Molecular characterization of mycobacterial species isolated from tuberculous lesions seen in bovine carcasses at post mortem: a case study of three abattoirs in the Accra Region of Ghana.

By

Samuel Kumah Atiadeve BSc. (Hons.), Dip. Bis. Admin. and Mgt. (ICM, UK)

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Faculty of Biosciences

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DECLARATION

I hereby declare that this submission is my own work towards the award of the MSc and that, to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any other degree of the University, except where due acknowledgement has been made in the text.

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Supervisor(s) Name Signature Date

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Head of Dept. Name Signature Date

ABSTRACT

Tuberculosis in cattle poses a serious public health threat and yet there is very little information on its extent in Ghana. A study to screen and characterize suspicious carcasses at post-mortem in three selected abattoirs in Accra was conducted. A total of 2,886 cattle slaughtered between June and October, 2009 were examined at post-mortem. One hundred and fifty five (155) suspicious tissue samples were taken from 145 bovines and subjected to Ziehl-Neelsen (ZN)

microscopy and subsequently cultured on Löwenstein-Jensen (L-J) media. Isolates were harvested and subjected to ZN microscopy and then screened with the Capilia TB-Neo test and also characterized by a 13-probe Multiplex Ligation-dependent Probe Amplification assay (MLPA). The results revealed an apparent lesion prevalence of 5.0%. ZN microscopy indicated that 32.3% (50/155) of tissues were acid fast. Culture on L-J yielded 85.8% (133/155) isolates. One hundred and twenty-nine out of one hundred and fifty-five (129/155 or 97%) of these were acid fast by ZN microscopy. The Capilia TB-Neo test was performed on 93 of the isolates. Capilia TB-Neo test identified 11.8% (11/ 93) as members of the *Mycobacterium tuberculosis* Complex (MTBC) and 88.2% (82/93) as Non tuberculous mycobacteria (NTM). A further characterization of isolates using MLPA revealed that 3.8% (5/130) were *M. africanum* and 6.2% (8/130) were *M. bovis*. Surprisingly, 38.5 % (50/130) were classified as MTB (not *M. bovis* or *M. africanum*). Mixed infection was also identified in 42.9% (3/7) bovines. A comparison of MLPA and Capilia TB-Neo tests using the latter as a "Gold Standard" revealed that MLPA has a sensitivity and specificity of 72.7% and 39.7% respectively. The positive predictive value (PPV) and negative predictive value (NPV) were 16.3% and 90.0% respectively. The data obtained indicates that contamination of beef by TB is prevalent and therefore could pose a serious health threat. More stringent management and control methods should be put in place to improve beef quality.

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DEDICATION

To my mum and dad

LIST OF ABBREVIATIONS

AA	Accra Abattoir
AFB	Acid Fast Bacilli

AIDS	Acquired Immuno-Deficiency Syndrome
BCG	Bacille-Calmette Guérin
BLN	Bronchial Lymph Node
bp	Base pair
BTB	Bovine Tuberculosis
CCT	Comparative Cervical Tuberculin Test
CFT	Caudal-Fold Test
CRD	Cardiac Tissue
DNA	Deoxyribonucleic Acid
dNTP	deoxyribonucleic Triphosphates
DOTS	Directly Observed Treatment Short Course
DST	Drug Susceptibility Testing
ELISA	Enzyme-linked Immunosorbent Assay
g	Gramme
G+C	Guanine plus Cytosine
GAEC	Ghana Atomic Energy Commission
HD	Head

HIV	Human Immuno-deficiency Virus
HYB	Hybridization
INH®	Isoniazid Resistance
IS6110	Insertion Sequence 6110
KIT	Royal Tropical Institute, Amsterdam, The Netherlands
KNUST	Kwame Nkrumah University of Science and Technology.
LIG	Ligation
LIV	Liver
L-J	Löwenstein-Jensen
LN	Lymph Node
LT	Lung Tissue
MDR-TB	Multiple Drug Resistant Tuberculosis
MELT	Melting
MIX	Mixture
ml	Millilitre
MLN	Mesenteric Lymph Node
MLPA	Multiplex Ligation-dependent Probe Amplification

MRC	Medical Research Council
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* Complex
NALC	N-acetyl-L-cystein
NTM	Non-Tuberculous Mycobacteria
OTT	Official Tuberculin Test
PCR	Polymerase Chain Reaction
PSLN	Pre-Scapular Lymph Node
RAMSRI	Radiological and Medical Sciences Research Institute
RIF®	Rifampicin Resistance
RIVM	National Institute of Public Health and The Environment, Bilthoven, The Netherlands
RLN	Retro-Pharyngeal Lymph Node
RNAse	Ribonuclease
rRNA	ribosomal Deoxyribonucleic Acid
RXN	Reaction
SCT	Single Cervical Tuberculin Test
sddH_2O	Sterile Double Distilled Water

SLN	Supra-Mammary Lymph Node
SNP	Single Nucleotide Polymorphism
SS+	Sputum Smear-Positive
TA	Tema Abattoir
TB	Tuberculosis
TbD1	*M. tuberculosis* Specific Deletion 1
TST	Tuberculin Skin Test
TU	Tulaka Abattoir
UD	Udder
WHO	World Health Organization
ZN	Ziehl-Neelsen

CHAPTER 1

1.0 INTRODUCTION

Tuberculosis (TB) is an infectious disease of warm-blooded animals caused by an acid-fast bacillus (AFB) belonging to the *Mycobacterium tuberculosis* Complex (MTBC). Diseases caused by the members of this complex are characterized by the formation of granulomatous lesions (tubercles) mostly in the lungs and its associated lymph nodes. Bovine Tuberculosis (BTB) which refers to tuberculosis infection in cattle is also of considerable concern due to the fact that humans are susceptible to *Mycobacterium bovis*, the causative agent of the disease (Kleeberg, 1984). Tuberculosis is considered a threat due to its contagious nature and can also lead to economic losses in an infected herd (Kent and Kubica, 1985). Even though *Mycobacterium tuberculosis* is the cause of TB in humans, an unknown proportion is as a result of *Mycobacterium bovis* infection (Acha and Szyfres, 1987). The trans-species relationship of *Mycobacterium bovis* between cattle and humans underlies the zoonotic nature of the disease.

In most countries in the world, the relevance of tuberculosis to public health and the importance of the disease to the economy has been established (Konhya *et al.*, 1980; Brosch *et al*., 2002). In view of the devastating consequences of tuberculosis, The World Health Organization (WHO), declared the disease a world health emergency in 1995.

Members of MTBC are pathogenic and consist of a group of closely related bacteria belonging to Mycobacteriaceae, a family of more than hundred different types of bacterial species (Noaki *et al.*, 2000). The members of the MTBC comprise *Mycobacterium tuberculosis, Mycobacterium bovis, Mycobacterium bovis BCG, Mycobacterium microti, Mycobacterium africanum* ,

Mycobacterium pinnipidii and *Mycobacterium carnetti*. Broadly, there are tuberculous mycobacteria and non-tuberculous mycobacteria (NTM). They are characterized by 99.9% similarity at the nucleotide level and have identical 16S rRNA sequence (Brosch *et al.*, 2002). They however differ largely in their pathogenicity, phenotypes and type of host (Wayne and Kubica, 1986).

Non-Tuberculous Mycobacteria (NTM) on the other hand, are ubiquitous organisms, found mainly in the environment i.e. soil and water and have also been reported to only cause disease in individuals with an underlining medical condition (particularly in immuno-compromised individuals) (Cassidy, 2006).

Tuberculosis has plagued mankind for a very long time and evidence is available to show that the disease affected both humans and animals long before recorded history. Globally, the incidence of TB is largely under-reported resulting in the under-estimation of the disease. Nonetheless, the resurgence of the disease barely three decades ago has brought to the fore its opportunistic relationship with HIV/AIDS (Daniel, 2006).

There are 22 high TB-burden countries in the world which constitute an estimated 80% of all new TB cases annually (WHO, 2008). In Africa, the disease is considered a major problem because of abject poverty as TB incidence continues to increase year after year. In 1990 for instance, the estimated number of TB prevalence on the continent was 829, 377 out of which 212,228 died. In 2006 however, the estimated number of TB prevalence shot up drastically to 2,807,688 killing 639,089 of the infected people (WHO, 2008). The WHO reported that Ghana

recorded approximately 47,632 new TB cases in 2007 killing 9% of the registered 7,786 patients even before they could finish their treatment (WHO, 2008).

Bovine tuberculosis is an economically important zoonosis affecting many countries in the developing world; and Ghana is no exception. A survey carried out in the Dangme-West District of the Eastern Region in Ghana revealed that the prevalence of bovine tuberculosis disease in some kraals investigated was 50% even though the total average prevalence was 13.8% (Bonsu *et al.*, 2000). Also, Ankugah (2000) reported that the prevalence of bovine tuberculosis in the Ho district of Volta Region was 3.1% even though it was 5.9% in a cluster.

Management of transmission of bovine tuberculosis is traditionally done either by adopting strategies to eradicate the disease or control its spread. An eradication programme aims at eliminating all infected animals through surveillance of the disease. This, however, is capital intensive and cannot be supported by most developing countries. However, data collected from the abattoir provide a good and efficient alternative for a disease surveillance system (Brown and Anda, 1998).

In Ghana, the Ministry of Food and Agriculture through its Veterinary Services Division has a policy to examine all cattle slaughtered at the various abattoirs for the presence of granulomatous lesions suggestive of bovine tuberculosis infection in an effort to control the spread of the disease from cattle to humans. Cattle with localized infections at post-mortem i.e. infection in

one organ and/or its associated lymph node only have the affected part trimmed off and the carcasses passed for human consumption. However, in animals with generalized infection i.e. infection in the entire carcass, the whole animal is deemed unwholesome and therefore condemned (Personal communication).

Several diagnostic procedures exist for screening TB in both humans and animals even though treatment of the disease is mostly prohibited in animals due to its contagious nature (American Thoracic Society, 1990). In humans, the screening of the disease is categorized into conventional (Acid fast smear microscopy, chest radiography and mycobacterial culture) and non-conventional molecular based methods. In terms of animal health, most of the diagnostic procedures available for screening *M. bovis* infection are field based. They are however slow, cumbersome and do not produce results in good time. Consequently, there is the need to adapt a more rapid, safer and verifiable method to diagnose the disease and the Polymerase Chain Reaction (PCR) provides a very good approach (Saiki *et al.,* 1985; Tortoli *et al*., 2001). Here, the presence of *Mycobacterium tuberculosis* DNA (MTB DNA) in cultured isolates can be determined in forty eight hours even though inhibitors in tissue samples could interfere with PCR performance (Haddad *et al.,* 2004; Singh *et al.,* 2004). A major challenge of PCR-based procedures is that they require highly trained personnel with adequate equipment (Richter *et al.,* 2004). Capilia TB-Neo test, an immuno-chromatographic assay therefore provides a simple and rapid procedure to distinguish between MTBCs and NTMs. Although some Capilia TB-Neo negative samples have been isolated in Japan (Hirano, 2004), the immuno-chromatographic assay has a relatively high sensitivity and specificity of 99.2% and 100% respectively (Abe *et al.,* 1999).

1.1 JUSTIFICATION

The consumption of beef and other dairy products continues to be a major source of protein to people in Ghana. Therefore, their quality and safety should be an important consideration in public health management. The identification and isolation of *M. bovis* in bovine tissue samples have therefore become imperative since TB is now a major problem in Ghana (www.USAID.com) and information on bovine tuberculosis is at best, scanty. Cosivi *et al*. (1998) reported that there is a direct correlation between the infection of *M. bovis* in cattle and the presence of the disease in humans and practically all non-pulmonary TB cases are as a result of bovine tuberculosis infection (Schwabe, 1984). The danger posed by *M. bovis* in the realm of public health therefore cannot be over-emphasized. A big challenge is the lack of information at the genetic level about the etiology and epidemiology of *Mycobacterium tuberculosis* strains, particularly *M. bovis*, in Ghana.

This study is therefore an attempt to shed light on the prevalence and nature of MTB (particularly *M. bovis*) in beef. Data and other information to be generated by this study can be employed to design effective strategies to control and manage the disease.

1.2 PROBLEM STATEMENT

In Ghana, beef and other protein-based food items are either not cooked or grilled well, e.g. khebab before consumption, thereby posing serious health questions about the safety of the food.

The quality of beef from the abattoir is therefore of utmost importance in public health safety. Bonsu *et al.* (2000) reported that there is a high level of ignorance about the bovine tuberculosis disease among cattle farmers in the Dangme-West district of the Greater Accra Region resulting in milk not being pasteurized before consumption and thereby increasing the risk of gastrointestinal tuberculosis infection. It follows therefore that information on the disease in the region is inadequate.

Due to the potentially infectious nature of bovine tuberculosis, coupled with the lax regulation governing cattle movement and the permit regime across the country, it is important to have reliable information on the disease at the point of slaughter. Metropolitan abattoirs provide an ideal and controlled environment as a monitoring point for the screening of beef samples at post mortem. Indeed, at the abattoirs selected for the research, post mortem inspection is carried out using the naked eye. However, a system of bovine TB control based on meat inspection at the abattoir is such that a possibility of not detecting incipient lesions on the beef exists (de Kantor *et al.*, 1987; Whipple *et al.*, 1996).

1.3 RESEARCH OBJECTIVES

1.3.1 Main objective

The main objective of this research is to use a molecular based method and an immuno-chromatographic assay to characterize *M. bovis* strains in cultured bovine tissue samples collected at post-mortem at three metropolitan abattoirs in the Greater Accra Region of Ghana and to determine the prevalence of the disease.

1.3.2 Specific objectives

- To identify the presence of Acid Fast Bacilli (AFB) in bovine tissue samples with lesions suggestive of bovine tuberculosis using Ziehl-Neelsen Microscopy.

- To distinguish between *Mycobacterium tuberculosis* Complex organisms and Non-Tuberculous Mycobacteria from cultured isolates using Capilia TB-Neo test.

- To use a Multiple Ligation-dependent Probe Amplification (MLPA) assay to characterize acid fast isolates obtained from the beef samples.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 BRIEF HISTORY AND EPIDEMIOLOGY OF TUBERCULOSIS

Tuberculosis, also known as the 'white plague', has always been a disease of both human and animals for a long time. *M. tuberculosis*, the causative agent of TB in humans is thought to have originated over 150,000 years ago and is responsible for most mortalities than any other known

pathogen (Daniel, 2006). However, contemporary members of the MTBC are known to have evolved from a common ancestor roughly 15,000-35,000 years ago (Gutierrez *et al*., 2005). Also, documents exist to show the presence of the disease in China, India and Egypt as far back as 2,300, 3,300 and 5,000 years ago respectively (Daniel, 2006).

TB is infectious and an active TB individual infects an average of between ten and fifteen people each year once the disease is not treated. Over two billion people the world over are infected with the TB bacilli (WHO, 2008). One million seven hundred and seventy seven thousand people died from TB infection out of the 9.27 million (139 per 100,000 population) new cases reported in 2007. Out of this staggering number, 1.37 million (19.7 per 100,000) representing 14.8% were HIV-positive. The WHO (2008) also reported that only 4 million (61 per 100,000 population) of that number were identified as smear positive cases. Also, an estimated 500,000 of these cases were attributed to Multi-Drug Resistant-TB (MDR-TB), a form of TB infection that is caused by MTBC strains resistant to both Isoniazid and Rifampicin, both first line anti-tuberculosis drugs (CDC, 2006).

Research conducted in Africa revealed that an estimated 85% of the cattle population and 82% of humans on the continent stayed in BTB endemic areas (Cosivi *et al.*, 1989; Michel, 2002). However, in Sub-Saharan Africa for instance, although an estimated two million new cases are recorded annually, the number attributable to *M. bovis* is still not known (Daborn, 1992).

2.2 THE TUBERCLE BACILLI

All *Mycobacteria* are non-motile rods and do not form spores. They have an unusually high level of G+C (61-70) in their DNA with high levels of mycolic acids in their cell walls. The tubercle bacilli have a waxy coat made up of lipids which makes them resistant to injury. The high lipid content of the cell wall is also implicated in the slow growth rate of the mycobacterium as it could limit nutrient intake by the cell. This explains why the disease takes a long time to develop and can also stay dormant in a host for its lifetime. However, they cannot tolerate long exposure to sunlight, heat and general dry conditions and can only depend on their host to multiply (www.TuberculosisTextBook.com).

2.3 TUBERCLE MORPHOLOGY

Microscopically, it is reported that discriminating between *M. tuberculosis* and *M. bovis* using conventional methods is difficult (Rodriguez *et al.*, 1995). The waxy nature of the tubercle cell wall makes it impermeable to aniline dyes unless phenol is added. When the tubercle bacilli, stained with auramine or carbol-fuchsin, are viewed under a microscope, they appear as slightly curved or straight rods. However, any dyed tubercle bacillus is usually uneven and faintly coloured as a result of some loss in the organism's content but improper staining may also cause this situation. The shape and size of the bacilli is influenced by the age and growth conditions of the culture. They are 1-10 μm in length (often 3-5 μm) and 0.2-0.6 μm in width. The bacilli are also acid fast, implying that they are resistance to decolourlization by an acid alcohol after it had been stained with an aryl methane dye e.g. carbol-fuchsin (www.TuberculosisTextBook.com). Factors such as specific mycobacteriophages infection (Frank *et al.*, 1992; Gangadharam and

Stager, 1976), cell cutolysis or trauma and cell exposure to antibiotics that target bacterial cell wall synthesis e.g. isoniazid (Mohammed *et al*., 2004) can affect the acid fastness of the organism. The bacillus can be kept for a long time at temperatures between 2 to 70ºC but thawing affects its viability as it requires proper adaptation to resume synthesis and growth (Kim, 1979). On a growth media, the bacilli show a number of characteristics and identification is based on colony morphology, growth rate and pigment production. Typically, *M. bovis* grown on an egg based media such as Lowenstein-Jensen (with Pyruvate or Glycerol) is tiny (<1mm), translucent or opaque, smooth, pyramidal and dysgonic. NTMs on the other hand are often smooth, pigmented and usually grow within seven days (Duffield *et al.*, 1989).

2.4 BOVINE TUBERCULOSIS

Bovine tuberculosis is caused by a non-photochromogenic bacillus called *M. bovis*. The pathogen affects all warm-blooded animals including humans even though cattle are the natural host of the bacilli. Horses and sheep are highly resistant to the disease (Radostits *et al.,* 2000; Theon *et al.*, 2006). Although the disease is now rare in developed countries, it is fairly common in developing countries (Cosivi *et al*., 1998). This is partly due to the poor animal husbandry systems and practices. At present though, the possible transmission of *M. bovis* to humans in developing countries is receiving some attention due partly to the fact that, it has been realized that, in most rural settings, humans and cattle live in close proximity and can therefore be a source of disease transmission (Fritsche *et al.*, 2004).

2.4.1 Infection

In BTB infection, the level of bacterial load in cattle is critical in bringing about disease. Factors that contribute to infection, rate of disease development or progression include the amount or level of the initial bacterial dose, immunity of the cattle, the kind of conditions creating the aerosol and the age of the infected cattle (Munroe *et al.,* 2000). Although the natural dose levels may vary from one animal to the other, inducing the dose with a bacterial concentration of between 3×10^2 and 2×10^7 bacilli, done through the trachea, can cause the disease (Cassidy *et al.,* 1998; Walravens *et al.,* 2001; Palmer *et al.,* 2002).

