DETECTION AND MOLECULAR IDENTIFICATION OF *MYCOBACTERIUM BOVIS* IN CATTLE SLAUGHTERED AT THE TUDUN-WADA ABATTOIR KADUNA, NIGERIA.

BY

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DECLARATION

I declare that the work in this dissertation entitled "DETECTION AND MOLECULAR IDENTIFICATION OF *MYCOBACTERIUM BOVIS* IN CATTLE SLAUGHTERED AT THE TUDUN-WADA ABATTOIR KADUNA, NIGERIA" has been carried out by me in the Department Of Veterinary Public Health And Preventive Medicine, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria under the supervision of Dr. M Bello and Professor C.A. Kudi. The information derived from the literature used in the text has been duly acknowledged in the text and a list of references provided. No part of this dissertation has been previously presented for another degree or diploma at this or any other Institution.

Kanneng Rita DUWONG

| --- | --- | --- |
| Name of student | Signature | Date |

CERTIFICATION

This dissertation entitled "DETECTION AND MOLECULAR IDENTIFICATION OF *MYCOBACTERIUM BOVIS* IN CATTLE SLAUGHTERED AT THE TUDUN-WADA ABATTOIR KADUNA, NIGERIA" by Kanneng Rita DUWONG meets the regulations governing the award of the degree of Master of Science (M.Sc) in Veterinary Public Health and Preventive Medicine of the Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to the memory of my late parents Da Richard Gyang Pwajok and Ngo Christiana Yop Pwajok, thank you for making me the woman I am today.

ABSTRACT

Bovine tuberculosis (bTb) is an important neglected zoonosis in Nigeria. Though endemic in the country, meat inspection at abattoirs serves as the main surveillance tool of the disease. A study was carried out with the aim of detecting and identifying *Mycobacterium bovis* from infected cattle slaughtered at the Tudun-Wada abattoir in Kaduna North Local Government Area of Kaduna State. A total of 514 tissue samples were collected from carcasses of cachetic, apparently healthy and animals with suspected tuberculous lesion. The samples were obtained from organs/tissues of the lungs, lymph nodes and small intestines. The samples were processed and examined using the Ziehl Neelsen staining technique. Positive samples to Acid Fast Bacilli (AFB) were subjected to multiplex Polymerase Chain Reaction (m-PCR) to identify the species involved. A structured piloted questionnaire was administered to selected stake holders to determine their level of knowledge, attitude and practice to *Mycobacterium* infection. Results obtained showed that out of 514 tissue samples examined, 56(10.89%) were positive by Ziehl Neelsen staining technique. These positive samples were further subjected to multiplex-PCR, out of which 23(4.4%) samples presented with amplicons similar to *Mycobacterium bovis (M. bovis)*. The results further revealed that the disease had a higher detection rate in female animals (11.77%), than in males (7.14%). There was no significant association established between the sex of the animals sampled and the infection (X^2=1.756, P=0.1851). The age of the animals sampled had no significant association with the presence of the infection in the animals sampled (X^2=1.627, P=0.4432), but detection of the disease was observed to be higher in animals within age group 5-7 years, than age group 2-4 years, and those above 7 years. Prevalence of bTb based on breed of cattle was 8.8% in White Fulani and 16.54% in Red Bororo breeds respectively, and results showed significant association of the infection to the Red Bororo breed (X^2 = 6.269, P =

0.0123). Samples that were positive for Acid Fast Bacilli included 3 from Lungs only, 1 from Lymph nodes only, 51 from pooled Lungs and Lymph nodes only and 1 from pooled Lungs, Lymph nodes and Small intestine only. Knowledge of the disease was generally good (72.0%) within the study area. In conclusion, bTb was observed to be present in cattle carcasses destined for human consumption, thus providing useful information on zoonotic risk of bovine tuberculosis in the study area.

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ABBREVIATIONS

bTb	Bovine tuberculosis
FAO	Food and Agricultural Organization
M. bovis	*Mycobacterium bovis*
HIV	Human Immunodeficiency Virus
AIDs	Acquired Immune Deficiency Syndrome
BCG	Bacille de Calmette-Guerin
MDR	Multi-drug resistance
XDR	Extreme drug resistant strains
TDR	Total drug resistant
ZN	Ziehl-Neelsen
mPCR	multiplex Polymerase Chain Reactions
AFB	Acid Fast Bacilli
OIE	Office International des Epizootics
MTC	Mycobacterium tuberculosis complex
NTM	Non-tuberculous Mycobacterium

WHO	World Health Organization
CD	Cluster of Difference
ELISA	Enzyme Linked Immunosorbent Assay
FPA	Fluorescence Polarization Assay
CFU	Colony Forming Unit
BCS	Body Condition Score
RD	Region of difference

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study.

Bovine tuberculosis (bTb) is a chronic, infectious and contagious disease of zoonotic importance of humans, domestic and wild animals (Radostits *et al.*, 2007). The disease is an endemic disease of cattle in Nigeria (Alhaji, 1976; Fasanmi, 1977, Koundoun, 1987; Ayanwale and Alonge, 1988; Alaku and Morupa, 1993; Aliyu and Kalra, 1993; and Igbokwe *et al.,* 2001). It is characterized by the formation of granulomas in tissues and organs, more significantly in the lungs, lymph nodes, intestines, liver and kidneys (Shitaye *et al*., 2007), and despite intensive efforts over the decades to eradicate the disease, bTb continues to be a significant problem globally (Phillips *et al.,* 2003; Pollock *et al.,* 2005).

Though primarily a disease of cattle, bTb has been seen to infect and cause disease in camels, pigs, sheep, goats, horses, dog, cats, badgers, lions, elephant, deer and primates (man inclusive), (Ayele *et al*.,2004). The distribution of bovine tuberculosis is worldwide with a serious effect on animal production. Prevalence data on animal tuberculosis in developing countries is generally scarce, though information on bTb occurrence and control measures exist (Theon *et al.,* 1995, FOA., 1993). Of all the nations in Africa, only seven (Algeria, Burkina Faso, Cameroon, Morocco, Namibia, South Africa, and Zambia) apply disease control measures as part of a test- and -slaughter policy and consider bTb as a notifiable disease (Cosivi *et al*., 1998). The remaining forty eight (48) African countries control the disease inadequately or not at all (FAO., 1993, 1994; Cosivi *et al*., 1998).

The impact of bovine tuberculosis in agriculture greatly influences the dynamics of agricultural economics by causing monetary and time losses (Pollock *et al.*, 2005), thus affecting international trade of both animals and animal products (Biet *et al.*, 2005). Bovine Tb, apart from being a chronic and contagious disease, is also highly zoonotic to humans who have become increasingly susceptible to the disease caused by *Mycobacterium bovis* (Kleeberg, 1984) due to its ability to cross over the specie barrier and the presence of other debilitating diseases like diabetes and HIV/AIDS, which tend to reduce the host immunity.

Mycobacterium bovis is a member of the *Mycobacterium tuberculosis* complex (Radostits *et al.*, 2007). Collins and Grange (1983) list *M. tuberculosis, M. africanum, M. microti, M. carnetti* and *M. ulcerans* as the other members of the *Mycobacterium tuberculosis* complex.

The bacterium causing bTb can be transmitted congenitally through the umbilical veins or post-natally through the alimentary, respiratory, genital or cutaneous routes (Jubb *et al.*, 1985). The direct correlation between *M. bovis* infection in cattle and the disease in the human population has been well documented in developed countries, but as for developing countries, information is still scanty (Cosivi *et al.*, 1995). This lack of data according to Bolognesi (2007), relates to its perception as an animal disease. However, the infection currently poses a major concern in the human population, especially in developing countries where humans and animals are seen to share common or same dwelling environment, especially in rural areas as seen with the Nomadic Fulani settlements of Nigeria (Shitaye *et al.*, 2007).

Indirectly, Man acquires the disease from animal sources by ingestion of contaminated/infected meat and meat products from slaughtered cattle, and consumption of

unpasteurized/contaminated milk (Cosivi *et al.,* 1998; Radostits *et al.,* 2000; Ayele *et al*., 2004; Theon *et al.,* 2006). The disease due to *M.bovis* can be transmitted from animal to animal, animal to man, rarely man to animal, and man to man (Grange and Collins., 1987). The disease primarily affects the respiratory tract but can also spread to other parts of the body like the lymph nodes, uterus, liver and intestine (Shitaye *et al*., 2006; *Bovine tuberculosis*, 2009). The primary route of transmission is the exchange of respiratory secretions between infected and susceptible animals. This can be achieved through nose-to-nose contact or by the inhalation of aerosol droplets that have been exhaled by an infected animal and by oral ingestion of contaminated feeds (Skuce *et al*., 2011).

Various factors affect the efficiency in which *M. bovis* is spread within a cattle herd. These include factors such as source of infection, route of transmission of the agent, persistence of *M.bovis* in infected animals after death and survival of *M.bovis* in the environment (Aranaz *et al*., 2004). Previously, it was believed that the number of infected animals shedding the organism, as well as the number of susceptible animals present within a herd can have an impact on the transmission of the bacteria. For example, the more animals within a herd that are shedding *M. bovis*, the greater the chances of a susceptible animal coming into contact with an infected animal (Shirimi *et al*., 2003). However, Durnez *et al.* (2009) reported a higher prevalence of bovine tuberculosis in the extensive than in the intensive system of management. Cattle infected with bovine Tb may shed bacteria in their faeces, urine and milk, but these are felt to be a minor source of bacterial transmission (Neil *et al.,* 1991).

Survival of *M. bovis* in the environment is primarily affected by exposure to sunlight and it can survive at temperatures between 12.2 ^{o}C and 23.9 ^{o}C for up to 18 to 32 days (Duffield

and Young, 1985). Under laboratory conditions, *M. bovis* has been isolated for up to 8 weeks from various feeds kept at 23.9 ^{o}C and 14 weeks from various feeds kept at -1.7 ^{o}C to 12 ^{o}C (Duffield and Young, 1985). However, under field conditions, it is difficult to isolate *M. bovis* from pastures grazed by animals known to be infected with bovine Tb (Duffield and Young, 1985).

Population growth has led to congested living conditions, which makes it easy for diseases to be spread in the environment. Humans having co-infection of Tb with HIV/AIDs, diabetes, high blood pressure and widespread development of drug resistant strains have further complicated the spread and effects of the disease in Africa (Daborn and Grange, 1993; Cosivi *et al.*, 1998; Corbett *et al*., 2003; WHO, 2009), because these diseases/conditions tend to decrease immunity of the host thereby making them more susceptible to the disease and other opportunistic infections.

Disease caused by *Mycobacteria* often develop very slowly and may take months to years to develop (Alan *et al*., 2012). Early infections in cattle are often asymptomatic, but as the disease progresses, bTb causes a slight fever, rough hair coat, swollen lymph nodes atimes with abscesses. There is dyspnoea with cough and lethargy (Pollock *et al*., 2006), while the disease in humans is characterized by prolonged coughing, general body weakness, wasting away (emaciation), night sweats and fever (Theon *et al*., 2009).

1.2 Statement of the Research Problem.

Bovine tuberculosis is a major problem in Nigeria. Abubakar *et al,* (2011) cited poor governance, planning, accountability, commitment and failure to conduct research in bovine tuberculosis as the main obstacles in controlling the disease in the country. Also, the human-animal interface has become a great problem due to an overlap of their territories caused by over population (Aranaz *et al*., 2004), thus bringing humans into close contact with wild and domesticated animal species which may harbour the disease. The disease, being zoonotic in nature, has a wide array of population at risk which includes livestock, livestock farmers, herdsmen, butchers, veterinarians, immune-compromised individuals, and this facilitates the spread of the disease within the population.

For most populations, Poor eating habits like consumption of poorly processed meat and unpasteurized contaminated milk (Liss *et al.,* 1994) and the living culture of sharing of same habitat by man and animals with accompanied poor ventilation, all contribute to the spread of the disease.

Lack of adequate veterinary services which leads to inadequate, poor, or no meat inspection at all at the slaughter places, favour the spread of zoonotic tuberculosis (Shitaye *et al.,* 2007). Diagnosis of the disease in Nigeria usually stops at the microscopic smear examination level, hence the species involved in causing the disease are not known thereby hampering further studies on disease outbreaks (Abubakar *et al*., 2011).

In Nigeria, zoonotic tuberculosis due to *M. bovis* is said to account for 5% of all cases of tuberculosis in human and up to 3% of all cases in children less than five (5) years of age (Ofunkwu, 2006). This constitutes an important public health concern (Cadmus *et al*., 2008;

Theon *et al*., 2009; Ehizibolo *et al.,* 2011). The disease has also been observed to have varying prevalence rates across the states of the Federation (Cadmus, (2003) with a prevalence of 8.30%; Aliyu *et al.,* (2009) with a prevalence of 4.05%; Abubakar *et al* (2011) with a prevalence of 14%). Controlling the disease within the country and West African region as a whole is quite difficult due to the presence of porous borders, sharing of grazing and watering points and lack of proper or total absence of quarantine measures (Ayele *et al.* 2004).

Bovine Tb is also a major opportunistic infection in HIV- Infected persons, diabetic, cancer and chronic renal disease patients, and this creates an additional public health threat to the population (Cosivi *et al*., 1995; Daborn *et al.,* 1996).

Despite the documented endemicity of bovine tuberculosis in Nigeria, there is no control or eradiation programme in place and though there have been research carried out on the disease, very little is still known about the true epidemiology of the disease in the country Alhaji, (1976); Ayanwale, (1988); Dusai and Abdullahi, (1994); Abubakar, (2007); Danbirni *et al* (2010).

1.3 Justification of the Study.

A number of foods (meat inclusive) have been reported to have a high incidence of bacterial contaminations in Nigeria, of which *Salmonella* species, *Listeria* species, *Mycobacteria* and *E. coli* form the contaminants (Nkanga and Uriah, 1981; Okonkwo, 2011). Kaduna state apart from being known for its academic institutions is also an agrarian society. This brings

animals and humans in close association at rural and pastoral settings. Thus in the process, it is common to see people coming into contact with bacterial contaminants along with unpasteurized milk, especially 'nono' (Abubakar, 2007; Ofukwu *et al*., 2008).

Reports of mixed infections of Tb with HIV/AIDS in humans have been made by Zubairu and Musa (2009). For example, mixed infections have been reported in Kano (10.5%), Ilorin (40%) and Ibadan (32.8%).

