Epidemiology and Public Health Implications of *Mycobacterium tuberculosis* complex and Non-tuberculous mycobacteria in Cattle and Humans in Oyo State, Nigeria

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CERTIFICATION

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LIST OF ABBREVIATIONS

TB	Tuberculosis
BTB	Bovine tuberculosis
MDR-TB	Multidrug-resistant tuberculosis
WHO	World Health Organization
HIV	Human Immunodeficiency Virus
AIDS	Acquired Immune Deficiency Syndrome
ATS	American ThoracicSociety
BTS	British Thoracic Society
HRCT	High-Resolution Computed Tomography
CDC	Center for Disease Control
DOTS	Directly Observed Treatment, Short-course
NTBLCP	National Tuberculosis and Leprosy Control Program
MTC	*Mycobacterium tuberculosis* complex
NTM	Non tuberculous mycobacteria
MAC	*Mycobacterium avium* Complex
IUATLD	International Union against Tuberculosis and Lung Diseases
AFB	Acid- Fast Bacilli
Z-N	Ziehl – Neelsen
PCR	Polymerase Chain Reaction
MGIT	Mycobacterial Growth Indicator Tubes
MODS	Microscopic Observation Drug Susceptibility
NRA	Nitrate Reductase Assay
PE	Proline-glutamic acid
PPE	Proline-proline-glutamic acid
BCG	Bacillus Calmete Guerin
VNTR	Variable Number Tandem Repeat
PGRS	Polymorphic GC-Rich Repetitive Sequence
DR	Direct Repeat
MIRU	Mycobacteria Interspersed Repetitive Unit.

RFLP	Restriction Fragment Length Polymorphism
PPD	Purified Protein Derivatives
MDG	Millennium Development Goals
HBCs	High Burden Countries
HPLC	High-Performance Liquid Chromatography
PRA	PCR-restriction enzyme analysis
RIVM	theRijksinstituut voor Volksgezondheid en Milieu
COPD	chronic obstructive pulmonary disease
CF	Cystic Fibrosis
NALC	*N*-acetyl l-cysteine
SNP	Single-Nucleotide Polymorphisms
LSP	large-sequence polymorphisms
L-J	Lowenstein-Jensen Medium
HBM	Health Belief Model
OIE	World Organisation for Animal Health

ABSTRACT

The risks of humans contracting bovine tuberculosis (BTB) and other mycobacterial infections abound in Nigeria. This could be attributed to the close association between farmers and cattle, unhealthy meat processing practices by butchers, consumption of unpasteurized milk and milk products, and poor knowledge of the disease. The circulating *Mycobacterium tuberculosis* complex and non-tuberculous mycobacteria in livestock workers and animals are largely unknown. Confirming the sources of exposure in humans will help to guide the direction of prevention and control of the diseases. This study aimed at determining the prevalence of tuberculosis and characterising the mycobacterial species in cattle, livestock workers as well as identifies risk factors associated with the infection in Oyo State.

A cross-sectional study was conducted among cattle and livestock workers in the five local government areas (LGA) in Ibadan metropolis, Iwajowa, Ibarapa North, Kajola, Oyo West and Akinyele LGAs. These sites are characterised by cattle population and dairy activities.Fresh milk from 269 pastoral cattle; 295 cheese "*wara"*; 150 fermented milk "*nunu"*; 124 nasal secretions and 124 faecal samples of slaughtered cattle; with 93 sputum samples from livestock workers collected by multistage sampling were analysed for the presence of *Mycobacterium* species using conventional culture method, multiplex polymerase chain reaction (PCR) and restriction enzyme analysis-*hsp*65. Furthermore, a survey to investigate the knowledge, attitude and practices with regards to BTB using pre-tested structured questionnaires was conducted on 124 livestock workers (Herdsmen = 42, Cattle traders = 42 and Butchers = 40) via multistage sampling technique. Data were analysed using chi square and odds ratio at P= 0.05 level of significance.

Mycobacterium species were isolated from 8.3%, 0.3%, 5.7%, and 1.6% fresh milk samples, cheese, nasal swabs and faecal samples respectively then 2.2% from sputum. Multiplex PCR revealed five strains of *M. africanum* (fresh milk = 2; cheese = 1 and sputum = 2) and a high prevalence (86.8%) of non-tuberculous mycobacteria (NTM): 24 from fresh milk, seven from nasal secretions and two from faeces. Diverse strains of NTM were also obtained (*M. gordonae* =16; *M. fortuitum* =12; *M. senegalense* =8; *M. avium* =1). Significant association was observed between isolation of mycobacteria

and types of sample; with fresh unpasteurised milk being 26 times more likely to have mycobacteria isolated (OR: 26.2; 95% CI 3.5 – 195.7). Livestock workers were knowledgeable about BTB transmission, with their occupation significantly affecting their knowledge. However, most livestock workers (70%) consume unpasteurised milk, 30% of butchers consume raw meat, and all agreed to consumption, selling or sharing of suspected infected animal products to the public.

The isolation of *M. africanum* and predominance of non-tuberculous mycobacteria highlight their significance in the epidemiology of tuberculosis. Also, the isolation of *M. senegalense* an emerging infectious agent in fresh milk and nasal secretion of cattle has been established. Public health enlightenment of livestock workers on risk of consuming unpasteurized milk or milk products, raw or undercooked meat and meat products and precautions when handling infected animals is recommended.

Keywords: Bovine tuberculosis, *Mycobacterium senegalense*, non-tuberculous mycobacteria,*Mycobacterium africanum*, livestock workers.

Word count: 488

DEDICATION

This research work is dedicated to the three great men in my life: my beloved father late Mr Yohanna Azgaku (JP) who believed in the education of the girl child, my late brother Mr Sunday Yohanna Azgaku who supported him all the way and lastly my loving husband Pastor Christopher Ujah Agada who encouraged me to explore my potential.

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CHAPTER ONE

INTRODUCTION

1.1 Introduction

Tuberculosis (TB) is an infectious, chronic and debilitating disease caused by the acid-fast, rod-shaped bacillus *Mycobacterium tuberculosis* which typically affects the lungs (pulmonary TB) but can affect other sites as well (extra-pulmonary TB) (WHO 2001). The disease can be spread via aerosol when people with pulmonary TB expel bacteria through coughing (Zayas *et al*., 2012). In general, a relatively small proportion of people infected with *M. tuberculosis* will develop TB disease (WHO, 2013). The reason for this is because of the immune response inherent in the system. The immune response against TB is very important in the outcome of infection against *M. tuberculosis.* Generally, the immune system is able to react aptly against several infections as in the case of TB; however, in the presence of immunocompromising diseases like human immunodeficiency virus (HIV), the likelihood of developing the disease increases considerably (Hernandez-pando *et al*., 2007).

Tuberculosis persists as a major cause of death worldwide and it's of significant public health concern. It causes ill-health among millions of people each year and ranks as the second leading cause of death from infectious diseases worldwide, after the human immunodeficiency virus (HIV) (WHO, 2011).

In 2012, 8.6 million new cases of TB were estimated (13% co-infected with HIV) and 1.3 million people died from TB, including almost one million deaths among HIV-negative individuals and 300 000 among people who were HIV-positive (WHO, 2013). Although the TB mortality rate has decreased by 45% since 1990 and the world is on track to achieve the global target of a 50% reduction by 2015;the African and European regions are however not on tract (WHO, 2013).

Mycobacterium tuberculosis complex (MTC), a group of genetically very closely related (but distinct) mycobacteria comprises of seven members including *M. tuberculosis, M. bovis, M. africanum, M. microti, M canetti, M. caprae and M. pinnipedi*(Aranaz*et al*., 2003; Cousins *et al*., 2003; Smith*et al*., 2006). Infection of humans with any of the organism of the MTC can result in the disease known as TB(de la Rua-Domenech *et al*., 2006). *M. tuberculosis* is known to account for most cases of human TB; however, in West Africa about 50% of the TB in humans is reported to be caused by *M. africanum*(de Jong, *et al*., 2010a; de Jong *et al*., 2010b) as well as an unknown proportion by *M. bovis* (Acha and Szyfres, 1987). According to the review by de Jong *et al.,* (2010b), the prevalence of *M. africanum* varies from 21% among smear-positive pulmonary TB patients in Senegal, 31% in Mali and Burkina Faso to 66% in Benin based on biochemical speciation. On the other hand, based on molecular genotyping results, the prevalence in Benin is 39% and in Ghana 21%.In Cameroon the prevalence may be declining; though 30 years ago 56% of TB was caused by *M. africanum* based on biochemical speciation (Huet *et al*., 1971), however, a study in 2003 using molecular methods (Niobe-eyangoh *et al*., 2003) reported the prevalence of only 9%. Futhermore, genetic assessment by spoligotyping of 565 Mycobacterium tuberculosis complex strains collected from the Western Region of Cameroon between 2004 and 2005 confirmed the establishment of the "Cameroon family" as the leading cause of tuberculosis in 45.9% of cases and evidenced the rapid quasi extinction of *M. africanum* (Koro Koro*et al*., 2013). The prevalence of *M. africanum* appears highest in Guinea Bissau, where 51% of smear-positive TB was caused by *M. africnum* 2, based on spoligotyping patterns (Källenius *et al*., 1999).In Nigeria; 11.8% of the 60 MTC strains cultured from humans were identified as *M. africanum* (Cadmus *et al*., 2006) and it has been isolated from cervical aspirates of children with tuberculous lymphadenitis as well as from HIV positive individuals(Cadmus *et al*., 2006; Cadmus *et al*., 2009; Cadmus *et al*., 2011).

Mycobacterium bovis,the causative agent of bovine tuberculosis (BTB), has a wide range of target organs (lungs, gastrointestinal tract, mammary gland, kidney and reproductive organs) in bovine and other mammalian hosts (wild animals, domestic animals and humans) (Phillips *et al*., 2002; Rupp *et al*., 2007). The zoonosis caused by *M. bovis* in developed countries is now considered a sporadic disease since control of

the disease in cattle was achieved and pasteurization of dairy products became extensively practiced (Torgerson and Torgerson, 2008; Michel *et al*., 2010). However; in developing countries like Nigeria *M. bovis* infection still constitutes a major public health threat (Ayele *et al*., 2004). *M. bovis* have been isolated from dairy farm workers and tuberculous patients in Ethiopia (Shitaye *et al*., 2007). In Tanzania, Cleaveland *et al*., (2007)reported 10.8% prevalence, indicating that the country is a high BTB risk area.

In Nigeria, the endemic nature of BTB has been established in the cattle population (Alhaji, 1976; Ayanwale, 1989; Shehu, 1992; Cadmus *et al.,* 2010a). The isolation of *M. bovis* along with otherNTM species in fresh and sour milk has been reported as well as across the country (Alhaji, 1976; Shehu 1988; Kolo, 1991; Abubakar, 2007; Cadmus and Adesokan, 2007; Ofukwu, 2008; Okayeto *et al*., 2008). Furthermore, Cadmus *et al* (2006; 2010b) and Damina *et al*.,(2011) have isolated *M. tuberculosis* and *M. africanum* in addition to *M. bovis* from slaughtered cattle with BTB; and also from raw milk of trade and pastoral cattle in parts of Nigeria. Epidemiological studies based on mycobacterial culture in Nigeria revealed approximately 3.9-15% of culture positive isolates of *M. bovis*from patients with pulmonary and extra-pulmonary TB (Idigbe *et al*., 1986; Garba, *et al*., 2004; Mawak *et al*., 2006). However, molecular characterization of MTC strains isolated from humans in Ibadan indicated that 5% were *M. bovis*(Cadmus, 2006). While recent studies among livestock workers in Ibadan has shown evidence of occupational exposure and infection with MTC, two of the seven strains isolated were *M. bovis*(Adesokan *et al*.,2012).

Humans are most often infected with *M. bovis* from infected livestock and this remains a serious public health concern in some countries (Kubica, *et al.*, 2003; Adesokan *et al*., 2012). Additionally, human-to-human transmission of *M. bovis* and the occurrence of multidrug resistant (MDR) *M. bovis* have also been documented, raising infection control concerns in health care settings (van Soolingen *et al.*, 1995; Blazquez*et al*., 1997;Gibson*et al*., 2004; Sunder *et al*., 2009).*M. bovis* has two different routes of transmission: airborne transmission in older patients with pulmonary disease and food-borne exposure in younger patients with extrapulmonary disease, via contaminated unpasteurized dairy products (Hlvasa *et al*., 2008). The link between drinking milk from diseased cows and the development of scrofula, cervical lymph node TB, was

established in mid-19th century when more than half of all cervical lymphadenitis cases in children were caused by *M. bovis*(Ashimwe, 2008). Infection acquired through ingesting of *M. bovis* is more likely to result in non-pulmonary forms of disease. Though uncommon, human TB due to *M. bovis* is still a public health threat to both the medical and veterinary professions and there is the need to maintain careful surveillance (Grange and Yates, 1994; de la Rua-Domenech, 2006).

