Evaluation of incidence of *Mycobacterium tuberculosis* complex associated with soil, hayfeed and water in three Agricultural facilities in Amathole District Municipality in the Eastern Cape Province, South Africa.

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SUMMARY

Mycobacterium bovis and other species of *Mycobacterium tuberculosis* complex (MTBC) can result to a zoonotic infection known as Bovine tuberculosis (bTB). MTBC has members that may contaminate an extensive range of hosts, including wildlife. Diverse wild species are known to cause disease in domestic livestock and are acknowledged as TB reservoirs. It has been a main study worldwide to deliberate on bTB risk factors as a result some studies focused on particular parts of risk factors such as wildlife and herd management. The objectives of this study were to design questionnaires from commercial farms and smallholding farms; isolate and identify MTBC from collected samples using culture and PCR assays recovered from Fort Hare, Middledrift and Seven star dairy farms; and assessing genotypic drug resistance through detection of mutations conferring resistance to INH and RMP associated with first line treatment for MTBC infection. Questionnaires were administered to thirty (30) smallholding farm owners in the two villages (kwaMasele and Qungqwala) and three (3) three commercial farms (Fort Hare dairy farm, Middledrift dairy farm and Seven star dairy farm). Detection of *M. tuberculosis* complex was achieved by Polymerase Chain Reaction using primers for *IS6110*; whereas a genotypic drug resistance mutation was detected using Genotype MTBDR*plus* assays.

Nine percent (9%) of respondents had more than 40 cows in their herd, while 60% reported between 10 and 20 cows in their herd. Relationship between farm size and vaccination for TB differed from forty one percent (41%) being the highest to the least five percent (5%). The highest number of respondents who knew about relationship between TB cases and cattle location was ninety one percent (91%). Approximately fifty one percent (51%) of respondents had knowledge about wild life access to the farms. Relationship between import of cattle and

farm size ranged from nine percent (9%) to thirty five percent (35%). Cattle sickness in relation to farm size differed from forty three (43%) being the highest to the least three percent (3%); while thirty three percent (33%) of respondents had knowledge about health management. Respondents with knowledge about the occurrence of TB infections in farms were forty eight percent (48%). The frequency of DNA isolation from samples ranged from the highest forty five percent (45%) from water to the least twenty two percent (22%) from soil. Fort Hare dairy farm had the highest number of positive samples forty four percent (44%) from water samples; whereas Middledrift dairy farm had the lowest positive from water, seventeen percent (17%). Twelve (22%) out of 55 isolates showed resistance to INH and RMP that is, multi-drug resistance (MDR) and nine percent (9%) were sensitive to either INH or RMP. The mutations at *rpoB* gene differed from 58% being the highest to the least (23%). Fifty seven percent (57%) of samples showed a S315T1 mutation while only 14% possessed a S531L in the *katG* gene. The highest *inh*A mutations were detected in T8A (80%) eighty percent and the least was observed in A16G (17%). The results of this study reveals that risk factors for bTB in cattle and dairy farm workers is a serious issue abound in the Eastern Cape of South Africa; with the possibility of widespread dissemination of multidrug resistant determinants in MTBC from the environment.

DECLARATION

I , Athini Ntloko, hereby declare that the work contained in this project is my own original work and that I have not previously, in it's entirely or in part, submitted it at any other University for a degree.

……………………………………

DEDICATION

This research work is firstly dedicated to the **Almighty God** to whom I owe my life and who has given me the **grace** to be what I am today. To him be all the glory, honour and adoration for His mercy and favour towards me. I also dedicate to my lovely parents, dearest son and all my siblings.

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LIST OF FIGURES

Figure 3.1: Respondent's knowledge about size of cattle in relation to herd size………… 62

Figure 3.2: Respondent's knowledge in relationship between farm size and vaccination…...63

Figure 3.3: Respondent's knowledge about TB cases……………. ……………………… 64

Figure 3.4: Respondent's knowledge about wild life in relation to herd size……………... 65

Figure 3.5: Respondent's knowledge about import of cattle……………………..…….. 66

Figure 3.6: Respondent's knowledge about cattle sickness in relation to farm size………. 66

Figure 3.7: Respondent's knowledge about health management in relation to farm size…. 68

Figure 3.8: Responses about experiences of TB infections in farms……………………… 69

Figure 4.1: A map showing study sites of the Municipalities in the Eastern Cape……….. 82

Figure 4.2: Showing sampling site of water collection……………………………………. 83

Figure 4.3: Showing sampling site of soil collection………………………………………84

Figure 4.4: Showing sampling site of cattle feeding on hayfeed………………………….. 84

Figure 4.5: Amplification product from the primer pair of the *IS6110* insertion sequence...89

Figure 5.1: Representative gel of amplification product from mutations of *rpo*B gene and *Kat*G gene…………………………………………………………… . ………………………103

LIST OF TABLES

Table 4.1: Samples showing a positive *IS6110* from three different farms and different samples………………………………………………………………………………. 89

Table 5.1: Mutations in *rpo*B gene conferring RMP resistance…....................................... 104

Table 5.2: Mutations in the *kat*G gene conferring INH resistance…………………. ……. 104

Table 5.3: Mutations in the *inh*A promoter region conferring INH resistance……………. 105

Table 5.4: Mutations in different samples at Fort Hare dairy farm……………………….. 106

Table 5.5: Mutations in different samples at Middledrift dairy farm……………………… 108

Table 5.6: Mutations in different samples at Seven star dairy farm……………………….. 109

Table 5.7: Drug susceptibility pattern of samples performed by the Genotype MTBDR*plus* ……………………………………………………………………………………………109

Table 5.8: RMP resistance in different samples from three different dairy farms ……….. 110

Table 5.9: INH resistance in different samples from three different dairy farms…………..110

TABLE OF CONTENTS

Contents

SUMMARY i
DECLARATION iii
DEDICATION iv
ACKNOWLEDGEMENTS v
LIST OF FIGURES vi
LIST OF TABLES vii
TABLE OF CONTENTS viii
CHAPTER ONE 1
1.1 GENERAL INTRODUCTION 1
1.2 PROBLEM STATEMENT 3
1.3 HYPOTHESIS 5
1.4 SCOPE OF DISSERTATION 6
REFERENCES 8
CHAPTER TWO 13
LITERATURE REVIEW 13
2.1 Tuberculosis and the *Mycobacterium tuberculosis* complex 13
2.2 History of *M. bovis* as a pathogen 14
2.4 Herd- level risk factors 21
2.5 Environmental persistence of *M. bovis* and influence of climate 31
2.6 Drug-resistant TB in South Africa 32
2.7 TB treatment regimens 34
ABSTRACT 56
3.2 MATERIALS AND METHODS 60
3.2.1 Primary data 60
3.2.2 Data collection and instruments 61
3.2.3 Data analysis 61
3.4 DISCUSSION 79
REFERENCES 86
CHAPTER FOUR 89
ABSTRACT 89
4.1 INTRODUCTION 91

4.2 MATERIALS AND METHODS 92
4.2.1 Study sites 92
4.2.2 Sample collection 93
4.2.3 Decontamination of the Samples 95
4.2.4 Culture of the samples 96
4.2.5 Acid- fast staining 96
4.2.6 DNA amplification 96
4.3. RESULTS 98
4.3.1 Culture 98
4.3.2 Staining 98
4.3.3 Molecular detection of MTBC 98
4.4. DISCUSSION 102
REFERENCES 106
CHAPTER FIVE 109
ABSTRACT 109
5.1 INTRODUCTION 110
5.2 MATERIALS AND METHODS 112
5.2.1 Genotype Resistance Detection 112
5.2.2 GenoType® MTBDRplus Assay 113
5.3 RESULTS 114
5.3.1 Mutation conferring resistance to RMP and INH 116
5.3.2 Overall drug resistance pattern 122
5.4 DISCUSSION 128
5.5 CONCLUSION AND RECOMMENDATIONS 135
REFERENCES 136
CHAPTER SIX 145
6.1 GENERAL DISCUSSION 145
6.2 CONCLUSION AND RECOMMENDATIONS 147
REFERENCES 148

CHAPTER ONE

1.1 GENERAL INTRODUCTION

Tuberculosis (TB) is an infectious disease that is commonly caused by *Mycobacterium tuberculosis complex.* TB predominantly affects the lungs of individuals and animals (FSAI, 2003; Du *et al*., 2011); nonetheless, it may further distress organs in the lymphatic system, central nervous system and circulatory system among others (Boukary *et al*., 2011)**.** Tuberculosis is a transferable disease that has unique scientific and uncontrolled features. The devastating fact is that it is also found in animal classes that could be used for nutrition such as milk and meat for individual ingestion including cattle, sheep, goats and deer. The dominant pathogen is *Mycobacterium tuberculosis*, and is connected with human tuberculosis (FSAI, 2003). Tuberculosis leads to severe mortality to the farm cattle businesses and to communal health. *Mycobacterium bovis* is an antique origin of tuberculosis that infects individuals through ingestion contaminated particles or in- take of uncooked milk from cattle farms that have the disease (Du *et al*., 2011).

Mycobacterium bovis and other species of *Mycobacterium tuberculosis* complex (MTBC) can result to a zoonotic disease known as Bovine tuberculosis (TB). MTBC has members that may infect an extensive range of hosts, thus involving wildlife (Modise, 2012; Richomme *et al*., 2013). Diverse wild species are known to cause disease in domestic livestock and are acknowledged as TB reservoirs. The issue of bTB risk factors has been debated worldwide. Some studies focused on particular parts of risk factors such as wildlife and herd management. According to supplying masses, feeding structures and animal interactions, farming practices are considerably different in developed and undeveloped countries (Ayele *et al*., 2004). As

prevalence in livestock decreases, the significance of wildlife TB reservoirs rises since reduction of the disease in wildlife hinders suppression in home species (OIE Terrestrial manual, 2009). Monitoring TB in wildlife under these conditions has turned to be a significant tool for initiating complete suppression systems (Richomme *et al*., 2013).

M. bovis is considered as one of the species of *M. tuberculosis* complex containing almost similar 16S RNA sequences and in excess of 99. 9% similar characteristic of their genome sequences (Ventura *et al*., 2007). This included *M. caprae*; although it was discovered to be a main microbe of goats in particular nations and bacteria of hairy seals and marine lions known as *M. pinnipedii* (Delahay *et al*., 2007). The above mentioned two new species are recognized to be cause of animal diseases. *M. caprae* is specifically identified as a main source of bovine tuberculosis in central Europe (Good and Duignan, 2011). The infection initiated by *M. caprae* is considered to be fundamentally related compared to the one produced by *M. bovis* and diagnosis is done using same tests (Corbett *et al*., 2006). Experimental confirmation of Bovine tuberculosis in livestock is a rare experience countries with tuberculosis eradication programmes such as skin test and removal and killing of sick cattle (OIE Terrestrial manual, 2009).

1.2 PROBLEM STATEMENT

Most pathogenic Mycobacterium species affecting man and animals are members of the *Mycobacterium tuberculosis* complex (MTBC) (Biet *et al*., 2005; Une and Mori, 2007). The domestication of animals by humans brought an era of understanding cross species infection or zoonotic nature of tuberculosis agents (Biet *et al*., 2005). The causative agents have since spread to all groups in the human population and constitute major threats to human health globally (Tan *et al.,* 2003; Ayele *et al*., 2004; Thoen *et al*., 2009).

In humans, TB co-infection with the Human Immunodeficiency Virus (HIV) and rapidly spreading Acquired Immune Deficiency Syndrome (AIDS) epidemic has significantly worsened the situation (Fatkenheuer *et al*., 1999; Larson, 2000; Corbett *et al*., 2002; Corbett *et al*., 2003; WHO, 2005; Corbett *et al*., 2006). Also, the widespread development of drug-resistant strains has complicated the treatment of TB in humans and significantly increased the cost associated with the use of multiple drug therapy (Thoen and Ebel, 2006; Thoen *et al*., 2009). Although TB is a major cause of human deaths, the real extent of human TB due to zoonotic agents is not known (O'Reilly and Daborn 1995; Ashford *et al*., 2001; Thoen *et al*. 2009). Over 70% (6 million) of humans co-infected with TB and HIV/AIDS live in sub-Saharan Africa (O'Reilly and Daborn 1995; Cosivi *et al*., 1998; Corbett *et al*., 2006) where bovine TB represents a potential health hazard to humans. Cow headers and farm workers may also be at risk of bovine TB because of its ability to infect both animals and humans.

The occurrence of disease caused by *Mycobacterium bovis* in livestock is rising in many countries including the UK and Ireland (Phillips *et al*., 2003; Smith *et al*., 2006). A large number of mammalian hosts are infected by the organism, and its removal becomes

complicated due to the occurrence of its reservoir in wildlife (Delahay *et al.,* 2007; Ward *et al.,* 2009). Considerable increase in interactions between wildlife and the middle host or their excreta has increased the risk of transmission to livestock overtime (Palmer *et al*., 2012).

This risk is heightened further by the possibility of cross-boundary transmission between cattle farms as seen in the British Isles (O'Reilly and Daborn 1995). *M. bovis* may enter the respiratory tract through ingestion of drinking water because of its survival ability in water (Michel *et al.,* 2007). The occurrence of extensive middle host is a huge factor in livestock for infection, however, it has been resolved that *M. bovis* disease in livestock is transferred in many ways and several of them can be restricted by proper farming (Phillips *et al*., 2003). The movement of deer on cattle farms, wildlife feeding, and livestock management practices, coupled with the ability of *M. bovis* bacteria to survive in natural environments including soil, water and hayfeed for a relatively long period (43 – 88 days) lead to increased risk of indirect transmission of *M. bovis* via environmental substrates (Fine *et al*., 2011). However, to the best of my knowledge, no reports have been published on risk factors associated with indirect transmission of *M. bovis* infection through environmental substrates (including soil, water and hayfeed) in cattle in the Eastern Cape Province of South Africa.

1.3 HYPOTHESIS

The hypothesis put forward for this study is that soil, hayfeed and water are key sources of *Mycobacterium tuberculosis* complex which can be transmitted to grazing animals in the Eastern Cape Province of South Africa. The close proximity of humans and domesticated animals can lead to the transmission of the organisms to humans, causing infection. The increasing occurrence of resistance in these causative agents of bovine TB, resulting from high levels of genetic mutations poses a public health risk due to rising failure of first-line drugs treatment.

1.4 SCOPE OF DISSERTATION

This dissertation comprises of six chapters. Chapter one: represents a general introduction to the topic of the dissertation, problem statement and hypothesis.

Chapter two: Literature review period. It encompasses background information on tuberculosis and *Mycobacterium tuberculosis* complex, history of *M. bovis* as a pathogen, risk factors, environmental persistence of *M. bovis* and influence of climate and drug resistant TB from humans in South Africa, TB treatment regimens. The chapter also outlines the methodologies used for detection of TB and its resistance.

Chapter three: assesses the understanding of bovine tuberculosis risk factors for infection control in the Eastern Cape Province of South Africa. Using appropriately designed questionnaires. The study sample comprised participants from three commercial dairy farms and two villages.

Chapter four: Focused on isolation, identification and confirmation of isolates using molecular techniques as well as molecular detection of *Mycobacterium tuberculosis* complex from soil, water and hayfeed samples obtained from three commercial dairy farms in the Eastern Cape.

Chapter five: focuses on assessing genotypic drug resistance through detection of mutations conferring resistance to INH and RMP associated with first line drugs in *M. tuberculosis complex* treatment using Genotype MTBDR*plus*. The mutations resistance to RMP and INH was observed from soil, water and hayfeed.

Chapter six: assembles the general discussion, conclusions and recommendations.

1.5 AIMS AND OBJECTIVES:

This study aims at determining the incidence of *Mycobacterium tuberculosis* complex that is associated with soil, hayfeed and water. Samples collected from dairy farms in the Eastern Cape Province. The specific objectives are as follows:

- To design questionnaires for assessing risk factors associated with MTBC in the Eastern Cape.
- To isolate and identify *Mycobacterium tuberculosis* complex from soil, hayfeed and water samples.
- To detect mutations conferring INH and RMP resistance associated with first line drugs in *M. tuberculosis* complex treatment using Genotype MTBDR*plus* assay.

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CHAPTER TWO

LITERATURE REVIEW

2.1 Tuberculosis and the *Mycobacterium tuberculosis* complex

Tuberculosis (TB) is caused by a group of closely related bacteria, collectively known as the *Mycobacterium tuberculosis* complex (MTBC) (Cole, 1998). TB in humans is mainly caused by *M. tuberculosis* and *Mycobacterium africanum*, a phylogenetic variant of MTBC limited to West Africa (de Jong *et al*., 2010). In addition, several animal-adapted members of MTBC exist, which affect a range of wild and domestic animal species (Smith *et al*., 2005). These include *M. bovis* which is a pathogen of cattle, *Mycobacterium caprae* found in sheep and goats, *Mycobacterium microti* from voles and *Mycobacterium pinnipedii* found in seals and sea lions. *Mycobacterium bovis* used to be a significant cause of human TB, primarily in children who consumed raw milk (Smith *et al*., 2006). However, *M. bovis* infections in humans decreased markedly following the introduction of pasteurization and meat-control practices (Skuce *et al*., 2011). Moreover, *M. bovis* does not easily transmit between humans and, similarly, while *M. tuberculosis* has been isolated from various animal species, including cattle, there is currently no evidence of animal-to-animal transmission of *M. tuberculosis* or *M. africanum*. Hence, the different members of MTBC appear to be best adapted to their particular host species. One particular member of MTBC deserves special mention: *Mycobacterium canettii,* which is part of the so-called 'smooth TB bacilli (Gutierrez *et al*., 2005). This somewhat enigmatic microbe was first described in the 1960s, and so far only about 60 isolates have been reported (Fabre *et al*., 2010).