Though there have been studies to determine the prevalence of tuberculosis caused by *Mycobacterium bovis* (Abubakar *et al.,* 2005), national data on the distribution of bTb, might not be available due to poor disease surveillance systems, unavailable diagnostic facilities, financial impediments, which in turn have led to inadequate implementation of control policies on bovine tuberculosis (Maher *et al.,* 1999; Igbokwe *et al*., 2001; Ehizibolo *et al.*, 2011).

The outcome of this study will provide useful information to both academic/government agencies towards policy formulation relating to bovine tuberculosis within Kaduna state and the nation at large.

1.4 Aim and Objectives.

1.4.1 Aim of the Study

The aim of the study was to detect and identify *Mycobacterium bovis* from infected cattle slaughtered at the Tudun-Wada abattoir, in Kaduna North Local Government Area of Kaduna state, Nigeria.

1.4.2 Objectives of the Study

The objectives of this study were to:

i. Determine the rate of recovery of *Mycobacterium* bacilli from suspected tissues of cattle slaughtered at the Tudun-Wada abattoir, through post mortem meat inspection and Ziehl-Neelsen staining techniques as preliminary diagnostic tools.

ii. Confirm and identify the species of *Mycobacterium* bacilli using molecular techniques.

iii. Determine the extent of awareness, of abattoir stakeholders to the danger posed by bTb through the administration of a questionnaire.

1.5 Research Questions

I. What is the detection rate of *Mycobacterium* bacilli in cattle slaughtered at the Tudun Wada abattoir in Kaduna?

II. Are Tb cases in cattle all due to *Mycobacterium bovis*?

III. Are abattoir stakeholders aware of the hazards of bovine tuberculosis?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Introduction

The genus *Mycobacterium* is an ancient one, and *M. tuberculosis* may have differentiated 15 300 - 20 400 years ago (Kim and Pimentel, 2002; Daniel, 2000). Most of the members of the genus *Mycobacterium* are water or soil organisms, and it is reasonable to suppose that the genus had its origins in such an environment (Daniel, 2000). Organisms from the genus *Mycobacterium* are also called acid fast bacilli (AFB) and are responsible for tuberculosis and leprosy with a number of saprophytic species causing opportunistic disease occasionally (Grange, 1987). There are approximately 50 species in the genus *Mycobacterium* which are divided into two major groups, the slow growers and rapid growers (Stahl and Urbance 1990). *Mycobacterium* is also divided into two groups based on their ability or inability to form tubercles (Metchock *et al*., 2005). The tuberculous forming *Mycobacterium* is grouped under the *Mycobacterium tuberculosis* complex (MTC) and includes *M. africanum, M. bovis, M. caprae, M. canetti, M. microti, M. pinnipedi,* and *M. tuberculosis* (Amanfu, 2006). *The M. tuberculosis complex* is generally considered a family of ‘‘ecotypes’’ of very closely related *Mycobacteria,* with each ecotype being adapted to cause tuberculosis in a specific host species or group, even though inter-species transmission can occur (Smith *et al*., 2006). The non-tuberculous *Mycobacterium* (NTM) includes *M. avium, M. intracellulare, M. scrofulaceum,* and *M ulcerance* (Metchock *et al.,* 2005). Unlike members of the *M. tuberculosis* complex, NTMs are rarely associated with invasive disease, but may

temporarily colonize the host and cause transient infection accompanied by non-specific stimulation of the host's immune system (Woods and Washington., 1987).

Bacteria of the genus *Mycobacterium* are non-motile and non-sporulating rods, with high lipid content in their cell wall, which is a similarity also seen in related genera like *Corynebacterium, Norcadia, Rhodococcus, Gordona, Dietzia and Tsukamurella,* thus giving rise to a phenotypic resemblance (Barrera, 2007). It has been asserted that *M. tuberculosis* evolved from *M. bovis,* making this evolutionary step in association with the domestication of cattle by humans (Stead *et al*., 1995; Hare., 1967). They are the most important species in the *Mycobacterium tuberculosis* complex (MTC) and commonly cause human and animal tuberculosis (Katale *et al*., 2012). These two species are so closely related genetically that they might as well be considered as single specie (Eisenach *et al.,* 1986). Historically, they had been distinguished by only a few biochemical differences and by greatly different host ranges but in the last decades, there has been major advances in understanding the genetic structure of *Mycobacterium*. As a result there are a series of molecular techniques like the Polymerase Chain Reaction (PCR), Ligase Chain Reaction (LCR), Transcription-Mediated Amplification (TMA) which can be used to distinguish members of the genus *Mycobacterium* (Sylvia *et al*., 2004). *Mycobacterium bovis* is pathogenic for many animals, although its historic host was almost certainly cattle, with the other species having acquired it from bovine sources. It has been argued that *M. bovis* is a more primitive species than *M. tuberculosis* because it carries only one copy of the transposon designated IS*6110* (Fomukong *et al*., (1994). However, single copy isolates of *M. tuberculosis* have been found in several locations in Asia by Fomukong *et al* (1994). They believed that the IS*6110* genetic element is an ancient one present in both species and antedating the differentiation of these

two species. The broader host range of *M. bovis* has also been used to argue that it is the more primitive specie (Haas *et al*., 1996).

2.2 Aetiology of Bovine Tuberculosis

Bovine tuberculosis is caused by *Mycobacterium* bovis. The organism microscopically appear as short to moderately straight or curved rods which appear pinkish when stained with the Ziehl Neelsen stain (Taylor *et al*., 2007). It is a Gram-positive, acid-fast, slow growing (16- 20 hours generation time), non-chromogenic, aerobic bacterium which on primary isolation grows poorly on glycerol-containing media (Garnier *et al*., 2003). It is mesophilic, having an optimal living temperature of 37^{o}C (Thierry *et al*., 2003). Unlike *M.tuberculosis, M.bovis* lacks pyruvate kinase activity, due to pykA containing a point mutation that affects binding of Mg^{2+} cofactor (Thierry *et al*., 2003). Twenty one days after inoculation on egg media and incubation at 37^{o}C yields small, rounded, white colonies with irregular edges and granular surfaces. Colonies on transparent oleic acid albumin agar appear as thin and flat colonies not easily emulsified in the absence of detergents (Taylor *et al*., 2007). The *M.bovis* genome contains 3,952 gene-encoding proteins and has a genomic sequence of 4,345,492 base pair length arranged in a singular circular chromosome (Garnier *et al*., 2003). Bovine tuberculosis (bTb) is a disease characterized by progressive development of specific granulomatous lesions or tubercles in lung tissue, lymph nodes or other organs (Ayele *et al*., 2004). In modern history, cattle serve as principle reservoir species for *M. bovis*, hence the name bovine tuberculosis (Anita *et al*., 2009).

Mycobacterium tuberculosis is the most common cause of human Tb, but the human burden of tuberculosis caused by the bovine tubercle bacillus is still largely unknown (Thierry *et al*.,

2003). *Mycobacterium bovis* infection in cattle and disease in the human population has been well documented in industrialized countries, whereas little information is available from developing countries (Collins and Grange., 1983; Cosivi *et al*., 1998). It is also known to cause tuberculosis in humans, a fact which has raised renewed concerns regarding the zoonotic risk for humans, especially those living at the animal-human interface (Michel *et al*., 2010).

2.3 World Distribution of Bovine Tuberculosis.

Most of the members of the genus *Mycobacterium* are water or soil organisms, and it is reasonable to suppose that the genus had its origins in such an environment (Ramzi *et al*., 2014). Distinct species have emerged over evolutionary time, and have been subject to selection pressures leading to differentiation (Daniel, 2000). The earliest evidence for tuberculosis in the Americas comes from the studies of Peruvian mummies carried out by Allison *et al* (1973), Arriaza *et* al (1995), and Salo *et al* (1994). Their work firmly establishes the presence of tuberculosis in the arid deserts of Peru and northern Chile, where mummification was widely practiced, during the first millennium of the common-era. *M. bovis* has a predilection for causing tuberculosis of the bones and lymph nodes in humans (Taylor *et al*., 1999). This has led some to conclude that all of the bone tuberculosis observed in paleologic specimens, including that from the Americas, had bovine origin (Taylor *et al*., 1999). Hare (1967), for example, asserts that all tuberculosis prior to about 4000 years ago was due to *M. bovis*, and Manchester (1984) accepts this. Haas and Haas (1996); Bates and Stead (1993) and Stead (1998) all accepted the view that early human

tuberculosis was due to *M. bovis.* This line of thinking offers further support for the thought that *M. tuberculosis* originated by mutation from *M. bovis* (Daniel, 2000).

The European badger (*Meles meles*) is implicated as a reservoir of *Mycobacterium bovis* (bovine Tb) infection of cattle in Britain and Ireland (Delahay *et al.,* 2009). Since the early 1970's the badger has been implicated in the transmission of *M. bovis* to domestic cattle in the United Kingdom and Ireland (Muirhead *et al*., 1974). There is compelling circumstantial evidence for the involvement of badgers in cattle herd Tb breakdown (Krebs 1997), particularly in the South-West of England where the problem was concentrated (MAFF 1996). Disease spread at the national level is due to both cattle movements and other factors (Wint *et al*., 2002; Woodroffe *et al*., 2006). While recent changes in government policy in the United Kingdom have introduced tuberculin testing for cattle prior to movement from herds deemed at high risk, the relative importance of cattle movement remains to be assessed (Goodchild & Clifton-Hadley 2001). Movements of infected cattle have been shown to pose a clear risk to the spread of the disease (Goodchild & Clifton-Hadley 2001; Gopal *et al*. 2006) and cattle movements are a significant predictor of the distribution of bTb (Gilbert *et al*. 2005). In some high-risk areas of Great Britain, *M. bovis* is also widespread in badgers (Gallagher & Clifton-Hadley 2000; de la Rua-Domenech *et al.* 2005) and they are implicated as a wildlife reservoir, as is the case for wildlife carrying *M. bovis* elsewhere (Morris *et al.,*1994; Griffin *et al.,* 1996 and Delahey *et al* 2009). Evidence that cattle-to-badger transmission is important is sparse (Woodroffe *et al*., 2006). However, if true, seedings via cattle infections may contribute to the broadening of high-risk areas.

Mycobacterium bovis infections in cattle herds in the United States is not common. *M. bovis* is endemic in white-tailed deer (*Odocoileus virginianus*) in the northeastern portion of

Michigan and northern Minnesota, and sporadic import of the disease from Mexico. Only the white-tailed deer has been confirmed as a maintenance host in the Michigan outbreak of bovine tuberculosis, although other mammals such as raccoons (*Procyon lotor*), opossums (*Didelphis virginiana*), and coyotes (*Canis latrans*) can serve as spill-over and dead-end hosts (Witmer *et al*., 2010). The fact that white-tailed deer are a maintenance host for *M. bovis* remains a significant barrier to the US nationwide eradication of the disease in livestock. In 2008, 733,998 licensed deer hunters harvested approximately 489,922 white-tailed deer in attempts to control the disease spread. These hunters purchased more than 1.5 million deer harvest tags. The economic value of deer hunting to Michigan's economy in the drive to eradicate TB is substantial. For example, in 2006, hunters spent US$507 million hunting white-tailed deer in Michigan (O'Brien *et al*., 2011).

Bovine tuberculosis in Asia accounts for 62% of global cases of bTb (Cosivi *et al*. 1998). Annual mortality due to all strains of tuberculosis in South-East Asia is estimated by the WHO to be 480,000 (Amanfu, 2006; Cosivi *et al*. 1998). China is the fifth largest producer of milk under Intensive production setups, thus coping with the overwhelming demand of risk factor for ingestion transmission of bovine tuberculosis can be a challenge (Chowdhury *et al*., 2002). Of 36 Asian nations, 16 (China, India, Thailand, North Korea, South Korea, Turkey, Singapore, Iran, Oman, United Arab Emirate, Bangladesh) reported sporadic/low occurrence of bovine Tb, and one (Bahrain) described the disease as enzootic, while ten of them (Mongolia, Sri Lanka, Malaysia, Indonesia, Philippines, Taiwan, Laos, Syria, Jordan and Yemen) did not report bovine Tb. The remaining nine (Iraq, Saudi Arabia, Oman, Afghanistan, Pakistan, Nepal, Bhutan, Singapore and Tajikistan) did not have data (Cosivi *et al*., 1998). Within the Asian region, seven countries (South Korea, Iran, Turkey, Jordan,

Malaysia, Indonesia and Spratly Islands) applied disease control measures as part of a test-and-slaughter policy and considered bovine Tb notifiable (Cosivi *et al*., 1998). In the remaining 29 countries, bovine Tb is partly controlled or not controlled at all (FAO, 1994). Of the total Asian cattle and buffalo populations, 6% and less than 1%, respectively, are found in countries where bovine Tb is notifiable and a test-and-slaughter policy is used. Furthermore, 94% of the cattle population and more than 99% of the buffalo population in Asia are either only partly controlled for bovine Tb or not controlled at all. Thus, 94% of the human populations live in countries where cattle and buffaloes undergo no control or only limited control for bovine Tb.

Out of 34 Latin American and Caribbean countries, only 12 (Brazil, Ecuador, Paraguay, Uruguay, Costa Rica, Nicaragua, Honduras, Mexico, Puerto Rico, Cuba, Turks And Caco And Grenada) reported bovine Tb as sporadic/low occurrence, seven of them (Dominican republic, Venezuela, Peru, Bolivia, Chile, Argentina and Guatemala) reported it as enzootic, and one (Dominican Republic) described occurrence as high. Twelve countries (Columbia, panama, Anguilla, Antigua and Barbuda, Aruba, Barbados, British Virgin Island, Guadeloupe, Martinique, Montserrat, St Kitts and Nevitts and St Lucia) did not report bovine Tb (Cosivi *et al*., 1998). No data were available for the remaining two countries (Guyana and Suriname). In the entire region, 12 countries (Argentina, Brazil, Peru, Uruguay, Paraguay, Venezuela, Panama, Guatemala, Cuba, Trinidad and Tobago, El Salvador and Falkland Islands) apply disease control measures as part of a test-and-slaughter policy and consider bovine Tb as a notifiable disease. In the remaining 22 nations, the disease is partly controlled or not controlled at all (Cosivi *et al*., 1998).

Currently, *M.bovis* accounts for only 1% of all human Tb in developed countries as compared to 10% in the developing world (Etchechoury *et al*., 2010).