Apart from the global threat of MTC, non-tuberculous mycobacteria (NTM) are also emerging as significant public health challenge worldwide. Non tuberculous mycobacteria are environmental opportunistic pathogens that are natural inhabitants of the soil (Brooks*et al*., 1984) and drinking water (Falkinham 1980; Falkinham *et al*., 2001). They are emerging pathogens causing opportunistic infections in humans and animals and have been implicated in chronic enteropathies, pulmonary as well as dermatological infections in animals (Falkinham, 2002; Cadmus *et al*., 2010c; Ayana *et al.*, 2013). Nontuberculous mycobacteria have also been associated with a variety of problems in humans including pulmonary, lymph node, skin, soft tissue, skeletal, and disseminated infections as well as nosocomial outbreaks related to inadequate disinfection/sterilization of medical devices (Saiman, 2004; Katoch, 2004; Onyejepu *et al*., 2006; Chimara *et al*., 2008).

Nigeria with a population of about 170 million people is ranked thirteenth among the twenty countries with the highest TB burden in the world and has the second highest HIV/AIDS burden in Africa (WHO, 2012 and 2013). In 2011, a case notification of 47,436; 33,034 and 3,793 of smear positive, smear negative and extrapulmonary cases respectively was reported (WHO, 2012).It relies mostly on smear microscopy for TB diagnosis like every other low income countries of the world (Njoku, 2005) which is not adequate for the diagnosis of TB.

The primary diagnostic test for TB in developing countries including Nigeria is smear microscopy. Though this method is cheap and easy to perform, however, it is greatly limited by its inability to identify the specie of mycobacteria causing the disease. It is important to identify the species so as to distinguish between strict human and zoonotic TB and to initiate an appropriate therapy (Djelouadji*et al*., 2008). The risks of humans contracting bovine tuberculosis (BTB) and other mycobacterial infections abound in

Nigeria. This could be attributed to the close association between farmers and cattle, unhealthy meat processing practices by butchers, consumption of unpasteurized milk and milk products, and poor knowledge of the disease. The circulating MTC and NTM in livestock workers and animals are largely unknown coupled with the increase in reported cases of NTM worldwide and the challenges of HIV/AIDS in developing countries. Confirming the sources of exposure in humans will help to guide the direction of prevention and control of these diseases.

1.2 Justifications for the study:

1. Despite the fact that cultural practices such as close association between farmers and animals, fattening of cattle in close proximity to the homes of farmers, processing of offal from diseased carcasses with bare hands by butchers and the consumption of unpasteurised milk and milk products that can facilitate the transmission of *M. bovis* infection between cattle and humans abound in Nigeria, zoonotic TB have received very little attention.

2. In developed countries, the introductions of milk pasteurization and tuberculin (PPD) positive cattle eradication programmes have greatly reduced the incidence of human and bovine diseases caused by *M. bovis* (Caffrey, 1994).In Nigeria, pasteurization of milk is not enforced; humans are therefore at the risk of contracting the disease through the consumption of unpasteurized milk.

3. Many humans particularly livestock workers, have little knowledge of BTB and lack of knowledge about the disease have been documented to result in delay in care seeking and could consequently result in the further spread of the disease.

1.3 Research questions:

a. Is the milk and milk products of cattle consumed in Oyo state sources of zoonotic tuberculosis infection to humans?
b. What are the incriminating members of the *Mycobacterium tuberculosis* complex found in the milk and milk products sold to humans in Oyo state?

c. Do infected cattle excrete the bacterial in their nasal secretions and feces thereby serving as sources of infection to humans working closely with them?
d. Could the increase in the incidence of human tuberculosis be as a result of lack of knowledge about bovine tuberculosis by humans?

1.4 Aim of the study

The study aimed at determining the prevalence of tuberculosis and characterising the mycobacterial species in cattle, livestock workers as well as identifies risk factors associated with the infection in Oyo State.

1.5Objectives of the study were to:

1. Assess the contamination of milk and milk products consumed in Oyo State by mycobacterial species found in cattle.
2. Determine if *M. bovis* is excreted in nasal secretions and faeces of slaughtered cattle with positive pathological lesions in Bodija Abattoir and the risk factors for the excretion of the organisms.
3. Determine the prevalence of BTB among the occupationally exposed livestock workers/groups comprising: the producers who are the herdsmen, the marketers and the processors (butchers) and assess the degree of their awareness, knowledge and the practices with regards to BTB.

CHAPTER TWO

LITERATURE REVIEW

2.1 Historical background

Tuberculosis (TB) has a long history; it was present before the beginning of recorded history and has left its mark on human creativity, music, art, and literature; and has impacted greatly on the advance of biomedical sciences and healthcare (Dezieck, 2011). Its causative agent, *M.tuberculosis*, may have killed more persons than any other microbial pathogen (Daniel, 2006).Tuberculosis has afflicted humans and animals since ancient times. The disease was described in Italian writing 2000 years or more before Christ was born and was also found to be evident in Neolithic man from various skulls and other bones recovered from different parts of the world (Salo *et al*., 1994).

The disease was prevalent in Egypt and Rome (Zink*et al*., 2003 and Donoghue*et al*., 2004) and known to be present in America before Columbus (Salo*et a*l.,, 1994; Konomi*et al*., 2002 and Sotomayor*et al*., 2004), and also in Borneo beforeany European contact (Donoghue*et al*., 2004). The earliest DNA-based documentation of the presence of the *M. tuberculosis* complex organisms was accomplished in a subchondral articular surface from an extinct long-horned Pleistocene bison from Wyoming, US, which was radiocarbon-datedat 17,870 +/- 230 years before the present (Rothschild*et al*., 2001). The German microbiologist, Robert Koch discovered the causative organism, the tubercle bacillus in 1882; in 1890 he developed the tuberculin test for the diagnosis of the disease. In 1924, a vaccine called Bacillus Calmete Guerin (BCG) for individuals exposed to the disease was developed(Locht, 2010).

Tuberculosis in cattle, which was also called 'Pearl-disease', attracted attention thousands of years ago and the early meat inspection regulations in various countries were concerned with this form of the disease (Salo*et al*., 1994).

The danger of eating meat from TB-infected cattle existed in mosaic laws and the German regulations banning the sale of TB meat (Collins and Grange, 1983).

When Robert Koch unveiled the mystery of TB in March 1882 with the discovery of the tubercle bacillus, he believed that the same organism caused the bovine and human TB. Commenting on the source of the disease in human beings; he (1882) wrote that, "One of these sources and certainly the most important, is the expectorant of individuals with tuberculosis, another is tuberculous of domestic animal, notably cattle". This statement led to considerable increase in interest in the prevention of infection by inspection of meat and heat treatment (pasteurization) of milk (Palmers and Waters, 2011).

2.2 Global TB burden and distribution

Globally, an estimated 9.0 million people developed TB and 1.5 million died from the disease, 360 000 of whom were HIV-positive in 2013 (WHO, 2014). TB is slowly declining each year and it is estimated that 37 million lives were saved between 2000 and 2013 through effective diagnosis and treatment (WHO, 2014). TB is present in all regions of the world and the Global Tuberculosis Report 2014 includes data compiled from 202 countries and territories (WHO, 2014).

Of the estimated 9 million people who developed TB in 2013, more than half (56%) were in the South-East Asia and Western Pacific Regions. A further 25% were in the African Region, which also had the highest rates of cases and deaths relative to the population. India and China alone accounted for 24% and 11% of total cases, respectively (Table 2.1).

An estimated 1.1 million (13%) of the 9 million people who developed TB in 2013 were HIV-positive. The number of people dying from HIV-associated TB has been falling for almost a decade. The African Region accounts for about four out of every five HIV-positive TB cases and TB deaths among people who were HIV positive (Table 2.1).

The 2015 Millennium Development Goal (MDG) of halting and reversing TB incidence has been achieved globally, in all six WHO regions and in most of the 22

high TB burden countries (HBCs). Worldwide, TB incidence fell at an average rate of about 1.5% per year between 2000 and 2013 (WHO, 2014).

Globally, the TB mortality rate fell by an estimated 45% between 1990 and 2013 and the TB prevalence rate fell by 41% during the same period. Progress needs to accelerate to reach the Stop TB Partnership targets of a 50% reduction by 2015 (WHO, 2014).

Two out of six WHO regions have achieved all three 2015 targets for reductions in TB disease burden (incidence, prevalence, mortality): the Region of the Americas and the Western Pacific Region. The South-East Asia Region appears on track to meet all three targets. Incidence, prevalence and mortality rates are all falling in the African, Eastern Mediterranean and European Regions but not fast enough to meet targets (WHO, 2014).The 22 HBCs as presented in Table 2.1 accounted for 82% of all estimated incident cases worldwide.

Table 2.1Estimated epidemiological burden of TB, 2013.

	population	Mortality[b]		HIV-positive TB mortality		Prevalence		Incidence		HIV-positive TB incident cases	
Afghanistan	30 552	13	8.4-16	<0.1	<0.1-0.1	100	54-170	58	51-65	0.2	0.2-0.2
Bangladesh[c]	156 595	80	51-110	0.2	0.1-0.2	630	330-1000	350	310-400	0.4	0.2-0.5
Brazil	200 362	4.4	2.5-6.8	2.1	1.5-2.7	110	54-200	93	83-110	13	13-13
Cambodia	15 135	10	6.3-14	0.6	0.5-0.8	110	91-130	61	55-67	2.3	2.1-2.6
China	1 385 567	41	40-43	0.7	0.2-13	1 300	1 100-1 500	980	910-1 100	4.5	4.3-9.9
DR Congo	67 514	46	22-53	6.4	0.2-2.4	370	190-610	220	200-240	16	9.8-75
Ethiopia	94 101	30	16-47	5.6	3.6-8.0	200	160-240	210	180-260	22	19-32
India[d]	1 252 140	240	150-350	38	31-44	2600	1 800-3 700	2 100	2 000-2 300	120	100-140
Indonesia[e]	249 866	64	36-93	3.9	2.2-6.2	680	340-1 100	460	410-520	15	8.7-20
Kenya	44 354	9.1	5.5-12	9.5	7.5-12	130	69-200	120	120-120	48	47-50
Mozambique	25 834	18	9.4-26	38	27-51	140	78-230	140	110-180	81	64-100
Myanmar	53 259	26	16-38	4.3	3.3-5.3	250	190-320	200	180-220	17	16-18
Nigeria	173 615	160	68-270	85	47-140	570	430-730	590	340-880	140	81-220
Pakistan	182 143	100	45-170	1.0	0.5-1.6	620	520-740	500	370-650	2.6	1.2-3.4
Philippines	98 394	27	25-29	<0.1	<0.1-<0.1	430	380-490	290	269-350	0.3	0.2-0.3
Russia Federation	142 834	17	17-18	1.4	1.0-1.9	160	74-290	130	120-140	7.9	6.9-9.1
South Africa	52 776	25	15-38	64	47-83	380	210-520	450	410-520	270	240-310
Thailand	67 011	8.1	4.9-12	1.9	1.3-2.4	100	48-170	80	71-90	12	10-13
Uganda	37 579	4.1	2.2-6.6	7.2	5.0-9.9	58	32-91	62	56-73	32	29-38
UR Tanzania	49 253	6.0	3.4-8.2	6.1	4.8-7.5	85	45-140	81	77-84	30	29-31
Viet Nam	91 680	17	12-24	2.0	1.2-2.9	150	79-350	130	110-160	9.4	8.0-12
Zimbabwe	14 150	5.7	3.6-7.4	22	11-27	58	33-89	78	67-91	56	48-66
High-burden countries	**4 484 710**	**960**	**810-1 100**	**300**	**250-350**	**9 300**	**8 200-11 000**	**7 400**	**7 100-7 800**	**910**	**820-990**
AFR	927 371	390	300-500	300	250-350	2 300	2 400-3 200	2 600	2 300-2 900	870	790-960
AMR	970 821	14	12-17	6.1	5.5-6.8	370	290-460	280	270 300	32	31-33
EMR	616 906	140	90-210	1.8	1.3-2.4	1 000	880-1 200	750	620-890	5.1	4.0-6.4
EUR	907 053	38	37-39	3.8	3.2-4.4	460	350-590	360	340-370	21	20-22
SEAR	1 855 068	440	330-550	48	42-55	4 500	3 500-5 700	3 400	3 200-3 600	170	150-190
WPR	1 858 410	110	100-120	4.8	3.7-5.9	2 300	2 000-2 500	1 600	1 500-1 700	23	19-26
Global	**7 135 628**	**1 100**	**980-1 300**	**360**	**310-410**	**11 000**	**10 000-13 000**	**9 000**	**8 600-9 400**	**1 100**	**1 000-1 200**

Source: WHO Report 2014

Best estimates are followed by the lower and upper bounds of the 95% uncertainty interval. Numbers in thousands[a]

a Numbers for mortality, prevalence and incidence shown to two significant figures. Totals (HBCs, regional and global) are computed prior to rounding.

b Mortality excludes deaths among HIV-positive TB cases. Deaths among HIV-positive TB cases are classified as HIV deaths according to ICD-10 and are shown separately in this table.

c Estimates of TB disease burden have not been approved by the national TB programme in Bangladesh and a joint reassessment will be undertaken following completion of the prevalence survey planned for 2015.

d Estimates for India have not yet been officially approved by the Ministry of Health & Family Welfare, Government of India, and should therefore be considered provisional.

AFR: WHO Africa Region; AMR: WHO Region of Americas; EMR: WHO Eastern Mediterranean Region; EUR: WHO European Region

SEAR: WHO South East Asia; WPR: WHO Regional Office Western Pacific

Numbers in thousands except where indicated.[a].