2.4.2 Aetiology

Different strains of the tubercle bacilli have been isolated in both humans and animals suffering from tuberculosis. Most of the implicated bacilli are members of the MTBC. These include *M. tuberculosis, M. africanum, M. bovis, M. bovis BCG, M. microti, M. carnetti and M. pinnipidii. Mycobacterium africanum* is however subdivided into two groups to indicate their geographical origins in West Africa. Subtype-1 which is related more closely to *Mycobacterium bovis* and the subtype 2 with its geographical origin from East Africa is related rather closely to *Mycobacterium tuberculosis* (David *et al*., 1978). Two sub-species of *Mycobacterium bovis* also exist namely *M. bovis subspecies Caprae* which is susceptible to Pyrazinamide and *M. bovis Subsp. Bovis* which is resistant to Pyrazinamide (Niemann *et al*., 2002), a first line anti-tuberculosis drug.

2.4.3 Pathogenesis

After infection, the tubercle bacilli are phagocytosed by macrophages in the alveoli and are destroyed. A primary focus is formed which is mediated by cytokines. The resulting product which is made up of degenerated and dead macrophages is then surrounded by epitheliod cells, granulocytes, lymphocyte and later giant cells to form a complex. However, in an immuno-compromised animal, this process is not efficient and the infection spreads (Liebana *et al.*, 2008).

2.4.4 Lesion formation

The development of BTB in cattle may or may not lead to the formation of visible lesions in cattle tissues even though the presence of visible lesions is crucial in determining the extent of progression of the disease in cattle. Classical BTB is characterized by the formation of localized bacterial lesions called tubercles or granulomas. The tubercles could be yellowish and calcified (with gritty texture), caseo-calcarous or caseous (cheese-like pus) and are often encapsulated (OIE, 2005). These tubercles are often seen in the lungs and lymph nodes, especially those associated with the respiratory system and head. However, they can also be seen in the liver, spleen and the lining of the body cavities. Tubercles are rarely seen on the male sexual organ but they are occasionally seen on the female genitals. In cases where the disease is widely disseminated, several small tubercles can be seen on almost every organ of the cattle. Whipple *et al*. (1996) screened 30 dairy cattle from a BTB infected kraal and reported seeing visible lesions within mediastinal, deep cervical, trachea-bronchial, retropharyngeal, sub-iliac, paratoid, lung and tonsilar tissue of the animals.

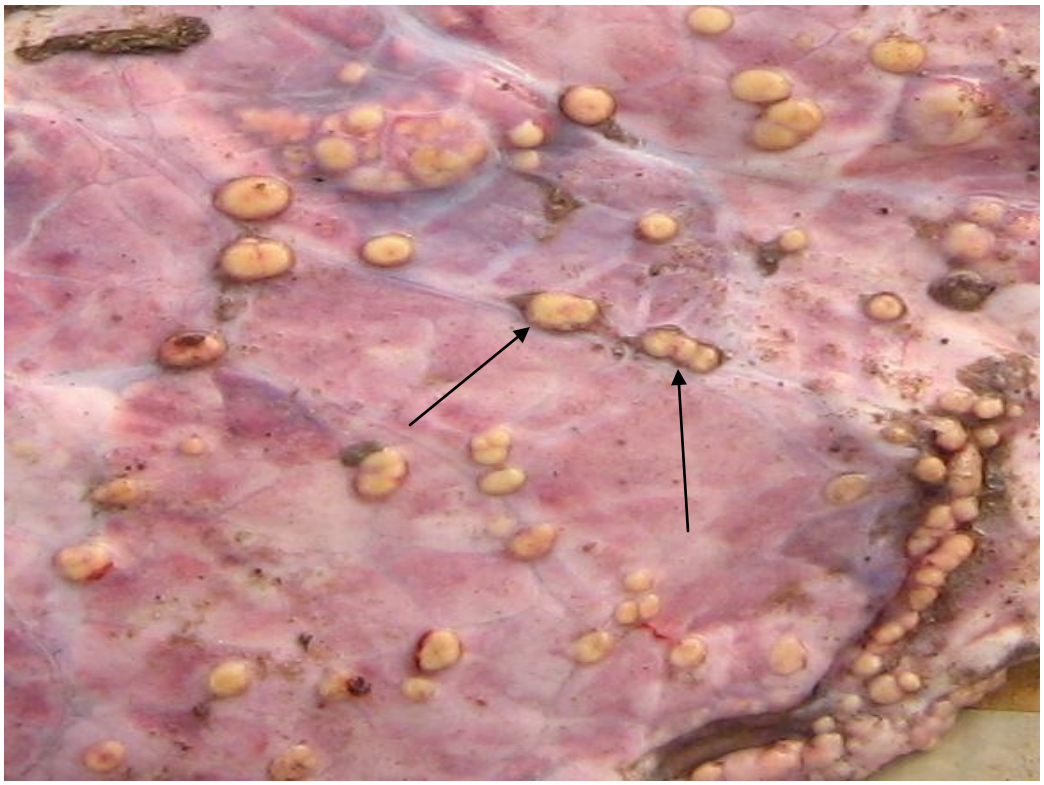

Figure 1: Picture of tubercles (Yellowish) seen in a Lung tissue at the Tema Abattoir.

2.4.5 Pathological manifestation at ante- and post-mortem

The BTB disease is chronic and seldom shows any sign/symptoms until the disease is advanced and this can take years to happen. The pathogen can affect any part of the cattle and clinical signs presented by the infected animal can give an indication of the location of the lesions and the extent of *M. bovis* infection. Clinical signs of the disease in cattle at ante-mortem include a gradual loss of weight in spite of adequate food intake/nutrition, weakness, low grade fever, enlarged lymph nodes, breathing difficulties and a persistent cough (that is if the lungs are infected). Varying prevalence rates based on the presence of gross visible lesions in carcasses at post-mortem have been reported. For instance, prevalence rates of BTB infection in meat of 5.2% (Ameni and Wudie, 2003), 4.5% (Teklu *et al.*, 2004) and 3.5% (Shitaye *et al.*, 2006) have

been reported in various abattoirs in Ethiopia. The different prevalence rates from one abattoir to the other probably might be as a result of the type of production system used i.e. the intensive dairy method for example is likely to record high infection rate as against the pastoral system (Ameni *et al.,* 2006; Shitaye *et al.,* 2006). It is also worth mentioning that studies carried out in Ethiopia suggest that not all cattle infected with BTB exhibit signs of tubercles at the point of slaughter (Asseged *et al.,* 2004; Teklu *et al.,* 2004).

Figure 2: The arrow shows a pre-scapula lymph node just in front and a little above the foreleg.

2.4.6 Transmission of bovine tuberculosis

The start and transmission of *M. bovis* infection are somewhat complicated and depend largely on the immunity levels, general health and age of the infected animal (Munroe *et al.,* 2000). The route of BTB infection is primarily two, that is through inhaling aerosols of *M. bovis* secreted from the lungs (respiratory route) and secondly, through the ingestion of infected carcass or contaminated milk (alimentary route) even though a break in the skin can be a likely source of infection (Mausner and Kramer, 1994).

M. bovis infection can be from animal to animal, animal to human (zoonosis), human to animals (reverse zoonosis) and human to human. However, the human to human transmission rarely happens in immuno-competent persons as the pathogen is rather known to spread occasionally through small clusters of people especially alcoholics and HIV-infected persons (www.TuberculosisTextbook.com).

2.5 BOVINE TUBERCULOSIS IN HUMANS

TB infection due to *M. bovis* is known to exist in up to 3% of all humans even though in Eastern Europe, a lower incidence of approximately 0.02% has been reported (Cvetnic *et al.,* 2000). In developing countries, where BTB is not properly controlled, the disease is common in children who drink or handle contaminated milk (Bonsu *et al.,* 2000). Adults can begin to show symptoms of the disease due to a possible re-activation of an old infection. Also, in people with HIV infection, poorly controlled diabetes and underweight, the disease can re-occur (Brosch *et al.,* 2002).

In Sub-Saharan Africa, where almost 2 million people are infected with TB, it is not clear what number is as a result of *M. bovis* infection (Daborn, 1992). Nevertheless, there is a lot of research done to indicate the spread of the disease in pastoral communities where there is usually a close contact between livestock and humans (Mposhy *et al.*, 1983).

2.6 DRUG RESISTANT TUBERCULOSIS

The discovery of multidrug-resistant TB (MDR-TB) strains which the World Health Organization defines as any resistance to at least rifampicin (RIF) and isoniazid (INH) complicates the control of the disease. Drug resistance strains often develop when there is a repeated treatment failure in individuals leading to mutations in mycobacterial DNA (Van Rie *et al.*, 1999). Individuals infected with these MDR-TB strains need to be entered into alternative treatment regimen involving second-line drugs that are more toxic, more costly and less effective. The problem of multidrug-resistant tuberculosis (MDR-TB) strains has also been recently reported. MDR-TB strains infection is defined as having resistance to at least three of the six main classes of second-line drugs (aminoglycosides, polypeptides, fluoroquinolones, thioamides, cycloserine, and paraaminosalicylic acid) (CDC, 2006). This situation now constitutes an emerging threat to the control of the disease and further spread of drug resistance strains, especially in HIV-infected patients (Gandhi *et al*., 2006).

Genotypic methods for drug resistance screening in TB look for the genetic determinants of resistance and involve two basic steps: nucleic acid amplification such as PCR which is carried

out to amplify the sections of the *M. tuberculosis* genome known to be altered in resistant strains and a second step of assessing the amplified products for specific mutations correlating with drug resistance (García de Viedma, 2003; Palomino, 2005).

2.7 TREATMENT OF TUBERCULOSIS

Treatment for tuberculosis started in 1944 when the first anti-tuberculosis drugs, streptomycin (SM) and para-aminosalicylic acid (PAS) were discovered. Since then, several drugs have been introduced into TB treatment to reduce the time from the initial 18-24 months to six months. Recently, two approaches have been used to address the TB problem in humans worldwide. This include the DOTS- Directly Observed Therapy Short Course (WHO, 2008) which promotes case detection and treatment of the disease and secondly, research/diagnostic laboratories which work to provide useful information on TB diagnoses, surveillance, specie-specific identification, drug susceptibility testing (DST) and genotyping which can be very useful in TB epidemiology studies (Noaki *et al.*, 2000).

The DOT short course regimen uses a combination of specific anti-tuberculosis drugs to treat the disease within 6 to 8 months (WHO, 1994). The objective is to first target and destroy rapidly growing pathogens in the lungs and secondly, to eliminate the semi-dormant pathogens in the other tissues. The initial treatment phase consists of three or more drugs (pyraminamide, isoniazid, rifampicin and streptomycin/ethambitol) which are taken for two months. The second phase consists of fewer drugs i.e. rifampicin and isoniazid which are taken for 4 to 7 months. In instances where drug resistance develops, the treatment is varied to include new combinations of second line drugs containing at least three drugs that have not been previously used. These

include polypeptide capreomyserine, aminoglycosides, prothioamide, ethionamide and fluoroquinolones (CDC, 2006).

2.8 DIAGNOSIS OF TUBERCULOSIS INFECTION

2.8.1 Diagnosis of tuberculosis in human

Diagnosis of the disease is done by taking sputa or fine needle aspirates from patients with pulmonary TB where the respiratory tract is infected or patients with extra-pulmonary TB where other parts of the body are infected. Routine identification and isolation of the tubercle bacilli in the laboratory can be categorized as conventional (Acid Fast Smear Microscopy, chest X-rays and culture of decontaminated samples) and non-conventional, which is molecular based. However, the success or otherwise of a particular test result is dependent on the extent of disease prevalence as well as the sensitivity and specificity of the test procedure (Mausner and Kramer, 1994).

2.8.2 Zeihl-Neelsen Microscopy

2.8.2.1 Acid fast smear microscopy

Acid fast smear microscopy is an important preliminary step for the diagnosis of active TB on either clinical samples or prepared tissues especially in resource-limited countries. Currently, two procedures are used for acid-fast staining namely carbol-fuchsin (Ziehl-Neelsen and Kinyoun) and fluorochrome (auramine O or auramine-rhodamine) staining. The procedure involves the smearing of a sample on a micro-slide and heat-fixing it. It is stained with a

primary dye and decolourized using an acid alcohol. Finally, the slide is counter-stained to distinguish between the acid fast bacilli and the background.

Smear preparation may be done either by the direct or concentration method or from culture (Kent and Kubica, 1985). The concentration method involves the centrifugation and sedimentation of the sample after homogenization and is known to be more efficient than the direct method where smears are prepared directly from the sample (Apers *et al.*, 2003). A major advantage of acid fast bacilli (AFB) smear microscopy is that it is inexpensive, rapid and technically simple. However a setback to the AFB smear microscopy is that, whilst a positive smear result can be obtained with a 1×10^4 AFB's per ml of a sample, approximately 60% of positive result is obtained with 1×10^3 AFB's per ml of sample (Pfyffer, 2003). Another disadvantage of the procedure is its inability to discriminate between members of the MTB Complex and NTM species coupled with the fact that it can identify other organisms such as Norcardia and even cell debris as acid fast, giving a false positive result (Kent and Kubica, 1985). However, it is estimated that the overall sensitivity of AFB smear microscopy is between 22-78% (Kasai *et al.*, 2000).

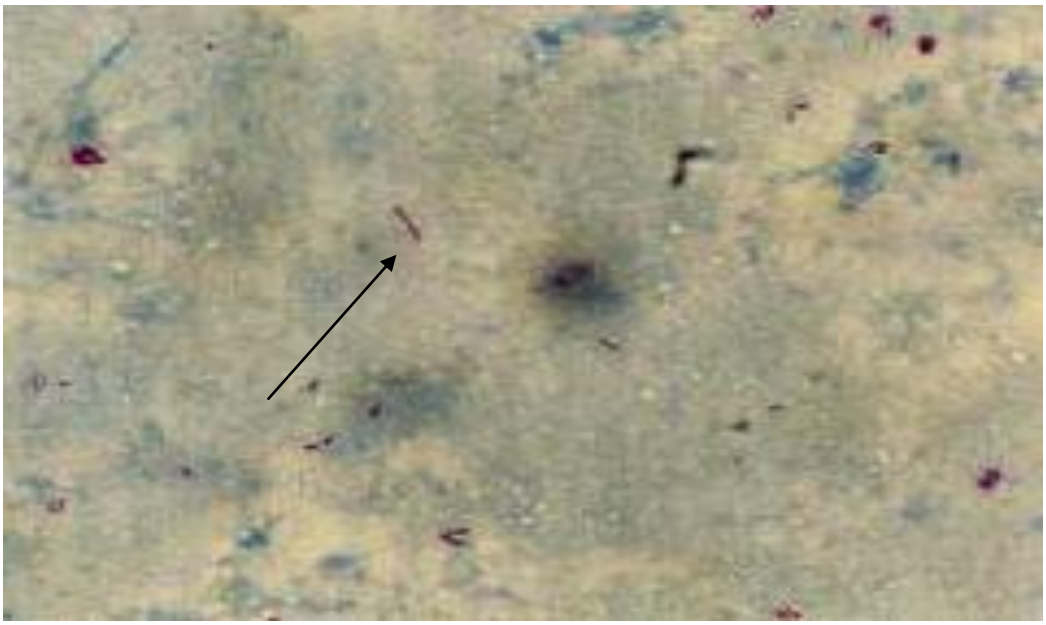

Figure 3: Acid-fast staining with arrow showing an acid-fast bacillus.

2.8.3 Mycobacterial culture

Mycobacterial culture is generally regarded the "gold standard" for the confirmation of TB as it provides a definitive test for diagnosing the disease (Taylor *et al.*, 2001). Before cultivation, the sputa or tissue specimen must be decontaminated to prevent the mycobacteria from being outgrown by other contaminating bacteria. The most commonly used decontamination reagents or procedures are: (i) NALC (ii) NaOH and (iii) Oxalic acid.

2.8.4 Decontamination

2.8.4.1 N-acetyl-L-cysteine-sodium hydroxide-sodium chloride

This method is one of the most common methods used in laboratories worldwide. It uses N-acetylcysteine for mucus digestion and sodium hydroxide and sodium chloride as the decontaminant (Della Latta, 2004).

2.8.4.2 Sodium hydroxide

This method uses sodium hydroxide at concentrations ranging between 2 and 4 % to digest and decontaminate the specimen. Here, each laboratory determines the lowest concentration for optimal digestion and decontamination (Della Latta, 2004).

2.8.4.3 Oxalic acid

This method is also used on tissue samples and is specially recommended to decontaminate clinical specimens that may be contaminated by *Pseudomonas aeruginosa* e.g. pulmonary specimens from cystic fibrosis patients and urine specimen (Della Latta, 2004).

2.8.4.4 Ogawa-Kudoh

This is a very simple and practical decontamination method that does not require specimen centrifugation before culturing. Hence, it does not require complex laboratory facilities and so can be undertaken in the field. The procedure was discovered by Kudoh and uses sodium hydroxide as the digestant/decontaminant and usually inoculated in a modified Ogawa media (Kudoh, 1974).

Several media exist for the cultivation of mycobacteria and these can be categorized into egg-based (Lowenstein-Jensen and Ogawa), egg-potato based, agar-based (Middlebrook 7H10,

M7H11, blood agar) and liquid based media (Middlebrook 7H9 and Dubos). Other semi-automated (BACTEC 460TB System) or automated (BACTEC MGIT 960, MB/BacT/Alert 3D system) liquid culture systems are also available and can reduce the detection time of mycobacteria growth drastically to a mean of 10-12 days even though, traditionally, mycobacteria take 3-5 weeks to grow (Hanna *et al.,* 1999; Piersimoni *et al.,* 2001; Woods *et al.,* 1997; Kubica and Wayne, 1994).

One major advantage of culture is that it is more sensitive than AFB Smear Microscopy as it can detect as low as 10 bacilli/ml of digested concentrated sample (American Thoracic Society, 1990). However, it is not 100% sensitive as false negative result may be realized especially in instances where the tubercle bacilli infection is at its early stages (Duffield *et al*., 1989).

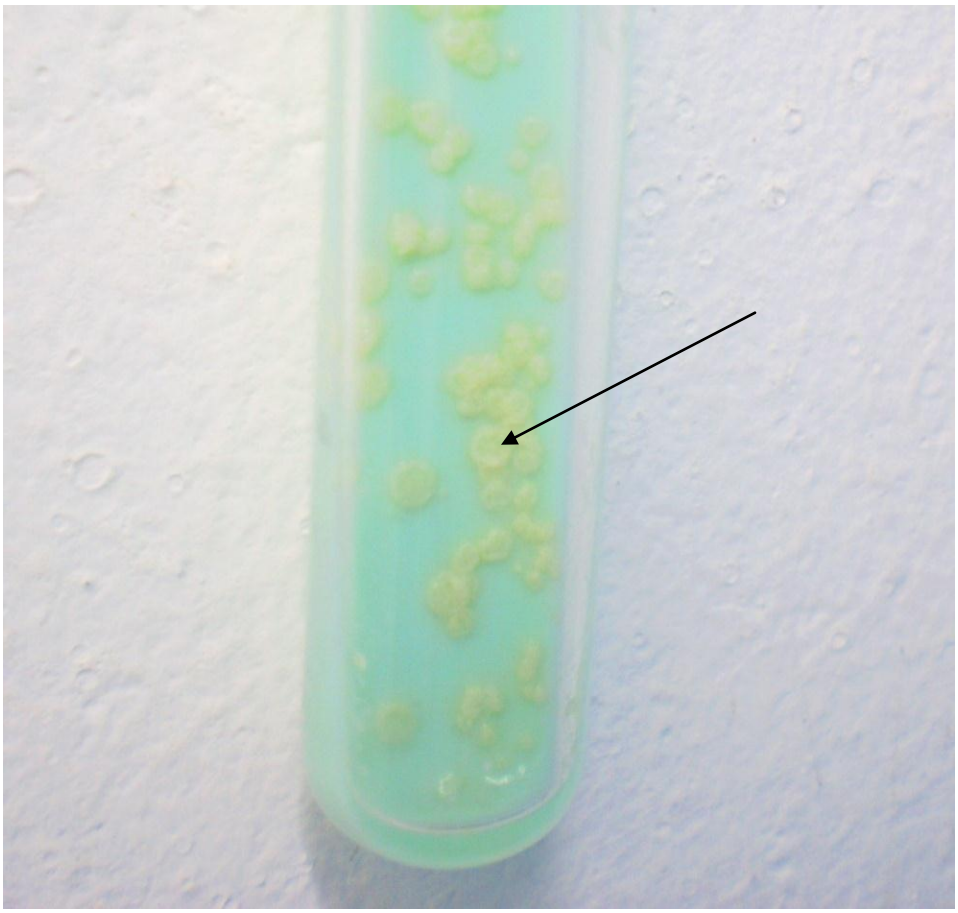

Figure 4: The arrow shows colony morphology of *M. bovis* grown on Lowenstein-Jensen media.

