria and will remain as asymptomatic. Such animals can uphold the disease in the farm as they might also later abort when they reach production phase or repeat the process (Plommet *et al*., 1973). Infected animals decrease milk production and increase somatic cells (SC) (Xavier, 2009), and the pathogens are hided in the milk due to association with interstitial mastitis with intralesional *B. abortus* (Meador *et al*., 1989). After calving or abortion, there is a high number of organisms shedding within the first 10 days from infected animal contaminating the environment.

Introducing new animals in the herd has a high impact with regard to increasing or introducing the infection to the herd. The foundation of infection can be from aborted foetus, foetal membrane, vaginal discharge and milk/milk products from an infected animal. As for water, feed and pasture play a secondary role (Acha and Szyfres, 2001).

1.2 Problem statement

Communal farms are not able to join the Brucella control scheme according to the Animal Disease Act 35 of 1984 but are able to send animals to abattoirs for slaughtering. In addition, among commercial farmers registered with the brucellosis scheme, between two brucellosis testing. It happens that some positive animals disappear from farms and are usually sold at auctions or sent to abattoirs; and the Animal Disease Act 35 of 1984 is not always fully applied. The true incidence of brucellosis in South Africa is unknown since the rate of the incidence was >0.2 per 100 000 population in study made in 1956 to 1959. Between 1977 and 1984 Department of Health found the annual incidence rate to be <0.1 and 0.3 per 100 000 population no update is made on national incident rate (Seleem *et al*., 2010; Pappas *et al*., 2006; Taleski *et al*., 2002; Schrire et al.,1962)

1.3 Research questions

Are all animals slaughtered in abattoirs across the country and in the North West Province free from Brucellosis or is there any risk for consumers and workers at abattoir?

1.4 Aim of the study

The aim of this study was to detect and isolate *Brucella abortus* and to isolate other pathogenic Gram negative bacteria among animals slaughtered in normal line abattoirs in the North West Province.

1.5 Objectives of the study

The objectives of this study were to:

1 Isolate and identify *Brucella abortus* from infected carcasses slaughtered in the normal chain in selected abattoirs around the North West Province.

2 Molecularly characterise them and confirm their genetic profile using polymerase chain reaction.

CHAPTER 2

LITERATURE REVIEW

2.1 Background

Worldwide, livestock plays an important role in human life as a critical resource and in the provision of meat, milk, nutrition and income. It also symbolises different cultural values. Rural areas depend on livestock as a source of income. Livestock contributes more than 30% of the agricultural gross domestic product and 19% of export earnings. High morbidity and mortality rate have a high impact in economic development due to the ineffective control measures with regard to diseases (Perry *et al.*, 2001). Infertility and abortions cause major losses in animal production. An abortion represents a loss of expected additional milk and meat, wastes breeding time and results in additional costs due to the special diet and care required for pregnant animals. While brucellosis is a well-known infectious cause of abortions, other less well-known causes of abortions or infertility include bovine viral diarrhoea, leptospirosis, trichomonas and campylobacteriosis, among others. Detection of these diseases can be through serum specific antibodies; however, this method is likely to give false positive and false negative results. Abortions can, therefore, cost the producer and the State a great deal of money (Faine, 1994; Njiro *et al.*, 2011).

Brucellosis is one of the trans-boundary diseases of animals affecting the economy (Gul and Khan, 2007), with more than 500,000 animals and humans cases being reported worldwide (Pappas *et al.*, 2006). The disease is more common in countries with poor standard health programme due to reinfection and delays in implementing measures increases the cost unlike developed countries such as the United State of America, New Zealand, Canada, Japan and Israel that managed to control and eradicate the disease (Refai, 2000). Countries such as Great Britain have managed to eradicate the disease through strict control of the disease and pasteurization of milk products. This has led many countries to restrict movement and to implement control measures. Despite a successful scheme put in place for more than 3 decades, Brucellosis remains a serious concern in South Africa and has an impact on the economy. Most rural areas in South Africa are considered as resource-poor areas with weak infrastructure, high rate of unemployment and subsistence farming dominates other agricultural activities. Implementation of information about disease control schemes in South

Africa is essential but very little is known about the prevalence of important zoonotic and production diseases in cattle.

2.2 Brucella species and types of animal

In the family of *Brucellaceae,* the *Ochrobactrum* is the nearest phylogenetic neighbour of Brucella. Historically, *Brucellae* are distinguished according to their host tropism, traits and pathogenicity. Before, the genus consisted of six classical species, nowadays, several new species have been discussed. Some classical species are as follows: *Brucella melitensis* biovars (1-3 isolated from goats and sheep); *Brucella abortus* biovars (1-6 and 9 mainly from cattle and other *bovidae spp*); *B. suis* biovars (1-3 for pigs- biovars 4 for reindeer and biovars for small rodents); *B. canis* (for dogs); *B.ovis* (for sheep) and *B. neotomae* (for desert wood rats). There are currently new additional species such as *B. pinnipedialis*, found in seals, *B. ceti* found in whales and dolphins (Foster *et al*., 2007). *B. microti* is found in red foxes and vole (Scholz *et al*., 2008; Scholz *et al*., 2009) as well as species that was isolated from the breast of a human implant wound (*B. inopinata* strain) with unknown animal reservoir (Scholz et al., 2010). *B. inopinata spp* was discovered from an Australian patient during lung biopsy of a patient with chronic harsh pneumonia (Tiller *et al*., 2010) and other different strains were found from native rodents and non-human primates in North Queensland and Australia (Tiller *et al*., 2010; Schlabritz-Loutsevitch *et al*., 2009).

2.3 Three major cells targeted by Brucella bacteria

The bacteria mostly targets dendritic, trophoblasts and macrophages cells. The bacteria needs to pass through the mucosal walls of the digestive and respiratory tract in order to reach these cells where they are engulfed and by local macrophages and dendritic cells, whereafter they migrate to lymphoid and reproductive organs (Andreson *et al*., 1986; Ackermann *et al*., 1988)

2.3.1 Macrophage cells

Macrophage cells get attacked by Brucella through phagocytosis, thus necessitating a reasonable recruitment of actin filaments when Brucella and receptors interact on the surface of the macrophage cell membrane (Campbell *et al*., 1994). Fat bundles are rich in cholesterol in the cell membrane of macrophages and contribute in bacterial internalisation, thus participate in leading intracellular transfer of bacteria (Kim *et al*., 2004; Lapaque *et al*., 2006). After bacterial internalisation, Brucella containing phagosome cooperates with early

and late endosomes. Most of the phagocytosed Brucella bacteria are destroyed by the action of bactericidal mechanisms of free extremists of nitric oxide, oxygen and enzymes inside phagolysosomes, however, some bacteria resist these mechanisms and replicate after transient fusion with the lysosome (Starr *et al*., 2008; Celli *et al*., 2003; Jiang *et al*., 1993; Celli *et al*., 2005). Bacteria are not hurt because of the acidification of Brucella containing phagosome. However, it causes countenance of bacterial genes that are vital for intracellular existence during the early phases of infection (Boschiroli *et al*., 2002; Porte *et al*., 1999).

2.3.2 Dendritic cells

Dendritic cells (DCs) are other phagocytes for which Brucella has a marked tropism, are more efficiently infected than macrophages. Bacteria are capable of surviving and replicating in DCs similarly to macrophages, although intracellular growth tends to be more prominent in DCs (Billard *et al*., 2005). Furthermore, bacteria can inhibit maturation of DCs compromising DC antigen presentation and cytokine secretion (Salcedo *et al*., 2008; Billard *et al*., 2007; Cirl *et al*., 2008). As an outcome, DCs have two chief features which convert them in brilliant carriers for Brucella, which is high acceptance for bacteria development and migratory properties, and could maintain the spread of pathogens (Billard *et al*., 2005). The behaviour of pathogens with regard to these cells, differs according to their host species.

2.3.3 Trophoblastic cells

Trophoblastic cells have a high concentration of steroid hormones and erythritol, and helps brucella to grow during the last three months of pregnancy (Samartino *et al*., 1996). In ruminants, trophoblastic cells are the main cell targeted by brucella in the last stage of gestation (Meador *et al*., 1989; Xavier *et al*., 2009). Abortion or weak offspring is a result of high volume of cells replicating fast in the placenta and could infect the foetus (Xavier *et al*., 2009; Samartino, 1993). Abortion is influenced by hormonal changes in infected placentas whereby, the level of prostaglandin increases (Verger *et al*., 1987), estrogen and cortisol and decreases the level of progesterone, thus simulating what happens during parturition (Gorvel *et al*., 2002).

Table 1.1: Brucella *spp* with their types of host and biovars

Brucella *spp*	**Types of biovars**	**Types of host**
B. abortus	1-6, 9	Cattle, bison, buffalo, elk, yak, camels
B.Melitensis	1-3	Sheep, goats, cows, camels
	3	Nile catfish, dogs
B.suis	1	Horses
	1-3	Pigs, wild boar
	2	European hare
	4	Caribou reindeer
	5	Rodents
B.canis		Canines
B.ovis		Rams
B.neotomae		Rodents
B.ceti		Whales, dolphins, porpoises
B.pinniopedialis		Seals
B.microti		Common voles, red foxes, (soil)
B.inopinate		Unknown
Baboon isolates		Baboons
B02		Unknown
Rodent isolates		Rodents
Frog isolates		African bullfrogs

The first *Brucella spp* discovered in 1887 was called *Microccus melitensis*, named after a been identified in the Mediterranean region (Malta) from military soldier been diagnosed with fever hence the Malta fever, and the species renamed Brucella melitensis(Cutler et al., 2005; Christopher *et al*., 2010)

Brucella melitensis is reported throughout the world and ovis are primary carriers of this *spp*. An outbreak of *B. melitensis* was reported in South Africa around 1965 from sheep in Limpopo and Mpumalanga. Later, around 2007, a sporadic outbreak was reported in wild

animals in three provinces (Kwa-Zulu Natal, Gauteng and North West). *Brucella melitensis* is also present in Mexico, some areas of Asia and certain African countries. Northern Europe, South-East Asia, Australia, New Zealand and Canada are areas believed to be free from *B. melitensis*. Clinical signs by bucks, sheep and goats are abortions, orchitis, mastitis, lameness, hygroma and chronic uterine lesions. It is unlikely to see all infected goats aborting. The pathogen may hide in the environment which may results in exposure for other animals that are susceptible to the spp and human beings who are around the area (Poester *et al*., 2013). The disease is prevalent in countries where goats are a significant part of the animal industry, and milk is a mutual source of human brucellosis through oral route or direct contact.

Brucella abortus has about seven different biovars (1, 2, 3, 4, 5and 9) and the most widely known worldwide is biovar 1. The most frequent clinical sign in animals is abortion. Cattle are affected by *B. abortus*, however, other Brucella species can also infect bovine such as *B. suis* (typically not allied with clinical signs) and *B. melitensis* when they graze/share in the same grazing field with infected pigs and sheep/goats, respectively. About 25% of milk is estimated to be reduced in the milk production infected herd (Acha *et al*., 2003). In countries such as Brazil, *B. abortus* causes infection in goats (Lilenbaum *et al*., 2007). Chronic infection in cattle is usually caused by *B. suis* in the mammary gland and this bacterial *spp* affect only cattle with no signs of abortion (Ewalt *et al*., 1997).

Abortion in females is caused by *Brucella abortus* between the 5th and 9th month of pregnancy, caused largely by inflamed placenta and the percentage varies from 30% to 80%. Other signs could be of weak calves, which can be related to higher neonatal mortality rates complemented by fibrinous and necrotising placentitis (Xavier *et al*., 2009) connected to involuntary orienting response of *B. abortus* for trophoblastic cells that are able to grant permission for intercellular growth of the pathogen (Carvalho Neta *et al*., 2008). This bacterium is present in the mammary gland, lymph nodes, foetal fluid and vaginal discharge of infected animals. Asymptomatic bulls develop orchitis which can be accompanied with vesiculitis and epididymitis and in a chronic case, it could cause testicular fibrosis and infertility in both sexes (Plommet *et al*., 1971; Plommet *et al*., 1973; OIE 2010).