2.4 Epidemiology

2.4.1 Epidemiology of bovine tuberculosis in Africa.

There is a lack of reliable field survey data on animal diseases in Africa (Benkirane, 1993). An analysis of more than 300 *Mycobacterium* strains originating from human sputum was conducted in Egypt to estimate the presence of *Mycobacterium bovis* in human Tb (El-Sabban *et al.*, 1992). Nearly 5% of these strains were found to be *M. bovis*. This incidence of the bovine type was attributed to the fact that most of the patients lived near the Cairo abattoir and some were actually abattoir employees. From the information available it is possible to make some general observations on the epidemiological situation concerning bovine Tb in Africa. In terms of susceptibility, it is established that Zebu cattle are more resistant to Tb than imported European breeds that have higher productivity (Martin *et al*., 2012). This may be a consequence of closer confinement, longer life span and the stress demand of greater productivity among dairy cows. In 1993, the presence of bovine Tb at herd level was reported only by five countries that include South Africa (34 outbreaks), Algeria, Morocco, Ghana and Madagascar (Cosivi *et al.*, 1995).

In Africa, bovine Tb primarily affects cattle. However, infection in other farm and domestic animals, such as sheep, goats, pigs, dogs and cats, is not uncommon (Cousins and Florisson, 2009). Although bovine Tb in cattle is widespread in Africa, some member States fail to report the annual prevalence and incidence of the disease to the OIE, while others tend to report the disease sporadically, at intervals of several years (FAO, 1993). Bovine Tb

represents a potential health hazard to both animals and humans in Africa, as nearly 85% of cattle and 82% of the human population live in areas where the disease is prevalent or only partially controlled (Anonymous, 1994; Theon *et al*., 1995). In Africa, as in most developing countries, *Mycobacterium bovis* infection remains an uninvestigated problem. Tuberculosis due to *M. bovis* is still a neglected disease in animals as well as in human populations, especially in the sub-Saharan countries where it is less studied (Boukary *et al*., 2011)**.**

Sub-Saharan Africa covers large expanses of arid territory where dairy cattle production is limited due to inadequate forage. In these areas goats are commonly kept for milk and meat production, and are locally known as 'poor man's cows' due to their adaptability to the high temperatures and efficient conversion of inedible roughage to edible animal protein (http://www.heifer.org.za/blog/detail/the-poor-mans-cow-the-goats-of-zusiphe-a-valuable-source-of-nutrition.). Contact between goats and cattle at pasture and in huts at night are common, and may expose them to infection with *M. bovis.* Similarly, in Spain, Garcia-Marine (1993) reported isolating *M. bovis* from goats housed with cattle at night to protect them from wild carnivores.

In Africa, the true extent of bovine Tb has not been adequately evaluated due to economic constraints, but the presence of the disease has been adequately established in various countries from research work carried out in different regions of the continent (Ayele *et al.*, 2004). The African buffalo (*Syncerus caffer*) has established itself as a maintenance host for *M. bovis* in two of South Africa's largest conservation areas, namely the Kruger National Park (KNP) and the Hluhluwe iMfolozi Park (HiP) (De Vos *et al* .,2001; Michel, 2002).

Bovine tuberculosis poses a serious threat to free-ranging wildlife and domestic animals, as well as having significant zoonotic potential (Michel, 2002). Control and eradication

programmes for this disease have focused mainly on tuberculosis in domestic cattle because they are the traditional hosts and have economic importance (de Lisle, 2002). Bovine Tb also poses a threat to trade in animals and their products (Clifton-Hadley *et al*., 1995). Once infected, many wild animals have shown the potential to act as reservoirs of infection for both domestic cattle and other valuable wildlife species (de Lisle, 2002). The brush-tail possum (*Trichosurus vulpecula*), European badger (*Meles meles*), Bison (*Bison bison*), African buffalo (*Syncerus caffer*), Kafue lechwe (*Kobus leche*) and white-tailed deer (*Odocoileus virginianus*) can all act as maintenance hosts for bTb, allowing the persistence of the infection in wildlife and enabling the horizontal transmission of the pathogen between species (de Lisle, 2002). In addition, some species act as 'spillover' hosts or 'dead-end' hosts (Keet, 2000).

2.4.2 Epidemiology of bovine tuberculosis in Nigeria.

Nigeria is ranked second in Africa and fourth in the world based on prevalence of bovine tuberculosis (WHO, 2004). Reporting the disease is not mandatory, and there is no active surveillance programme in place. Therefore the true status of bovine Tb in the country remains unknown (Cadmus *et al.*, 2004). Although, the current status on the actual prevalence rate of bovine tuberculosis at a national level is unknown, but from the limited survey/research which have been reported over the last 30 years in the country, the prevalence of bovine tuberculosis due to *M. bovis* ranges from 2.5% in 1976 to 14% in 2007 which shows that the prevalence of the disease has been on the increase (Abubakar *et al*., 2011). The epidemiology and public health significance of bovine Tb in Nigeria remain largely unknown. This might be due to the poor governance, planning, accountability,

commitment and failure to conduct research in bovine tuberculosis (Abubakar *et al*., 2011). However; few laboratories in the country are capable of differentiating *M. bovis* from *M. tuberculosis* and other members of the *M. tuberculosis Complex* (MTC) (Cadmus *et al*., 2004). The problem of *M. bovis* in Nigeria may, to some extent, mimic the pre-eradication period in Europe before the 1960s where the prevalence of bovine Tb in the human population was relatively high (Pavlik *et al*., 2002). Nigeria is one of the African countries where bTb is considered a protruding disease of animals (Cadmus *et al*., 2007). Abubakar (2007) attributed the prevalence of the disease in the country to the advent of rural and some urban inhabitants still consuming potentially contaminated unpasteurized and soured milk and their products. Bovine tuberculosis caused by *M. bovis* is a zoonosis in which both natural and anthropogenic movement of animals has influenced its epidemiology (Kruse *et al*., 2004).

Ofukwu (2006) in a study carried out in hospitals in Benue State reported that out of a total of 124 pus, urine and sputum samples collected from patients, 2.4% were identified as *M.bovis*, while 82.3% were attributed to *M. tuberculosis*. They also revealed the presence of *M. bovis* in milk samples ("nono") collected from a market in Benue State.

2.5 Risk Factors associated with Bovine Tuberculosis.

There are certain factors that make bovine tuberculosis a highly contagious disease. These may be viewed from the animal, human and the environmental perspectives respectively (Humblet *et al*., 2009).

2.5.1 Animal perspective.

***2.5.1.1 Age*:** One of the main individual risk factors identified by numerous studies in both developed and developing countries is the age of animals (Humblet *et al*., 2009). In a 1996 cross-sectional study, which included more than 2 000 animals issued from 200 herds, Irish authors observed that calves were less likely to be positive reactors than older animals (Griffin *et al*., 1996*). Mycobacteria* have the ability to remain in a latent state for a long period before reactivation at an older age (Pollock and Neil, 2002). Thus Animals might get infected at a young age, but only express the disease clinically when they are adults.

***2.5.1.2 Breed*:** Studies conducted in Africa also identified the animals' breed as a risk factor for a positive skin test. Studies carried out suggested that imported breeds, used to improve the dairy industry in tropical areas, may be less resistant to bTb compared to indigenous breeds, like the zebu (Omer *et al*., 2001). The difference in susceptibility between breeds is likely to be related to differences in management as imported dairy animals are generally kept under intensive conditions (Elias *et al*., 2008).

***2.5.1.3 Immune status*:** Immuno-suppression is a predisposing factor for a number of diseases (De la Rua-Domenech *et al*., 2005). Thus, the risk of becoming infected with *M. bovis* also seems to increase, though it has not been scientifically demonstrated in cattle to date (Menzies and Neill, 2000). Susceptibility to *M. bovis* may be as well enhanced in cattle infected with immunosuppressive viruses such as bovine viral diarrhea or immunodeficiency virus (De la Rua-Domenech *et al*., 2005).

***2.5.1.4 Gender*:** Gender has only been mentioned as a risk factor in studies carried out in Africa. There are diverse opinions regarding its influence on the susceptibility to *M. bovis* infection. A cross-sectional study conducted in Tanzania from 1994 to 1997, which included 5,692 indigenous and 244 exotic cattle, revealed that male cattle were significantly more affected by bTb than female animals (Kazwala *et al.*, 2001). Male cattle are mostly used as oxen, which are kept longer in the herd than females. Due to this particular longevity, it is more probable that they get in contact with infected cattle from other affected herds and in turn get infected. This would imply that between-herd contact is a major source of bTb transmission (Kazwala *et al*., 2001). From 2006 to 2007, a cross-sectional study on 1,470 animals in Uganda revealed significantly more females positive to the skin test than males (Inangolet *et al*., 2008). Gender-linked factors are probably related to management practices or behavioral habits where males and females are managed differently, both in developed and developing countries. In developed countries, dairy cows usually reach an older age than males because of their role in calving and milk production.

***2.5.1.5 Body condition*:** The body condition score (BCS) relies on palpation of the sharpness, muscle and fat covering the backbones and lumbar processes and is determined on a 1 (emaciated) to 5 (obese) scale (Edmonson *et al.*, 1989). In 1996, Cook and his co-workers linked a low BCS to an increased risk of a positive skin test result in their cross-sectional study including 2,226 animals in Zambia (Cook *et al*., 1996). During a matched case-control study including 80 chronic bTb herds carried out in 1990 in Ireland, Griffin *et al* (1993) demonstrated that an animal's resistance to tuberculosis was reduced by a shortage of feed and/or an unbalanced diet. However, this study was carried out over a three month

period hence might not truly reflect a picture of the situation in reality. It has been shown that animals suffering from a clinically advanced bTb often present a low BCS as a result of the long-lasting pathological process (Kazwala *et al*., 2001).

2.5.2 Human perspective.

2.5.2.1 Migration: Migration and people's travelling habits are additional risk factors for the spread of *M. bovis*. Since bTb is present in most developing countries where surveillance and controls are often inadequate or unavailable, international migration plays a key role in the spread of infectious diseases (Cosivi *et al*., 1998). In many developing countries, milk and dairy products are still consumed un-pasteurized, and the risk of *M. bovis* transmission remains likely (Amanfu, 2006). Human to cattle transmission of *M. bovis* has been described whereby a foreign-born person, contaminated in his/her childhood and clinically expressing the disease, represents a risk if he comes in contact with a bTb-free herd (Theon *et al*., 2006). Also wild animal movements take them across borders and these trans-border movements of wildlife can occur naturally or be human-induced via legal importations to zoological parks or animal reintroduction for conservation programmes, this in turn facilitate the transmission of the disease (Humblet *et al*., 2009). Movement of cattle within and between countries and continents certainly facilitates the worldwide distribution of bovine tuberculosis. Also animals purchased from a high bTb incidence area and introduced in a low bTb incidence region have also been seen to increase the risk of a herd's contamination (Carrique-Mas *et al*., 2008).

***2.5.2.2 Humans as reservoir and susceptibility due to health challenges*:** The occurrence of *M. bovis* in the human population is a persistent, though insufficiently quantified feature in developing countries (Humblet *et al*., 2009). The isolation rate of *M. bovis* from symptomatic human patients in specific studies was 13.8% in Mexico (Perez-Guerrero *et al*., 2008), 6.9% in Uganda (Oloya *et al.,* 2008), 5% in Nigeria (Cadmus *et al*., 2006), 0.5% in Taiwan (Jou *et al*., 2008) and between 0 and 2.5% in 10 Latin American countries (De Kantor *et al*., 2008). Although the proportion of *M. bovis* causing human tuberculosis is very low compared to *M. tuberculosis*, its potential impact on population groups at the highest risk should nevertheless not be underestimated (Humblet *et al*., 2009). Exposure to aerosol-borne infection with *M. bovis* from cattle remains highest in farmers, veterinary staff, rural and slaughterhouse workers, while in developing countries, ethnicity, cultural and religious practices as well as socio-economic factors have been identified as additional contributors to an increased occurrence of *M. bovis* infections in humans and subsequently animals (Michel *et al*., 2010).

It has been observed that individuals with the human Immunodeficiency Virus (HIV) show a high susceptibility to members of the *Mycobacterium tuberculosis* complex, especially in countries where Acquired Immune Deficiency Syndrome (AIDS) poses a public health challenge (WHO, 2009). Also, other debilitating conditions such as cancer, diabetes and chronic ailments decrease host resistance to diseases; thus making co-infection with tuberculosis to more readily happen (WHO, 2009).

2.5.3 Environmental perspective.

***2.5.3.1 Pasture*:** The importance of environmental contamination in the transmission of bTb to healthy cattle might differ between developed and developing countries. Infection of cattle through environmental contamination is probably negligible, because the required infective dose is high: 107 bacilli would be necessary for oral contamination, as suggested by O'Reilly and Daborn (1995), whereas an aerosol containing only one bacillus was shown to contaminate 4–7 month old calves in an English study carried out in 1988 (Neill *et al*., 1988). Morris *et al* (1994) concluded that contaminated feed and pasture play a negligible role in the transmission of the disease to cattle because *M. bovis* does not survive long enough on fomites to be infective, and also because animals are not commonly exposed to a high enough doses to become infected orally (Morris *et al*., 1994).

The survival time of *M. bovis* in soil has been estimated in different studies. Wray (1975) argues that the availability of nutrients is the limiting factor. Sunlight causes desiccation, while ultraviolet light may strongly influence survival in tropical areas where adequate levels of moisture are necessary for survival (Wray, 1975). Kelly and Collins (1978) later suggested that the major factors influencing survival in soil and on pasture as temperature, moisture, and pH, exposure to sunlight, dissolved oxygen, presence of naturally occurring antibiotics in the soil, natural micro flora and types of micro floral associations. Soil ecology is also of major importance in the environmental survival of *M. bovis* (Artois *et al*., 2004) which has also been seen to persist in slurry-treated soil for up to two years (Kelly and Collins., 1978) though experimental conditions should nevertheless be considered carefully since they differ from field conditions.

***2.5.3.2 Water*:** Watering points must be mentioned as potential risk factors Animals from different herds all come and drink from the same watering points thus making it easy for diseases to spread from infected animals to susceptible ones. When cattle drink, splashing occurs, and could favour the entry of bacilli through routes other than the digestive tract like the respiratory tract through inhalation of contaminated droplets (Phillips *et al*., 2003). Flooding has also been suggested as a propagating factor of *M. bovis* in the environment (Cleaveland *et al*., 2007).