2.8.5 Diagnosis of tuberculosis in cattle

Clinically, animals with bovine TB look normal until later stages where they show progressive emaciation. After slaughter, a thorough visual inspection is carried out to identify the presence of lesions suggestive of BTB infection. In Ghana, meat inspection is a very important means of

controlling BTB. In the field however, the tuberculin skin test is recognized internationally for the initial diagnosis of BTB. This test includes Official Tuberculin Test (OTT) e.g. Caudal-Fold Tuberculin Test (CFT), Comparative Cervical Tuberculin Test (CCT), Cervical Tuberculin Test (CT) and Single Cervical Tuberculin Test (SCT). The gamma-interferon assay and enzyme-linked immunosorbent assay (ELISA) also plays a supplementary role in BTB control, (Palomino, 2005; Frank *et al.*, 1992) although the confirmation of the disease must necessarily be done through culture.

2.8.6 Capilia TB-Neo test (Immuno-chromatographic assay)

Capilia TB-Neo assay is a simple and rapid immuno-chromatographic test which is based on the MPB64 antigen identification discovered in 1984 (Nagai *et al.,* 1991). The antigen is one of the over 200 proteins secreted by members of the MTBC during Mycobacterial culture (Anderson, 1991; Li *et al.,* 1993). The Capilia TB-Neo test was therefore designed to identify members of MTBC (Abe *et al.,* 1999). The efficiency of this test depends largely on the adequacy of the MPB64 antigens in the test sample. The principle underlining the test is based on the reaction of monoclonal antibodies obtained from mouse against MPB64 antigens secreted by a bacillus belonging to the MTBC, with the exception of some *M. bovis* BCG sub strains (Nagai *et al.,* 1999; Yamaguchi *et al.,* 1989).

The test plate is made up of an anti-MPB64 monoclonal antibody which is conjugated with gold colloids and then immobilized on a nitro-cellulose membrane. It has a specimen placing area, reagent area, a developing area and a reading area. The reading area is made up of a Control (C)

and Test (T) coloured bands. A positive test result is therefore indicated by a red-purple coloured band if the C and T bands appear within 15-60 minutes of dispensing the sample in the specimen placing area. A negative test result is indicated by a red-purple coloured band on only the Control (C) band. A red-purple band produced by the T band only indicates an invalid test result and must be repeated.

Although the reported sensitivity and specificity of the Capilia TB-Neo assay of 99.2% and 100% respectively are high, some confirmed MTBC bacilli have proved negative in Japan as a result of the inability of the isolate to produce the MPB64 antigen due to a mutation in the gene (Hirano, 2004).

2.8.8 Polymerase chain reaction

The Polymerase Chain Reaction (PCR) is a molecular-based *in-vitro* method that is used to amplify either a single strand of a gene or only a few copies into millions of a specific gene using DNA polymerases. DNA polymerases elongates and make copies of single-stranded DNA starting from short stretches of double stranded DNA. The strands in the double-stranded DNA are first separated by heating. Two synthesized short oligonucleotides called primers, each complementary to a given sequence in each of the strands of the targeted DNA, are hybridized to the separated DNA strands by cooling. One of the daughter DNA strands is then extended by DNA polymerase using nucleotide building blocks called deoxyribonucleotides. A chain reaction is then achieved by repeating this sequence of activity over and over again, say, 35 cycles, until a sufficient number of copies of the target DNA sequence is obtained. The use of

specific DNA probes and PCR for diagnosis require detailed examination of the molecular genetics of the organism, especially for the identification of those DNA sequences that are specific for the organism in question. During the last decade or so, several such unique sequences have been reported for MTBC (Hance *et al.,* 1989; Eisenach *et al.,* 1990; Hermans *et al.,* 1990).

Although PCR on direct tissue sample is feasible, allowing for diagnosis within two days, its overall performance can be adversely affected by the presence of inhibitors like protein (De Wit *et al.,* 1990; Clarridge *et al.,* 1993; Manigiapan *et al.,* 1996; Rodriguez *et al*., 1999). This therefore follows that running PCR with mycobacterial colonies obtained from culture is more reasonable (Sakamoto, 1997). Nonetheless, the PCR technique still offers a rapid diagnosis for several different diseases and has been reported to be immensely beneficial in detecting *M. bovis* in bovine samples (Kolk *et al.,* 1992; Kox *et al.,* 1994; Liebana *et al.,* 1995; Wards *et al.,* 1995; Vitale *et al.,* 1998; Zanini *et al.,* 1998; Romero *et al.,* 1999). The PCR technique gives a moderate to good sensitivity of between 65%-90% with a comparatively higher specificity of between 98% and 99% (Livingstone, 2001).

2.8.9 Multiplex ligation-dependent probe amplification (MLPA)

MLPA was developed in 2002 to screen human DNA. However, it has recently been used for bacterial genotypic studies to identify mycobacterial strains (Erlandson *et al.,* 2003; Hogervorst *et al.,* 2003). The assay is used to simultaneously genotype and identify drug resistant strains in one multiplexed assay (Bergval *et al.,* 2007). The multi-faceted nature of MLPA makes it a

good candidate for the study of TB epidemiology. Since drug resistance in *M. tuberculosis* is as a result of point mutations, molecular characterization using MLPA will be extremely beneficial as the assay is based on a single nucleotide polymorphism (Riska *et al.,* 2000; Sreevatsan *et al.,* 2001; Arnold *et al.,* 2005; Filliol *et al.,* 2006). Several genotypic markers have therefore been synthesized to characterize the various strains of mycobacteria e.g. TbD1, *IS6110, KatG* and *gyrA* and to also determine the mycobacterial's ability to be adaptive e.g. ogt, mutT2, mutT4. Markers are also available to determine their resistance to drugs e.g. *inhA, rpoB, embB, KatG*. Whilst the specificity is determined by how well the MLPA probes are ligated to the target gene, its efficiency depends on the PCR amplification (Bergval *et al.,* 2007).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 CHEMICALS, REAGENTS AND EQUIPMENT

Chemicals and reagents used for the research work include the following: sodium hydroxide, sodium citrate, sodium chloride, sodium dihydrogen phosphate, potassium chloride, potassium dihydrogen phosphate, disodium hydrogen phosphate which were all obtained from Sigma Chemical Corporation, USA. Others include BBL Mycoprep Specimen Digestion/Decontamination Kit which was obtained from Becton, Dickson and Company, USA. Basic Fuchsin and Methylene Blue were obtained from Eurostar Limited, UK. Absolute alcohol

and Phenol Red were also obtained from Sigma-Aldrich, USA. Sulphuric Acid was obtained from BDH, UK. Agarose gel powder, ethylene diamine tetra-acetic acid dihydrate (EDTA), Tris-borate and ethidium bromide were also obtained from Sigma Chemical Corporation, USA. Reagents used for the Multiplex Ligation-dependent Probe Amplification were from the Royal Tropical Institute, Holland. These include a DNA Probe mix, Hybridization mix, Ligation mix and a PCR mix.

The Carbol-fuchsin stained slides were examined using a Leica CME microscope (Bufallo NY, USA). Centrifugation, Vortexing and Incubation were carried out in an Eppendorf Micro Centrifuge 5415C, Heidolph Vibramax Vortex Mixer and a GFL Water Bath respectively. All Polymerase Chain Reaction were carried out in a Dyad Peltier Thermal Cycler (BIO-RAD Richmond, Calif.). Agarose gel electrophoresis was run on EC320 Mini Gel Electrophoresis System, USA and gel pictures viewed on a Vilber Lourmat UV Trans-illuminator. Gel pictures were taken with an Olympus digital camera (Olympus imaging Corp, Indonesia) connected to an Alpha Digidoc Gel analysis system (Alpha-Innotech/Cell Biosciences, USA).

3.2 STUDY SITES

The Greater Accra Region is home to four approved abattoirs and one Livestock market (i.e. Tulako livestock market) which serve as a conduit for cattle distribution in the southern part of Ghana. The Current study was carried out in two (2) government approved metropolitan slaughter houses located in the Greater Accra Region namely the Tema and the Accra Abattoirs. However, a third slaughter slab constructed purposely for the emergency slaughter of livestock at

the Tulako livestock market was also included in the study. On daily bases, the Tema Abattoir (TA), Accra Abattoir (AA) and Tulako Abattoir (TU), on average, slaughter twenty five (25), eighty (80) and fifteen (15) cattle respectively even though the facilities have the capacity to slaughter more.

Livestock merchants buy mostly cattle and other animals such as sheep and goat from different parts of the country i.e. Bawku, Yendi and Ada with some even going as far as Burkina Faso and Ivory Coast to buy and transport the livestock to the Tulako livestock market where they are in turn purchased by the local butchers and sent to the various abattoirs for slaughter. The geographical location or the origin of the animals could not be immediately determined largely due to the multiple selling-on and the general lapse in the cattle movement permit system in the country, leading to unreliable and poor documentation. At the abattoirs and in a kraal, Ministry of Health certified Veterinary Officers perform ante-mortem examinations on either all or a selected few by checking on the animals stress level, hair colour, sex and other relevant body conditions. Post-mortem inspections are then carried out after slaughter where the carcasses are examined for suspected tuberculous lesions, indicative of tuberculosis infections.

3.3 SAMPLE COLLECTION

For the present study, 155 tissue samples with gross visible lesions were collected from 145 bovines comprising 73 bulls and 72 cows out of an approximate total of 2886 bovines screened between June-October, 2009. Majority of these bovines were crossbreeds even though some were also local *Bos indicus* breeds. During inspection, whole animals are condemned only if

there is a generalized tuberculous lesion invasion. However, if lesions are observed within only one organ alone and any associated lymph nodes, then only the affected parts are declared unfit for human consumption. The rest of the carcass is then salvaged. Although the focus was to pick one sample per animal, a few bovines contributed two or three pathological samples. In all, a total of 108, 36 and 11 pathological samples were taken from the Accra, Tema and the Tulako Abattoirs. Pathological samples were taken from the lungs, udder, head and other associated thoracic and lymphatic lymph nodes namely bronchial, retro-pharyngeal, pre-scapular, mesenteric, and supra-mammary lymph nodes.

At the slaughter house, approximately 4 grammes of lesion samples were taken into a small sterile plastic container of length 4.7 cm and breath 4.1 cm and covered with a tight fitting lid. They were labelled according to the tissue type, sex and the date of sample collection. The samples were then temporary stored on ice and transported in an ice chest for the laboratory analysis at the Clinical and Cellular Centre of The Radiological and Medical Sciences Research Institute (RAMSRI) of the Ghana Atomic Energy Commission (GAEC). At the Centre, the samples were stored at -20^{o}C prior to processing.

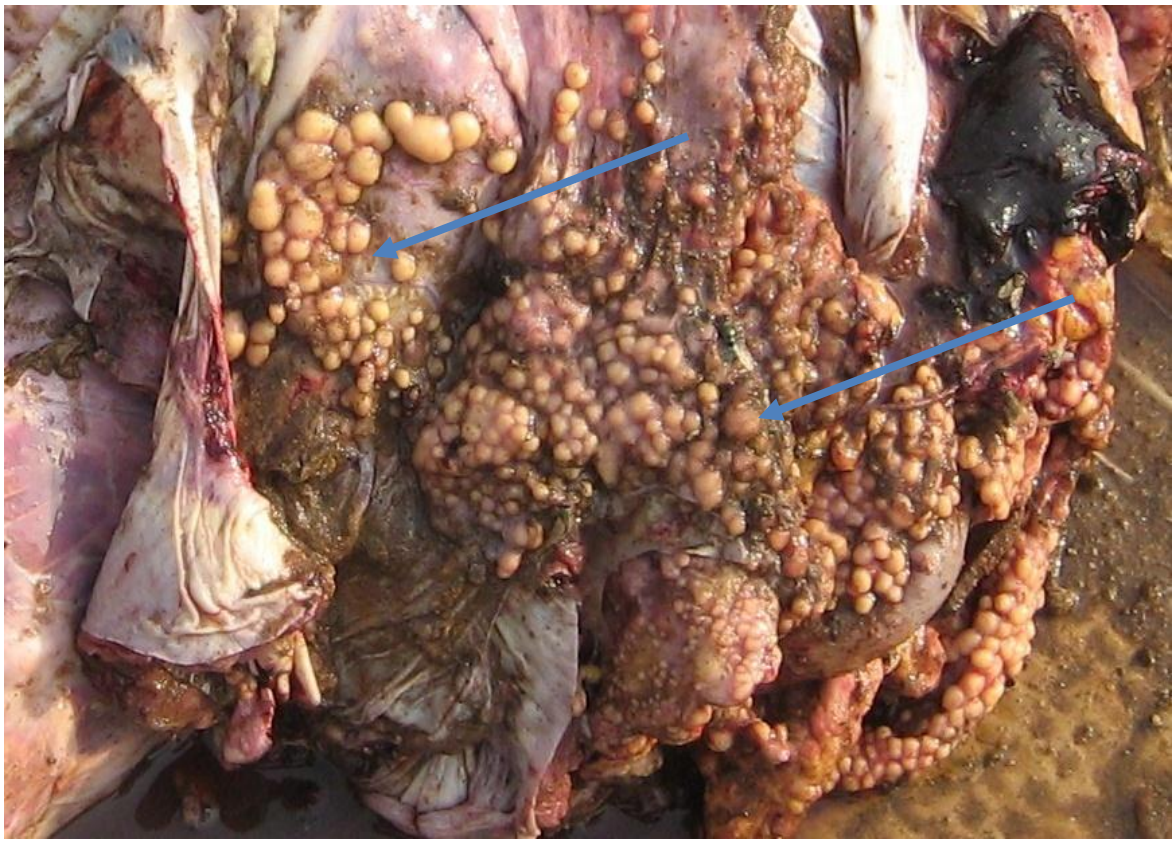

Figure 5: Lung tissue with visible lesions (indicated with blue arrows)

3.4 ACID FAST MICROSCOPY

All pathological samples were subjected to Acid Fast Smear Microscopy as a first line screening tool using Ziehl-Neelsen (ZN) staining.

3.4.1 Sample preparation prior to smearing

The stored pathological samples were defrosted in a biohazard cabinet for processing. Sterile scalpel blades were used to sort through the pathological tissue samples, discarding as much fat and connective tissue as possible.

3.4.2 Smear preparation

New glass microscopic slides cleaned with 70% alcohol were labelled using the sample identification number, date of smear preparation as well as the series number i.e. **A**, which corresponds with the tissue sample treatment batch before mycobacterial concentration by centrifugation (tissue sample decontamination).

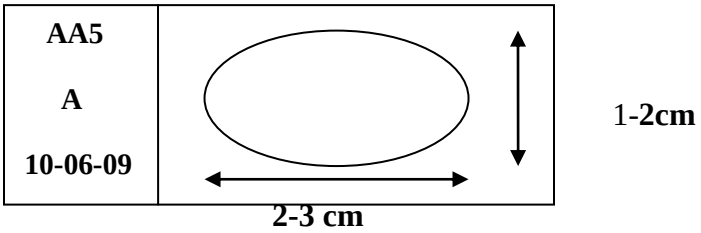

Figure 6: Diagram indicating how the glass microscopic slides were labeled.

AA 5 represents the sample identification number whilst **A** represents the series of tissue smear preparation. The date **10-06-09** represents date of smear preparation.

Using a 0.1 µl plastic loop, the tissue homogenate were spread over a 1-2 cm by 2-3 cm area on the glass microscopic slides in an oval shape.

3.4.3 Smear fixing

The smeared glass microscopic slide was allowed to air-dry and heat-fixed using an electric flat plate warmer at 80ºC for 10 minutes.

3.4.4 Ziehl-Neelsen microscopy

In small batches of between 5 and 8, heat-fixed slides were arranged on a metal rack over a sink. The slides were then flooded with Carbol-fuchsin ensuring that enough stain was added to keep

the slides covered. Using a bunsen burner, the bottom of the slides were heated slowly until the first appearance of vapour. The slides were allowed to stand for 5 minutes and rinsed with a running tap water and made to drain. The slides were flooded with 20% sulphuric acid and the smears allowed to decolourize for 5 minutes. The slides were then again rinsed with water and excess water drained. Finally, the smear was flooded with the counter stain, a 0.3% methylene blue for 30 – 60 seconds. The slides were rinsed thoroughly with water and tilted on a rack to drain and allowed to air-dry before examining under the light microscope. Positive control used was C100 (MTB) and the negative control was water. The positive control was kindly donated by the National Institute of Health and the environment (RIVM), Bilthoven, The Netherlands.

3.4.5 Microscopic examination of slides

To begin, all light conducting surfaces, lenses and mirrors were cleaned using a Whatman lens tissue paper. The mirror, condenser, diaphragm and light were then adjusted to focus light towards the objective lens. The nosepiece was rotated to allow an initial low power objective lens i.e. 10X over the condenser. The slides were then mounted sequentially on the stage and the course knob and light adjusted till the object was in focus. The fine adjustment knob was turned until a fine focus was achieved. The nosepiece was now turned and a higher power objective lens i.e. 100X was selected (ensuring that the objective lens did not touch the slide). A drop of immersion oil was applied to the smear and the slide moved gradually over the stage for observation. A straight or slightly curved pink or pale red rod on a blue background indicated the presence of acid-fast bacilli whereas a negative result was characterized by only a blue background.

3.5 CULTURE

All 155 pathological samples were processed for mycobacterial culture as follows: In a Biohazard hood, approximately 1 g of the visible lesion (often from areas where they were available or otherwise, they were randomly taken) was sliced into smaller bits using sterile scalpel blades and transferred into a sputum container and homogenized using sterile pipette tips. The homogenate was then again transferred into a 15 ml centrifuge tube and made up to the 5 ml mark with sterile double distilled water. This was then decontaminated by using a modification of the protocol described by Kubica *et al.* (1963). Described briefly: 5 ml of the decontamination solution (consisting of equal volumes of 1 M NaOH and 0.1 M $Na_3C_6H_5O_7$. $2H_2O$, 0.01% N-acetyl-L-cystein and 0.5% w/v Phenol red solution) was added to an equal volume of the homogenate. The tubes were then quickly set on a gyratory shaker in a horizontal position at room temperature for 20 minutes to kill all contaminating bacteria and about 80-90% of the mycobacteria present. A neutralization buffer (0.15 M $NaHPO_4$, pH=5.0) was added drop-wise using a Pasteur pipette with occasional shaking until a 'salmon colour' was achieved, signaling the end point of neutralization. The samples were then concentrated by centrifugation for 30 minutes at 3000 xg. The supernatant was carefully decanted into a 5% phenol solution (disinfectant) leaving behind the pellet. The pellet was then re-suspended in about 300 µl of Phosphate Buffered Saline (140 mM NaCl, 2.6 mM KCl, 10.1 mM Na_2HPO_4, and 1.7 mM KH_2PO_4). An approximately 2-3 drops of the re-suspended pellet was then inoculated onto two Lowenstein-Jensen (L-J) slants (one containing glycerol to support mainly *M. tuberculosis* growth and the other pyruvate, to support *M. bovis* growth). The inoculated media were then incubated at 37^{o}C by first inclining horizontally for seven days and thereafter made to stand

vertically. The slants were checked for fast growers after one week and then weekly readings were made for two months. Growth identification on the culture media was based on macroscopic characteristics such as colony morphology, pigment production and the mycobacterial growth rate. L-J media with no evidence of mycobacterial growth after 8 weeks were recorded as negative and discarded appropriately. Already decontaminated samples were further decontaminated and re-inoculated onto a new L-J media if the culture media became contaminated or collapsed during the period of incubation. All mycobacterial colonies were confirmed for the presence or absence of an acid-fast bacilli using Ziehl-Neelsen Microscopy. Mycobacterial isolates were finally harvested into 1.5 ml sterile eppendoff tubes containing Middlebrook 7H9 liquid media and stored at -20ºC for molecular work.