Brucella canis can, also affect dogs, cause reproduction problems and infect human beings. The bacterium can reproduce itself and continue in host cells with other cells that have the same capacity (Fichi, 2003). Dogs are the mutual host but there are seldom chances that dogs can be infected by other *Brucella spp* such as *B.abortus, B. suis or B melitensis*. Mostly, signs

of abortion can be seen in kennelled dogs and bitches. Stillbirths and failure to conceive are common signs and other signs that may result are spondylitis, epididymitis, periorchitis and prostatitis in male dogs. Urine can be a source of transmission in both sexes. Exposure of the bacteria can be up to 18 months in the environment. The case of *B. canis* has never been reported since its first isolation was found in 3 dogs in the Western Cape Province, South Africa (Henton, 2010).

B. ovis infection causes ovine brucellosis in sheep found in different parts of the world such as South Africa, Australia, North and South America, New Zealand and parts of Europe (Burguess *et al*., 1982). Brucella in sheep is divided into two as follows: Ram epididymitis, caused by non-zoonotic agent *B. ovis*; and classical brucellosis, which is zoonotic (Acha *et al*., 2003). Early infection in ewe occurs after mating with a ram with poor semen quality, low sperm concentration and abnormal sperm (Cameron *et al*., 1976). The primary sign of *B. ovis* in rams that are sexually active is epididymitis and, sometimes, abortion in ewes, and development of lesions in the epidymis, either unilaterally or bilaterally. Lesions may occur bilaterally during examination and may be felt along the progression of the disease (Lawrence *et al*., 1961). Most rams show no signs of the disease even if they are infected and can hide the bacteria in the semen for a long period of time and spread the disease in the farm (Burguess *et al*., 1982). Direct contact between rams in the same farm can occur through contact between the infected and the susceptible (Brown *et al*., 1973). During gestation in ewes, *B. ovis* unusually causes abortion, accompanied by placentitis in the first 30 days and weak lambs with high rate of neonatal mortality is seen in other cases (Meineshagen *et al*., 1974)

Brucellosis in pigs is one of the crucial diseases caused by *Brucella suis,* with about three types of biovars (biovars 1, 2 and 3). These biovars differ around the country of occurrence (Olsen *et al*., 2012). Asia and America are found to be endemic with only these two types of biovars (1 and 3) and the two cause a serious reproductive problem in pigs and diseases in humans (Olsen *et al*., 2012). Biovars 2 are mostly found in Europe constitute one of the initial causes of abortions, infertility and high economic impact in pig production/farms, and infections in humans (EFSA, 2009). Spondylitis is a mutual sign that goes together with paralysis and abscesses are seen in bones and joints during post mortems (Poester *et al*., 2013). Reindeer/Caribou and Moose are naturally affected by biovars 4 and serve as reservoirs for other animals that can be affected by *B. suis spp* (Forbes *et al*., 1991).

In the 1990s, Brucella strains were isolated from several marine animals such as whale (Balaenoptera acutorostrata), seal (Phoca vitulina), dolphins (Tursiops truncatus; Delphinus delphis; Lagenorhynchus acutus; Stenella coeruleoalba) and other species (Ewalt *et al.*, 1994; Foster *et al.*, 1996). They were classified as *B. maris* and *Brucella spp* are now putatively allocated into two species: *B. ceti* from cetaceans and *B. pinnipedialis* seals (Foster *et al.*, 2007). With reported cases associated with neurological disorders in humans, marine isolates are found to have the capability of infecting humans (Sohn *et al.*, 2003; Hernandez-Mora *et al.*, 2008). It is believed that transmission to humans can be through direct or indirect contact with marine mammals or ingestion of meat from infected animals. Yet, there are few reports of humans with the disease caused by marine isolates in which there was no indication of contact of the patient with marine mammals (Sohn *et al.*, 2003; McDonald *et al.*, 2006)

There are some pathological developments in marine species and Brucella infections include abortion, hepatic, histiocytic inflammation, discospondylitis, meningitis, neurological signs and abscesses in the skin (Foster *et al.*, 1996). Meningoencephalitis has been described as the most reliable histological change in dolphins with neurological signs to *Brucella spp* (Hernandez-Mora *et al.*, 2008, Gonzale *et al.*, 2002). Transmission of Brucella in marine mammals can be by direct contact, through mucosa and injured skin through the oral route when there is ingestion of infected meat products (Foster *et al.*, 2002). Transmission to the foetus can be considered by vertical or horizontal route of infection, since the detection of Brucella can be isolated in milk and foetal tissues of dolphins (Hernandez-Mora *et al.*, 2008; Miller *et al.*, 1999). Furthermore, marine Brucella species are capable of infecting terrestrial mammal species (Rhyan *et al.*, 2001).

Brucella species was also reported in a baboon colony in the second trimester of the gestation period after they experienced two stillbirth cases (Schlabritz-Loutsevitch *et al.*, 2009). In baboons, it appears to be less pathogenic species of Brucella and was reported after being discovered in 1947 as *B. neotamoe* (Stoenner *et al.*, 1957) known to infect desert wood rats in natural conditions in the USA and since then, no other cases have been reported. *B. microti* was isolated in 2000 as a new Brucella isolate from common voles (*Microtus avails*) infected in South Moravia, Czech Republic (Scholz *et al.*, 2008). With regard to wild red foxes (*Vulpes vulpes*), *B. microtia* was isolated from mandibular lymph nodes in Austria (Scholz *et al.*, 2009).

2.4 Brucellosis in humans (Undulant fever/ Malta fever)

The disease is primarily one for animals but humans can contract it accidentally (Grove *et al*., 2003). During the 19th and 20th centuries, human beings on the island of Malta were the first to be affected by the disease (Maadi *et al*., 2011). Brucellosis in human is one of serious problematic public health diseases, and referred to as undulant fever.

There are currently about half a million cases of human infections that have been reported worldwide and because of unclear clinical symptoms of the disease (Brucellosis), the estimated number should be ten times higher. The disease is a serious threat to humans and has been established to be connected with farm workers, veterinarians, veterinary pharmacists, animal attendants, abattoir workers and laboratory attendants (Young, 1983). The seroprevalence of the disease in India was found to be as high as 6.3% in veterinarians, 7.9% in veterinary pharmacists, 8.8% in animal attendants, 20.0% in laboratory workers, 10.5% in dairy farmers and 6.4% in abattoir workers (Bedi *et al*., 2007; Deepthy *et al*., 2013).

There are five Brucella species that cause infection in humans such as *B. melitensis*, which is connected with occupationally contact or consumption of poorly prepared milk products (Corpel *et al*., 1997), *B. suis, B. abortus, B. canis* (Acha *et al*., 2003) and *B. ceti* found in marine (Brew *et al*., 1999; McDonald *et al*., 2006; Sohn *et al*., 2003). Infection in humans sometimes, is due to contact with the bacteria when working/helping infected animals during dystocia or in abattoirs, even through the oral and respiratory routes. As few as 10–100 bacteria can be able to cause infection in humans through aerosol and is very effective given the reasonably low concentrations of organisms (Maloney, 2001). This route of infection has brought attention to this old disease and has serious health and safety implications (Maloney, 2001). Infections can lead to spontaneous abortions, miscarriage, premature birth and intrauterine foetal death in pregnant women but with no birth defects (Mili *et al*., 1993; Khan *et al*., 2001; Kose *et al*., 2014). There is however, a low rate of mortality in untreated persons; the estimated rate of fatality does not tend to be higher than 2% to 5%. The cause of deaths is usually through endocarditis or meningitis (Sauret *et al*., 2002). Mistreatment or misdiagnoses of the disease are because mostly the disease is likely to be confused with malaria, typhoid fever and other diseases with febrile syndromes (Bax *et al*., 2007).

In some areas, the rate transmission from animals to humans is subjective of endemicity of animal infection, farming systems, hygienic standards and milk products such as cheese or unpasteurised milk can be a source of infection in humans. Safety measures should also be

practised when handling infected and cultured sample in the laboratory (WHO, 2004; Alton *et al*., 1988). *Brucella abortus* (S19) and *B. melitensis Rev. 1* live animal vaccines is known to cause the disease in humans. It is rare to find a transmission from direct contact from person-to-person but breastfeeding mothers can infect the child if infected with brucella (Arroyo *et al*., 2006; Kato *et al*., 2007). Recently other routes of transmitting the disease have been identified such as through blood transfusion (Akcakus *et al*., 2005), sexual transmission (Lim *et al*., 2005) and direct spread from infected persons in the same household (Sofian *et al*., 2007; Almnueef *et al*., 2004).

In a country such as Nigeria, Brucellosis should be suspected if a human patient is diagnosed to have an acute febrile reaction; such a person should be treated for Brucellosis (Ofukwu *et al* 2007; Diaz *et al*., 2011). The disease in humans is extensive in Nigeria mostly in an occupationally exposed groups. Selling and eating of the gravid uterus are common between meat handlers, and with traditional doctors using gravid uterus in traditional medicines (Crawford *et al*., 1990; McEntee, 2012). It was reported that the status of Brucellosis in different districts of Nigeria, with the prevalence of bovine brucellosis was from 0.2 and 80 % (Ducrotoy *et al*., 2014) and the prevalence of the disease in abattoirs and institutional areas in the southern part of Nigeria was between 3.7 and 38,8 % (Cadmus *et al*., 2010, 2013).

In countries with of poor sanitation facilities, and where safety precautions in abattoirs and slaughter slabs are not observed, there is increased risk of human exposure to brucellosis. For example, in some abattoirs in Nigeria, workers do not use gloves to protect themselves but use their bare hands to handle infected organs and carcasses from suspect or diseased animals (Cadmus and Adesokan, 2007).

In Botswana it has been reported that the practice of processing bush meat in household represents an important *Brucella spp* exposure risk to the community. Unsafe butchering and consumption of meat from an infected animal that is not well cooked can lead to the transmission of the bacteria to humans (Alexander *et al*., 2012).

South Africa is an endemic country of brucellosis and most people in rural areas are unaware of it as a zoonotic disease due to insufficient knowledge. The rate of infection of humans in South Africa is unknown (Godfroid *et al*., 2004). Clinical signs are undulant fever, pneumonia, endocarditis, meningitis, anorexia, polyarthritis, chills, weakness (Sauret and Vilissova, 2002), orchitis and prostatitis (Acha *et al*., 2003).

2.4.1 Treatment for human Brucellosis

For the treatment of Brucellosis in humans, limited antibiotics are active to be used against organisms if they enter the host and for these drugs to be effective, they should be often used together. The chance of the incidence of the disease to relapse is from 5% to 40%. Examples of such antibiotics are as follows: trimethoprim-sulphamethoxazole, quinolones, tetracycline, chloramphenicol and rifampicin (Montejo *et al*., 1993; Al-Tawfiq, 2008).

2.4.2 Control of Brucellosis in humans

Currently, there is no effective Brucella vaccine to prevent the disease in humans and hence the need for such a vaccine. The Soviet Union, in the past, widely used *B. abortus S19* as a vaccine for humans, but *B. melitensis Rev.1* and *S19* were inappropriate for human vaccination because of their ability to cause infection in human (Vershilova, 1961; Spink *et al*., 1962).

Prevention of Brucellosis in humans is achieved by controlling infection animals and greater care when handling animals, vaccine and foods that are suspect. Vaccination of humans is still in progress but nothing has yet been proved (Lawinsky *et al*., 2010; Corbel, 1997).

2.5 Mixed herd of animals

Mixing of animals during the grazing period may cause infection in animals that are not infected to easily get exposed to the disease from multiple sources such as contact with infected animals with a discharge and aborted fetus. Mixing animals in the farming area can be a risk factor as Brucella can be transmitted between different species of animals, however, goats and sheep are rarely infected with *B. abortus* (Ocholi *et al*., 2004). Wild pigs can be a carrier of *B. abortus* for more than 25 years (Stoffregen *et al*., 2007).

2.6 Brucellosis in wild animals

This disease is not often reported in wildlife. *Brucella abortus* and *B. suis spp* are likely to be detected in wildlife worldwide in animals such as bison, foxes, wild boars, feral pigs, African buffalos, European hare and waterbuck. Cases of *B. melitensis* in wild animals are rare (Davis, 1990). *Brucella suis* was eradicated in domestic pigs for years but the presence of this disease is reported to be at a low rate. The European hare is the reservoirs for the breeding of the disease. In feral pigs, infection is reported regularly in Hawaii, Queensland and Australia,

where the distribution was due to releases of infected feral pigs with *B. suis*. (Abou-Eisha 2000).

In the Middle East, most *B. melitensis* infections were due to contact of a one-humped camel with sheep and goats. The organism was isolated from camel milk and is a serious public health concern (Abou-Eisha, 2000).