2.2 History of *M. bovis* as a pathogen

M. bovis is a source of TB in cattle. Above 40% of milk cows were infected with *M. bovis* between 1930 and 1939 in the United Kingdom (Skuce *et al.,* 2011). Numerous cows were reserved in huge towns that are not far to offer municipal residents with treated milk. Many of them were strictly restricted in undeveloped publicized cow houses since there could be possible circumstances resulting in infection dispersing from the lungs then to other parts of the body such as the udder (Fabre *et al*., 2010). *M. bovis* spread easily to humans through ingestion of unpasteurized (untreated); and a lot of cows had udders that were infected with tuberculosis, making it an issue of serious health concern (PAHO, 2011). In 1950 a required TB abolition plan, involving tuberculin testing of animals was developed (Cosivi *et al*., 1998). From the 19th century meat inspection was implemented initially to discover animal infection and organisms such as tuberculosis and *Taenia saginata* were discovered as significant causes of zoonoses (OIE Terrestrial manual, 2009).Van Rhijn *et al*., 2008 explained the significance of meat inspection as an inhibition exercise against the supply of diseased meat that could infect human beings with the pathogen. The elimination of clearly unusual yields from the meat series, and encouraging in the detection and suppression of certain diseases of livestock. Traditional meat inspection methods can be easily achieved by elimination of clearly unusual yields. Meat inspection has an important function in the monitoring of diseases in national flocks (OIE Terrestrial manual, 2009). The most vital and utmost productive restrictive exercise to inhibit diffusion of zoonotic tuberculosis via milk is pasteurization or additional active heat treatment of milk preceding human consumption (FSANZ, 2009).

A secure guarding body against milk-borne transmission of *M. bovis* has pointed out databases that can assist in indicating symptoms of infected cattle from the onset and remove cattle

infected with *M. bovis* by making sure that diseased livestock are eliminated from dairy flocks after they are detected (FSAI, 2003). The utilization of unpasteurized milk in farms is a potential threat to human health in relation to transmission of *M. bovis* infection (Oliver *et al*., 2009). Pasteurization of milk is an effective and well-established intervention to control the transmission of *M. bovis* and other infectious agents contained in milk and other dairy products. The use of minor-rule home pasteurization components might decrease the danger of milk-borne infection with *M. bovis* when individuals ingest milk from their farms (FSIS, 1998). Cheese is the best corporative milk product prepared from unheated milk, although the outcome of cheese production in relation to the viability of *M. bovis* in the final product is not well-defined. The detection of *M. bovis* in milk and milk products has validated the zoonotic effect of this organism (Weyer *et al*., 1999). Therefore, cheese produced from unpasteurized milk may be a conduit for *M. bovis* infection in humans (FSAI, 2003).

Approximately 85% of farm animals and 82% of people in Africa live in regions where TB infection is common (Cosivi *et al*., 1998). In India and other developing countries, there are limited reports regarding the occurrence of *M. bovis* in livestock and livestock products. There are many reports on TB transference and some Mycobacterial diseases transmitted from farm animals to humans (FSIS, 1998). Milk and meat are main sources of protein and other nutrients, however they are ruined by bacterial agency and discovery of *M. bovis* in these samples. Additionally the presence of microorganisms in these samples are compulsory to be inspected. Thus the disease survives as an extensive public health issue (Weyer *et al*., 1999).

2.3 Risk factors of MTBC

2.3.1 Age

In both developed and developing countries, one of the major characteristic risk factors recognized by several studies is the age of animals. As publicized by a number of cross-sectional studies from Tanzania, Zambia and Chad, the age of animals rises with time of being at risk; mature animals are more likely to be revealed than juveniles (Cook *et al*., 1996; Kazwala *et al*., 2001; Cleaveland *et al*., 2007; Inangolet *et al*., 2008; Munyeme *et al*., 2008). A cross-sectional study in 1996 which evaluated more than 2 000 parameters in 200 flocks revealed that there is less potential for young cows than matured animals to get infected with TB Animal infection becomes more pronounced when they become fully developed (Griffin *et al*., 1996). Mycobacteria are capable of latency for extended periods before it reacts when the animal is at an adult stage (Pollock and Neil, 2002). However, it has not been suggested as yet by scientists that a proper latent status do occur in cattle (van Rhijin *et al*., 2008).

2.3.2 Gender

In studies carried out in Africa, gender was revealed as a risk factor for *M. bovis* in farm animals (Griffin *et al*., 1996). Opinions vary concerning the influence of gender on the susceptibility to *M. bovis* infection. In Tanzania, a cross-sectional study from 1994 to 1997 that integrating 5 692 home-grown and 244 foreign cattle, revealed that male cattle are considered to be more affected by bTB compared to female cattle (Cook *et al*., 1996). Male cattle are commonly used in the herd than females which are predominantly used as oxen. It is high risk to come in contact with infected animals from other flocks due to frequent use, which may increase the potentials of infection transmission. This implies that contact between herds is a major risk factor in the

transmission of bTB (Kazwala *et al*., 2001). A study carried out in Uganda from 2006 to 2007, on 1 470 cattle revealed that more male cattle tested positive to skin test in comparison to their female counterpart (Inangolet *et al*., 2008). Male and female cattle are treated in different ways in both developed and developing countries where female cattle are used for milk-production and males for ploughing. Milk producing female cattle reach early maturation stage than males in developed countries, since they usually function in both birthing and dairy production (Humblet *et al*., 2009).

2.3.3 Breed

A case study carried out in Africa has reported that the breed of cattle is a major risk factor in the transmission of TB (Katale *et al*., 2013). Another study carried out in 1998 on 1 813 cows from 494 concentrated milking farms in Eritrea found that introduced cattle breeds were less affected with bTB in contrast to home-grown breeds (e.g. zebu), which is used for milk production in Steamy region (Omer *et al*., 2001). During their 2005–2006 survey on 1 572 milking cows in Ethiopia, Elias *et al*. (2008) reported that bTB sickness rate was relatively higher amongst introduced animals. The distinction in management is probably the reason for the observed differences in susceptibility among breeds (Elias *et al*., 2008). This implies that breed, as a risk factor requires verification during research. An additional issue worthy of consideration with regards to the breed of the animal, is that inconsistency of the skin test response might exist (Katale *et al*., 2013). If established, this inconsistency could mean that analytical tests have to be properly designed to unravel the response of animals to TB test according to breed.

2.3.4 Body condition

The body condition score (BCS) is definite on a 1 (emaciated) to 5 (obese) scale and depends on scratching of the acuteness, muscle and backbones that are covered by fats and dorsal region (Edmonson *et al*., 1989). In their cross-sectional study involving 2 226 cattle in 1996, Cook *et al*. (1996) reported that low BSC is associated with an increased risk of positive skin test in Zambia. As recommended by a cross-sectional study of 5 692 indigenous cattle from 1994 and 1997 in Tanzania, cattle distressing from bTB usually show poor BCS, thus representing a longer lasting preventive progress; positive animals to skin test might experienced a low BCS as an outcome of the critical period of bTB (Kazwala *et al*., 2001). These case studies were accepted throughout a specific stage period. Scientists do not have an idea about the initial status of cattle in cross-sectional studies therefore it is not achievable to differentiate a low BCS as a risk factor (Costello *et al*., 1998). The BCS of an animal is normally linked to its nutritional value (Griffin *et al*., 1993).

2.3.5 Immune status

Immunosuppression is a prejudicing aspect for a large number of infections. As a result, the threat of being affected by *M. bovis* has also risen (Menzies *et al*., 2000). Vulnerability to *M. bovis* might also be complicated in animals infected by immunosuppressive viruses such as viral bovine diarrhea or immunodeficiency viruses (de la Rua-Domenech *et al*., 2006). Additional investigation could assist to explain further factors in this region.

2.3.6 Genetic resistance and susceptibility to bTB

In cattle, the significance of the genetics of bTB defence has evolved only for interrogation purposes. A low-dose *M. bovis* test could be caused by genetic mechanisms of non-definite immunity that are not specific to the lungs (Phillips *et al.*, 2002). An ordinary defensive-associated macrophage protein 1gene (NRAMP 1 gene) was reported to be present in a host that is defensive to tuberculosis and brucellosis in mice (Morris, 2007); however, a similar association could not be confirmed in cattle (Barthel *et al.*, 2000). A real-time qRT-Polymerase Chain Reaction approved the classification of some heredity that are identified at minor stages in animals affected with bTB in 2007 (Meade *et al.*, 2007). The focal point on inheritance factors of bTB are too new to categorize which mechanisms are involved and an investigation still need to be done to really examine their significance in cattle. Additional investigations are essential to explore the possible usage of heredity factors as constant biomarkers of bTB disease.

2.3.7 Vertical and pseudo-vertical transmissions

Turkish scientists, during 2007 illustrated the direct spread of *M. bovis* from a contaminated weir transfer to her calf through uteral inheritance disease (Ozyigit *et al.*, 2007). As recommended by Italian scientists in 1998, intake of infected milk is an alternative route of bTB spread that occurs from cattle to young ones (Zanini *et al.*, 1998). This risk factor has extra significance in countries where rule methods are less valuable. Pseudo-direct transmission has also been declared as a potential risk factor during direct contact between cattle and their young ones (grooming) (Phillips *et al*, 2003). However, in developed countries

and where normal testing programmes are implemented, and outbreaks are uncommon, the hazard may be measured as significant.

2.3.8 Auto-contamination

During the rumination process, a cow becomes contaminated during the oral way and may release extra infected splashes (Phillips *et al*., 2003). The cattle could inhale these infected splashes and consequently contracting the disease; only one bacillus can spread to infect many other cattle via this route (Neill *et al*., 1998).

2.4 Herd- level risk factors

2.4.1 History of bTB outbreak in the herd and human antecedent of tuberculosis in the household

In 2004, an Irish study recommended by a conservative group showed that 6 757 flocks were exposed to bTB and 10 926 herds were not exposed to bTB (Olea-Popelka *et al*., 2004). Another study in Ireland involving not less than 2 000 individuals (200 flocks) was, however, unsuccessful in exhibiting the eventual cause of bTB disease in relation to the major herd-level risk factor (Ayele *et al*., 2004). Griffin *et al*. (1993) explained that this study's failure to show the cause of bTB disease could be because it mostly incorporated meat selling cattle where the yield is essential for source of proteins in humans. According to Ayele *et al*. (2004) and Grange and Yates (1994), humans can infect animals in the same way that animals can infect humans. These authors reported that where citizens regularly urinate on pastures such as in Africa, an individual with analytical genitourinary tuberculosis who urinates on a grazing land during emitting period of *M. bovis* tuberculosis could infect cattle. In 1996, a cross sectional study involving 2 226 animals in Zambia revealed occurrences of tuberculosis from individuals who were in contact with a herd, and this was acknowledged as a risk factor through the discovery of a positive animal from skin test (Corner *et al*., 1990). Cattle infection by an individual releasing *M. bovis* was reported in Switzerland in 1998 were stated (Fritsche *et al*., 2004).

2.4.2 Herd size

Studies in industrialized and developing nations belonging to some parts of the globe acknowledged herd volume as a major bTB herd-level risk factors (Cook *et al*., 1996; Griffin *et al*., 1996; Munroe *et al*., 1999, Kaneene *et al*., 2002; Olea-Popelka *et al*., 2004; Cleaveland *et al*., 2007, Porphyre *et al*., 2008). The more cattle there are on a farm, the greater the possibility that one of them will obtain the disease. In 1999, a study revealed that many herds normally graze on a wide area that has higher chances of accommodating flocks from nearby areas, thus increasing the hazard of spread between herds of cattle (Cleaveland *et al*., 2007).

The higher the chances of having a reactor, the more animals should be skin tested (Monaghan *et al*., 1994). Between 2000 and 2003 in England, two relative case-control studies (a study of continuous herd occurrence integrating 50 incident cattle farms with similar restrictions and one more study of a temporary herd occurrence integrating 58 incident herds and 121 restriction cattle farms) linked flock volume with risk factors that are associated with farm supervision such as: herd income charges, mass supplied, farm selling and foodstuffs (Reilly and Courtenay, 2007). A category of management that manipulates touch among cattle was examined in this study. The findings were that transmission that is aerogenic was definitely the main route of infection in animals (Costello *et al*., 1998).

2.4.3 Type of cattle industry or enterprise

Cattle without a housing pen are more vulnerable to disease that those that are housed (Artois *et al*., 2004). Nonetheless, Munroe *et al.* (1999) reported that there was hazardous distinction between dairy and 151 milking cows and 477 meat cow studied in Canada between 1985 and 1994. Nonetheless, beef flocks had large numbers than dairy flocks. Throughout a conservative group study from 1980 to 2004 in New Zealand the milking cow flocks were well-known as being in extra danger involving 430 animals affected with bTB (Porphyre *et al*., 2008). Distinction in conditions of management are possibly responsible for the observation. In 1997, modelling of bTB transmission in New Zealand showed that assembling of cattle through milking raises chances of bTB transmission due to fact that some of the milking parlours may result in re-infection (Barlow, 1997).

2.4.4 Management

Management structure can be classified in terms of in contact among animals and infected environmental causes, as well as between cattle and wildlife. Consequently, a review of the risk factors has been shown. However, in order to implement defensive and restrictive measures, large-range studies are necessary to categorize obviously the supervision rehearsal listed out as one of the factors at risk (Kaneene *et al*., 2002).

2.4.5 Intensity of the farming system and housing of cattle

From developed and undeveloped countries management practice is different. More dairy cows are being introduced towards improving milk production in developing countries and are generally reserved under demanding circumstances (Cosivi *et al*., 1998). In areas where demanding dairy systems are run, the peak prevalence of bTB is usually increased. In developed aerogenic transmission of bTB appears to govern in these demanding systems (Menzies and Neill, 2000). In Michigan, the use of rented/united bulls further advanced the threat of bTB-prologue in a flock of 18 bTB-infected farms (Kaneene *et al*., 2002). In Ethiopia, it was reported that the seriousness of bovine TB was considerably advanced (containing considerably complex interferon-gamma (IFN-c) stages and further critical injuries) in animals reserved in the house with a larger inhabitant mass compared to animals reserved on grazing land, thus contrasting the outcomes of non- feeding against open feeding between 54 of Holstein and 37 of zebu cows (Ameni *et al*., 2006). Pressure caused by excess numbers or dietary dissimilarity among housed and grazing cattle was declared as contributing to the increase in the disease. A report from Ireland illustrated a relation involving recurring bovine TB and the existence of partitioned houses in 1993 that is linked with demanding milk cattle farms (Menzies and Neill, 2000).

For cattle, compartment accommodation is considered to be very traumatic, hence it complicates the bovine TB vulnerability (Ameni *et al*., 2006) Ethiopian scientists in a case study of 1 869 cows, explained that underprivileged accommodation and supervision is implicated in the decreased capacity of animals to fight bovine TB (Elias *et al*., 2008). In Ireland, Costello *et al*. (1998) in an elongated study showed that broken down aeration might assist in bovine TB transmission as well as immature steers reserved in feed lots for a 12 month

period. In the housing pen of cattle, the paucity of sanitation influences the creation of microbes. During 2001, an Eritrean case study showed that farm volume (surface) on 1 813 animals from 494 herds had been a factor at risk for bovine TB; however, no information was supplied on how farm volume measured could translate into threat (Omer *et al*., 2001). As reported in the UK, regarding conditions of quantity of holdings and length limit, farm size has an influence on bTB risk (Morris *et al*., 1994). Nonetheless, herd size might possibly be correlated to farm size and outsized farms having a lot of cattle (Costello *et al*., 1998).

2.4.6 Manure

A case-control study in 1993 reported that cattle can definitely become infected during the digestive process or after consumption of infected aerosols through the dispersing of slurry and dispersing of slurry on grazing land. There are no previous storage introduced an advanced possibility of bTB incidence in the herd on 160 Irish farms (Wray, 1975). When slurry is disperse in two months and introduce grazing then cattle are possible at risk for bTB (Griffin *et al*., 1993). Nonetheless, cattle that graze on man-made pastures are at high risk of infection due to transmission of organisms from the manure that supplements the growth of the pastures (Morris *et al*., 1994). Given that the existence of organisms might be short-lived during the dehydration season, mainly when reviewing the survival of the rainy and dehydrated seasons in Africa, dispersing of slurry may be of special importance (Wray, 1975). It would be as such motivating in Africa to examine the survival of *M. bovis* in slurry.

2.4.7 Feeding, supplementary feeding and feed storage

In developed countries, nourishing routines have been considered as risk factors for bovine TB. A cross sectional study n Ireland involving 160 farms established that an individual-feed fodder method cause additional traumatic experience for cattle (Griffin *et al*., 1993). In 2004, a double case-restriction study from the UK, reported that nourishing fodder might complicate the hazard of an established occurrence (Kaneene *et al*., 2002). Continuous outbreaks in the UK and feeding maize fodder, grass feed or molasses was reported as risk factors for short periods (Reilly and Courtenay, 2007). In two English farms observed in 1999, fodders were confirmed to be attracted to badgers (Garnett *et al*., 2002). Wildlife (deer and badgers) can access cattle feed in the cases above. However, managing the wildlife factor is to significantly investigate the potential suggestions of feeding behaviour as a risk factor of bTB disease .In two case-restricted reviews approved from UK in 1995 and 1999; amplified nourishing was discovered to reduce the chances of short-lived bovine TB occurrence on 229 herds (Reilly and Courtenay, 2007).