***2.5.3.3 Human-animal interface*:** Transmission of *M.bovis* at the livestock-wildlife or human-animal interface occurs essentially because of an overlap in their territories (Aranaz *et al*., 2004). Many developing countries have intensified their livestock production to meet the growing demand for food security, which has led to a higher risk of transmission for both *M. tuberculosis* and *M. bovis* at the human–livestock interface (Aranaz *et al*., 2004). It may be speculated that in some countries more cattle contract tuberculosis from humans than vice versa (Michel *et al*., 2010). This may be due to people clearing newer land for agricultural purposes, and in the process encroaching on wildlife habitat, which makes disease transmission much easier (Humblet *et al*.,2009) . Specifically, boundary regions in protected areas are used increasingly for grazing of livestock and agriculture which corresponds with areas where the remaining population of wildlife has been concentrated by this land-use pressure (Etter *et al.,* 2006).

***2.5.3.4 Management system*:** Contact between cattle, and also between cattle and contaminated environmental sources and wildlife, play a vital role in the spread of bovine

tuberculosis (Humblet *et al*., 2009). Management practices differ in developed and in developing countries. In developing countries, Holstein cattle are increasingly being imported in order to improve milk production and are usually kept under intensive conditions. The highest incidence of bTb is generally found in areas where intensive dairy systems are practiced (Cosivi *et al*., 1998). Costello *et al* (1998), suggested in a longitudinal study carried out in Ireland including young steers kept in feedlots for a one-year period, that defective ventilation may facilitate the transmission of bTb, and that lack of hygiene encourages proliferation of pathogens in general. Farm size, in terms of number of holdings and boundary length, has been implicated as a worldwide bovine tuberculosis risk factor (Humblet *et al*., 2009). Nevertheless, herd size could probably be related to farm size, with larger farms having more cattle.

Also traditional practices such as sleeping in the same house as animals, lack of knowledge regarding the disease and its risk, HIV/ AIDS, raw milk consumption and poor ventilation of houses all contribute in buttressing the risk factors for the disease (Mfinanga *et al*., 2004). In sub Saharan Africa, active competition exists between large scale commercial enterprises and smaller/less regulated holdings that frequently ignore safety standards for hygiene and product quality, thus increasing the risk of zoonotic tuberculosis (Etter *et al*., 2006).

2.6 Transmission routes of Bovine Tuberculosis

The best evidence of the transmission route of *Mycobacterium bovis* to cattle is the pattern of lesions observed in slaughtered animals. In Great Britain in the late 1990s, the tissue samples with visible lesions from tuberculin reactor cattle submitted for mycobacterial culture were mainly from the broncho-mediastinal and head lymph nodes, suggesting that the route of

infection was principally via the respiratory tract (Phillips *et al.*, 2003). In the case of ingested organisms some mesenteric lymph node lesions would also be expected, but are not normally observed. Some of the routes of transmission of bovine tuberculosis include Inhalation route, ingestion/oral route and Cutaneous/mucosa route.

2.6.1 Inhalation route

The prevalence of *M. bovis* infection in cattle, whilst a potential health risk, is not currently a widespread problem in countries where milk is pasteurized and cattle are regularly tested with tuberculin and reactors slaughtered, but is a cause for concern in individuals at high risk, such as abattoir workers and farmers (Grange and Yates, 1994). Infection of animals with *M. bovis* almost always occurs by inhalation of aerosols or consumption of feeds containing the bacillus (Grange, 2001). The organism may be transmitted by aerosol or droplets of exudates containing bacilli which have been coughed up by animals with advanced cases of the disease (Theon *et al*., 2009). Respiratory transmission has been responsible for the spread of infection in domestic and wild bovidae, as well as in animals held in captivity. Also aerosols generated during the handling of tuberculous carcasses in abattoirs also pose a risk of infection. In Australia, five cases (four pulmonary and one renal) occurred among 3000 Australian abattoir workers over a 2 year period (0IE, 1986).

2.6.2 Ingestion/ oral route

Digestive lesions suggest oral contamination by affecting animals or humans eating contaminated carcasses, which is a characteristic of mostly carnivorous species (Bruning *et al*., 2001). The capacity of excretion, etiology and ecology of wild animals, as well as the prevalence of the disease determine the role of wild animals as reservoir for *M. bovis* (Artois

et al., 2004). Those who consume contaminated milk are infected via the tonsil or intestinal mucosa so they may present with enlarged cervical or mesenteric lymph nodes, sometimes with abscess or sinus formation (Grange, 2001). The use of raw milk in the production of cheese and other dairy products is a potential public health risk associated with tuberculous cattle, as viable *Mycobacteria* (including *M. bovis*) have been found to survive in unpasteurized cheeses (Holsinger *et al*., 1997). Naive cattle can acquire the infection from pasture several weeks after it has been grazed by manifestly infected cattle (Schellner, 1956). *Mycobacteria* survive readily in water and this is the major source of human non-tuberculous mycobacterioses (Dailloux *et al.,* 1999), these could be implicated in tuberculous cases observed in rural areas where humans and animals drink from same watering holes. *Mycobacterium bovis* bacilli can survive for up to approximately 400 days in running water (Briscoe, 1912).

2.6.3 Cutaneous/mucosal route

A third and much less common route of infection is by traumatic inoculation into the skin (Humblet *et al*., 2009). This was once an occupational hazard to those handling contaminated meat and the local lesion was termed the 'Butcher's Wart' (Grange and Yates, 1994). In the past, cutaneous or mucosal transmission was an occasional source of localized skin, tendon and lymph node lesions, otitis and conjunctivitis in milkers, those regularly dressing carcasses of tuberculous animals and veterinarians exposed during surgical interventions or necropsies (Ashford *et al*., 2001). The transmission of infection between wildlife and hunters (humans) can occur through the entry of the organism via cuts in the skin or alimentary system (Fanning and Edwards., 1991).

2.7 Economic significance of Bovine Tuberculosis.

Animal disease outbreaks pose significant threats to livestock sectors throughout the world, both from the standpoint of the economic impacts of the disease itself and the measures taken to mitigate the risk of disease introduction (Perry and Randolph, 2003). In the developing world, livestock diseases have broader, more pronounced effects on markets, poverty, and livelihoods, given the diversity of uses of livestock and complexity of livestock value chains (Perry and Grace, 2009). How long a disease occurs and when it occurs can both influence its economic impact.

Currently large amounts of public resources are channeled into preventing and controlling bTb in developed countries of the world and even in some developing countries like South Africa (Cosivi *et al*., 1998). Pasteurization as a means of preventing *M.bovis* infection in humans has the cost implication of using an energy source to heat up the milk to such temperatures that render it safe for human consumption.

In a study carried out by Ejeh *et al* (2014) on financial losses, number and weight of edible cattle organs condemned on annual basis for a five-year period (2008–2012) showed that a total of 1,935 (3046.50 kg) edible organs valued at N2.91 billion Naira ($1.82 x 10^4) were condemned

Bovine Tb is responsible for the condemnation of a significant amount of all meat inspected. In 1991, Egypt condemned meat and viscera estimated at a value of nearly US$5 million (El-Taweel, 1992). Similarly, in Côte d'Ivoire, in 1992 nearly 50 percent of all whole bovine carcasses were condemned and 46 percent of all partial carcass condemnations were attributed to Tb contamination, while during the same year only one outbreak of the disease was officially reported.

2.8 Pathogenesis of *Mycobacterium Bovis* Infection.

Much of the information that is available today on the pathogenesis of bovine tuberculosis has been acquired from experimental infection of cattle with *M. bovis*. Thus pathological changes occur mainly in the lungs and associated lymph nodes, but the upper respiratory tract has been implicated in a significant number of cases (Neill *et al*., 1988). The process by which infection gets to the exposed animal can lead to a range of disease outcomes which are not fully understood (Neill *et al*., 1988). Earlier experimental infections of *M. bovis* infection in cattle were carried out through subcutaneous and intravenous challenge routes (Waddington and Ellwood, 1972), which resulted in severe systemic disease, which did not reflect the pathology observed in natural infection. Similarly, experimental oral infection with high doses of the organism (Pollock *et al*., 2006), resulted in intestinal lesions, which are not commonly seen in cattle in developed countries where eradication programmes are in place. Direct inoculation of *M. bovis* into the nasal cavity, tonsil or the trachea is highly effective at inducing infection, although the pathology may be atypical of natural infections (Pollock *et al*., 2006).

The host protective immunity against infection depends on the activation of cell mediated immune response (Charles *et al*., 2009). Following deposition of the bacilli onto the respiratory surface, the bacilli are engulfed by phagocytic cells of the innate and acquired immune system (Phillip *et al*., 2003). Immunity to *Mycobacteria* is characterized by a cell-mediated immune response where infected macrophages become surrounded by a zone of lymphocytes to form a granuloma (Wedlock *et al*., 2002).

Microscopically, lesions are observed earlier at days 7 and 11 post-infection, with macrophage giant cells (containing acid-fast bacilli) and neutrophilic debris observed

(Charles *et al*., 2009). As lesion development progresses, there are more extensive necrosis, consisting of intact and degenerate neutrophils, macrophages and lymphocytes. Some mineralization and fibrosis may also be seen. . Gross lesions are seen in the upper and lower respiratory tract and associated lymph nodes at 14 days post infection (Cassidy *et al*., 1998). It is characterized by the development of granulomas primarily in the lungs, lymph nodes, and occasionally in other organs. If the infection is not contained, caseous lesions develop which interfere with lung function. The disease course is chronic with progression over a number of years (Cassidy *et al*., 1998). Lesions grossly start as small granulomas, pale yellow in color and with a caseous core, 14 days post infection. There are large numbers of acid-fast bacilli present. Some CD2+ cells are seen within the degenerate neutrophil core and by day 21 post-infection CD2+ cells are also present within the macrophage mantle surrounding the necrotic area (Charles *et al*., 2009). Prior to day 12, only small numbers of B-cells may be seen. However, by day 42, clusters are observed on the periphery of the lesion and are associated with lymphoid cells (Charles *et al*., 2009).

In conditions where the host response is not sufficient to eliminate the *Mycobacterium* bacilli, due to low CD4+ T-cells count resulting from stress, caused by a disease like HIV, or due to old age, the bacilli may become re-activated and thus transmit infection (Charles *et al*., 2009).

2.9 Prevention and Control of Bovine Tuberculosis.

The cornerstone of Tb control in cattle and other species is the accurate detection and removal of animals infected with *Mycobacterium bovis* (De la Rua-Domenech *et al*., 2005). Infection of cattle with this organism is usually chronic and can remain subclinical for a long period (De la Rua-Domenech *et al*., 2005). Importantly, infected cattle can become infectious long before they exhibit any obvious clinical signs or lesions typical of Tb detectable even with the most careful veterinary examination (De la Rua-Domenech *et al*., 2005). Even if present, the clinical signs of Tb in cattle are not pathognomonic. As a result, effective ante mortem surveillance for bovine Tb must primarily rely on the detection of infected cattle at an early stage by the use of sensitive immunodiagnostic tests (Adams, 2001).

The key measure of diagnostic test accuracy is the relationship between sensitivity and specificity, which determines the false-positive and false-negative proportions. None of the tests currently available for the diagnosis of bovine Tb allow a perfectly accurate determination of the *M. bovis* infection status of cattle (Anderson *et al*., 2000). Although various factors can reduce the sensitivity and specificity of the skin tests, these remain the primary ante mortem diagnostic tools for Tb in cattle, providing a cost-effective and reliable means of screening entire cattle populations (Anderson *et al*., 2000).

Tuberculosis can be effectively eradicated from many developed, developing countries and regions with the implementation of sound programmes. These programmes may involve regular tuberculin skin testing and removal of reactors, coupled with slaughterhouse surveillance for undetected infections, repeat testing and culling of infected herds, cattle movement restrictions to prevent introduction of infected animals and occasional slaughter

of entire herds with intractable breakdowns (De la Rua-Domenech *et al*., 2005). Other in vitro assays for Tb, such as the antibody ELISA tests (Hanna *et al*., 1992), the lymphocyte proliferation assay, the fluorescence polarization assay (FPA) (Surujballi *et al*., 2002) and the ‘‘electronic nose’’ (Fend *et al*., 2005) have been developed for use in cattle, but their application remains confined to the experimental setting or to very specific field situations. Newer research tools are rapidly emerging for the characterization of bovine immune responses. Numerous primer sets are available for characterizing bovine cytokine/ chemokine/transcription factor responses to bTb via real-time PCR techniques (Thacker *et al*., 2007) and bovine immune microarrays (Almeida *et al*., 2007; Machugh *et al*., 2009).

Other preventive/control measures under consideration or newly implemented include additional control measures to limit risks with imported animals. Enhanced programmes to mitigate wildlife reservoir risks, re-evaluation of options to manage bTb-affected herds/regions, modernization of regulatory framework(s) to re-focus control efforts, and consideration of emerging testing technologies (i.e. improved or new tests) for use in bTb control/eradication programs should be implemented (Schiller *et al*., 2010). Traditional slaughter surveillance and test/removal strategies will likely be augmented by incorporation of new technologies and more targeted control efforts (Buddle *et al*., 2002) Early studies using BCG to vaccinate cattle against tuberculosis gave variable results and early field trials conducted in cattle were disappointing (Buddle *et al*., 2002). The apparent failure of BCG to protect cattle in field trials may be attributed to several factors, which include the use of high doses of BCG (equivalent to 108–1010 CFU) and it is now known that lower doses are more effective at stimulating cellular immunity to *Mycobacterial* antigens (Bretscher, 1992). In addition, vaccination of cattle was often carried out in areas with a very high prevalence of

bovine tuberculosis and animals may have been exposed to *M. bovis* prior to vaccination. A disadvantage of using BCG in cattle is that vaccination may induce reactivity in the intra-dermal tuberculin test (Bretscher, 1992).

Extensive husbandry practices are widespread, with cattle moving from place to place searching for grazing pasture and water. This is made pronounced during drought, thus posing a challenge in control of the disease, but eradication at national level is possible if attention is given at policy level and if there is clear identification of goals of the policy that guide actions/sequences of actions that are required within the programme to accomplish these goals (Collins., 2006).

CHAPTER THREE

3.0 MATERIALS AND METHODS.

3.1 Study Location.

The research was conducted in Kaduna-North Local Government Area of Kaduna state (Fig 3.1). The state is located in central Northern Nigeria particularly in the North-Western geo-political zone of the country. It is divided into 23 Local Government Areas (LGA), and is located between latitude $10^{o}\,19^{/}$ N and longitude $7^{0}\,45^{/}$ E and has an estimated total land mass of 46,053 km^2 (http:www.ramalanyero.com/Kaduna/#tabs). It is bordered by Nassarawa, Niger, Kano, Katsina, Plateau, Bauchi, as well as Abuja. The state is divided into 3 geo-political zones, and has a total population of over 6 million (2006 census). It has over 60 ethnic groups and the major languages spoken including Bajju, Gbagyi, Jaba, Hausa, Fulani and Gwandara to mention a few (http:www.ramalanyero.com/Kaduna/#tabs). About 60% of the state's population is involved in agriculture of which animal rearing plays a huge part.