In South Africa, a few species of wild animals have tested serologically positive for Brucellosis but the species (African buffalo, impala, eland, waterbuck, zebra and hippotamus) are probably of slight importance in the epidemiology of bovine Brucellosis in Southern Africa due to scarce contact between cattle and wildlife. Abortions are less common in water buffalo cows than cattle (Borriello *et al*., 2007). *Brucella abortus* was detected in the cotyledons of pregnant buffalos in slaughter houses and few cases of abortion in bison have been reported in Southern Africa (Gradwell *et al*., 1977). In the Kruger National Park, 23% of African buffalo were serologically positive for Brucellosis (Herr *et al*., 1981).

Mortality is rare in the case of adult animals but is usually seen in young ones in a herd; about 30% to 80% rate of mortality can be due to abortion and could lead to loss of cattle production on the farm and is less common in water buffalo cows (DAFF manual, 2004).

2.7 World distribution of *Brucella spp*

There are different species of Brucella worldwide and they differ in geographic distribution. A species that is mostly found universally is *Brucella abortus* which affects cattle farming, however, in New Zealand, Australia, Japan, Canada, Israel and parts of Europe, the species has been wiped out (Seleem *et al*.,2010). Since bison and elk are not in a closed system they continue to act as a reservoir for *B. abortus* in the Greater Yellowstone Area, in the United States of America. Brucella is still an issue in the Greater Yellowstone Area (GYA) that surrounds the Yellowstone and Grand Teton National Parks but was eradicated from domestic cattle (Clifford, 2008). In Florida and in the Mid-West because of feral pigs, *B. suis* is also problematic to cattle and other livestock (Leiser *et al*., 2013). In Guatemala and Panama both *B. suis* and *B. abortus spp* were found to be endemic (Pappas *et al*., 2005).

Even if the prevalence of Brucella is not known in presently it still affects the whole of the African continent and is customarily measured to be endemic in North Africa, with heavy consequences on public health, food security and food safety (Hotez *et al*., 2012).

Bovine Brucellosis is also endemic in most countries of South America and affects the production of livestock. Argentina is one of the countries in South America and it is estimated that about 50% of dairy farms are reported to be affected (Aznar et al., 2014). Eradication programmes in France, Sweden and Germany were effectively accomplished to as an outcome of satisfactory surveillance and eradication programme. However, in Spain and Portugal, the programme is still ongoing and has been found to be endemic (Pappas et al., 2005). The country with the highest Brucella incidences worldwide is Greece. Syria (in the Middle East), has the highest Brucella incidence reported and the continent is historically endemic (Pappas *et al*., 2005).

Brucella in China has shown that the disease is spread to the Southern provinces but there is an ongoing work to reverse this trend (Memish and Balkhy, 2006).

2.8 Occupational hazards

Since workers are being exposed to many risks in their daily work, reports suggest that occupational hazards are the main cause of morbidity and mortality among workers (Driscoll *et al*., 2005). Typically, in developing countries, the rate of blood borne and other communicable diseases is increasing due to occupational hazards (Susoinc *et al*., 2007) among abattoir workers, which can be either by iatrogenic or infectious agents such as bacteria, viruses, fungi and parasites or even toxins produced by these organisms (Ann *et al*., 2006). Human behaviour could endorse infection in workers if repeatedly exposed to contact with sick animals or trade of live/wild animals (Environmental Health Washington, 2004). The exposure control plan (ECP) is implemented by employers for any occupational hazard occurence and they will either identify risk, assessment of workers, training of workers and implementation of safe work procedures (Banjo *et al*., 2013).

2.9 Risk factors of brucellosis in humans

The prevalence of this disease varies according to sexual maturity, as old animals show a high incidence of infection rate (Abubakar *et al*., 2010). However, there is a debate about mature females as they show a high rate than male animals and are likely to be infected (report made by different workers in Punjab (India) (Aulakh *et al*., 2008). The danger in the prolonged seronegative phase is that infected dams may infect the calves at a time when they are born. In most communities, they make home-made milk products and consumption of

such milk poses a risk of infection with human Brucellosis (Sofian *et al*., 2008). Is has been reported that when the season changes, the rate of abortion tends to be high from February to July during delivery due largely to the disease (Shang *et al*., 2002). The percentage of prevalence in humans is high in summer, representing about 39,5% (Salari *et al*., 2003). The presence of Brucellosis in farm animals is said to be a factor in limiting animal growth and the success of livestock in Nigeria (Mai *et al*., 2012). The common route of the infection in abattoir/ livestock workers is through inhalation of the bacteria since most of the workers do not use or wear face masks or protective clothing. Workers are sometimes exposed through and when handling carcasses or wasted foetus (Aworh *et al*., 2013).

In Uganda, it was reported that in Kampala city, the number of annual cases of the incidence was largely due to the fact that people were buying milk contaminated with *B. abortus*, and the number was estimated to be 5.8 per 10,000 people and 12, 6% in the informal market (Makita *et al*., 2010). Lake Mburo National Park in Kiruhura District is considered the milk basin of Uganda and because of the close relationship between humans, domestic and wild animals and the high touristic activities in the area, it poses a serious risk (Makita *et al*., 2011). Movement of animals in trade practice, overcrowding of animals and unhygienic practices were reported in India as risk factors (Chand and Chhabra, 2013; Saini *et al*., 1992). Mixing of animals and sharing of pasture and water points could be a factor since the bacteria can be spread around (Kadohira *et al*., 1997; Kabagambe *et al*., 2001; Omer *et al*., 2000; Gumaa *et al*., 2014). Lack of information about vaccinating calf is the main setback which increases many problems (Singh *et al*., 2015).

In some cases goats are mistakenly vaccinated with RB51 which may result in abortion or stillbirth in pregnant goats (Villa *et al*., 2008; Herrera *et al*., 2011); vaginal discharge of *B. melitensis* could also spread the bacteria (Herrera *et al*., 2011).

2.10 Pathogenesis and the ability of the agent to cause disease

The incubation period of Brucella infection is 1 to 4 weeks and the pathogen is then shifted from the lymph nodes, different organs and body systems, eventually expressing various clinical signs and symptoms. The virulence of Brucella infection and the bacterium's evading of the immune system remain to be clarified and resolved (Gorvel *et al*., 2008). Its intracellular survival within polymorphonuclear and mononuclear phagocytes, escaping phagosome–lysosome fusion and the immune response, is facilitated by factors such as its

ability to produce urease, which offers protection from stomach acid, Brucella-containing vacuoles, where the bacteria can survive, LPS and Cu/Zn superoxide dismutase.

2.10.1 Mechanism of *B. abortus* pathogenesis

Pathogenesis of *B. abortus* and its mechanisms have virulence factors that are essential for invasion (Guzmán-Verri *et al*., 2001) and intracellular existence (Moreno and Moriyón, 2001), that can tolerate the organism to reach its intracellular replication site (Detilleux *et al*., 1990; Pizarro-Cerdá *et al*., 1998, 1999). In addition, the Brucella molecular mechanisms have no classical virulence factors such as exotoxins, cytolysins, capsule, fimbria, flagellum, plasmids, lysogenic phages, antigenic variation, endotoxic lipopolysaccharide (LPS), and inducers of host cell apoptosis (Moreno and Moriyón, 2001). Rough strains of Brucella are not efficient to invade host cells than smooth strain signifying that the LPS O chain plays a part in virulence and some of the rough strains are certainly virulent (Sola-Landa *et al*., 1998; Ko and Splitter, 2003). Due to low immunogenicity, Brucella LPS was originally known as a virulence factor and alternative complement pathway activation is prevented (Sangari and Aguero, 1996). Confirmation of the role of LPS was by chromosomal alteration of the O chain that rendered Brucella more prone to complement-mediated bacterial lysis (Allen *et al*., 1998) and to kill bacteria of peptides such as defensins and lactoferrins (Lapaque *et al*., 2005).

In addition, Brucella LPS was long said to be a weaker inducer of the immune response than of enterobacterial endotoxins (Keleti *et al*., 1974). Cellular apoptosis is inhibited by the LPS O chain, avoiding immune response activation (Jimenez de Bagues *et al*., 2004; Pei and Ficht, 2004; Pei *et al*., 2006). It is important that Brucella LPS plays a more significant role in virulence while the organism is in the extracellular environment prior to invading host cells (Ko and Splitter, 2003). However, *B. abortus* rough mutant strains have a lower strength to survive intracellularly than smooth strains while the LPS O chain is important for entry and early intracellular stage of Brucella in macrophages (Porte *et al*., 2003; Lapaque *et al*., 2005). At the time of internalisation *B. abortus* depends on a two-component guiding system named BvrR/BvrS, which is needed for recruitment of GTPases and looking after the outer membrane. Thus, bvrS–bvrR organism is damaged for the invasion of non-phagocytic cells and intracellular survival (Lopez-Goni et al., 2002). The two components of this system are BvrS (a sensor protein and one of the histidine-kinase superfamily) and BvrR (which is a controller protein). Both components control the look of outer membrane proteins (Omp)

participating in the invasion of host cells (Lopez-Goni *et al*., 2002; Guzman-Verri *et al*., 2002). *B. abortus* strains with bvrR and bvrS organism lack the capability to recruit GTPases of the Rho subfamily, mainly Cdc42, which is needed for actin polymerisation and invasion of host cells. Furthermore, mutants invade host cells and are stimulated artificially by enzymatic treatments, the mutants are more liable to the host cells killing mechanisms. Attenuation in intracellular existence, in this case, is triggered by the incapacity of the mutants to prevent phagosome–lysosome fusion (Sola-Landa *et al*., 1998; Lopez-Goñi *et al*., 2002). Depletion of Brucella cyclic b-1,2-glucans synthetase results in absence of cyclic b-1,2-glucans, which are constituents of outer membranes that are necessary for the existence of *B. abortus* in mice and intracellular reproduction in HeLa cells (Briones *et al*., 2001). Cyclic b-1,2-glucans are needed during the early intracellular stage of Brucella infection since they avoid phagosome–lysosome fusion even though they are not essential for the trafficking of Brucella to the RER.

The Brucella type VI emission system is programmed by the virB operon (Comerci *et al*., 2001; Delrue *et al*., 2001), which is made of 12 genes, i.e. virB1 through virB12. An orthologue T4SS was first recognised in the plant pathogen Agrobacterium tumefaciens, and later recognised as an important virulence mechanism of *B. abortus* needed for an intracellular increase of the organism (O'Callaghan *et al*., 1999; Hong *et al*., 2000; Sieira *et al*., 2000). The virB encoded T4SS is necessary for intracellular increase of *B. abortus* in both phagocytic and non-phagocytic cells such as of HeLa cells (O'Callaghan *et al*., 1999; Sieira *et al*., 2000; Comerci *et al*., 2001; Delrue *et al*., 2001). Even if molecular mechanisms by which the T4SS system influences on *B. abortus* is not clear, apparently secreted effectors play a role in the biogenesis and maturation of the *B. abortus* having vacuole, and transport of *B. abortus* to its intracellular site of replication (Delrue *et al*., 2001; Comerci *et al*., 2001; Boschiroli *et al*., 2002), with indication that the system is obligatory for fusion of the autophagosome-like vacuole with the RER (Arellano-Reynoso *et al*., 2005). Trial infection of mice and cultured cells with strains of *B. abortus* with a defective T4SS results in intracellular killing the mutant strains fail to reach the RER (O'Callaghan *et al*., 1999; Hong *et al*., 2000; Sieira *et al*., 2000; Comerci *et al*., 2001; Delrue et al., 2001; Sun *et al*., 2002; Watarai *et al*., 2002; Den Hartigh *et al*., 2004, 2008; Kim *et al*., 2004; Celli, 2006). Even though the T4SS are totally needed for intracellular survival and replication of *B. abortus* (Hong *et al*., 2000; Boschiroli *et al*., 2002; Celli, 2006), apparently the system does not play any part during invasion and the primary steps of infection of host cells (Celli, 2006).

Brucella T4SS is as well vital for determined infection in mice and to impel the host immune response (Rolán and Tsolis, 2007, 2008; Roux *et al*., 2007). It is also necessary to cause inflammatory and immune responses during Brucella infection in mice. *B. abortus* lacking useful T4SS is incompetent in stimulating the appearance of pro-inflammatory genes and types I and II interferon (IFN) response as created by the wild type strain in the spleen (Roux *et al*., 2007).

