CHAPTER ONE

INTRODUCTION

1.1. Introduction

Tuberculosis (TB) is an ancient infectious disease that has been infecting different populations around the globe and it has also been considered as one of the most successful human and animal disease. TB found in animals such as cattle and other known bovids is known as bovine tuberculosis (Michel *et al*., 2010). Bovine tuberculosis (BTB) is an infectious disease found in cattle mainly caused by *Mycobacterium bovis* (*M. bovis*) (Michel *et al*., 2010). *M. bovis* is a member of the *Mycobacterium tuberculosis* complex (MTC) together with *Mycobacterium tuberculosis* (*M. tuberculosis*), *Mycobacterium africanum* (*M. africanum*), *and Mycobacterium canetti* (*M. canetti*) where the natural host is humans; whereas *Mycobacterium caprae* (*M. caprae*), *Mycobacterium microti* (*M. microti*) *and Mycobacterium pinnipedii* (*M. pinnipedii*) usually have animals as their natural host (Szewzyk *et al*., 1995; Huard *et al*., 2003). BTB was first reported in South Africa in 1880 (Hutcheon, 1880). BTB is a major threat to the economy as livestock farmers are concerned due to the low productivity of dairy products and to the international trade of animal products. *M. bovis* has also been isolated from humans and wildlife animals (Cosivi *et al*., 1998; Renwick *et al*., 2007) and infection to wildlife has been reported as one of the risk factors for BTB in South Africa at the wildlife-livestock interface (Hlokwe *et al*., 2011 and Michel *et al*., 2006). The consequences of the national BTB control introduced in 1969 in the USA (Myers and Steele, 1969) and eradication scheme showed a significant decrease in the prevalence of the disease (Cosivi *et al*., 1998). Moreover there was a sharp increase in the prevalence of BTB from 4.4% to 16% and 27% to 38.2% found in the central and south regions respectively between 1992 and 1998 (Gibson *et al*., 1998).

More than 50 million cattle were estimated to be infected with BTB worldwide (Lyashcheko *et al*., 2006). BTB can be transmitted through consumption of contaminated milk and through close proximity with the infected cattle thereby becoming a major threat to humans. There is an

estimated 0.5-1.5% of cases responsible for all the human TB cases reported in certain developed countries (Chen *et al*., 2009). However, in developing countries there is a higher prevalence of 5-10% of human *M. bovis* infection due to the poor BTB control schemes (Jeon *et al*., 2010).

The MTC is generally considered a family of ecotypes of very closely related Mycobacteria, with each ecotype being adapted to cause tuberculosis disease in a specific host species or group, even though inter-species transmission can occur (Smith *et al*., 2006). In contrast to the earlier hypothesis that tuberculosis has evolved from an originally animal disease to a human disease (Diamond, 2002), new findings indicate that in fact tuberculosis first emerged in humans and was subsequently transmitted to animals (Wirth *et al*., 2008). Recent studies suggest that the common ancestor of the *M. tuberculosis* complex emerged from its progenitor perhaps 40,000 years ago in East Africa (Gutierrez *et al*., 2005; Wirth *et al*., 2008). Some 10,000–20,000 years later, two independent clades evolved, one resulting in *M. tuberculosis* lineages in humans, while the other spread from humans to animals, resulting in the diversification of its host spectrum and formation of other *M. tuberculosis* complex member species, including *M. bovis* (Gutierrez *et al*., 2005; Wirth *et al*., 2008). This adaptation to animal hosts probably coincided with the domestication of livestock approximately 13,000 years ago (Michel *et al*., 2010).Evidence in the form of skeletal lesions compatible with Pott's disease and especially the use of PCR-based DNA techniques date the occurrence of early documented cases of tuberculosis in both humans and animals to at least 3000 BC (Taylor *et al*., 2005). Pathognomonic bone lesions indicative of tuberculosis in bovids were found in skeletons of ice-age representatives of this genus but the link to hominids is currently unclear (Rothschild and Martin, 2006).

In modern history, cattle served as principal reservoir species for *M. bovis* (Muller *et al*., 2009), hence the name bovine tuberculosis. This term is also commonly used to describe *M.bovis* infection in other species including wildlife and humans to demonstrate the bovine source of the infection (Muller *et al*., 2009). Movement of cattle within and between countries and continents certainly facilitated the worldwide distribution of bovine tuberculosis (Muller *et al*., 2009), although the ultimate origin of *M. bovis* is unknown. However, progress has been made in our

understanding of the population structure of *M. bovis* through the use of the PCR-based spoligotyping and VNTR typing methods, which allowed the identification of clonal complexes of *M. bovis* dominant in larger geographic locations (Muller *et al*., 2009). Recently, a clonal complex of strains of *M.bovis* named African1 (Af1) that is geographically localized to the Central-West African region has been described (Muller *et al*., 2009). Strains of Af1 were normal in this region and appeared to have nearly reached fixation in some areas of Central-West Africa. The most likely explanation for this observation is an introduction of *M. bovis* into cows that were originally naive to tuberculosis (Muller *et al*., 2009). It might be that other groups are likely to be geographically localized to other regions of the world (Muller *et al*., 2009). Recent advances in our understanding of the population structure of *M. bovis* notwithstanding, the actual origin of these clonal complexes remains unknown (Michel *et al*., 2010).

M. bovis has also been identified in humans in most countries where isolates of mycobacteria from human patients have been fully characterized (O.T.M, 2009). The incidence of pulmonary tuberculosis caused by *M. bovis* is higher in farm and slaughterhouse workers than in urban inhabitants (O.T.M, 2009). The transmission of *M. bovis* to humans via milk and its products is eliminated by the pasteurization of milk. One of the results of bovine tuberculosis eradication programmes has been a reduction in disease and death caused by bovine tuberculosis in the human population (O.T.M, 2009). Although cattle are considered to be the true hosts of *M. bovis*, the disease has been reported in many domesticated and non-domesticated animals (O.T.M, 2009). Isolations have been made from buffaloes, bison, sheep, goats, equines, camels, pigs, wild boars, deer, antelopes, dogs, cats, foxes, mink, badgers, ferrets, rats, primates, South American camelids, kudus, elands, tapirs, elks, elephants, sitatungas, oryxes, addaxes, rhinoceroses, possums, ground squirrels, otters, seals, hares, moles, raccoons, coyotes and several predatory felines including lions, tigers, leopards and lynx (De Lisle *et al*., 2001; O'Reilly and Daborn 1995).

1.2. Organism

The MTC belongs to the kingdom bacteria of the phylum actinobacteria in the order of actinomycetales (Cole *et al*., 1998). The MTC organisms are obligate aerobes which grow optimally in tissues with high oxygen content, such as the lungs hence TB is a respiratory disease (Saiman, 2004). They are also facultative intracellular pathogens usually infecting mononuclear phagocytes and are slow-growing aerobes with a generation time of 12 to 18 hours (Madison, 2001). They are hydrophobic with high lipid content in the cell wall. The cell wall complex contains peptidoglycan, but of composed of complex lipids (Aralakol, 2008). Over 60% of the mycobacterial cell wall is lipid and the lipid fraction of MTC's cell wall consists of three major components, mycolic acids, cord factor, and wax-D (Brennan, 2003). Because the cells are hydrophobic and tend to clump together, they are impermeable to the usual stains such as the Gram's stain unless the stain is combined with phenol and are known as "acid-fast bacilli" because of their lipid-rich cell walls, to various basic dyes unless the dyes are combined with phenol (Draper and Daffe 2005). Once stained, the cells resist decolorization with acidified organic solvents (www.microbiologybites.com/blog/about).

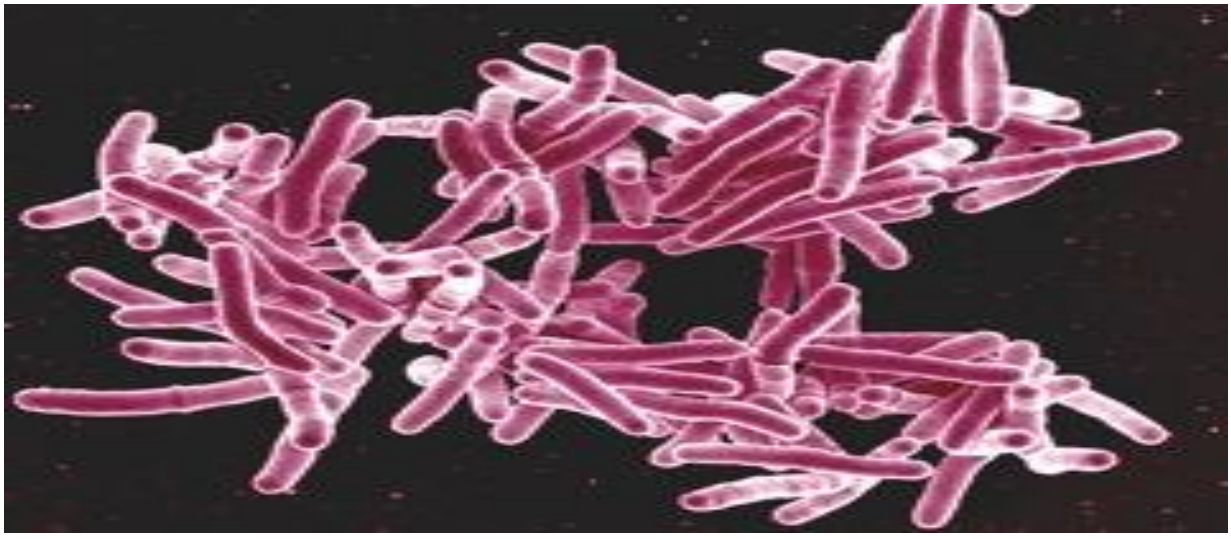

Figure 1.1 Tuberculosis organism under scanning electron micrograph (http://www.cbc.ca/health/story/2006/03/17/tb-who060317.html?ref=rss)

1.3. Pathogenesis

It is usually characterized by formation of nodular granuloma known as tubercles. Although commonly defined as a chronic debilitating disease, bovine tuberculosis can occasionally assume a more progressive course (Collins and Grange, 1983). Any body tissue can be affected, but lesions are most frequently observed in the lymph nodes (particularly of the head and thorax), lungs, intestines, liver, spleen, pleura, and peritoneum (O.T.M, 2009). It should be noted that other members of the *M. tuberculosis* complex, previously considered to be *M. bovis,* have been accepted as new species despite identical 16SrRNA sequences and over 99.9% identity of their genome sequences (Boddinghaus *et al*., 1990). These include *M. caprae* (Aranaz *et al*., 2003) (in some countries considered to be a primary pathogen of goats) and *M. pinnipedii* (Cousins *et al*., 2003), a pathogen of fur seals and sea lions. These two new species are known to be zoonotic. In central Europe, *M. caprae* has been identified as a common cause of bovine tuberculosis (Prodinger *et al*., 2005). Disease caused by *M. caprae* is not considered to be substantially different from that caused by *M. bovis* and the same tests can be used for its diagnosis (Cousins, 2001). In countries with tuberculosis eradication programmes, clinical evidence of tuberculosis in cattle is seldom encountered because the intradermal tuberculin test enables presumptive diagnosis and elimination of infected animals before signs appear (Cousins, 2001). Prior to the national tuberculosis eradication campaigns, however, clinical signs associated with tuberculosis were commonly observed (Cousins, 2001). In many cases, the course of the infection is chronic and signs may be lacking, even in advanced cases when many organs may be involved (O.T.M, 2009). When present, clinical signs vary; lung involvement may be manifested by a cough, which can be induced by changes in temperature or manual pressure on the trachea. Dyspnoea and other signs of low-grade pneumonia are also evidence of lung involvement (Prodinger *et al*., 2005).

1.4. Host Pathogen Interaction

When *M. tuberculosis* infection has been inhaled by a non-infected person, it is taken up by the lungs and goes into inactivated alveolar macrophages this will in turn prevent the phagosomes needed for lysosomal enzymes to kill the bacteria. This bacterium carries UreC which prevents

acidification of the phagosomes which is needed to kill the bacteria (Jasmer *et al*., 2002). When the bacteria haven't been digested, it replicates in the cells and ultimately kills them too and will then spread to other cells of the body. Most disease symptoms and damage to the body is a result of immune responses to the bacterium. Eventually activated macrophages engulf and kill the bacteria while cytotoxic T-lymphocytes kill *M. tuberculosis*-infected cells. If the bacterial load is small at this time the bacteria are destroyed with minimal tissue damage (Tufariello *et al*., 2003; Flynn and Chan, 2003). However, if the bacterial load is high, production of inflammatory cytokines, activation of the complement pathways, and the hydrolytic enzymes and toxic oxygen radicals produced by macrophages lead to considerable tissue death (Jasmer *et al*, 2002).

If the initial foci of infection are small and the localized accumulations of activated macrophages are less than 3 millimeters, the activated macrophages usually contain and kill the *M. tuberculosis*. However, when larger granuloma develop in areas with more tissue necrosis, they become encased in fibrin and the bacteria are protected from macrophage killing. In this state the *M. tuberculosis* can remain dormant for years and be reactivated if immune defenses weaken as a result of aging, immunosuppressive diseases, or immunosuppressive treatments. The formation of granuloma is actually the result of cell-mediated immune responses attempting to wall-off and localize infections that the body cannot effectively remove with macrophages (CDC, 2000).

Dyspnoea and other signs of low-grade pneumonia are also evident of lung involvement. In advanced cases, lymph nodes are often greatly enlarged and may obstruct air passages, the alimentary tract, or blood vessels. Lymph nodes of the head and neck may become visibly affected and sometimes rupture and drain. Involvement of the digestive tract is manifested by intermittent diarrhea and constipation in some instances (Prodinger *et al*., 2005). Extreme emaciation and acute respiratory distress may occur during the terminal stages of tuberculosis. Lesions involving the female genitalia may occur. Male genitalia are seldom involved. At necropsy, tubercles are most frequently seen in bronchial, mediastinal, retropharyngeal and portal lymph nodes and may be the only tissue affected. In addition, the lung, liver, spleen and

the surfaces of body cavities are commonly affected. Early nodular pulmonary lesions can often be detected by palpation (O.T.M, 2009).

Although TB in humans is curable, it remains a major global health problem (WHO, 2012). It poses a great risk in health among millions of people each year and ranks as the second leading cause of death from an infectious disease worldwide, the first being the human immunodeficiency virus (HIV) (WHO, 2009). It typically affects the lungs, generally a small percentage of people infected with *M. tuberculosis* develop TB disease; however people with compromised immune systems, such as people living with HIV, malnutrition or diabetes, or people who use tobacco, have a much higher risk of falling ill (WHO, 2010).