A Michigan retrospective corresponding case survey from 18 farms infected with bovine TB confirmed that giving hay on the land, preferably in feeding animals, and giving free hay, preferable in packages, had been linked to an heightened danger of bovine TB (Kaneene *et al*., 2002). Mainly concerning silage, feeding storage appears to promote transmission of bovine TB. Studies performed in the UK illustrated that badgers emerged to be a cause of infection for fodder with urine, faeces or sputum infected with *M. bovis* and considered as a chief wildlife source of *M. bovis* (Garnett *et al*., 2002; Reilly and Courtenay, 2007). Throughout an examining study of two English farms in 2002, wild animals were regularly noticed visiting

cattle farm construction to nourish straight from amenities of animals and feed storage fences (Garnett *et al*., 2002).

2.4.8 Cattle-to-cattle transmission via the faeco-oral route

In demanding controlled systems, aerogenic transmission is significant. It is useful to mention at this point the function of faecal-oral transmission from one cattle to another. Several authors claim that *M. bovis* contamination can be doubtful when obtained straight from ingesting grass infected by other animals because cows do not regularly feed where animals defecate (Phillips *et al*., 2003). However, mycobacteria might continue to exist in the environment within faeces under suitable climatic conditions (Gopal *et al*., 2006). A study carried out from four to seven months, in a set of five calves contaminated intra-nasally with *M. bovis* confirmed that in more infected animal, pathogens do not survive and are often expelled in cow faeces (Neill *et al*., 1988). Nevertheless, it is not possible in field circumstances to identify the exact mycobacterial load leading to cattle infection; these interpretations were prepared under experimental circumstances.

2.4.9 Introduction of cattle in a herd: purchase

Studies from UK, Michigan, Italy and Tanzania reported the entrance of the affected cattle into a bTB liberated flock as the only main factor initiating the infection (Marangon *et al*., 1998; Shirima *et al*., 2003; Johnston *et al*., 2005; Gopal *et al*., 2006, Kaneene *et al*., 2006). In north east (NE) England the cause of bTB was found in 31 herds that had been purchased during the occurrence of bTB between 2002 and 2004 and also the nine flocks that were purchased

during the 2001 foot and mouth disease (FMD) outbreak (Gopal *et al*., 2006). A Canadian study between 1985 and 1994 demanded examination for potential mixing of cattle that are positive for TB or else supplied farms with positive cattle and established that flocks examined during epidemic outbreaks posed serious threats if they were bought from farms with positive cattle herds (Munroe *et al*., 1999). It might be attractive to measure the most suitable testing to categorize positive animals when buying them in order to assess their compatibility with methods for the initiation of fresh animals. As stated in the epidemiological scene, it useful to utilize and apply the skin test or IFN-c as suggested.

2.4.10 Movements of animals

Between 1985 and 2003 in a study evaluating cattle movements in Great Britain, relocation of cattle was reported as being a significant risk factor in the spread of TB (Gilbert *et al*., 2005). Since revealed in 31 herds that showed evidence of bTB infection, molecular typing between 2002 and 2004 confirmed that animal movements were accountable for the majority of bTB epidemics reported in NE England (Gopal *et al*., 2006). If animals are relocated from a widespread region to a bTB-liberated one, this precise factor has a strong impact (Munroe *et al*., 1999, Gopal *et al*., 2006).

Animals scuff in grazing land when combined by some farmers throughout the raining season. Some herds are collected in movable crowds which join together through the dry season alongside in grazing areas and watering sources (Oloya *et al*., 2007). A Zambian cross-sectional study involving 106 herds in three pastoral areas from 2003 to 2004, showed that transhumance was linked to improved bTB status (Munyeme *et al*., 2008).

2.4.11 Other domestic species

Although no transfer of *M. bovis* to cattle has been reported, bTB infection has been illustrated in dogs (Gay *et al*., 2000) and cats (Wilesmith and Clifton-Hadley, 1994). In countries where pig farming is relatively advanced; they are not a possible risk factor on cows (Gilbert *et al*., 2005). Since previously monitored cases reported that goats have been extremely vulnerable to *M. bovis* disease in Spain and western Wales (Malone *et al*., 2003), they might obstruct bTB eradication plans. Epidemics of caprine, *M. bovis* tuberculosis have only been relatively irregular (Gutierrez and Garcia, 1999; Seva *et al*., 2002, Alvarez *et al*., 2008; Crashaw *et al*., 2008). The threat of infecting bTB-free cattle herds must not be allowed by bringing in infected goats. However, bovine infection is uncommon to other domestic species and is regularly linked to cattle cases (Malone *et al*., 2003 and Houlihan *et al*., 2008). In addition, horses are vulnerable to *M. bovis* disease (Seva *et al*., 2002). In areas such as Camargue, southern France where in contact exists among horses and cows, this might be a possible cause of disease (Alvarez *et al*., 2008). The risk to domestic cattle in New Zealand exists where deer farming is extensively exercised (Lugton *et al*., 1997). It is necessary in developed countries to allocate a bTB rank to herds and to increase the bTB observation in farmed red deer; the potential suggestion of farmed red deer should be considered where bovine TB is recognized in a formerly OTF cows flock (Houlihan *et al*., 2008). In the epidemiology, extra investigations of bTB might discover the possible function of domestic species. Since they are regularly in close contact with each other in developed nations, dogs, cats, goats and sheep must be supervised, however, in developing countries, every type of animal should be given attention (Garcia, 1999).

2.4.12 Wildlife

A large variety of wild animals can be infected by *M. bovis* (Morris *et al*., 1994; O'Reilly and Daborn, 1995; de Lisle *et al*., 2001). Through ingesting infected remains of grasses; digestive wounds propose oral infection in mainly carnivorous species, which is a way of spreading the infection. The incidence of the infection shows the character of wild animals as a source for *M. bovis* due to the presence of microbes in the ecology and ecosystem of natural animals (Artois *et al*., 2004). Since the relationship among nourishment and spread over hosts is active, however, the edge among these host types is not visibly well-known (Delahay *et al*., 2001). In a case study of 4 180 natural animals with 16 diverse species done by British scientists, it was reported that spread over hosts might transfer the contamination to other host inhabitants which was carried out from twelve milking farms along with 8 experiencing a current record of cows with bovine TB and four controls. It was reported that spread over hosts might transfer the contamination to other host inhabitants (Mathews *et al*., 2006). Disease can multiply and spread as hosts increases, as was observed lately in France (Morris *et al*., 1994). Throughout the 2005/2006 hunting season, 33 contaminated red deer were captured and had wounds with a huge capability of circulating bTB (Zanella *et al*., 2008).

For inhabitants of European badger of United Kingdom and Ireland, bTB is widespread (Cheeseman *et al*., 1988; Griffin *et al*., 1993; Denny and Wilesmith, 1999). In New Zealand, brush tail possums have been the principal reservoir of bovine TB amongst natural host (Morris and Pfeiffer, 1995). In the entire world *M. bovis* was cut off from diverse types of cervids (white-tailed deer) in Michigan (Schmitt *et al*., 1997; Kaneene *et al*., 2002), red deer in New Zealand (de Lisle *et al*., 2001), muntjac, roe, cherry and bare deer from the United Kingdom

(Gunning, 1985; Delahay *et al*., 2001; Delahay *et al*., 2007), cherry and bare deer from Ireland (Quigley *et al*., 1997), cherry deer from France (Zanella *et al*., 2008) or in Spain (Aranaz *et al*., 1996; Gortazar *et al*., 2005).

In some states such as Spain, France and Italy, the wild pig namely, Sus scrofa, has been acknowledged as a TB carrier (Aranaz *et al*., 1996; Parra *et al*., 2003; Zanella *et al*., 2008). In Spain, the model of wounds examined indicates the potential of the wild boar as a reservoir (Parra *et al*., 2006). In Kruger National Park, South Africa, the African buffalo (Syncerus caffer) has been reported as a reservoir of *M. bovis* (de Vos *et al*., 2001; Rodwell *et al*., 2001; Michel *et al*., 2007). Among buffaloes, the infection has extended broadly and other mammalian species have developed into spreading over hosts, including predators such as lions (Michel *et al*., 2006).

2.5 Environmental persistence of *M. bovis* and influence of climate

Facts are debatable on the occurrence of *M. bovis* from cattle that are not housed. The report of Menzies and Neill (2000) stated that the pathogen has an ability of living in the surroundings; during normal circumstances the diseases can be transmitted to animals for only some weeks. Morris *et al* (1994) claimed that infected nourishments have an insignificant role in livestock affected by the disease because animals are not normally fed largely sufficient quantity to become affected, and the infection does not exists long enough on fomites to be affected. The survival period of *M. bovis* from several studies has been evaluated. Wray (1975) disagrees that the accessibility of the nutrients is the limiting component. Survival in tropical areas may be strongly affected by daylight as a source of dehydration and invisible brightness. Kelly and

Collins (1978) proposed the main components affecting existence in soil and on grazing land as: high temperature, humidity, pH, exposure to sunlight, decreasing oxygen and existence of logically prevailing antibiotics in the environment.

New Zealand scientists in 1992 to 1993 reported that sources of water should be considered as possible risk factors as regions found near water reservoirs are usually humid and as such provides suitable growth conditions for *M. bovis*. The entry of the pathogen into the respiratory tract by ingestion of infected water could be favoured when a cattle drinks by splashing. It has been shown that running water can be contaminated directly by excited animals (Phillips *et al*., 2003). In Africa, this may cause certain hazards when many herds are using the same water points, and that could increase possible infection. As reported by Cleavelend *et al*. (2007) in Tanzania, a cross-sectional survey of 1 549 animals positive for TB was revealed, in Africa it is recognized as a circulating cause of disease in the surroundings. Nevertheless, risk factors require additional verification regarding the existence of *M. bovis* in the environment (Phillips *et al*., 2003). In a study carried out in New Zealand in 1992 to 1993, the survival time of the pathogen in the environment was described by Jackson *et al*. (1995).

2.6 Drug-resistant TB in South Africa

South Africa is home to one of the world's worst HIV epidemics and is related to one of the highest burdens of TB. In 2005, it was estimated that 285,000 cases of TB was reported in the country, representing the seventh highest total number of incidence in the world and the second highest total in Africa (WHO, 2007). Fifty-eight percent of patients with TB were coinfected with HIV (Davies *et al*., 1999). Of significant concern was the fact that in South Africa, the TB

cure rate (54%) was the lowest among the 10 countries with the highest TB burden (WHO, 2007).

Compared with most sub-Saharan African countries, South Africa has an advanced public health infrastructure and far greater capacity for TB drug-resistance surveillance (Victor *et al*., 2007). Between 1965 and 1991, a total of 25 annual surveys of drug resistance were conducted by the Tuberculosis Research Institute of the South African and the Medical Research Council (MRC) (Weyer and Kleeberg, 1992). In 1995, the MRC's TB research program was suspended because of budgetary constraints. Several smaller drug-resistance surveys, including two provincial surveys, were undertaken, but national data were not collected again until 2001. The data from these interim surveys, described below, suggested that the prevalence of MDR-TB remained low in the mid-1990s but began to increase in the latter half of the decade (Wilkinson *et al*., 1996 and Davies *et al*., 1999).

Smaller, localized studies of TB drug resistance in the mid- 1990s found generally low rates of MDR-TB among patients with newly treated TB (1%) and 3% among patients with previously treated TB (Wilkinson *et al*., 1996; Davies *et al*., 1999 and Churchyard *et al.,* 2000). TB drug resistance was not associated with HIV infection, and primary drug resistance appeared to be limited, with acquired resistance playing a dominant role (Weyer and Kleeberg, 1992). Between 1993 and 1997, an outbreak of TB resistance to 4 first-line drugs namely isoniazid, rifampicin, ethambutol, and streptomycin affected at least 21 individuals in Cape Town, none of whom were infected with HIV (WHO, 2013). More recently, in Western Cape, the emergence of outbreak-associated MDR-TB strains with patterns of resistance to pyrazinamide, streptomycin, and ethambutol has been reported (Louw *et al*., 2006 and Victor *et al*., 2007). The most recent and systematic national estimates of drug resistant TB were based

on a survey of 9 provinces performed between 2001 and 2002 by the Tuberculosis Lead Programme of the MRC in South Africa, which is part of the global network of supranational reference laboratories assembled by the Global Project on Anti-tuberculosis Drug Resistance Surveillance of the WHO (Weter *et al*., 2004). Worldwide, MDR-TB was detected in 1.6% of isolates recovered from patients with newly diagnosed TB and in 6.6% of isolates recovered from patients with previously treated TB (Davies *et al*., 1999 and Churchyard *et al*., 2000).

Among patients with newly diagnosed TB, the prevalence of MDR-TB ranged from 0.9% in Western Cape to 2.6% in Mpumalanga (WHO, 2013). The prevalence of MDR-TB among previously treated patients with TB ranged from 3.9% in Western Cape to 13.7% in Mpumalanga. In KwaZulu-Natal, the prevalence of MDRTB among patients with newly diagnosed TB (1.7%), as well as that among patients with previously treated TB (7.7%), was similar to the national average because of population size and TB prevalence (Louw *et al*., 2006 and Victor *et al*., 2007); the largest number of cases was estimated to have arisen in KwaZulu-Natal. In the five years since the last formal survey was conducted, it is likely that the number of cases of drug resistant TB has increased substantially in South Africa, in parallel with the increase in the number of TB cases which is supported by the dramatic increase in the HIV epidemic (Weyer *et al*., 2004).

2.7 TB treatment regimens

First and second-line anti-TB drugs are performed in humans for the treatment of all types of TB which is referred to as schemo therapy procedures (WHO, 2013). A first-line anti-TB drug is composed of isoniazid (INH), rifampicin (RMP), pyrazinamide (PZA), ethambutol (EMB) and streptomycin (STM) (Davies *et al*., 1999). However, the main two frequently used drugs

for management of TB are INH and RMP. When handled accurately, first-line anti-TB drugs are harmless and successful (WHO, 2013). In an effort to decrease the spread of drug-resistant TB in villages, efficient management of MDR-TB is crucial. Recently, a collection of drugs for the management of MDR-TB treatment procedure have been checked (Migliori *et al*., 2010).

Concerning to the emergence of MDR-TB, TB maintenance and handling is presently very difficult. MDR-TB is treated using the following drugs: the aminoglycosides such as amikacin (Am) and Kanamycin (Km); polypeptides such as capreomycin (Cm), viomycin and enviomycin; fluoroquinolones such as ciprofloxacin (Cip), levofloxacin (Lfx), ofloxacin (Ofx), moxifloxacin (Mxf) and gatifloxacin; and thioamides such as ethionamide (Eto), prothionamide and cycloserine (Cs), and P-aminosalicylicacid (PAS) which are called second-line anti-TB drugs (WHO, 2013). Second-line anti-TB drugs, in contrast to first-line anti-TB drugs, are not very effective, they require proper administration for a sufficient period and may be poisonous and very expensive (Migliori *et al*., 2010). Third-line anti-TB drugs include: clofazimine (Cfz), linezolid (Lzd), amoxicillin/clavulanate (Amx/Clv), thioacetazone (Thz), imipenem/ cilastatin (Ipm/Cln) and high-dose isoniazid which are mediators with uncertain functions in drug-resistant TB treatment (WHO, 2013).These drugs are not administered in cattle infected with TB (WHO, 2007). Cattle are diagnosed with TB are killed to prevent transmission among farm animals since contact between animals may create opportunities of bTB risk transmission (WHO, 2013).

2.8 Methodologies used for detection of TB and its resistance

2.8.1 Acid fast bacilli

The observation of acid fast bacilli (AFB) in a stained smear examined under a microscope is often the first evidence of mycobacterial disease (Kent and Kubica, 1985). Furthermore, staining of decontaminated samples using the Z-N staining technique and microscopy remains the only diagnostic proof of MTBC (Boehme *et al*., 2005). Microscopy of AFB is a simple, cheap and a rapid method, but is neither specific nor sensitive enough when compared to culture and can detect bacilli only when the number of microorganisms is greater than 10^4 cells /mL. Microscopy clearly has many advantages when it comes to speed and feasibility, and if sensitivity could be improved it has the potential to become an even more valuable tool for National TB Control Programmes (NTPs) around the world (Uddin *et al*., 2013).

2.8.2 Culture

Culture is usually required for the laboratory confirmation of TB and it enables specific identification of the mycobacterial pathogen. This is accomplished after the tubercle bacilli have been recovered on primary isolation media (Kubica, 1984). The bacilli can be recovered using either solid media such as Lowenstein-Jensen and agar-base such as Middlebrook media. The detection of MTBC by bacterial culture can take 8-12 weeks for visible colonies and this enables longer period for identification of the mycobacterial pathogen (Boehme *et al*., 2005). Culture, although offers improved sensitivity, is time consuming and requires high cost safety facilities. Despite these limitations, culture has been recommended as a cost effective method

for assessing and monitoring the prevalence of drug resistant TB in any TB control Program (IUATLD, 1998).

2.8.3 Molecular identification using PCR assays

PCR is the most well-developed molecular technique up to now, and has already shown satisfactory potential in clinical applications such as specific or broad-spectrum pathogen detection, evaluation of emerging novel infections, surveillance, early detection of biothreat agents, and antimicrobial resistance profiling. PCR-based methods may also be cost effective relative to traditional testing procedures. Further advancement of technology is needed to improve automation, optimise detection sensitivity and specificity, and expand the capacity to detect multiple targets simultaneously (multiplexing) (Yang and Rothman, 2004).