In addition, a virB mutant strain persists longer in B- and T-cells hit mice than in control mice (Rolan and Tsolis, 2007), while the T4SS is needed for premature response of cytokines such as interleukin (IL)-12 and IFN c that helps the T-helper cell type 1 (Th1) polarisation of the immune response (Rolan and Tsolis, 2008).

2.11 Diagnosis and challenges Brucellosis

It is difficult to diagnose brucellosis since the disease shows a variety of the manifestation of clinical signs. Accurate and fast diagnosis test is confirmed only by laboratory test and if misdiagnoses are done or failure to treat, high fatality cases are seen (Dahouk *et al*., 2007). The gold standard used to diagnose is by isolation of Brucella from blood, bone marrow, cerebrospinal fluids or lymph nodes (Acha *et al*., 2003; Glynn and Lynn, 2008; Mantur and Mangalgi, 2004). A diagnostic test commonly used to detect acute infection is serum agglutination test but there are other tests such as the indirect enzyme-linked immunosorbent assay, Rose Bengal test and Coombs test. The two tests used in chronic cases are the Complement Fixation tests and 2-Mercaptoethanol (Acha *et al*., 2003; Orduna *et al*., 2000).

The most accurate diagnosis of human Brucellosis is specifically by doing laboratory tests. For patients (e.g abattoir workers, farmers and others) who are likely to be infected with Brucellosis, they require a combination of several approaches such as taking medical history, clinical examination, routine haematological and biochemical laboratory tests, radiological investigation and Brucella-specific culture, serological and molecular tests. It’s important to note that most of the times, haematological tests findings are not specific for the diagnosis of human Brucellosis, and some tests have advantages and limitations guarantees when interpreting results.

2.11.1 Diagnosing Brucella from different tissues and blood

Isolation of the pathogen from tissues and blood is the ultimate method for diagnosis of Brucellosis. The number of bacteria from definite infected samples differs in according to the

stage of the disease when the disease is in the acute or chronic stage (Al Dahouk *et al*., 2003). It is assumed that if the number of feasible bacteria present in the blood of infected patients is lower, then the volume of a sample is important and timing of detection is contrariwise linked with the concentration of organisms in the blood sample (Yagupsky *et al*., 1999). The presence of bacteria in the blood usually takes place early in the progression of the disease and because of the presence of the bacteria in the blood of a patient they experience fever and chills, which are the signs of the infection (Kadanali *et al*., 2009). During the first two (2) weeks of clinical signs, the rate of isolation is much higher at the time during the pyrexia phase (Memish *et al*., 2000) and more samples of blood increase the rate of exposure. The sensitivity rate in acute cases can be from 80 % - 90 % and much lower in chronic cases ranging between 30% and 70% successful isolation rate which is greatly influenced by the technical method used (Espinosa *et al*., 2009; Franco *et al*., 2007).

With regard to patients with chronic diseases, the possibility of isolation of the bacteria can be enhanced using tissue samples from the affected spot, and use of selected media like Farrell's medium can be useful (Farrell *et al*., 1974). Blood cultures for the detection of *Brucella spp* have not been proved to be more sensitive than bone marrow culture at any stage of the disease (Gotuzzo *et al*., 1986; Mantur *et al*., 2008) and the method has proved its effectiveness in patients treated with antibiotics. Bone marrow aspiration and biopsy procedure should be regulated to specific cases since the procedure is painful (Gotuzzo *et al*., 1986).

Brucellae can be detected in the blood of infected patients four days after infection or even less (Cetin *et al*., 2007), but in other cases, it is recommended for the incubation period to be long at least four weeks, with intermittent subculturing (Yagupsky *et al*., 1999). Enrichment of the bacteria can further increase the isolation rate of Brucellae from blood samples using blood clot culture techniques or lysis centrifugation (Epinosa *et al*., 2009; McDonald *et al*., 2006). The lysis centrifugation system is the most efficient method recognised because of the independence of the stage of the disease. In both blood samples and sterile body fluids, time can be reduced to two days for detection (Cetin *et al*., 2007; Epinosa *et al*., 2009; Mantur *et al*., 2004). When isolates of facultative intracellular pathogens shell, vial culture is appropriate when demanding cultivable Brucellae from clinical specimens with very low numbers of cultivated Brucellae (Rovery *et al*., 2003).

2.11.2 Serological diagnosis of human Brucellosis

In humans, serological testing is confirmed to be a fast, safe and more sensitive test favoured in routine clinical practice. Diagnostic titers can be noticed after acute infection and can take months or years despite healing accomplishment, agglutination titres is ≥ 1:160 or more (Ariza *et al*., 1992). The detection of antibodies even without signs or background of being exposed to the disease remains questionable (Al Dahouk *et al*., 2011). Treatment follow up can be by serological test, which is an initial diagnosis of human Brucellosis. Negative results can take place when a serological test is done particularly at an early stage of the disease therefore after one or two weeks repeating of laboratory test should take place in suspicious cases (Al Dahouk *et al*., 2003).

2.11.3 Serum agglutination test

Serum agglutination test was a preferred method for human Brucellosis. Even now it is considered for serological diagnosis (Al Dahouk *et al*., 2003) as a gold standard assay for human Brucella. Labour-intensive and time consuming classic tube agglutination test is replaced by more a test-like slide, plate and card agglutination test for routine clinical laboratories, an example of a card test the Rose Bengal Test (RBT) (Ruiz-Mesa et al., 2005). The RBT antigen is built on 8% antigen suspension of *B. abortus* strain 1119-3 (United States Department of Agriculture). In most endemic countries, RBT is used as a rapid screening in crisis cases but is not effective enough in patients repeatedly exposed to the mediator (Ruiz-Mesa *et al*., 2005). More serological tests need to be performed to avoid RBT false-positive results (Diaz *et al*., 2011).

2.11.4 Coombs test

Coombs test is used to detect incomplete or nonagglutinating antibodies as a complement to the serum agglutination test, especially in chronic cases and at relapse. Coombs test has proved to be the best tool choice when serum agglutination test (SAT) results are either inconclusive or negative (Casanova *et al*., 2009), but both the CT and SAT are labour-intensive and time-consuming. For an alternative option, the Brucellacapt, a single step immunocapture assay can be an option for the detection of total anti-brucella antibodies. Brucellacapt titres are good because of infection marker in the independent stage of the disease.

2.12 Brucellosis control

In South Africa, Brucellosis is controlled under the Animal Disease Act 35 of 1984. The Directorate of Veterinary Services introduced the Bovine Brucellosis Scheme in 1979 for all commercial farms to be registered under the Brucellosis scheme and animals are regularly tested by the state veterinary services. Animals found positive after three consecutive tests, are branded and sent to the abattoir for the last slaughter. On negative farms, a vaccination programme is installed and animals vaccinated using two accepted vaccines: the S19 and RB 51. Although commercial farmers under the scheme can sell positive animals to an abattoir and/or to other farmers and still be registered under Brucellosis scheme, the strategic way to eradicate or control infection in an animal is by vaccinating and all infected animals should be eliminated (Briones *et al*., 2001).

a) Strain 19 vaccine

Brucella abortus strain 19 (S19) vaccine is commonly and widely used in the world against bovine Brucellosis. The vaccine has the ability to protect cattle against abortion or infections (McDiarmid, 1997). *Brucella abortus S19 vaccine* is good for immunity to reasonable challenges by virulent *B. abortus* or *B. melitensis* organisms. *S19* is one of the live vaccines used in female cattle at the age of 3 to 6 months of age with a normal single dose of $5–8 \times 10^{10}$ possible organisms subcutaneously and for adults cattle, the dose is decreased to 5×10^{9} from 3×10^{8} organisms subcutaneously. Some cases of S19 vaccination, it is either the vaccine strain is excreted with milk or the animal aborts if pregnant and the development of persistent antibody titres in vaccinated animals with reduced dose. To avoid this complication vaccination procedure to increase protection chances is when the vaccine is given to cattle of any age either by one or two doses of 5×10^{9} viable organisms, not by subcutaneous route but given by the conjunctival route against both *B. abortus* (*Nicoletti et al*., 1978) and *B. melitensis* (Jiménez de Bagües *et al*., 1991). Without experiencing antibody response, high risks of abortion and excretion in milk when vaccinating adult cattle. When using strain S19 for vaccination and for eradication policy to be successful at the time of test and slaughter, there must be rigid control of animals to be vaccinated based on their age. Use of S19 indicates that production of antibodies depends on the age of the animal at the time of vaccination (Morgan, 1969).

b) RB51 vaccine

The RB51 vaccine is approved but there has been disagreement on the performance of the vaccine because unlike strain S19, RB51 interferes with serological diagnoses. In other countries, the vaccine is officially used in preventing Brucellosis in cattle since 1996 (Moriyon *et al*., 2004). Different countries use different methods in applying the vaccine. In the United States of America, they start to vaccinate calves from between 4 and 12 months of age subcutaneous with a dose of 1–3.4 × 10^{10} viable organisms and for cattle, from the age of 12 months the dose is 1–3 × 10^{9} viable organisms but vaccination is done only under authorisation from the State or Federal Animal Health Officials (USDA, 2003). Other countries vaccinate calves with a different dose. Some suggest doses be given at 1–3.4 × 10^{10} dose and repeated after 12 months of age onwards to boost immunity (Verger, 1985). When *B. abortus strain* RB51 is given in a right/correct dose intravenously in cattle, it induces the infection of the placenta and placentitis in supreme vaccinated animals but still, some will excrete the vaccine in milk. Both the strain S19 and RB51 after observation, the record states that pregnant cattle should not be vaccinated because of a chance of abortion unless given at a low dose of (1 × 10^{9} colony-forming units [CFU]) in the late pregnant animal. The main challenge is that if a dose in a pregnant animal is reduced, during vaccinating, calves may not be protected against *B. abortus* (Verger, 1985).

The best and available vaccine used to control the disease in ovine and caprine is *B. melitensis* strain Rev 1, which should be administered at the standard dose by conjunctival route and precautions taken when handing the vaccine since it is highly infectious to human(Adones et al., 2008; Blasco, 1997; Garin-Bastuji et al., 1998). To reduce abortion in ovine and caprine Rev 1 can be given at times of pregnancy and the antibody reaction to the vaccination cannot be distinguished to the infection witnessed after field infection which delays control programmes (Adones *et al*., 2008; Blasco, 1997; Garin-Bastuji *et al*., 1998). Good measures of husbandry, together with vaccination programmes, are likely to succeed in the eradication of Brucella (Morgan, 1969). Rev 1 is not effective in a cow with *B. melitensis*. Immunisation of lamb and kids with Rev 1 is at the age of 3 and 5 months with a single dose subcutaneously route; the standard dose is between 0.5 × 10^{9} and 2.0 × 10^{9} viable organisms. When using *B. melitensis* Rev 1, care should be taken seriously as it can contaminate the environment. The side-effects of abortion or the excretion of the vaccine in milk are avoided by giving full dose conjunctively at the time of lambing and kidding or before they start to mate (Blasco, 1997).

c) Live Brucella vaccines

Live Brucella vaccines have shown that they can give the best defensive response against infections and abortions (Yang et al., 2013). The development of these new live Brucella vaccines takes into account, security issues for both the animal host and humans administering the vaccine. The vaccines provide a better protective response against both the infection and abortion than conservative livestock vaccines. When developing a live vaccine, care should be taken to generate a strain which can appropriately persist in the host to aggravate an appropriate adaptive immune response, while simultaneously exhibiting reduced virulence to render it safe (Yang et al., 2013; McGiven et al., 2015).

d) Subunit vaccines

Subunit vaccines use a purified protein or DNA approach to stimulate immune responses in animals (Yang *et al*., 2013). Subunit vaccines are safe to use since they are not a live vaccine, they are not infectious and cannot cause a disease such as the case is with a live vaccine. They also have the ability to protect against more than one Brucella species because they have extensive gene homologies (Yang *et al*., 2013). The antigen in subunit vaccine is confirmed to have the potential (Gomez *et al*., 2013). The vaccine contains the plasmid DNA, OMPs and LPS for *B. ovis*. The vaccine is multivalent because of the genes (Dhama *et al*., 2008; Da Costa Martins *et al*., 2010). One dose of subunit vaccine was required to be protective but it has been proved that the antigen is generally less effective than the live vaccine (Yang *et al*., 2013).