3.6 CAPILIA TB-NEO TEST (Immuno-chromatographic assay)

Ninety three mycobacterial isolates were carefully selected based on their growth rates, pigment formation and colony morphology and subjected to Capilia TB-Neo test for culture confirmation of *Mycobacterium tuberculosis* Complex organisms. This test was based on a slight modification of the protocol developed by TAUNS Laboratories Inc, Numazu, Japan (Abe *et al*., 1999). Briefly described: 200 ul of the extraction buffer (0.1% Tween-80 Phosphate Buffered Saline) was dispensed into a 1.5 ml sterile eppendoff tubes. One loopful (0.1 µl) of bacteria obtained from mycobacterial colony was suspended in the extraction buffer and vortexed. In instances where the colony had already been harvested into M7H9 liquid media, 40 µl of that was suspended in 60 µl of the extraction buffer and vortexed. The resultant suspension was used as specimen for Capilia TB-Neo Test. Using a pipette, approximately 80-100 µl of specimen was dropped on the specimen placing area of the Capilia TB-Neo test plate. The reading was then

made between 15 and 60 minutes. A positive reading is indicated by the presence of a purple-red colour line at the reading areas of both the control band (C) and the test band (T). Likewise, a negative result is indicated by the presence of the purple-red colour line at only the control band (C) and not the test band (T). Here again, the positive control (C100) and the negative controls (C687 and C809) donated by RIVM, Bilthoven, The Netherland, were used.

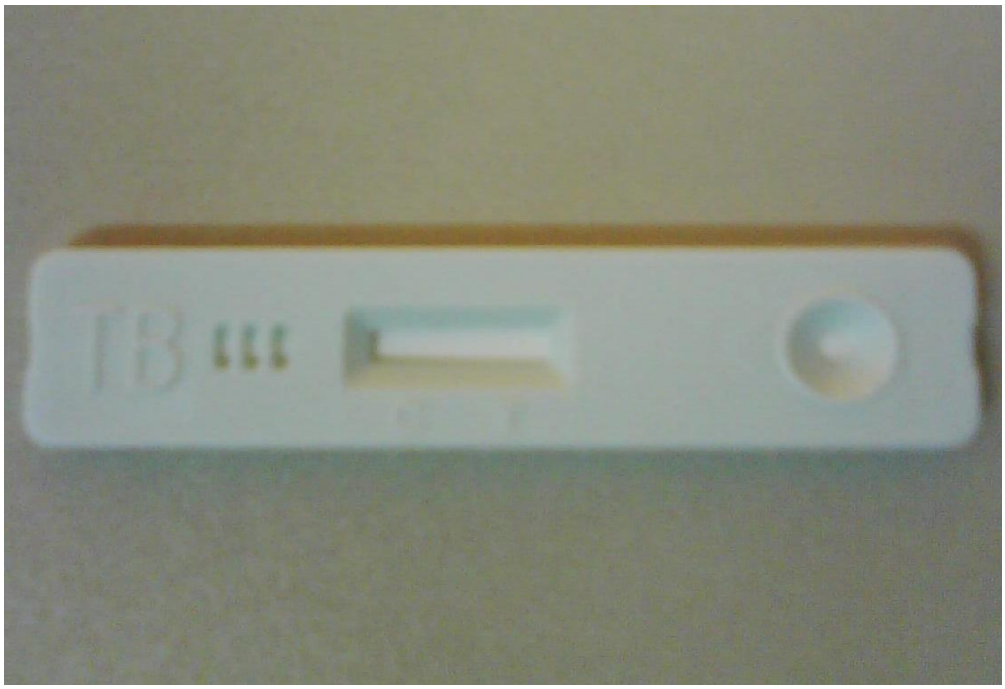

Figure 7: Capilia TB-Neo Strip (before test)

Figure 8: Capilia TB-Neo strip showing MTBC negative results

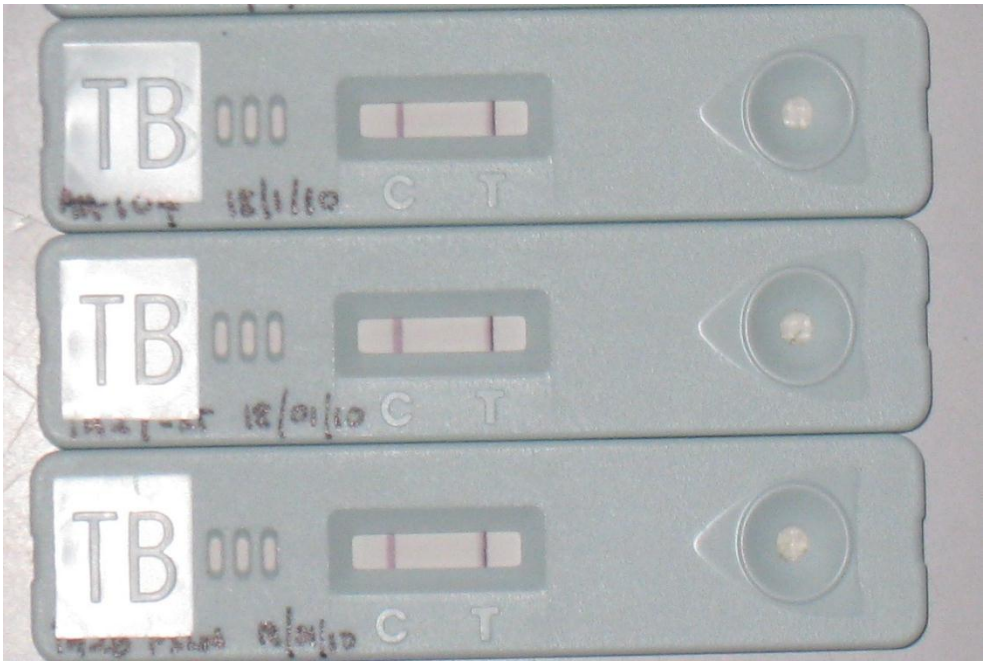

Figure 9: Capilia TB-Neo strip showing MTBC positive results

3.7 MYCOBACTERIAL DNA ISOLATION

For each sample (isolate) in Middlebrook 7H9 media, an aliquot of 150 µl of cell suspension was pipetted into a sterile 1.5 µl eppendoff tube and the tip discarded into a 5% phenol solution. The sample was then centrifuged for 3 minutes at 13000 rpm and the supernatant carefully removed so as not to disturb the pellet formed.

One hundred and fifty microlitres of lysis buffer (10 mM TRIS-HCL, 1m M EDTA and 1% Triton X-100) was added to the cell suspension and vortexed for 10 seconds. The tubes were then placed on a covered heating block and incubated at 95ºC for 30 minutes. The cell lysates were then mixed for 10 seconds and centrifuged for 10 minutes at 14000 rpm. One hundred and thirty five microlitres of the supernatant were carefully transferred into a correspondingly labelled sterile 1.5 ml eppendoff tubes (ensuring that the extraneous cellular pellets formed is not disturbed). They were stored in labelled tube boxes at 4ºC prior to molecular work.

3.8 MULTIPLEX LIGATION-DEPENDENT PROBE AMPLIFICATION (MLPA)

A standard MLPA protocol developed by the Medical Research Council (MRC) of The Netherlands with slight modifications was adopted for the analysis (Bergval *et al*., 2007). The assay was divided into two parts and carried out in two consecutive days.

3.8.1 Day one

The assay for the first day involves DNA melting ("MLPA MELT") and DNA Probe Hybridization ("MLPA HYP"). All DNA amplifications were carried out in a final volume of 50 µl in 0.2 ml Biozym reaction tubes (with the tubes for DNA melting and hybridization labelled with a black marker and tubes for Ligation and PCR labelled with a red marker). The "HYB MIX" which is made up of an equal volume of Probe mix and MLPA buffer was prepared and vortexed based on the number of lysates to work on (Table 1).

Table 1: Preparation of hybridization mixtures

COMPONENT ("HYB MIX")	VOLUME PER REACTION (µl)
Probe mix	1.5
MLPA buffer	1.5
Total	3.0

Table above shows how the hybridization mixture was prepared (MLPA=Multiplex ligation-dependent probe amplification; "HYB MIX" = Hybridization mixture).

An aliquot (3 µl) of each lysate was added to 2 µl of double distilled molecular biology grade water. In a Dyad Peltier Thermal Cycle, the 'MLPA MELT' program was selected and

executed. The "MLPA MELT" program is as follows: Heat at 98ºC for a period of 10 minutes and then cool to 25ºC. An aliquot (3 µl) each of the "HYB MIX" was added to the melted DNA to a volume of 8 µl. The reaction tubes were then set back into the thermal cycler. The reaction mix was heated at 95ºC for one (1) minute and then incubated at 60ºC for sixteen hours (overnight).

3.8.2 Day two

The assay for the second day involves Product Ligation ("MLPA LIG") and Amplification ("MLPA PCR") using a Ligation mix ("LIG MIX") and Polymerase Chain Reaction mix ("PCR MIX"). The "LIG MIX" (Table 2) and "PCR MIX" (Table 3) were prepared on ice prior to product ligation and amplification.

Table 2: Preparation of ligation mixture

COMPONENT ("LIG MIX")	VOLUME PER REACTION (µl)
Ligase-65 buffer A	3.0
Ligase-65 buffer B	3.0
Water	25.0
Ligase-65	1.0
Total	32.0

Table above shows how the ligation mixture was prepared (" LIG MIX"=Ligation mixture).

Table 3: Preparation of Polymerase Reaction Chain mixtures

COMPONENT ("PCR MIX")	VOLUME PER REACTION (µl)
10xSALSA PCR buffer	4
Water	31.5
SALSA PCR Primers	2
SALSA enzyme dil. Buffer	2
SALSA Taq. Polymerase	0.5
Total	40

Table above shows how the polymerase chain reaction mixture was prepared ("PCR MIX" = Polymerase Chain Reaction mixture; dil=dilution).

Using a repeater pipette, 30 µl aliquots of the "LIG MIX" was added to the hybridization product (from day one) and an additional 2 µl more to a volume of 40 µl. The reaction tubes were then

set back into the PCR machine. The mixture was heated at 54ºC for 15 minutes followed by 98ºC for 5 minutes to inactivate the ligase-65 and rapidly cooled to 0ºC (ice bucket) (Table 2). Here, only the probes that were completely hybridized to the targeted gene would be ligated.

For DNA amplification (PCR), 40 µl each of the "PCR MIX" were put into another set of reaction tubes corresponding to the labelled strip from day one (Table 3). The reaction tubes containing ligation products were removed from the PCR machine and aligned with the new tubes which already contained the PCR mix. Ten microlitres each of the ligated products were aliquoted into the reaction tubes containing 40 µl of the "PCR MIX" and incubated at 60ºC for 20 minutes. This was followed by 35 cycles of denaturing at 95 ºC for 30 seconds, annealing at 60ºC for 30 seconds and an extension at 72 ºC for 1 minute. The PCR products were then held at a final elongation step of 72ºC in the thermal cycler for another 20 minutes. Four positive sample controls namely *M. tuberculosis* (P26), *M. africanum* (P65), *M. bovis*, INH® (P123), RIF® (P69) and Multi-drug resistance (MDR) strains (N168) were included in the analysis. Two negative controls, *M. gordonnae* (NTM) and molecular biology grade water were also included. The positive and negative sample controls were donated by RIVM, Bilthoven, The Netherlands.

3.9 AGAROSE GEL ELECTROPHORESIS

All polymerase chain reaction products were analysed by running an agarose gel electrophoresis. The gels were stained with ethidium bromide at a final concentration of 0.5 µg/ml. To run the gel, 15 µl of the amplicons were mixed with 5 µl of the loading buffer (50 ml 100% glycerol, 50 ml 10 X TE buffer and 0.125 g Orange 'G'). The gels were then run using 2% agarose (w/v) at 7

V/cm for 45 minutes. After electrophoresis, the gels were observed on a UV-trans-illuminator and pictures of DNA bands taken with an Olympus digital camera coupled to an AlphaDigidoc gel analysis system. Based on the photograph taken, the DNA band profile was analysed on the basis of their alignment on the gel by comparing the band sizes (i.e. in base pairs) of the gel products with 100 bp DNA marker (Bioron GmbH, Ludwigshafen, Germany).

Table 4: Summary of MLPA probes used

PROBE	TARGET-SPECIFIC SEQUENCE	TARGET	SIZE (bp)
RD9	CCGCCGAAAATTACTACCGGAGC**A**gcgcccgtgtcgtccacggctgcgattattgcct	Genotype marker only present in modern TB	130
KatG-315	caccggaaccggtaaggacgcgatcacca**C**CGGCATCGAGGTCGTATGGACGAACACCCC	INH resistance marker	160
inhA-15	CGATTTCGGCCCGCCGCGGAGA**T**gataggttgtcggggtgactgccacacagcc	INH resistance marker	178
16SrRNA	CACGGGATGCATGTCTTGTGGTGGAAA**A**gcgctttagcggtgtggatgagcccggc	16SrRNA gene, MTBC specific	202
rpoB-531	GTTGACCCACAAGCGGGGACTGT**T**ggcgctgggccggcggtcttgtcacgt	RIF resistance marker	256
rpoB-526T	caacccgctgtcggggttgacc**T**ACAAGCGCCGACTGTCGGCGCTGGGCC	RIF resistance marker	274
IS6110	GTCGAACTCGAGGCTGCCTGCCTACTACGC**T**caacgccagagaccagccgccggctgaggtctcagat	Insertion element*IS6110*,MTBC specific	301
mutT2-58	CCCGAGAGCTCGCCGAAGAACTG**C**gactcgaggtcgccgacctcgcggtgg	Genotypic marker, specific for Beijing 2	355
ahpC-46	GGCGATGCCGATAATATGGTGT**A**atatatcacctttgccctgacagcgacttcacggc	Genotypic marker, specific for Beijing 2	364
ogt-12	CGCACCATCGATAGCCCCATCGG**A**ccattaaccctggccgggcatggctcg	Genotypic marker,	373

	gtgttga	specifc for CAS1 spoligotype	
Ogt-15	taccgcaccatcgatagccccatcgggccattaaa**G**CCTGGCCGGGCATGGCTCGGTGTTGA	Genotypic marker, specifc for Haarlem	382
TbD1	GCGGTCGCGGGATTCAGCGTCTA**T**cggttgcacggcatcttcggctcgcacgaca	Absent in modern *M. tuberculosis* strains	418

Table above indicates the probes used, sequence, targets and their base pairs. The specific sequence of probes comprise sequences targeted by the synthetic probe which is indicated by uppercase and M 13-derived probes indicated by lowercase. The sequence of the ligated site/SNP is also indicated in bold.

CHAPTER FOUR

4.0 RESULTS

4.1 COLLECTION OF TISSUE SAMPLES WITH LESIONS

A total number of 2886 bovines were routinely examined at post-mortem between June and October, 2009 at the selected abattoirs and organ tissues with gross visible lesions suggestive of tuberculosis collected. This represents 2420, 425 and 41 samples screened at the Accra Abattoir, Tema Abattoir and the Tulako Abattoir respectively. Out of this number, 145 bovines were found to possess gross visible lesions suggestive of bovine tuberculosis infection. Based on these, the apparent lesion prevalence, at post mortem, was found to be 5.0% (Table 5 appendix).

A total number of 155 tissue samples were collected from 73 bulls and 72 cows at the three abattoirs. A few bovines provided more than one sample each. Results of the total number of bulls and cows are represented on Figure 10. Also, results of the total lesion distribution at the three abattoirs are represented on Figure 11. Figure 12 indicates the types of lesion tissues sampled whilst figure 13 represents a breakdown of all lymph nodes sampled.

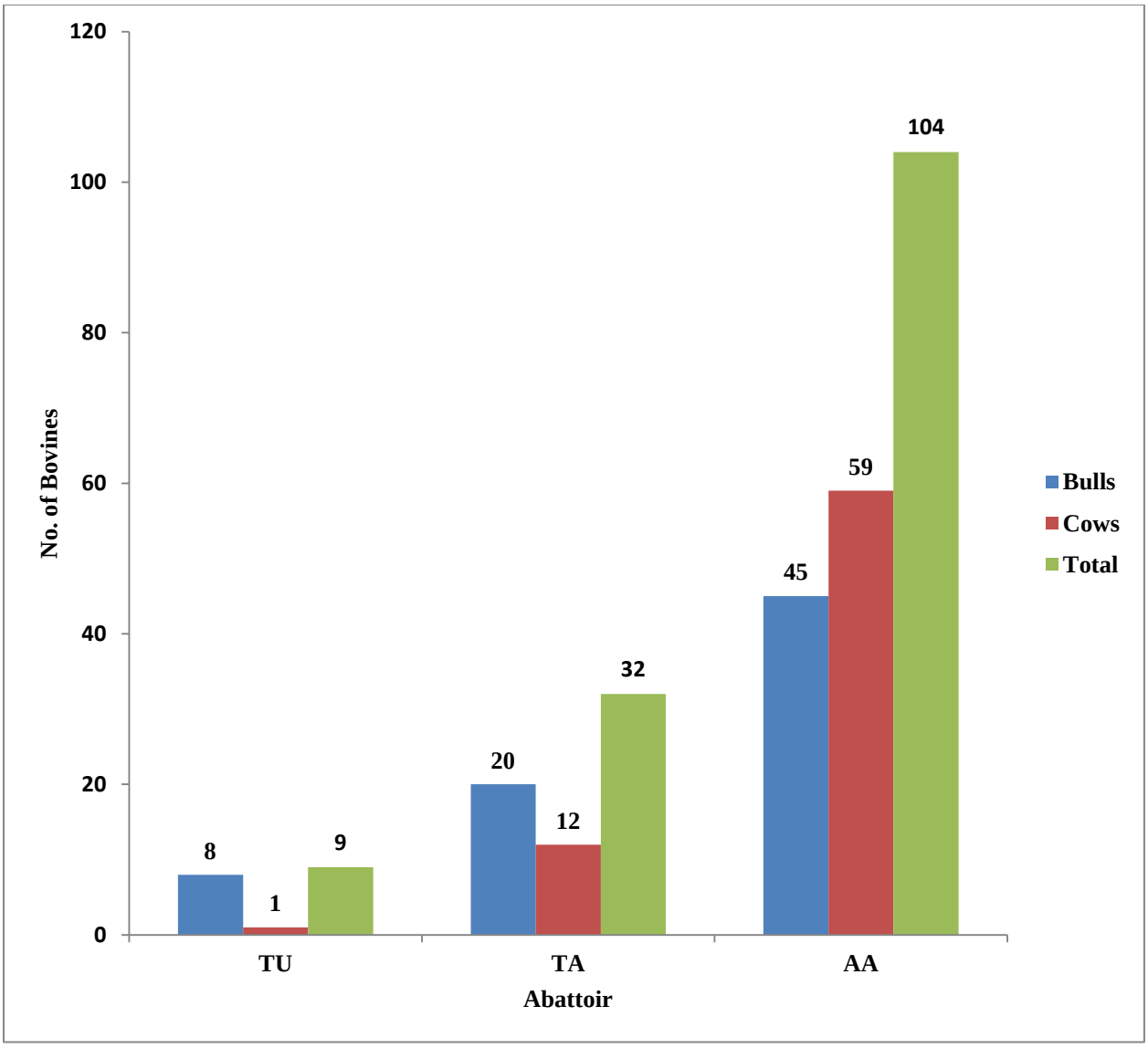

Figure 10: Total number of bulls and cows obtained from three abattoirs in the Greater Accra Region.