PCR sensitivity ranges from 70-90% and its specificity varies between 90 and 95% but in smear positive cases, its sensitivity is greater than 95% while in smear negative cases it is only 50-60% (Hermans *et al.,* 1991). The strength of the PCR assay is that it provides rapid results (within hours) than detection of MTBC by bacterial culture, which can take up to 12 weeks; and is not hindered by contaminating organisms that can overgrow a bacterial culture (Boehme *et al*., 2005). PCR also gives information about the presence and distribution of MTBC in the environment much longer after the initial evaluation of contamination than mycobacterial culture does (Yang and Rothman, 2004). In TB epidemiologic investigations of farms and wildlife sites, PCR-based assays may be useful for testing in parallel with bacterial culture to enhance the detection of MTBC in the environment (Hermans *et al*., 1991).

2.8.4 First line TB drugs

First-line anti-tuberculosis agents use to be more effective, and were necessary components of any short course therapeutic regimen (Crofton *et al*., 1997) in the treatment of human TB. These first-line drugs represent the standard treatment of TB as recommended by the World Health Organization (WHO) and includes four antibiotics; rifampicin (RIF), isoniazid (INH), pyrazinamide (PZA), and streptomycin (STR) or ethambutol (EMB) (Crofton *et al*., 1997; Mushtaque and Chowdhuri, 1999).

The bactericidal activity of both RIF and INH in killing *M. tuberculosis* makes these drugs most effective for standard treatment of TB (Telenti, 1997).They show a high early bactericidal activity on rapidly proliferating bacteria (Mitchison, 2000) though each separately is less efficient in killing semi-dormant bacteria (Crofton *et al*., 1997).

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CHAPTER THREE

Understanding bovine tuberculosis risk factors for infection control in the Eastern Cape, South Africa

ABSTRACT

Questionnaires are popular and fundamental tools for acquiring information on public knowledge. The objective was to develop a valid and reliable questionnaire that would measure the understanding of bTB risk factors for infection control. Samples from this study comprised participants from three commercial dairy farm: Fort Hare dairy farm, Middledrift dairy farm and Seven star dairy farm, to whom questionnaires were administered. In addition, two villages (kwaMasele and Qungqwala in Peddie) with different smallholding farmers were also selected to participate in the study. The questionnaire was administered to thirty (30) farm owners in the two villages and the three (3) commercial farm owners. Respondents in this study that ranged from sixty percent (60%) (10-20 cows) to nine percent (9%) (more than 40 cows). Relationship between farm size and vaccination for TB differed from forty one percent (41%) to five percent (5%). The highest number of respondents who knew about relationship between TB cases and cattle location was ninety one percent (91%). Approximately fifty one percent (51%) of respondents had knowledge about wild life access to the farms. Relationship between import of cattle and farm size ranged from nine percent (9%) to thirty five percent (35%). Cattle sickness in relation to farm size differed from forty three (43%) to the least three percent (3%) while thirty three percent (33%) of respondents had knowledge about health management. Respondents with knowledge about the occurrence of TB infections in farms were forty eight

percent (48%) Bovine TB risk factors are often observed in smallholding farms than commercial farms; this observation is often linked to poor management. Commercial farmers have access to facilities, and more often than not, the owners have agricultural training; hence they appear to be more familiar with bovine TB management to control transmission.

3.1. INTRODUCTION

Bovine tuberculosis (bTB) is a zoonotic disease caused by *Mycobacterium bovis* and closely related members of the *Mycobacterium tuberculosis* complex (MTBC). Members of the MTBC can infect a broad range of hosts, including wildlife. In particular for *M. bovis*, different wild species have been recognized as TB reservoirs in different areas of the world, becoming a source of infection for domestic livestock and thus, impeding the eradication of tuberculosis in cattle (Corner, 2006). In France, the first cases of wildlife TB were detected in red deer (Cervus elaphus) and in 2001 wild boar (Sus scrofa) was identified in Eurasian and Brotonne-Mauny forest found in Normandy in the North-West (Richomme *et al*., 2013). In 2006, the TB prevalence in this small area reached 24% in red deer, which appeared to act as the primary host, and 42% in wild boar which appeared to act as a spillover host (Zanella *et al*., 2007). In 2003, a red deer was also found infected in Côte d’Or where grouped cases of wild boar and badgers (Meles meles) have been regularly identified since 2007 (Elias *et al*., 2008). Wildlife has been identified as a risk factor for bTB in cattle all over the world (Richomme *et al*., 2013).

In developed regions, countries mainly involved in bTB research are found in Western Europe (United Kingdom, Ireland, Spain, Italy and France) North America (USA and Canada) and New Zealand (Humblet *et al*., 2009). Management practices differ in developed and in developing countries. In developing countries, Holstein cattle are increasingly imported in order to improve milk production and are usually kept under intensive conditions (Corner, 2006). The highest incidence of bTB is generally found in areas where intensive dairy systems are practiced (Cosivi *et al*., 1998). Dairy production in developed countries follow a trend towards increased intensification on a smaller number of larger production units, which implies

increased contact between animals and thus an enhanced risk of bTB transmission (van Arendok and Liinamo, 2003). In a cross-sectional study involving 1 869 cattle, Ethiopian scientists observed that poor housing and management could result in the reduction of an animal's resistance to bTB (Elias *et al*., 2008). The aim of this study was to gather information relating to farm owners and investigate if environmental factors for the survival and spread of bovine tuberculosis (bTB) exist in the farms

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3.2 MATERIALS AND METHODS

3.2.1 Primary data

The study sample comprised participants from three commercial dairy farms (Fort Hare dairy farm, Middledrift dairy farm and Seven star dairy farm) to whom questionnaires were administered. In addition, two villages (kwaMasele and Qungqwala in Peddie) with smallholding farmers were also selected to participate in the study. The questionnaire was administered to thirty (30) farm owners in the two villages. In this study, self-administered questionnaires wherein the researcher reads questions out to the respondents and then ticks their responses was considered the relevant method for data collection because there was high level of illiteracy among farmers and cattle herders; and it took approximately 10 minutes to complete the questionnaire, which was generally well received by the respondents. The following guidelines as suggested by Babbie et al. (2001) were considered prior to the implementation of this survey:

- Appearance and demeanour of the researcher;
- Familiarity with the questionnaire;
- Following questionnaire wording exactly;
- Recording responses exactly; and
- Probing for response.

Respondents were selected based on practice and involvement in agricultural activities. Their willingness to participate in the research also determined their selection. Respondents were informed of the objectives of the study and were further informed about the confidentiality of the study during the data collection process before questionnaire administration. Structured, interviewer-administered questionnaires were used to acquire information about farmers' perceptions regarding the farm management that could lead to high risk of bovine tuberculosis.

The use of interviewer-administered questionnaires was to ensure that information was obtained from illiterate respondents. The questionnaire was composed of closed ended questions to make the coding of the responses easier and to extract as much information as possible from the respondents without taking too much of their time.

3.2.2 Data collection and instruments

This study used quantitative data which was elicited through self-administered questionnaires. The required information included general information such as size of cattle in different farms that included small size (herds between 10-20), middle size (herds between 30-40) and large size herds (more than 40 herds), how often vaccination is given for different diseases in cattle, knowledge of TB cases, wildlife access, import of cattle, cattle sickness into different diseases, health management in cattle, and occurrence of TB infection. The study visited identified farms where different species of animals could be found and to investigate farmer's opinions about the spread and risk of bTB amongst the livestock. The commercial farms were located in urban areas and smallholding farms in rural areas. Ethical clearance approval, REC-270710-028-RA was obtained from the University of Fort Hare at Govan Mbeki Research Development Centre.

3.2.3 Data analysis

After collecting and gathering data, it was captured and encoded in the form of spread sheets in Microsoft Excel and exported to SPSS software. The study made use of descriptive statistics in analysing the data collected to determine farm management from the farm owners that could

create high chances of risk that might result to high prevalence of bTB. Interpretive analysis used was simple statistics, tables and graphs

3.3 RESULTS

The number of respondents on the question of size of cattle in relation to herd size differed from 60% being the highest to the least (9%). All of respondents in commercial farms located in urban areas had large herd size (more than 40 cattle) and a majority of respondents in smallholding farms located in rural areas had small herd size (herds between 10-20 cattle) and middle herd size (herds between 30-40 cattle) (Figure 3.1).

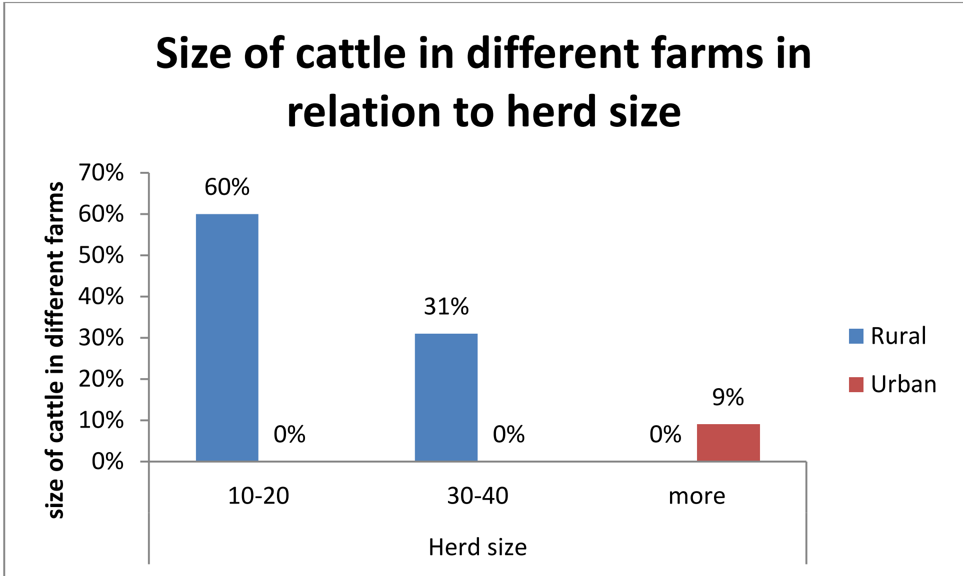

Figure: 3.1. Respondent's knowledge about size of cattle in relation to herd size in commercial and smallholding farms.

The number of respondents in relationship between farm size and vaccination differed from 41% being the highest to the least 5%. Respondents in commercial farms give vaccination once in 12 months in a lifetime and smallholding farms remembered that vaccination was given once in 18 months and 24 months in a lifetime (Figure 3.2).

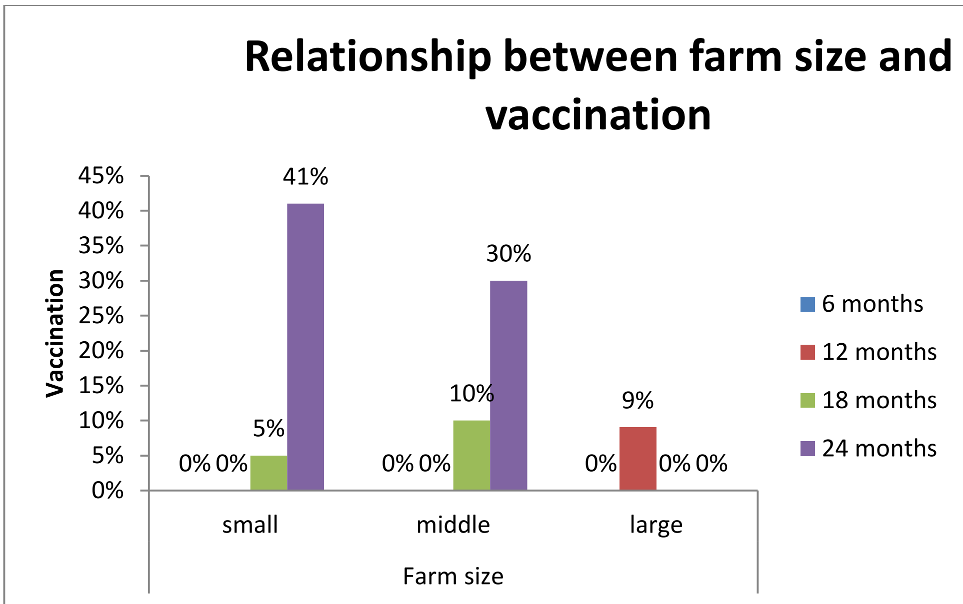

Figure: 3.2. Respondent's knowledge on relationship between farm size and vaccination in commercial and smallholding farms.

The number of respondents in the relationship between TB cases and cattle location differed from 91% being the highest to the least 9%. A Majority of respondents in smallholding farms said no and only 9% of respondents said yes in smallholding farms (Figure 3.3).

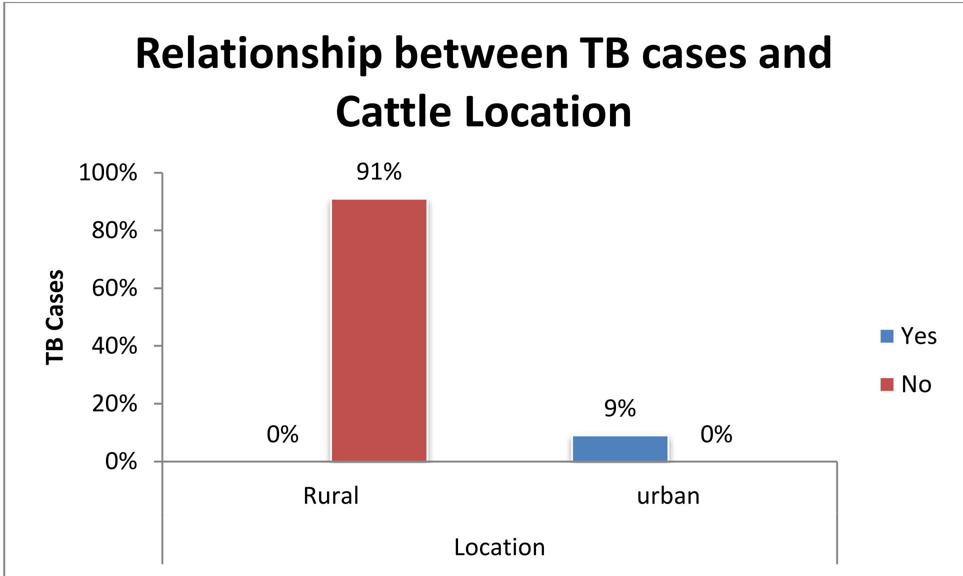

Figure: 3.3 Respondent's knowledge about TB cases in commercial farms located in urban areas and smallholding farms located in rural areas.

The number of respondents to question on wild-life versus herd size differed from 51% being the highest to the least 9%. All of the respondents in both commercial and smallholding farms said yes about wildlife access and no respondents said no (Figure 3.4).

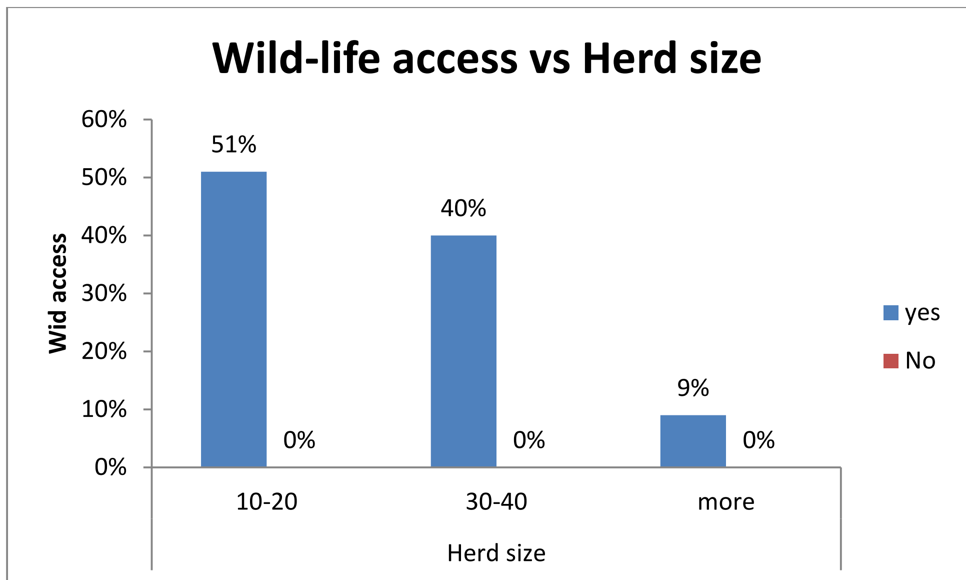

Figure: 3.4 Respondent's knowledge about wild life access in relation to herd size in commercial farms and smallholding farms.

The number of respondents in relationship between import of cattle and farm size differed from 35% being the highest to the least 9%. A majority of respondents said no in regards to import of cattle in smallholding farm (small size and middle size) and all respondents in commercial farms (large size) said yes about import of cattle (Figure 3.5).