1.5. Epidemiology of TB

Tuberculosis (TB) remains one of the world's most serious afflictions even though its eradication was predicted by the end of the twentieth century. It affects a significant proportion of the world population. Globally, one person out of three is infected with the bacillus. Eighty percent of estimated TB cases worldwide each year occur in only 22 countries, including India, Pakistan, South Africa, and Brazil, and are designated by the World Health Organization as high-burden countries (WHO, 2003). The World Health Organization reported 9.2 million incident cases worldwide in 2007 (WHO, 2008) and 1.7 million death occurs from TB annually while Smith *et al*., (2004) reported that developing countries account for over 90% of the tuberculosis burden. Every year, according to WHO (2007; 2009), about 8.8 million people develop TB and 1.8 million die from it worldwide. Sub-Saharan Africa, much of Asia, and in some Eastern European countries recorded highest incidences of tuberculosis where rates typically exceed 100 per 100 000 population (WHO, 2007). It was also reported that the incidence of TB is increasing by approximately 0.4% per year globally; this increase is higher in countries of sub-Saharan Africa and the former Soviet Union (WHO, 2003). In most high-income countries, the overall incidence of tuberculosis is relatively static at 10 cases per 100 000 or less (WHO, 2003).

There has been a significant change in the distribution of the disease in high-income countries, with ethnic minority groups accounting for an increasing proportion of cases (WHO, 2003) and immigrants from high burden countries that harbor the organism having latent TB. Immigration is changing the epidemiology of TB all over the world. The number of TB cases in the population born in the United States has declined while the number of cases in the foreign-born population has increased. In 1986, 21.6% of the cases reported in the United States were among foreign-born individuals and in 1992; the proportion had risen to 61% (CDC, 1995). Mexico accounted for the largest proportion (23%) of foreign-born U.S. patients (McKenna *et al*., 1995; CDC, 1998). In 1996, 83% of the cases of TB among foreign-born Hispanics in the United States were reported in the states bordering Mexico (McKenna *et al*., 1995). Within the U.S border state of Texas, the incidence of TB is higher in countries along the border than elsewhere in the state (Escobedo and Cosio 1997; Taylor *et al*., 1999). Although TB is transmitted in three ways mentioned above, it is not as easily transmitted, as are certain other airborne infectious diseases (Riley, 1967; 1983). War and social upheaval have played a role in the spread of TB beyond endemic zones (Hoa *et al*., 2004). The increase in the global burden has resulted in the WHO in 1993 declaring TB a Global emergency (Blumberg, 1995).

1.5.1. South African TB epidemiology

In South Africa, a country with almost 49 million inhabitants, the incidence of TB in 2000 was estimated at >300 cases per 100,000 population, with an estimated 21,594 (22%) new sputum smear-positive cases (DOH, 2006). South Africa has cities that are relatively attractive economic destinations in the Southern African region. However, poor and under-serviced rural areas such as in Limpopo, KwaZulu-Natal, Eastern Cape and Mpumalanga Provinces (Table 1.1) provide fertile grounds for the disease to flourish. Tuberculosis in South Africa affects mostly those who were discriminated against under apartheid legislation. As a result, wide variances in tuberculosis incidence, depending on race, were found prior to 1994 (WHO, 2007). Incidence ranged from less than 20/100,000 in the white community to 400-600/100,000 in black and colored communities (DOH, 2001). KwaZulu-Natal, Eastern Cape, Northern Cape and Western Cape provinces have the highest number of cases of TB. These provinces also had incidences of TB which were higher than the national average (Barr *et al*., 2004).

Table 1.1. MDR-TB cases per province 2004-2007

Province	**2004**	**2005**	**2006**	**2007 (Q1)**
E Cape	520	601	927	315
F State	131	171	226	47
Gauteng	662	711	794	168
KZN	308	1014	2806	1134
Limpopo	86	58	84	21
Mpumalanga	156	156	177	25
N West	126	180	201	38
N Cape	126	161	203	44
W Cape	1163	1253	1298	348
S Africa	3278	4305	6716	2140

South African National Department of Health, 2006. Keys; E. Cape= Eastern Cape, F. State = Free State, KZN = Kwazulu Natal, N. West = North West, N. Cape = Northern Cape, W. Cape = Western Cape, S. Africa = South Africa, Q1=First Quarter.

There has been a total number of new and relapse cases of 343715 and total number of cases notified is 389974 in 2011 in South Africa. There have also been reported cases of MDR-TB in 2011, a total number of 49304 cases reported. In 2011 there was a number of 323440 (83 %) of TB patients with known HIV status, 211800 (65 %) HIV positive patients, 161298 (76 %) HIV positive TB patients on co-trimoxazole preventive therapy (CPT) and 92376 (44 %) HIV-positive TB patients on antiretroviral therapy (ART) (WHO, 2012).

1.5.2. Tuberculosis and HIV/AIDS

Largely associated with poverty and an inadequate health service response, the TB epidemic in sub-Saharan Africa remained at a relatively constant level until the onset of the impact of the

HIV epidemic in the 1980s (Giardi *et al*., 2000; WHO, 2002a). The human immunodeficiency virus (HIV) pandemic is the world's leading public health emergency, with a particularly severe impact on sub-Saharan Africa. It is destroying the health of Africans, the economies of African nations and their prospects for development. HIV infection is also fuelling TB epidemic by weakening the immune system and increasing the susceptibility of patients to TB (Datiko *et al*., 2008). The HIV epidemic has posed major and almost insurmountable challenges to TB control efforts across the world (Godfrey-Faussett *et al*., 2002; Ghiya *et al*., 2009). TB and HIV/AIDS represent a deadly combination, since they are more clinically devastating together than either disease presenting alone (Harries and Graham, 2004; Sharma *et al*., 2004). TB is more difficult to diagnose and progresses more rapidly in the HIV-positive population (Lawn, 2009; Low, 2009).

In 2011, 1.1 million (13%) of the 8.7 million people who developed TB worldwide were HIV-positive; 79% of these HIV-positive TB cases were in the African Region. WHO's recommended package of collaborative TB/ HIV activities to reduce the burden of TB/HIV includes HIV testing for TB patients; CPT and early initiation of ART for HIV-positive TB patients; and screening for TB among people living with HIV and provision of IPT to those eligible for it. Substantial progress in the implementation of collaborative TB/HIV activities has occurred since WHO recommendations were first issued in 2004, and further progress was evident in 2011. The percentage of notified TB patients with a documented HIV test result in the African Region rose from 60% in 2010 to 69% in 2011; 46% of those tested in 2011 were HIV-positive, ranging from 8% in Ethiopia to 77% in Swaziland. Worldwide, 40% of TB patients notified in 2011 had a documented HIV test result, up from 33% in 2010 and more than ten times the level of 2004. In 2011, 79% of TB patients known to be HIV-positive were provided with CPT, and 48% were started on ART, similar to levels achieved in 2010. More work remains to be done to ensure that all HIV-positive TB patients are rapidly started on ART, in line with WHO recommendations. Their progress on treatment should also be closely monitored. In 2011, 3.2 million people enrolled in HIV care were reported to have been screened for TB, up 39% from 2.3 million in 2010. Of those without active TB disease, 0.45 million were provided with IPT, more than double the number started on IPT in 2010 (mostly the result of progress in South Africa). The

scale-up of collaborative TB/HIV activities saved a total of 1.3 million lives between 2005 and the end of 2011 (WHO, 2012).

1.6. *M. tuberculosis* Genome

The complete genome of *M. tuberculosis* H37Rv was published in 1998 (Cole *et al*., 1998). Its size is 4.4 million base pairs, with 3959 genes; 40% of these genes have had their function characterized, with possible function postulated for another 44% figure 1.2. Within the genome are also six pseudogenes. The genome of *M. tuberculosis* is GC rich (65.6 %). The genome contains 250 genes involved in fatty acid metabolism, with 39 of these involved in the polypeptide metabolism generating the waxy coat. Such large numbers of conserved genes show the evolutionary importance of the waxy coat to pathogen survival. This was updated four years later and the function of 2058 genes (52%) was predicted (Camus *et al*., 2002) Fig.1.3.

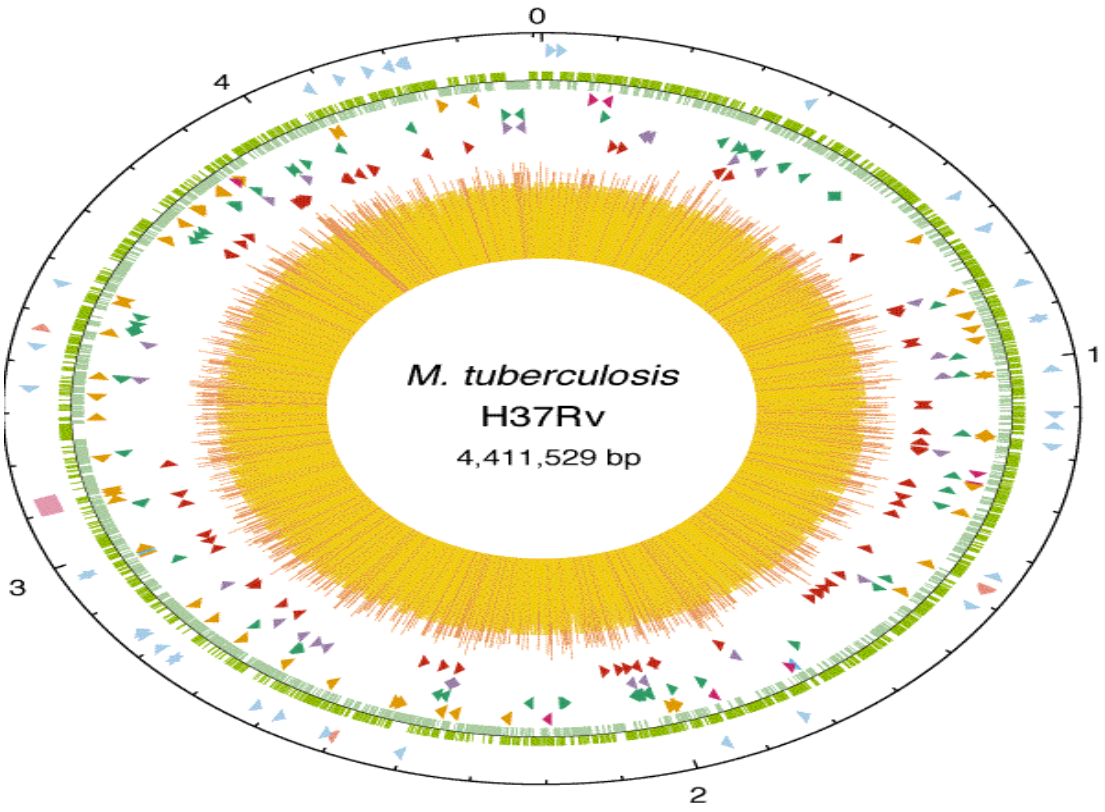

Figure 1.2 A diagrammatic representation of the *M. tuberculosis* H37Rv genome (Cole *et al*., 1998).

1.6.1. Phylogeny

According to the SpolDB4 (web link)database, the MTC is grouped into 62 genetic lineages where *M. canetti*, *M. caprae* and *M. microti* all have 1 lineage each, *M.pinnipedii* has 2 lineages, *M. bovis* has 3 lineages, *M.africanum* has 4 lineages and *M. tuberculosis* has 50 lineages (Brudey *et al*., 2006)

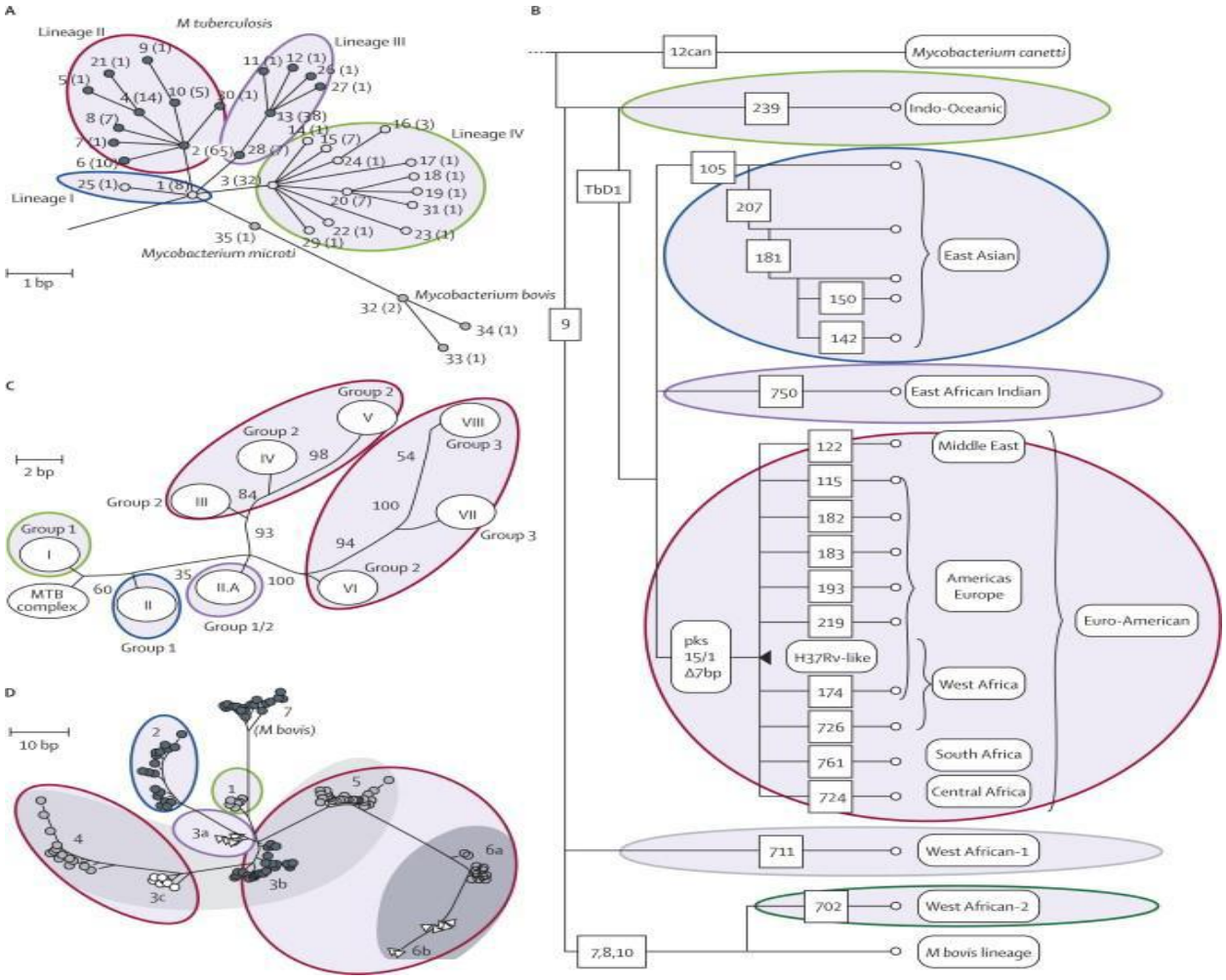

Figure 1.3 Global Phylogeny of *M. tuberculosis*. A: Groups I-IV adapted from Baker 2004. B six phylogeographic groups adapted from Gagneux (2007). C: Nine groups with corresponding Principal Genetic Groups 1-3 (Srrevatsan 1997) adapted from Gutacker (2006). D: nine groups and *M. bovis* adapted from Filliol (2003). Colored areas indicate corresponding groups of strains (Gagneux & Small 2007).