TU=Tulako Abattoir; TA=Tema Abattoir; AA=Accra Abattoir.

In all, gross visible lesions suspected of bovine tuberculosis infection were collected from 8 bulls and 1 cow at the Tulako Abattoir, 20 bulls and 12 cows at the Tema Abattoir and 45 bulls and 59 cows from the Accra Abattoir (Fig 10).

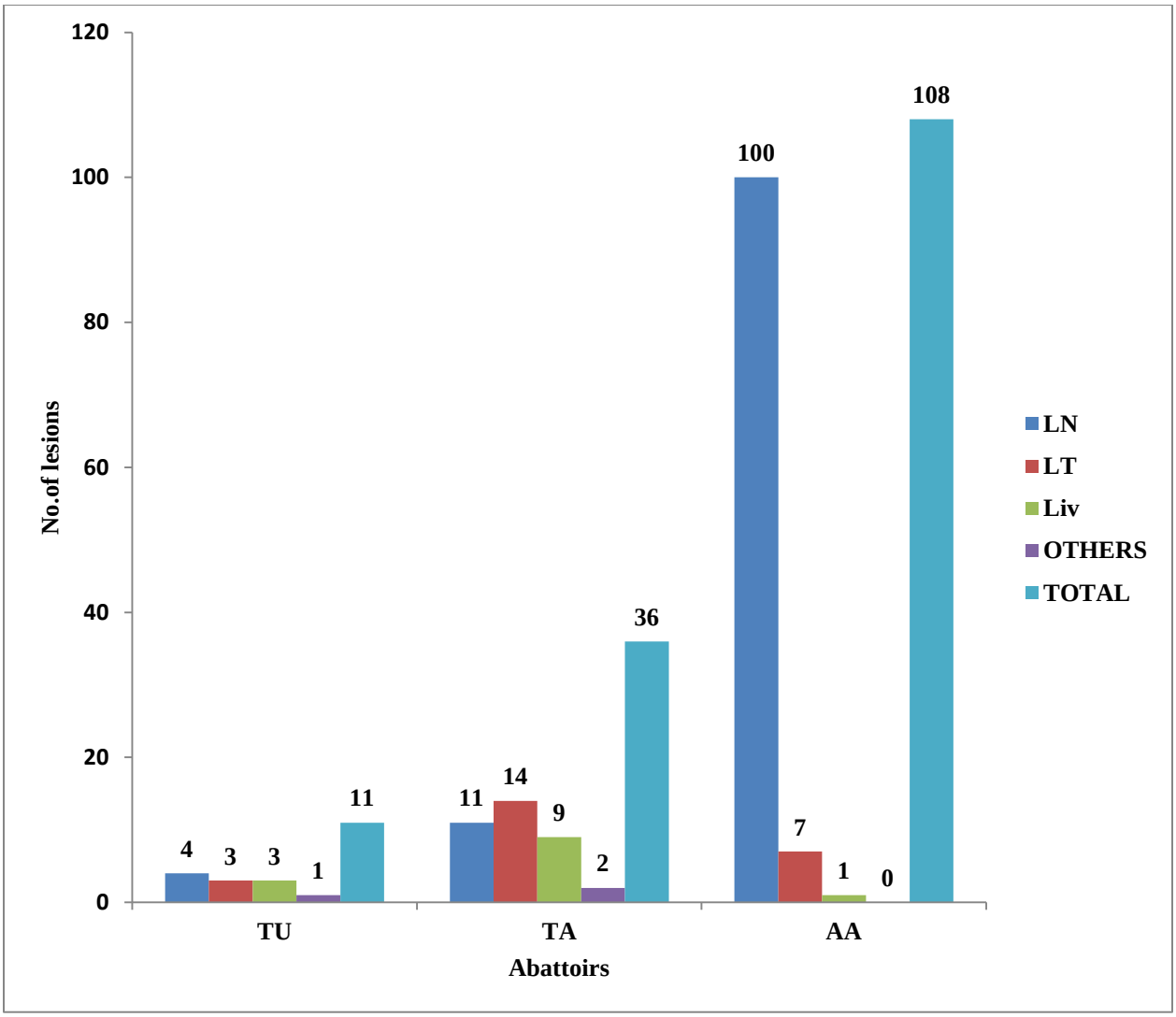

Figure 11: Various tissue types collected at post-mortem at the three abattoirs. Other bovine tissues include head, cardiac and udder.

TU=Tulako Abattoir; TA=Tema Abattoir; AA=Accra Abattoir; LN=Lymph nodes; LT=Lung tissue; Liv=Liver.

In terms of lesion distribution, gross visible lesions comprising lymph nodes, lung tissue, liver and others such as the cardiac, udder and head tissues were collected from the three abattoirs (Fig. 11). The Accra Abattoir contributed the most i.e. 108/155 (69.7%) followed by the Tema Abattoir i.e. 36/155 (23.2%) and then the Tulako Abattoir i.e. 11/155 (7.1%).

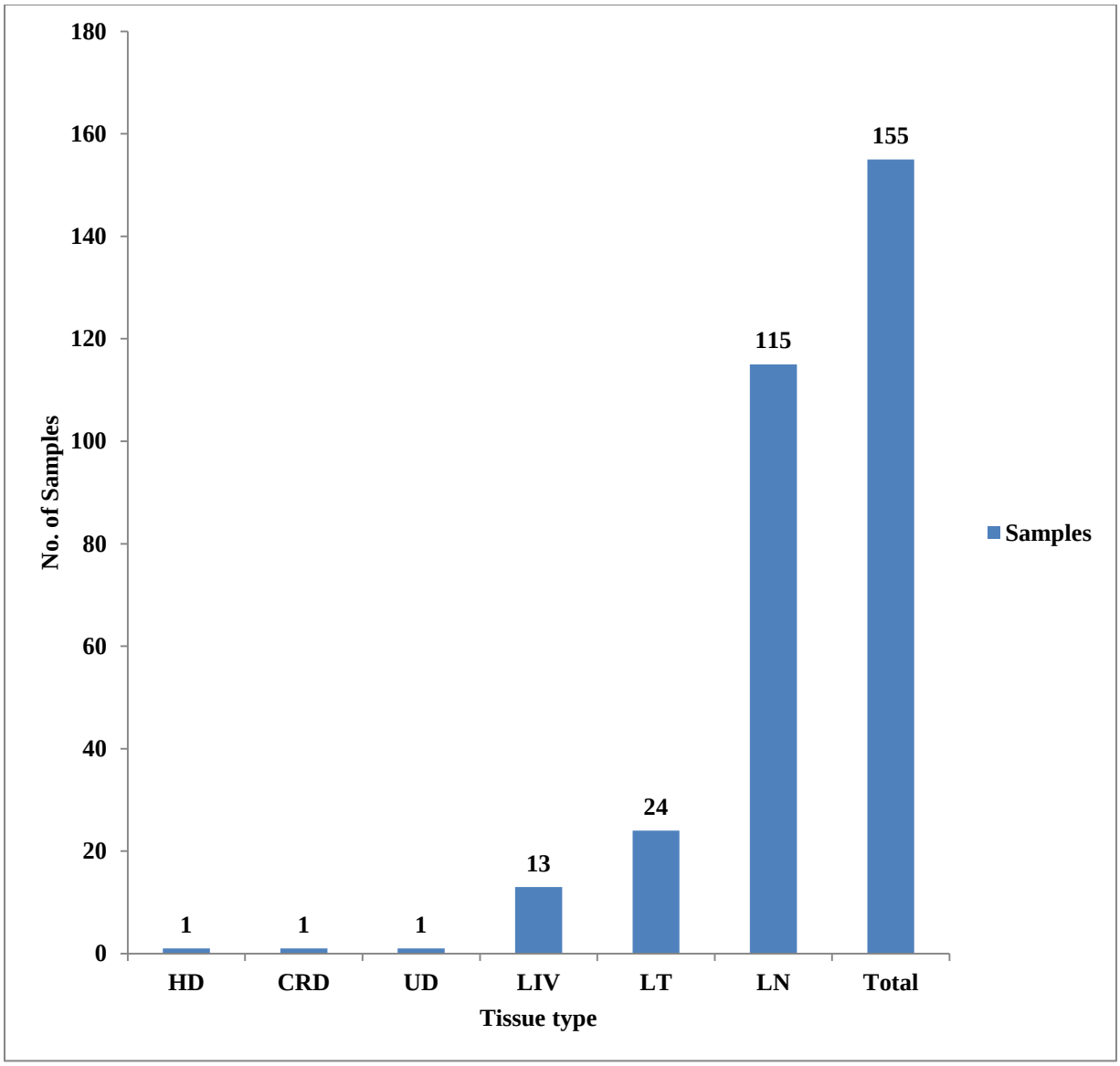

Figure 12: Types of tissue collected at post-mortem.

HD=Head tissue; CRD=Cardiac tissue; UD=udder tissue; LIV=Liver tissue; LT=Lung tissue; LN=Lymph nodes.

In all, 115/155 (74.2%) of the lesions were from lymph nodes. Twenty four out of one hundred and fifty-five (24/155 or 15.5%) were lung tissues and thirteen of one hundred and fifty-five (13/155 or 8.4%) were from liver tissues. The head, cardiac and udder tissues contributed one tissue sample each thus 1/155 (0.6%).

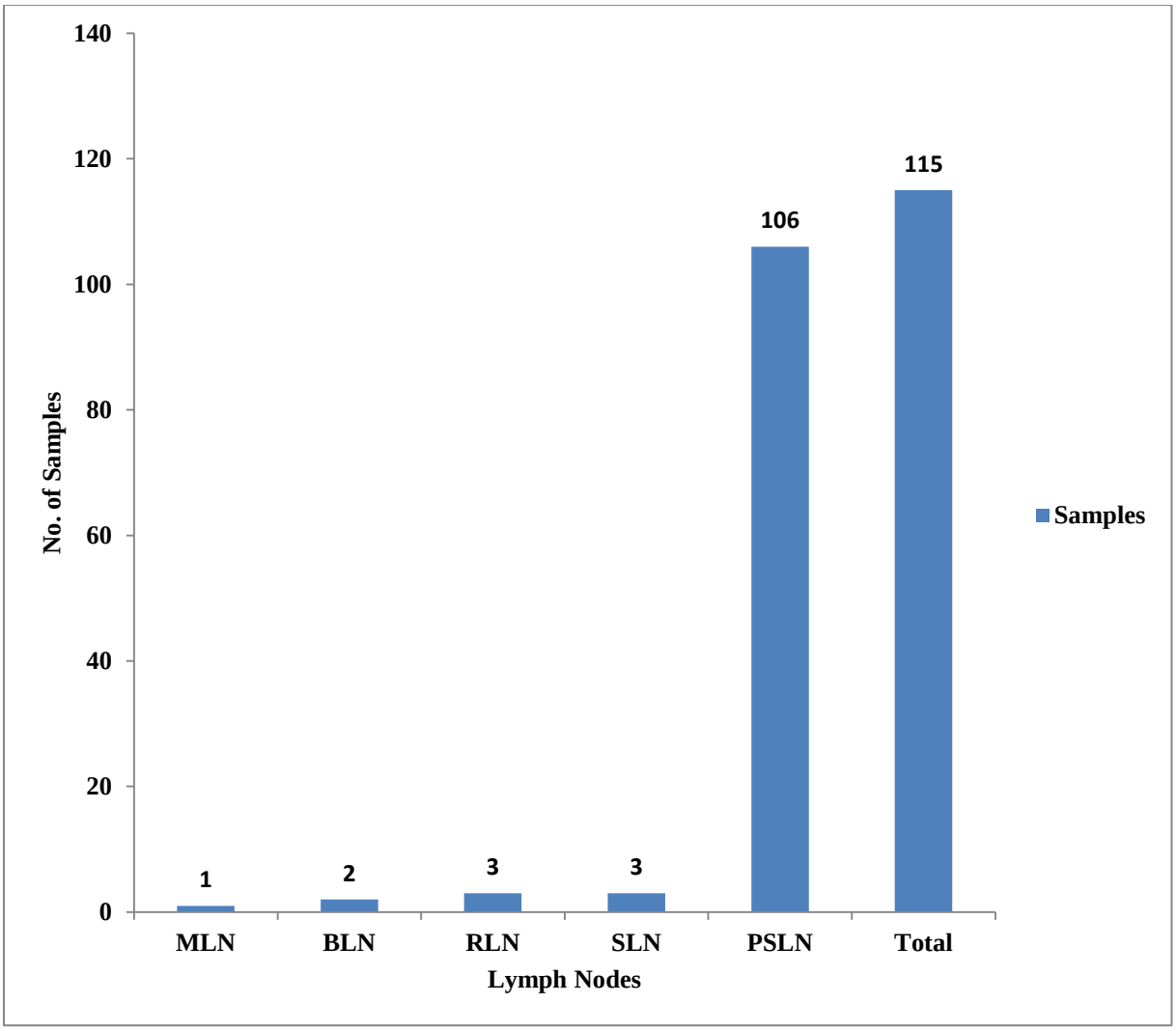

Figure 13: Types of lymph nodes collected at Post-mortem at the three abattoirs.

MLN=Mesenteric lymph node; BLN=Bronchial lymph node; RLN=Retropharyngeal lymph node; SLN=Supra-mammary lymph node; PSLN=Pre-scapular lymph node.

A breakdown of the types of lymph nodes sampled shows that 106/155 (68.4%) were from pre-scapular lymph nodes whilst the supra-mammary lymph node and retro-pharyngeal lymph nodes contributed 3/155 (1.9%) each. Also, 2/155 (1.3%) was obtained from the bronchial lymph node whilst 1/155 (0.6%) was obtained from the mesenteric lymph node.

4.2 LABORATORY ANALYSIS

4.2.1 Microscopy

For the laboratory analysis, all 155 tissue samples obtained from a total number of 145 bovines were subjected to ZN Microscopy. ZN Microscopy results from all bull and cow samples are represented in Figure 14. Figure 15 indicates ZN microscopy results of all bovines obtained from the three abattoirs. Results of ZN microscopy of all tissue types screened are also depicted in Table 6 appendix. A breakdown of positive and negative ZN microscopy results of all lymph nodes samples is also represented in Table 7 appendix. The positive and negative ZN microscopy results of 138 individual tissue samples obtained from each animal are also depicted in Table 8 appendix. Figure 16 shows positive and negative ZN microscopy results of all bovines from which two or three tissue samples were obtained.

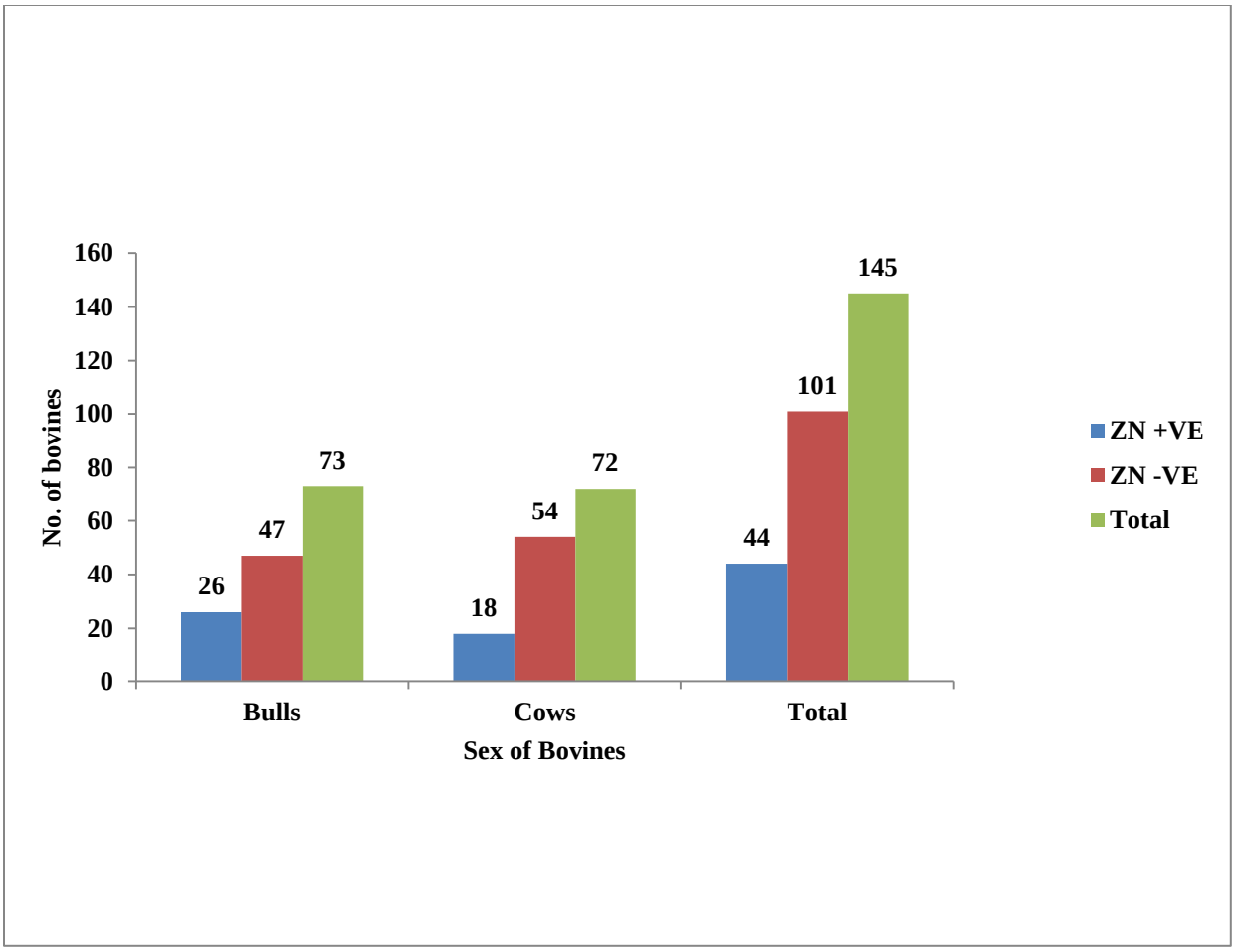

Figure 14: ZN microscopy results of all bull and cow samples.

ZN+VE=Ziehl-Neelsen Positive; ZN-VE= Ziehl-Neelsen Negative.

Figure 14 indicates the sexes of the bovine samples and the number that proved positive or negative by ZN microscopy. Out of the 73 bulls screened, 26 (35.6%) tested positive and 47 (64.4%) tested negative. Likewise, out of the 72 cows screened, 18 (25.0%) tested positive and 54 (75.0%) were negative. In all, 44/145 (30.3%) bovines tested positive by ZN whilst 101/145 (69.7%) bovines were negative.