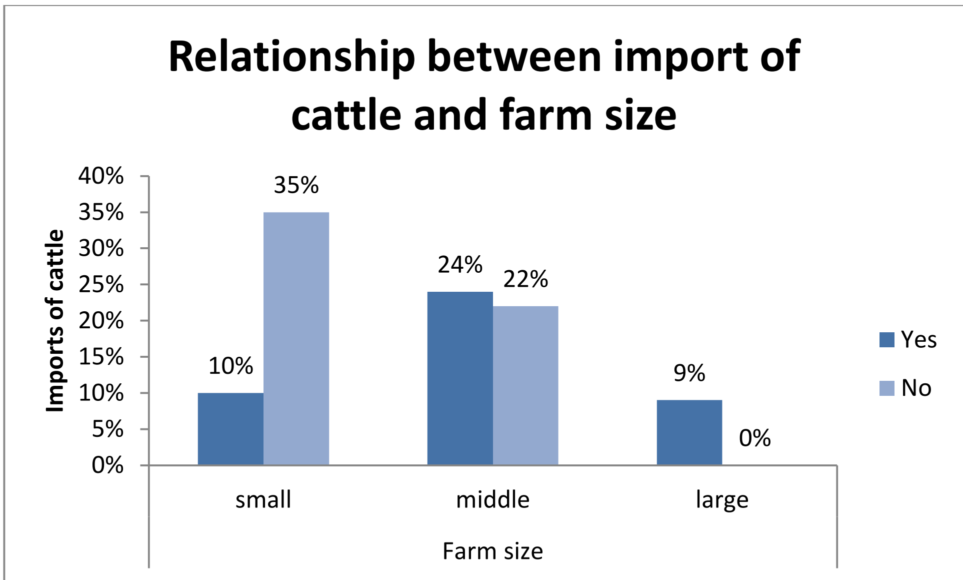

Figure: 3.5. Respondent's knowledge about import of cattle in commercial farms and smallholding farms.

The number of responses of cattle sickness (anaplasmosis) in relation to farm size differed from 43% being the highest to the least 9 %. A majority of respondents in both commercial farms and smallholding farms said yes and were familiar with anaplasmosis sickness in cattle and minority said no (Figure 3.6).

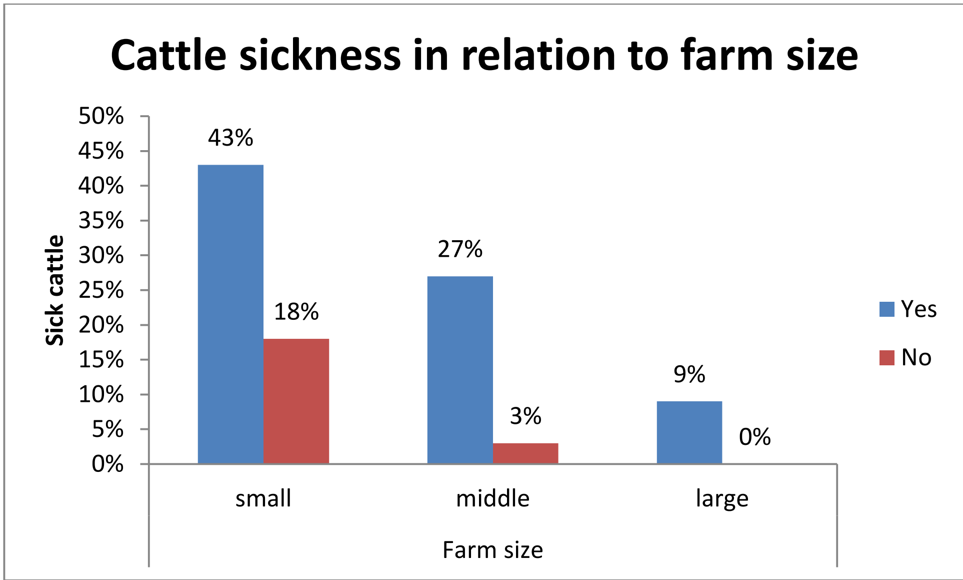

Figure: 3.6 Respondent's knowledge about cattle sickness in relation to farm size in commercial and smallholding farms

The number of responses to questions on health management in relation to farm size differed from the highest 33% to the least 3%. A majority of respondents in smallholding farms practice by culling while minority by vaccination and all three commercial farmers practice by vaccination (Figure 3.7).

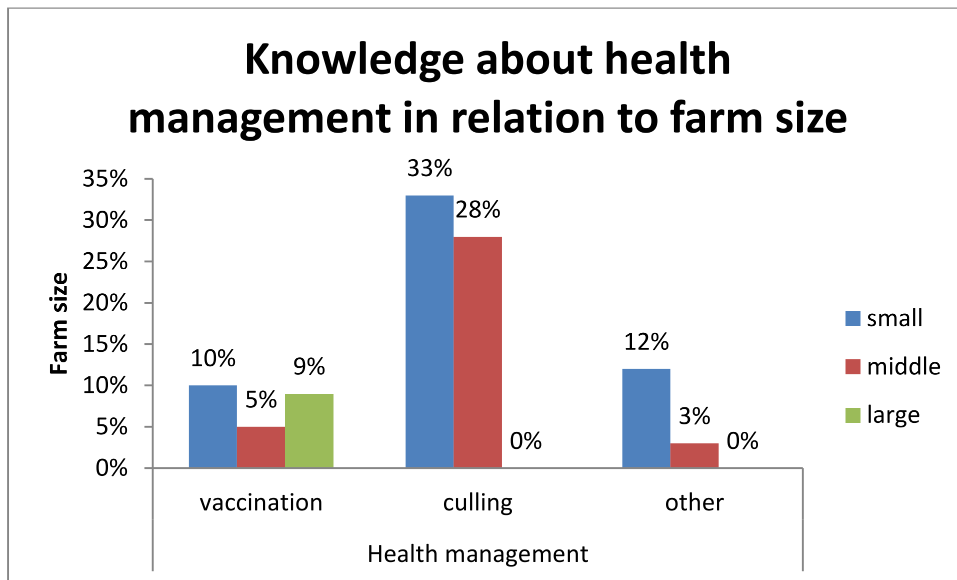

Figure: 3.7 Responses to knowledge about health management in relation to farm size in commercial and smallholding farms.

The responses to questions of TB infection in farm differed from 9% to 48%. A Majority of respondents in smallholding farms which represents small size and middle size said no about occurrence of TB infection in cattle and minority said yes while in commercial farms which represent large size, all respondents had knowledge about TB infection. (Figure 3.8).

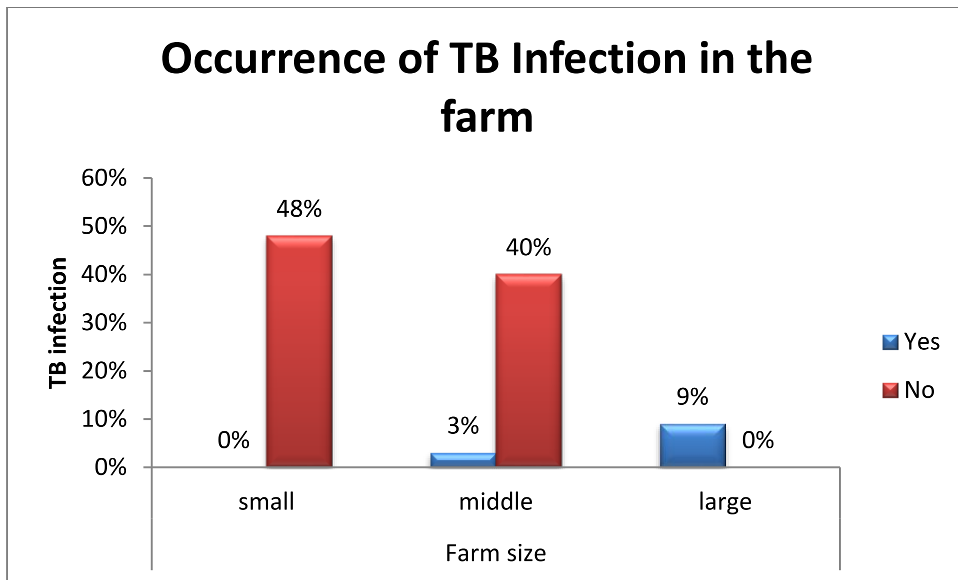

Figure: 3.8 Responses to questions on experiences of TB infections in farms.

3.4 DISCUSSION

Commercial farms are usually described as more capital-intensive and less labour intensive than small family farms whereas smallholding farming refers to the use of small pieces of land without using advanced and expensive technologies for production of crops and livestock (Katale *et al*., 2013). The commercial farms and smallholding farms were located in urban and rural areas respectively.

According to the results of this study, smallholding farmers represented farmers with small herd size (herds between 10-20); whereas farms of 30-40 cows were said to be middle sized. However, a large number of herd size in commercial farms may create opportunities for bTB risk if farm management is not reliable; this can also be the case in smallholding farmers whereby infection can be due to lack of farming skills. Humblet *et al*. (2010) reported that the higher the density of the animal population, the higher the probability of close contacts between them and the highest incidence of bTB is generally observed in areas where intensive farming is practiced (Figure 3.1). Moreover, smallholding farmers normally face challenges due to climate change, limited financing options, and inadequate access to health and nutritious food, and as a result, it is very difficult for them to develop to a larger herd size (van Arendok and Liinamo, 2003).

Vaccination is administered to create immunity in animals and prevent diseases. The vaccination programme chosen for farm animals depends on the management system, the location of the farm and history of the herd, and whether or not the disease is confirmed (NAOH, 2007). Most young farm animals are usually more susceptible to infection, so they are frequently given vaccines (NAOH, 2006). In this study results showed that 9% of

commercial owners carry out vaccination every 12 months. Five percent (5%) and 41% of respondents from smallholding farms mentioned that vaccination was done every 18 months and 24 months respectively (Figure 3.2). The study also showed 30% and 10% of respondents from the middle sized (herds between 30-40) indicating that vaccination was done in every 24 months and 18 months respectively (Figure 3.2). The respondents in smallholding farms mentioned that vaccination was given for other diseases like anaplasmosis; instead of tuberculosis since they do not have knowledge about TB in cattle. Adwoa *et al*. (2014) reported in Ghana that commercial farmers frequently give animals vaccination.

Inappropriate mixing of cattle, when giving vaccination, may create risk of bovine TB transmission between farm animals (NAOH, 2006). However, farm animals from commercial farms may not suffer from the infection since vaccination is done frequently, unlike in smallholding farms. Furthermore, commercial farmers are frequently aware of prevalent infections so they rapidly prevent or control any disease from spreading between animals and smallholding farmers lack of knowledge regarding diseases common to farm animals and have shortage of facilities to control and prevent infections.

The results showed that smallholding farms were not aware that their farms had history TB whereas all respondents in commercial farm reported history of TB cases. This study was not similar with results in Tanzania where 60% of respondents had history about TB cases in commercial farms. The commercial farmers have access to assets, and they take a lot of responsibility to do test for diagnosis of the disease, which increase chances to be aware of cattle that are affected with TB. On the other hand, smallholding farmers are unaware of infected cattle since they lack knowledge about TB infection in cattle and also lack access to

resources to perform test for diagnosis of TB. Smallholding farms are commonly owned by families, and often produce livestock for consumption. They also market, albeit minimally, as a source of income to provide for the family (Chawatama *et al*., 2005)

Wild animals are commonly known as a source of bovine tuberculosis to farm animals, and the infection is transferred through feeding because some wild animals have access to animal feed. It becomes very difficult for farmers to prevent contact between farm animals and wild animals since this usually occurs in the field where the cattle feed. Moreover, most farm owners especially in smallholding farms are not aware that bTB infection is transmitted from wild animals to their livestock. The results obtained in this study showed that 51% of respondents in small size (herds between 10-20) and 40% of those from the middle size (herds between 30-40) farms had knowledge about wildlife access to their farms and also all commercial farm owners responded. Katale *et al*. (2013) reported that 81% of respondents were aware of wildlife access in their farms, a result contradictory to our study.

The most common wild animal that usually had contact with farm animals was namely deer, and there are different kinds of deer such as the white tailed deer, red deer and cherry deer; but the most common is the white tailed deer (Windsor, 2008). The boar was the second most common wild animal, and this wild animal species is normally located in fields of rural areas. In this study, both commercial farms and smallholding farms reported challenges regarding wild animal access to their farms. In Belgium, the influence of wildlife densities on the emergence of bTB outbreaks was observed (Humblet *et al*., 2010).

Importing of cattle is the introduction of new herds to another farm, and this results in an increase of the herd size. This study showed that 9% of respondents of commercial farms import cattle. Ten percent (10%) respondents of the small size (herds between 10-20) and 24% of the middle size (herds between 30-40) import cattle (Figure 3.5). The commercial farmers practice importation of cattle which are used for breeding to produce calves and this may result to bTB transmission. A study performed in Britain showed animal movement as insignificant risk factor for bovine TB (Humblet *et al*., 2010). However, in rural areas where smallholding farmers are found, they sell or buy bulls for draught purposes than for meat production. Pen *et al*. (2012) stated that cattle selling and buying occurs most commonly between farmers with female cattle. Female cattle are bought for cow-calf production, and in smallholding farms, breeding programmes are lacking. Windsor (2008) stated that rural farmers have less knowledge of husbandry and do not make much profit to advance husbandry.

Cattle sickness may influence farm size because if vaccine is not given, the cattle may end up dying and that will decrease the number of cattle. The results obtained in this study showed respondents of 43% of the small size (herds between 10-20) and 27% of the middle size (herds between 30-40) in smallholding farms reporting that anaplasmosis was a common sickness. Respondents (9%) from the large commercial farms also mentioned anaplasmosis sickness. Results of this study are not similar to that of a Chinese study where (32.35%) of respondents mentioned anaplasmosis in cattle (Zhang *et al*., 2013). Some species of *Anaplasma* having zoonotic significance have been reported in 7.5% in Egyptian farmers with close proximity to domestic livestock such as *A. Phagocytophilum* (Ghafar and Eltablawy, 2011). Most respondents from both commercial and smallholding farms had experience of anaplasmosis in their livestock; however small holding farmers did not find it easy to determine the type of the disease the cattle are suffering from due to lack of education. Anaplasmosis can be spread

among cattle by blood, for example by stinging insects, red legged tick and dehorning and injecting animals with the same needle. Farm owners should take quick action for sick cattle, and the medicine should be used for the disease when necessary. It is encouraged to give medicine to cattle once the disease has been diagnosed.

Health management is a major criteria in the improvement of livestock industry generally. Livestock health problems could cause obstructions to running a business in livestock because particular diseases decrease production and increase morbidity and mortality (Duvel and Stephanus, 2000; Mwacharo and Drucker, 2005; Chawatama *et al*., 2005). The results of this study revealed that 9% of respondents of commercial farms, practice health management in cattle by vaccination (Figure.3.2). In smallholding farms respondents mentioned that health management is practised by vaccination and culling. Respondents from smallholding farms reported that they practice health management by vaccination and culling. A study in the United Kingdom reported that infected cattle with bovine TB were removed by culling and it has also been exploring the efficacy of cattle vaccination (Good and Duignana, 2011). Smallholding farmers practice health management by culling or any other measurement like burying, and most respondents had no knowledge about TB infection in cattle due to poor education and as result only 10% was giving vaccination for the disease. Consultation with Veterinarians for health programmes that decrease the spread of disease to new born calves is a very crucial intervention measure (Duvel and Stephanus, 2000). This health precaution is rarely practiced in smallholding farms as compared to commercial farms.

Results of this study revealed that all respondents of the commercial farm owners reported experience of TB infection in their farms (Figure 3.8). No respondent of the small size (herds

between 10-20) farm reported experience of TB infection while respondents of 3% of the middle size (herds between 30-40) farmers reported experience of TB infection in smallholding farms. These results are similar with the ones in Tanzania where the awareness of respondents to bovine TB was very low and 64.8% of the respondents had never heard about bovine tuberculosis (Katale *et al*., 2013). Since in commercial farms there is high intensive population of farm animals where there are different species, contact between animals may result to incidence of bovine TB (Good and Duignana, 2011). On the other hand, incidence of TB may not be identified in smallholding farms because of small population of farm animals and few farms had different species (Drucker, 2005). However, commercial farmers have advanced innovative practices and technology in farm management, and therefore may not be easily affected by TB infection. Commercial farms are situated in developed environments where they have access to several facilities such as water, veterinary doctors, and space for keeping and controlling their cattle (Zhang *et al*., 2013. Climate change and variability have may adverse effects on livestock production through increasing diseases and insects which become more abundant as a result of changes in climatic conditions; these insects attack livestock and transmit infection to livestock vectors such as ticks.

3.5 CONCLUSION AND RECOMMENDATIONS

This study found that there was no breeding program to manage the genetic improvement in smallholding cattle production systems. Another point is that risk factors exist as common factors that affect both smallholding and commercial farms. This is because both rural and urban farmers have no control over these risk factors, but damage through dangerous insects can be minimized through use of pesticides that do not harm cattle (Ghafar and Eltablawy, 2011). These include calving, weaning, shipping, working or moving the cattle, and extreme weather conditions (Chawatama *et al*., 2005). Stress can reduce an animal’s ability to resist infectious agents. It is advisable to give extra attention to the animals’ health after a period of stress.

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CHAPTER FOUR

Detection of *Mycobacterium tuberculosis* complex from soil, water and hayfeed obtained from three commercial dairy farms in the Eastern Cape.