2.13 Other bacteria that might be isolated from carcasses and can be of public hazard

Enterococcus: The *Enterobacteriaceae* group are of epidemiological concern as most of them are pathogenic and may cause serious infections and or food poisoning (Al-Mutairi, 2011). In the past, *enterococcus* was considered the most central bacterial causing nosocomial infections in humans (Hunt, 1988; Tacconelli *et al*., 2008; Vankerckhoven *et al*., 2008). Currently, the species have developed exact contrivances of resistance that have made *enterococcal* infections hard to treat. *Enterococcus* is usually found in meat and poultry by-products and could be one of the routes of exposure to humans. *Enterococci* can cause gastroenteritis in immune-compromised humans since it is present in vegetables, water and cheese through contamination, and the bacteria can invade the intestines of both domestic and wild animals during grazing (Giraffa, 2002). The presence of *enterococci* in food products is

considered an indication of poor hygienic conditions during production and processing. In food processing, *enterococci* is used to extend shelf life, for example, fermented meats and cheese (Centeno et al., 1996. Cocolin *et al*., 2007) so is used as the probiotic and a starter in these products (Gaggia *et al*., 2010)

Two *enterococcal spp* found to be increasingly important pathogens internationally are *E. faecalis* and *E. faecium*. Due to of their life-threatening nature, they have the ability to cause infections, including bacteraemia, infective endocarditis and mortality within 30 days (Arias *et al*., 2008). Due to the resistance of the bacteria, it is difficult to treat, since they have acquired resistance to other antimicrobial agents such as Quinolones, Macrolides, Tetracyclines, Streptogramins and Glycopeptides (Arias *et al*., 2008; Murray, 1990). *Enterococcal species* prefer different conditions such as *E. faecium* and *E. faecalis* in the gastrointestinal tract of humans and *E. mundtii* and *E. casseliflavus* in plant sources. The species are necessary for microflora of both human and animal sources (klein, 2003). Infections caused by *enterococcal spp* are hepatobiliary sepsis, surgical wound infections, bacteraemia and urinary tract infections (Poh *et al*., 2006).

Enterococcus spp have three virulence factors that determine their capacity to cause infections as a bacteria (capability to colonise the gastrointestinal tract, which is the normal habitat; capability to stick to urinary tract epithelia, oral cavity epithelia and human embryo kidney cells and the capability to stick to a range of extracellular matrix proteins, as well as thrombospondin, lactoferrin and vitronectin (Franz *et al*., 1999).

Staphylococcus: Many *staphylococcal species* are bald since they do not harm humans and animals when found to reside in their skins (Guran and Kahya, 2015). *Staphylococcus* is one of the food-borne diseases and poisonous bacteria from the staphylococcal family. *Staphylococcus aureus* is common on the skin and mucous membranes of both humans and animals (Tong *et al*., 2015). Food-borne disease is the number one problem world-wide; because of its affects on public health and the economy. Food contamination with pathogens has raised public health concerns since some of these pathogens produce toxins (Bryan, 1982). *Staphylococcus aureus* has been identified in red meat, poultry and their products from several countries (Genigeorgis, 1989; Wieneke *et al*., 1993). Red meat is slightly involved in *staphylococcal* food poisoning, however, both frozen and fresh poultry and their products are most likely to be contaminated with *S.aureus* (Waldroup, 1996). *Staphylococcus aureus* has the ability to cause more than one infection in human beings (Bergdoll, 2000).

Infection in humans is by ingestion of highly contaminated food with *staphylococcal toxin* e.g *exfoliative toxins*, *staphylococcal enterotoxins* and other toxins produced by the genus of *staphylococcus* (Dinges *et al*., 2000; Le Loir *et al*., 2003).

Reports in countries such as the United States of America reveal that up to 185000 cases of food poisoning were caused by *Staphylococcus aureus* enterotoxins every year and 0.94% hospitalised annually (Wieneke *et al*., 1993).

Clostridium: Clostridium pathogens can cause disease in humans and animals. *Clostridium spp* can survive both standard cooking and food processing methods by forming spores if the condition is not favourable (Liu *et al*., 2011). Clinical signs and symptoms can be absent from infected hosts with clostridia (Keessen *et al*., 2010). Regardless of this, in some patients, symptoms may vary from uncomplicated watery diarrhoea to bloody diarrhoea or stiff and flaccid muscles (Songer *et al*., 2010; Loo *et al*., 2005; Willey *et al*., 2008). According to Oksana et al. (1998), *C. histolyticum* has not been isolated before in patients with endocarditis or bacteremia. *C. histolyticum spp* is one of the histotoxic clostridia and is known to be involved in myonecrosis. In hospitals and other healthcare facilities, the rate of illness caused by *Clostridium difficile* is said to be increasing in the community (Hensgens *et al*., 2012).

Clostridium difficile and *Clostridium perfringens* type A are able to produce heat-resistant spores. These species are mostly isolated in foods, especially in meats, vegetables and shellfish (Weese, 2009; Rupnik *et al*., 2010; Hoover *et al*., 2013). Cooked meat that is linked to *C. perfringens* food poisoning and spores can survive cooking temperatures when left at temperatures between 12 and 50 degrees. Those that will stay, will grow and start to multiply in the intestine and form toxins causing gastroenteritis (Grass *et al*., 2013). A report on the rate of prevalence reveals that *C. difficile* is commonly found in the intestines of different animals due to consumption of food contaminated with the bacteria (e.g cattle, pigs, sheep, and birds, as well as dogs and cats) (Hensgens *et al*., 2012; Koene *et al*., 2012). There are few antibiotics that have been found to be successful in decreasing infections caused by *C. difficile* (Vonberg *et al*., 2008). Using soap and water to wash hands can help reduce spores better than using alcohol-based hand sanitizers (Jabbar *et al*., 2010).

CHAPTER 3

METHODOLOGY

3.1. Sampling areas

Samples were collected from five different abattoirs in the North West Province, South Africa as follows: Vryburg; Zeerust; Koster; Potchefstroom and Stella.

3.2 Sampling method

Animal selection was based on simple random sampling. Only non-branded cows were considered and targeted for sampling. Samples were collected for five (5) consecutive days and collection done on 10% of the total number of carcasses of animals slaughtered daily.

3.3 Collection of tissues

Sample collection was done from September 2015 – February 2016. A total of 236 samples were collected using sterile knife and gloves (Table 3.1). The following-tissues were collected from different animals: Lymph nodes, Spleen, Mammary glands, Amniotic fluids and Uterus/Placenta. The samples were labelled and packed in different plastic bags. The plastic bags were transported to the Animal Health Laboratory, North West University in a cool box containing ice. All the samples were kept at 2-8 °C until further analysis.

Table 3.1: Number of samples collected from each abattoir

	Name of abattoir collected from					
Type of samples	Vryburg	Zeerust	Stella	Potchefstroom	Koster	Total
Spleen	3	7	12	6	9	37
Lymph nodes	16	14	7	9	6	52
Uterus	20	19	19	18	11	87
Placenta	25	0	11	10	14	60
Total	64	40	49	43	40	=236

3.4 Isolation of bacteria

3.4.1 Precautionary and safety measures

To minimise contamination in the laboratory, biosecurity measures were followed according to biosafety level 3 practices. Protective clothing were used at all times, such as a laboratory coat, gloves, safety glasses or eye shields and face masks during the processing and

manipulation of specimens. Before and after use the biosafety cabinet area was cleaned with an appropriate disinfected 70% Ethyl or F10. All samples were handled with great care and labelled as potentially hazardous.

3.4.2 Test procedures

3.4.2.1 Preparation of test materials

Materials required to process the specimens were cleaned, sterilised and made available on the bench before the experiment. All samples to be tested and culture media were prepared and left in a safety cabinet for at least 30 minutes to reach room temperature before the procedure. The tissue samples were cut under biological safety cabinet class 2 (model, 4BI020158. South Africa).

3.4.2.2 Tissues culture and isolation of Brucella abortus

- Morphological identification of isolated samples.

The culture and identification of *Brucella spp* were done according to the method described by Plackett (1986). Brucella agar media were used to culture the bacteria. Lymph nodes, spleen, mammary glands, amniotic fluids and uterus/placenta samples and fats were removed. Samples were dipped in ethanol and then flamed to burn off the ethanol and cut into several pieces each about 1cm³, using sterile forceps and scalpel blades, placed in a zip plastic bag and a volume of 5ml sterile saline added. The plastic were placed in a Stomacher®400 Circulator machine for 1 minute. Fluids from the specimen were swabbed in media plates.

Fluids were placed onto the Brucella agar media and incubated at 10% CO_2 for 48 hours. Plates not showing the colonies (Negative cultures) were further incubated for 72 hours. For confirmation, positive Brucella control strains obtained from the Potchefstroom Laboratory (SANAS accredited) were cultured using the same conditions.

Cultured plates were monitored on a daily basis. The first evidence of growth was noticed by a tiny colony resembling droplets of moisture which appeared after 48 hours of incubation. The plates were sub-cultured on a fresh media for 2-3 days. After the first 72 hours of incubation, colonies should be large and stained smear should show the characteristic of coccobacillus forms. Plates were discarded as negative after 8-10 days of incubation.

3.5 Primary Biochemical test

- ***Gram staining***

Gram staining method was used to distinguish between the two key categories of bacteria (Gram positive and Gram negative). This step in Gram stain is a very vital preliminary step and a key procedure in identification. Gram staining was performed using standard methods (Fawole and Oso, 2004; Purkayastha *et al*., 2010). Staining was done as follows: a single colony was picked up from a pure culture with a sterile wire loop and smeared on the slide; few drops of sterile water were added and the slide heated for few seconds; crystal violet was applied on the slide for two minutes and the slide was decolourised using 95% ethanol.

Following crystal violet, few drops of iodine solution were applied on the slide for few seconds and the slide washed again with running tap water. Few drops of 95% ethanol were applied on the smear slide till for few seconds, then the slide was washed again with running tap water. Finally, few drops of safranin solution were dropped on the slide as a counter stain for 1 minute. The slide was then washed with running tap water. The slide was blotted and dried in air and visualised using a light microscope (Axio star plus, Serial. 3108001956) and immersion oil.

- ***Catalase test***

The catalase test was used to determine the presence of the catalase enzyme, which degrades toxic hydrogen peroxide (H_2O_2) in cells. A pure colony was transferred onto the surface of a microscopic slide using a sterile inoculum loop. A drop of 3% hydrogen peroxide was added and the slide observed for the presence of bubbles. Generally, the test reaction is very fast. This test is particularly important for Gram positive bacteria, *Staphylococcus* spp. and *Micrococcus* spp. Catalase negative results showed *Streptococcus* spp. and *Enterococcus* spp. The presence of bubbles after 10 seconds is an indication that the sample is catalase positive (Montso and Ateba, 2014).

- ***Oxidase test***

Oxidase test is used to differentiate between oxidase negative *Enterobacteriaceace* and oxidase positive *Pseduomadacaece*. This test was performed using the Oxidase Test reagent™ (Mast Diagnostics, Neston, Wirral, U.K) as recommended by the manufacturer. The oxidase is based on the principle that tetramethyl-p-phenylenediamine is oxidised by bacterial cytochrome in the presence of atmospheric oxygen to form a purple coloured compound. A pure culture of each bacterium was singled out using a sterile wire loop and

smeared on an oxidase strip paper (Microbact identification kits, MBO266A). After 5-10 seconds, the formation of a purple or blue colour indicates an oxidase positive result and a delayed positive result appears after 10-60 seconds. A negative result shows no changes in colour on the strip paper (Ateba and Setona, 2011).

3.6 Identification of the isolates

3.6.1 *Molecular identification:*

Identification of bacterial isolates in the present study was based on 16S-rDNA gene sequence analysis (Fakruddin and Mannan, 2013; Kirk *et al*., 2004). These techniques involved DNA extraction, PCR amplification, Agarose gel electrophoresis and phylogenetic investigation.