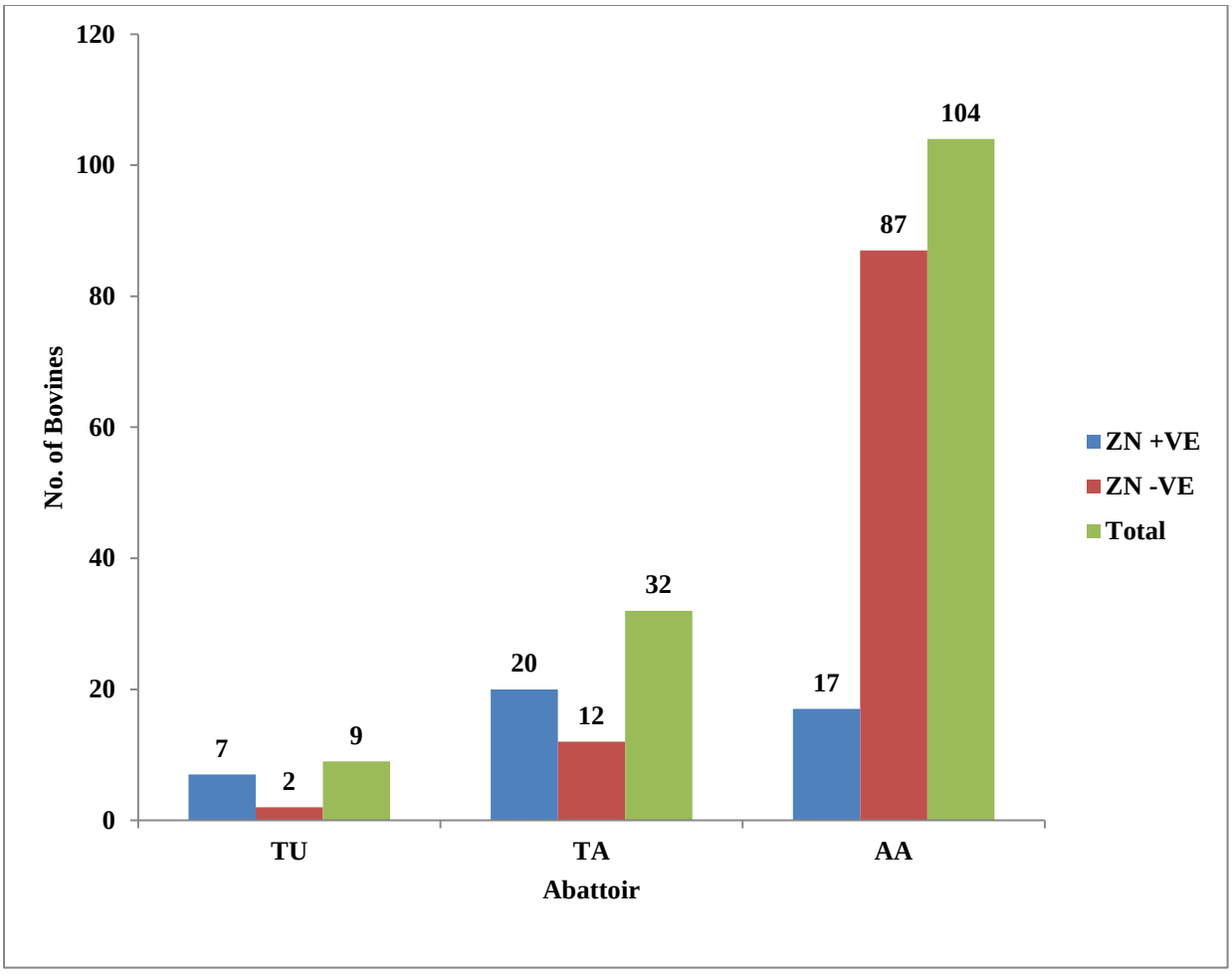

Figure 15: ZN microscopy results of all bovine samples from the Abattoirs.

TU=Tulako Abattoir; TA=Tema Abattoir; AA=Accra Abattoir; ZN+VE=Ziehl Neelsen Positive; ZN-VE= Ziehl Neelsen Negative.

Figure 15 indicates the ZN microscopy results of all bovine samples from the three abattoirs. Out of the 104 bovines from which samples were collected at the Accra Abattoir, 17 (16.4%) tested positive and 87/104 (83.7%) tested negative. Analysed tissue samples taken from the Tema Abattoir showed that 20/32 (62.5%) were positive whilst 12/32 (37.5%) tested negative. At the Tulako Abattoir, out of the 9 bovine samples, 7 (77.8%) tested positive and 2 (22.2%) tested negative.

In all, lymph nodes represented the highest number of tissue samples that were positive by ZN microscopy i.e. 26/155 (16.8%) followed by lung tissues i.e. 14/155 (9.0%) and then liver i.e. 8/155 (61.5%). One sample each taken from the head and cardiac tissues were found to be positive by ZN microscopy whilst the udder sample was negative (Table 6 appendix).

In terms of a breakdown of the lymph nodes screened, out of the 106 pre-scapular lymph nodes screened with ZN microscopy, 19 (17.9%) tested positive and 87 (82.1%) tested negative. One (1/3 or 33.3%) out of the three supra-mammary lymph nodes screened tested positive and 2 (66.7%) tested negative. All three (3/3 or 100%) of the retropharyngeal lymph nodes screened tested positive. Also, all two (2/2 or 100%) of the bronchial lymph nodes screened tested positive. The only mesenteric lymph node screened also tested positive (Table 7 appendix).

A sample each was obtained from the 138 bovines. One hundred and thirty eight bovines contributed one tissue sample each at post-mortem. Out of the 107 lymph nodes taken from these bovines, 22 (20.6%) tested positive for ZN microscopy and 85 (79.5%) were negative. Eleven lung tissues i.e. 11/20 (55.0%) tested positive whilst nine i.e. 9/20 (45.0%) tested negative. Six liver tissues i.e. 6/10 (60%) tested positive whilst 4 i.e. 4/10 were negative. The only tissue taken from the udder tested negative by ZN microscopy (Table 8 appendix).

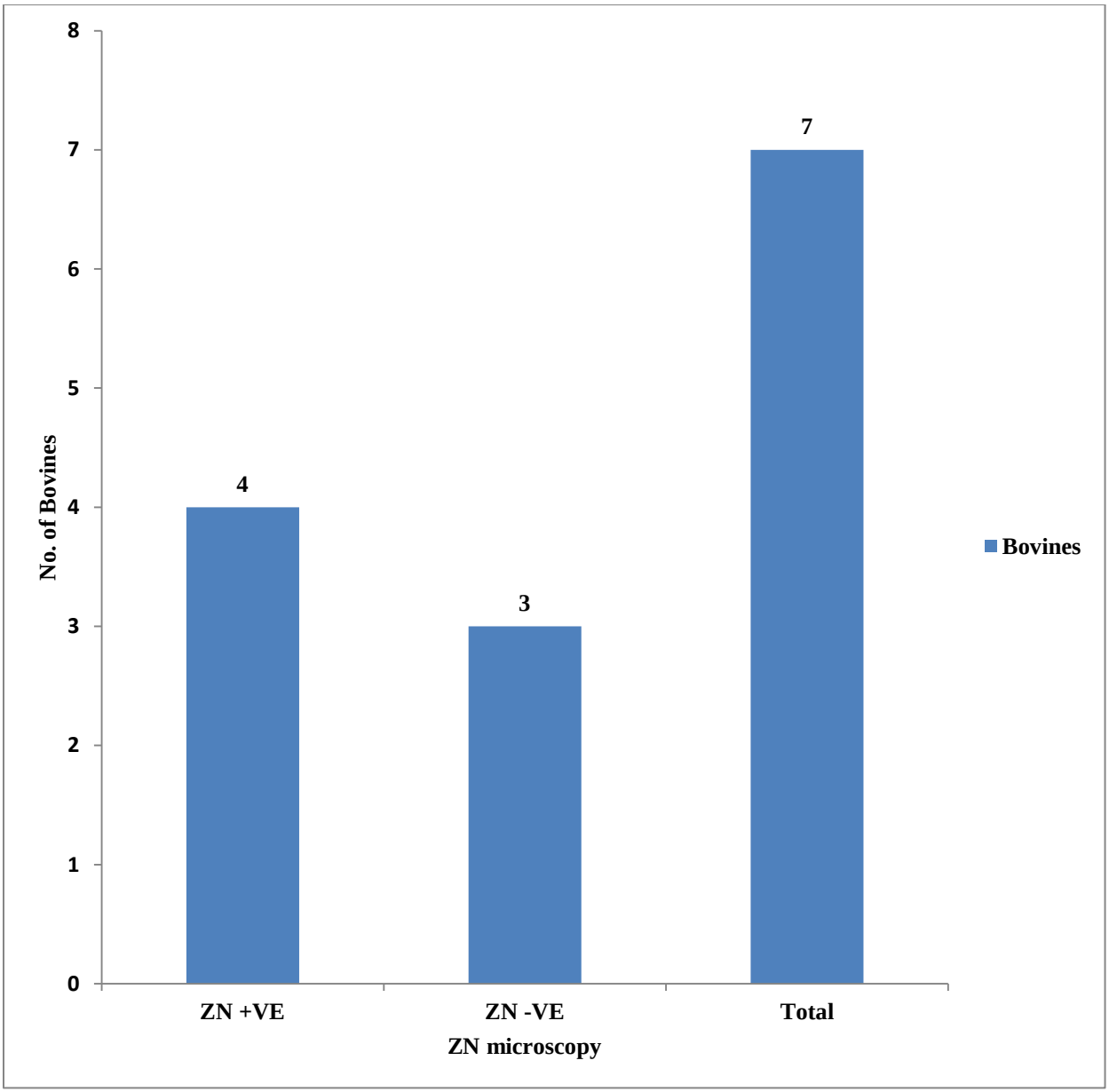

Figure 16: ZN microscopy results of all bovines inspected at Post-mortem from which two or three tissue samples were obtained.

ZN+VE=Ziehl-Neelsen Positive; ZN-VE= Ziehl-Neelsen Negative.

Figure 16 depicts all the ZN microscopy results of seven bovines from which two or three tissue samples were obtained during post-mortem inspection. In all, four i.e. 4/7 (57.1%) tested positive and three i.e. 3/7 (42.9%) tested negative by ZN microscopy.

4.3 MYCOBACTERIAL CULTURE

A total of 155 tissue samples from suspected tuberculous lesions obtained were processed and subjected to culture on two Löwenstein-Jensen media each (one enriched with glycerol and the other with pyruvate). Löwenstein-Jensen medium with glycerol supports the growth of *M. tuberculosis* and Löwenstein-Jensen medium with pyruvate supports *M. bovis* growth. Consequently, culture isolates could be harvested at a higher frequency on Lowenstein-Jensen medium enriched with pyruvate (often with dysgonic colonies) than Lowenstein-Jensen medium with glycerol. A summary of all collected samples with lesions, samples cultured and isolates contaminated is shown on Table 9. Figure 17 indicates all culture positive isolates.

In all, 105/155 (67.7%), 33/155 (21.3%) and 9/155 (5.8%) from the Accra, Tema and Tulako Abattoirs were cultured respectively. Eight i.e. 8/155 (5.2%) however were contaminated and subsequently discarded (Table 9 appendix).

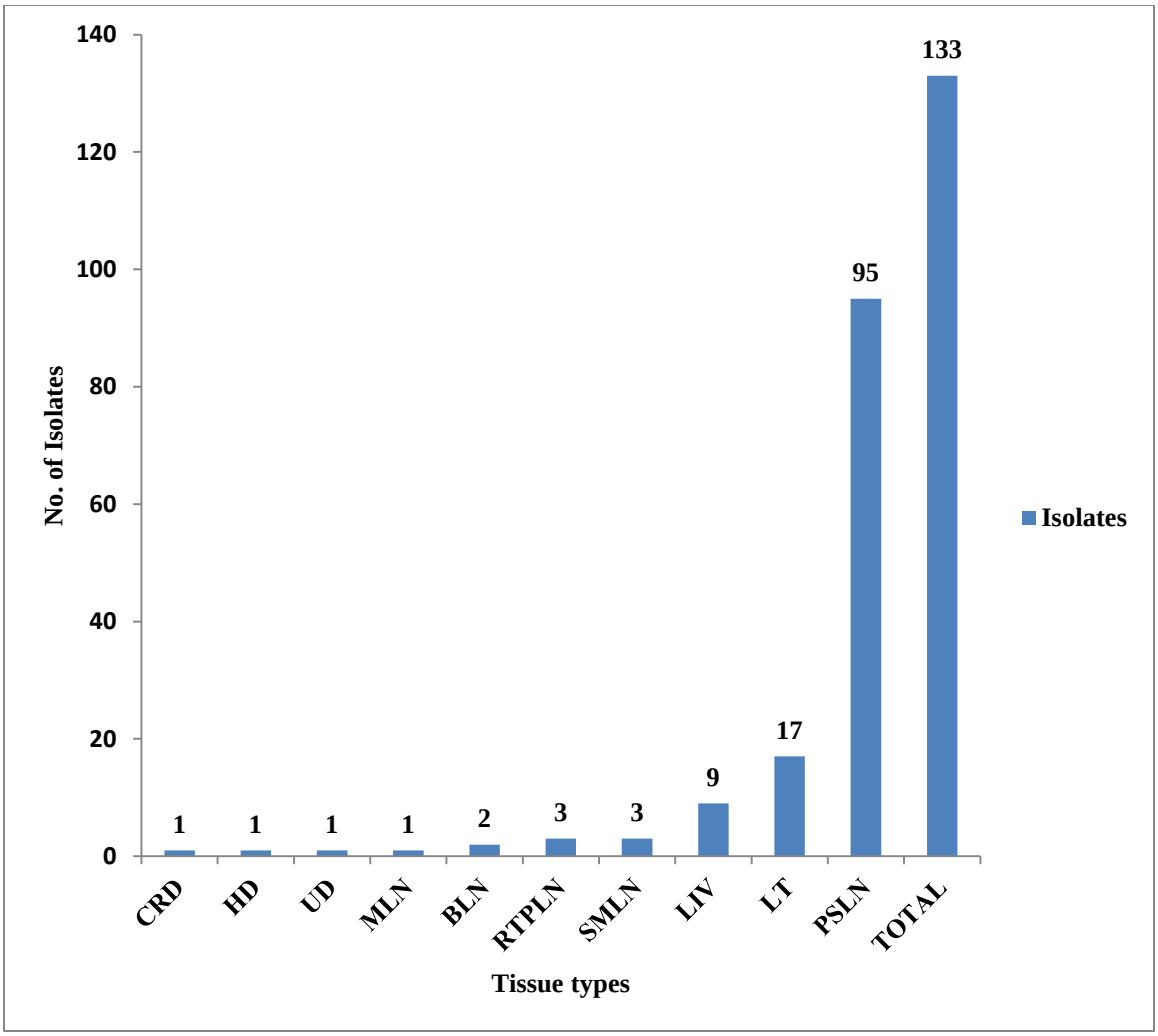

Figure 17: Tissue distribution of all culture positive isolates.

CRD=Cardiac tissue; HD=Head tissue; UD=Udder tissue; MLN=Mesenteric lymph node; BLN=Bronchial lymph node; RTPLN=Retropharyngeal lymph node; SMLN=Supra-mammary lymph node; PSLN=Pre-scapular lymph node; LIV=Liver tissue; LT=lung tissue.

Figure 17 shows a breakdown of all tussue types inoculated on L-J media that grew. Out of the 133 culture isolates, 95 (71.4%) were from pre-scapular lymph nodes, 17 (12.8%) from lung tissues, 9 (6.8%) from liver , 3 (2.3%) each from supra-mammary and retro-pharyngeal lymph nodes and 2 (1.5%) from bronchial lymph nodes. An isolate each i.e 1/133 (0.6%) from the cardiac, head, udder and mesenteric lymph node were also recorded.

4.4 CAPILIA TB-NEO TEST

Ninety three culture isolates were selected based on the mycobacterial's rate of growth, morphology as well as colour on L-J media and subjected to Capilia TB-Neo test. In all, 11 (11.8%) were MTBC by Capilia TB-Neo test and 82 (88.2%) were NTMs (Table 10 appendix). A breakdown of the result indicates that, out of the 64 culture isolates from the Accra Abattoir that were subjected to Capilia TB-Neo test, 3 (4.7%) were MTBC and 61 (95.3%) were NTMs. Of the 22 culture isolates from the Tema Abattoir, 8 (36.4%) were MTBC and 14 (63.6%) were NTMs. All the 7 culture isolates from the Tulako Abattoir were however NTMs (Table 11 appendix).

4.5 MULTIPLEX LIGATION-DEPENDENT PROBE AMPLIFICATION

A total of one hundred and thirty culture isolates were subjected to MLPA assay using 13 probes designed for MTB genotyping and drug resistance testing. A summary of a representative MLPA result, probe by probe, is shown on Table 12 appendix. Out of the 130 culture isolates analyzed, 63 (48.5%) were members of the MTBC whilst 49 (37.7%) were NTMs. However, 18/130 (13.9%) produced inconclusive results (Table 13 appendix). A breakdown of that result indicates that 8/63 (12.7%) were *M. bovis* and 5/63 (7.9%) were *M. africanum.* Surprisingly, most of the isolates analyzed i.e. 50/63 (79.4%) were *M. tuberculosis* (Table 14 appendix). In terms of tissue type, out of the 85 culture isolates from lymph nodes analyzed, 32 (37.7%) were *M. tuberculosis* and 6 (7.1%) were *M. bovis.* Also, out of the 15 culture isolates from lung tissues that were analyzed, 10 (66.7%) were *M. tuberculosis* and 1 (6.7%) was *M. bovis.* Of the

12 culture isolates from the other tissues (head, udder, liver) that were analyzed, 8 (66.7%) were *M. tuberculosis* and 1 (8.3%) was *M. bovis* (Table 15 appendix).

A breakdown of the MLPA result, abattoir by abattoir, indicates that, out of the 80 culture isolates that were analyzed from the Accra Abattoir, 31 (38.8%) were *M. tuberculosis* and 2 (2.5%) were *M. bovis.* Out of the 24 culture isolates that were analyzed from the Tema Abattoir, 11 (45.8%) were *M. tuberculosis* and 6 (25.0%) were *M. bovis.* All 8 (100%) of culture isolates analyzed from the Tulako abattoir were *M. tuberculosis* (Table 16 appendix).

Results of seven bovines from which 2 or 3 tissue samples were obtained indicated that 4 (57.1%) had single mycobacterial infection whilst 3 (42.9%) had mixed mycobacterial infection (Table 17 appendix).

A comparison of the MLPA result and the Capilia TB-Neo test revealed a high relative sensitivity of 72.7% and a low relative specificity of 39.7%. The positive and negative predictive values were 16.3% and 90.0%, respectively (Table 18 appendix).

Below are pictures of 2.0% (w/v) agarose gel electrophoresis run on the samples incorporating the positive sample controls i.e. MTB (P26), *M. africanum* (P65), INH® strain (P123), RIF® strain (P69) and an MDR strain (N168). The negative control was molecular grade biology water.