The State has 2 distinct seasons, the dry season and rainy season with vast expanse of land of fertile land for growing food and cash crops like ginger, potatoes, Shea-nut, millet, rice aside from animal husbandry.

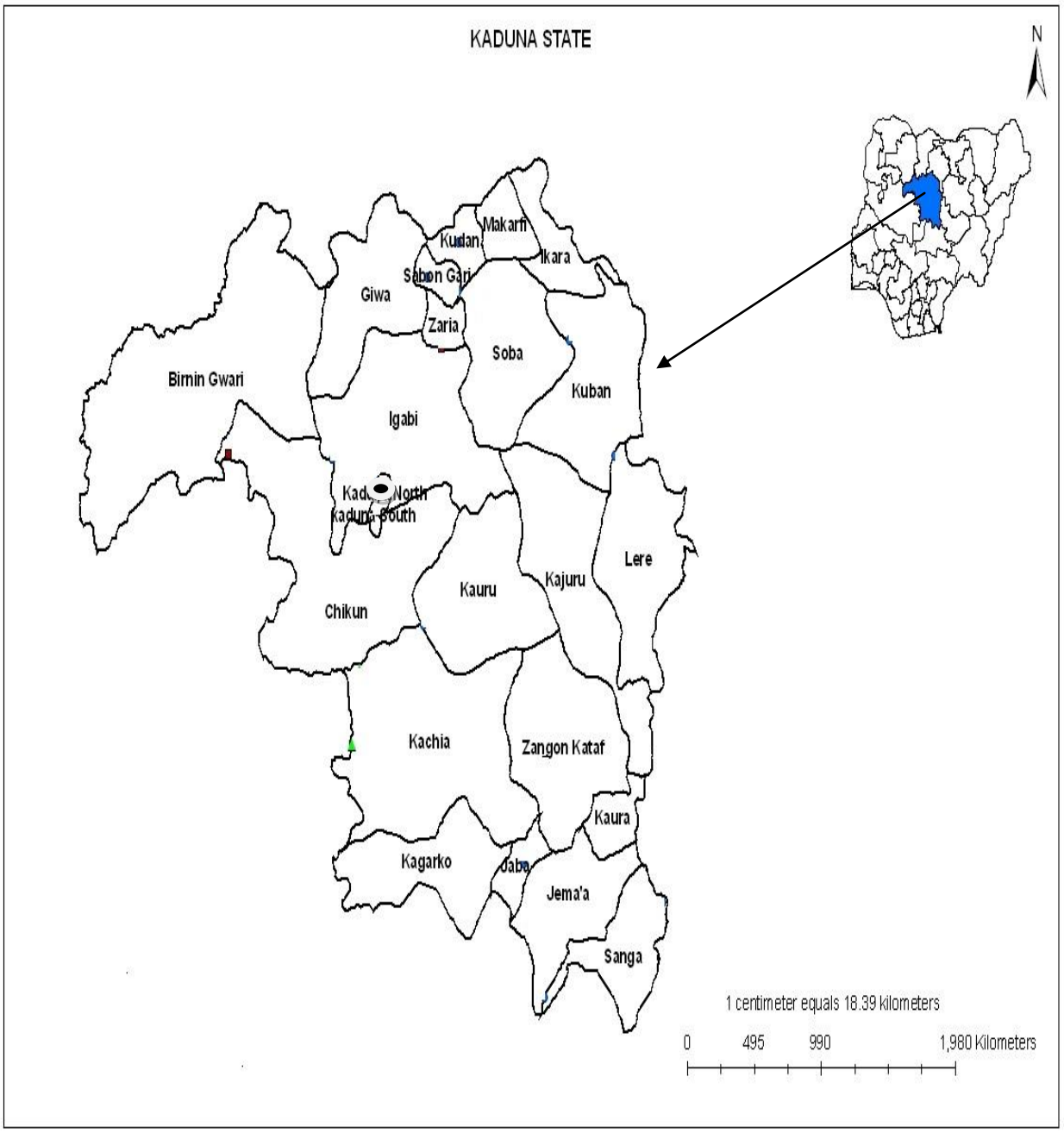


Fig. 3.0: Map of Kaduna State, Nigeria, showing Local Government Areas.

Key = Kaduna-North Local Government Area.

3.2 Study Design:

A cross-sectional study was carried out over a period of 7 months (May – November), to detect suspected *Mycobacterium* lesions in suspected bovine tissues and subsequent identification of agents using Ziehl Neelsen staining technique and confirmation of the agent using molecular process (mPCR) at the Tudun-Wada abattoir in Kaduna-North Local Government Area of Kaduna State. Purposive selection was the criteria for abattoir selection, while cross-sectional selection was the criteria for animal selection.

3.3 Source of Samples:

Sampling was carried out in the Tudun-Wada abattoir, which is a state owned abattoir presently under the management of a private company (Kada Payless). Samples collected were mainly lungs, lymph nodes and intestinal tissue samples. The abattoir slaughtered an average of 120 cattle, 50 goats and 15 sheep daily at inception, but these numbers are much lower today. Cattle slaughtered in the abattoir were sourced from cattle markets within the state and from other cattle markets in Bauchi, Jigawa and Katsina states. The common breeds encountered at slaughter were White Fulani and Red Bororo. Meat from the abattoir were supplied to the major markets within the state, which included the Tudun-Wada market, Central market, Railway station market, Barnawa market and Sabo market all within Kaduna metropolis. The abattoir was chosen due to the large number of cattle slaughtered, and the wide area it supplied meat and meat products to. Consumers of meat and meat products from the abattoir included veterinarians, butchers, animal rearer's, hawkers, meat seller's and the general public.

3.4 Sample Size

The sample size was determined as described by Mugo (2008) using the formula

$$N = \frac{Z^2pq}{d^2}$$

Where

Z = appropriate value for the standard normal deviate far the desired confidence 1.96

N – Sample size

q = 1 – p

p = Prevalence = 14% (Abubakar, 2007)

d = degree of precision (3%) = 0.03

$$N = \frac{1.96^2 \times 0.14 (1- 0.14)}{0.03 \times 0.03} = \frac{3.8416 \times 0.14 \times 0.86}{0.0009} = \frac{0.4135}{0.0009} = 513.9$$

This was the minimum sample size collected using a precision of 3%. A total of 514 tissue samples were therefore collected for the purpose of this research.

3.5 Sampling Procedure:

Purposive sampling was used in obtaining tissue samples from slaughtered cattle. Slaughtered cattle were grouped into 3 age groups as 2-4, 5-7, and above 7 years old respectively to aid in prevalence calculation for each age group.

3.5.1 Post mortem meat inspection.

Animals sampled were selected based on the following criteria:

- Cachetic animals brought to the abattoir for slaughter. These were animals showing shallow paralumber fossa with no fatty tissues under the skin of the head and tail, skin may be supple or fairly supple, spine prominent with horizontal processes having sharp or fairly rounded ends , and showing ribs which can be identified individually without palpation. Every tenth animal encountered during slaughter presenting these signs was sampled.

- Animals with lesions suspected of being bovine tuberculosis in any organ or tissue after slaughter.

- Lymph nodes from apparently healthy cattle. These were animals presenting completely filled folds and cavities with patches of fats evident, ribs are not visible, spinal and horizontal processes cannot be felt and have a completely rounded appearance. Every tenth animal encountered during slaughter presenting these signs was sampled.

In all the above cases, post mortem examination was carried out in conjunction with the personnel in charge of meat inspection in the abattoir during the course of the sampling. It involved methods routinely used for meat inspection like visual examination, palpation, incision of suspected organs, and observation of gross atypical granulomatous lesions in affected organs carried out early in the morning during slaughter. Samples collected from suspected animals with Tb lesions included lungs, lymph nodes and intestinal tissues, while only the lymph nodes were collected from cachetic and apparently healthy animals. All animals sampled were aged according to the method described by Ron *et al* (2003).

3.5.2 Transportation of Samples.

Samples collected were placed in 0.9% normal saline in sterile screw-capped containers and transported to the Veterinary Public Heath and Preventive Medicine Laboratory, where they were frozen at -20^{o}C until they were processed.

3.5.3 Questionnaires.

A total of 250 structured, piloted questionnaires were administered on abattoir staff, butchers, meat hawkers and buyers/customers who came into contact with the abattoir, to assess the level of their knowledge on bovine tuberculosis. Knowledge of respondents was graded on a scale of 0-12 marks, with 0-5 being poor knowledge and 6-12 being good knowledge using the Guttman scale for grading qualitative data (Guttman, 1944). Knowledge was tested based on the sex, age, educational status, activity/occupation and contact with the abattoir by the respondents.

3.6 Laboratory Procedures

3.6.1 Tissue processing.

The stored tissue samples were thawed to room temperature to facilitate easy processing. The tissues were processed using the following steps:

i. A small portion of the tissue was used for the microscopic examination for the presence of *Mycobacterium* bacilli. The rest was returned to the freezer for the multiplex PCR technique if such samples were positive for *Mycobacterium* bacilli.

ii. The tissues after debridement were properly homogenized using a make-shift mortar and pestle (100mls sample bottle and 10ml syringe plunger).

iii. The homogenized tissues were then sieved. The extract was centrifuged and a loop full of the sediment was placed on a clean, labeled grease free glass slide and spread thinly using a sterile pipette to make a thin smear of about (1cm x 2cm). They were then labeled according to the samples.

iv. The smeared slides were air-dried and the tissue heat-fixed by gently passing them over the flame of a bunsen burner with the smeared side up so as to firmly attach the specimen to the slides and preserve the morphology of the bacteria during staining.

v. The slides were stained with carbol-fuschin and heated gently until fumes appeared to ensure the cell wall of the *Mycobacterium* organism if present, pick up the dye, and then allowed to stand for 5 minutes.

vi. The stained slides were then rinsed in flowing tap water to remove excess stain from the slides. The slides were then decolorized with 3% alcohol for 10 minutes till the red color faded to a light pink colour.

vii. The decolorized slides were rinsed again in water and counter-stained using methylamine blue for 1 minute.

viii. The slides were further rinsed in running water to remove excess colour and air dried.

3.6.2 Microscopic examination.

After staining, slides were examined as follows:

i. The slides were mounted on an optical light microscope (x100) and observed under oil immersion objective, to detect acid – fast bacilli (AFB).

ii. Results showing pinkish, straight or slightly curved rods, which occurred singly or in a cluster, indicated the presence of tuberculous bacilli. The background appeared blue due to the methylene blue dye.

Only samples inferred to be positive by Ziehl-Neelsen staining technique were used for the multiplex- PCR technique.

3.6.3 DNA extraction from tissue samples.

The method of extraction of DNA used was that of Janice *et al* (1997), with a slight modification.

i. Another portion of the tissues in 3.6.1 above was also homogenized for this process. The homogenated tissue sample was collected into a 1.5ml microfuge tube at room temperature and transported to the DNA-LABS Laboratory, in Angwan-Sarki, Kaduna.

ii. In the DNA-LABS, using an applicator stick, the homogenate was dispersed in a mixture of 400µl of lysis buffer and 25 µl proteinase K in the 1.5ml tube, and placed on a heated block at 65^{o}C for 1 hour. It was vortexed using a Deluxe Mixer (American Scientific Product) at 15 minute intervals to ensure proper breakdown of the tissues.

iii. 400µl of phenol-chloroform, according to Del Paortillo *et al* (1991), was added to the mixture and vortexed for 10 seconds and then centrifuged at room temperature at 12,000 G for 10 minutes.

iv. The supernatant was harvested into a fresh micro-centrifuge tube and 400µl of clear chloroform added and the content vortexed for 15 seconds before re-centrifuging for 5 minutes at room temperature at 12,000 G.

v. Using a micro-pipette, the clear top layer was decanted into new eppendorf tubes to which 1000µl of 100% ethanol and 40µl of 3M sodium acetate was added and the tubes inverted for 5 seconds and stored at -20^{o}C for 24 hours for alcohol precipitation of the DNA.

vi. The eppendorf tubes containing the alcohol/DNA mix was then centrifuged at 4^{o}C at 12000G for 10 minutes.

vii. The top layer containing the 100% ethanol was then decanted and 1000µ l of 70% ethanol added to rinse the DNA pellet at the bottom of the eppendorf tube. This was also centrifuged at 12000G for 5 minutes at 4^{o}C.

viii. The 70% ethanol was then decanted, leaving behind a purified DNA pellet which was allowed to air dry by leaving the tubes open.

ix. Following air drying, the purified DNA was suspended in 60µl of ultra pure water and an aliquot was tested by multiplex PCR using a Programmable Thermal Control Machine (PTC-100^{TM}) from MJ Research Incorporation Company.

Primers used (table 3.1) were designed by Boineer Corporation South Korea, and targeted the RD1, RD4, RD9 and RD12 nucleic acids in the cell wall of the *Mycobacterium bovis* organism (Brosch *et al.,* 2002).

Table 3.1 Primers and their sequences used for multiplex PCR.

Primer sequences	RD	*M. tuberculosis*	*M. bovis*	*M. africanum*
AAGCGGTTGCCGCCGACCGACC	1	Present	Present	Present
CTGGCTATATTCCTGGGCCCGG	1	(146bp)	(146bp)	(146bp)
GAGGCGATCTGGCGGTTTGGGG	1			
ATGTGCGAGCTGAGCGATG	4	Present	Absent	Present
TGTACTATGCTGACCATGCG	4	(172bp)	(268bp)	(172bp)
AAAGGAGCACCATCGTCCAC	4			
CAAGTTGCCGTTTCGAGCC	9	Present	Absent	Absent
CAATGTTTGTTGCGCTGC	9	(235bp)	(108bp)	(108bp)
GCTACCCTCGACCAAGTGTT	9			
GGGAGCCCAGCATTTACCTC	12	Present	Absent	Present
GTGTTGCGGGAATTACTCGG	12	(369bp)	(306bp)	(369bp)
AGCAGGAGCGGTTGGATATTC	12			

Warren *et al*., 2006.

Key: RD= Regions of Difference.