3.6.2 Genomic DNA extraction

A commercial Zymo Research kit (Zymo-Research fungal/Bacterial Soil Microbe DNA, D6005 USA supplied by Bio lab, South Africa) was used for the extraction of DNA samples according to the manufacturer's instructions. Accordingly, the isolates were transferred into nutrient broth (TSA), incubated at 37°C for 24 hours. The inoculum products were then transferred into 15 mL conical tube and centrifuged at 15000 rpm for 10 minutes, the pellets were collected and used for DNA extraction as follows: pellets were suspended in 750 µL lysis solution; disrupted with a disruptor gene (Inquaba biotech, mode SID258, USA) and vortexed at 14.000 rpm for 14 minutes followed by centrifugation at 10.000 rpm for 1 minute. Four hundred µL of the upper aqueous phase was aliquoted into a new zymo-spin IV™ and centrifuged at 7000 rpm for 1 minute. 12.000 µL of buffer was added to the filtrate and then 800 µL of the mixture transferred into a new collection tube (Zymo-spin IIC™) and the tubes centrifuged at 10.000 rpm for 1 minute. The filtered DNA were pre-washed by adding 200 µL DNA pre-wash buffer and the tubes centrifuged at 10.000 rpm for 1 minute. 500 µL of DNA wash buffer was added to the new collection tube (Zymo-spin IIC™) and the tubes centrifuged at 10.000 rpm for 1 minute. Finally, 100 µL of DNA elution buffer was added to elute the DNA in a sterile 1.5 mL micro-centrifuge tube.

3.6.3 Amplification of 16S rDNA

Amplification of 16S rDNA gene was performed using an DNA Engine DYAD Peltier thermal cycler (Bio Rad. USA). The PCR of endophytic bacteria was conducted using Oligonucleotide primers for PCR assay of *B. abortus* IS711 repetitive Genetic primer, forward (5'-GACGAACGGAATTTTTCCAATCCC-3') and the Reverse (5'-

TGCCGATCACTTAAGGGCCTTCAT-3') and also by using the Universal primers, forward 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and the reverse 1492R (5'-TGA CTG ACT GAG ACG TTG CGA-3'). These primers were commercially synthesised by Inqaba biotechnical Industrial (Pty) Ltd (Pretoria, South Africa). A total volume of 25µL containing: 12µL PCR Master Mix, 1µL template DNA, 10µL nuclease free water and 1µL of each oligonucleotide primer were prepared and mixed in PCR tubes (Ngoma *et al.*, 2013; Ngoma et al., 2014). Thermocycling (Bio-Rad T100™ thermal cycler, Singapore), conditions consisted of an initial denaturation at 95^0 C for 5 minutes, followed by 35 cycles, denaturation at 95^0 C for 1.15 minutes, annealing at 55.5^0C for 2 minutes, and two extensions at 72^0 C for 2 minutes and a final hold at 4^0C.

3.6.4 Agarose gel electrophoresis

PCR products were resolved in 1% agarose gel in 25ml 1x TAE buffer. The agarose gel was prepared as follows: 500g of agarose gel power was weighed and 490ml of 1% TAE buffer added and the bottle mixed thoroughly. The mixture was heated using a microwave for 5 minutes and the gel allowed to cool down to 40˚C and agarose gel stained in 0.5 ml of ethidium bromide. A 1 kb DNA ladder (Fermentas Thermo Scientific, Waltham, USA) was used to determine molecular size. Electrophoresis was performed at 80 voltage and 400 MA for 90 minutes. Gel was then visualised under UV light at 420mm wavelength using a Bio-Rad Chemi Doc™ MP imaging system (Bio-Rad. USA) to confirm the expected size of the product. The presence of a single bright band (DNA bands) indicates successful amplification. Following electrophoresis, the PCR products obtained were sent to Inquaba, biotechnology in Pretoria, for sequencing.

3.6.5 DNA sequencing

The purified PCR fragments of the 16S rDNA of the bacterial isolates were sent to INQABA biotechnology, Pretoria, South Africa for sequencing. The sequences and chromatograms were observed with Bio-systems; forward and reverse sequences were compared and corrected for conformity. Blast programme tools were applied to search for the sequences as described by (Altschul *et al.*, 1997) to find the closest match for each in the Gen-Bank. The closest sequences were then downloaded and aligned with the original sequences using clustal (Thompson *et al.*, 1994) and edited via Bio-edit for all to have the same length. The edited sequences were compared using maximum parsimony in the programme DNA pair (Felsentein *et al.*, 1989). Results were then, showed as bootstrapped (1000 boot strap).

3.6.6 Phylogenic tree

Sequencing results obtained were analysed and corrected with Bio-Edit sequence alignment according to Ngoma *et al*. (2013). Multiple alignments of the sequences were performed with Mafft programme 6.864, against corresponding nucleotides sequences retrieved from Gen-Bank. Evolutionary distance matrices were created according to the methods developed by Tamura *et al*. (2011). The aligned 16S rDNA gene sequences were used to construct a phylogenetic tree and the establishment of phylogenetic relationship at the genus and species level neighbour joining analysis implemented with MEGA 5.10 package (Tamura *et al*., 2011) for minimum evolution; maximum likelihood (Fitch, 1986); unweighted pair Group Method with Arithmetic Mean (UPGMA) and maximum parsimony (Rzhetsky and Nei, 1992). Bootstrapping was done with 1000 replications (Saitou and Nei, 1987). Putative chimeric sequences were identified with the chimera Buster 1.0 software. Manipulation and tree editing were performed using tree view (Page, 1996).

CHAPTER 4

RESULTS

Two hundred and thirty six tissues samples were collected and cultured on the Brucella media. About 94 plated samples were able to grow in the CO_2 incubator at 37 degrees and Biochemical tests (Oxidase and Catalase) were performed. From the 94 isolates that were able to show, selection of isolates were made according to the morphological relation (colour, rough, smooth) and the area of the same abattoir. Fifty four isolates and 40 plated samples were excluded when during DNA and PCR confirmation.

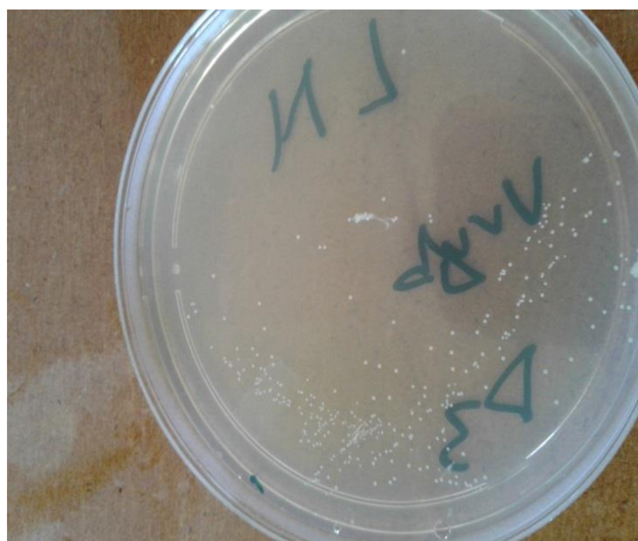

Figure 4.1: Plates showing bacterial growth after sub-culturing on new *Brucella abortus* plates and placed in the CO_2 incubator for 4 days at 37 degrees

PRELIMINARY RESULTS FOR BIOCHEMICAL TEST.

The aim of the study was to isolate *Brucella abortus* strain, thus biochemical tests were conducted on samples that showed growth to elements that constituted the focus of the study and the results are summarised in Table 4.1. Results showed that the following plates showed growth under CO2 incubation: 50; 50; 44; 36 and 22 % respectively of isolates obtained in samples from Vryburg, Potchefstroom, Koster, Zeerust and Stella.

Three major strains (*Bacillus spp., Coccobacillus spp* and *Streptococcus spp.*) were identified based on the morphology and other biochemical characteristics.

Table 4.1: Summary of results of biochemical tests in all gram negative bacterial isolates identified using the biochemistry methods

Biochemical test	Zeerust n=22	Potchefstroom n=18	Koster n=16	Vryburg n=16	Stella n=22
Gram negative	36%	50%	44%	50%	22%
Oxidase	41%	44%	37%	44%	45%
Catalase	18%	100%	19%	21%	0%
CO_2 growth	23%	83%	19%	25%	14%

Table 2.2: Summary of the occurrence (%) per organ and per abattoir where Bacilli, Coccobacilli, Staphylococcus and Cocci strains were morphologically identified stained blue on oxidase and reacted positively on catalase reactions

Type of samples	Morphology	Zeerust n=22	Potchefstroom n=18	Koster n=16	Vryburg n=16	Stella n=22
Spleen	Bacillus	5%	0	0	6%	5%
	Coccobacillus	16%	0	18%	6%	9%
	staphylococcus	0	6%	0	0	0
	Coccus		6%	0	0	5%
Lymph nodes	Bacillus	0	6%	6%	6%	0
	Coccobacillus	5%	0	0	6%	0
	coccus	5%	6%	0	0	0
	streptococci	0	0	0	0	5%
Uterus	Bacillus	0	6%	0	6%	0
	Coccobacillus	9%	11%	18%	0	9%
	Coccus	0	6%	0	0	0
Placenta	Bacillus	0	0	0	6%	0
	Coccobacillus	5%	0	0	13%	0
	cocci	0	6%	0	0	0

Table 4.3: Number of samples from each abattoir that were positive suspect of Brucella after running the biochemical tests and taken for confirmation by running the molecular work using different primers

	Name of abattoir collected from					
Type of samples	**Vryburg n=16**	**Zeerust n=22**	**Stella n=22**	**Potchefstroom n=18**	**Koster n=16**	**Total**
Spleen	2(13%)	3(14%)	3(14%)	2(11%)	3(19%)	13(71%)
Lymph nodes	2(13%)	2(9%)	1(5%)	2(11%)	1(6%)	8(44%)
Uterus	1(6%)	2(9%)	3(14%)	4(22%)	3(19%)	13(70%)
Placenta	3(19%)	1(5%)	0%	1(6%)	0%	5(30%)

Total	8 (51%)	8(37%)	7(33%)	9(50%)	7(44%)	=39(100%)

Table 4.4 Summary of isolated strains per organ sampled confirmed by PCR from Stella

Organs isolated from	Isolated organisms
Spleen	*Bacillus spp.*
	Staphylococcus aureus
	Enterococcus gallinarium
Lymph nodes	*Staphylococcus aureus*
	Enterococcus faecalis
	Enterococcus durans
Uterus	*Clostridium spp*

Table 4.5: Summary of isolated strains per organ sampled confirmed by PCR from Potchefstroom

Organs isolated from	Isolated organisms
Spleen	*Lactococcus garvieae*
	Streptococcus dysgalactiae
	Clostridium spp X3
Uterus	*Vagococcus spp*
	Lactobacillus spp
	Clostridium spp

Table 4.6: Summary of isolated strains per organ sampled confirmed by PCR from Zeerust

Organs isolated from	Isolated organisms
Spleen	*Lactococcus spp*
	Staphylococcus aureus
	Enterococcus
	Peptostretococcus russellii
Lymph nodes	*Enterococcus casseliflavus*
	Enterococcus faecalis
	Lactobacillus spp
Uterus	*Enterococcus spp.*
	Clostridium histolyticum
Placenta	*Enterococcus spp*
	Clostridium histolyticum

Table 4.7: Summary of isolated strains per organ sampled confirmed by PCR from Koster

Organs isolated from	Isolated organisms
Uterus	*Staphylococcus aureus*
	Enterococcus spp.
	Enterococcus spp.
Spleen	*Clostridium spp*
	Streptococcus spp.

Table 4.8: Summary of isolated strains per organ sampled confirmed by PCR from Vryburg

Organs isolated from	Isolated organisms
Spleen	*Enterococcus faecalis*
	Enterococcus spp
Lymph node	*Staphylococcus agnetis*
	Enterococcus faecalis
Placenta	*Enterococcus hirae*
	Aneurinibacillus spp
Uterus	*Bacillus cereus*

Table 4.9: Frequency summary of bacterial pathogens isolated from different abattoirs in the North West Province

Micro-organisms	Number of bacteria isolated	Percentage
Enterococcus spp	14	35%
Clostridium spp	9	23%
Staphylococcus spp	5	12%
Bacillus spp	2	5%
Lactococcus spp	2	5%
Peptostreptococcus spp	1	3%
Aneurinibacillus spp	1	3%
Lactobacillus spp	2	5%
Vagococcus spp	1	3%
Streptococcus spp	2	5%
Total	39	100%

Table 4.10 Frequency (%) of bacteria isolated from carcasses from different abattoirs confirmed by PCR

Micro-organisms	Stella n=7	Potchefstroom n=8	Zeerust n=11	Koster n=5	Vryburg n=7
Enterococcus spp.	8%	0	13%	5%	10%
Clostridium spp.	3%	10%	5%	3%	0
Staphylococcus spp.	5%	0	2%	2%	2%
Bacillus spp.	3%	0	0	0	3%
Lactococcus spp.	0	3%	3%	0	0
Peptostreptococcus spp.	0	0	3%	0	0
Aneurinibacillus spp.	0	0	0	0	3%
Lactobacillus spp.	0	3%	3%	0	0
Vagococcus spp.	0	3%	0	0	0
Streptococcus spp.	0	3%	0	3%	0
Total	19%	22%	29%	13%	18

Molecular identification of isolates

Thirty-nine isolates were subjected to molecular analysis, and showed a 1% (w/v) agarose gel, representing 16S rDNA gene fragments (Figures 4.1 and 4.2). The desired 1 kb base pairs fragments were obtained after running the gel on electrophoresis for 65 minutes. After running the gel, an automatic UV trans-illuminator (UV tec, Sigma, Germany) was used to view bands of the genomic DNA and photographed using a Bio profile gel documentation system. The purpose was to check the presence or absence of DNA bands and confirm successful extraction from both genomic DNA and the Bio gel documentation system. The bands below (Figures 4.2 and 4.3) were seen only from the universal primers with regard to *B. abortus* IS711 primers were absent, indicating the absence of the Brucella bacteria.