ABSTRACT

Tuberculosis (TB) continues to be a worldwide disease that contributes to the challenges of health problems in nations. Millions of people are diagnosed with the disease each year and TB is the second highest infection that leads to death amongst infectious diseases in countries worldwide. In this study 330 samples were collected from Fort Hare dairy farm, Middledrift dairy and Seven star dairy farm investigated in Eastern Cape, South Africa. One hundred and fifty water samples were collected from three points in various locations of specific site such as rivers, streams and ponds that serve as source of drinking water for the cattle which included upper stream, middle stream and lower stream; 90 soil samples were collected from three randomly selected points where cattle are normal located; 90 hayfeed samples were collected from three randomly selected points where cattle feed and used for analysis. Fifty water samples were collected from three selected points of rivers, ponds and streams at each farm on a weekly basis for four weeks; 30 soil samples were collected from three randomly selected points at each farm on a weekly basis for four weeks; 30 hayfeed samples were collected from three randomly selected points at each farm on a weekly basis for four weeks. The samples were processed using the modified Petroff's method and cultured on Lowenstein Jensen media and incubated at 37°C for 8 to 12 weeks. DNA was extracted directly from processed samples using boiling method for PCR identification. Fifty five isolates were positive for MTBC using PCR assay. Isolation of DNA samples ranged from 25(45%) in water samples to 12(22%) in

soil samples. Fort Hare dairy farm had the highest number of MTBC samples 11(44%) from water samples while Middledrift dairy farm had the lowest MTBC positive from water, 2(17%). Water and soil were found to be risk factors for the transmission of bovine tuberculosis and revealed the fact that farm practices needs to be improved.

Keywords: Tuberculosis, water, soil, Polymerase Chain Reaction (PCR), *Mycobacterium tuberculosis* complex

4.1 INTRODUCTION

Bovine tuberculosis (bTB) is an infectious disease caused by *Mycobacterium bovis*, a member of the *Mycobacterium tuberculosis complex* (MTBC), which includes the closely related *M. tuberculosis*, the major causative agent of human tuberculosis (TB) (van Soolingen *et al*., 1994). Worldwide TB caused about 2 million deaths and about 9 million new cases are reported annually, with sub-Saharan Africa having the highest annual risk of infection with TB (Corbett *et al*., 2003). Globally, *M. bovis* accounts for 3.1% of all human TB cases (Cosivi *et al*., 1998). However, the extent of *M. bovis* involvement in the global TB burden in Africa is still largely unknown. This can be partly explained by the fact that in humans, TB due to *M. bovis* is indistinguishable from TB caused by *M. tuberculosis* in terms of clinical signs, radiological and pathological features (O'Reilly and Darbon, 1995). Although cattle are considered to be the main hosts of *M. bovis*, the disease has been reported in many other species, including humans, other domesticated animals, and wildlife (deLisle *et al*., 2002). Therefore, the risks encountered by cattle may also be the same in humans.

Indeed, little information on risk factors of disease transmission to cattle, between cattle and from cattle to humans, is available from an African context. Most information is extrapolated from experiences in developed countries. For Africa, the most comprehensive studies done so far have been in Tanzania (Kazwala *et al*., 2001; Ayele *et al*., 2004 and Mfinanga *et al*., 2004) and Uganda (Oloya *et al*., 2007). In countries where bovine TB is uncontrolled, most human cases occur in young persons and results from drinking or handling contaminated milk (Cosivi *et al*., 1998). This study therefore is one of the few that investigates bovine tuberculosis risk factors in the Eastern Cape Province of South Africa. The aim of this study was to isolate and identify MTBC from water, soil and hayfeed using molecular and culture based techniques.

4.2 MATERIALS AND METHODS

4.2.1 Study sites

Amadlelo Project has established four dairy farms known as the Fort Hare dairy trust, Middledrift dairy farm and Seven star dairy trust farm. Fort Hare dairy was established in 2007 and has 800 cows, Middle drift was established in 2008 and has 600 cows and Seven star dairy trust farm was established 2009 and comprises 600ha of farmland and has 900 cows. Fort Hare dairy is found in the Eastern Cape Province in Alice with geographic coordinates of 32°46′59″ South and 26°49′59″ East. Middledrift dairy is also found in the Eastern Cape Province with geographic coordinates of 32° 49' 0" South, 26° 59' 0" East. Seven star dairy trust as well found in the Eastern Cape Province in Keiskammahoek with geographic coordinates are 32° 37' 0" South, 27° 7' 0" East.

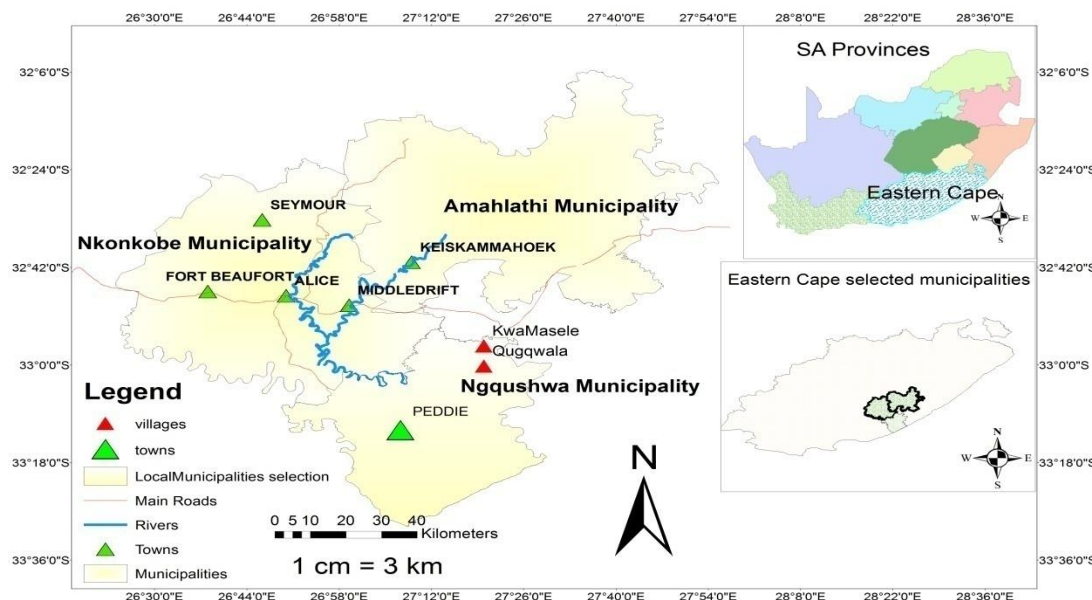

Figure 4.1 A map showing study sites of the Municipalities in the Eastern Cape Province.

4.2.2 Sample collection

In this study, 330 samples were collected from three commercial farms (Fort Hare, Middledrift and Seven star dairy trust farms) in Eastern Cape Province, South Africa on a weekly basis for a period of four months. One hundred and fifty (150) water samples were collected from three points in various locations of specific site such as rivers, streams and ponds that serve as source of drinking water for cattle which included upper stream, middle stream and lower stream, 50 water samples were collected from three selected points of rivers, ponds and streams at each farm on a weekly basis for four weeks (Figure 4.2). Ninety (90) soil samples were collected from three randomly selected points where cattle are normal located, 30 soil samples were collected from three randomly selected points at each farm on a weekly basis for four weeks (Figure 4.3). Ninety (90) hayfeed samples were collected from three randomly selected points where cattle feed, 30 hayfeed samples were collected from three randomly selected points at each farm on a weekly basis for four weeks (Figure 4.4). Separate large capacity sealed whirl-pack bag was filled with 10 g of soil and10 g of hayfeed. Ten millilitre (10 ml) of water was collected in capped 0.5 litre sterile plastic bottles that were capped .The samples were placed in a cooler box containing ice packs and transported to the laboratory within 8 hours of collection for analysis immediately on arrival.

Figure 4.2 Showing sampling site of water collection at Middledrift dairy farm.

Figure 4.3: Showing sampling site of soil collection at Fort Hare dairy farm.

Figure 4.4 Showing sampling site of cattle feeding on hayfeed at Seven star dairy trust

4.2.3 Decontamination of the Samples

Seven and half millilitre 7.5 mL of sterile water and 5 mL of 4% sodium hydroxide were added to the solid substrate (soil, hayfeed) of 10 g in the whirl-pack bags. The whirl-pack bags were placed in a Stomacher 80 laboratory blender for 30 s and samples were then milled and merged. Five millilitre (5mL) of 4% of sodium hydroxide was added to the water samples, and mixed with a vortex machine at high speed for 30 s. The samples were allowed to settle for 30 min positioned upright. Forty-five millilitre (45 mL) conical tube which contains 10mL of Decontamination Solution (20X Tris-citrate Buffer, 4% of sodium hydroxide and water) was mixed together with top 10mL of liquid from each processed samples. Samples were incubated at 37ºC for 75 min and mixed with a vortex machine. Sterile water was added to the 45 mL mark in each tube, mixed and centrifuged at 3,000 g for 20 min. Supernatant contained in tubes was decanted completely. A pipette was used to carefully remove all but 1–3mL of liquid from samples without a visible pellet. One mL of sterile water was added and mixed. A 0.5 mL of

sample was transferred to a 2.0 mL labelled cryogenic vial, and frozen at -80ºC and maintained for additional experiments (Fine *et al*., 2011).

4.2.4 Culture of the samples

Inoculation of decontaminated samples was done onto solid media slants and plates containing modified Middlebrook 7H11 agar (Becton-Dickinson) with sodium pyruvate and 7H11 containing glycerol (Becton-Dickinson). Plates and slants were incubated at 37ºC for 8–12 weeks and observed weekly for colony development. Growth of colonies appear within 3–6 weeks of incubation depending on the media used (Fine *et al*., 2011).

4.2.5 Acid- fast staining

Decontaminated samples were stained using Ziehl-Neelsen stain to validate the presence of acid fast bacteria. Smears were prepared and stained according to the Ziehl–Neelsen technique after bacterial growth was visible (Fine *et al*., 2011).

4.2.6 DNA amplification

4.2.6.1 Inactivation of pathogens

To inactivate the other bacterium organisms 0.5-ml aliquot of the decontaminated sample was incubated at 75ºC for 1 hour.

4.2.7.2 Bacterial lysis and DNA Extraction

The inactivated samples were centrifuged at 13,000 rpm in a microcentrifuge for 5 min and the supernatant was discarded. The pellet was washed with 1 ml of sterile distilled water and recentrifuged for 5 min. The pellet was resuspended in 100 ml of a proteinase K (0.1 mg/ml) solution, and the mixture was incubated the mixture for 1 h at 56ºC. The samples were boiled for 5 min after incubation and centrifuged for an additional 5 minutes after they were equilibrated to room temperature. The supernatant was finally transferred to a new 1.5-ml micro-centrifuge tube and stored at -20˚C (Adams *et al*., 2013).

4.2.7.3 DNA amplification by PCR

A PCR procedure was done using a mixture of the two PCR primer sets targeting IS*6110*. The PCR assay in the experiments enabled enhanced detection of MTBC DNA in processed environmental samples. PCR amplification was done with an initial 25µl reaction mixture containing 12.5µl Promega GoTag Green2 x Master Mix (Promega, Fitchburg, WI), 7.1µl water and 5µl DNA with 0.2µl each of forward primer CGTGAGGGCATCGAGGTGGC and reverse primer CCTGCGAGCGTAGGCGTCGG. The IS*6110* insertion sequence found in the MTBC genome targets this primer and an amplification product of 252 bp is produced. The initial denaturation and enzyme activation step of 94°C for 4 min was followed by 20 cycles of 94°C for 30 s, 67°C for 30 s, and 72°C for 30 s and a final extension step of 72°C for 5 min. A 1.5% agarose gel containing ethidium was used for gel electrophoresis of the PCR product. All PCR processing was ran with both positive and negative-control samples and standard precautions were taken to avoid false-positive results due to laboratory contamination (Adams *et al*., 2013).

4.3. RESULTS

4.3.1 Culture

There were no positive samples for MTBC when isolated using mycobacterial culture due to non-target organisms that took over the plates.

4.3.2 Staining

There were no acid-fast bacilli observed in all samples stained.

4.3.3 Molecular detection of MTBC

Fifty five samples were positive for *IS6110*. The amplification product of 252 bp was obtained (Figure 4.5).

1 2 3 4 5 6 7 8 9 10 11 12 13 14

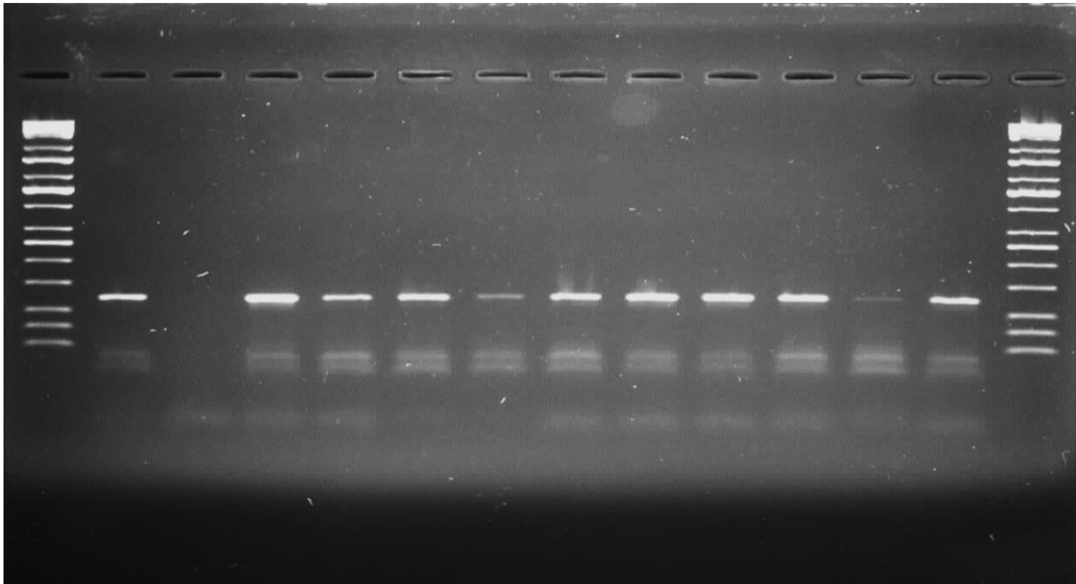

Figure: 4.5.: **Amplification product from the primer pair of the *IS6110* insertion sequence.**

Lane 1: Kappa universal DNA ladder, Lane 2: positive control (252bp), Lane 3: negative control, Lane 4: water sample -3, Lane 5: soil sample -12, Lane 6: hayfeed sample - 10, Lane 7: SS-8, Lane 8: WS-5, Lane 9: WS-20, Lane 10: HFS-30, Lane 11: WS-43, Lane 12: HFS-35, Lane 13: SS-39, Lane 14- DNA ladder. The molecular size expected for *IS6110* was 252 base pair.

Fifty five isolates were positive for MTBC using PCR assay. The results include 25(45 %) water samples, 12 (22%) soil samples and 18(33%) for hayfeed samples.

Table 4.1: Samples showing a positive *IS6110* gene from the three different dairy farms

Substrates	Total No. of positive samples	Fort Hare dairy farm(+)	Middledrift dairy farm(+)	Seven star dairy trust farm(+)
Water	25	11	6	8
Soil	12	6	2	4
Hayfeed	18	7	5	6

4.4. DISCUSSION

Mycobacterium bovis is known to be an intracellular microorganism; however, it continues to exist outside the host for extensive periods during suitable circumstances from the environment (Fine *et al*., 2011). There were no samples that were positive for MTBC on mycobacterial culture. This might be because of contamination and growth of other organism observed. In Michigan, it was reported that none of the samples had growth during mycobacterial culture due to the occurrence of contamination with mould and non-mycobacteria (Adams *et al*., 2013). Several efforts to isolate MTBC have also failed from alleged physically contaminated environmental samples by bacterial culture (Cooney *et al*., 1997, Witmer *et al*., 2010).

Molecular detection of MTBC indicates the existence of MTBC in the environment although it does not specify that viable cells do occur (Adams *et al*., 2011). The amplified samples in this study that were positive using PCR assay showed the bands at 252bp. Fifty five samples that were positive for the *IS6110* band where 25/55(45%) were from water samples,12/55(22%) from soil samples and 18/55(33%) from hayfeed samples. Fort Hare dairy farm showed 11/25(44%) of water samples, which is higher than 6/25(24%) in Middledrift dairy farm and 8/25(32%) in Seven star dairy trust farm. The study showed Fort Hare dairy farm with higher percentage of water samples positive for MTBC as compared to other two farms; the reason may be that cattle were not fenced away from natural water sources where it is possible to prevent MTBC transmission. A study in Michigan reported that MTBC was detected from water samples at 48 days in winter and soil was negative at 20 days during summer sampling period (Fine *et al*., 2011). Previous studies observed that animals splash water while drinking, and this might lead to the entrance of bacteria into the respiractory tract because of infected drops of water (Cleaveland *et al*., 2007). In the United State of America, it was confirmed that

cattle excrete their faeces on flooding water which may result in contamination (Humblet *et al*., 2009). In Africa, where most herds utilise similar water points, this might cause a certain risk factor of bTB transmission (Phillips *et al*., 2003). In the present study MTBC DNA was detected more in water samples using PCR assays.

More soil samples (6/12(50%)) were positive for MTBC DNA from Fort Hare dairy farm compared to 2/12(17%) in Middledrift dairy farm and 4/12(33%) in Seven star dairy farm. The observation could be because the moisture content was also not shown among the soil samples for survival of mycobacterium in Middledrift dairy farm and Seven Star dairy farm. Several studies reported that detection of MTBC in a soil becomes successful at 88 days during winter and spring sampling period (Fine *et al*., 2011). Previous studies suggested that MTBC is expected to persist in slurry-treated soil for two years.