3.6.4 Polymerase chain reaction (PCR) amplification

i. This amplification was carried out in a final volume of 1µl DNA template, 5µl Q-buffer, 2.5µl x 10 buffer, 2µl 25mM $MgCl_2$, 4µl x 10mM dNTPs, 0.5µl of each primer, 0.125µl HotStarTaq plus DNA polymerase (Qiaqen, Hilden, Germany) and made up to 20µl with water.

ii. DNA from *M. bovis* isolate sourced from prior research work carried out at the DNA-LABS Laboratory and sterile distilled water was used as positive and negative PCR controls respectively.

iii. Amplification was performed in the following sequence:

a. Initial denaturing of DNA at 94^oC for 15 minutes

b. Denaturing at 94^o C for 1 minute

c. Annealing at 62^o C for 1 minute.

d. Extension at 72^oC for 1minutes

e. Final extension at 72^oC for 10 minutes. (Warren *et al*., 2006).

3.6.5 Electrophoresis.

After amplification, 20µl of the amplified PCR product was separated by electrophoresis in 3% agarose gel, containing 0.5 µg/ml of ethidium bromide. The gel was loaded with the DNA mix, a 100bp Plus DNA ladder, DNA from *M. bovis* isolate as positive control and sterile distilled water as negative control. Electrophoresis was carried out at 6V/cm for l hour. An image documentation system for visualization of DNA bands (Warren *et al*., 2006) was used, and a sample was inferred to be *Mycobacterium bovis* positive, *Mycobacterium tuberculosis* positive, or *Mycobacterium africanum* positive upon detection of amplicons signifying the presence or absence of RD1, RD4, RD9 and RD12.

3.7 Data analyses

Data obtained from the study was presented using tables and bar charts. Data were analyzed using SPSS version 17.0, Inc, Chicago, Illinois, USA. $P<0.05$ was regarded as significant. Chi square was used to test association between variables that included age, breed, and sex of animals with *Mycobacterium* bacilli while odds ratio was used to determine the strength of association amongst the mentioned variables, in relation to *Mycobacterium* bacilli infection.

The Detection Rate was calculated using the formula.

$$\text{Detection Rate} = \frac{\text{Number of Positive Samples}}{\text{Total Number of Sample Analyzed}} \times 100.$$

CHAPTER FOUR

4.0 RESULTS

From the study carried out, a detection rate of 10.9% for acid fast bacilli using Ziehl Neelsen staining technique was obtained. A detection rate of 4.4% for *M. bovis* was obtained using the multiplex PCR technique.

4.1 Detection rate of *Mycobacterium* bacilli in slaughtered cattle.

The results obtained from the study are presented in Tables 4.1 to 4.11 respectively.

The results revealed that out of 514 cattle sampled, 56 (10.9%) were positive for *Mycobacterium* bacilli using Ziehl Neelsen staining techniques (plate 4.1).

4.2 Detection rate of *Mycobacterium* bacilli based on the sex of cattle.

Table 4.1 shows the detection rate of *M. bovis* based on the sex of the animals examined. It showed that out of 416 samples obtained from female cattle, 49 (11.77%) were positive for *Mycobacterium* bacilli using Ziehl Neelsen staining, out of 98 male animals sampled, 7(7.14%) were positive for *Mycobacterium* bacilli using Ziehl Neelsen stain. Female animals did not show significant positivity than males ($p<0.05$) (Table 4.1).

4.3 Detection rate of *Mycobacterium* bacilli based on age grouping of cattle.

Animals aged 2-4 years accounted for 215 of the 514 animals sampled, out of which 19(8.84%) were positive, those aged 5-7 years accounted for 290 out of the 514 animals sampled, out of which 36(12.41%) were positive. Animals older than 7 years of age accounted for the remaining 9 animals out of the 514 animals sampled, with 1(11.11%) being positive (Table 4.2).

Table 4.1 Detection Rate of *Mycobacterium* bacilli based on sex of cattle.

Sex	**No. sampled**	**No. Positive (%)**	**OR**	χ^2	**P-Value**
Male	98	7(7.14)	0.5761	1.756	0.1851
Female	416	49(11.77)			
Total	**514**	**56(10.89)**			

Key: OR=Odds ratio.

Table 4.2 Detection Rate of *Mycobacterium* bacilli based on age of cattle.

Ages (Yrs)	No. sampled	No. Positive (%)	O R	χ^2	P-Value
2 – 4	215	19(8.83)	1.00	1.627	0.4432
5 – 7	290	36(12.41)	1.462		
□ 7	9	1(11.11)	1.289		
Total	**514**	**56(10.9)**			

Key: OR=Odds ratio.

4.4 Detection Rate of *Mycobacterium* bacilli based on breed of cattle.

From the Study, a total of 514 cattle were examined during slaughter. 375(72.97%) were White Fulani while 139(27.24%) were Red Bororo (Table 4.3). Of the 375 White Fulani cattle examined, 33(8.80%) had lesions that were positive for *Mycobacterium* bacilli, while 23(16.54%) of the Red Bororo were positive for the *Mycobacterium* bacilli. There was significant association between the Red Bororo breed and the occurrence of bovine tuberculosis in the study.

4.5: Distribution of tuberculous lesions in organs

Table 4.4 below shows the distribution of lesions in organs sampled and the acid-fast bacilli obtained by Ziehl Neelsen (ZN) staining. Fifty one (91.07%) of the 356 samples collected from Lungs and Lymph nodes only were positive using the Ziehl Neelsen staining techniques while one ((1.79%) out of the 146 samples obtained from lymph nodes only was positive using the same technique. Three (5.36%) of the 10 Lungs only samples as well as 1 (1.79%) out of the two Lungs, Lymph nodes and Small intestine only samples were also seen to be positive for acid-fast bacilli using the Ziehl Neelsen staining technique. From the results, the Lungs and Lymph nodes were the organ most commonly affected.

Table 4.3 Detection Rate of *Mycobacterium* bacilli based on breed of cattle.

Breed	No. sampled	No. Positive (%)	O.R	χ^2	P-Value
White Fulani	375(72.97%)	33(8.80)	0.4867	6.269	0.0123
Red Bororo	139(27.24%)	23(16.54)			
Total	**514(100%)**	**56(10.9)**			

Key: OR=Odds ratio.

Table 4.4: Distribution of tuberculous lesions in organs.

Tissues sampled	No sampled (%)	No positive (%)
Lungs only	10 (1.95)	3 (0.58)
Small intestine only	0 (0.00)	0 (0.00)
Lymph nodes only	146 (28.40)	1 (0.19)
Lungs and Small intestine only	0 (0.00)	0 (0.00)
Lungs and Lymph nodes only	356 (69.26)	51 (9.92)
Small intestine and Lymph nodes only	0 (0.00)	0 (0.00)
Lungs, Lymph nodes and small intestine only	2 (0.39)	1 (0.19)
Total	**514 (100)**	**56(10.9)**

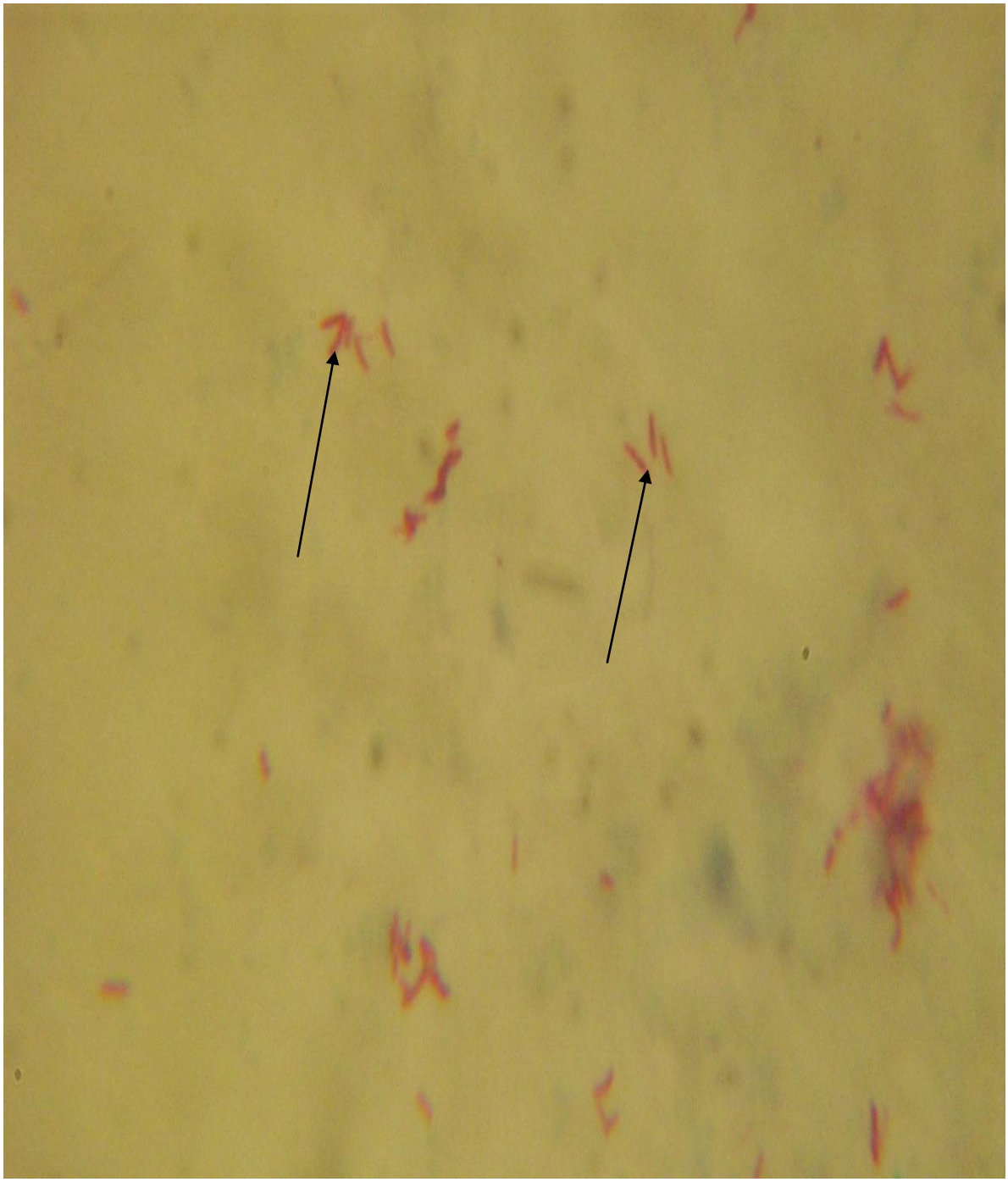


Plate I: Acid- Fast bacilli obtained after Ziehl Neelsen staining technique (X100) Magnification.

4.6 Deletion typing of *Mycobaterium species* isolated from organs/tissues.

Plates 4.1 and 4.2 showed the electrophoretic pattern obtained from multiplex-PCR. Out of the 56 isolates run, 23 (lines1,2,7,8,13,16,17,21,23,24,27,31,32,33,34,35,37,39,41,44,46,49 and 55) gave bands of 245 bp sizes corresponding to that of *M. bovis* control (lane P), while the others could be other species of *Mycobacterium* (Tables 4.5).

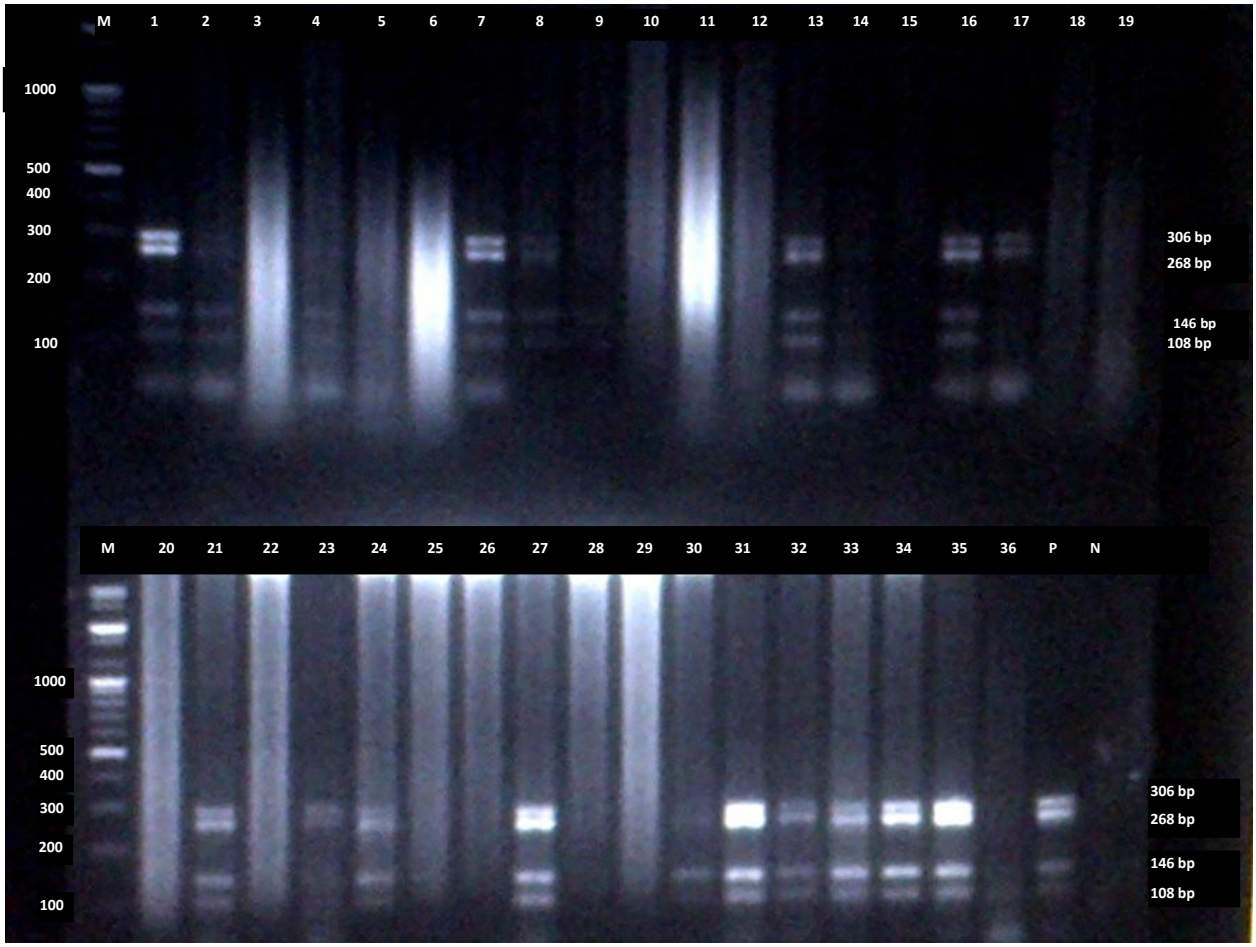


Plate II: Electrophoretic fractionalization of PCR products in 3% agarose. Lane M- molecular weight marker, lanes 1 – 36 = isolates, P- positive control, N- negative control.