2.3 Aetiology of tuberculosis

Tuberculosis is caused by *M. tuberculosis* and any other member of the *M. tuberculosis* complex(MTC); these include *M. tuberculosis*, *M.africanum*, *M. bovis* (along with the *M. bovis*-derived BCGvaccine strains), *M.microti*, *M.bovis* subsp. *caprae* (*M. caprae*), and "*M.tuberculosis* subsp. *canettii"* (Brosch *et al*., 2002;Mostowy *et al.*, 2002). The most common species of *Mycobacterium* associated with TB in humans is *M. tuberculosis* although an unknown proportion of cases are due to *M. bovis* (Cadmus, 2003).

2.3.1. *Mycobacterium tuberculosis*

M. tuberculosis bacilli are straight or slightly curved rods occurring singly and in occasional threads rods and ranging in size from 0.3-0.6 × 1-4 μm. They stain uniformly or irregularly, often showing banded or beaded forms. They are strongly acid-fast and acid-alcohol-fast as demonstrated by Ziehl-Neelsen or fluorochrome procedures. Growth tends to be in serpentine, cordlike masses in which the bacilli show a parallel orientation. Colonies of a virulent form are less compact (Sneath *et al.*, 1986).

On most solid media, *M. tuberculosis* colonies are rough, raised, and thick, with a nodular or wrinkled surface and an irregular thin margin; may become somewhat pigmented (off-white to faint buff or even yellow). Colonies on oleic acid albumin agar are flat, rough, corded, dry and usually non-pigmented. In liquid media lacking a dispersing agent, it forms a pellicle which, with age, becomes thick and wrinkled. In Dubos' Tween albumin medium, growth is diffuse, settling if undisturbed, but readily dispersed(Sneath *et al.*, 1986).

Generation time for *M. tuberculosis* in vitro under optimal conditions is 14-15 hours. Optimum temperature for growth is 37^0C, though some grow at 30-40^0C. Optimum pH is 6.4-7.0. Its growth is stimulated by incubation in air with 5-10% added CO_2 and by inclusion of glycerol to 0.5% in the medium. Bacilli grown under highly aerobic conditions die rapidly on abrupt shift to anaerobiosis; when allowed to grow and settle slowly through a self-generated oxygen gradient, they adapt a tolerance to oxygen deprivation, and exhibit synchronised growth on resuspension (Wayne and Lin, 1982).

Strain-to-strain differences in tubercle bacilli have been demonstrated by their different phage susceptibility patterns, but three major patterns are recognised (Bates and Mitchison, 1969; Rado *et al*., 1975).

M. tuberculosis produces TB in man, other primates, dogs and some other animals which have contact with man. Experimentally, from inoculums of 0.01 mg, it is highly pathogenic for guinea pigs and hamsters, but relatively non-pathogenic for rabbits, cats, goats, bovine animals or domestic fowls. Inocula of 0.001 mg are used to produce experimental disease in mice. Attenuation of virulence may occur spontaneously upon subculture in artificial media. Virulence can be maintained by selection of appropriate portions of growth on suitable media or by animal passage. Strains of *M. tuberculosis* isolated from patients from southern India may cause localised lesions in guinea pigs and these tend to regress. These strains produce catalase and are susceptible to both hydrogen peroxide and to isoniazid (Mitchison *et al*., 1963).

Streptomycin, p-aminosalicylic acid, isoniazid, ethambutol, rifampin and some other secondary drugs are used to treat TB. Spontaneous mutants resistant to one of these drugs may replace the parent strain if treatment is improper. Resistance to isoniazid is frequently accompanied by changes in other properties, such as loss of peroxidase and catalase activity and attenuation of virulence for guinea pigs (Middlebrook and Cohn, 1953; Middlebrook, 1954).

2.3.2. *Mycobacterium microti*

These organisms are rods and primary culture can grow on glycerol-free egg media in 28-60 days. They may adapt to tolerance to glycerol, their colonial morphology is variable and the optimum temperature for growth is 37^{o}C.

M. microti is the cause of naturally acquired generalised TB in the vole (Cavanagh*et al*., 2002). However, an increasing number of cases have also been reported in domestic and wild mammals (Rodriguez-Campos *et al*., 2014), such as cats (Rüfenacht *et al*., 2011; Xavier Emmanuel *et al*., 2007), pigs (Taylor *et al*., 2006) European wild boar (Sus scrofa) (Cavanagh *et al*., 2002),ferrets (Mustela putorius), badgers (Meles meles) (Xavier Emmanuel *et al*., 2007), New World camelids (Lama glama and Vicugna pacos) (Xavier

Emmanuel *et al*., 2007), squirrel monkeys (Saimiri sciureus) (Henrich *et al*., 2007), meerkats (Suricata suricatta) (Palgrave *et al*., 2012), and a dog (Deforges *et al*., 2004). Until now, 27 cases of M. microti infection in both immunocompetent (Niemann *et al*., 2000) and immunocompromised human patients (Horstkotte *et al*., 2001) have been described (Panteix *et al*., 2010), demonstrating its capacity for causing clinical illness and thus for being a potential zoonotic agent (Michele*et al*., 2015).

2.3.3. *Mycobacterium africanum*

M. africanum are rods with an average length of 3µm. When grown on egg medium at 37^{o}C, colonies are flat, dull and rough. Sodium pyruvate stimulates growth in egg medium. Growth is homogeneous in Dubo's medium with Tween 80, and granular in Youman's medium with bovine serum. Colonies in Lebek agar extend 15mm below the surface of the medium (OIE, 2008).

M. africanum was first isolated from sputum of a TB patient in Senegal and is a cause of human TB in tropical Africa. In guinea pigs 0.01 and 1 mg injected subcutaneously exhibit irregular pathogenicity, of lower order than *M. tuberculosis* of normal virulence. Generalised lesions are seen by the third month. It produces limited virulence on intravenous injection of 0.01 mg to rabbits (Castets *et al*., 1968, 1969).

David *et al*. (1978) reported that a large series of African strains exhibited more phenotypic heterogeneity than strains of *M. tuberculosis* and *M. bovis* from other areas, but noted that individual strains of *M. africanum* clustered with one or the other of these two species. They also noted that sub clustering behaviour reflected the geographic region within Africa from where they were isolated.

2.3.4. *Mycobacterium bovis*

M. bovis is commonly known as bovine tubercle bacillus and was first distinguished from other mycobacteria through the work of Smith (1898) and Koch (1901).

They are short to moderately long rods. On primary isolation, growth is very poor on glycerol-containing media, although repeated subculture permits adaptation to growth on such media (Beste*et al*., 2009). Furthermore, freshly isolated cultures of *M. bovis* are

microaerophilic; inocula dispersed into liquid, semisolid or solid agar media grow in the medium but not on the surface, as distinguished from *M. tuberculosis* which is highly aerobic (Schmiedel and Gerloff, 1965). On repeated subculture, *M. bovis* will adapt to aerobic growth. Dilute inocula on egg media yield small, rounded, white colonies, with irregular edges and a granular surface after 21 days or more of incubation at 37^{o}C. Colonies on transparent oleic acid albumin agar are thin, flat, generally corded; not easily emulsified in absence of a detergent (Cernoch*et al*., 1994).

Strains usually lose catalase on acquiring resistance to isoniazid. Originally isolated from tubercles in cattle; generally more pathogenic for animals than is *M. tuberculosis*. *M. bovis* is the primary aetiological agent of bovine tuberculosis. Smith (1898) clearly differentiated it from other types of tubercle bacilli. It has a wider range of pathogenicity for different animal species than any of the other species in the genus (Rich, 1951).

It produces TB in cattle, both domestic and wild ruminants, man and other primates, carnivores including dogs and cats, swine, parrots and possibly some birds of prey (Cernoch*et al*., 1994). Experimentally, it is highly pathogenic for rabbits, guinea pigs and calves; at least moderately pathogenic for hamsters and mice; slightly pathogenic for dogs, cats, horses and rats; not pathogenic for most fowl. Loss of virulence for guinea pigs and rabbits and loss of catalase activity accompany a loss of sensitivity to isoniazid as for *M.tuberculosis* (Cernoch*et al*., 1994).

Characteristics of *Mycobacterium bovis*

M. bovis is a slow growing non-photochromogenic acid-fast bacillus. In general, the bovine tubercle bacillus is short (1-3um long) relatively plump and not as beaded as the human type but shows solid staining. *M. bovis* is Gram positive while its cell wall composition is similar to Gram-negative organisms (Kotani *et al.*, 1959). The wax D-component which plays a decisive role in inducing delayed type hypersensitivity of *M.bovis* differs from that of *M. tuberculosis* in lacking peptides, a fact which apparently makes it unsuitable for use as an adjuvant (Alhaji, 1976). English workers frequently use the terms eugonic (heaped up growth indicates an aerobe) and dysgonic (a flat sheet of growth indicates a microaerophil) in referring to the ease with which mammalian bacilli

can be cultivated, the first term being equivalent in general to the human and the latter to the bovine type. The human type develops a little faster than the bovine on cultures (Hagan and Bruner, 1951a). *M. bovis* grows slowly on solid or liquid media than *M. tuberculosis*, especially on primary isolation.*M. bovis* can be differentiated from the other species of the genus *Mycobacterium* based on series of biochemical tests. It is microaerophilic, nitrate reduction negative or weakly positive, niacin production negative, weakly catalase positive, susceptible to thiophen 2-carboxylic acid hydrazide (5mg/1) and resistant to pyrazinamide (60mg/1) (Cernoch*et al*., 1994).

Inclusion of a dye, usually malachite green, in egg-based media enables colonies of mycobacteria to be more readily seen against a contrasting background and also tends to inhibit the growth of certain contaminants in primary cultures (OIE, 2008). The growth of *M. bovis* is inhibited by glycerol. Theobald Smith (1905) as cited by Hagan and Bruner (1951b) early pointed out a growth character by which the mammalian strains generally may be differentiated from each other. This depends upon the fact that the human type utilises glycerol very actively whereas utilisation by the bovine type is limited. When cultivated upon a broth containing 3% glycerol, human types produce enough acid to maintain a terminal acidity, whereas bovine types leave the medium alkaline. Smith tested his fully developed cultures with phenolphthalein. When the reaction is acid, there is a strong probability that the stain belongs to the human type; when alkaline, it is of the bovine type. When the original glycerol content of the medium is below 1%, both typeswill produce an alkaline terminal reaction (Smith, 1905 as cited by Hagan and Bruner, 1951b). An egg medium with pyruvate replacing glycerol is favourable for growth, but the bacilli can be grown either on egg or agar base medium. The colonial morphology of *M. bovis* varies with the medium for instance, primary colonies on Middlebrook's 7 H – 10 medium, are colourless, flat, irregular, rough and dull, while on Stonebrink's medium they are white, moist and convex resembling those of *M. avium*(OIE, 2008).

An important property of virulent bovine tubercle is their ability to form cords when grown in liquid culture medium. These cords are consistently demonstrated in smears. Lipids or lipid complexes present in the cell wall of virulent tubercle bacilli appear to

contribute to the formation of these "rope-like" cords, which represent bacilli arranged in parallel form (Thoen and Bloom, 1995). Cord factor, a glycolipid, extracted with petroleum ether from viable tubercle bacilli, identified as trehalose-6,6 dimycolate does not induce tuberculin sensitivity, but it does inhibit the migration of leukocytes and is reportedly leukotoxic (Noll *et al*., 1956).

2.4 Bovine tuberculosis (BTB)

Bovine tuberculosis (BTB) is an infectious disease of cattle caused by *M.bovis* and is characterized by the formation of tubercles in any tissue/organ of the animal. It is zoonotic, being transmitted to humans by an aerogenous route and/or through consumption of infected milk and other cattle products.

BTB is a disease of both economic and zoonotic importance and has resulted in the adoption of country-wide control programmes in developed countries. Developing countries, in contrast, know little of the disease's prevalence, particularly as the contribution of BTB to human TB has received limited attention. Major constraints, including lack of resources to study the disease pattern and implement control measures, have led to the current situation in Africa(Sharp andDaborn, 1995).

The disease is a chronic contagious respiratory disease of cattle which spreads horizontally within and between species, by aerosol and ingestion (O'Reilly and Daborn, 1995). It is occurring in almost all developed and developing nations of the world. The incidence of the disease is not only higher in the developing nations but also in the absence of any national control and eradication program. It is also increasing worldwide particularly in the Asian, African and Latin American countries (Bonsu *et al*., 2001).*M. bovis*, the cause of BTB and *M. tuberculosis*, the cause of classical human TB, are genetically and antigenically very similar and cause identical clinical disease in humans (Dankner *et al*., 1993). There is considerable and continuing public health significance of *M. bovis* infection in humans and animals and the disease has emerged as a major zoonotic problem in many African countries (WHO, 1994a). In humans, *M. bovis* is the major cause of extra-pulmonary TB like TB of gastrointestinal tract and TB of cervical and mesenteric lymph nodes, the peritoneum, and the genito-urinary tract (Bonsu *et al*., 2001;

Dankner *et al*., 1993). In countries where bovine milk is not pasteurized before consumption, BTB has emerged as the single major cause of extra-pulmonary human TB.