1.6.2 Evolution

Although there is limited genetic diversity between strains (Gutierrez *et al*., 2005), the differences that have been observed have been useful in the investigation of the global evolution of the *M. tuberculosis* complex. Deletions and point mutations can be used in the studies of the global evolution of the organism (Supply *et al*., 2003). The *kat*G gene and the mutations in this gene can confer resistance to isoniazid in *M. tuberculosis* while mutations in the *gyr*A gene, which encodes DNA gyrase, may confer resistance to fluoroquinolones (Takiff *et al*., 1994). A study by Sreevatsan and colleagues on the analysis of the genes implicated in drug resistance in over 800 geographically diverse isolates from the *M. tuberculosis* complex identified three distinct groups of strains (Sreevatsan *et al*., 1997). These groups were referred to as principal genetic groups 1, 2, and 3 have been identified on the basis of 2 single-nucleotide polymorphisms (SNPs) that occur in the *katG* and *gyrA* genes (Sreevatsan *et al*., 1997). Principal genetic group 1 *M. tuberculosis* was found to be evolutionarily old and was allied with the *M. tuberculosis* complex "ancestor," *M. microti, M. africanum,* and *M. bovis*. Principal genetic groups 2 and 3 of *M. tuberculosis* were thought to have phylogenetically followed from group 1. It was surmised that the common precursor organism of the *M. tuberculosis* complex possessed codon *kat*G463 CTG (Leu) and *gyr*A95 ACC (Thr). Members of the *M. tuberculosis* complex that possessed these codons were considered to be the oldest and members were termed to be in Principal Group 1(Sreevatsan *et al*., 1997). Genomic comparisons between the completed genome sequence of *M. tuberculosis* H37Rv (Cole *et al*., 1998) and *M. bovis* have revealed a number of large sequence polymorphisms (LSPs) that appear to distinguish virulent *M. bovis* isolates relative to *M. tuberculosis* H37Rv (Behr *et al*., 1999; Gordon *et al*., 1999 and Salaman *et al*., 2000). To date, only deletions relative to the sequenced strains of *M. tuberculosis* (H37Rv and CDC1551) have been identified (Sreevatsan *et al*., 1997).

Comparative genomic analysis showed 14 regions of difference (RD1-14), ranging in size from 2 to 12.7kb, that were present in *M. tuberculosis*, but absent in *M. bovis* BCG (Behr *et al*. 1999; Gordon et al. 1999). In addition, six deletions were identified in the reference strain *M. tuberculosis* H37Rv compared with other members of the *M. tuberculosis* complex (RvD1-5 and the *M. tuberculosis* specific deletion 1 (TbD1) (Brosch *et al*., 1999; Gordon *et al*., 1999).

Geographically distributed isolates from the *M. tuberculosis* complex were examined for the presence or absence of TbD1, a number of regions of difference (RD) as well as single nucleotide polymorphisms (SNPs) in the gene sequences of *kat*G, *gyr*A, *oxy*R, *pnc*A and *mmp*l6. Results showed that *M. tuberculosis* as well as other members of the *M. tuberculosis* complex had evolved from a common precursor (Brosch *et al*., 2002). Strains that possessed TbD1 were termed ancient whilst those lacking the region were labeled modern. Additionally, the assumption that *M. tuberculosis* had evolved from *M. bovis* was discredited. This finding was also confirmed by Mostowy and colleagues (Mostowy *et al*., 2002).

Further analysis of well-characterized laboratory strains, as well as clinical isolates has contributed to the study of the evolution of the *M. tuberculosis* complex (Sreevatsan *et al*, 1997). In addition to the presence or absence of TbD1 and the SNPs at *kat*G463 and *gyr*A95, other SNPs and long sequence polymorphisms (LSPs) have been investigated. Baker and colleagues (Baker *et al*., 2004) investigated SNPs in *kat*G and *gyr*A, as well as a further five genes (*rpo*B, *oxy*R, *ahp*C, *pnc*A, and *rps*L). As sSNPs are neutral, they become fixed in a bacterial population and may be used to study its evolution.

Epidemiological data showed that there were strong associations between the groups and the country of birth of the patients. Groups I, II and III were associated with South East Asia, Europe and the Indian subcontinent, respectively. Group IV strains were globally disseminated, but were negatively associated with Europe. Large proportions of these Group IV strains possessed only one copy of IS6110 and were more likely to be drug susceptible. This, combined with the presence of TbD1 strongly suggested that this group was more closely related to the common ancestor than the other groups (Baker *et al*., 2004).

As a result, strains representative of *M. bovis* clades which had evolved through clonal expansion in a restricted geographical location were subsequently shared between geographically distinct countries with political and economic ties (Muller *et al*., 2009). Examples have been documented

in Algeria, Mali and South Africa where VNTR typing revealed a link between local *M. bovis* isolates and those described in France and the United Kingdom, respectively (Michel *et al*., 2008; Sahraoui *et al*., 2009). Intensification of the dairy industry in combination with movement of cattle (Gilbert *et al*., 2005) has contributed to the transmission of *M. bovis*, especially in the absence of suitable control measures. Cattle trade between neighbouring countries and trading partners probably lead to the regional dispersal of *M. bovis* and to the dominance of strains in large areas (Fig.1.4) (Diguimbaye- Djaibe *et al*., 2006; Cadmus *et al*., 2006; Muller *et al*., 2008).

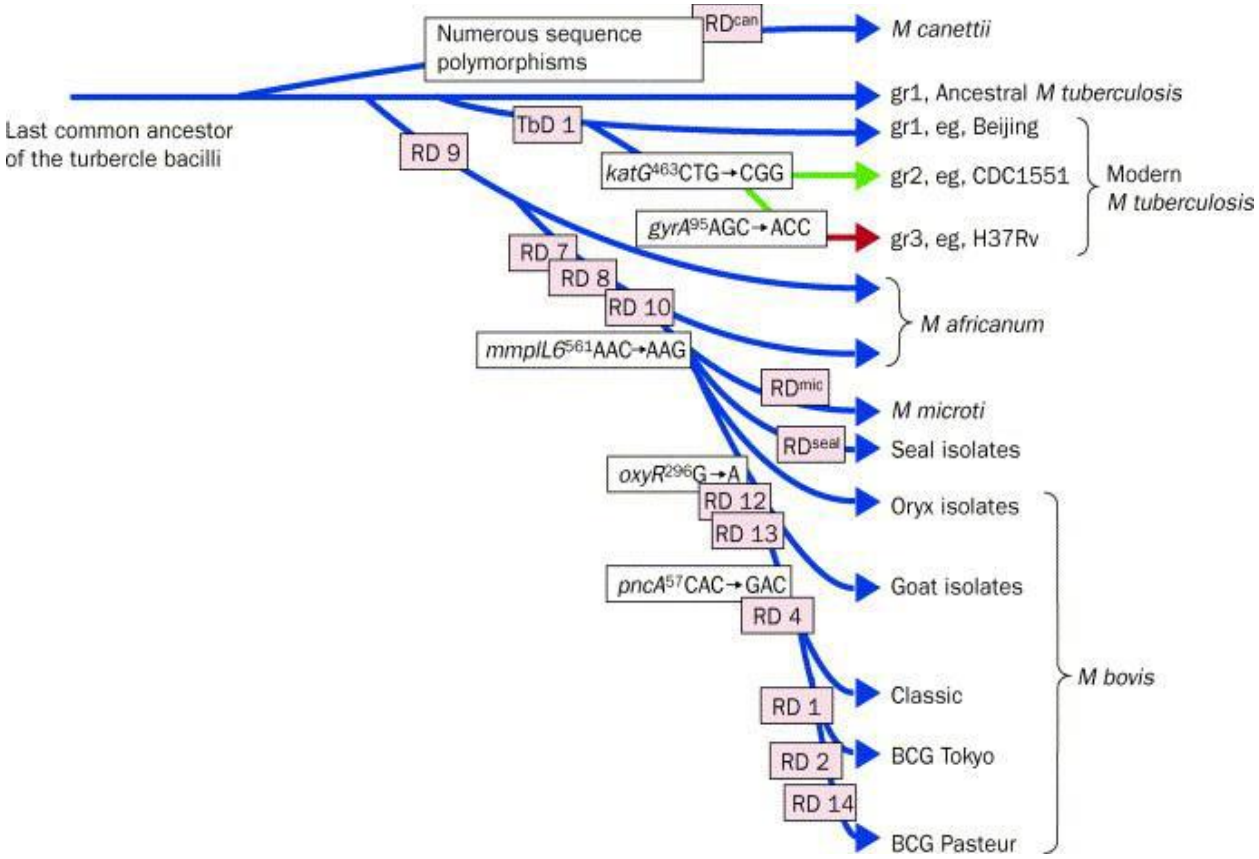

Figure 1.4 Diagram of the evolution of *M. tuberculosis* complex. Principal Groups 1, 2 and 3 are shown towards the top of the figure and are indicated by blue, green and red arrows, respectively. (Donoghue *et al*., 2004).

1.6.3. Drug resistant *Mycobacterium tuberculosis.*

Short-course chemotherapy forms the backbone of antitubercular chemotherapy (Kochi *et al*., 1993). However, the emergence of new strains of MTC resistant to some or all current antituberculous drugs contributes to the increased death rate (table 1.1). The resistance is attributed to inconsistent drug supply, patient non-compliance and weak tuberculosis-control infrastructure. In health care settings, delayed recognition of drug resistance, which results in delayed initiation of effective therapy, is one of the major contributing factors to MDR-TB outbreak (WHO, 2010). This can lead to a vicious cycle of inadequate treatment (fig. 1.5), the generation of tuberculosis-drug resistance, and transmission of resistant strains. People who have primary drug resistance and who are infected with a strain of tuberculosis that is already resistant frequently fail treatment with drug regimens designed for use against drug sensitive disease and become progressively more resistant and difficult to cure (Mukherjee *et al*., 2004).

Table 1.2: Antituberculosis drugs and the gene(s) involved in their resistance

Drug	Gene(s) involved in drug resistance
Isoniazid	Enoyl acp reductase (*inhA*)
	Catalase-peroxidase (*katG*)
	Alkyl hydroperoxide reductase (*ahpC*)
	Oxidative stress regulator (*oxyR*)
Rifampicin	RNA polymerase subunit B (*rpoB*)
Pyrazinamide	Pyrazinamiadase (*pncA*)
Streptomycin	Ribosomal protein subunit 12 (*rpsL*)
	16s ribosomal RNA (*rrs*)
	Aminoglycoside phosphotransferase gene (*strA*)
Ethambutol	Arabinosyl transferase (*emb A, B* and *C*)
Flouroquinolones	DNA gyrase (*gyr A* and *B*)

Some countries have already been labeled MDR-TB hot spots, where a substantial proportion of incident tuberculosis is MDR-TB (WHO, 2007). TB is currently treated with an initial intensive 2-month regimen comprising multiple antibiotic isoniazid, rifampicin, ethambutol or streptomycin and pyrazinamide (Fig. 1.5) to ensure that mutants resistant to a single drug do not emerge (MMRW, 1993). Limpopo Province in South Africa gives patients Rifafour (consisting of rifampicin 150 mg, isoniazid 75 mg, pyrazinamide 400 mg, ethambutol 275 mg) for two months; rifampicin and isoniazid are administered for the next 4 months to eliminate persisting tubercle bacilli (Personal communication, 2004). The emergence of strains resistant to either of these drugs causes major concern, as it leaves only drugs that are far less effective, have more toxic side effects, and result in higher death rates, especially among HIV infected individuals (Rattan *et al*., 1998). Rattan defines the phrase MDR state in mycobacteriology as simultaneous resistance to at least RMP and INH with or without resistance to other drugs (Rattan *et al*., 1998).

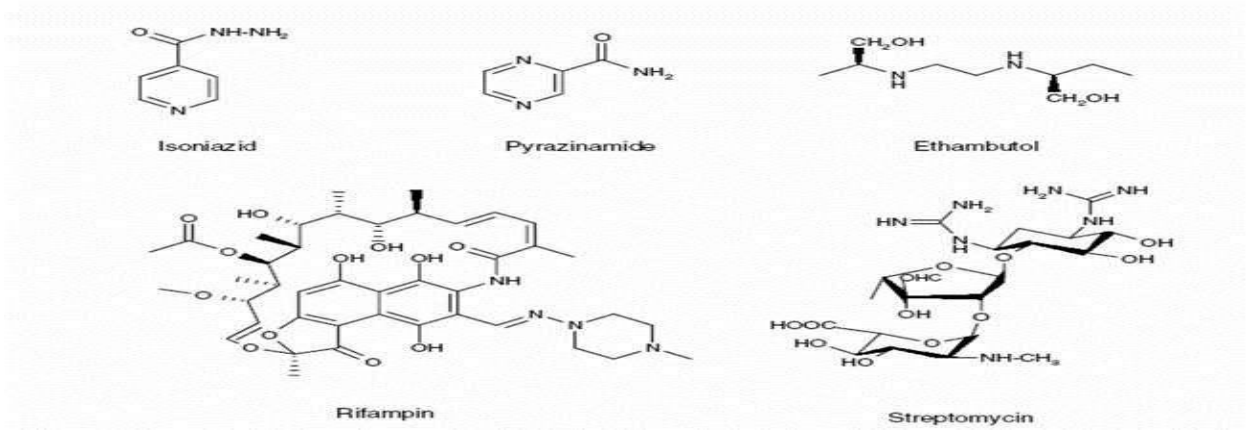

Figure 1.5 Chemical structures of five first line drugs used for the treatment of tuberculosis.

Even though there is an unequal distribution of drug resistance between poor and rich countries, the problem is global. The regions where drug-resistant TB is more prevalent lack the resources to implement adequate measures to control even the susceptible types of the disease. Reviews (Cohn *et al*., 1997, Espinal *et al*., 2001) have reported an increasing prevalence of primary multidrug resistant tuberculosis in Latvia (1998: 9.0%), Estonia (1998: 14.1%), The Dominican Republic (1994-1995: 6.6%), Ivory Coast (1995-1996: 5.3%), Argentina (1994: 4.6%), Russia

(Ivanovo Oblast) (1998: 9%), Iran (1998: 5.0%) and Henan, China (1996: 10.8%). South Africa‘s neighbors Botswana (1995-1996), Lesotho (1994-1995), and Swaziland (1994-1995) have reported encouraging results of 0.2%, 0.9%, and 0.9% respectively. Acquired multidrug resistance of higher than 20% was reported in Guinea (1998: 28.1%), Latvia (1996: 54.4%), Mexico (1997: 22.4%) Italy (1999: 33.9%), Russia (Ivanovo Oblast) (1998: 25.9%), Tomsk Oblast (1999: 26.7%), Estonia (1998: 37.8%), Iran (1998: 48.2%), Sierra Leone (1997: 23.1%), Argentina (1994: 22.2%), and Spain (Barcelona) (1995-1996: 20.5%). Again acquired MDRTB was low in Botswana (1998: 9.0%), Mozambique (1999: 3.3%), Lesotho (1994-1995: 5.7%), and Swaziland (1994-1995: 9.1%) (Cohn *et al*., 1997, Espinal *et al*., 2001). However, in the present day, MDR-TB is a problem (WHO, 2009)

Genetic and molecular analyses of drug resistance in MTC suggests that the bacilli usually acquire resistance either by alteration of the drug target through mutation (Spratt, 1994; Soini and Musser, 2001) or by titration of the drug through overproduction of the target (Davis, 1994). MDR-TB results from accumulations in individual drug target genes. However, resistance to a drug does not confer any selective advantage to the bacterium unless it is exposed to the drug. During exposure to drugs, there is a selective pressure for such resistant mutants. Under these circumstances, the sensitive strains are killed and the drug resistance mutants flourish. The development of MDR is based on a sequence of such mutations giving resistance to one drug or a group of drugs (e.g. rifamycins) one at a time. In many other pathogenic bacteria, resistance plasmids can potentiate a rapid change from wild type susceptibility to MDR. Such extrachromosomal genetic elements can transfer resistance to several unrelated antibacterial substances in one single step (Levy, 1992 and Levy, 1997). However, this has never been reported in MTC and so sudden development of multiple resistances in a strain does not take place (Petrini and Hoffner, 1999).