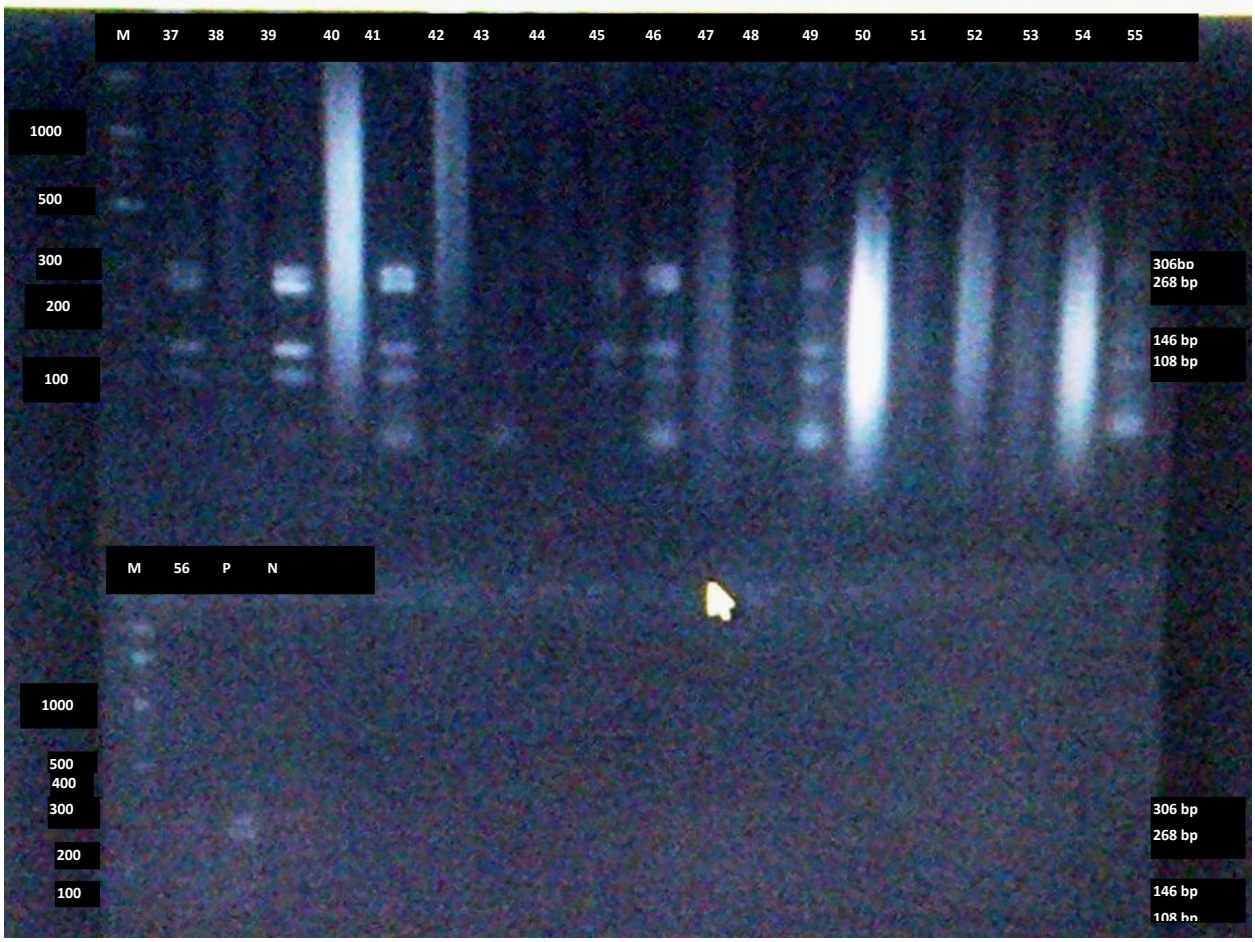


Plate III: Electrophoretic fractionalization of PCR products in 3% agarose. Lane M- molecular weight marker, lanes 37 – 56 = isolates, P- positive control, N- negative control.

Table 4.5 Deletions targeting region of differences (RDs)

Mycobacterium Specie	RD1	RD4	RD9	RD12
M. bovis	**23**	**-**	**-**	**-**
M. tuberculosis	**-**	**-**	**-**	**-**
M. africanum	**-**	**-**	**-**	**-**
Total	**23**	**-**	**-**	**-**

Key: RD= Region of difference, - = Nil

4.7 Knowledge of Tb based on sex of the respondents

Twenty four (30.7%) of the 78 male respondents indicated they had poor knowledge of bovine tuberculosis while 54 (69.2%) of them said they had good knowledge of the disease. Similarly 11 (23.4%) of the 47 female respondents had poor knowledge of the disease while 36 (76.6%) of them had good knowledge of the disease as seen in Table 4.6.

4.8 Knowledge of Tb based on age of the respondents

Seven (41.18%) of the 17 respondents within age group 16-20 indicated they had poor knowledge of bovine tuberculosis while 10 (58.82%) of them said they had good knowledge of the disease. Similarly 8 (21.05%) of the 38 respondents within age group 21-25 indicated they had poor knowledge of the disease while 30 (78.95%) of them said they had a good knowledge of the disease. Out of 26 respondents within age group 26-30, 4 (15.38%) said they had poor knowledge of bovine tuberculosis while 22 (74.62%) of them indicated they had good knowledge of the disease. For respondents within age group 31-35, 4 (26.67%) of the total 15 respondents indicated they had poor knowledge of the disease while 11 (73.33%) said they had good knowledge of bovine tuberculosis. Similarly 2 (20.0%) of the 10 respondents within age group 36-40 indicated they had poor knowledge of the disease while 8 (80.0%) of them said they had good knowledge about bovine Tb. For the 19 respondents within age group 40 and above, 10 (53.63%) of them said they had poor knowledge of the disease while the remaining 9 (47.37%) indicated they had good knowledge on bovine tuberculosis as shown in Table 4.7.

Table 4.6 Knowledge of Tb based on sex of respondents

Sex	Total No	Poor Score N (%)	Good Score N (%)	OR	χ^2	P-Value
Male	78	24 (30.77)	54 (69.23)	0.6875	0.7891	0.3744
Female	47	11 (23.40)	36 (76.60)			
Total	**125**	**35 (28.0)**	**90 (72.0)**			

Key: OR=Odds ratio, N=Number

Table 4.7 Knowledge of Tb based on age of the respondents

Age	Total number	Poor Score N (%)	Good Score N (%)	OR	χ^2	P-Value
16-20	17	7 (41.18)	10 (58.82)	1.00	10.48	0.0628
21-25	38	8 (21.05)	30 (78.95)	2.625		
26-30	26	4 (15.38)	22 (74.62)	3.850		
31-35	15	4 (26.67)	11 (73.33)	1.925		
36-40	10	2 (20.0)	8 (80.0)	2.800		
>40	19	10 (53.63)	9 (47.37)	0.630		
Total	**125**	**35 (28.0)**	**90 (72.0)**			

Key: OR=Odds ratio, N= Number

4.9 Knowledge of Tb based on respondent's educational status

Three (75.0%) of the 4 respondents with no formal education indicated they had poor knowledge on bovine tuberculosis while 1 (25.0%) had good knowledge of the disease. Out of a total of 14 respondents with primary education, 10 (71.43%) said they had poor knowledge about the disease as compared to the remaining 4 (28.57%) who indicated they had good knowledge of bovine tuberculosis. Similarly 11 (28.95%) of the 38 respondents with secondary education indicated they had poor knowledge on the disease, while 27 (71.05%) showed good knowledge on Tb. For the 8 respondents with adult education, 5 (62.50%) of them had poor knowledge of the disease while 3 (37.50%) indicated they had good knowledge on bovine tuberculosis as shown in Table 4.8.

4.10 Knowledge of Tb based on respondent's activity/ occupation.

Out of the 9 respondents designated as animal scientist, 1 (11.11%) showed poor knowledge on bovine tuberculosis while 8 (88.89%) exhibited good knowledge about the disease. Sixteen of the respondents were veterinarians and 0 (0%) of them had poor knowledge to the disease while 16 (100%) of them had good knowledge on bovine tuberculosis. Nineteen respondents were butchers and 9 (47.37%) of them exhibited poor knowledge about the disease while 10 (52.63%) of them had good knowledge about bovine tuberculosis. Of the 7 animal rearer's interviewed, 1 (14.29%) had poor knowledge on bTb while the remaining 6 (85.71%) said they had good knowledge about it. Questionnaires were administered to 6 meat sellers and 4 (66.67%) indicated they had poor knowledge on bovine tuberculosis, the remaining 2 (33.33%) had good knowledge on the disease. Eight (42.11%) of the 19 IT

students said they had poor knowledge of bTb while 11 (57.89%) presented with good knowledge on the disease condition. Three (8.82%) of the 34 consumers interviewed indicated a poor score knowledge about the disease while the remaining 31 (91.18%) of them had good knowledge about bovine tuberculosis. From a total of 15 hawkers, 9 (60.0%) of them exhibited poor knowledge about the disease while the remaining 6 (40.0%) of them showed good knowledge of bovine tuberculosis (Table 4.9).

Table 4.8 Knowledge of Tb based on respondent's educational status

Educational Status	Frequency	Poor Score N (%)	Good Score N (%)	OR	χ^2	P-Value
No formal education	4	3 (75.0)	1 (25.0)	1.00	32.20	0.0001
Primary	14	10 (71.43)	4 (28.57)	1.200		
Secondary	38	11 (28.95)	27 (71.05)	7.364		
Tertiary	61	6 (9.84)	55 (90.16)	27.50		
Adult education	8	5 (62.50)	3 (37.50)	1.800		
Total	**125**	**35 (28.0)**	**90 (72.0)**			

Key: OR=Odds ratio, N= Number.

Table 4.9 Knowledge of Tb based on respondent's activity/ occupation.

Activity/ occupation	Total Number	Poor Score N (%)	Good Score N (%)	OR	χ^2	P-Value
Animal Scientist	9	1 (11.11)	8 (88.89)	1.00	31.83	<0.0001
Veterinarian	16	0 (0)	16 (100)	5.824		
Butcher	19	9 (47.37)	10 (52.63)	0.1389		
Animal Rearer	7	1 (14.29)	6 (85.71)	0.7500		
Meat seller	6	4 (66.67)	2 (33.33)	0.0625		
IT Students	19	8 (42.11)	11 (57.89)	0.1719		
Consumers	34	3 (8.82)	31 (91.18)	1.292		
Hawker	15	9 (60.0)	6 (40.0)	0.0833		
Total	**125**	**35 (28.0)**	**90 (72.0)**			

Key: OR=Odds ratio, N= Number.

4.11 Knowledge of Tb based on years of respondents contact with the abattoir.

Three (8.33%) of the 36 respondents in lower than 1 year group had poor knowledge of bovine tuberculosis while 33 (91.67%) of them said they had good knowledge of the disease. Similarly 13 (28.26%) of the 46 respondents within group 1-5 had poor knowledge of the disease while 33 (71.74%) of them had good knowledge of the disease. Within group 6-10, 4 (26.67%) of the total 15 had poor knowledge about bovine tuberculosis while the remaining 11(73.33%) of them displayed good knowledge on the disease. Six (54.55%) of the respondents in group 11-15 had poor knowledge about the disease while 5 (45.45%) had good knowledge about bovine tuberculosis. For the 17 respondents in group 16 years and above, 9 (52.94%) of them had poor knowledge about the disease while the remaining 8 (47.06%) had good knowledge about the disease (Table 4.10).

Table 4.10 Knowledge of Tb based on years of respondents contact with the abattoir.

Groups (Yrs)	Total Number	Poor Score N (%)	Good Score N (%)	OR	χ^2	P-Value
< 1	36	3 (8.33)	33 (91.67)	Ref	16.01	0.003
1 – 5	46	13 (28.26)	33 (71.74)	0.2308		
6 – 10	15	4 (26.67)	11 (73.33)	0.2500		
11 – 15	11	6 (54.55)	5 (45.45)	0.07576		
> 16	17	9 (52.94)	8 (47.06)	0.08081		
Total	**125**	**35 (28.0)**	**90 (72.0)**			

Key: OR=Odds ratio, N= Number.

CHAPTER FIVE

5.0 DISCUSSION

The detection rate of *Mycobacterium* bacilli using the Ziehl Neelsen (ZN) staining technique for this study yielded 10.9%, which was lower than the prevalence of 14.3 % reported in a survey carried out in Kaduna and Abuja by Abubakar (2007), and also lower than the prevalence of 17.5% reported by Danbirni *et al* (2010), but much higher than the prevalence of 4.4% obtained by Damina *et al*. (2011) in Plateau State, 2.8% reported by Igbokwe *et al*. (2001) in Borno and Yobe states and 0.5% by Bello *et al*. (2009) in the Zango abattoir, Zaria. . The difference in prevalence obtained may be entirely or in-part due to the type of diagnostic technique used, since it is known that the Ziehl Neelsen method is not specific for tuberculous bacilli because it requires 10^3 to 10^4 cells per ml of specimen and detects only acid-fast bacilli (Ojo, 2009). This makes the ZN method less dependable than the DNA based methods such as PCR. Also, the higher prevalence may be due to the fact that animals were obtained from different sources and herds thus having a higher tendency to have a higher prevalence than that obtained from animals from a single herd. Another reason for the difference in prevalence from other research findings may be due to the type of samples collected, since some of the work were carried out on fresh milk, serum and macerated tissues (Ofunkwu *et al*., 2008; Danbirni *et al*.,2010; Awah-Ndukum *et al*.,2012). Another reason for difference in prevalence may be due lack of thoroughness by meat inspectors during meat inspection at the abattoir, some pathological lesions are completely missed due to lack/unassisted command on the part of the veterinary staff over the rough behaviors of butchers and meat traders (Awah-Ndukum *et al*., 2012). Also, there have been reported cases of infected organs being smuggled out of the abattoirs and sold to the public, with some

consumers making special request for these diseased organs (Damina *et al*., 2011). The detection of Tb lesions in slaughtered cattle can be affected by other infections other than *M.bovis*, such as parasites, non-specific reactions (Shitaye *et al*., 2006), and other irregularities of abattoir meat inspections (FAO, 1994), and lesions may be confused with those caused by *Norcadia*, *Corynebacterium* and other granuloma causing organisms (Grist, 2008). Discrepancies in prevalence may also be attributed to the sample sizes and study designs (Katale *et al*., 2013).