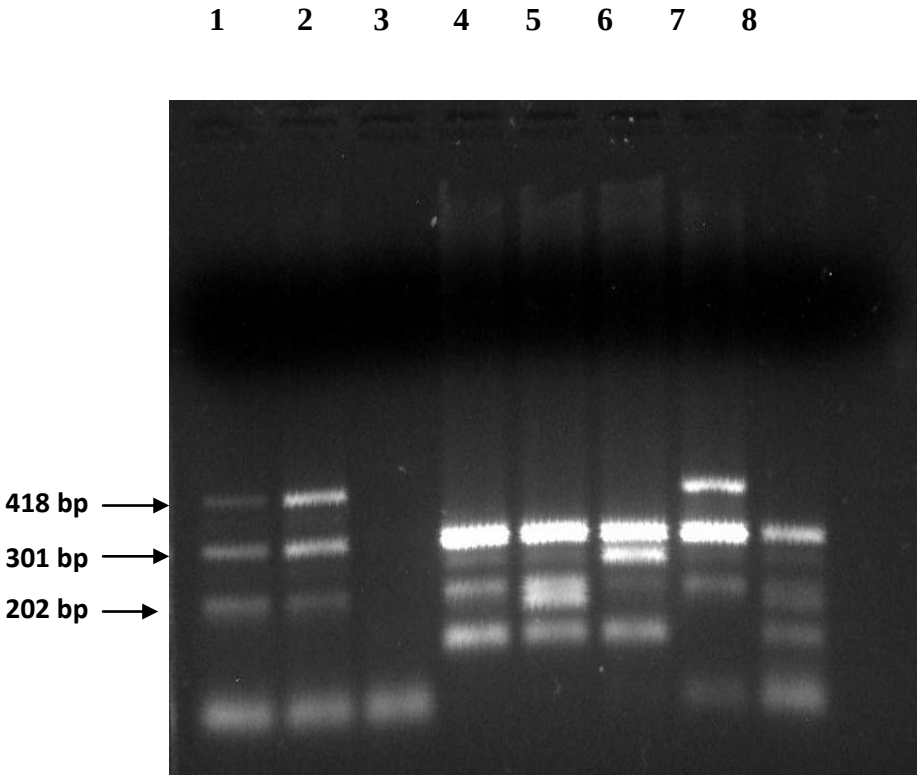

Figure 18a: An ethidium bromide-stained 2% (w/v) agarose gel electrophoresis analysis of MLPA products.

There were amplifications in well 1 and 2 (three bands) indicating the presence of *M. bovis*.

Legend: Lane 1: Test sample TA5PSLN (bovine isolate from pre-scapular lymph node lesion).

Lane 2: Test sample TA30PSLN (bovine isolate from retro-pharyngeal lymph node lesion).

Lane 3: Negative control (Water).

Lane 4: P26 (Positive sample control-MTB).

Lane 5: P123 (Positive sample control-INH®MTB).

Lane 6: P69 (Positive sample control-RIF®MTB).

Lane 7: P65 (Positive sample control-MTB).

Lane 8: N168 (Positive sample control-MDR-TB).

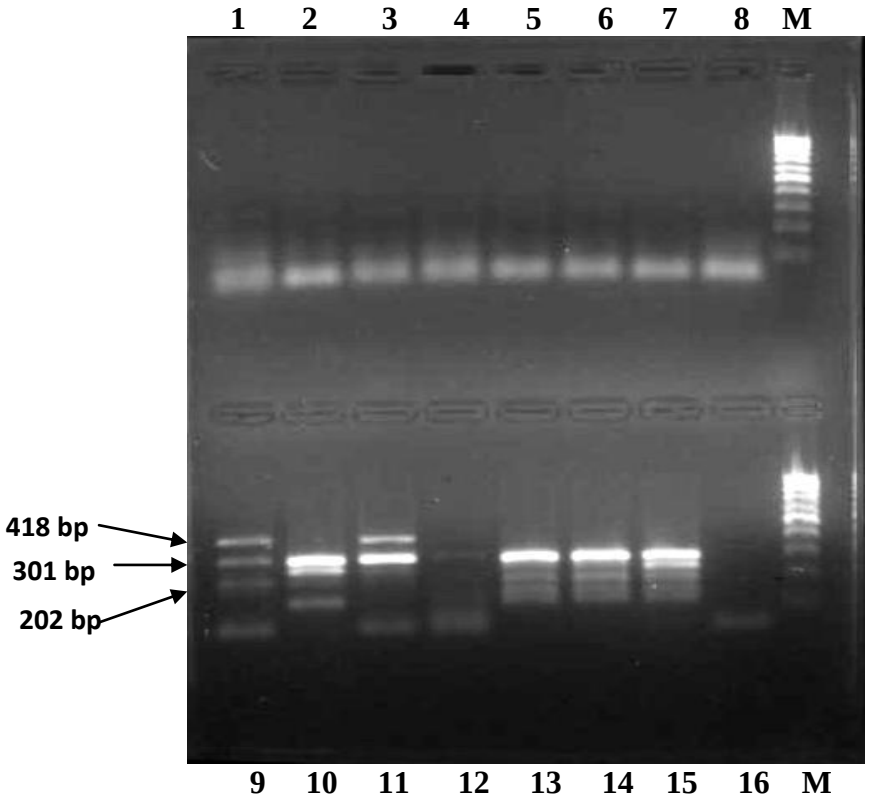

Figure 18b: An ethidium bromide-stained 2% (w/v) agarose gel electrophoresis analysis of MLPA products.

There were no amplifications in well 1-8 indicating the absence of MTBC organisms. Well 9 shows 3 bands indicating the presence of *M. bovis*.

Legend: Lanes 1 – 8: Test samples indicating the absence of MTBC by MLPA.

Lane 9: Test sample AA20RPN (bovine isolate from retro-pharyngeal lymph node lesion).

Lane 10: P69 (Positive sample control-RIF®MTB).

Lane 11: P65 (Positive sample control-MTB).

Lane 12: Test sample TA9LT (bovine isolate from lung lesion).

Lane 13: Lib2a (Suspected MDR isolate from Liberia commissioned by WHO Office Accra, Ghana).

Lane 14: Lib3a (Suspected MDR isolate from Liberia commissioned by WHO Office Accra, Ghana).

Lane 15: Lib4a (Suspected MDR isolate from Liberia commissioned by WHO Office Accra, Ghana).

Lane 16: Negative control (Water).

Lane M: DNA 100 bp Marker (Bioron GmbH, Ludwigshafen, Germany).

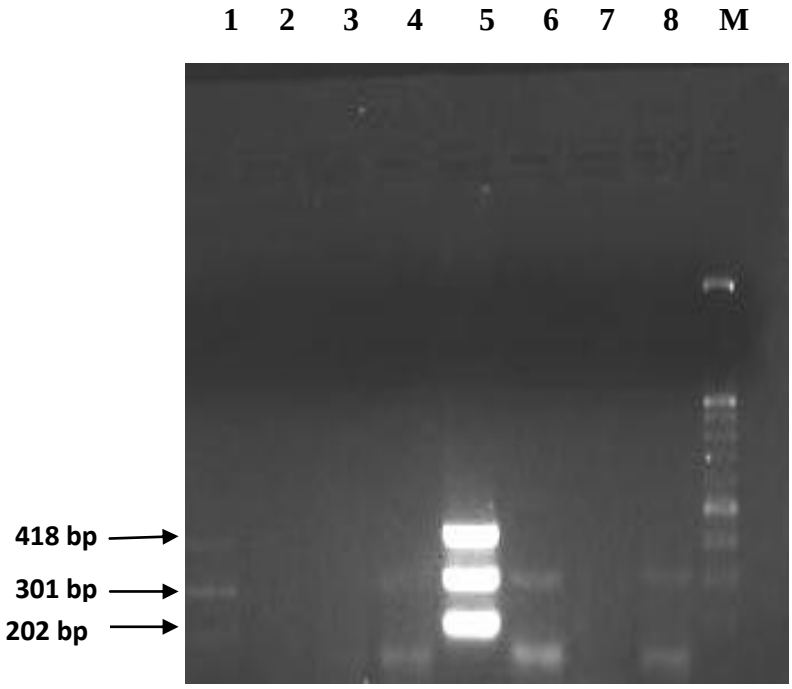

Figure 18c: Another ethidium bromide stained 2% (w/v) agarose gel electrophoresis analysis of MLPA products.

There were amplifications in well 5 indicating the presence of *M. bovis*). Even though there were amplifications (one band each) in well 1, 6 and 8, the result was inconclusive.

Legend: Lane 1: Test sample AA104PSLN (bovine isolate from pre-scapular lymph node lesion showing three bands).

Lane 2: Negative control (water).

Lane 3: Test sample AA31PSLN (bovine isolate from pre-scapular lymph node lesion).

Lane 4: Test sample 27PSLN (bovine isolate from pre-scapular lymph node lesion).

Lane 5: Test sample 20RT (bovine isolate from retropharyngeal lymph node lesion).

Lane 6: Test sample 13PSLN (bovine isolate from pre-scapular lymph node lesion).

Lane 7: Test sample 12PSLN (bovine isolate from pre-scapular lymph node lesion).

Lane 8: Test sample 9PSLN (bovine isolate from pre-scapular lymph node lesion).

Lane M: DNA 100bp Marker (Bioron GmbH, Ludwigshafen, Germany).

CHAPTER FIVE

5.0 DISCUSSION

This is the first time MLPA has been used to characterize *M. bovis* strains isolated from bovine tissue samples in Ghana. The intra-dermal tuberculin skin test has however been used to screen cattle in dairy farms (Ankugah, 2000; Bonsu *et al.,* 2000). Bovine tissue examination based on ZN microscopy has also been reported in Ghana (Adu-Bobi *et al.,* 2009).

An almost equal number of bulls and cows (73 verses 72) were involved in this study. In contrast, Bonsu *et al.* (2000) reported that twice as much cow was infected with bovine tuberculosis than in bulls and heifers.

The result of this study indicates an apparent lesion prevalence of 5.0% (145/2886) which is slightly higher than apparent lesion prevalence found at post mortem in an Ethiopian Abattoir where Stefan *et al.* (2009) reported of an a apparent lesion prevalence of 4.7%.

Lesions in lymph nodes were the most prevalent (115/155 or 74.2%) followed by lung tissue (24/155 or 15.5%) and Liver (13/155 or 8.4%). Fewer lesions were found in the head, udder and the heart. Pritchard (1988) reported that even though bovine tuberculous lesions are often found in the pulmonary region, other organs can equally be affected. This finding was confirmed by Gutierrez and Garcia (1993). The high percentage of lymph node samples (115/155 or 74.2%) is not uncommon as Milian-Suazo and colleagues (Milian-Suazo *et al.,* 2000) reported a high percentage of lymph node involvement in mycobacterial infection. Forty four bovine samples (44/145 or 30.3%) tested positive by Ziehl-Neelsen microscopy. In terms of tissue distribution,

although the number of lesions seen in lymph nodes were higher than those in the lungs and liver, the presence of acid fast bacilli was strongly associated with lung tissue (14/24 or 58.33%) and liver (8/13 or 61.5%) compared to the lymph nodes (26/115 or 22.61%). A possible explanation for the low ZN positive results in the lymph nodes, most of which were not associated with the thoracic region, is the low rate of mycobacterial survival in the central caseation environment of the lymph node (Cassidy, 2006) or the loss of bacterial structure as a result of some immune response that occur in response to infection by mycobacteria, a condition which is evident by the inflammation of the granuloma (Gutierrez and Garcia, 1993).

A breakdown of the ZN microscopy results of all lymph node samples indicated that acid-fastness was strongly associated with isolates from lymph nodes of the thoracic region as all bronchial and retropharyngeal lymph nodes tested positive for AFBs (Table 7 appendix). The presence of visible lesions in an organ may not always be linked with mycobacterial infection as lesions could be caused by other parasites or intracellular agents and that can potentially mislead a meat inspector during post mortem examinations (Asseged *et al.*, 2004).

The identification of acid fast bacilli in bovine tissue samples using ZN microscopy is widely considered as a first-line screening method in resource-limited countries. Using the procedure, the number of acid fast bacilli identified on the microscopic slides varied markedly from one tissue type to the other. Few slides had scanty bacilli i.e. one or two per slide whilst other slides had relatively high numbers i.e. >10 bacilli per field. It must be noted that the efficiency of any

routine abattoir meat inspection is largely dependent on the time, work load and diligence on the part of the meat inspector (Corner *et al.,* 1990).

Even though variable numbers of tissue samples were taken from the three abattoirs, ZN microscopy identified acid fast bacilli in 17/104 (16.4%) bovines at the Accra Abattoir compared to the 20/32 (62.5%) positives taken from the Tema Abattoir and also the 7/9 (77.8%) positives taken from the Tulako Abattoir (Fig. 15). In total, ZN microscopy identified acid-fast bacilli in 32.3% of all lesion samples from the three abattoirs. This was however lower than the 71.7% reported in a similar study in the Kumasi Metropolitan Abattoir (Adu-Bobi *et al*., 2009). The findings of the current study however give some insight into the efficiency of the post mortem examinations at the three abattoirs. It reveals the likelihood that a lesion sample taken by a meat inspector truly contains acid fast bacilli was higher at the Tulako livestock market followed by the Tema Abattoir and then the Accra Abattoir. Tulako livestock market is an emergency transitional slaughter facility, ostensibly started to cater for weak and very sick animals.

The total number of visible lesions that grew on the culture media was found to be 133/155 (85.8%) (Fig. 17). This result is higher than the 60% culture yield obtained from cultured bovine tissue samples from abattoirs in Brazil (Nassar *et al.,* 2007) and in Britain where 50% yield was reported (Liebana *et al.,* 2008). In terms of cultured tissue distribution, Out of the 133 culture isolates obtained, 95 (71.4%) were from lymph node lesions (Fig. 17). This was less than 84% of culture isolates obtained by Milian-Suazo and co-workers (Millian-Suazo *et al.,* 2000). The same study showed that all nine pre-scapular lymph nodes cultured grew to yield isolates,

indicating the high culture yield associated with pre-scapular lymph nodes. It has been suggested that the rate of culture yield largely depends on the type of decontamination procedure used (Haddad *et al.*, 2004). However, recovering or isolating *M. bovis* from bovine tissue is fraught with a lot of technical difficulties even though culture is regarded as the 'Gold Standard' in determining the tuberculous infection. The bacilli are slow growing and therefore take between 8-12 weeks for colonies to mature on a solid egg-based media. Sample preparation involves the maceration and homogenization of tissue before decontamination using standard protocol. In our case, homogenization and maceration were done manually and therefore obtaining a positive culture could be dependent on the efficiency of the process. Growth colonies turn to be dysgonic rather than eugenic in their morphology, making it very difficult to count. The presence of very low viable microbial load as well as fast growers can potentially hinder the growth of *M. bovis* on a culture media. The isolation of fast growers such as *M. kansasii, M. smegmatis, M. fortuitum, M. gastri* and others in bovine tissue samples is therefore not uncommon (Milian-Suazo *et al.*, 2000).

To differentiate members of the *Mycobacterium tuberculosis* Complex (MTBC) from the Non-Tuberculous Mycobacteria (NTM), Capilia TB-Neo assay was performed. Based on colony morphology, growth rate and colour, 93 isolates were selected and used for the analysis. The assay, which is a simple but rapid immuno-chromatographic test and requiring no specialized instrumentation, was used in detecting MTBC in 11/93 (11.8%) of the isolates examined (Table 10 appendix). This is however lower than the results of a study carried out by Noaki *et al.* (2000) where they reported 50% positive results with clinical isolates using the Capilia TB-Neo test. The low sensitivity in this current study may be due to the limited production of MPB64

antigen by the culture isolates (Hasegawa *et al.*, 2002). Another possible explanation may be that the MTBCs might have been outgrown by the fast growers due the neutralization step incorporated during sample decontamination (Kubica *et al*., 1963).

Multiplex Ligation-dependent Probe Amplification (MLPA), a molecular based assay, was also performed to further characterize all Capilia TB-Neo positive strains. This assay, which is made up of markers for genotyping and drug resistance determination was able to distinguish *Mycobacterium bovis* from other related mycobacterial strains such as *Mycobacterium tuberculosis*, *Mycobacterium africanum* and NTM. The reliability of this assay has been demonstrated by using a reference panel of well characterized mycobacterial strains (Bergval *et al*., 2007). Amplification products with band sizes 202 bp (16S rRNA), 301 bp (IS6110) and 418 bp (TbD1) on the electrophoretic gel indicate the presence of *Mycobacterium bovis* strain in the isolate (Fig. 18a). Amplification products with band sizes 130 bp (RD 9), 202 bp (16S r RNA) and 301 bp (IS6110) also indicate the presence of *Mycobacterium tuberculosis* strain in the isolate. Also, amplification products with band sizes 130 bp (RD 9), 202 bp (16S r RNA), 301 bp (IS6110) and 418 bp (TbD1) indicate the presence of *Mycobacterium africanum* in the isolate (Table 12 appendix). No amplification product was observed for NTMs.

Out of a total number of 130 isolates typed, 63 (48.5%) was found to be members of the MTBC (Table 13 appendix). However, 18/130 (13.9%) of the isolates could not be identified. The reason may be that the DNA template lacked the sequence complementary to the primers and probes used in assay or the DNA template might have mutated.

Surprisingly, *Mycobacterium tuberculosis,* the strain that causes tuberculosis in humans was identified in 50/112 (79.4%) of the culture isolates (Table 15 appendix). This is higher than that found in a similar study in Ethiopia where 8/171 (4.7%) of *Mycobacterium tuberculosis* was isolated (Stefan *et al.,* 2009). *Mycobacterium bovis* and *Mycobacterium africanum* were isolated from only 8/112 (12.7%) and 5/112 (7.9%) bovines respectively (Table 14 appendix). It has been reported that *Mycobacterium tuberculosis* isolated in bovine samples is an indication that infected animals with active tuberculosis living in close contact with humans can be a source of disease transmission (Ayele *et al.,* 2004; Erwin *et al.,* 2004; Fritsche *et al.,* 2004). Likewise, it is known that any individual with active tuberculosis and spreading the disease can infect an animal when exposed to it (Fritsche *et al.,* 2004). Sjogren and Hillerdal (1978) also reported that there have been numerous instances where the disease has been transmitted from humans to cattle and inferred that this mode of transmission is more likely in individuals with smear-positive pulmonary tuberculosis.

Forty nine (49/130 or 37.7%) of the culture isolates typed using MLPA were found to be Non-tuberculous mycobacteria (Table 15 appendix). The presence of NTM in an isolate was strongly associated with lymph nodes. The 49/130 (37.7%) of NTMs isolated in the present study is consistent with a similar study in Ethiopia where 53/171 (31.0%) of NTMs were identified. Also, several species of NTMs have been previously identified in various studies in Africa suggesting that the species are found in cattle herds (Diguimbaye-Djaibe *et al.,* 2006; Michael *et al.,* 2007).

The current study also revealed that out of the seven bovines from which two or three tissue samples were taken and analyzed with MPLA, 4 (57.1%) were as a result of a single strain infection whilst 3 (42.9%) were mixed infections (Table 17 appendix). A breakdown of all the bovines with mixed infection shows that, one of the cattle was infected with *Mycobacterium tuberculosis* and *Mycobacterium bovis.* Another bovine was infected with *Mycobacterium tuberculosis* and NTM and the last was infected *Mycobacterium bovis* and NTM. Liete *et al.* (1998) reported that in addition to *Mycobacterium tuberculosis* mixed infection in bovines, other NTMs such as *Mycobacterium fortuitum* and *Mycobacterium avium* Complex strains can also be a source of infection. The identification of mixed mycobacterial infection especially *Mycobacterium tuberculosis* and *Mycobacterium bovis* in bovine suggest that the public is at risk of TB infection.

Characterization or speciation of the NTMs was not done. Out of a total number of 68 samples, 8 were MTBC by both Capilia TB-Neo and MLPA assays**.** Also, 27 out of the 68 samples were NTMs by both methods. Forty-one (41) out of the 68 isolates were NTMs by Capilia TB-Neo test but MTBC by MLPA. Three were also found to be MTBC by Capilia TB-Neo test and NTM by MLPA (Table 18 appendix). Capilia TB-Neo test could not identify all MLPA positive isolates probably due to the rather low mycobacterial load in the isolates. The minimum detection sensitivity of Capilia TB-Neo assay is 1.2×10^6 CFU/ml of isolate (Nagai *et al.*, 1991).