Our understanding of the molecular mechanisms for resistance of MTC to anti-mycobacterial agents has increased very significantly. On epidemiological grounds, drug resistance has been divided into three broad categories, which are; primary drug resistance, where drug resistant

bacilli are isolated from previously untreated patients. ii) Acquired drug resistance, where drug resistant bacilli are isolated from patients who had originally susceptible bacilli and iii) initial drug resistance, denoting drug resistance in patients who deny a history of previous chemotherapy (WHO, 2009). For each of the first line drugs at least one or more genes have been identified in which specific mutations lead to a resistant phenotype (WHO, 2010). However, there is still much to be understood before the full picture can be defined. MTB is naturally resistant to many antibiotics (WHO, 2010). This resistance is due mainly to the cell envelope acting as a permeability barrier, but many potential resistance determinants are also encoded in the genome. Resistance can be caused by a variety of mechanisms: (i) the presence of an enzyme that inactivates the antimicrobial agent; (ii) the presence of an alternative enzyme for the enzyme that is inhibited by the antimicrobial agent; (iii) a mutation in the antimicrobial agent‘s target, which reduces the binding of the antimicrobial agent; (iv) post-transcriptional or posttranslational modification of the antimicrobial agent‘s target, which reduces binding of the antimicrobial agent; (v) reduced uptake of the antimicrobial agent; (vi) active efflux of the antimicrobial agent; and (vii) over-production of the target of the antimicrobial agent. In addition, resistance may be caused by a previously unrecognized mechanism. On the other hand, a gene that is not expressed in vitro may be expressed *in vivo* (Hatful, 1993).

1.6.3.1. Rifamycins

Rifamycins inhibit the RNA polymerization by interacting with DNA-dependent RNA polymerase. Rifamycins are a group of compounds that not only have antimicrobial effects on mycobacteria, but also towards Gram-negative and Gram-positive bacteria. They are also important groups of drugs for treatment of leprosy. Since they were discovered, hundreds of derivatives have since been isolated. The most important rifamycin for treatment of TB is rifampicin (RMP) discovered in the 1960‘s. Other rifamycin of importance are, rifapentine, rifabutin (RFB), rifalazil (KRM-1648), and rifamycin T9, some of which show higher bactericidal effects against TB than RMP. Most resistance mutations give rise to cross-resistace to other rifamycins. RMP is active against *M. tuberculosis in vitro* at concentration of 1μg/mL and is most efficient in inhibiting actively replicating bacteria. *In vivo* it is regarded that RMP is

not only effective in curing active TB, but also regarded to inhibit further spurts of metabolism among latent bacteria (Mitchison and Nunn, 1986).

1.6.3.1.1. Mechanism of resistance to rifampicin.

The MTC RNA polymerase is a complex oligomer composed of five different subunits (α, α', β, β', σ encoded by *rpoA, rpoB, rpoC, rpoD* part of the gene, respectively) that are highly conserved among bacterial species (McClure and Cech, 1978). When RMP binds to the β subunit, the core can still assemble to the DNA and the first phosphodiester bond can be formed. However, RMP then blocks the further formation of transcripts of three to four base pairs, and the elongation of RNA is inhibited (Artsimovitch and Vassylyev, 2006). The β-subunit of the RNA-polymerase is encoded by the 3.5 kbp *rpoB* gene. In *M. tuberculosis*, most resistance mutations involve single nucleotide substitutions in this gene, and insertions or deletions are seen (Ramaswamy and Musser, 1998). Although being a highly essential gene, these mutations do not seem to affect the redundancy of the RNA-polymerase. Instead, these mutations are thought to mainly alter the amino acids to which the RMP molecule presumably binds (Sensi and Grassi, 2006). Recently, from structural studies of the *Thermus aquaticus* and*Thermus thermophilus*, a theory about rifamycin-binding was introduced (Artsimovitch and Vassylyev, 2006). Rifamycins are classed into two groups depending on the side chains. Thus mutations in the RNA polymerase can circumvent rifamycin binding in three ways; through (i) steric hinder, (ii) reduced affinity and (iii) allosteric modulation. The latter would not inhibit binding of the drug but distort an inhibitory signal of the drug (Artsimovitch and Vassylyev, 2006).

The association of the RNA polymerase B (*rpoB*) subunit gene with resistance to rifampicin has been documented previously and subsequent reports from various groups have confirmed this association in clinical isolates of *M. tuberculosis* (Hoffner *et al*., 1988). Rifampicin (RMP) inhibits the DNA-dependent RNA polymerase. Mutation in a single gene seems responsible for almost every case of drug resistance. In at least 96% of studied clinical rifampicin (RMP) resistant *M. tuberculosis* strains, mutation in the 81-bp region of the *rpoB*-gene referred to as Rifampicin Resistance Determinant Region (RRDR) (Fig. 1.6) has been demonstrated (Heep *et*

al., 2001). Since the β sub-unit of the RNA polymerase is the target for rifampin (RMP), a substitution or deletion in the *rpoB*-gene leading to a target modification, also causes a high-level resistance to RMP and in many cases, other rifamycins. Since the rate of such mutations is low, single resistance to rifampin (RMP) is uncommon. Therefore, if an isolate is shown to be RMP-resistant it is very often an indication that it is MDR (Green *et al*., 2008).

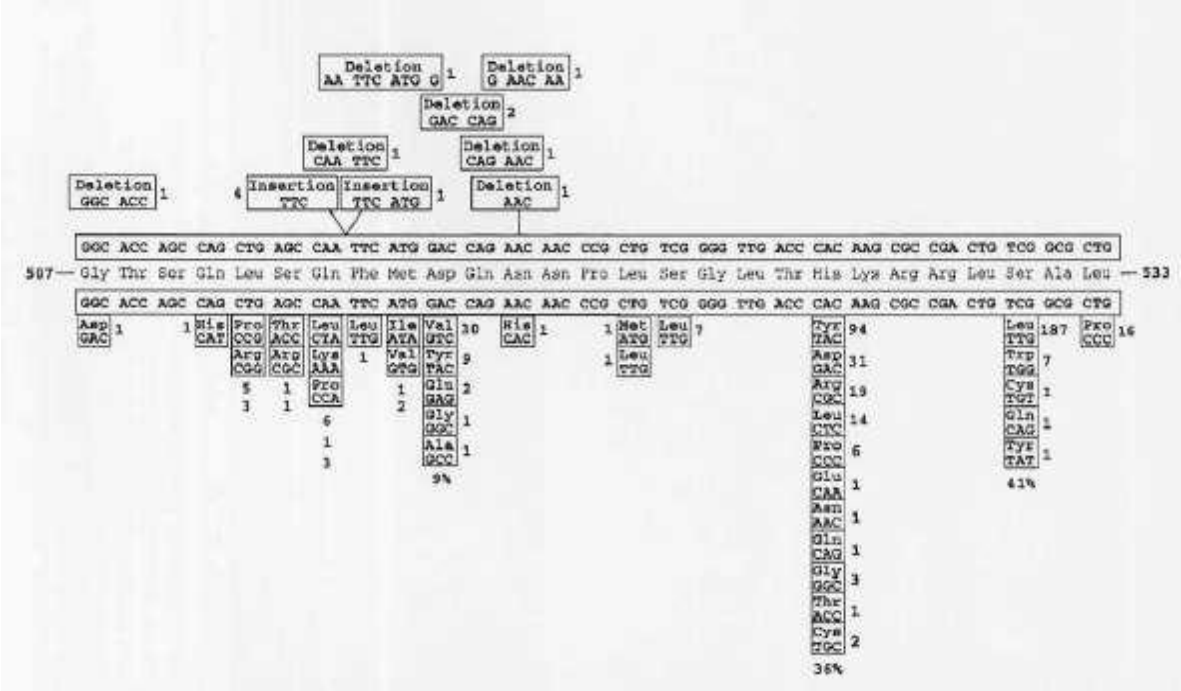

Figure 1.6 Mutations located in the 81-bp core region between codon 507 - 533 of the *M. tuberculosis rpoB* gene. Common mutations in the *rpo*b gene. Top panel shows the nucleotide changes (deletions) that are similar to previously reported mutations. The middle panel depicts the corresponding amino acid changes within the RNA polymerase b subunit. The bottom panel indicates nucleotide changes and their amino acids. Frequency and type of mutations are indicated for each codon. The codon numbers are designated on the basis of alignment of the translated *E. coli rpoB* sequence with the homologous part of the translated *M. tuberculosis* sequence. The figure is adopted from the review article by Ramaswamy and Musser, (1998).

1.6.3.2. Isoniazid (isonicotinic acid hydrazide)

Isoniazid (INH) or isonicotinic acid hydrazide is a synthetic bactericidal agent, first produced in the early 1900‘s, but was not utilized as an antituberculous agent until 1952. Presently, it is the prophylaxis of choice due to its low cost per dose, relatively low frequency of hepatotoxicity (Nolan *et al*., 1999), and reasonable bioavailability (Gurumurthy *et al*., 1999). Isoniazid enters mycobacterial cells via passive diffusion across the bacterial envelope. The organism, using the gene *KatG*, produces the enzyme catalase peroxidase that activates the INH drug making it toxic (fig 1.7 and 1.8). *Kat*G is a gene whose physiological role is protective, combating the low pH found during the "oxidative burst" in human phagocytes, where liberated O_2 radicals are converted to H_2O_2 within the phagosome. *KatG* activity eliminates this via a"deceptively simple reaction" (Loewen *et al*., 2000) where hydrogen peroxide is converted to water and oxygen using the enzyme catalase-peroxidase. Fascinatingly, this same protective enzyme is implicated in susceptibility to INH. Specifically, INH is a prodrug that requires cellular activation by *KatG* producing a reactive species with antimicrobial action. Furthermore, the catalase-peroxidase-activated isoniazid binds to and inhibits the activity of the mycobacterial fatty acyl enoyl-ACP reductase encoded by the *inh*A gene (Johnsson *et al*., 1995), the enzyme considered to be the target of isoniazid action.

In *E. coli*, both a constitutively expressed *katE*-encoded catalase and a hydrogen peroxide inducible *kat*G-encoded catalase peroxidase are present (Lowens *et al*., 1985), while in *M. tuberculosis* the *kat*G encoded catalase-peroxidase is constitutively expressed and no *kat*E-type catalase has been identified (Diaz *et al*., 1974). Isoniazid (INH) resistance also involves other genes found in *M. tuberculosis*. The main target of isoniazid (INH) is mycolic acid synthesis, and mutations in the *katG* and *inhA* genes result in resistance. Deletion or mutation of the *katG* gene leads to high-level isoniazid (INH) resistance and renders the bacteria catalase-negative. The mutation rate leading to INH resistance is 100 times higher than that responsible for rifampin (RMP) resistance (Fig 1.7). Thus, isoniazid (INH) resistance is often the first demonstrated modification in the wild type susceptibility of *M. tuberculosis*. It is often followed by resistance to other drugs and should be seen as a warning flag.

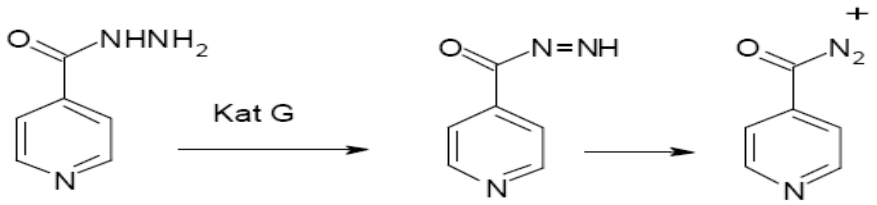

Figure 1.7 Potential metabolic activation mechanism for Isoniazid. *Kat G* mediates 2 electron transfers to produce an activated Isoniazid intermediate(s). It is this reactive intermediate that is capable of intracellular acylation of nucleophiles in *M. tuberculosis*, thereby facilitating toxic effects.

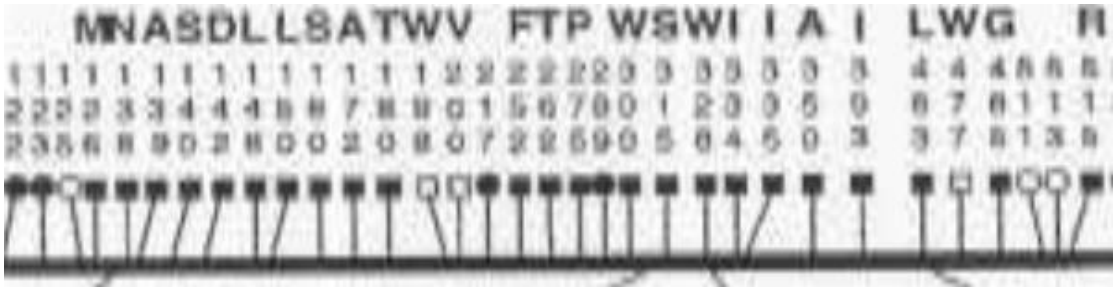

Figure 1.8 Polymorphism in the *Kat*G protein identified in INHR *M. tuberculosis.* The variant amino acids are numbered vertically. The single-letter amino acid abbreviations are used. The KatG 463 Leu→Arg substitution commonly occurs in natural polymorphism that is not associated with INH resistance. The figure is adopted from the review article by Ramaswamy and Musser (1998).

1.6.3.2.2. Streptomycin

Streptomycin is an aminocyclitol glycoside antibiotic that is widely used in tuberculosis therapy. The exact mechanism of action of streptomycin has not been extensively investigated in mycobacteria, but in *E. coli* the antibiotic bind to 16S rRNA, interferes with the proofreading

step in translation, inhibit translational initiation thereby perturbing protein synthesis (Bercovier *et al*., 1986). Most bacteria express aminoglycoside-modifying enzymes as a common mechanism of aminoglycoside-aminocyclitol resistance, and these enzymes are generally encoded by resistance plasmids. However, clinically significant aminoglycoside-modifying enzymes have not yet been described in mycobacteria. Even though rRNA operons in most eubacteria are many, slowly growing MTB and *M. leprae* have one copy (Bercovier *et al*., 1986).