In developed countries, BTB in animals is a rarity with occasional severe occurrences in small groups of herds. However, in 46% of African, 44% of Asian and 35% of the South American and the Caribbean countries, sporadic occurrences and (particularly in Africa 11%) enzootic occurrences of BTB have been reported (Cosivi *et al*., 1998). The actual impact of animal BTB on human health is generally considered low in developed and developing countries, which may be based on the rare identification of *M. bovis* isolates from human patients (Amanfu, 2006). In addition, the occurrence of BTB due to *M. bovis* in humans is difficult to determine accurately because of technical problems in isolating the micro-organism (Collins and Grange, 1983). In most advanced countries of the world, the disease in farmed animals is now relatively well controlled and supplementary precautions of regulated meat inspection and milk pasteurization have minimized the risk of human infection from *M. bovis*. Where human tuberculosis caused by *M. bovis* is encountered in countries of the developed world, it is relatively rare and estimated to be at around <1% of all tuberculosis cases (Grange, 2001). In such instances, infection is often seen in the elderly, who have or have had agricultural associations, and disease has probably arisen from reactivation of dormant lesions. Aside from the farming community, abattoir workers are occupationally amongst those at highest risk, potentially contracting infection from aerosols generated through handling carcasses from infected cattle, resulting in pulmonary tuberculosis or more severe non-pulmonary manifestations following dissemination. Meat handlers are also prone to accidental *M. bovis* inoculation through the skin, resulting in self-limiting lesions known as Butcher's Wart (Neil *et al*., 2005).

There is now a greater awareness of zoonotic risks amongst these occupational groups, as there is amongst other risk groups such as veterinarians and zoo employees. Definitive statements about the public health risk from BTB in developing countries cannot be made with such confidence. The precise epidemiology of this disease in animal and human populations in these countries has not been established and the contribution of *M. bovis* to human TB in most instances therefore remains largely unknown (Neil *et al*., 2005).

However, the correlation between the prevalence of *M. bovis* infection in humans and in local cattle populations in Africa highlights the potential threat of BTB to humans in such countries (Daborn *et al*., 1996). Currently, the BTB in humans is becoming increasingly important in developing countries, as humans and animals are sharing the same micro-environment and dwelling premises, especially in rural areas (Amanfu, 2006). Prevalence data on BTB infection in Africa is scarce;though there is sufficient evidence to indicate that it is widely distributed in almost all African countries and is even found at high prevalence in some animal populations (WHO, 1994b; Ayele *et al*., 2004; Zinsstag *et al*., 2006a). Thus BTB is still a great concern in many developing countries and in Nigeria, the endemic nature of human TB has long been documented (Cadmus *et al*., 2010a; Cadmus *et al*., 2010b).

2.5. Epidemiology of Bovine tuberculosis

Environmental and animal reservoirs of *M. bovis*

M. bovis is considered to be an obligate intracellular pathogen whose most efficient way of infection is direct animal contact (Pollock and Neil, 2002). However, experimental evidence has shown that *M. bovis* can survive for long periods outside an animal host in an environment directly or indirectly contaminated by discharges of infected animals, suggesting other possible ways of transmission (Biet *et al*., 2005).

Yet in cattle, the natural host of *M. bovis* and the main source of human spread, transmissionvia the oral route or even the respiratory route by inhalation of dust particles in fields where no wildlife reservoir are implicated in transmission to livestock, would play a less important role since the excretion of the organisms in faeces even from heavily infected cattle occurs irregularly and at a low frequency (Menzies and Neil, 2000). There are no records of human infection by *M. bovis* coming from a direct environmental source, revealing that this way of transmission is not the most important one for this pathogen.

Physiological characteristicsfor environmental survival

The success of tubercle bacilli as pathogenscomes mainly from its ability to persistin the host for long periods and cause diseaseby overcoming host immune responses(Flynn and Chan, 2001). Nevertheless, the possibilityof surviving for long periods in theenvironment

is explained by the mycobacterialimpermeable cell wall (Brennan and Nikaido, 1995) and slowgrowth (Gonzalez-y-Merchand *et al*., 1997). In contrast, other featuresrender these species more sensitive to environmentalsurvival, like a more enhancedpH sensitivity of the *M. tuberculosis* complex(MTC) compared to *Mycobacterium avium-intracellulare* complex (MAC) species (Chapman and Bernard 1962; Cotter and Hill, 2003). Genomiccomparisons between MAC and MTC members will not only allow explainingdifferences in virulencedeterminants between these two mycobacterialcomplexes but also the disparities inenvironmental survival factors.

Animal reservoir:**Wildlife as a source of *M. bovis***

Domestic and non-domestic animals may be considered either as maintenance (or reservoir) hosts or non-maintenance (orspill-over) hosts for BTB.In reservoir host species, infectioncan persist through horizontal transferin the absence of any other source of*M. bovis* and may as well be transmitted tothe susceptible hosts. In contrast, spill-overhosts become infected with *M. bovis* butthe infection only occurs sporadically orpersists within these populations if a truemaintenance host is present in the ecosystem.If the source of infection is removed,the prevalence for this disease is reducedand it can only be maintained in the longterm by re-infection from another source(Haydon *et al*., 2002).

A main trait of *M. bovis* is its broad hostrange, actually the largest of any member ofthe MTC. *M. bovis* causesdisease in a wide range of domestic but alsofree-range and farmed wildlife animals aswell as in humans (de Lisle *et al*., 2002). Only a small proportionof these animal species that become infectedcan act as maintenance hosts of this organism.Table 2.2 is a non-exhaustive list summarizing two reviews byG.W. de Lisle (de Lisle *et al*., 2001; de Lisle *et al*., 2002), which describes*M. bovis* inreservoirs and spill-over wildlife species aswell as their distribution.

Table2.2 List showing the distribution of *M. bovis* in wildlife host

Species	Epidemiological status	Route of transmission	Countries
African buffalo (*Syncerus caffer*)	Maintenance host	Respiratory	Uganda
Baboon (*Papio ursinus*)	Spill over	Oral/respiratory	Kenya
Badger (*Meles meles*)	Maintenance host	Respiratory	Ireland/England
Bison (*Bison bison*)	Maintenance host	Respiratory	United States of America/Canada
Black bear (Ursus americanus)	Spill over	Oral	United States of America
Bobcat (*Felis rufus*)	Spill over	Oral	United States of Ameeica
Brushtail possum (*Trichosurus vulpecula*)	Maintenance host	Respiratory	New Zealand
Cheetah (*Anisonyx jubatus*)	Spill over	Oral/respiratory	South Africa
Coyote (*Canis latrans*)	Spill over	Oral	United States of America
Deer (*Cervus elaphus*)	Maintenance host/spill over	Respiratory/oral	New Zealand
Feral pig (*Suis scrofa*)	Maintenance host/spill over	Oral	Italy, Spain, Australia, Hawaii, New Zealand
Ferret (*Mustela putorius*)	Maintenance host/spill	Oral	New Zealand
Greater kudu (*Tragelaphus strepsciceros*)	Spill over	Scarification/oral	South Africa
Leopard (*Panthera pardus*)	Spill over	Oral/respiratory	South Africa
Lion (Panthera leo)	Spill over	Oral/respiratory	South Africa
Raccoon (*Procyon lotor*)	Spill over	Oral	United States of America
Red fox (*Vulpes vulpes*)	Spill over	Oral	England/United States of America
Warthog (*Pahcochoerus aethiopicus*)	Spill over	Oral/respiratory	Uganda
White-tailed deer (*Odocoileus virginianus*)	Spill over	Oral/respiratory	United States of America, Canada

Source: Biet *et al.*,2005.

It is worth investigating which factors render species as a maintenance host. Physiopathogenesis of BTB,that is the capacity of excretion,ethology (for example gregarious or not gregariousbehaviour) and ecology (alimentarybehaviour, population density and interactionswith other species) determine their capability to participate in a particular biotope as an *M. bovis* reservoir (Biet *et al*., 2005).

Physiological characteristics for host adaptation

Although the course of infection, clinical signs and development of disease can vary within different host species, it can be presumed that certain essential physiological characteristics are common for successful infection in any susceptible host. The analysis of the complete genome sequence of *M. bovis*(Garnier *et al*., 2003) provides a means to dissect these characteristics.

To begin with, the cell wall protects the bacteria from harsh environments but also promotes intracellular persistence (Brennan and Nikaido, 1995).The ability to infect and persist in the macrophage by inhibiting phagosome-lysosome fusion, creating a privileged compartment and remaining sequestered away from the terminal endocytic organelles, is central to the success of the pathogen (Pieters, 2003). The presence of acidic, glycine-rich proteins (PE and PPE families) also found in *M. leprae* (Cole *et al*., 2001)and *M. marinum* (Ramakrishnan *et al*., 2000) whose genes are involved in virulence are worth mentioning. Another important genetic factor implicated in the attenuation of the *M. bovis* BCG strainis the lack of the RD1 locus (Ramakrishnan *et al*., 2000), which isinvolved in a novel described secretion system(Ramakrishnan *et al*., 2000).

Latency is another important aspect oftubercle bacilli pathogenesis. The molecularbasis for the persistence phenotype andthe pertinent host immune mechanisms thatcontribute to the maintenance of tuberculosislatency are just beginning to be understood.The bacillus releases peripheral cell walllipids into their host cells, which inducethe granulomatous response (Smith, 2003). This representsactive manipulation of the host's responseto ensure the maintenance of the infection (Smith, 2003).

The granuloma appears as a balance structurethat walls off the infection and limitsits metastasis. However, the very prisonthat limits spread could well restrict thecapacity of the host to activate the macrophagesrequired to kill the bacteria (Russell, 2003).

Spread of bovine tuberculosis in domestic livestock

Within domesticated animals, cattle, farmed buffalo and goats are consideredreservoir hosts of *M. bovis*, while pigs, cats, dogs, horses and sheep are considered spill-over hosts(Cousins*et al*., 2003; Biet *et al*., 2005).The realisation that wildlife is infected with*M. bovis* may result in apparent failure programmesto eradicate the infection fromcattle (Delahay *et al*., 2002). Knowledge of wildlife tuberculosisthrough appropriate surveillance programmesin feral animal populations maybe important in the research strategies forthe total elimination of livestock tuberculosis.

2.6. Transmission of bovine tuberculosis

Tuberculosis is primarily a respiratory disease but it can also spread to other parts of the body. The primary route of transmission of infection within and between species is by the airborne route and is facilitated by close, prolonged contact between infected and healthy humans or animals through the exchange of respiratory secretions (O'Reilly and Daborn 1995; Kempf *et al.*,2005). However, other routes of transmission such as congenital and vertical transmission have been recorded (Collins and Grange 1983; Neil *et al*., 1994). Transmission of TB can be animal-to-animal, animal-to-human, human-to-animal as well as human-to-human (Collins and Grange, 1983).

2.6.1. Animal to animal transmission of bovine tuberculosis

In cattle as well as in other animal hosts, the route of transmission of *M. bovis* can be deduced by the pattern of lesions observed in slaughtered animals. Animals with lesions restricted to the thoracic cavity are presumed to have been infected by the inhalation of aerosols, while those with lesions in mesenteric lymph nodes are thought to have acquired the infection by ingestion (Pollock and Neil, 2002). In cattle on extensive system of management, the majorities of lesions are found in the upper and lower respiratory tract and associated lymph nodes. Thus, it is considered that the inhalation of *M. bovis* is the most probable route of infection (Neil *et al*., 1994). In fact, the development of TB lesions

which invade the airways is thought to be required to facilitate active excretion and aerosol spread of *M. bovis* (Menzies and Neil, 2000).

Respiratory excretion and inhalation of *M. bovis* is considered to be the main route through which cattle-to-cattle transmission occurs in bovines (Neil *et al*., 1988). Droplets of contaminated water, eructation while ruminating, infected pastures, or inhalation of contaminated dust particles can also be an alternative way of aerogenous infection (Neil *et al*., 1994). This is, in fact, suspected to be the most likely way cattle could get infected in a contaminated environment by badger excretions (Phillips *et al*., 2003). Ingestion of *M. bovis* directly from infected animals or from contaminated pastures, water or fomites is considered secondary to respiratory spread, as deduced from the minor presence of mesenteric lesions in cattle cases (Menzies and Neil, 2000). Congenital infections and vertical transmission to calves as well as genital transmission are uncommon in regions where intensive eradication programmes operate.

Intensive livestock farming promotes close contact between animals, favouring the spread of *M. bovis* (Alhaji, 1979; O'Reiley and Daborn 1995; Shirima, 2003). Extensive livestock farming, however, especially transhumance with no housing system, raises the question as to how BTB transmission can take place. Close contact between animals occurs for example at water points such as ponds, wells, and streams. In Africa, grazing animals usually gather at night for protection from predators. Vaccination and artificial insemination centers, dipping tanks, auction stations, market places and transportation are the commonest animal gathering places, and again are sites where transmission of infection could easily occur (Ayele *et al*., 2004). Due to the high ambient temperature in tropical zones, animals tend to concentrate under trees or other shaded areas for parts of the day, preferring to graze early in the morning and late in the afternoon (Ayele *et al*., 2004). Possibly the most dangerous spots for nose-to-nose or mouth-to-mouth contact between animals are salt supplementing points. Therefore, while extensive farming is safer than zero level grazing systems to prevent disease transmission, some of the above situations stimulate the dangers of intensive farming in relation to disease transmission (Ayele *et al*., 2004).