The comparison revealed a test sensitivity, which is a measure of the true positive results that were positive by the test (MLPA), of 72.7% (Table 18 appendix). The Capilia TB-Neo test was

used as the 'Gold standard'. The relatively high test sensitivity suggests that MLPA was efficient in detecting MTBC strains in bovine tissue samples. However, the specificity which is a measure of the true negative results that were negative by the test (MLPA) was found to be 39.7%. Even though the Positive Predictive Value (PPV), which is the probability that a positive test result is truly positive was low i.e. 16.3%, the Negative Predictive Value (NPV) which is defined as the probability that a negative test result is truly negative was rather high i.e. 90.0% (Table 18 appendix). The significance of PPV and NPVs obtained is that when MLPA is used as the only assay for MTBC characterization, only 16.3% of all isolates it identifies as MTBC will be truly MTBC and 90.0% of all isolates it identifies as NTM will be truly NTM.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

The prevalence of cattle with lesions observed at post-mortem at the three abattoirs is 5.0% (145/2886). This indicates a higher prevalence of bovine tuberculosis in beef at abattoirs in the Greater Accra Region than 3.1% obtained in the Volta Region of Ghana. Tissue samples with lesions that were found to contain acid-fast bacilli were low (50/155 or 33.2%).

Capilia TB-Neo test used for the differentiation of the MTBC and NTM identified only 11/93 (11.8%) of the isolates as members of the *Mycobacterium tuberculosis* Complex. It was however found to be simple, fast and required little processing and no instrumentation for the analysis. The only drawback was that it required the availability of grown cultured isolates for the testing.

Multiplex Ligation-dependent Probe Amplification (MLPA) identified as high as 112/130 (86.2%) of the cultured isolates examined as MTBC or NTM. Surprisingly, most of the MTBC organisms detected by MLPA were found to be *Mycobacterium tuberculosis* which is the etiological agent for TB in humans. The assay also identified mixed infections in three of the bovines analyzed. The mycobacterial isolates lacked rifampicin and isoniazid resistance genes when they were analysed by MLPA. This result ought to be confirmed by *in vitro* drug susceptibility test. However, this has not been confirmed by an *in vitro* PCR-based drug susceptibility test such as the Hain MTB-DR+ test.

6.2 RECOMMENDATIONS

Based on the results, the following recommendations are being made:

1. The Ministry of Food and Agriculture should immediately consider implementing a test and slaughter policy as early as possible because accurate identification and subsequent

removal of bovine tuberculosis infected animals are crucial to the control of the disease (De la Rua-Domenech, 2006; Gormley *et al.,* 2006).

2. The government should also take steps to adopt a cattle slaughter-infection-compensation policy which will adequately compensate butchers or cattle owners whose animals have been found to be infected during the cattle examination process. This will help them to coorperate fully in the control of the disease in Ghana. The inclusion of an insurance policy may be helpful. This insurance policy may have cattle traders paying a fixed sum (or a percentage of fees charged) as a premium on every animal slaughtered. Approximately 75% of the average cost of an animal may be paid back or reimburse to the merchants in the event that his/her carcass is confiscated to be condemned after post mortem examination.

3. To prevent the possible transmission of the disease from cattle to agriculture or abattoir workers, infrastructure as well as Safety Management practices at the three abattoirs should be improved. The procedure used to dispose off infected carcasses must also be reviewed as birds allowed to feed on these infected carcasses can be the disease reservoir. All this could be achieved through education, as good knowledge and regular attention of cattle owners to their health and economic consequence are crucial in any bovine tuberculosis control program (Gormley *et al*., 2006). A Good Laboratory Practice (GLP) culture should be strictly adhered to in abattoirs in Ghana.

4. There is also the need for the Veterinary Service of Ghana to set up a functioning laboratory at the three abattoirs as against the current practices where suspected lesions will have to be transported in a commercial vehicle to a central laboratory several kilometers away for the confirmation of the disease.

5. The Veterinary Services of Ghana should also enforce regulations governing cattle movement in the country.

6. The identification of mycobacterial strains by well equipped research institutions and the various disease surveillance activities should be integrated into the activities of the Veterinary Services Division of the Ministry of Food and Agriculture (MoFA), Ghana. This will ensure the implementation of a thorough and effective control program.

Based on the results of the current research, it is being suggested that further research should be done in the following areas:

1. Investigation based on mere observation of activities at the abattoirs should be carried out to determine the risk of transmission of bovine tuberculosis from cattle to humans.

2. Molecular based assays should be developed using gene probes to directly indentify mycobacterial strains in bovine tissue samples. This will ensure a rapid determination of bovine tuberculosis infection compared to the several weeks required for mycobacterial culture before molecular analysis is carried out.

3. Another Capilia TB-Neo assay should be developed to differentiate between the members of *Mycobacterium tuberculusis* Complex.

4. A random collection of beef samples without visible lesions in the market should be included in any further studies to determine the safety and quality of meat consumers purchase. This type of study should be supported by a structured questionnaire.

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APPENDIX I

SOLUTIONS FOR MICROSCOPY

Stock Carbol-Fuchsin (A)

3 g of Basic fuchsin

100 ml Ethanol

5% Phenol Solution (B)

5 ml of melted Phenol crystals

95 ml distilled water

Ziehl-Nelseen (ZN) Working Carbol-Fuchsin Solution (C)

10 ml of solution A

90 ml of solution B

0.3% Methylene Blue Solution

0.3 g methylene Blue

100 ml distilled water.

Solution should be filtered before use.

20% Sulphuric Acid Solution

20 ml concentrated Sulphuric acid (H_2S0_4)

80 ml of distilled water

Preparation of solution should be in a fumed chamber.

DECONTAMINATION SOLUTIONS

1M Sodium Hydroxide

10 g of NaOH

250 ml distilled water.

0.15 Sodium Hydroxide

0.6 g of NaOH

100 ml distilled water

0.1 M Sodium Citrate

7.35 g of $Na_3C_6H_5O_7.2H_2O$

250 ml distilled water

0.15 M Sodium Dihydrogen Phosphate (pH=5.0)

9.0 g of NaH_2PO_4

500 ml distilled water

0.5% Phenol Red

0.5 g phenol red

100 ml 0.15 NaOH

MYCOBACTERIAL DNA ISOLATION

Lysis Buffer: 10 mM TRIS-HCl (pH=8.0) / 1 mM EDTA / 1%Triton X-100

0.2 ml Tris-HCl

0.02 ml 1mM EDTA

0.1 ml Triton X-100

1 X PHOSPHATE BUFFERED SALINE (pH=7.4)

140 mM Sodium Chloride

8.19 g of NaCl

1000 ml distilled water

2.6 mM Potassium Chloride

0.2 g of KCl

1000 ml distilled water

10.1 mM Disodium Hydrogen Phosphate

1.79 g of Na_2HPO_4

1000 ml distilled water

1.7 mM Potassium Dihydrogen Phosphate

0.23 g of KH_2PO_4

1000 ml distilled water

0.1% TWEEN 80 PHOSPHATE BUFFERED SALINE (pH=7.6)

0.85% NaCl

4.25 g of NaCl

500 ml distilled water

0.1% Tween 80

0.5 g of Tween 80

500 ml distilled water

10 mM Na_2HPO_4

0.71 g of Na_2HPO_4

500 ml distilled water

Middlebrook 7H9 Liquid Media

0.47 g of 7H9 Broth
90 ml sdH_2O } Autoclave at 121°C for 10 minutes

200 ml glycerol + 10 ml ADC Enrichment

RUNNING BUFFER

1 x Tris Borate EDTA Buffer (pH 8.3)

108 g Tris

55 g boric acid

20 ml 0.5 M EDTA (pH=8.0)

1000 ml distilled water

2.0% Agarose Gel

1200 g (1.2g) agarose gel powder

60 ml (1x TBE Buffer)

3 µl Ethidium Bromide (0.5 µl/ml)

APPENDIX II

For Sample Collection

Sample identification number	Pathology	Sex	Total daily slaughter	Collection date

* Sample identification number- a unique number assigned to each tissue sample based on the site of sample collection (abattoir) and the number of tissue taken.

For TB Culture Records

Culture Number						
Date						
Observation for culture Examination						
	Glycerol			Pyruvate		
	Morphology/Colour		Growth	Morphology/Colour		Growth
	Rough, Smooth			Rough, Smooth		
	Eugonic, Dysgonic			Eugonic, Dysgonic		
	Buff,White,Yellow,Orange			Buff,White,Yellow,Orange		
Rapid growth						
within 4 days						
1 Week						
2 Week						
3 Week						
4 Week						
5 Week						
6 Week						
7 Week						
8 Week						
Sub-culture						
Staining of colonies:		AFB		Positive/Negative		
		Confirmation		None/Present		
Memo:						

For Laboratory analysis

Tissue	ZN Microscopy of inoculums	Culture on LJ	ZN Microscopy of Culture isolates	RD9	katG-315	inhA-15	16S rRNA	rpoB-531	rpoB-526G	rpoB-526T	IS6110	mutT2-58	ahpC-46	ogt-12	ogt-15	TbD1	Speciation by MLPA	Capilia Test	Remarks
				130bp	160bp	178bp	202bp	256bp	265bp	274bp	301bp	355bp	364bp	373bp	382bp	418bp			

Decision matrix

	Capilia TB-Neo test		
	"Gold Standard"		
MLPA Results	MTBC	NTM	Total
MTBC	A	C	A+C
NTM	B	D	B+D
Total	A+B	C+D	A+B+C+D

Legend

MLPA: Multiplex ligation-dependent probe amplification.

MTBC: *Mycobacterium tuberculosis* Complex.

NTM: Non-tuberculous mycobacteria.

Sensitivity = A/A+B (100%),

Specificity = D/C+D (100%).

Positive Predictive Value (PPV) = A/A+C (100%).

Negative Predictive Value (NPV) = D/B+D (100%).

APPENDIX 111

Table 5: Apparent lesion prevalence of animal samples at the three abattoirs.

Abattoir	Animals examined	Animals with lesions
AA	2420	104
TA	425	32
TU	41	9
Total	2886	145

Table above shows the total number of bovines examined at Post-mortem and that found to have gross visible lesions between June and October, 2009 at the three abattoirs (TU=Tulako Abattoir; TA=Tema Abattoir; AA=Accra Abattoir).

Table 6: ZN -positive and -negative microscopy results of all tissue samples.

Tissue types	ZN +VE (%)	ZN –VE (%)	Total
LN	26 (22.6)	89 (77.4)	115
LT	14 (58.3)	10 (41.7)	24
LIV	8 (61.5)	5 (38.5)	13
HD	1 (100)	0 (0)	1
CRD	1 (100)	0 (0)	1
UD	0 (0)	1 (100)	1
Total	50	105	155

Table above indicates results of ZN microscopy of all tissue types screened (LN=Lymph nodes; LT=Lung tissue; LIV=Liver tissue; HD=Head tissue; CRD=Cardiac tissue; UD=udder tissue).

Table 7: Positive and Negative ZN microscopy results of all lymph node samples

Lymph nodes	ZN +VE (%)	ZN –VE (%)	Total
PSLN	19 (17.9)	87 (82.1)	106
SLN	1 (33.3)	2 (66.7)	3
RLN	3 (100)	0 (0)	3
BLN	2 (100)	0 (0)	2
MLN	1 (100)	0 (0)	1
Total	26	89	115

Table above represents a breakdown of ZN microscopy results of all lymph node samples (PSLN=Pre-scapular Lymph node; SLN=Supra-mammary lymph node; RLN=Retropharyngeal lymph node; BLN=Bronchial lymph node; MLN=Mesenteric lymph node).

Table 8: ZN microscopy results of 138 individual samples each obtained from an animal.

Tissue types	ZN +VE (%)	ZN –VE (%)	Total
LN	22 (20.6)	85 (79.5)	107
LT	11 (55.0)	9 (45.0)	20
LIV	6 (60.0)	4 (40.0)	10
UD	0 (0)	1 (100)	1
Total	39	99	138

Table above indicates positive and negative ZN microscopy results of 138 individual tissue samples obtained from each animal at post-mortem (LN=Lymph nodes; LT=Lung tissue; LIV=Liver tissue; UD=Udder tissue).

Table 9: Summary of collected lesioned samples, number cultured and isolates contaminated.

Abattoirs	Samples with lesion (%)	Samples cultured (%)	Isolates contaminated (%)
AA	108 (69.7)	105 (71.4)	3 (37.5)
TA	36 (23.2)	33 (22.5)	3 (37.5)
TU	11 (7.1)	9 (6.1)	2 (25.0)
Total	155	147	8

Table 9 above indicates the total number of samples collected with lesions, the number cultured and that which showed contamination on L-J media (AA=Accra Abattoir; TA=Tema Abattoir; TU=Tulako Abattoir).

Table 10: Results of Capilia TB-Neo test (N=93)

	MTBC	NTM	Total
Number	11	82	93
Percentage (%)	11.8	88.2	100

Table 10 above indicates the number of isolates that were MTBC positive and NTM negative by capilia TB-Neo test (MTBC=*Mycobacterium tuberculosis* Complex; NTM=Non-tuberculous mycobacteria).

Table 11: Capilia TB-Neo test

Abattoir	MTBC (%)	NTM (%)	Total

AA	3 (4.7)	61 (95.3)	64
TA	8 (36.4)	14 (63.6)	22
TU	0 (0)	7 (100)	7
Total	11	82	93

Table 11 above indicates the total number of MTBC and NTM results by Capilia TB-Neo test of isolates from the three abattoirs. (MTBC=*Mycobacterium tuberculosis* Complex; NTM=Non-tuberculous mycobacteria; AA=Accra Abattoir; TA=Tema Abattoir; TU=Tulako Abattoir).

Table 12: MLPA results of 10 isolates typed from the three abattoirs.

ISOLATES	RD9	katG-315	inhA-15	16S rRNA	rpoB-531	rpoB-5312	rpoB-526T	IS6110	mutT2-58	ahpC-46	ogt-12	ogt-15	TbD1	MLPA RESULTS
	130 bp	160 bp	178 bp	202 bp	256 bp	256 bp	274 bp	301 bp	355 bp	364 bp	373 bp	382 bp	418 bp	
1	■			■				■						*MTB*
2	■			■				■						*MTB*
3				■				■					■	*M. bovis*
4				■				■					■	*M. bovis*
5	■			■				■					■	*M. africanum*/Ancestral TB
6	■			■				■					■	*M. africanum*/Ancestral TB
7														NTM
8														NTM
9								■						**Inconclusive**
10								■						**Inconclusive**

The table above indicates how the MLPA results were recorded according to each marker (■ indicates the presence of a band; MLPA=Multiplex ligation-dependent probe amplification; MTB=*Mycobacterium tuberculosis*; *M. bovis=Mycobacterium bovis*; *M. africanum=Mycobacterium africanum*; NTM=Non-tuberculous mycobacteria; RD9, *KatG-315*, *inhA-15*, 16SrRNA, *rpoB-531*, *rpoB-526G*, *rpoB-526T*, IS6110, *mutT2-58*, *ahpC-46*, *ogt-12*, *ogt-15* and TbD1 are all molecular markers included).

Table 13: Summary of MLPA results of 130 genotypes

	MTBC	NTM	Inconclusive	Total
Number	63	49	18	130
Percentages (%)	48.5	37.7	13.9	100

Table 13 above indicates MTBC positive and NTM negative as well as inconclusive results by MLPA. Inconclusive refers to gel pictures showing isolates with either one or two bands. (MTBC=*Mycobacterium tuberculosis* Complex; NTM=Non-tuberculous mycobacteria).

Table 14: Breakdown of MLPA results for 63 MTBC-positive isolates

	M. bovis	*M .africanum*	*MTB*	Total
Number	8	5	50	63
Percentage (%)	12.7	7.9	79.4	100

Table indicates a breakdown of the MLPA-positive isolates (*M. bovis=Mycobacterium bovis*; *M. africanum=Mycobacterium africanum*; *MTB=Mycobacterium tuberculosis*).

Table 15: MLPA results according to tissue types

Tissue types	*M .bovis* (%)	*MTB* (%)	*M. africanum* (%)	NTM (%)	Total
LN	6 (7.1)	32 (37.7)	4 (4.7)	43 (50.6)	85
LT	1 (6.7)	10 (66.7)	0 (0)	4 (26.7)	15
Others	1 (8.3)	8 (66.7)	1 (8.3)	2 (16.7)	12
Total	8	50	5	49	112

Table 15 indicates a breakdown of all tissue types that were identified using MLPA (*M. bovis=Mycobacterium bovis*; *MTB=Mycobacterium tuberculosis; M. africanum=Mycobacterium africanum;* NTM=Non-tuberculous mycobacteria; LN=Lung nodes; LT=lung tissue. Others include MLPA results for head, udder and liver tissues).

Table 16: Results of Mycobacterial genotypes identified by MPLA.

Abattoir	*M. bovis* (%)	*M. africanum* (%)	*MTB* (%)	NTM (%)	Total
AA	2 (2.5)	4 (5.0)	31 (38.8)	43 (53.8)	80
TA	6 (25.0)	1 (4.2)	11 (45.8)	6 (25.0)	24
TU	0 (0)	0 (0)	8 (100)	0 (0)	8
Total	8	5	50	49	112

Table indicates the various mycobacterial genotypes identified from the three abattoirs with the Accra Abattoir contributing the highest followed by the Tema Abattoir and Tulako Abattoir in that order (*M. bovis=Mycobacterium bovis*; MTB=*Mycobacterium tuberculosis; M. africanum=Mycobacterium africanum;* NTM=Non-tuberculous mycobacteria).

Table 17: Results of MLPA on all bovines from which more than a sample was taken

Cattle	LT	LIV	RLN	CRD	HD	Left PSLN	Right PSLN	BLN
A	**MTB**	**MTB**	***M. bovis***					
B	**MTB**		**-**		**NTM**			
C							**NTM**	***M. bovis***
D						**NTM**	**NTM**	
E						***M. africanum***	***M. africanum***	
F	***M. bovis***	***M. bovis***						
G	**MTB**	**MTB**		**MTB**				

The table represents single and mixed mycobacterial strain infection from seven bovines that contributed two or three samples for laboratory analysis. In all, 4 bovines had single infections whilst 3 had mixed infections (A, B, C=Mixed infection; D, E, F, G=Single infection; LT=lung tissue; LIV=Liver tissue; RLN=Retropharyngeal lymph node; CRD=Cardiac tissue; HD=Head tissue; PSLN=Pre-scapular Lymph node; BLN=Bronchial Lymph node; *M. bovis=Mycobacterium bovis*; *MTB=Mycobacterium tuberculosis; M. africanum=Mycobacterium africanum;* NTM=Non-tuberculous mycobacteria).

Table 18: Decision matrix

	Capilia TB-NEO Test results 'GOLD STANDARD'					
MLPA Results	**MTBC**	**NTM**	**Sensitivity**	**Specificity**	**PPV**	**NPV**
MTBC	8	41	72.7%	39.7%	16.3%	90.0%
NTM	3	27				
Total	11	68				

Table 18 indicates a comparison of the 'Gold Standard' (Capilia TB-Neo test) with the MLPA assay. (MLPA=Multiplex ligation probe amplification; MTBC=*Mycobacterium tuberculosis* Complex; NTM=Non tuberculous mycobacteria; PPV=Positive Predictive Value; NPV=Negative Predictive Value).