The practical implication of the observation of one 16S rRNA gene copy in the slowly growing mycobacteria is that single nucleotide changes can result in antibiotic resistance (dominant behavior), whereas in *E. coli*, a bacterium with seven 16S rRNA gene copies, or *M. smegmatis,* with two copies (Fig.1.9), nucleotide changes in a single rRNA gene are not expected to confer resistance (recessive behavior) (Domenech *et al*., 1994).

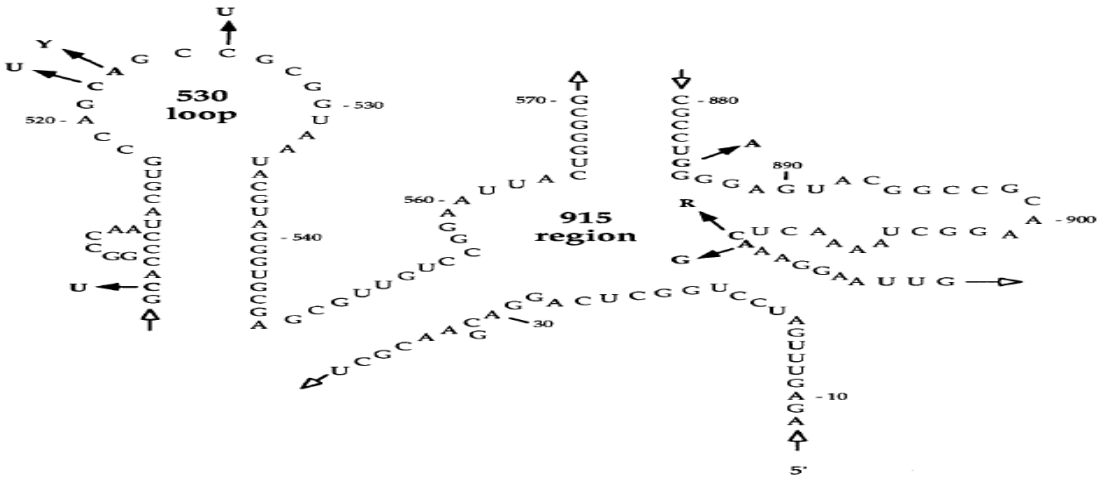

Figure 1.9 Mutations located in 16S rRNA associated with STR resistance in *M. tuberculosis*.

The drawing is based on a model structure of *E. coli* 16S rRNA. The nucleotide numbering system used is based on the publication of Finken *et al*. (1993), and this system is used to

maintain continuity with previously reported mutation data. Note that the position designations do not represent the actual MTC positions. For the actual MTB positions, subtract 10 from the 530 loop region numbers and subtract 8 from the 915 region numbers. The mutations associated with STR resistance are indicated by the solid arrows and are described in the references cited: position 491, position 512, positions 513 and 516 (Finken et al., 1993), position 903 (Honore and Cole, 1993), and position 904.Y 5 U or C; R 5 A or G.The target for streptomycin (STR) resistance is the ribosomal proteins (Hoffner *et al*., 1988). Mutations in genes *rpoL*, *rrs* and *strA*, involved in the synthesis of these proteins have been shown to be related to development of streptomycin (STR) resistance. It has been shown by Kahila and co-workers in 1971 that, streptomycin has two major phases of action: (a) the initiation which involves protein synthesis which probably represents effective streptomycin on the membrane resulting in the entrance of the drug into the cell, (b) the lethal phase, which involve further attack not involving protein synthesis. It should be noted that such mutations render the strain resistant only to streptomycin (STR) not to the other amino-glycoside agents, such as kanamycin and amikacin (AMK). It has been reported that streptomycin (STR) resistant clinical isolates are generally susceptible to AMK (Hoffner *et al*., 1988).

1.6.3.2.3 Ethambutol (EMB)

There are three contiguous genes encoding putative target(s) for EMB in *M. tuberculosis* and *M. smegmatis*. These genes were subsequently cloned, sequenced, and characterized (Telenti *et al*., 1997; Mokrousov *et al*., 2002). Two of these genes were similar to the *embA* and *embB* genes described in *M. avium*, and the third one was termed *embC.* These genes are likely to be organized as an operon in the order *embC*, *embA*, and *embB*. Therefore, it is reasonable to expect that these genes are transcribed as a single polycistronic mRNA from a unique promoter, but this has yet to be shown. The *embCAB* gene cluster was initially identified in an EMB resistant strain of *M. smegmatis* (Telenti *et al*., 1997) and was subsequently characterized in *M. tuberculosis* (Telenti *et al*., 1997; Tracevska *et al*., 2004)

Majority of clinical *Mycobacterium tuberculosis* isolates resistant to EMB have mutations on the *embB* gene and thus the EmbB protein has been proposed as the main target for EMB. The Emb proteins are predicted to be integral membrane proteins with 11–13 transmembrane domains and a large carboxyl-terminal globular region of external location. These proteins may also operate, at least in part, as proteins involved in transporting lipoarabinomannan (LAM) and arabinogalactan (AG) arabinan precursors across the plasma membrane. In this case, the Emb proteins would work in close relation with the true arabinosyltransferases so far unidentified or might be bifunctional proteins with both transferase and transport activities. The mechanism of action of ethambutol (EMB) resistance has long been thought to be interference with the cell wall polysaccharides (Björn *et al*., 1999). However, studies that are more recent show that the primary site of action is arabinan biosynthesis blocking the formation of the cell wall (Mikusova *et al*., 1995). Based on this fact, it has been shown that ethambutol weakens the cell wall and other drugs are then able to act on the organism. Over expression of the *emb*, proteins coded by the genes *emb a*, *b* and *c* are related to ethambutol resistance (Telenti *et al*., 1997).

1.6.3.2.4 Pyrazinamide (PZA)

Although discovered in the 1950‘s several obstacles surrounding PZA still need to be solved. PZA was discovered to be effective against *M. tuberculosis* in the mouse models, but found to have little or no *in vitro* effect under normal culture conditions (Zhang and Metchison, 2003). However, through its introduction in physical therapy relapse-rates were strikingly reduced and PZA has since greatly contributed to shortening of TB therapy from 9-12 months to 6 months (Saltini, 2006). In contrast to other anti-tuberculous drugs, PZA seems to be active against semidormant bacilli (Heifets and Lindholme-Levy, 1992). In line with this, it also been shown that the drug has a greater activity in an anaerobic environment (Wade and Zhang, 2004) and against stationary phase culture (Zhang *et al*., 2003). PZA is exclusively active against the MTC members, with the exception of *M. bovis*, whose intrinsic PZA-resistance is a characteristic feature for this member (Zhang *et al*., 2003).

1.7. Prevention of tuberculosis

Ventilation dramatically dilutes the concentration of infectious droplet nuclei. Wherever possible, opening windows during and after cough-inducing procedures or in hospital wards is one of the most, if not the most, efficient means of reducing the probability of exposed persons becoming infected. Wearing surgical masks by exposed persons is likely to be of low efficiency, because most masks neither filter out particles of less than 5 μm, nor do they generally fit snugly enough around both mouth and nose. In industrialized countries, the emergence of multidrug-resistant tuberculosis, and exposure of staff in hospitals with often poor ventilation and usually little to no direct exchange with fresh outdoor air, has led to the recommendation for staff to wear a special mask, called a high efficiency particulate air-filter respirator (CDC, 1990). This mask is designed to filter out particles in the droplet nucleus size of 1 μm to 5 μm. It costs several times the price of usual surgical masks, and data on how snugly they fit to prevent droplet nuclei from entering between skin and mask are scarce.

1.8. Problem Identification

The host range of MTC is very broad and it can cause TB in various domestic and wild animals and humans. As a result bovine TB is a disease causing significant losses to the decrease in milk production and meat production. MTC, therefore, poses a public health threat due to consumption of raw milk and considerable economic losses due to animal production losses and abattoir condemnation of infected carcasses. Although no estimates have been given for the disease burden in the whole country, evidence of infection generally detected as post-mortem findings during slaughter (Asiimwe, 2008) suggests that the disease burden is high. There is also a problem with the emergence of resistant tuberculosis and little has been done especially drug resistance MTC from raw milk isolates. Moreover a recent study by Silaigwana has reported high drug resistance for specimens isolated from unpasteurized cattle milk (Silaigwana *et al*., 2012). These findings warrant urgent intervention from other researchers to investigate the resistance profiles of MTC isolated from raw milk. There is also a substantial lack of knowledge on the distribution, epidemiological pattern in cattle and zoonotic importance of bovine tuberculosis. As such, fundamental questions regarding the molecular aspects of MTC in South

Africa have not yet been addressed. Questions include the genetic diversity and degree of geographical sub-structuring of MTC isolates from raw milk. Molecular techniques that can be used to answer these questions are available (van Embden *et al*., 1993; Kamerbeek *et al*., 1997). This study therefore used these techniques to elucidate the molecular aspects of MTC in the Eastern Cape region, South Africa.

1.8.1. Justification

Bovine tuberculosis not only poses a great threat to public health in developing countries, including Africa (Ayele *et al.*, 2004), but also leads to great economic losses, and in South Africa research on control of animal tuberculosis has not received much attention as human tuberculosis has. Bovine tuberculosis is endemic in many African countries (Asiimwe, 2003), but economic constraints preclude the use of skin test and slaughter control strategies, which have proved effective in the developed world. In many African settings, domestic animals are an integral part of human social life and in those cases the risk factors for *M. bovis* infection in both animals and humans are close contact, food hygiene practices and HIV/AIDS infection (Cosivi *et al.*, 1998). Control policies have not been enforced due to cost implications, lack of capacity and infrastructure limitations (Cosivi *et al.*, 1998; Ayele *et al.*, 2004).

This study will go a long way to answer key questions on the molecular epidemiology of MTC in a high disease burden country and the genetic diversity. This will also help to determine the distribution pattern of MTC strains in the country and thus could help in policy formulation regarding the control of animal tuberculosis in the Eastern Cape Province, South Africa

1.8.2. Hypotheses

Consumption of raw milk of cows may lead to tuberculosis caused by the *Mycobacterium tuberculosis complex.*

1.8.3. Research Aims

The study is aimed at exposing the dangers associated with raw milk in a setting where milk is normally consumed unpasteurized especially in rural regions where people have their own cows and milk them. The study will investigate the molecular characterization of the *Mycobacterium tuberculosis* complex from raw milk of cows in the Eastern Cape.

1.8.4. Objectives

To achieve this aim, the objectives that follow will be pursued:

- To isolate and detect *Mycobacterium tuberculosis* (MTC) complex from raw milk
- To identify and differentiate the members of the MTC
- Determine the molecular susceptibility profiles of MTC isolates from the Eastern Cape Region.
- Perform Spoligotyping on the isolates.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Mycobacterium Tuberculosis* Complex (MTC)

2.1.1 *Mycobacterium tuberculosis*

Mycobacterium tuberculosis is a human pathogenic bacterium which belongs to the genus Mycobacteria and is the major causative agent of human tuberculosis. First isolated by Robert Koch in 1882 (Kaufmann *et al*., 2005) and is the most successful bacteria which existed 5000 years ago and has infected almost a third of the population of the world. It consists of obligate aerobes, facultative intracellular pathogens usually infecting mononuclear phagocytes. They are also slow growing, hydrophobic acid-fast bacilli. For antibacterial activity the following drugs are isoniazid, rifampicin, and streptomycin and for inhibiting the development resistance isoniazid, rifampicin and ethambutol are used. Phenotypically, *M. tuberculosis* can be identified using analyses such as nitrate reductase, niacin production and resistance to thiophe-2-carboxylic acid hydrazide (TCH) and pyrazinamide (PZA) (Hoffner *et al*., 1993 and Niemann *et al*., 2002). Genotypically, using spacer digonucleotide typing, *M. tuberculosis* has been further classified into different phylogenetic lineages (Gagneux and Small 2007). These lineages are spread around the world and demonstrate differences in their distributions and concentrations to certain populations (Brudey *et al*., 2006).

2.1.2 *Mycobacterium bovis*

Mycobacterium bovis which leads to bovine TB is a zoonotic disease with potential public health and socio-economic significance as it can affect international trade in animals and animal products. The primary sources of infection for humans are consumption of unpasteurized milk and close association between humans and infected animals (Abubakar *et al*., 2011). They are Gram negative rods, non-spore forming, and non-motile, slightly curved, acid-fast staining aerobic slow growing organisms (Jenkins, 2008). They are thiophe-2-carboxylic acid hydrazide

(TCH) sensitive, almost universally resistant to PZA, niacin test negative, and nitrate test negative. Most *M. bovis* infections are extra-pulmonary TB cases with rare cases of pulmonary TB. The clinical signs of the disease in humans are indistinguishable from those occurring due to infection with *M. tuberculosis*. *M. bovis* can be distinguished from *M. tuberculosis* on the basis of epidemiology, phenotype and some genetic markers. *M. bovis* does not produce niacin, does not reduce nitrate and is sensitive to TCH but resistant to PZA (Niemann *et al.*, 2002).

2.1.3 *Mycobacterium africanum*

M. africanum is the term historically used to describe two lineages of the MTC that cause an important proportion of pulmonary tuberculosis (TB) in West and Central Africa 1, 2. *M. africanum* was first isolated in 1968 from a Senegalese patient suffering from pulmonary tuberculosis (Castets *et al*., 1968). *M. africanum* has also been found in several parts of Africa, representing about 60% of clinical isolates from patients also suffering from pulmonary tuberculosis (Haas *et al*., 1997; Viana-Niero *et al*., 2001). Two distinct subgroups of *M. africanum* have been described using biochemical characterisation and have been identified having an immediate position between *M. tuberculosis* and *M. bovis* (Kallenius *et al*., 1999 and Viana- Bissau *et al,* 2001). *M. africanum* subtype I geographically originating from West Africa and *M. africanum* subtype II geographically originating from East Africa , together they cause up to half of human tuberculosis. *M. africanum* subtype II has distinct phenotypes compared to *M. tuberculosis* such as lower progression in exposed contacts, despite a similar rate of transmission. Infection with *M. africanum* responds to regular TB treatment. *M.africanum* (subtype I) can be distinguished from the other members of the MTC by genomic deletion analysis of region of difference RD9 and spoligotyping where it lacks spacer 7-9 and spacer 39 (Viana- Niero *et al*., 2001; Brudey *et al*., 2009).

2.1.4 *Mycobacterium microti*

First described in humans in 1998 in immunocompromised patients *M. microti* was later on reported to cause disease in immunocompetent individuals (Frank *et al*., 2009, Niemann *et al*.,

2000; van Soolingen *et al*, 1998). Tuberculosis in the wild vole, or field mouse (*Microtus agrestis*), was discovered by Wells in 1937 (Greenwald *et al*, 2003; Greenwald *et al*, 2009), and this epizootic disease was found to be rather common among these animals in the United Kingdom, with a prevalence ranging from 9 to 31% (Greenwald *et al*., 2003). The causative agent was named *M. tuberculosis* subsp. *muris* (Animal Health Division, 1986), and later this species was designated *Mycobacterium microti* and classified as a member of the *M. tuberculosis* complex (Gormley *et al*., 2006).