2.6.2. Animal-to-human transmission of bovine tuberculosis

In industrialized countries, the incidence of TB due to *M. bovis* in human is almost at zero level as a result of pasteurisation of milk and milk products and eradication of BTB in cattle population (Radostits, *et al*., 2007). However, in developing countries especially Africa, the disease in animals can be widely distributed in regions where control measures are not applied or are conducted sporadically and pasteurisation is rarely practised (Cosivi *et. al.,* 1998), and hence transmission to humans. The direct correlation between *M. bovis* infection in cattle and disease in the human population has been well documented in industrialized countries as well as in developing countries (Cosivi *et al.*, 1998, Cook *et al*., 1996; Ameni and Erhikum 2007). Pulmonary TB due to *M. bovis* is more common in rural dwellers, as a result of inhalation of dust particles or bacteria-containing aerosols shed by infected animals, while urban dwellers acquire the infection via the gastrointestinal route and develop extra-pulmonary TB (Daborn *et al*., 1996). In countries with a relatively high prevalence of BTB in cattle, abattoir and farm workers are the groups most exposed to infection (Ayele *et al*., 2004).

Infection of humans may occur by the inhalation of aerosols or through the consumption of contaminated milk. The aerosols are the result of animal excretion but can also be produced by handling lesioned carcasses (Neil *et al*., 1988). This route of infection leads to respiratory TB. Current economic and social globalization has created greater opportunities for the spread of zoonotic disease such as TB. When considering the revival of TB in countries previously declared to be free of the disease, it is worth noting the statement by Grange: 'we are now learning the hard way that none are safe until all are safe' (Grange, 1995).An HIV infection result in humans becoming much more susceptible to all forms of TB; and it is estimated that 50% or more of new cases are related to prior HIV infection (Raviglione *et al*., 1995). This not only poses a risk for other humans but also results in cows being exposed to far higher levels of *M. tuberculosis* and other mycobacteria than was previously the case. Mixed infections of different *M. tuberculosis* strains in the same patient have also been demonstrated (Warren *et al*., 2004). The cycle of TB infection between HIV positive workers and cows, has yet to be fully explored.

The primary sources of infection for humans are consumption of unpasteurised milk and close association between humans and animals (Coetzer and Tustin, 2005). Rural inhabitants and some urban dwellers in Africa still consume unpasteurised and soured milk potentially infected with *M. bovis.* Milk-borne infection is the main cause of non-pulmonary TB in areas where bovine TB is common and uncontrolled (Ayele *et al*., 2004). The agro-pastoral system of farming in Africa also exposes the farmer to the mycobacteria which may be present in the faeces excreted by infected animal often used as manure to fertilize the farmlands (Ayele *et al*., 2004). Traditionally in Africa, cows suckle their calves and are milked at the same time. Usually the cow is separated from the calf for about 12 hours, either at night or, more usually, during the day and milked as the calf is returned to her. An infected cow could produce milk containing mycobacteria, or cough infected droplets in the direction of the milker. Milk is seldom pasteurised in pastoral societies, and, even if soured, can still contain infective levels of mycobacteria (Kazwala, 1997; Coetzer and Tustin, 2005). Animals in traditional African farming systems are seldom culled and there is a greater chance for chronic TB in older cows, particularly those subjected to stress (Michel *et al*., 2004).

2.6.3. Human-to-animal transmission

Fritshe *et al*., (2004) reported a scenario of TB in cattle exposed to a patient infected with *M. bovis,* where the strain isolated in the cattle and the patient were identical. The human patient, a 72-year old farmer was reported to have been exposed and contaminated from a farm during childhood. The role of humans in infecting cattle with bovine TB was reviewed by Torning in 1965 (van Soolingen *et al*., 1994). Sjögren and Hillerdal (1978) cited several examples of human-to-cattle transmission, and stressed the potential danger that patients with smear-positive pulmonary TB due to *M. bovis* may pose to animals. However, reports of human infection of cattle are rare (O'Reilly and Daborn, 1995).The genitourinary tract in humans is a site of non-pulmonary TB due to *M. bovis*; genitourinary TB may appear to be of little importance to epidemiologists in studying human infection, but this route of infection from man to cattle had been documented(Grange and Yates, 1994). Grange and Yates reported that farm workers urinating in cowsheds may represent a source of infection for animals (Grange and Yates,

1994). An analogous situation is thought to occur in rural Africa, where patients with genitourinary TB may urinate on pasture; animals craving salt preferentially graze on this grass and may succumb to infection. Due to hand milking, the contact between cow and human is also much closer and there is a good possibility that droplet-mediated transmission of TB could occur – an infected human could exhale contaminated particles into the bucket of milk or at the cow(Grange and Yates, 1994).

2.5.4. Human-to-human transmission

Mycobacterium bovis is pathogenic for humans, but its pathogenicity may be less than that of *M. tuberculosis*.This assumption comes from the findings that there is a very low incidence of human-to-human transmission and the likelihood of endogenous reactivation occurring years or decades after the initial infection (Pasquali, 2004). Person to person spread of *M. bovis* has been recorded, but the relative contribution of this mode of transmission to the overall problem of TB has not been investigated in detail. Human TB caused by *M. bovis* as a result of human-to-human transmission was reported in the Netherlands in 1994 (van Soolingen *et al.*, 1994). Evidence of transmission of *M. bovis* between humans is considered rare and largely anecdotal, and the rate of transmission seems insignificant compared to animal-to-animal or animal-to-human infection (O' Reilly and Daborn, 1995). Though this mode of transmission is considered less efficient than that of *M. tuberculosis* (van Soolingen, 2001), transmission amongHIV-infected humans, where immunosuppression increases the susceptibility of the host to infection, may be different. *M. bovis* has been isolated from HIV-infected individuals in some industrialised countries, with an additional serious complication of high primary resistance to isoniazid, streptomycin and pyrazinamide (Guerrero *et al*., 1997).In France, there was a report of human-human transmission of TB caused by *M. bovis* in immunocompetent patients (Sunder *et al*., 2009).

2.7. Distribution and global situation of bovine tuberculosis.

2.7.1 Prevalence and distribution in the human population

In the period 1986–2005, several other national surveys on BTB in humans were published, confirming certain stability in its incidence. In France, in 2000-2005, *M. bovis*TB cases accounted for 2.2% of all mycobacterial isolates (Mignard*et al*., 2006). In

the Australian population, during 1970–1994, *M. bovis* was isolated from around 1% of human TB cases (Cousins and Dawson, 1999), while in Madagascar, a survey performed in urban areas found prevalence of *M. bovis* cases limited to 1.3% of pulmonary and extrapulmonary TB cases (Rasolofo-Razanamparany *et al*., 1999). Australian investigators, based on the frequency of BTB cases diagnosed, had suggested in 1989 that BTB should be considered an occupational hazard in abattoir workers, where the disease is transmitted by inhalation causing pulmonary TB (Robinson *et al*., 1988).

In 1988 investigators from Quebec, Canada, published a case report entitled: ‘‘*Mycobacterium bovis* sepsis in an infant with human immunodeficiency virus infection’’. At least on the basis of the present search list, the article was the first one that reported *M. bovis* in an HIV-infected patient (Houde and Dery, 1988). In the 1990s and 2000s, main causes of concern related to human *M. bovis* in industrialized countries have been:

(a) The reemergence of this infection in humans carried by immigrants from regions where BTB is still prevalent (Dankner *et al*., 1993; Dankner and Davis, 2000; LoBue *et al*., 2003; Rodwell *et al*., 2010). Analysis of *M. bovis* disease by age and ethnic group, and further supported by classical and molecular epidemiology studies performed in the United States and Mexico, linked the origin of these infections to ingestion of raw-milk products, such as soft cheeses, from Mexican dairy herds (Dankner *et al*., 1993; Dankner and Davis, 2000; Milian *et al*., 2000; Besser *et al*., 2001; LoBue *et al*., 2003). Several of these studies also found an association between human *M. bovis* disease and HIV co-infection(LoBue *et al*., 2003; CDC 2005; LoBue and Moser, 2005; Hlavsa *et al*., 2008; Rodwell *et al*., 2008). With Mexican immigration beyond the Border States (California, Arizona, Texas and New Mexico) increasing dramatically in recent years, the potential for finding cases of human *M. bovis* in other parts of the country has also increased. This is demonstrated by a report from New York City of 35 cases of human *M. bovis* disease detected between 2001 and 2004, almost all of whom were adults born abroad (primarily Mexico) or children of Mexican-born parents (Winter *et al*., 2005). Most of these cases were associated with ingestion of unpasteurized soft cheeses imported from Mexico.

(b) Occurrence of *M. bovis* disease by reactivation or primary infection in HIV-infected patients, including, outbreaks of multidrug-resistant (MDR) *M. bovis* strains among hospitalized HIV-infected patients (Blazquez *et al*., 1997; Samper *et al*., 1997; Rivero *et al*., 2001; Hughes *et al*., 2003). Transmission from patients with infectious pulmonary *M. bovis* disease to immune-competent contacts also appears to occur (LoBue *et al*., 2004a, b).

(c) Epizootics in domesticated and wild mammals that could transmit the infection to man (Fanning and Edwards, 1991; Liss *et al*., 1994).

(d) Airborne acquired *M. bovis* infection in animal keepers and meat industry workers (Thompson *et al*., 1993).

Two mycobacterial species related to *M. bovis* were described in the 1990s. One of these was primarily cultured from goats (*M. caprae*), identified in Spain, Germany and Austria. In Germany: it was found to be responsible for one-third of human *M. bovis* associated TB cases (Gutierrez *et al*., 1997; Aranaz *et al*., 1999; Kubica *et al*., 2003). The other new species described was originally isolated from seals (*M. pinnipedii*) in Australia, Argentina and Uruguay. Seals appeared to be the natural host, although these bacteria were identified as the cause of disease in humans (Cousins *et al*., 2003).

2.7.2. Prevalence and global distribution of bovine tuberculosis in cattle

Data on animal TB prevalence is generally scarce asit is difficult to measure the precise incidence of the disease in some countries and only qualitative assessment often not supported by laboratory or field investigations, are submitted (Amanfu, 2006; Banoo *et al*., 2010).Official data (2004) reported by member countries of the OIE are summarized by Amanfu (2006) is summarized in table 2.2 below.

Table 2.3. Summary of bovine tuberculosis data reported by member countries of World Organisation for Animal Health (OIE)

Region	Number of countries reported to OIE	Declared TB detection in cattle	Declared absence TB	Do not report on presence or absence of TB
Africa	51	26	7	18
Americas	43	21	19	3
Asia	45	20	20	5
Europe	51	15	35	1
Oceania	19	1	18	0

Source: Amanfu, 2006.

2.8. Bovine tuberculosis andHIV/AIDS

Tuberculosis is a major opportunistic infection in HIV-infected persons (Raviglione *et al*., 1995). Chretien (1990) referred to the combination of HIV-infection and TB as the 'Cursed Duet'. Kitching (1993) also referred it as 'a deadly combination'. As a pathogen of high virulence, TB tends to develop early in the course of HIV infection; usually preceding other AIDS defining conditions (Festenstein & Grange, 1991). And it is one of the very few HIV/AIDS-related diseases that is transmissible to other human beings, whether HIV positive or not and in some cases animals (Daborn and Grange, 1993).

TB in HIV-infection is caused by *M. tuberculosis*; however some cases are due to the bovine tubercle bacillus *M. bovis* which is transmissible from man to animals principally by the aerogenous route, although the majority of cases in man are non-pulmonary (Daborn and Grange, 1993).

BTB (*M. bovis*) infects man, but frequently does not manifest itself clinically until old age, but it was suggested that any host immunological factor that protected humans from developing post-primary disease or preventing man to man transmission would be abrogated in HIV/AIDS patients (Daborn and Grange, 1993; Grange 1995). In developing countries where there is an existing pool of BTB in the cattle and wild life population, together with high prevalence of HIV in human population the consequences are likely to be uncontrollable (Kitching, 1993). A high prevalence of the virus in a pastoralist community could establish a complete cycle of transmission of TB involving cattle and their keepers. This, in turn could have a devastating effect on such a community (Daborn and Grange, 1993).

TB cases due to *M. bovis* in HIV positive persons also resemble disease caused by *M. tuberculosis*. They manifest as pulmonary diseases, lymphadenopathy, or more profoundlyin the immunosuppressed a disseminated disease (Cosivi *et al*., 1998).*M.bovi*have been isolated from HIV-infected persons in industrialized countries. In France, *M. bovis* infection accounted for 1.6% of TB cases in HIV- positive patients and all isolated strains were resistant to isoniazid (Dupon and Ragnaud, 1992). While in San Diego; California; one of 24 adults with pulmonary TB and 11 of 24 adults with non-pulmonary TB due to *M. bovis* had AIDS. And one of 25 children; a 16 year old boy with

abdominal TB, was HIV- positive (Dankner *et al*., 1993). It has been described at the US/Mexico border. Hospital outbreak of TB by *M. bovis* with clear evidence of human-to-human transmission occurred in Paris (Bouvet *et al.*, 1993).