Fort Hare dairy farm showed 7/30(23%) hayfeed samples to be positive for MTBC, 5/30(17%) amplification of *IS6110* in Middledrift dairy farm and 6/30(20%) for Seven star dairy farm .In this study, from three farms not much difference of percentages was identified and it is because wild animals are commonly found in farms and have access to hayfeed where farm animals feed. It was also observed that hay was purchased from a single supplier. If contamination occurred from the supplier, it will be carried to those three farms. The studies conducted in Michigan confirmed that MTBC strain was found to persist for about to 12 weeks on feed (Fine *et al*., 2011). In this study combined results for three farms showed 18/90(20%) of hayfeed had *IS6110*. An Irish study in 1993 reported that a self-feed silage system was more stressful for animals and that result in enhanced susceptibility to bTB (Witmer *et al*., 2010). In a study carried out in two English farms in 1999, silages were approved to be attractive to badgers

(Garnett *et al*., 2002). This could explain our results where badgers feed on the hayfeed meant for cattle undetected, thereby transmitting the organisms to hayfeed.

It is essential to use both molecular techniques and bacterial culture for identification of MTBC in environmental samples. To detect MTBC by bacterial culture takes longer time to produce results as compared to PCR assay which has the ability to provide results in a few hours (Adams *et al*., 2013).

4.5. CONCLUSION AND RECOMMENDATIONS

In this study water, soil and hayfeed were identified as risk factors for MTBC in cattle in Fort Hare, Middledrift and Seven star dairy farms. Polymerase Chain reaction-based assays is valuable for determining MTBC in the environment although it does not tell if the organism is viable or not. Improvement of biosecurity in farms is needed to decrease bovine TB risk from the herd level to preventing transmission among farm animals.

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CHAPTER FIVE

Assessing genotypic drug resistance through detection of mutations conferring resistance to INH and RMP associated with the first line drugs in *M. tuberculosis complex* treatment using Genotype MTBDR*plus* assay.

ABSTRACT

Multidrug-resistant (MDR) TB has been known to be an important health crisis with recent difficulties of restricting its spread. Genotype® *Mycobacterium tuberculosis*-multidrug resistant *plus* (MTBDR*plus*) assay kit was used to detect mutation on genes *rpo*B, *kat*G and *inh*A *i*n DNA isolates from 55 isolates. Twelve out of 55 (22%) isolates showed resistance to INH and RMP and (9%) were sensitive to either INH or RMP. The mutations at *rpo*B gene showed fifty eight percent (58%) which was the highest when compared to twenty three (23%). Fifty seven percent (57%) of the samples showed a S315T1 mutations while only 14% possessed a S531L in the *kat*G gene. The most *inh*A mutations detected were T8A (80%) and the least was A16G (17%). These observation illustrate the need for further investigations to develop a more rapid and specific assay for the detection of MDR MTBC to be used as screening in prevalent areas.

Keywords: Multidrug resistance, Genotype MTBDR*plus* assay, Multiplex PCR, Gene, Mutation

5.1 INTRODUCTION

Tuberculosis (TB) is caused by a group of closely related bacteria, collectively known as the *Mycobacterium tuberculosis* complex (MTBC) (Cole, 1998). TB in humans is mainly caused by *M. tuberculosis* and *Mycobacterium africanum* (de Jong *et al*., 2010). In addition, several animal-adapted members of MTBC exist, which affect a range of wild and domestic animal species (Smith *et al*., 2005). These include *M. bovis* which is a pathogen of cattle; *Mycobacterium caprae* found in sheep and goats; *Mycobacterium microti* from voles and *Mycobacterium pinnipedii* found in seals and sea lions (Du *et al*., 2011). *Mycobacterium bovis* is an antique cause of tuberculosis that infects individuals through ingestion of contaminated particles or in- take of uncooked milk from infected cattle (Du *et al*., 2011). *Mycobacterium bovis* used to be a significant cause of human TB, primarily in children who consumed raw milk (Smith *et al*., 2006). It was a major public health problem when it was transmitted to humans in milk from cattle (Morris *et al*., 1995). The introduction of pasteurization of milk and milk products helped to eliminate this problem. In Horred, Sweden the public health significance of consuming milk containing *M. bovis* was well illustrated in an outbreak of tuberculosis that occurred in 1994 (O'Reilly and Darbon, 1995). Previous studies also identified meat infected with *M. bovis* to be cause of tuberculosis in humans (Kazwala *et al*., 2001).

In humans, TB co-infection with the Human Immunodeficiency Virus (HIV) and rapidly spreading Acquired Immune Deficiency Syndrome (AIDS) epidemic has significantly worsened the situation (Fätkenheuer *et al*., 1999; Larson, 2000; Corbett *et al*., 2002; Corbett *et al*., 2003; WHO, 2005; Corbett *et al*., 2006). Also, the widespread development of drug-resistant strains has complicated the treatment of TB in humans and significantly increased the

cost associated with the use of multiple drug therapy (Thoen and Ebel, 2006; Thoen *et al*., 2009). Although TB is a major cause of human deaths, the real extent of human TB due to zoonotic agents is not known (O'Reilly and Daborn 1995; Ashford *et al*., 2001; Thoen *et al*. 2009). Over 70% (6 million) of humans co-infected with TB and HIV/AIDS live in sub-Saharan Africa (O'Reilly and Daborn 1995; Cosivi *et al*., 1998; Corbett *et al*., 2006) where bovine TB represents a potential health hazard. The aim of this study was to detect mutations conferring resistance to isoniazid (INH) and rifampicin (RMP) using Genotype MTBDR*plus*.

5.2 MATERIALS AND METHODS

5.2.1 Genotype Resistance Detection

5.2.1.1 DNA isolation

GenoLyse® kit (Hain Lifescience, Germany) for bacterial DNA extraction was used according to manufacturer's instruction. Five hundred microliters of sediment were transferred in Eppendorf tube of 1500 µl. Suspension was centrifuged at 10,000g in aerosol-tight rotor for 15 min. Supernatant was discarded. One hundred microliters of lysis buffer was added to the sediments and vortexed to homogenize. The suspension was inactivated at 95 °C for 5 min. One hundred microliters of neutralization buffer was added to the preparation. The inactivated suspension was centrifuged at 13,000g for 5 min. The DNA contained in the supernatant was transferred into a fresh tube. A negative control was included in each run of environmental substrate sample decontaminated for DNA extraction (N'guessan *al.*, 2014).

*5.2.1.2 Amplification of the rpo*B *and kat*G *genes*

Genotype MTBDR*plus* assay version 2.0 (Hain Lifescience, Nehren, Germany) was performed as recommended by the manufacturer. The amplification mixture contained 35 µl of primer-nucleotide Mix B, 10 µl of Mix A, 5 µl of 10X polymerase incubation buffer, 2 µl of $MgCl_2$, 3 µl of molecular water, 0.2µl of Ampli*Taq* Gold polymerase, and 5 µl of extracted chromosomal DNA solution. Amplification parameters used were as follows: 15 min of denaturation at 95 °C, followed by 20 cycles of 30 s at 95 °C and 2 min at 65 °C, followed by

30 additional cycles of 25 s at 95 °C, 40 s at 53 °C, and 40 s at 70 °C with a final extension step of 8 min by 1 cycle at 70 °C (N'guessan *et al*., 2014).

5.2.2 GenoType® MTBDRplus Assay

GenoType® MTBDR*plus* kit (Hain Life science, Nehren, Germany) was used to perform Drug susceptibility testing and identification of mutations conferring resistance to RMP and INH, according to the manufacturer's 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. Thermal cycler MyCycler TM (Bio-Rad, Cape Town, South Africa) was used to perform amplification. The protocol 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 (N'guessan *et al*., 2014). 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) was used for detecting INH resistance (Ashford *et al*., 2001). When all WT probes stained positive and no mutation band formed, the result was interpreted as susceptible to the respective antibiotic (N'guessan *et al*., 2014). 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.

5.3 RESULTS

The bands observed for *rpo*B gene amplification of RMP yielded 165 bp PCR products compared to DNA size marker in agarose gel electrophoresis. The bands observed for *kat*G gene amplification of INH yielded 170 bp PCR products (Figure 4.1).

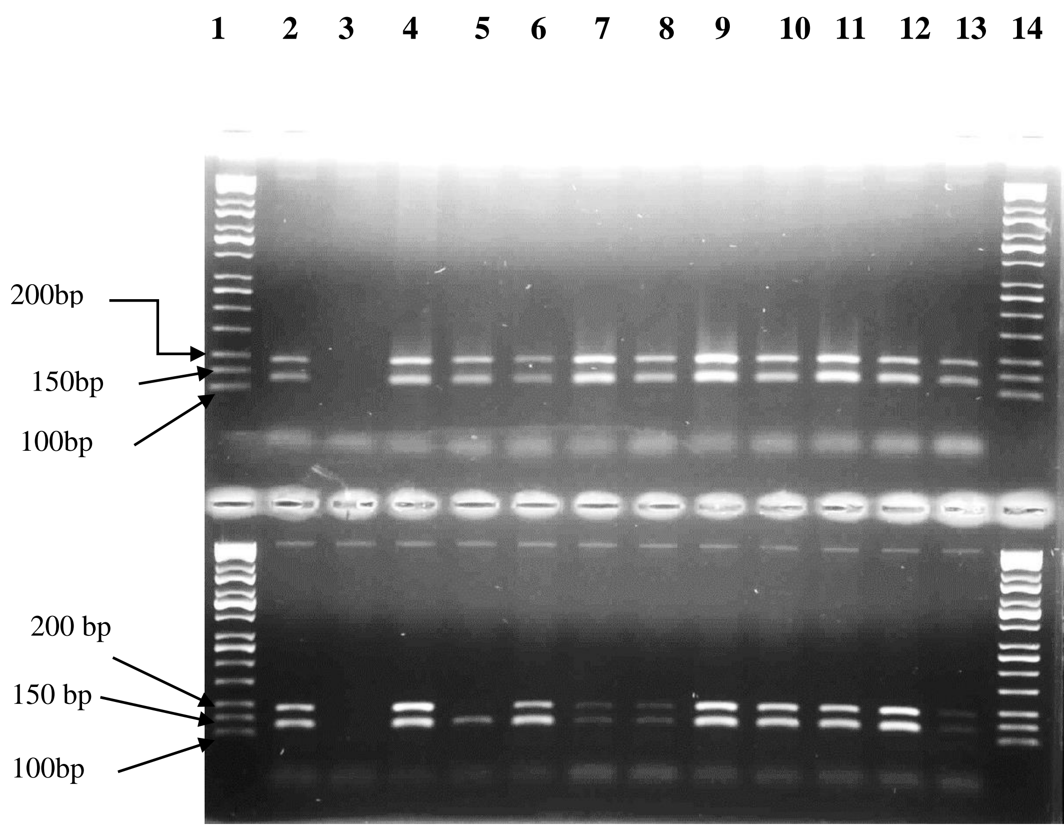

Figure 5.1: Representative gel of amplification product from mutations of *rpo*B gene and *Kat*G gene

Upper Lanes: Lane1-Kappa universal ladder, Lane2-positive control, Lane3-negative control, Lane4-13-*rpo*B gene amplification of RMP yielded 165 bp, Lane14- Kappa universal ladder. Lane4-8 carry wild probes and Lane10-13 carry mutant probes. **Lower Lanes:** Lane1-Kappa universal ladder, Lane2-positive control, Lane3-negative control, Lane4-13-*kat*G gene amplification of INH yielded 170bp, Lane14-Kappa universal ladder. Lane4-9 carry mutant probes and Lane10-13 carry wild probes.

5.3.1 Mutation conferring resistance to RMP and INH

The mutations at *rpo*B gene for RMP resistance ranged from 80% being the highest for H526Y (water sample) to the least 0% for H526V (soil sample), H526Y (soil and hayfeed samples) and S531L (hayfeed sample). The mutations at *kat*G gene for INH resistance ranged from 100% being the highest for S315T2 (hay feed sample) to the least 0% for S315T2 (soil and hayfeed samples). The mutations at *inh*A region promoter for INH resistance ranged from 50% being the highest for A16G (water sample) to the least 0% for A16G (hayfeed sample) (Table 5.1).

Table 5.1 Mutations in *rpo*B, *kat*G and *inh*A genes from different samples collected at Fort Hare dairy farm.

Mutations	Water	Soil	Hayfeed
RMP-*rpo*B gene			
D516V	10/15 (67%)	1/15 (7%)	2/15 (13%)
H526Y	8/10 (80%)	0 (0%)	0 (0%)
H526V	2/6 (33%)	0 (0%)	2/6 (33%)
S531L	2/9 (22%)	1/9 (11%)	0 (0%)
INH-*kat*G gene			
S315T1	5/20 (25%)	3/20 (15%)	2/20 (20%)
S315T2	5/5 (100%)	0 (0%)	0 (0%)
INH-*inh*A region			
C15T	10/25 (40%)	4/25 (16%)	3/25 (12%)
A16G	3/6 (50%)	2/6 (33%)	0 (0%)
T8C	7/15 (47%)	1/15 (7%)	1/15 (7%)
T8A	12/28 (48%)	2/28 (7%)	2/28 (7%)

***rpo*B gene-** D516V total: 15, H526Y total: 10, H526D total: 6, S531L total: 9

***kat*G gene**- S315T1 total: 20, S315T2 total: 5

***inh*A region**- C15T total: 25, A16G total: 6, T8C total: 15, T8A total: 28

The mutations at *rpo*B gene for RMP resistance for DNA samples from Middledrift dairy farm ranged from 22% for S531L (water sample) being the highest to the least 0% for D516V (water sample), H526Y (soil sample), H526D (all samples) and S531L (soil and hayfeed samples). The mutations at *kat*G gene for INH resistance ranged from 15% being the highest for S315T1 (water sample) to the least 0% for S315T1 and S315T2 (all samples). The mutations at *inh*A promoter region for INH resistance ranged from 20% being the highest to the least 0% for A16G and T8C (Table 5.2).

Table 5.2: Mutations in *rpo*B, *kat*G and *inh*A genes from different samples collected at Middledrift dairy farm.

Mutations	Water	Soil	Hayfeed
RMP-*rpo*B gene			
D516V	0 (0%)	2/15 (13%)	0 (0%)
H526Y	2/10 (20%)	0 (0%)	0 (0%)
H526D	0 (0%)	0 (0%)	0 (0%)
S531L	2/9 (22%)	0 (0%)	0 (0%)
INH-*kat*G gene			
S315T1	3/20 (15%)	2/20 (10%)	0 (0%)
S315T2	0 (0%)	0 (0%)	0 (0%)
INH-*inh*A region			
C15T	2/25 (8%)	1/25 (4%)	1/25 (4%)
A16G	0 (0%)	0 (0%)	0 (0%)
T8C	3/15 (20%)	0 (0%)	2/15 (13%)
T8A	5/28 (18%)	1/28 (4%)	2/28 (7%)

***rpo*B gene-** D516V total: 15, H526Y total: 10, H526D total: 6, S531L total: 9

***kat*G gene**- S315T1 total: 20, S315T2 total: 5

***inh*A region**- C15T total: 25, A16G total: 6, T8C total: 15, T8A total: 28

The mutations at *rpo*B gene for RMP resistance for DNA samples at Seven star dairy trust ranged from 33% being the highest for H526D (water sample) and S531L (hayfeed sample) to the least 0% for D516V (all samples), H526Y (all samples), H526D (soil and hayfeed samples) and S531L (soil sample). The mutations at *kat*G gene for INH resistance ranged from 15% being the highest for S315T1 (soil sample) to the least 0% for S315T1 (all samples) and S315T2 (hayfeed sample). The mutations at *inh*A promoter region for INH resistance ranged from 17% being the highest for A16G (water sample) to the least 0% for C15T (hayfeed sample), A16G (soil and hayfeed samples) and T8C (soil and hayfeed samples) (Table 5.3).

Table 5.3 Mutations in *rpo*B, *kat*G and i*nh*A genes from different samples collected at Seven star dairy farm.

Mutations	Water	Soil	Hayfeed
RMP-*rpo*B gene			
D516V	0 (0%)	0 (0%)	0 (0%)
H526Y	0 (0%)	0 (0%)	0 (0%)
H526D	2/6 (33%)	0 (0%)	0 (0%)
S531L	1/9 (11%)	0 (0%)	3/9 (33%)
INH-*kat*G gene			
S315T1	2/20 (10%)	3/20 (15%)	0 (0%)
S315T2	0 (0%)	0 (0%)	0 (0%)
INH-*inh*A region			
C15T	2/25 (8%)	2/25 (8%)	0 (0%)
A16G	1/6 (17%)	0 (0%)	0 (0%)
T8C	1/15 (7%)	0 (0%)	0 (0%)
T8A	3/28 (11%)	1/28 (4%)	1/28 (4%)

***rpo*B gene-** D516V total: 15, H526Y total: 10, H526D total: 6, S531L total: 9

***kat*G gene**- S315T1 total: 20, S315T2 total: 5

***inh*A region**- C15T total: 25, A16G total: 6, T8C total: 15, T8A total: 28

5.3.2 Overall drug resistance pattern

The Genotype MTBDR*plus* Assay when used for detection of INH and RMP resistance showed 35/55 (64%) being the highest for INH resistance to the least 5/55(9%) for RMP & INH. Drug resistance detection rate at Fort Hare dairy farm ranged from 46% being the highest for RMP resistance to the least 0% for RMP&INH sensitivity. Drug resistance pattern at the Middledrift dairy farm ranged from 31% being the highest for RMP and INH to the least 0% for MDR. Drug resistance pattern at the Seven star dairy farm ranged from 80% being the highest for RMP&INH sensitivity to the least 0% for MDR (Table 5.4).

Table 5.4 Drug susceptibility pattern of samples performed by the Genotype MTBDR*plus* Assay.