M. microti differs from other *M. tuberculosis* complex strains in its S-shaped cell morphology, its slow growth in vitro, and its distinct host-specific pathogenicity for laboratory animals (Cousins., 2001; Cousins *et al*., 1998 and Greenwald *et al.*, 2003). It was found to be difficult to distinguish from *M. tuberculosis*, *M. africanum*, or *M. bovis* based on biochemical properties (Gormley *et al.*, 2006). In subsequent studies, *M. microti* was also detected in a limited number of other mammalian species, i.e., the bank vole (*Clethrionomys glareolus*) (Greenwald *et al.*, 2003), the wood mouse (*Apodemus sylvaticus*) (Greenwald *et al.*, 2003), the shrew (*Sorex araneus*) (Greenwald *et al.*, 2003), cats and pigs (Clifton-Hardley and Wilesmith, 1991; Coad *et al.*, 2007), and a zoo llama (*Lama vicugna molina*) (Corner *et al*., 2006).

A morphologically similar organism, the "dassie bacillus," was isolated in South Africa from the Cape hyrax, or dassie (*Procavia capensis*) (Aranaz *et al*., 1999, Cousins and Florrisson 2005 and Garnier *et al*., 2003), but this isolate differed from the vole bacillus in not being virulent for mice. Recently, various repetitive genetic elements have been identified in *M. tuberculosis* complex bacteria, and these have been used to differentiate clinical isolates of *M. tuberculosis*, *M. africanum*, and *M. bovis* (Cousins *et al*., 2003). *M. microti* strains display characteristic IS6110 banding patterns and spoligotypes distinct from other MTC strains (van Soolingen *et al*., 1998). *M. microti* gives a negative response to purified protein derivative (PPD) skin test and interferon-gamma (IFN- γ) release assays (Frank *et al*., 2009)

2.1.5 *Mycobacterium canetti*

Mycobacterium canetti was first described in 1969 by Georges Canetti from a French farmer and was further described in 1977 by van Soolingen when he reported a case of lymph node TB in a 2year old Somali child (Goguet de la *et al*., 1997; Kamerbeek *et al*., 1997. This species causes very rare cases of TB and is so far only found in humans and the natural reservoir is still unknown (Michel *et al*., 2010). *M. canetti* has smooth and glossy colony at the first stage of isolation and loses the smoothness or reverts after subsequent sub-culturing. Differentiation using biochemical tests is difficult and time consuming. The most rapid differentiation of *M. canetti* from other MTC isolates is by PCR restriction analysis of the hsp65 gene and by RD12 analysis (Aranaz *et al*., 2003).

2.1.6 *Mycobacterium caprae*

M. caprae was first described as the causative agent of TB in goat but it has been found in other animals too (Aranaz *et al*., 2003) such as cattle (Prodinger *et al*., 2002; Erler *et al*., 2004; Boniotti *et al*., 2009), pigs (Pavlik *et al*., 2002), red deer (Pavlik *et al*., 2002),and wild boars (Erler *et al*., 2004). Some studies have even reported isolates in humans (Kubica *et al*, 2003 and Prodinger *et al*., 2002) for a long time this species was considered as *M. bovis* and *M. bovis* BCG (Aranaz *et al*., 1999). Later on *M. caprae* has been differentiated from *M. bovis* because of the different sequence in the *gyr*B gene and susceptibility to PZA (Aranaz *et al*., 1999). For some time *M. caprae* was thought to be *M. bovis* because of the similar results that were being obtained from the biochemical tests done on *M. bovis* and *M. bovis* BCG. Later on *M. caprae* has been differentiated from *M. bovis* because of the different sequence in the gyrB gene and susceptibility to PZA. In contrast to the other MTC members *M. caprae* harbours most of the deletions such as RD9, RD7, RD8, RD10, RD5, RD6, RD12 and RD13 (Aranaz *et al*., 1999).

2.1.7 *Mycobacterium pinnipedii*

Mycobacterium pinnipedii was isolated from Australian fur seal in May 1990 and March 1991, inhabiting the Southern Australian coastal waters (Smith, 2003). Similar organisms have been subsequently recovered from the same species in Argentina, Uruguay, Great Britain and New Zealand and from a Brazilian tapir (Bigi *et al*., 2005). They are acid/alcohol-fast, non-spore-forming, non-motile bacilli with loose cord formation. Colonies are dysgonic, rough, flat and non-photochromogenic. Isolates are negative for nitrate reduction and generally negative for niacin accumulation; some isolates demonstrate low to medium reactions for niacin. Pathogenic in guinea pigs and rabbits; the apparent incidental infection of a human, bovine and tapir indicates that they may have a wide host range. The growth of *M. pinnipedii* can be enhanced by media that contains sodium pyruvate. Cases of transmission have been reported in humans who are in close contact with marine animals (Thompson *et al*., 1993). *M. pinnipedii* are closely related to *M. bovis* but they are different in their loose cord formation, lack of the MPB70 antigen which is detectable in *M. bovis* and they are susceptible to PZA (Cousins *et al*., 2003).

All isolates contain the sequences *IS6110, IS1081*, *MPB70* and *mtp40,* yet fail to produce detectable MPB70 antigen. The seal isolate spoligotypes form a cluster that is clearly different from those of all other members of the MTC. The isolates are susceptible to isoniazid, rifampicin, streptomycin, ethambutol and paraminosalicylic acid. As *M. bovis* they have deletions in RD3, RD7, RD 8, RD 9 and RD 10 regions but have intact at RD 4, RD 5 and RD 6 regions. *M. pinnipedii* can be distinguished from the species of the MTC by regional deletion analysis of PiD1 and PiD2 loci (Bigi *et al*., 2005).

2.2 Genotyping Characterization

Genotypic characterization which is also known as molecular characterization involves all the molecular techniques used to determine whether a strain causing the disease in one patient is the same as the one causing disease in another person. These molecular methods are applied in order to demonstrate whether transmission has occurred or not (Ghebremichael, 2010). These

techniques include DNA extraction, DNA amplification, and drug susceptibility testing and spoligotyping analysis.

2.2.1 DNA extraction

DNA extraction is a method used to collect DNA for subsequent molecular analysis. There are some steps involved in the extraction of DNA. These include breaking the cells open which is commonly known as cell lysis, this is done so as to expose the DNA (Schneegurt *et al*., 2003). This is followed by the removal of lipids and proteins and precipitating the DNA with an alcohol. There are different types of DNA extraction methods; some methods have been packaged into kits such as the ZR Fungal/Bacterial DNA Kits.

2.2.2 Drug susceptibility testing

Drug susceptibility of M. tuberculosis at molecular level can be detected by use of mutations and genes related to drug action. It is determined by the metabolic inhibition induced by the drug detection of genetic mutations using molecular techniques (Lin *et al*., 2000; Torres *et al*., 2000; El-Haji *et al*., 2001; Kim *et al*., 2001; Mokrousov *et al*., 2002; Lebrun *et al*., 2003 and van der Zanden *et al*., 2003). There are various molecular methods which have been reported that are used to detect gene mutations related to resistance, including PCR-based methods. However, not all resistance-related genes for the different antituberculosis drugs and their sites of mutation have been found, except for *rpo*B gene mutations which confer resistance to RMP. These molecular methods usually require primary amplification and thus when they are used on a routine basis for long periods of time; they are not free from false results due to contamination of the DNA or PCR amplicons. The GenoType® MTBDR*plus* kit (Hain Life science, Nehren, Germany) is commonly used. It is a molecular genetic assay for identification of resistance to rifampicin and/or isoniazid of the *Mycobacterium tuberculosis* complex, it involves the amplification and hybridization of the specimens used in the assay (Barnard *et al*, 2007).

2.2.3 Spoligotyping

Spoligotyping is a molecular typing method for MTC and it is also a PCR based spacer oligonucleotide typing. This method was proposed as an alternative to hybridization based fingerprinting methods for diagnosis and epidemiology of tuberculosis (Kamerbeek *et al*., 1997). It detects variability in the direct repeat region in the DNA of the MTC, this region is present in all MTC strains in a unique locus which contains well conserved 36bp repeats interspersed with non-repetitive short spacer sequences of 34-43 bp. Strains vary in the number of DRs and in the presence or absence of particular spacers and *M. bovis* characteristically lacks spacers 39 to 43 in the spoligotype system (Kamerbeek *et al*., 1997). Spoligotyping is a rapid and reliable supplementary tool for molecular epidemiological analysis of TB (Kamerbeek *et al*., 1997).

To visualize the DNA polymorphism in the direct repeat region of the MTC strains, the spacer sequences are amplified by PCR using biotin labeled primers. The PCR products are then denatured and hybridized perpendicular to 43 oligonucleotides which are covalently bonded to a spoligo membrane. It can be applied directly to detect and type MTC bacteria in clinical samples. It is a good technique to differentiate and type the species of MTC and at the same time identifies different phylogenetic lineages of strains of MTC. It can be used to determine the degree of clustering and relatedness since strains bearing the same spoligotype pattern are assumed to be a set of isolates derived from a single ancestral cell (Smith *et al*., 2003).

The degree of differentiation achieved by spoligotyping is higher than that of 1S*6110* RFLP for strains with low 1*S6110* copy numbers such as *M. bovis*, but for strains with high 1S*6110* copy numbers like most *M. tuberculosis* strains, RFLP is a more discriminative test (De La Salmoniere *et al*., 1997). Spoligotypes and the numerical output of spoligotyping are not exclusive to one *Mycobacterium tuberculosis* complex member nor are they restricted as strains can waver from the expected minimal consensus spoligo pattern for their *Mycobacterium tuberculosis* complex subspecies (Huard *et al*., 2006). The generation of a DNA fingerprint by RFLP (usually about 3 months) has also been a major obstacle to using RFLP information especially in predicting links between patients. This drawback can be overcome by using spoligotyping, which can be

performed within one or two days (Kamerbeek *et al*., 1997). Although this method provides digital typing data, it is only measuring variability in a single locus and does not generally provide sufficient discrimination for outbreak investigation (Kremer *et al*., 1999). The use of spoligotyping alone overestimates the number of epidemiological links and thus has to be used in association with another rapid fingerprinting technique (De la Salmoniere *et al*., 1997).It doesn't need any sophisticated software which doesn't need large amounts of extracted and purified DNA can be done on non-viable bacteria. The results from this assay technique can be converted to octal values compared to the international database SpolDB3 and assigned phylogenetic lineages. It also has calculates probabilities using the Multivariate Bernoulli Mixture model.

CHAPTER THREE

MATERIALS AND METHODS

3.1.1 METHODS

3.1.1 Study site

We collected milk samples on three different dairy farms in the Eastern Cape. The farms are located in Alice, Middledrift and Komga named University of Fort Hare Dairy trust, Middledrift Community dairy trust and Komga dairy farm respectively. There are at most 800 cows in these farms with a big land for the cows to feed. The land is divided into several portions with fences where they have divided the cows. The cattle share the water and feeding sources in the farms. The ethical clearances are not needed as the farms used are commercial farms.

3.1.2 Milk sample collection

A total of one hundred and twenty (120) milk samples were collected from three different farms selected around the Eastern Cape from three different breeds of cattle. Farm 1 and Farm 2 are 20 km apart and farm 3 approximately 200 km from the other two farms. These milk samples were collected into 40 ml sterile universal containers and then placed in cool boxes with ice bags. If not used immediately they were stored at -20 C.

3.1.3 Decontamination of milk samples

The specimens were decontaminated using a modified method of Petroff (Al- Saqur et al., 2009). Briefly 10 ml of milk was pippeted into 10 ml decontamination solution (7% NaCl in 4 % NaOH) in a centrifuge tube and mixed by shaking before it was centrifuged at 10 000 rpm for 10 minutes. The supernatant was discarded and the pellet kept for further analysis.

3.1.4 DNA extraction

DNA was isolated using the ZR Fungal/Bacterial DNA MiniPrepTM kit. Briefly, a 100 mg of the decontaminated pellet was suspended in 200 µl of sterile distilled water. The mixture was then pipetted into a ZR BashingBead^{TM} lysis tube into which 750 µl of lysing solution was added. The mixture was then vortexed for 5 min. The ZR bashing tube was secured in a 2 ml tube holder and centrifuged at 10 000 xg for 5 min. Four hundred microliters of the collected solution was transferred into a Zymo-Spin^{TM} IV spin filter secured in a collection tube and then centrifuged at 7000 rpm for 1 min. The DNA binding buffer (1200 µl) was then mixed with the filtrate to bind DNA. Approximately 800 µl of the mixture was transferred to a Zymo-spin IIC column and centrifuged for 1 min at 10 000 xg. The flow through was discarded and 200 µl of the pre-wash buffer was added to the Zymo-Spin IIC column in a new collection tube and centrifuged at 10 000 xg for 1 min. Approximately 500µl of the wash buffer was added to the Zymo-spin column IIC in a new collection tube and centrifuged for 1 min at 10 000 xg. The Zymo-spin IIC column was then transferred to a 1.5 µl microcentrifuge tube, then 100 µl of DNA elusion buffer was added (to elute the bound DNA) and centrifuged at 10 000 xg for 30 sec.

3.1.5 Amplification/ Detection

Amplification of bacterial DNA was done using the Seeplex® MTB Nested ACE detection assay (Seegene Inc, Korea) according to the manufacturer's instructions using a MyCycler thermal cycler (Bio Rad, Cape Town, South Africa). The amplification protocol involved the first PCR (1 cycle at 94 °C for 15 min; 40 cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s; 1 cycle at 72 °C for 5 min) and a nested PCR (1cycle at 94 °C for 15 min; 30 cycles at 94 °C for 30 s, 62 °C for 30 s, 72 °C for 30 s; 1 cycle at 72 °C for 5 min). The amplicons were run on 2% agarose gel, at 110 V for 90 min. The gel was thereafter visualized under Alliance 4.7 transilluminator (UVITEC Limited, Cambridge, UK).

3.1.6 Drug susceptibility test

GenoType MTBDRplus

GenoType® MTBDR*plus* kit (Hain Life science, Nehren, Germany) was used according to the manufactures instructions. Briefly, 5 µl of DNA was amplified with hot-start *Taq* DNA polymerase (Qiagen, Pretoria, South Africa) using biotinylated primers provided in the kit. Amplification was performed using thermal cycler MyCycler^{TM} (Bio-Rad, Cape Town, South Africa) following the protocol that consisted of 1 cycle at 95 °C for 15 min (*Taq* activation cycle), 10 cycles of denaturation at 95 °C for 30 s and primer annealing at 58 °C for 2 min, 40 cycles of denaturation at 95 °C for 25 s, primer annealing at 53 °C for 40 s and extension at 70 °C for 40 s, followed by a 1 cycle of final extension at 70 °C for 8 min. Subsequent hybridization steps were performed using hybridization trays (Hain Lifescience, Germany) according to the manufacturer's instructions. Eight *rpoB* wild-type probes (WT1-WT8) and 4 mutant probes (MUT1, MUT2A, MUT2B and MUT3) were used for detecting RIF resistance. One *katG* wild-type (*katG* WT) and 2 mutant probes (MUT1 and MUT2); plus 2 *inhA* wild-type (WT1 and WT2) and 4 mutant probes (MUT1, MUT2, MUT3A and MUT3B) were used for detecting INH resistance. When all WT probes stained positive and no mutation band formed, the result were interpreted as susceptible to the respective antibiotic. The absence of a band for at least one of the WT probes indicated resistance to the respective antibiotic, according to the manufacturer's instructions.