2.9. Zoonotic tuberculosis and public health

2.9.1. Close physical contact

Close physical contact between humans and potentially infected animal is present in some communities, especially in developing regions. In many African communities cattle are an integral part of human social life; they represent wealth and are at the centre of many events (Ayele *et al*., 2004). Also about 65% of Africans, 70% of Asian, and 26% of Latin American and Caribbean populations are agricultural workers (Cosivi *et al*., 1998) meaning that large population of these regions are exposed to BTB. Animal-to-animal transmission is most commonly by the respiratory routes (de la Rua-Domenech, 2006). Therefore, a potential risk of contracting BTB via respiratory route also exist for humans regularly handling animal reservoirs of *M. bovis* or their carcasses these are: cattle and deer farmers, stockmen, veterinarians, meat inspectors, slaughter house and knacker yard operatives, hunters, game and zoo keepers, TB diagnostic and research laboratory personnel (Ayele *et al*., 2004; de la Rua-Domenech, 2006). There are several reports supporting the view that BTB is an occupational zoonosis in those professionals and that exposure to *M. bovis* aerosols from infected animals is the mechanism for human transmission; as summarized in Table 2.3 below(de la Rua-Domenech, 2006).

Before the eradication of BTB in Great Britain, cases of BTB in man were more prevalent in the rural than the urban areas(de la Rua-Domenech, 2006). A relatively higher prevalence in rural areas was reported in other European counties and was thought to be the result of direct aerogenous infection from diseased cattle (Grange and Yate, 1994). The cutaneous/mucosal transmission is extremely in the industrialized countries however; may be common in the developing nations. It was an occasional source of localized skin, tendon and lymph node lesions, otitis and conjunctivitis in milkers, those who regularly dress carcasses of tuberculous animals and veterinarians exposed during surgical interventions or necropsies (de la Rua-Domenech, 2006).

Table 2.4 Reports in the medical and veterinary literature of zoonotic TB due to presumed occupational exposure to *M. bovis* aerosols from infected cattle and other animals.

Setting	Country	Reference
Three adult men and two women with active pulmonary or pleural TB, all of them were abattoir workers. Evidence of transmission to several contacts.	South Australia	Robinson *et al*., 1988
Outbreak of bacteriologically confirmed pulmonary TB in abattoir workers.	Queensland, Australia	Georghiou *et al*., 1989
Of 150 bacteriologically confirmed cases of human Tb due to *M. bovis* occurring during 1970-1994, 37 had worked in the meat industry, 22 were farm workers and 3 had been employed in laboratories handling tissues specimens for the bovine TB eradication campaign.	Australia	Cousins and Dawson, 1999
TB outbreak with an initial prevalence of skin test reactions of 21% in humans exposed to *M. bovis*-infected elk, nine skin test conversions and one case of active pulmonary disease.	Alberta, Canada	Fanning and Edwards, 1991
High prevalence of skin test reactors (and one conversion) amongst operators of rendering plant and abattoir processing tuberculosis deer from two herds depopulated in Ontario.	Ontario, Canada	Liss *et al*., 1994
Outbreak of *M. bovis* infection in 7 of 24 zookeepers exposed to a tuberculous rhinoceros.	Louisiana, USA	Dalovisio *et al.*, 1992
Of 10 patients diagnosed with zoonotic TB in a hospital between 1986 and 1990, two were young veterinary students.	Barcelona, Spain	Sauret *et al*., 1992
Isolates of a goat-adapted strain of *M. bovis* found in three TB patients linked with goat farming. One of the patients was resident in a rural area where goat farming was common, the second worked in an abattoir, and the third was a veterinarian with a history of contact with infected goat herds.	Spain	Gutierrez *et al*., 1997
High prevalence of tuberculin reactors (45.55%) and clinical TB (4.1%) in veterinarians working with infected herds. It could not be established whether all cases were due to *M. bovis*.	Italy	Moda *et al*., 1996
Bacteriologically confirmed *M. bovis* infection diagnosed in 1999 in two young siblings living on a small beef cattle holding sustaining a bovine TB breakdown. History of infected badgers in the locality. Neither patient had knowingly drunk unpasteurized milk or traveled abroad. One of them would assist when cattle were restrained for veterinary examination or testing. The second case may have resulted from intrafamilial spread	Gloucestershire, England	Smith *et al.*, 2004

Culled from de la Rua-Domenech, 2006.

2.9.2. Food hygiene practices

Milk contaminated by *M. bovis* has been regarded for a long time as a source of infection of TB from animal to humans (Acha and Szyfres, 1987). In developing countries where BTB is common and uncontrolled; milk borne infection is the principal cause of cervical lymphadenopathy (scrofula), with abdominal and other forms of non-pulmonary tuberculosis (Cosivi *et al*., 1998). Food hygiene practices could play a major role in controlling these forms of TB, but it is difficult to institute in the developing countries.

Of the total milk produced in sub Saharan Africa 90% is consumed fresh (Walshe *et al*., 1991; Abubakar *et al*., 2011). Even though it has been stated that Africans generally boil milk and that souring process destroy *M. bovis* (Walshe, *et al*., 1991), other sources contradict these statements (Barrera and de Kantor, 1987). *Mycobacterium bovis* was isolated from seven (2.9%) of 241 samples of raw milk in Ethiopia (WHO, 1994). Both *M. bovis* and *M. tuberculosis* have also been found in milk samples in Nigeria (ldrisu and Schnurrenberge, 1977;Abubakar, 2007; Ofukwu *et al*., 2008; Okayeto*et al*., 2008;Cadmus *et al*., 2010a) and in Egypt (El-Sabban*et al*.,1992). Therefore serious public health implication of potentially contaminated milk and milk products cannot be under estimated. Thus, the consumption of unpasteurized cows' milk poses a public health risk, in relation not only to *M. bovis* but too many zoonotic infections as well (de la Rua-Domenech, 2006).

Even though the main source of non-pulmonary TB is ingestion of contaminated raw milk and associated products, meat from infected cattle containing viable *M. bovis* can serve as a source of infection especially when eaten raw (Moda *et al*., 1996; Hambolu *et al*., 2013). Transmission of *M. bovis* to humans through the consumption of meat has not been documented as a public health risk during surveillance for TB in many developed countries over many decades (Anon, 2003). This is because TB lesions in the skeletal muscles are very rare and observed in animals with advanced infection which could be detected during routine tuberculin screening and during postmortem examination. However, the risk posed by consumption of undercooked meat of tuberculous animals may be marginally greater in developing countries, where *M. bovis* infection in animals

are prevalent but veterinary controls (including meat inspection) are only sporadically applied (Cosivi *et al*., 1998; Ayele *et al*., 2004).

2.10. Bovine tuberculosis: the status in Nigeria

Nigeria has a population of about 173 million peopleand is rated thirtheenth amongst the TB burdened nations of the world (WHO, 2014).The case notificationin 2013 was: 52,811 bacteriologically confirmed pulmonary TB; 33,873 clinically confirmed cases of pulmonary TB in addition to 5,313 extrapulmonary TB (WHO, 2014).She has the second highest HIV/AIDS burden in Africa (WHO, 2012 and 2013). Details of the estimates of the burden of the disease in the country in 1990-2013 are given in table 1.4 below. Though total prevalence is declining every year, the high number (5,313) of extrapulmonary TB cases reported in 2013 is disturbing (WHO, 2014).This is because70% of the country's work force is in the agricultural sector (Cadmus *et al.*, 2004) and it has been reported that in countries where BTB is endemic in the cattle population and surveillance and control are not enforced, 15% of cases of tuberculosis in humans may be caused by *M. bovis* (Ashford*et al*., 2001).

Table 2.5 Estimates of the burden of disease caused by tuberculosis (1990-2013) in Nigeria

		MORTALITY (EXCLUDING HIV)		PREVALENCE (INCLUDING HIV)		INCIDENCE (INCLUDING HIV)	
Year	**Population (Millions)**	**Number (thousands)**	**RATE[a]**	**Number (Thousands)**	**RATE**	**Number (Thousands)**	**RATE**
1990	97	38 (21-58)	39 (22-60)	270 (110-520)	277 (109-531)	130 (109-180)	131 (151-189)
1995	110	60 (48-74)	55 (44-67)	420 (180-690)	378 (163-627)	210 (170-250)	188 (151-226)
2000	125	94 (75-120)	75 (60-82)	660 (290-1100)	527 (233-879)	340 (270-410)	272 (218-326)
2005	141	120 (96-150)	87 (68-108)	880 (390-1400)	621 (279-1026)	460 (370=550)	327 (262-393)
2006	144	120 (94-150)	83 (65-103)	870 9390-1400)	600 (272-989)	460 (370=550)	319 (255-383)
2007	148	120 (91-150)	79 (62-99)	860 (390-1400)	579 (264-949)	460 (370=550)	311 (249-373)
2008	151	120 (91-140)	77 (60-95)	850 (390-1400)	562 (257-922)	460 (370=550)	303 (242-363)
2009	155	110 (89-140)	73 (53-91)	830 (380-1400)	537 (247-876)	460 (370=550)	295 (240-351)
2010	158	33 (11–68)	21 (7.2–43)	320 (110–690)	199 (70–438)	210 (99–360)	133 (63–228)
2011	162	27 (6.1–64)	17 (3.7–40)	280 (71–620)	171 (44–382)	190 (90–330)	118 (55–204)
2012	168	27 (1.6-86)	16 (0.95-51)	270 (43-710)	161 (25-420)	180 (85-310)	108 (50-186)
2013	173	160 (68–270)	94 (39–156)	85 (47–140)	49 (27–78)	570 (430–730)	326 (246–418)

[a] Rates per 100 000 population.Source: collation from World Health Organization reports (WHO, 2010, 2011, 2012, 2013 and 2014).

2.10.1. Bovine tuberculosis in the animal population in Nigeria

In Nigeria, the presence of bovine tuberculosis can be traced back to 1932 (Alhaji, 1976). Past studies have confirmed the prevalence and the endemicity of tuberculosis in indigenous cattle population (Alhaji, 1976; Ayenwale, 1986). Prevalence rates of 0.49% and 1.29% have been reported in slaughtered cattle in Sokoto State by Dusai and Abdullahi (1994) and Ajogi *et al.,* (1995) respectively. And in a similar studies conducted in Oyo and Osun States in the south-western Nigeria and Maiduguri in the north-eastern part of the country reported prevalence rates of 1.43%, 1.22% and 1.26% correspondingly (Ogundipe and Ajiboye, 1997; Ishola *et al.*, 2001; Egbe-Niyi*et al*., 2000). Wekhe and Berepubo (1989) reported a prevalence of 8.2% in the study carried out in an eastern abattoir of the Nigeria; while Cadmus (2006), confirmed the prevalence of 8.8% in Bodija abattoir in the west.

There has been a rise in the prevalence and the existence of foci of infection of the disease as demonstrated by the tuberculin testing results of cattle; Alhaji (1976), Ayewanle (1984), Shehu (1988), Cadmus *et al*., (2004), Abubakar (2007), Okaiyeto *et al*., (2008), Cadmus *et al*., (2010a) and Ibrahim *et al*.,(2012) where individual prevalence rates of between 1%-16.65% and herd prevalence of 45.45% were reported. The isolation of the causative agent along with other mycobacteria species in fresh and sour milk have been reported as well across the country (Alhaji 1976; Shehu, 1988; Okolo 1992; Abubakar 2007; Cadmus and Adesokan, 2007;Ofukwu*et al*., 2008; Okayeto *et al*., 2008).

Various organs especially lungs and lymph nodes of suspected tuberculous cattle from abattoirs and slaughter houses have yielded *M. bovis* in most of the cases, with a small number of other mycobacteria such as *M. tuberculosis* and *M. africanum* (Cadmus *et al*., 2006, 2007; Abubakar 2007).*M.bovis* have been isolated from the nasal secretions of tuberculin positive cattle by Ayewanle (1989) while Ibrahim *et al*., (2012) in a study conducted in Jigawa State ascertained that tuberculin reactivity is significantly associated with respiratory signs. Thus emphasizing inhalation as the route of transmission as the *Mycoacterium* is excreted in the nasal discharge and can be transmitted to humans through the cough sprays.

The disease was reported in other animal species in Nigeria. A rare case of TB of the skin was reported in a horse in Sokoto State (Garba *et al.*, 2001). *M. bovis* was isolated from the horse which was presented to the Veterinary Teaching Hospital of the Usmanu Danfodio University Sokoto with a main complain of circumscribed deep seated wound located on the cheek. The infection resulted to cross-infection to the horse boy who reacted positively to tuberculin test.

2.10.2. Bovine tuberculosis in humans in Nigeria

The occurrence of BTB due to *M. bovis* in humans is difficult to determine accurately in Africa because of technical problems in isolating the microorganism (Collins and Grange, 1983). Currently BTB in humans is becoming increasingly important in developing countries like Nigeria as humans and animals are sharing the same micro-environment and dwelling premises especially in rural areas (Abubakar, 2007). The association of TB with the HIV/AIDS pandemic and in view of the high prevalence of HIV/AIDS in Nigeria, the human TB due to *M. bovis* is likely to change (Amanfu, 2006).