Numbers in Figures 4.2 and 4.3 irrespectively represent bacterial isolates and species in Table 4.11.

L 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 L

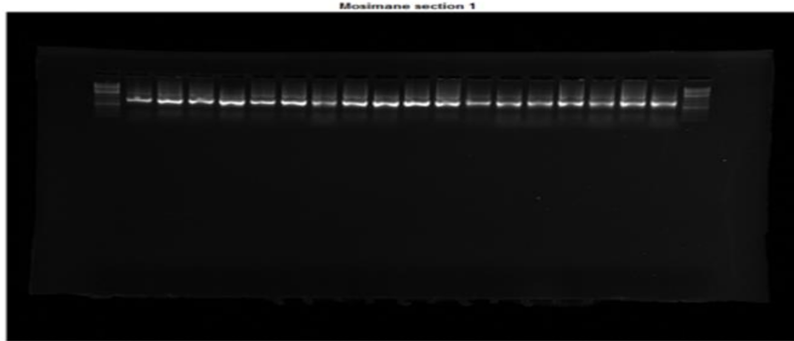

Figure 4.2: Picture of Gel electrophoresis of PCR products amplified from bacterial isolate 1-18

Lane 1-*Enterococcus faecalis*, Lane 2-*Clostridium histolyticum*, Lane 3-*Enterococcus spp*, Lane 4-*Enterococcus spp*, Lane 5-*Lactococcus spp*, Lane 6-*Staphylococcus aureus*, Lane 7-*Staphylococcus aureus*, Lane 8-*Enterococcus faecalis*, Lane 9-*Enterococcus hirae*, Lane 10-*Clostridium spp*, Lane 11-*Staphylococcus aureus*, Lane 12-*Enterococcus faecalis*, Lane 13-*Clostridium histolyticum*, Lane 14-*Bacillus spp*, Lane 15-*Clostridium spp*, Lane 16-*Clostridium spp*, Lane 17-*Peptostretococcus russellii* and Lane 18-*Staphylococcus agnetis.*

L,19,20,21,22,23,24,25,26,27,28,29,30,31,32,33,34,35, L

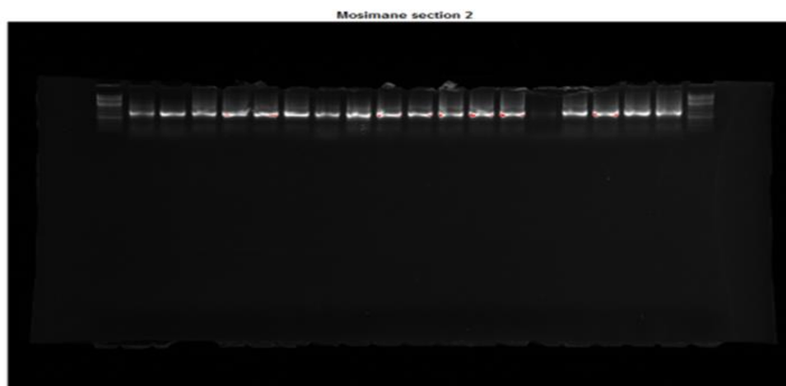

Figure 4.3: Picture of Gel electrophoresis of PCR products amplified from bacterial isolates 19-35

Lane 19-*Enterococcus faecalis,* Lane 20-*Enterococcus spp,* Lane 21-*Enterococcus gallinarium,* Lane 22-*Streptococcus dysgalactiae,* Lane 23-*Staphylococcus aureus,* Lane 24-*Clostridium spp,* Lane 25-*Enterococcus spp,* Lane 26-*Lactobacillus spp,* Lane 27-*Enterococcus durans,* Lane 28-*Enterococcus spp,* Lane 29-*Clostridium spp,* Lane 30-*Bacillus cereus,* Lane 32-*Lactococcus garvieae,* Lane 33-*Streptococcus spp,* Lane 34-*Enterococcus*

casseliflavus, Lane 35-*Vagococcus spp,* Lane 36-*Aneurinibacillus spp,* Lane 37-*Enterococcus spp,* Lane 38-*Clostridium spp* and Lane 39-*Clostridium histolyticum.* In Lane 30, no bands were found due to low bacterial concentration.

Confirmatory results of bacterial isolates by PCR and sequencing

The amplification and sequencing of the 16S rDNA gene were performed on all isolated bacteria. Thirty nine isolates were selected and subjected to PCR analysis. All isolates showed 79%−100% identity similarity to sequences already available on the NICB. All sequences obtained from INQABA were blasted using the 16S r-DNA Nucleotide BLAST and all isolates sent to gene Bank and accession numbers obtained for each (Tables 4.6-4.10). Results obtained confirmed the presence of strains of *Enterococcus spp.* such *as E. faecalis; Clostridium spp; Staphylococcus aureus; Streptococcus dysgalactiae;* and *Clostridium histolyticum*.

Table 4.11: All types of bacteria identified from samples collected

S/N	Isolate code of organisms identified	Reference from NCBI database	Percentage similarity (%)	Acc. No. in GenBank (PCR)
1	NWU 1	*Clostridium histolytium*	81	NR104889.1
2	NWU 2	*Clostridium sp*	82	MG592384.1
3	NWU 3	*Clostridium argentinense*	86	MF988717.1
4	NWU 4	*Clostridium histolyticum*	79	NR104889.1
5	NWU 5	*Enterococcus sp*	78	KP181650.1
6	NWU 6	*Staphylococcus aureus*	91	CP018629.1
7	NWU 7	*Staphylococcus aureus*	87	CP028189.1
8	NWU 8	*Staphylococcus agnetis*	85	CP009623.1
9	NWU 9	*Enterococcus gallinarum*	89	MG740795.1
10	NWU 10	*Enterococcus sp*	97	CP023515.1
11	NWU 11	*Streptococcus dysgalactiae*	91	KY118916.1
12	NWU 12	*Staphylococcus sp*	94	FJ752530.1
13	NWU 13	*Enterococcus sp*	81	KF826017.1
14	NWU 14	*Enterococcus gallinarum*	87	MG740795.1
15	NWU 15	*Streptococcus australis*	79	FJ823144.1
16	NWU 16	*Edwardsiella tarda*	80	FJ405291.1
17	NWU 17	*Staphylococcus aureus*	84	HQ752897.1
18	NWU 18	*Enterococcus faecalis*	83	MH158265.1
19	NWU 19	*Enterococcus faecalis*	90	HQ751967.1
20	NWU 20	*Clostridium argentinense*	86	CP014176.1
21	NWU 21	*Bacterium Te82A*	76	AY587798.1
22	NWU 22	*Clostridium bifermentans*	93	X73437.1
23	NWU 23	*Bacillus cereus*	73	KT626956.1
24	NWU 24	*Bacillus sp*	82	EU266059.1
25	NWU 25	*Bacillus sp*	78	JF701967.1
26	NWU 26	*Clostridium histolyticum*	79	NR104889.1
27	NWU 27	*Lysinibacillus fusiformis*	77	KX713168.1
28	NWU 28	*Enterococcus gallinarum*	80	MG74075.1
29	NWU 29	*Aneurinibacillus thermoaerophilus*	89	EU723620.1
30	NWU 30	*Peptostreptococcaceae bacterium*	93	AB298728.1

31	NWU 31	*Bacillus cereus*	86	MG557773.1
32	NWU 32	*Peptostreptococcaceae*	93	AB298728.1
33	NWU 33	*Clostridium sp*	87	JF312698.1

Molecular phylogenetic analysis using Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura *et al*., 1993). The tree with the highest log likelihood (-17696, 0220) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 59 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 243 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016).

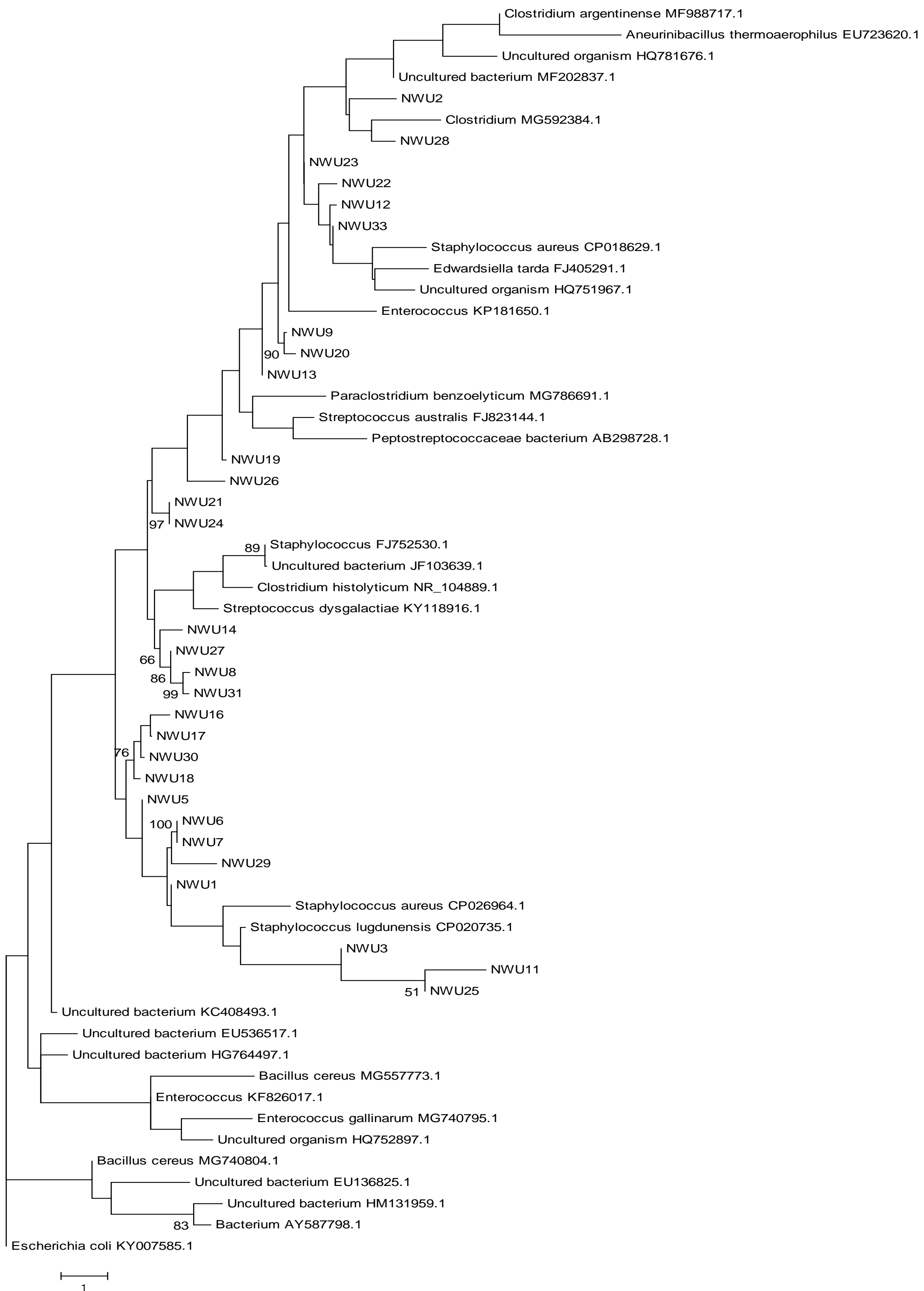

Figure 4.4: Phylogenic tree showing similarities between isolated strains among themselves

CHAPTER 5

Discussion

The aim of this study was to detect and isolate Brucella bovis and to isolate other pathogenic gram negative bacteria among animals slaughtered in normal line abattoirs in the North West Province. Results obtained showed that all samples collected and analysed were negative to Brucella *spp*, while suspecious bacteria isolated were mostly unrelated to the bacteria.