Antibiotic susceptibility pattern		**Number of Samples (+)**	**Fort Hare dairy farm**	**Middledrift dairy farm**	**Seven Star dairy farm**
RMP	Resistant	26/55 (47%)	12/55 (22%)	8/55 (15%)	6/26 (23%)
INH	Resistant	35/55 (64%)	15/55 (27%)	11/55 (20%)	9/55(16%)
RMP & INH	MDR	12/55 (22%)	12/55 (22%)	0 (0%)	0 (0%)
RMP & INH	Sensitive	5/55 (9%)	0 (0%)	1/55 (1.8%)	4/5 (7.2%)

MDR- Multi-drug resistance

Table 5.5 show RMP resistance in the three dairy farms according to sample type. RMP resistance in Fort Hare dairy farm ranged from 42% being the highest in soil samples to the least 25% for hayfeed samples. RMP resistance in Middledrift dairy farm ranged from 50% being the highest for soil samples to the least 25% for water and hayfeed samples. That of the Seven star dairy farm ranged from 50% being the highest for soil samples to the least 17% for water samples

Table 5.5 RMP resistance in the three different dairy farms according to sample type.

Samples	**Fort Hare dairy farm**	**Middledrift dairy farm**	**Seven star dairy farm**
Water	4/12 (33%)	2/8 (25%)	1/6 (17%)
Soil	5/12 (42%)	4/8 (50%)	3/6 (50%)
Hayfeed	3/12 (25%)	2/8 (25%)	2/6 (33%)

Table 5.6 show INH resistance pattern in the three dairy farms according to sample type. INH resistance in Fort Hare dairy farm ranged from 40% being the highest for soil and hayfeed samples to the least 20% for water samples. INH resistance for Middledrift dairy farm ranged from 36% being the highest for water and soil samples to the least 27% for hayfeed samples. That of Seven star dairy farm ranged from 56% being the highest for soil samples to the least 22% for water and hayfeed samples.

Table 5.6 INH pattern in the three dairy farms according to sample type.

Samples	Fort Hare dairy farm	Middledrift dairy farm	Seven star dairy farm
Water	3/15 (20%)	4/11 (36%)	2/9 (22%)
Soil	6/15 (40%)	4/11 (36%)	5/9 (56%)
Hayfeed	6/15 (40%)	3/11 (27%)	2/9 (22%)

5.4 DISCUSSION

Drug resistance in *Mycobacterium tuberculosis* complex occurs as random mutations in genes conferring resistance to antituberculosis drugs. Drug resistance can be evaluated quickly in a few hours using molecular assays such as the Genotype MTBDR*plus* assay that permits quick and definite identification of the common point mutations conferring resistance to Isoniazid (INH) and Rifampicin (RMP) resistance (Dahal *et al*., 2013). However, molecular methods designed to detect resistance in *M. tuberculosis* have some limitations. Since only the more frequent mutations related to RMP and INH resistance are detected by the Genotype MTBDR*plus* assay, the results must be confirmed by phenotypic methods. Although the common mutations predictive of resistance are well known for some drugs, in some cases the mutations identified are silent and are not always related to the acquisition of resistance (Yang *et al*., 2012). In addition, the exact ratio of resistant to susceptible bacilli that results in phenotypic resistance is unclear. This means that in practice, a molecular assay result can differ from the one obtained by a susceptibility proportion method, such as the method conducted with the Bactec 460TB system. The identification of a resistance mutation by a molecular test in clinical samples is clinically informative and useful, whereas the absence of a mutation in the target sequence analysed must be interpreted cautiously (Simon, 1999). MTBDR*plus* assay may be a very useful tool for the management of TB because it allows the identification of RMP and INH-resistant *M. tuberculosis* in both clinical samples and bacterial isolates.

Water is one of the key constituents required for extant and thriving of carbon based life form (Rani *et al*., 2013). Its pollution is one of the most challenging environmental issues and has become a global impediment for improving the quality of life in many communities (Anon,

1996). The major source of water pollution comes from the discharge of domestic and agricultural wastes, and untreated sanitary and toxic industrial effluents (Li *et al*., 2013). The presence of pollutants in water bodies can be pernicious to aquatic life as well as render it unsuitable as potable water sources for domestic usage (Simon, 1999). Sequel to pollution of freshwater environment is a life-threatening effect on man's healthy living (Yang *et al*., 2012).

The results obtained in this study revealed a high (33%) prevalence RMP resistance gene in water samples from Fort Hare dairy farm, which was higher than that observed in Middledrift dairy farm (25%) and Seven star dairy farm (17%). Although water showed a low level of RMP resistance, it remains a very important conduit for TB in cattle. D'Angelo *et al*. (2004) observed that *Mycobacterium avium* can be isolated from hospital water. Water released from domestic and industrial sources flow into rivers. Cattle utilizing water from these rivers containing contaminants such as fluids from nearby hospitals may end up getting sick. Mycobacterial infections linked to contaminated hospital water have been recognized for many years (Wallace *et al*., 1998).

Many studies have concentrated on resistance from clinical isolates in *Mycobacterium tuberculosis* complex. However, with water acting as a conduit for diseases, it is also proper to find out if the organisms isolated from this solvent also harbors antibiotic resistant traits. INH and RMP form part of the first-line drugs used in the treatment of tuberculosis in humans. Organisms showing resistance to both INH and RMP at the same time are said to be multi-drug resistant (Miotto *et al*., 2006). From this study a total of 22% of samples from Fort Hare farm were shown to harbour genes for resistance to both INH and RMP, while no resistance to both INH and RMP was observed in Middledrift and Seven star farms. The relatively high rate of

MDR in Fort Hare compared to other sampled sites may due to the fact that it is situated next to the waste water treatment plant and they use its water to clean milking parlour and milking utensils. River water within the vicinity of Fort Hare is used to water vegetables and grass used to feed cattle. This creates advanced health risk to people working at Fort Hare farm. Silaigwana *et al*. (2012) detected MDR isolates in raw milk from Fort Hare farm. In Cote d'Ivoire, MDR was observed in relapse cases from human specimen (N'guessan *et al*., 2014). In Nepal, it was reported that MDR isolates were observed and differed in all the ten distinct patterns of mutation (Dahal *et al*., 2013). In previous studies on human specimen, the prevalence of MDR–TB was reported to be high due to mutation that were detected by one probe of 4 *rpo*B gene mutations which was shown on the strip, and this was reported as high in South Africa (Huyen *et al*., 2010).

Approximately 20% of water samples from Fort Hare farm showed mutations in genes conferring resistance to INH gene only, which was lower than what Seven star dairy farm 22% and Middledrift dairy farm 36% (Table 5.6). Hospital wastes are washed away into rivers during flooding and pit may contribute to the results obtained. Water from the waste water treatment plant is used for crop irrigation as well as drinking water for animals. There has been an increase in the number of potentially pathogenic mycobacterial species whose transmission route is associated with water (Shelton *et al*., 1999).

At the genetic level, gene alterations can change the structure of a target protein via mutations in the coding region or the amount of the protein expressed by modulating gene regulation, both of which ultimately cause the anti-drug resistance in MTBC (Li *et al*., 2013). Resistance to RMP was observed in 47% of water samples from three farms in this study. Fort Hare dairy

farm showed resistance of 22% which was the higher as compared to 14% from Middledrift dairy farm and 11% from Seven star dairy farm. A South Vietnam study showed 93% of rifampicin strains from sputum specimen (Huyen *et al*., 2010) which has higher resistance than the present study. The overall mutation of D516V for *rpo*B in this study was 58% which is higher than other *rpo*B mutations observed from other two farms in this study. However, in water samples, the most frequently mutated *rpo*B codons were H526Y (80%), D516V (67%) and S531L (22%) from Fort Hare dairy farm, which is similar to observations reported elsewhere (Metcalfe *et al*., 2004; Hilleman *et al*., 2005; Jiao *et al*., 2007). This is an indication that there is no difference in the main mutation types and mutation rates of *rpo*B gene according to geographic location in this region. However, our study is different in a sense that, it was done on environmental samples, while other studies were conducted on clinical samples (Taniguchi *et al*., 1996, Yue *et al*., 2003). Water samples from Middledrift and Seven star dairy farms showed RMP-*rpo*B gene with mutations at codons H526Y (20%), S531L (22%) and H526D (33%), S531L (11%) (Table 5.3). This is not surprising as Middledrift and Seven star dairy farms are furthest from Alice. Fort Hare dairy farm is in Alice which is situated close to waste water treatment plant and a nearby hospital.

There is evidence of *Mycobacterium bovis* in soil (Sweeny *et al*., 2007); however, very little evidence exists of *Mycobacterium tuberculosis* complex isolated from soil. Detection of resistant genes to RMP was shown to be high in Middledrift (50%) and Seven star farms (50%) soil samples as compared to Fort Hare farm (42%) (Table 5.5). Hruska *et al*. (2011) suggested that soil is easily contaminated by fertilisation with manure of liquid dung or by water contaminated by animal faeces. Couternay *et al*. (2006) mentioned that the presence of pathogenic bacteria in slurry which is spread on pasture is a potential source of infection for

susceptible grazing animals. The detection of MTBC with RMP resistance from soil sample may pose a risk to both cows and humans working at the farms where this study was done. Mutations for *rpo*B observed from soil samples obtained from Fort Hare dairy farm were only two, D516V (7%) and S531L (11%) (Table 5.1). Middledrift dairy farm only showed one mutation D516V (13%) from soil sample (Table 5.1). Although this was the most abundant mutations in the previous study (Metcalfe *et al*., 2004; Hillemann *et al*., 2005; Jiao *et al*., 2007), they were very few mutations in soil samples. The reason for this could be the fluctuation of temperature degrading the DNA as well as the current carrying away most of the organisms from water to other villages; therefore fewer organisms were sprayed onto the field (Simon, 1999).

It was previously shown that piles of feed, set out to attract deer and improve their productivity and winter survival, are thought to contribute to the transmission of TB among white-tailed deer (Metcalfe *et al*., 2010) thereby increasing local density and contact between animals (Hillemann *et al*., 2005) and providing an opportunity for the indirect transmission of TB through contamination of the feed by infected deer shedding *M. bovis* in their saliva or nasal discharges. Hayfeed samples from Seven star dairy farm (33%) contained *rpo*B gene showing a high resistance to RMP as compared to Fort Hare dairy farm (25%), and Middledrift dairy farm (25%). Seven star farm uses hayfeed as a bedding material in winter and this might promote cross contamination. The presence of mycobacteria in bedding was also reported out by Piening *et al.* (1972) who isolated 67 strains. An observation was made concerning specific supplemental feeding practices that might be associated with an increasing risk for bovine TB in the study setting. Within the *rpo*B gene, mutations S531L (0%) was lower than mutation H526V (33%) and D516V (13%) from Fort Hare dairy (Table 5.1). However, mutations

observed from H526Y from water samples were higher than other mutations from soil and hayfeed. Similar results were reported in Russia by Nikolayevskyy *et al*. (2009).

The overall resistance to INH obtained in this study was (64%), the highest rate (43%) of mutation was detected from Fort Hare, while Middledrift (31%) and in Seven star dairy farm (26%) were lower. It is not surprising to find this high rate of INH resistance at Fort Hare farm since the same waste water treatment plant that has been shown to contain MTBC, is used to water plants and as drinking water for cows. The resistance rates in Fort Hare dairy farm are lower than those reported by Silaigwana *et al*. (2012) who reported 90.9% resistance. However, his study was done on milk, which can pose immediate risk to humans consuming the contaminated milk. A study in Spain showed 62.95% of samples had mutations in genes conferring INH resistance (Lacoma *et al*., 2008).

MTBC DNA isolated from soil samples showed 40% INH resistance signature from Fort Hare dairy farm. This is higher than the 36% detected in Middledrift dairy farm but lower than the 56% from Seven star dairy farm (Table 5.7). Cole (2002) revealed that there are more mycobacteria in soil and water. Mutations observed in *kat*G gene isolated from water sample obtained from Fort Hare Dairy farm were S315T1 (25%) and S315T2 (100%). These mutations are more frequent in Fort Hare dairy farm than in Middledrift S315T1 (15%), S3152 (0%) and Seven star dairy trust S315T1 (10%) and S315T2 (0%). Normally, mutation at codon 315 is said to be a high level mutation with a good chance of the organism in which the mutation was detected showing phenotypic resistance (Yue *et al*., 2013). From Kwazulu Natal these mutations were found in contrast with the present study (Kiepiela *et al*., 2000); whereas van Rei and collegues reported observations similar to results of this study in isolates from clinical samples in Western Cape (Talenti *et al*., 1993).

Low level mutation in the *inh*A region was also observed from water samples. The A16G (50%) mutation was the highest observed from Fort Hare dairy farm and it was higher than A16G (17%) mutations observed from Seven star dairy trust and A16G (0%) from Middledrift dairy farm. Other low mutations shown in the results section, may not cause phenotypic resistance, however, if detected in one organism, they may account for 40% of resistance to INH worldwide (Couternay *et al*., 2006).

However, water from the contaminated waste water treatment plant from Fort Hare dairy farm is used to water the feed before it is cut and stored. From this study, Fort Hare dairy farm (40%) showed the highest detection of INH resistance genes compared 22% in Seven star dairy farm and 27% in Middledrift dairy farm with reference to hayfeed samples. The higher percentage of INH resistance observed in Fort Hare dairy farm could be because of wild animals having access to hayfeed which may result in advance of mycobacteria that contain resistant genes and which may lead to cross contamination. The observation of the present study are lower than with the ones reported by Fine *et al*. (2011) in the study performed in Michigan showing 67%. Palmer *et al*. (2012) reported a large number of mammal species visiting farms and having close contact with farm animals and sharing feed. This could also contribute to an increase in prevalence of organisms causing diseases and their resistance to antibiotics in specific farms. All the farms in this study produce their own hayfeed. They store the hayfeed so that they can give to cows during drought and when they milk the cows.

5.5 CONCLUSION AND RECOMMENDATIONS

This is the first report to the best of my knowledge on the simultaneous detection and genotypic analysis of the *rpo*B and *kat*G genes isolated from environmental samples in the Eastern Cape Province of South Africa. Overall, 47% showed RMP resistance, 64% INH resistance and 22% were MDR. The observation illustrate the need for further investigations to develop a more rapid and specific assay for the detection of MDR MTBC to be used as a screening method in these areas where there is prevalent MTBC. Although no organism grew when cultured, these results cannot be overlooked. These observations also suggested that the drug resistance mechanisms of these first-line antituberculosis drugs were not completely clear and still need further research.

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CHAPTER SIX

6.1 GENERAL DISCUSSION

Mycobacterium bovis the causative agent of bovine TB, continues to circulate among livestock in the farms and wildlife and environmental substrates remain as risk factors for spreading bTB infection. The results of this study revealed that the insertion sequence *IS6110* that encode pathogenicity for MTBC was successfully amplified by PCR thus confirming that the isolates were pathogenic strains (Shelton *et al*., 1999). Water samples were found to create more chances of bovine TB transmission among cattle and some measurements must be taken into consideration to limit the high rate of bTB risk. There is high rate of bTB transmission in water because farm animals and wild animals normally share water sources (Humblet *et al*., 2009) and can this lead to bovine TB to cattle.

In regions where the occurrence of MTBC tuberculosis is prevalent, control measures must be practiced in domestic cattle and wildlife to prevent transmission. These measures necessitate the implementation of both offensive and defensive (preventive) measures. Hay- feed also creates risk of bTB transmission among cattle. Humblet *et al*. (2009) suggested improvement of farm management as essential in order to decrease bTB risks at the herd level such as secure feed storage, correct management of slurry spreading, secure feeding habits, and correct hygiene, including introducing a grazing system that limits wild animals having access to cattle feed and avoiding contact.

In commercial farms, according to respondents in this study have knowledge about history of TB cases and TB infection and diagnosis of TB are taken into consideration using skin test. On the other hand, smallholding farms according to respondents had no knowledge about bTB in cattle; hence they mentioned vaccination in cattle for other diseases like anaplasmosis, but not for TB .

This study revealed low levels of MDR from the three farms and that pose considerable health risk to local residents who rely on unpasteurized milk for nourishment. The resistance rate (22%) observed in Fort Hare dairy farm are lower than those reported by Silaigwana *et al.* (2012) who reported 90.9% resistance. However, Silaigwana and co-workers' study was done on milk, which can pose immediate risk to humans consuming the contaminated milk. Cattle utilise water sources in dams that contain human fluids from nearby hospitals and cattle excretion and this may result in chances of infection. This might be the reason why a slightly higher resistance was observed in Fort Hare dairy farm than other farms investigated in this study because it is situated close to waste water treatment plant and a nearby hospital (Victoria). Mycobacterial infections linked to contaminated hospital water have been recognized for many years (Wallace *et al*., 1998). The mutations were detected in all three farms with the samples identified respectively, hence, this assay is significantly needed to be applied in areas where there is advanced high risk of TB especially in Forte Hare dairy farm.

6.2 CONCLUSION AND RECOMMENDATIONS

The observations of this study show that water, soil and hayfeed are risk factors for MTBC in farm animals. The feed stores should be protected from wild animals, domestic dogs and cats to avoid interaction and sharing. The questionnaire responses highlighted that commercial farmers had moderate knowledge relating to risk factors for bovine TB in cattle and smallholding farmers were clueless about bTB risk in cattle. The cattle production of smallholding farmers was assessed as very low in terms of management and feeding.

The Genotype MTBDR*plus* assay represents a rapid, reliable, and easy to perform test for the simultaneous detection of RMP and INH resistance in *M. tuberculosis* complex. In the present study the detection of MDR is a big challenge and rapid diagnosis technique like Genotype MTBDR*plus* is essential to counteract such challenge.

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