Rural inhabitants and some urban dwellers in Nigeria still consume unpasteurized and soured milk potentially infected with *M. bovis*. In a study in four states in Northern Nigeria, Idrisu and Schnurrenberger (1977) reported that one of 10 mycobacteria isolated from sputum-positive cultures was *M. bovis* while Indigbe *et al*., (1986) reported the isolation of *M. bovis* from sputum samples of patients with pulmonary TB; of the 102 MTCisolates, 4 (3.9%) were *M. bovis*.Recent studies in Nigeria reveal a range of prevalence in some state of the country. Garba *et al*., (2004) in an investigation to determine the species of *Mycobacterium* involved in human TB in Sokoto State; reported that 39 (36.8%) of the 106 samples yielded *Mycobacterium* on culture and on further characterization 8 (7.6%) were *M. bovis* and 4 (3.8%) atypical mycobacteria which were principally from cases of extra-pulmonary tuberculosis, thus giving a prevalence of 7.6%. In a related study in Jos, Plateau State Mawak *et al*., (2006) discovered that 10 (15.4%) out of the 65 isolates from sputum of patients with persistent bronchopneumonia were *M. bovis*. While in Ibadan, south western Nigeria *M. bovis* was isolated from a child with cervical lymphadenitis (Cadmus *et al*., 2004).Also in a study to establish baseline for strains causing TB in Nigeria conducted in Ibadan, Cadmus *et al*., (2006)established that

13% of the disease in humans studied was caused by *M. africanum* and *M. bovis* rather than *M. tuberculosis*. Furthermore, in a bacteriological screening of cattle marketers in Akinyele International Cattle Market Oyo State, for the prevalence of *Mycobacterium*; 26 (37.14%)out of 70 people screened tested positive for TB by cultural isolationand biochemical characterization and showed that five (7.14%) of the isolates were *M. bovis*, however, deletion analysis confirmed only two (3.86%) isolates as *M.bovis* (Adesokan *et al*., 2012).

2.11.Factors responsible for the persistence of BTB in Nigeria

2.11.1 Bovine tuberculosis control in Nigeria:

The control of BTB in Nigeria is regulated by the Federal Ministry of Agriculture. However, the control policy of the Animal Disease (Control) Decree of 1988 is poorly or inadequately implemented (Abubakar *et al*., 2011). This is largely due to politico-economic reasons including high cost of sustainable test and slaughter of infected animals and the subsequent compensation to the farmers.In addition to this is the problem of social unrest due to political instability and ethnic wars especially between the Fulani herders and local farmers (Ayele *et al*., 2004; Abubakar *et al*., 2011).

Also contributing to this occurrence is the lack of quarantine and communication networks at the various control posts which enhances animal movement across state boundaries (Ayele *et al*., 2004). This is also buttressed by the fact that most control posts have turned into revenue collection points rather than checking points for disease surveillance. Most abattoirs are under-staffed lacking sufficient veterinary expertise. The uncooperative attitudes of butchers during meat inspection as a result of little or no compensation by the Government for condemned carcasses due to bovine tuberculosis coupled with illiteracy are major hinderances in the control of the disease in most African countries especially Nigeria (Cadmus *et al*., 2008).

2.11.2 Eating habit and standard of living:

Majority of the cattle in Nigeria are of dual purpose; they are raised for both milk productionsas well as for beef and are with the pastoralists, who own them as a sign of wealth, cohabit with them and also feed on their milk.Negassa and Jabbar (2008) noted that in smallholder mixed farming and pastoral systems, animals are kept for multiple

functions and sale or other forms of disposal are not a common phenomenon, rather sales are sporadic based on immediate cash needs.Within rural communities, cattle owners do not often sell cattle except during festive seasons and the beginning of the school year (Nkosi and Kirsten, 1993). There is a preference or tendency among cattle farmers to sell their cattle when they are old (Nkosi and Kirsten, 1993; Duvel, 2002) this is because the younger ones (females) are used for breeding purposes (Nkosi and Kirsten, 1993).Because these cattle that are kept for a long time with humans, they are able to TB transmits diseasesto them and vice versa. The risk of transmission of the disease from cattle to humans abound as there has been association between the keeping of cattle and the occurrence of both pulmonary and extra pulmonary TB in man (Grange and Yates, 1994; Ameni and Erkihun, 2007).

The agro-pastoral system of farming in Africa also exposes the farmer to mycobacteria which may be present in the faeces excreted by infected animal often used as manure to fertilize the farmlands (Lawal and Babalola, 2014). An infected cow could produce milk containing mycobacteria, or cough infected droplets in the direction of the milker. Milk is seldom pasteurised in pastoral societies, and, even when soured, can still contain infective levels of mycobacteria (Kazwala *et al*., 1998; Coetzer and Tustin, 2005). Animals in traditional African farming systems are seldom culled and there is a greater chance for chronic TB in old cows, particularly those subjected to stress (Michel *et al*., 2004).

Additionally, prior to sale, cattle are fattened in close proximity to the farmers' home and after being sold at the market; they are being slaughtered in nearby abattoirs where butchers use minimal protective clothing and process diseases carcasses with bare hands (Cadmus *et al*., 2008).The following factors contribute to the risk of contracting BTB: family owner of cattle, previous ownership of livestock, history of working with animals, living with relative that owns cattle; and consumption of unpasteurized milk and raw or poorly cooked meat (Abubakar *et al*., 2011).

2.11.3 Diagnosis of bovine tuberculosis

Diagnosis of TB in Nigeria most times ends at the smear level (Abubakar *et al*., 2011) and as a result of that, infection by *M. bovis* cannot be differentiated from that caused by *M. tuberculosis.* Consequently, cases of treatment failures or relapse often occur as *M.*

*bovis*is resistant to pyrazinamide which is widely used for the treatment of infections of *M. tuberculosis* complex in humans (Krauss *et al*., 2003). There are very few laboratories that carry out culture and isolation in Nigeria, and oftentimes it is mostly done for research purposes not for routine diagnosis. The inability to identify the specific species of the *Mycobacterium* causing TB in humans in the country has resulted in the lack of understanding of the extent of the involvement of BTB in the TBepidemic in the country.A major setback is the failure of the National TB control program to recognize the importance of BTB as a major public health problem, in addition to lack of collaboration between the veterinary and human doctors with regard to the control of the disease (Abubakar, 2007).

2.11.4 HIV/AIDS-associated TB due to *M. bovis*

Information on hand indicates that the incidence of TB in humans has risen as a result of HIV/AIDS epidemic (WHO, 2005; Shitaye *et al*., 2007). As the result of the HIV/AIDS epidemic, there has been increase in the incidence of BTB in humans as well (Cosivi *et al*., 1998; Ayele *et al*., 2004; Zinsstag *et al*., 2006).Tuberculosis and other mycobacterial infections are major opportunistic infections in HIV/AIDS infected individuals (Grange and Yates, 1994; Raviglione *et al*., 1995), while HIV/AIDSis a major predisposing factor for TB including reactivation of the disease. The current spreading pandemic of HIV/AIDSinfection in developing countries, especially where BTB is prevalent in domestic and wild animals, poses an additional serious public health threat (Grange and Yates, 1994; Cosivi *et al*., 1995; Pavlik *et al*., 2003; Ayele *et al*., 2004).

2.11.5 Illiteracy

The inability to read and write as well as the failure to utilize modern methods of communication (Ayele *et al*., 2004) is a major problem in Nigeria; and the limited knowledge of the rural dwellers,herders, farmers and the general public about theepidemiology of BTB, makes prevention and control programs difficult and often impossible to apply (Shitaye *et al*., 2007).

2.12. Non-tuberculous mycobacteria (NTM)

The non-tuberculous mycobacteria (NTM) are *Mycobacterium* species different from thosebelonging to the *M.tuberculosis* complex (e.g., *M. tuberculosis, M. bovis,M.*

africanum, and M. microti) and *M. leprae*(Dawson, 2000). NTM can be subdivided into many different species with varying rates of evolutionary divergence, variable biochemical characteristics,clinical presentation, clinical relevance and susceptibility to anti-mycobacterialagents (Buijtels, 2007). NTM are generally free-living organisms that are ubiquitous in the environment. These organisms are traditionally designated 'atypical mycobacteria' and this term was introduced by Pinner in 1935 (Pinner, 1935). Atypical mycobacteria have also been named 'anonymous-', 'non-tuberculous-', 'environmental-', 'saprophytic-', or 'opportunistic' mycobacteria, and 'mycobacteria other than tubercle bacilli' (Buijtels, 2007).

However, these 'atypical' mycobacteria are not merely, as the name seems to imply, variants of *M. tuberculosis*- the 'typical' mycobacteria (Gentry, 2005). In contrast, the atypical mycobacteria constitute an evolutionary divergent group of bacteria with a wide variability in biological features and in ability to cause disease in the humans and animals (Buijtels, 2007). Thus, atypical mycobacteria are not 'unusual' *M. tuberculosis* strains. The name 'saprophytes' seems to be incorrect, since it is not justified terming a micro-organism 'non-pathogenic' for man by means of characteristics such as the production of pigment, the rate of growth or the non-virulence in a guinea pig as was done early in the last century. During that time teaching held that a non-tuberculous acid-fast organism that did not produce disease in the guinea pig was a saprophyte.Moreover, a significant proportion of the atypical *Mycobacterium* species are able to cause clinical disease in man similar to what is caused by the *M. tuberculosis* complex bacteria(Buijtels *et al*., 2009). This implies that none of these terms has been universally accepted, but the designation 'non-tuberculous mycobacteria' (NTM) seems to be the most accepted term inthe international context. This designation has been endorsed by the American ThoracicSociety (ATS) in their 1990 statement on the diagnosis and treatment of disease causedby NTM (Wallace *et al*., 1990). The diseases caused by NTM have been termed 'mycobacteriosis' and 'opportunistic mycobacterial diseases'.

2.12.1. Classification of non-tuberculous mycobacteria

In 1954, Timpe and Runyon (1954) argued that the 'mycobacteria other than *M. tuberculosis'* cause disease in humans, and, subsequently, Runyon developed the first

classification of these organisms. The NTM were classified into four groups on the basis of growth rates, colony morphology, and pigmentation in the presence and absence of light (Runyon, 1959).This classification can be basically categorized into two namely: slow growing NTM (photochromogens, scotochromogens and nonchromogens orRunyon group I, II and III, respectively) and rapidly growing mycobacteria (Runyon group IV).Though now outdated, this classification allowed microbiology laboratories to more readily identify individual species of NTM, resulting in clearer characterization of distinct diseases or syndromes associated with these organisms. With the availability of 16S ribosomal DNA sequencing, high-performance liquid chromatography (HPLC), and 65 KD gene polymerase chain reaction-restriction fragment length polymorphism analysis (PRA), the number of new species of NTM has risen dramatically in recent years. This has given rise to the naming of species such as *M. genavense*, *M. interjectum*, *M. triplex*, *M. celatum*, and *M. lentiflavum*. At present over a 100 species are recognized in the genus *Mycobacterium* (Wolinsky, 1979; Brown-Elliott*et al*., 2002).

Worldwide, the most common NTM species causing human disease are the slowly growingmycobacteria of the *M. avium* complex (MAC), and *M. kansasii* (Johnson and Odell, 2014). Less commonly encounteredhuman pathogens are the slow growing bacteria of the species *M. marinum*, *M. xenopi*, *M. simiae*, *M. malmoense*, and *M. ulcerans*, and the rapidly growing bacteria of the species *M. abscessus*, *M. fortuitum*, and *M. chelonae*(Gentry, 2005). Certain relatively common laboratory isolates, such as *M. gordonae*, are important to be known by clinicians because they are almost invariably contaminants and not true pathogens. Traditional methods for speciation of mycobacterial isolates are based upon growth characteristics and biochemical test results, requiring 4-6 weeks for the whole identification procedure (Buijtels, 2007). Another disadvantage was the inflexibility of this procedure in discovering new species and subspecies. Test results that did not fit the established schematics of particular (sub-) specieswere seen as exceptional and irreproducible, and it was difficult to link such observations to mycobacterial groups, not previously recognized (Buijtels, 2007).

In the 1980s, the first molecular tests were introduced to distinguish between MTC bacteria and the NTM (Gen-probe, San Diego, California, USA) (Buijtels, 2007). Also

specific nucleic acid probes tests became available for recognition of the most commonly encountered clinical isolates of NTM, including *M. kansasii*, the *M. avium* complex, and *M. gordonae*. In the 1990s, 16S DNA sequencing was introduced and the identification of mycobacteria not only became less time consuming, but also much more robust. Andthe list of new species and sub-species is growing rapidly (Buijtels, 2007). Until 01 August 2007, 127 *Mycobacterium* species have been described (http://www.bacterio.cict.fr/m/mycobacterium. html). It is expected that in the near future the insight in the phylogeny of mycobacteria will grow significantly. Also many currently recognized species may be further divided in two or more new species and sub-species. Particular sub-species may have more clinical relevance than other sub-species (Buijtels, 2007). For example, on the basis of DNA polymorphism in the gene coding for the 16S-23S rRNA internal transcribed spacer, the species *M. kansasii*was recently divided in several sub-groupings. *M. kansasii* sub-group I is often associated with clinical disease, while the other subgroups are more likely to represent environmental bacteria (Buijtels, 2007).