The absence of Brucella *spp* here is unforeseen and is good news for consumers, even though a previous study in a similar South African rural setting also established a comparably low result (Bishop, 1984). The absence of detection can be explained by the impact of the Brucella Scheme currently going on in South Africa. In addition, this can be explained by the fact that ante mortem is properly handled at the abattoirs. This study is in consistent with that of Herr and Roux (1981),who, in a similar investigation did not obtain any consistent results.

The media was incriminated as being the cause of bad results. However they concluded that for dairy cows, examination (at slaughter) of samples for udder tissue from each quarter, pooled supra mammary lymph nodes and pooled iliac lymph nodes, can give an accurate indication of the presence of Brucella organisms (Herr and Roux, 1981).

In a study by Keitiretse Molefe (2016), in communal areas of the Ngaka Modiri Molema District, it was revealed that approximately 30% of animals that were presented with reproductive disorders (retained placenta, abortion and dystocia) were positive to Brucella. This confirms the hypothesis of this study that the population in communal areas are at risk, vulnerable as most slaughtering done for rituals and funerals in rural and communal areas are done without prior ante mortem and meat inspections. Due to the latent status of some animals, the disease can only be detected beyond 18 months of age (DAFF Brucellosis Manual, 2012). In addition, the major challenge might also be the methodology and the identification of the Brucella strains. Many laboratories might miss it due to requirements. Hence, Seleem *et al*. (2010) confirm that 15-70 % of cases of Brucellosis are missed due to low sensitivity of methodology used.

The presence of some other Enterobacter in this study could be explained by several factors such as cross contamination of carcasses and organs by digestive materials. While collecting samples, use of water might have been one of example of the cross contamination, while flushing off the dirt on the carcass could spread to other parts. Abdominal parts such as

uterus, placenta and mammary glands, disposing them in the same basket without cleaning all the carcass could have been another example of cross contamination. It is, therefore, advisable that stakeholders change their practices and use different baskets or do thorough cleaning for other carcass in order to reduce contamination in abattoirs. There is need to test water in abattoirs since other species of bacteria could be present in water, for example *enterococcus spp* and *staphylococcus spp* could be in water since water is considered one of the intrinsic parameters (Genigeorgis *et al*., 1989).

Brucellosis is considered a re-emerging zoonotic disease that affects animals and people throughout the world. Measures for controlling brucellosis are effective with high cost and developing countries lack funding to build infrastructure that could assist in sensitising and educating people on the risks of Brucellosis and train personnel on the collect and testing of samples at proper laboratories. In developed countries, there is a decrease in human brucellosis incidence following control of bovine Brucellosis. If all concerned parties (i.e. farmers, milk and milk products industry, breeding. companies, consumers and politicians) could take part in finding a solution, this could go a long way eradicating chances of Brucellosis. There is need for sensitisation and awareness campaigns among abattoir workers as well as training on possible zoonotic diseases and implementation of proper hygiene practices since various pathogenic organisms found in this study, are those that cause food-borne illnesses.

Since there is no vaccine to control the disease in humans, there is need to prevent the disease from affecting humans. Finding a vaccine for wild animal can be a great effort since there is also contact between domestic and wild animals. Wild animals are responsible for spreading the disease to domestic animals and human beings due to limited information on the pathology of Brucella from other wildlife.

There is need to conduct further studies in North West Province as the rate of out breaks of Brucellosis in 2014 stood at 295 cases reported, the use of different strains of primer and the machine (e.g PCR method) and the prevalence rate for each province is about 25% each year (DAFF, 2015), according to Mbizeni.

Molecular analysis based on 16s rDNA revealed a high frequency of occurrence of pathogenic bacteria, such as *Enterococcus spp (*35%), *Clostridium spp(23%), Staphylococcus spp (12%), Streptococcus spp (5%), Bacillus spp (5%), Lactococcus spp (5%),*

Peptostreptococcus spp (3%), Aneurinibacillus spp (3%), Lactobacillus spp (5%) and Vagococcus spp(3%) (Table 4.9).

The presence of other pathogenic bacteria in meat has remained broadly reported in different parts of the world (Kinsella *et al*., 2008) and is a public health concern in developing as well as developed countries (Saulat, 2012). About 13.8 million cases are estimated to cause food-borne illnesses due to known causes; approximately 30% are due to bacteria (Mead *et al*., 1999). Bacterial control in the meat processing industry is a basic component to assure meat safety and quality (Langsrud *et al*., 2006; Brightwell *et al*., 2006).

In this study, *Enterococcus spp* were isolated in 35% of samples and was higher than other bacterial species. It was highly isolated from Zeerust and Vryburg with 10% each, largely due to the high number of animals slaughtered each day than other abattoirs, followed by Stella (7%), Koster (5%) and in Potchefstroom *enterococcus spp* was not detected in samples analysed. A study by Olawale *et al*. 2014 revealed the occurrence of the presence of Enterobacteriaceae in raw meat and handlers in Egypt. *Enterococcus spp* can be found in different environments such as the intestinal tract, soil, water, plants, insects and mammals (Witte *et al*., 1999; Muller *et al*., 2001; Aarestrup *et al*., 2002). *Enterococcus spp* pathogen is a public health concern when found in food because the pathogen can cause infections in human beings. Even if they are normally found in the digestive tract, they are of medical importance (Olawale *et al*., 2014). Previous studies show that male sex and hypoalbuminemia are risk factors of enterococci infection (Gatta *et al*., 2012). It has been revealed that *enterococcuss spp* causes cellulitis, bloodstream and urinary infections (Murray, 1990; Sievert *et al*., 2010; Arias *et al*., 2012) and the bacteria is also associated with other problems such as endocarditis, the central nervous system, intra-abdominal, bacteraemia and pelvic infection (Franz *et al*., 1999). Enterococcus is found to be a causative pathogen in problematic urinary tracts (Spoorenberg *et al*., 2013) and from children with urinary tract disorder (Bitsori *et al*., 2005). In cattle, it causes diarrhea in calving and bovine mastitis dairy cows (Rogers *et al*., 1992). Bovine mastitis pathogens are from the environment and the transmission is from the environment to animals (Rossitto *et al*., 2002).

Clostridium spp isolated were 23% less than *Enterococcus spp*. Clostridium pathogens can pass on the disease to people and animals. Animals are primary host for clostridia. Some food products can transfer these pathogens when hygiene measures are poor (Jobstl *et al*., 2010; Loo *et al*., 2005; Barbut *et al*., 2007; Bauer *et al*., 2011; Keessen *et al*., 2011). When

consuming food contaminated with Clostridia endospores infections may occur (Jobstl *et al*., 2010) and these endospores are able to change into vegetative cells in the gastrointestinal tract of the host and end up producing strong toxins that have cytopathic effects on the epithelial cells (Songer *et al*., 2010; Loo *et al*., 2005; Willey *et al*.,2008). There is currently no information on the occurrence of Clostridium species in food products around the North West Province, South Africa.

Staphylococcus spp (12%) was high in Stella with 4,8%, Zeerust, Koster, Vryburg were at 1, 2% each and as for Potchefstroom, *staphylococcus spp* were not present while *Bacillus spp* (5%) was found in Stella (2,5%) and Vryburg (2,5%). These results correlate with the findings of Rane (2011), who in his study observed that improper food handling can lead to transfer of pathogenic bacteria such as *Staphylococcus aureus* from the human body and the environment into food. Both *B. cereus* and *S. aureus* have been identified to cause foodborne illnesses. The report states that *S. aureus* was isolated from food handlers in their nose and throat (Omoregbe *et al*., 1992) and from more than 50% of healthy humans. Poor hygiene can be connected with the results of contamination on meat with *B. cereus* and *S. aureus* (Okonko *et al*., 2009; Sobukola *et al*., 2009). With regard to *B. cereus*, since it has the ability to resist heat, it can be found in the air and form spores. The results are similar to those of Clarence *et al*. (2009); Mensah *et al*. (2002); and Sina *et al*. (2011) as poor handling was found to be the cause of contamination with *S aureus*. Few reasons that might have led to contamination in this study are such as packaging and humidity while transporting from the abattoir or time of preparing them as outlined by Genigeorgis *et al*. (1989). Studies conducted in abattoirs in the United States of America revealed that beef carcasses, after chilling, were contaminated with 2.68 log bacteria per cm^2 (McNamara, 1995).

Montville *et al*., (2012) conclude that human beings are the main reservoir for *S. aureus* because of their ability to make them grow on the skin, nose and animals hides. This could have been the cause since abattoir workers do not wear masks or gloves. *S. aureus* has been reported as being responsible for gastroenteritis after eating food with one or more enterotoxins (Jay, 2004). Poor refrigeration, poor sanitation and keeping food for a long period can cause *S. aureus* infections (Montville *et al*., 2012). A study in Botswana revealed that *S. aureus* was present in butcheries, especially in *biltong* (Matsheka *et al*., 2014).

Lactobacillus spp (5%) were found in Potchefstroom and Zeerust from the spleen and mammary lymph nodes. A review by Cannon *et al*. (2005) revealed that Lactobacillus species

are usually used to ferment food and as a probiotic. *Lactobacillus spp* are chemo-organotrophic and can grow under reduced oxygen but they need rich media to grow and live anywhere and be present in carbohydrates. Lactobacilli can be confused with some Gram-positive organisms such as Leuconostoc (Tommasi *et al*., 2008) and Streptococcus (Neef *et al*., 2003). *L. casei* and *L. rhamnosus* are the two most common Lactobacillus species associated with the disease (Cannon *et al*., 2005). Their presence, which did not have detailed similarities with strains in the Gene bank, could be explained by possible mutations or occurrence of new strains not identified in the past. This was also observed in the phylogenic tree as some strains did not have a link while some had a link with others.

In this study, molecular identification of strains revealed that some strains such as NWU 5;10; 12; 13; 21; 24; 25 and 33 were not yet fully identified and full similarities were not obtained from the Gene bank (Figure 4.4 and Table 4.11). This can be explained by environmental changes such as climate, movement of animals and populations, leading to mutations and appearance of new strains. This was an interesting finding which requires in-depth studies to identify and characterise them in order to predict the risk for both animals and consumers.

The results are important because they assure consumers of the risk of Brucellosis contracted through meat, but also raise an alarm on the possibility of the presence of other strains, which could even be pathogenic and cause diseases and outbreaks.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

The aim of this study was to detect and isolate *Brucella abortus* and to isolate other pathogenic gram negative bacteria among animals slaughtered in normal line abattoirs in the North West Province. To achieve these, samples were randomly collected from the main abattoirs in the North West Province and analysed.

Results obtained showed no Brucella contamination in samples analysed. The absence or non-isolation of the *Brucella spp* in samples analysed does not mean that this might not occur. Due to the prevalence in both commercial and communal herds and because of the current application of the Brucellosis Scheme, where only farms responding to defined criteria are allowed to join the scheme, it is concluded that regular monitoring in abattoirs and increased effort to eradicate Brucellosis from herds remain a priority to ensure food safety of consumers and safety of abattoir workers.

In addition, the results show contamination of samples analysed with pathogenic bacteria such as *Enterococcus spp., Clostridium spp., Staphylococcus spp., Streptococcus spp., Bacillus spp., Lactococcus spp., Peptostreptococcus spp., Aneurinibacillus spp., Lactobacillus spp. and Vagococcus spp,* and concentration in meat could lead to damage in different organs in the body of humans, thus causing a major risk in public health. Contamination could have been caused by cross contamination with digestive materials, equipment used and or water during slaughtering or by workers themselves. There is need for training of abattoir workers in meat hygiene to reduce contamination. Meat inspectors and environmental practitioners at abattoirs should ensure regular monitoring and request test of water and other equipment used in the abattoir as well as the strict separation of contaminated from clean organs to reduce contamination.

Isolation of unidentified strains in this study raises a major concern and requires that more investigations be done to examine and characterise these isolates in order to prevent possible outbreaks.

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