3.1.7 Spoligotyping

3.1.6.1. In Vitro Amplification of Spacer DNA by PCR

DNA samples were sent to the University of Stellenbosch for Spoligotyping and the method was done according to Kamerbeek et al., (1997). The chromosomal DNA of *M. tuberculosis* strain H_{37}Rv and *M. bovis* BCG were included as positive controls and water was used as a negative control. The reaction mixture consisted of 4 µl of primers DRa and DRb, 4 µl of dNTP mixture, 5 µl of 10x Super T buffer, 0.1µl of Super T polymerase, nuclease free water and template DNA (20ng). A drop of mineral oil was added to the tubes to prevent evaporation of the PCR-mix

during amplification. The tubes were then placed in a PCR-apparatus for amplification and the following temperature cycling was performed: 1x cycle of 96^{o}C for 3 min, followed by 20 cycles of 96^{o}C for 1 min; 55^{o}C for 1 min and 72^{o}C for 30 sec; then lastly 1x cycle of 72^{o}C for 5 min.

3.1.6.2. Hybridization with PCR Product and Detection

All buffers were pre-warmed before use. The following buffers were prepared from concentrated stocks using de-mineralized water for dilution: 250 ml of 2x SSPE/0.1% SDS at 60^{o}C; 250ml of 2x SSPE/0.5% SDS at 60^{o}C; 250 ml of 2x SSPE/0.5% SDS at 42^{o}C; 250 ml of 2x SSPE at room temperature (all these quantities are for one membrane). Approximately 20 µl of the PCR products were added to 150 µl of 2x SSPE/ 0.1% SDS. The diluted PCR product was then heat-denatured for 10 min at 99^{o}C and was then immediately cooled on ice. The membrane was washed for 5min at 60^{o}C in 250 ml of 2x SSPE/ 0.1% SDS. The membrane and support cushion was placed into the mini-blotter in such a way that the slots were perpendicular to the line pattern of the applied oligonucleotides. The residual fluid was then removed from the slots of the mini-blotter by aspiration. The slots were filled with diluted PCR product and hybridization was performed at 60^{o}C for 60 min on a horizontal surface. The samples were removed from the mini-blotter by aspiration and the membrane was taken using forceps. The membrane was washed twice in 250 ml of 2x SSPE/0.5% SDS for 10 min at 60^{o}C. The membrane was then placed in a rolling bottle and was allowed to cool down to prevent inactivation of the peroxidase in the next step. 2.5 µl of streptavidin-peroxidase conjugate (500 U/ml) were added to 10 ml of 2x SSPE/0.5% SDS and the membrane was incubated in this solution for 45 to 60 min at 42^{o}C. The membrane was then rinsed with 250 ml of 2x SSPE for 5 min at room temperature. For chemiluminiscent detection of the hybridizing DNA, the DNA was incubated for 1min in 20 ml Enhanced Chemiluminescence (ECL) detection liquid. The membrane was then covered with a transparent plastic sheet or Saran-wrap and a light sensitive film was exposed to the membrane for 20 min.

3.1.6.3. Statistical Analyses and Interpretation of Results

The prevalence of MTC DNA amongst cattle breeds, drug susceptibility and the frequency of mutations in the *rpo*B, *kat*G and *inh*A genes were respectively represent in percentages of the samples and the results from the spoligotype membrane autoradiograph were analyzed by recording the presence or absence of signals at the sites of DNA/DNA hybridizations. The presence of spacers was represented on film as black squares after incubation with streptavidin-peroxidase and ECL detection. The spacers were converted into octal values replacing a present spacer with 1 and the absent with 0. Results were entered into Excel spreadsheets and compared with the published spoligotyping database SpolDB3 and TB-Lineage software (Brudey *et al*., 2006) (http://tbinsight.cs.rpi.edu/run_spotclust.html) was used for TB-Lineage.

SPOTCLUST method results in simple binary pattern for each TB patient. It uses mixture models to identify families within MTC bacteria based on their spoligotyping patterns. It uses SpolDB3 prototype, Bernoulli mixture model for calculation of probability and also TB-Lineage models.

CHAPTER 4

RESULTS

4.1 Results

DNA was isolated from 25/120 (20.8 %) samples collected. The MTC DNA was detected in all the three different breeds (Friesland, Jersey and the Crosses) of cattle which were used for this study from three different dairy farms. Most positive MTC cattle were of the friesland breed with 15 positives and the least was the crosses with 4 positives (Table 4.1).

Table 4.1: prevalence of MTC DNA amongst different cattle breeds

Total herd	Breed	Sex	Number positive for MTB
40	Friesland	Female	15
40	Jersey	Female	6
40	Crosses	Female	4

The Seeplex® MTB Nested ACE detection assay (Seegene Inc., Seoul, Korea) used was able to show detection of 12/13 (92.3 %) DNA band with the internal band marker (520 bp) which marks the presence of the MTC in the DNA isolates (Figure 4.1).

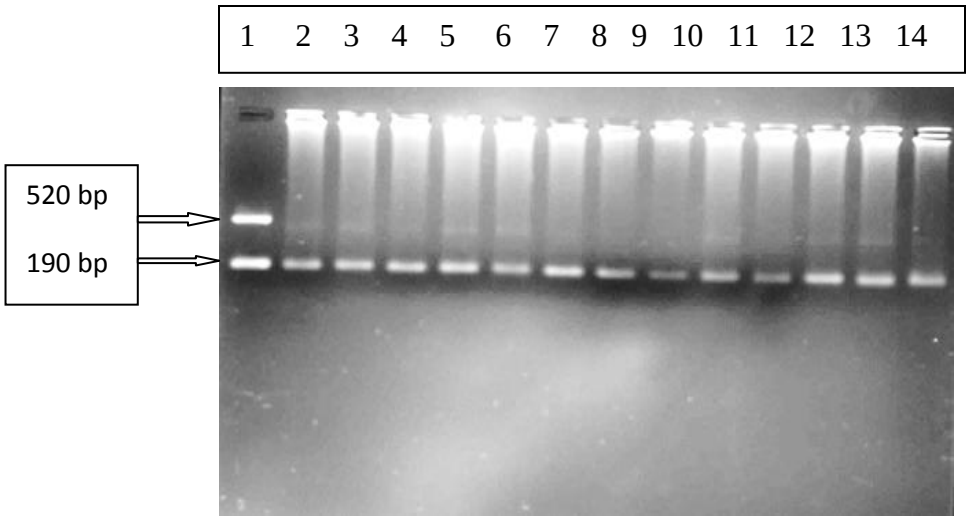

Figure 4.1 PCR results of the MTB Nested ACE detection assay, lane 1-internal control band (520 bp) and MTB band (190 bp). Lane 1 DNA marker, lane 2- 14 are milk samples.

During the experiment initially used the marker, negative control and positive control but those samples were negative to MTC DNA. After some time I then used only the marker and because of the more samples I had with one a 14-well gel apparatus.

Figure 4.2 shows the remainder of the amplification results from raw milk samples. The results display 100 % (13/13) positive results.

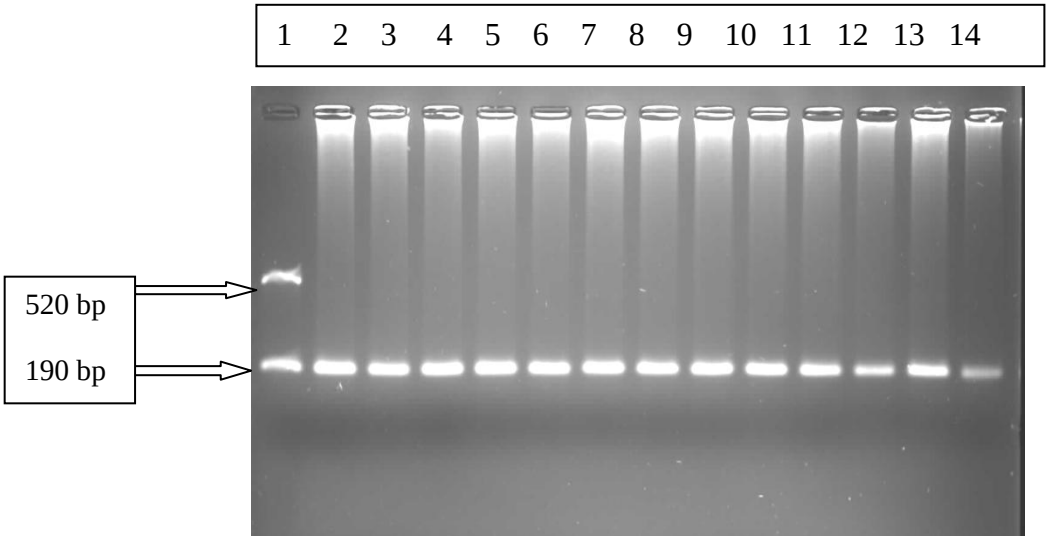

Figure 4.2 PCR results of the MTB Nested ACE detection assay, an internal control band (520 bp) and MTB band (190 bp). Lane 1 DNA marker, lane 2 - 14 milk samples. In the first lane the DNA marker was loaded, which shows a typical positive sample. Lane 2-14 shows the milk samples which have all been detected as positive as they display both the internal band and the MTB band.

GenoType MTBDR*plus* assay results which show that of the 100 % positive samples, 57.9 % of those samples were sensitive to both INH and RMP, whereas a total of 42.1 % were resistant to both INH and RMP. The samples that were detected as MDR were from all the four breeds used in the study in (Table 4.2).

Table 4.2 drug susceptibility assay

Antibiotic susceptibility pattern	Result	Genotype MTBDRplus assay Number of samples
RMP	Resistant	8 (42.1 %)
INH	Resistant	8 (42.1 %)
RMP&INH	MDR	8 (42.1 %)
RMP&INH	Sensitive	11 (57.9 %)

RMP=Rifampicin, INH=Isoniazid.

Five mutations were observed in this study, S531L mutation of the *rpo*B gene with (6/25) 24 % conferring resistance to RMP. Both mutations from the *kat*G gene were revealed with S315T1 mutation 24 % and S315T2 24 %. In the *inh*A gene three mutations were C15T mutation (6/25) with 24 %, T8C mutation (1/25) 0.4 % and the T8A (1/25) 24 % found with conferring resistance to INH shown in table 4.3 below.

Table 4.3 Frequency of mutations in the *rpoB*, *katG* and *inhA* gene

Mutation probe	Mutations analyzed	Number of isolates
***rpo*B**		
MUT1	D516V	0 (0%)
MUT2A	H526Y	0 (0%)
MUT2B	H526D	0 (0%)
MUT3	S531L	6 (24 %)
katG		
MUT1	S315T1	1 (0.4 %)
MUT2	S351T2	1 (0.4 %)
inhA		
MUT1	C15T	6 (24 %)
MUT2	A16G	0 (0 %)
MUT3A	T8C	2 (8 %)
MUT3B	T8A	0 (0 %)

The clonal structure based on Spoligotyping shows different pattern with no clear similarity among the investigated DNA isolates. The spoligotypes seem to be most if not all resembling Family33 strains; as described, a Family33 strain had spacers 33-34 absent (Ramachandran *et al*., 2011) and a few resembled *M. africanum* strain. Out of the 25 analyzed two of the samples had no amplification (Sample number 22 and 24). Fig. 4.3 and Table 4.4).

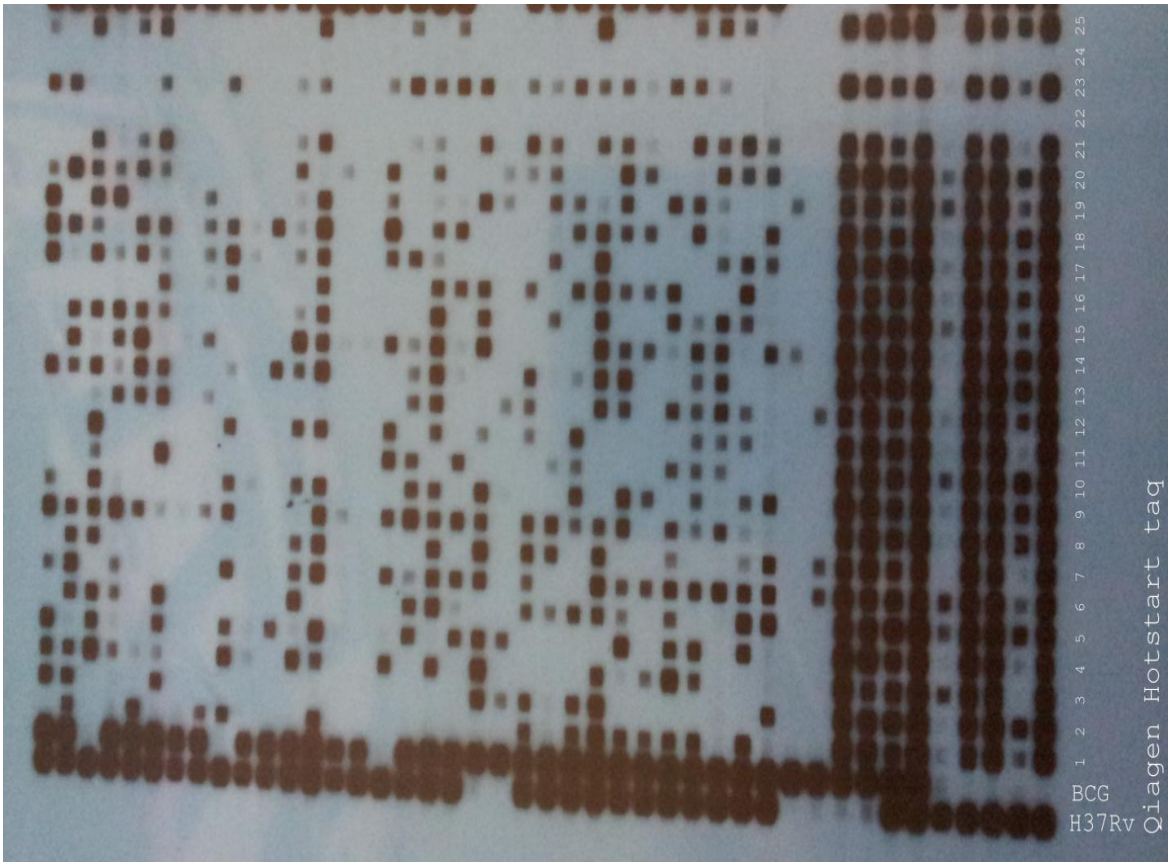

Figure 4.3 spoligotype patterns hybridization blot with the H37Rv and BCG as controls. H_{37}RV=Control, BCG=Control, 1-25, DNA isolated from milk samples.