The study also revealed a higher detection rate of acid-fast bacilli in female than in male animals. This could be due to the fact that in most herds, the number of female animals far surpasses the number of male ones and these female animals are usually taken to the abattoir for slaughter when they can no longer reproduce due to old age or are observed to be sick by the owners. This finding was in agreement with the study carried out by Damina *et al*. (2011) where he observed that more females were slaughtered in the abattoir than males and tuberculous lesions were present more in female than male animals. Cadmus *et al*. (1999) also reported that tuberculosis may be more in female cattle than in males due to a decrease in immunity brought about by pregnancy induced stress and lactation. It may also be due to the fact that male animals are usually sold off at a younger age than females, and since bovine tuberculosis is a chronic disease (Radostits *et al*., 2007), the young bulls do not survive long enough to manifest tuberculous lesions. Even though more females were infected than males, it was found not to be significant statistically.

From the study, it was also observed that a higher prevalence of *Mycobacterium* bacilli was encountered in animals of age group 5-7 years, than in those of age group 2-4 years. This might be due to the fact that older animals are known to have a reduced immune status as

they grow older, thus the variance in the prevalence based on age. Also it was observed from the results of the study that adult animals accounted for 36 out of the 56 animals which tested positive, this may be due to the fact that farmers generally have a lower tendency to sell off younger animals when hard pressed for money because it is believed that younger animals have more productive years ahead than adult or older animals which may have accrued mileage in their usefulness. For animals older than 7 years, the study revealed a lower number of animals with the disease and a detection rate of 11.11%. The low number of cattle may be attributed to the chronic nature of the disease and the fact that farmers retain female animals that may be productive thus the lower numbers seen in the abattoir. This agrees with the report of Belkhiri *et al*. (2009), who observed higher occurrence of bovine tuberculosis in older female animals and the possibility of the disease being exhibited as the age of the animal increased. The findings of the study also agrees with that of Awah-Ndukum *et al*. (2012), which had a higher value of the infection amongst adult/older cattle than in younger animals. This infers that the disease is more of a disease of older animals than young ones. Though older animals were observed to be more infected than younger ones, no statistical significance was established.

Based on the assessment of the relationship between breed and detection rate of *Mycobacterium* bacilli carried out in the study, it was observed that the Red Bororo breed had a significantly higher ($P<0.05$) occurrence of acid-fast bacilli (16.54%) than the White Fulani breed which had (8.80%). These finding agree with that of Awah-Ndukum *et al*. (2010), who found a higher prevalence of the disease in the Red Mbororo breeds than the Gaudali and White Fulani breeds.

During the course of the study, characteristic lesions of tuberculosis were mainly observed in the lungs, small intestine and the lymph nodes. The pooled samples from Lungs, Lymph node and Small intestine only and samples from the Lymph nodes only had the least (one) tuberculous lesion encountered respectively (1.79%). The Lungs and Lymph node only samples accounted for the most of the tuberculous lesions obtained 51 (91.07%), followed by the Lungs only samples which had 3 (5.36%). This higher occurrence of the lesions in the lungs and lymph nodes than in other tissues may be due the fact that the transmission of the disease is almost always by inhalation of aerosol, and the tuberculous bacilli are seen to localize in the lining of the respiratory tract (Grange, 2001). This is in agreement with the findings of Damina *et al*. (2011), and Igbokwe *et al*. (2001) who found a higher detection rate of tuberculous lesions in the lungs, followed by the lymph nodes then other organs. They also reported that bovine tuberculosis in Nigeria was mostly the pulmonary form involving the lungs and associated lymph nodes. The results also agree with the findings of Abubakar *et al*. (2012) who found a similar pattern of detection rate of tuberculous lesions in organs of camels. Hilty (2006) also reported that bovine tuberculosis is characterized by progressive development of granulomatous lesions in the lungs and lymph nodes than other organs. It was also observed that though gross lesions were seen and sampled at slaughter, none of the tissues/affected organs were condemned, the butchers became slightly aggressive at talks of condemnation which may be due to the fact that butchers are not compensated for any form of meat condemnation thus they bear the brunt of the loss entirely. This trend has also been reported by Ibironke and Fasina (2010), and Cadmus and Adeshokan (2009).

Based on the results obtained by multiplex-PCR, 23 of the 56 isolates were identified as *M. bovis*, due to the presence of the RD1 region of difference. This agrees with studies carried out by Ejeh *et al*. (2014) and Damina *et al*. (2011) where the presence of RD1 and the absence of RD4, RD9 and RD12 indicated a positive identification for *M.bovis*. The absence of RD4, RD9 and RD12 indicated that none of the isolates was *M. tuberculosis* or *M. africanum*. In *M. africanum*, RD1 and RD4 and RD12 are all present, while RD9 is absent. In *M. tuberculosis*, RD1, RD4, RD9 and RD12 are all present (Warren *et al*., 2006). Co-infection of *M. bovis* and *M. tuberculosis* have been observed by Cadmus *et al*. (2010) and Damina *et al*. (2011) in North Central Nigeria but co-infection was not encountered during this study. This could be that none of the cattle examined during the study had exposure to humans suffering from tuberculosis due to *M*. *tuberculosis*.

From the questionnaires, it was observed that 90 (72.0%) of the respondents had good knowledge about tuberculosis, while 35 (28.0%) of them had poor knowledge on tuberculosis. There was no significant association found between sex of the respondents and knowledge about Tb (P-value = 0.3744). There was also no significant association found between age groups and knowledge about Tb (P-value = 0.0628). A significant association between educational status and knowledge about Tb was observed with P-value <0.0001, where respondents having no formal education had a greater percentage of poor scores than those with primary, secondary, tertiary and adult education. These findings were in agreement to the findings of Awah-Ndukum (2010), who observed that butchers and cattle professionals with low level of formal education were most at risk of exposure to zoonotic bovine Tb. There was also a significant association observed between activity/occupation with knowledge about tuberculosis (P-value <0.0001), with those respondents having

professional expertise having better scores than those in unskilled labour. However, consumers were observed to have good score (91.18%) on Tb knowledge, which may be due to the fact that many of them might have been from skilled professions, and therefore better informed. This is similar to the findings of Hambolu *et al*. (2013), who observed a good knowledge about bovine tuberculosis amongst abattoir stakeholders in southwestern Nigeria. Between contact with the abattoir and knowledge about Tb, there was a significant association observed with P-value = 0.003, from respondents having less than a year contact with the abattoir exhibiting better knowledge of Tb than those who have been in longer contact.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS.

6.1 CONCLUSIONS

It was concluded from this study that *M. bovis* had been detected in some animals slaughtered within the Tudun-Wada abattoir/ Kaduna state, with a detection rate of 10.9% using the Ziehl Neelsen staining technique and 4.4% using the mPCR techniques. It was also observed that though more expensive, m-PCR method of diagnosis was a more sensitive method of diagnosis than detection of acid-fast bacilli by Ziehl Neelsen staining techniques. The presence of a positive sample from apparently healthy animals and the detection of *M. bovis* positive samples from animals slaughtered at the abattoir, goes further to reiterate the presence of the disease and the likelihood of the transmission of zoonotic disease from animals to humans, thus it becomes necessary to enlighten consumers on proper heat treatment when consuming meat and its resultant products. The detection rate of bovine tuberculosis obtained from this study suggest that unwholesome contaminated beef is likely being sold for human consumption, thereby reiterating the fact that infections from cattle to humans is possible.

The detection of suspected gross Tb lesions in cattle slaughtered at the abattoir increases the potential risk of the disease being contracted by the public especially abattoir workers and butchers, where minimal protective clothing are worn and meat is being practically processed using bare hands.

Knowledge/ awareness of the disease within the study area though being good, does not eliminate risk of disease spread among respondents.

6.2 RECOMMENDATIONS

In view of some of the finding during the course of this study, it becomes necessary to recommend that:

1. Meat inspection in the abattoir should be left in the hands of professionals to ensure that proper inspection and adequate detection of diseases is carried out to provide wholesome meat certified for human consumption.
2. A good animal documentation and identification of cattle should be put in place to aid in monitoring the source of tuberculous animals slaughtered in the abattoir (trace-back) and aid in pinpointing sources of infection/also to help with disease control at the herd level.
3. More enlightenment of abattoir stakeholders should be carried out to increase their awareness of hazards involved in dealing with zoonotic diseases, and adequate measures to take in preventing infections.
4. There is a need of compensation for organ/ tissue condemnation, by stakeholders in the cattle industry, to encourage butchers/ cattle owners to willingly relinquish diseased organs to the appropriate authority.

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APPENDIX I

QUESTIONNAIRE

DEPARTMENT OF VETERINARY PUBLIC HEALTH AND PREVENTIVE MEDICINE, AHMADU BELLO UNIVERSITY, ZARIA.

A STRUCTURED INTERVIEWER-ADMINISTERED RISK FACTOR ASSESMENT AND AWARENESS QUESTIONAIRE.

Dear sir/madam,

This questionnaire is part of an M.Sc Research work, aimed at finding out the risk factors and degree of awareness associated with bovine tuberculosis among staff, butchers and meat hawkers, practicing their trade within the abattoir. All findings will be treated with utmost confidentiality. Your truthful response to the questions in this questionnaire will go a long way in validating my work.
Thank you for your anticipated cooperation.

INSTRUCTIONS
Kindly tick (√) the box where options are given, and kindly provide short answers where the space is provided. Upon completion, kindly return the questionnaire to the person administering them. Thank you.

SECTION A: BIODATA

A1:SerialNumber.
…….

A2: Age of Respondent (yrs): 16-20 [ ], 21-25 [ ], 26-30 [ ], 31-35 [ ], 36-40 [ ], 40 and above[ ].

A3: Sex: Male [ ], Female [ ]

A4: Occupation: Veterinarian [ ], Animal scientist [ ], Butcher [ ], Hawker [ ], Animal rearer [ ], Others (specify) ……

A5: How long have you practiced this profession? .....

A6: How many people are there in your family? .....

A7: How many rooms are in your household? ……

A8: Do you have animals within your household? Yes [ ], No [ ]

A9: If yes, what specie? ……

SECTION B: AWARENESS OF TUBERCULOSIS (TB)

B1: How long have you been in contact with abattoirs? Less than 1 year [ ], 1-5 years [ ], 5-10 years [ ], 10-15 years [ ], 16 years and above [ ].

B2: Does your line of duty require protective clothing? Yes [ ], No [ ]

B3: If Yes, specify what type ……

B4: How often are these protective clothing used? .....

B5:Do you think people can contact diseases from ruminants (cattle, sheep and goats)? Yes [ ] No [ ]

B6: Why? (give reasons) ……...

B7: kindly list some diseases that you know can be contacted from ruminants? ……..…… ……...

SECTION C: KNOWLEDGE ABOUT TUBERCULOSIS (TB)

C1: Level of Education: None [ ], Primary [ ], Secondary [ ], Tertiary [ ], Adult Education [ ],Others (specify). ……

C2: Do you know of the disease Tuberculosis? Yes [ ], No [ ]

C3: If yes, what are the ways TB can be contacted?

1.....

2……..

3……

4…….

C4: Do you think TB is associated with contact? Yes [ ], No [ ].

C5: Can a person contact TB from animals slaughtered at an abattoir? Yes [ ], No [ ].

C6:If yes, how?
.....

C7: Do you know the signs of TB? Yes [ ], No [ ]

C8: If yes, list the signs you know
……
……

C9: How can you protect yourself from getting infected with TB?
……
……

C10: Is there a vaccination against TB? Yes [ ], No [ ].

C11: Do you think TB can be cured? Yes [ ], No [ ].

C12: What will you do if you encounter TB at slaughter? ……..……
……..

APPENDIX II

Table A1 Deletions targeting region of differences (RDs)

SAMPLE NO	RD1	RD4	RD9	RD12	COMMENT
DNA ladder					
1	+	-	-	-	*M.bovis*
2*	+	-	-	-	*M.bovis*
3	-	-	-	-	O.M
4*	-	-	-	-	O.M
5	-	-	-	-	O.M
6	-	-	-	-	O.M
7	+	-	-	-	*M.bovis*
8*	+	-	-	-	*M.bovis*
9	-	-	-	-	O.M
10	-	-	-	-	O.M
11	-	-	-	-	O.M
12	-	-	-	-	O.M
13	+	-	-	-	*M.bovis*
14	-	-	-	-	O.M
15	-	-	-	-	O.M
16	+	-	-	-	*M.bovis*
17*	+	-	-	-	*M.bovis*
18	-	-	-	-	O.M
19	-	-	-	-	O.M
DNA ladder					O.M
20	-	-	-	-	O.M
21	+				*M.bovis*
22	-	-	-	-	O.M
23*	+	-	-	-	*M.bovis*
24	+	-	-	-	*M.bovis*
25	-	-	-	-	O.M
26	-	-	-	-	O.M
27	+	-	-	-	*M.bovis*
28	-	-	-	-	O.M
29	-	-	-	-	O.M
30	-	-	-	-	O.M
31	+	-	-	-	*M.bovis*
32*	+	-	-	-	*M.bovis*
33	+	-	-	-	*M.bovis*
34	+	-	-	-	*M.bovis*
35	+	-	-	-	*M.bovis*
36	-	-	-	-	O.M
Positive control					
Negative control					

Table A1 Cont. Deletions targeting region of differences (RDs) continued.

Sample No.	RD1	RD4	RD9	RD12	COMMENT
DNA ladder					
37	+	-	-	-	*M.bovis*
38	-	-	-	-	O.M
39	+	-	-	-	*M.bovis*
40	-	-	-	-	O.M
41	+	-	-	-	*M.bovis*
42	-	-	-	-	O.M
43	-	-	-	-	O.M
44*	+	-	-	-	*M.bovis*
45	-	-	-	-	O.M
46	+	-	-	-	*M.bovis*
47	-	-	-	-	O.M
48	-	-	-	-	O.M
49	+	-	-	-	*M.bovis*
50	-	-	-	-	O.M
51	-	-	-	-	O.M
52	-	-	-	-	O.M
53	-	-	-	-	O.M
54	-	-	-	-	O.M
55*	+	-	-	-	*M.bovis*
56	-	-	-	-	O.M
Positive control					
Negative control					

RD – Region of difference. Positive control – *M.bovis,* Negative control – Distilled water.
KEY: O.M= Other species of *Mycobacterium*, + = Present, - = Absent, * = Faint bands.