Table 4.4 strain number, the octal values, spoligotype family, the lineage and the probability, for each spoligotype genotype family

Strain number	Octal value	Spoligotype family	Probability
M2011	623020027767661	Family33	0.999999945704819
M8128	230400277207571	Family33	0.999972740167026
M8313	740462412743761	Family33	0.999034210342194
M2157	511242701307661	Family33	0.999666530731643
M8244	350206172147771	Family33	0.999986173822038
M2503	502145647767771	Family33	0.999999997946746
M2621	300141264027761	Family33	0.999997875710678
M2588	763047676703671	Family33	0.999999990298405
M0946	501143213263671	Family33	0.994294925652542
M2501	110006430703771	Family33	0.999502497002811
M8405	101145250343661	Family33	0.999916390546073
M6541	070014615707671	*M. africanum*	0.99999881530445
M2524	730343046303671	*M. africanum*	0.999804194742262
M2178	262147103553771	Family33	0.999999999961999
M2106	370141263143771	Family33	0.999918638133135
M8155	013041657443671	Family33	0.999646095132054
M2592	733245417223771	Family33	0.999999945977255
M8171	542044304713771	Family33	0.999999999807257
M2486	770125163163771	Family33	0.999745058492467
M2613	130120655463661	*M. africanum*	0.998898672388814
M8155	604463737743671	*M. africanum*	0.999601374282115
M2625	530042402163671	*M. africanum*	0.999999932307603

CHAPTER FIVE

DISCUSSION

5.1 Discussion

Bovine tuberculosis is a chronic disease that is especially more prevalent in African countries and its existence has been reported in several livestock production settings across the country (Humblet *et al*., 2009). Its existence has raised concern for human and animal health as a result control policies have to be made which requires the availability of information based on scientific knowledge of the disease. As yet there has not been any sufficient BTB control policy; this resulted in a need for investigation of molecular characteristics of MTC strains involved in causing disease in cattle (Corner, 2006). This study investigated molecular characteristics of the MTC, which include the isolation, detection of MTC from milk samples, drug susceptibility profiles and identification using spoligotyping.

Although the ZR Fungal/Bacterial DNA MiniPrep™ is a very efficient in DNA isolation as it uses ultra-high density BashingBeads™ which are chemically inert, there were milk samples which were very fatty and the Kit could not isolate a clear DNA. This could cause an underestimation of the spread of BTB in the three farms investigated. This kit was able to isolated 25/120 (20.8 %) of DNA from 120 samples. We found out there was 15/40 (37.5 %) MTC DNA for the Friesland breed, 6/40 (15 %) MTC DNA for the Jersey breed and 4/40 (10 %) MTC DNA for the Crosses. Some studies have reported that the type of breed is one of the risk factors associated with BTB infection in cattle (Humblet *et al*., 2009). As opposed to other studies (Humblet *et al*., 2009), it is evident in our study that MTC can be detected from all three different breeds of cattle. Moreover the cattle were also from three different dairy farms.

The Seeplex MTB Nested assay used in this study was able to detect 20.8 % MTC DNA from milk samples. The main strength of the Seeplex® MTB Nested ACE detection assay is in the use

of multi-target PCR (IS*6110* and *mpb*64) for the specific detection of MTC only, which simultaneously amplifies IS*6110* and *MPB*64 DNA based on nested PCR. It therefore eliminates false positive and false negative results. Another factor which prevents false positive results is choosing of the buffer for gel electrophoresis. There is however one disadvantage of the assay, that it does not differentiate amongst members of the MTC. DNA isolated from the milk could represent any of the MTC members. However the organism known to cause TB in cattle is *M. bovis*, thus the DNA could represent *M. bovis* as the cattle in dairy farms live in close proximity, and it therefore raises concern on animal. Ameni and colleagues have reported the isolation and detection of the *M. tuberculosis* in cattle (Ameni and Erkihum., 2007; Ameni *et al*., 2011). This suggests that these cattle will continue infecting other cattle and workers' working with the cattle as the source of infection is not known.

One of the main significant risk factors associated with BTB identified in different studies in both developed and developing countries, is the age of animals (Cleaveland *et al*., 2007; Cook *et al*., 2001; Inangolet *et al*., 2008). The other factors includes the duration of exposure that increases with age; older animals are more likely to have been exposed than younger ones, as shown by several cross-sectional studies carried out in Tanzania, Zambia and Chad (Cook *et al*., 1996; Kazwala *et al*., 2001; Cleaveland *et al*., 2007; Inangolet *et al*., 2008 and Munyeme *et al*., 2009). In this study we did not report on the age of the cows from which samples were collected. However, the farms where the study was carried out had cows that are older than 3 years (personal communication, 2012). Different countries have different risk factors which vary according to countries; in Tanzania risk factors include herd size and the history of BTB in the herd (Cleaveland *et al*., 2007), in other countries different types of drinking water sources, areas of production, communal grazing, management systems and animal breed are the main risk factors for BTB infection (Humblet *et al*., 2009). Specific risk factors in South Africa remain unclear but the spillover from wildlife especially the African buffalo in the country's game parks has been identified as the main risk factor for infection in communal cattle herds (Michel *et al*., 2005). Observations made in this study include sharing drinking water sources and feeding sources which could be the possible risk factors as there were no statistics done. Another

possible risk factor is the type of breed found in this study as there was 12.5 % positive MTC DNA for the Frieslan breed which agrees with studies done by Humblet *et al*., 2009.

The identification and molecular characterization of different MTC species isolated from cattle is important for determining the threat of transmission of tuberculosis between humans and animals. Despite the low prevalence of mycobacterial species in cattle with 20.8 % from milk samples found in this study, their presence is still a public health threat and should encourage the increase in public health measures such as the pasteurization of milk, cooking of meat, and generally, the control of tuberculosis in domestic animals. The isolation of *M.bovis* from slaughter cattle in Uganda confirmed the presence of bovine tuberculosis and makes the risk of transmission of tuberculosis from animals to humans real (Asiimwe, 2008). This is true because of the food hygiene practices of people in our communities such as consumption of unprocessed dairy products, fresh blood and raw meat which are commonly consumed during traditional ceremonies. Silaigwana and colleagues (2012) showed that MTC is present in raw milk from the Eastern Cape, South Africa, although they didn't do any identification tests they suggested their DNA was of *M. bovis* or *M. tuberculosis* (Silaigwana *et al*., 2012).

Cattle with BTB may not show any clinical signs even during the advanced stages of the disease (Angela *et al*., 2006). This was also proven to be true in another study as all the cows used were in a good body condition but the results of the PCR assay showed that their milk was in fact infected with MTC (Angela *et al*., 2006). Moreover, the clinical signs may not be noticed by the cattle owners because of ignorance or lack of awareness (Strain *et al*., 2011). Furthermore, cattle with BTB shed bacteria via urine and other excreta, hence other cows grazing on the contaminated grass may ingest or inhale MTC (Angela *et al*., 2006). Additionally, calves are fed using milk from adult cows; therefore there is a possibility of infection through consumption of milk infected with MTC (Silaigwana *et al*., 2012). Therefore, the burden of BTB in cattle could be decreased by educating people about the disease, transmission of the disease, symptoms associated with BTB, how they can protect themselves from being infected and the necessary treatment of the disease if one is infected.

The emergence of multidrug-resistant tuberculosis which is defined as the resistance to both INH and RMP has raised a great public health concern. It also threatens the global antiTB control programs (WHO, 2012). The WHO has recommended the use of the GenoType MTBDR*plus* assay for the detection of MDR-TB (WHO, 2007). The weakness of the Genotype® MTBDR*plus* assay is that it only detected mutations in the *rpoB, kat*G and *inhA* genes; therefore, resistance to INH and RMP caused by mutations in other genes could not be detected in this study.

The common occurrence of the major RMP resistance in the S531L mutation 6/25 (24%) is similar with that found by other authors who reported 60 %, 63.2 % and 73.6 % which was also the most frequent mutation in their studies (Silaigwana *et al*, 2012; Huang *et al*, 2009 and Doris *et al*, 2007). Other mutations conferring resistance to RMP were found in the S315T mutation with 1/25 (0.4 %) which is found in the *kat*G gene, another study revealed 1.6 % of this mutation while a study from the Eastern Cape reported that there were no mutations found in the *katG* gene (Hillemann *et al*., 2007; Silagwaina *et al*., 2012).

The frequency of the INH resistant isolates in C15T mutation of the *inhA* gene in our study 6/25 (24 %) was slightly lower than that found in China (30.3 %) and very high when compared to the study conducted in Cape Town, South Africa (27 %) (Huang *et al*, 2009, Barnard *et al*, 2008). Additional mutations conferring resistance for INH were found to be T8C with 8 % and T8A with 0 %. These two last mutations are the least common mutations revealed in this study and these findings are very different from those obtained in another study with 60 % and 80 % respectively in the Eastern Cape (Silaigwana *et al*., 2012).

Overall there were 8/19 (42.1 %) of DNA isolates with mutations showing resistance to the both RMP and INH with 68 % of the DNA isolates susceptible to both RMP and INH. Our results are comparable with those obtained in a similar study done in Italy where 63.6 % INH and/or RMP resistant strains of *M. bovis* isolates in cattle (Sechi *et al*., 2001). All the 42.1 % resistant strains were characterized as MDR-TB strains. MDR-TB requires extensive treatment which should be

monitored by a trained nurse and is highly infectious (WHO, 2012). Although there is no TB treatment for cows, humans are going to suffer if transmission is not stopped.

Genotyping of MTC strains is very important if identification of the strain and transmission route is required. Spotclust represents a novel approach to advance global studies of MTC genotyping data. Spoligotyping is one of the techniques of genotyping that allows for the differentiation of various members of the MTC as each has its own characteristic spoligotype (van Soolingen *et al*., 1997).

What makes SPOTCLUST so effective is the fact that it uses mixture models to identify families within MTC bacteria based on their spoligotyping patterns. It incorporates biological information on spoligotype evolution without attempting to derive the full phylogeny of MTC (Vitol *et al*., 2006). Together with the SpolDB3-based model which is the old version and appropriate for spoligotypes will give a clear picture of the relatedness of the organisms. The belief is that spoligotypes develop by the deletion of spacers, but it is not clear in what order and how many spacers can be lost simultaneously; therefore, the distance can be probabilistically assessed as a probability of a "child" spoligotype having been evolved from a "parent" spoligotype by mostly losing but not gaining spacers (Vitol *et al*., 2006). We acknowledge that there are recently updated models with more spoligotype patterns which might have given better analysis. However, SPOTCLUST still remains a highly informative method which can be used for identification of strain family-specific signatures.

There are as far as nine major families that can be identified by the SPOCLUST which is based on the SpolDB3 model, these families are divided into 36 subfamilies (Vitol *et al*., 2006). The nine major spoligotyping-based families namely; *Mycobacterium africanum*, *M.bovis*, East African-Indian (EAI) Beijing, Haarlem, Latin American and Mediterranean (LAM), Central and Middle Eastern Asian (CAS), the European family X and a default family T (Filliol *et al*., 2002). Most of the spoligotyping patterns (78.3%) in our study resembled the signature of family33. In

the family33 it is only spacers 33 and 34 that are absent (Magana *et al*, 2011). There is also a recently described family MANU which is of Indian origin that also belongs to this family (Magana *et al*., 2011) althoughit was identified using a spoldb4 database for analysis of the spoligotypes. Magana and colleagues found similarities in five clades in their study namely Beijing, T1, EAI5, T2 and T3 (Magana *et al*., 2011) when compared with the spoldb4. Magana and colleagues reported that Family33 appears to gather spoligotypes with most spacers present and that could not find any other parent than were assumed (Magana *et al*., 2011).This is in agreement with our findings as most spoligotypes resembled this family although the spoligotypes are not similar, the spacers absent from the patterns are not in the same position e.g. in M2011 spacers 1, 3-5, 9-11 are absent; in M8128 spacers 3-5, 7-20 are absent and in M8313 spacers 4, 6-8 are absent but even though they do not have the same pattern they are all characterized as Family33 strains.

The SPOTCLUST didn't tell much about family33 as this is an older version for analyzing spoligotypes as recent versions contain more data and more strains for comparison worldwide. However, the lineage of the detected DNA was deduced. The family33 strains were of Indo-Oceanic origin when run in the TB-Lineage; this lineage includes a group of strains that have been referred to as "ancestral" due to the fact that they conserve the TbD1 genomic region which is deleted in the modern strains of *M. tuberculosis* (Brosch *et al*., 2002).

The other five 5/22 (22.7 %) DNA isolates displayed a spoligotyping pattern resembling that of *M. africanum*. The *M. africanum* is divided into two subtypes namely subtype I (West Africa 1) and subtype II (West Africa 2); the *M. africanum* strains isolated from this study belong to West African 2 origin. In the TB-Lineage there are mainly seven major MTC groups, these groups are further divided into two sub-groups the Modern and the Ancestral lineages. In the ancestral lineage there is Indo-oceanic, West Africa 1, West Africa 2 and *M.bovis*; in the modern lineage there is the East-Asian (Beijing), Euro-American and East- African Indian (Shabbeer *et al*., 2011; Aminian *et al*., 2009). From our study there is the Family33 strain and *M.africanum*, both these strain fall under the ancestral lineage.

To the best of our knowledge, this is the second research done which reports MDR isolated from cattle, and the first which reports Family33 and *M. africanum* strains isolated from three different dairy farms in the Eastern Cape, South Africa. Since these cattle are from the rural areas where people have no knowledge of bovine tuberculosis in unpasteurized milk and continue to consume this milk, this poses a great risk to their health as the cattle not only have TB but also harbor MDR-TB and the Ancestral strains with *M. africanum* which has human as a natural host.

M. africanum was first isolated from humans in 1968 from a patient who suffered from pulmonary tuberculosis (Castets *et al*., 1998). In the Western Cape, South Africa Demers and colleagues reported that *M. africanum* was not the major cause of human tuberculosis (Demers *et al*., 2010). As this is the first study in the Eastern Cape to report *M.africanum* in cattle, more research is required to determine the major cause of TB in the Eastern Cape.

The outcomes of our study showed that molecular methods for detection of MTC can be applied directly on milk samples without the need for culturing, which is time consuming. In the study we also found out that the Family33 strain is highly prevalent amongst cattle from the selected farms in the Eastern Cape. Therefore there is a need for effective control measures of BTB as it poses a significant risk to public health due to its zoonotic potential and awareness of the MDR-TB prevalent in cattle in the Eastern Cape and for dairy farmers to take the necessary precautions to protect those working in close proximity with the cattle. The method of spoligotyping will enable the rapid identification of emerging strain families and epidemiology of bovine tuberculosis.

CHAPTER 6

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