2.12.2. Infections with non-tuberculous mycobacteria

Non-tuberculous mycobacteria are ubiquitous in the environment and can colonize or infect people and animals(Al-anazi *et al*., 2014). In contrast to *M. tuberculosis* and *M. leprae*, species that affect only mammals, NTM form an integral part of the natural environment and may also prevail in certain man-made environments, thereby exposing susceptible individuals leading to colonization and infection (Buijtels, 2007). NTM act like saprophytes, commensals, and symbionts and are common inhabitants of a wide variety of environmental reservoirs throughout the world, including natural and municipal water, soil, aerosols, protozoans, domestic- and wild animals, milk- and food products (Buijtels, 2007). NTM may be abundant in certain natural surroundings or niches, where climatological factors are advantageous for their growth (Pruden *et al*., 2013).

There are geographical variations in the distribution of specific NTM organisms (Contreras *et al*., 1988), most likely reflecting regional differences in the mycobacterial habitats. However, only a very small proportion of all human-mycobacteria interactions progress to outright mycobacterial infection. Such progression is much more common in

immuno-compromised patients, especially those with AIDS (Arasteh *et al*., 2000). The relatively low incidence of infection due to NTM, despite high levels of exposure, suggests that NTM generally have a low pathogenicity for man (Albelda *et al*., 1985; Miller *et al*., 1993). The ubiquitous distribution of NTM contributes to the difficulties in interpretingpositive culture results. Most NTM can inhabit body surfaces or secretions without causing disease (Wali et al., 2008). Thus, the mere isolation of NTM from respiratory specimens in itself may not only indicate harmless colonization, but also infection (Buijtels, 2007 and Wali *et al*., 2008). Furthermore, NTM can potentially contaminate smears and cultures during the acquisition, transportation and laboratory processing of specimens, which may lead to incorrect diagnosis of tuberculous disease (Parsons *et al*., 2011).

The clinical manifestations of the infections vary among the NTM species, and distinguishing between colonization or contamination and true infection is complicated. Some terms used in this thesis are defined below as seen in the literature (Buijtels, 2007). Colonization, this refer to the repeated recovery of viable NTM organisms from properly collected clinical specimens, in the absence of clinical manifestations, tissue invasion or other sign of damage to the host (Buijtels, 2007). The NTM multiplieson a body surface without evoking an immune response. The term ‘infection’ refers to a disease state in which there are signs or symptoms suggesting a pathologic process: NTM have invaded the body tissues (Buijtels, 2007). The term ‘disease’ will be used to indicate the clinical manifestation of damage that results from the host-NTM interaction (Buijtels, 2007). This is the case in a symptomatic patient who does meet the diagnostic criteria in the guidelines formulated by the American Thoracic Society (ATS) or British Thoracic Society (BTS), which includes pathology on the chest radiograph or chest high-resolution computed tomography (HRCT) scan, repeated sputum samples, bronchial wash or lung biopsy positive for NTM and exclusion of other disorders (BTS, 2000; Griffith *et al*., 2007). These terms (colonization, infection and disease) are used to describe the host-microbe relationship and have been in use for nearly a century. Over the years the concept of host-pathogen interaction changed and the definitions for these terms were revised. In the literature, the definitions of these terms are often not clarified. In several publications the

difference between rates of colonization and rates of infection or disease are not evident. This may lead to confusion and may overestimate the clinical importance of NTM.

In broad terms, the following main categories of clinical symptoms caused by NTM can be distinguished: pulmonary disease, superficial lymphadenitis, skin and soft tissue infection, anddisseminated disease. Pulmonary disease accounts for up to 90% of all cases. Furthermore, NTM are occasionally involved in nosocomial infections and pseudooutbreaks(Wallace *et al*., 1998; Phillips and von Reyn, 2001). Unfortunately, official figures on infections due to NTM cannot be given because they are mostly not incorporated in surveillance programs. In The Netherlands, one focal centre performs the secondary laboratory diagnosis of tuberculosis and mycobacterioses; theRijksinstituut voor Volksgezondheid en Milieu(RIVM). At the RIVM in the year 2005 nearly 600 isolates of NTM were received, and the actual number of NTMisolates in Netherlands may be much higher. In most of the concerned cases there was a clinical ground underlying the decision to culture mycobacteria. The magnitude of clinical relevance of isolation of particular NTM is currently under investigation at the RIVM, in collaboration with University Lung Centre Dekkerswald in Nijmegen.

Non tuberculous mycobacteria infections of the lung often occur in the context of pre-existing lung disease, especially chronic obstructive pulmonary disease (COPD), bronchiectasis, pneumoconiosis, cystic fibrosis, and a history of tuberculosis (Yeager *et al*., 1973; Davidson, 1989; ATS, 1997). As a result, the clinical manifestations of NTM-associated lung disease are often similar to those of the underlying disease and are also present in patients with NTM lung disease who do not have pre-existing pulmonary disease. Progressive pulmonary disease is primarily caused by *M. avium* complex (MAC) and *M. kansasii*. Other species which cause lung disease include *M. abscessus*, *M. fortuitum*, *M. xenopi*, *M. malmoense*, *M. szulgai*, *M. simiae*, and *M. asiaticum* (ATS, 1997). Differences in geographic distribution play a prominent role in the epidemiology of NTM pulmonary disease. *M. xenopi* is relatively more common in Europe, Great Britain, and Canada, while *M. malmoense* is relatively more common in Scandinavia and Northern Europe (Wolinsky *et al*., 1979; Wolinsky *et al*., 1968).

Cervical lymphadenitis, especially in children younger than 5 years of age, is caused mostly by MAC and *M. scrofulaceum* in the United States, and, in Northern Europe mostly by *M. malmoense*. However, the most common cause of this infection in Europe is *M. tuberculosis*. Skin and soft tissue infections usually occur from percutaneous inoculation (e.g. trauma or surgery) and are caused primarily by *M. marinum* and *M. ulcerans* and the rapid growers *M. chelonae* and *M. fortuitum*. Chronic granulomatous infections of bursae, joints, tendon sheaths and bones are seen after direct inoculation of NTM through accidental trauma, surgical incisions, puncture wounds or injections. Disseminated infections of NTM are rare and most commonly caused by *M. chelonae*, *M. abscessus* and *M. fortuitum*. A review of the literature from the early 1990s identified worldwide 54 cases over 14 years (Ingram *et al*., 1993). The diseases occur most commonly in patients with profound immuno-suppression and presented as multiple subcutaneous nodules (pseudo erythema nodosum) or abscesses that drained spontaneously (Wallace *et al*., 1992). The majority of disease due to NTM in HIV-infected patients is caused by MAC. In these patients, disseminated disease is usually reported, although localized forms of MAC infection are currently being reported with the widespread use of more effective antiretroviral therapies.

2.13. Global burden of NTM infection

Although reports listing the significance of NTM differ in various geographic parts of the world, there does seem to be a definite geographic distribution for some organisms. In the United States, NTM lung disease is most commonly attributable to *M. avium* complex, with *M. kansasii* being second (O'Brien *et al*., 1987). In the United Kingdom, *M. kansasii* is the pathogen most commonly associated with NTM lung disease in England and Wales, while *M. malmoense*is the most commonly encountered NTM in Scotland. *M. xenopi* predominates in Southeast England (BTS, 2000). In Japan, the most common cause of NTM pulmonary diseaseis *M. avium* complex, followed by *M. kansasii* (Tsukamura *et al*., 1988). The distribution of NTM and the incidence of disease caused by them are not yet fully known in most parts of the world (Buijtels, 2007). The reported rates of NTM colonization and infection are likely to be underestimated, with the former probably less

accurate than the latter, given that people without significant symptoms are not likely to have intensive diagnostic work-up for possible NTM infection (Buijtels, 2007).

The lack of systematic reporting of NTM infection in most nations limits the ability to derive accurate estimates of incidence and prevalence of colonization and infection (Marras and Daley, 2002). NTM disease is clearly a major problem in certain groups, including patients with underlying lung disease and also in individuals with impaired immunity (Marras and Daley, 2002). North American rates of colonization and infection have been reported to range from approximately 1-15 per 100,000 and 0.1-2 per 100,000, respectively (Buijtels, 2007). Generally, similar rates have been reported in European studies, with the exception of extremely high rates in an area of the Czech Republic where mining is dominant (Kubin *et al*., 1980). These studies have also shown marked geographic variability in prevalence. Rates in Japan and Australia were similar to those reported in Europe and North America. On the whole, the impression seems to be that NTM infections in humans are on the increase. However, most data reporting high rates of infection with NTM come from Northern European countries or the United States (Buijtels, 2007). In African countries, despite the fact that environmental exposure to NTM is very high in that part of the world, infections with NTM seem to be very rare, even among patients with AIDS (Morrissey *et al*., 1992). Though, not much data is available to date regarding NTM infection in Africa.

2.13.1Non tuberculous mycobacteria infection in Africa

The only available population-based studies in the continent of Africa have been conductedin South Africa and they report extremely high rates of infection (Buijtels, 2007). These are generally limited to select populations. The results of mycobacterial studies of sputum cultures from large random samples of South African native people were reported in two studies (Arabin *et al*., 1979; Fourie *et al*., 1980). The sputum from 1,196 Zulus in Natal, South Africa, was obtained and examined for the presence of mycobacteria (Arabin *et al*., 1979). No clinical information was collected. Sputum was obtained either spontaneously or induced by mechanical irritation of the epiglottis. Nine and 17 samples were culture positive to*M. tuberculosis* and NTM respectively, translating into prevalence rates for *M. tuberculosis* of 750 per 100,000 and prevalence rates of

colonization/infection for NTM of 1,400 per 100,000. In the other study,aerosol-induced sputum from 2,230 Xhosa people in the Transkei region was obtained (Fourie *et al*., 1980). Ninety specimens grew *M. tuberculosis* and 150 grew NTM, yielding prevalence rates for *M. tuberculosis* of 4,300 and prevalence rates of colonization/infection for NTM of 6,700 per 100,000. According to the authors there was no evidence that the isolated NTM presented with any health threat. Moreover, 79% of the NTM cultured were manifested as 1-colony isolates. Therefore, the 150 NTM represented probably colonization.Both studies utilized population-based methods to determine the prevalence of pulmonary mycobacterial infection. However, the selected population had an extremely highrate of tuberculosis, probably resulting in bronchiectasis and leading to NTM infection. It is doubtful whether these prevalence rates can be generalized to other populations.

Two South African NTM studies focussed on a population of gold miners (Corbett *et al*., 1999a; Corbett *et al.*, 1999b). The records of a cohort of HIV-negative gold miners, investigated for suspected pulmonary mycobacterial disease between 1993 and 1996, were reviewed (Corbett *et al.*, 1999b). Annual rates of NTM infection/disease were found to be 101 per 100,000 with the two most common organisms causing 66 (*M. kansasii*) and 12 (*M. scrofulaceum*) per 100,000, respectively. Interestingly,MAC made up for only 6% of all isolates and the rate of MAC disease was not reported. The same group consequently presented data on another cohort of gold miners, this time including HIV-positive patients (Corbett *et al*., 1999a). Rates of NTM disease were somewhat lower than in the earlier report (37 *M. kansasii* and 8.8 *M. scrofulaceum* per 100,000, respectively). In Ethiopia the prevalence of pulmonary tuberculosis was assessed in out patients in Addis Ababa (Bruchfeld *et al*., 2002). Of 509 consecutive outpatients evaluated on the basis of a clinical suspicion of pulmonary tuberculosis 168 (33%) were culture positive for *M. tuberculosis* and two isolates classified as *M. avium* complex.An overview of literature on prevalence and incidence rates of NTM colonizationinfectiondisease in Africa is presented in table 2.5.

Table 2.6: Overview of literature on prevalence/incidence rates of NTM colonization/infection/disease in Africa.

Setting	Study	Year	Size	Specimen	Smear positive	Culture result		Incidence/prevalence/proportion of colonization, infection or disease NTM	Ref
						Mtb	NTM		
South Africa, Transkei	Random sample rural population	1977	2230	Aerosol induced sputum	47 (36 *M. tuberculosis* and 6 NTM in culture)	90	150	Prevalence of colonization/infection: 6,700 per 100,000	Fourie *et al.*, 1980
South Africa, Welkom	Cohort of HIV-neg gold miners with *M. kansasii* or *M. scrofulaceum* in sputum	1993-1996	202 with *M. kansasii*, 41 with *M. scrofulaceum*	Sputum spontane-ously	164 with *M. kansasii* and 35 with *M. scrofulaceum* in culture	-	297 NTM: 202 *M. kansasii*, 41 *M. scrofulaceum*, 18 *M. avium-intracellulare* complex, 36 nonpathogenic NTM species	Incidence infection/disease: 101 per 100,000 for most common NTM or 125 per 100,000 for all NTM	Corbett *et al.*, 1999
Ethiopia, Addis Ababa	Out patients with clinical suspicion of pulmonary tuberculosis	1996	506	Sputum spon-tenouesly	91 with *M. tuberculosis* in culture	168	2 *M. avium* complex (one smear positive)	Proportion patients colonized/ infected: 0.4% (2/509)	Bruchfeld *et al.*, 2002
Kenya	Rural and urban population adults	1994-1996	281 clinical episodes of acute	Sputum or percutaneous transthoracic	23 (of which 3 remained culture neg) of the 53	26	11 *(M. fortuitum/M. chelonae* (3), *M. szulgai* (2),	Proportion patients colonized/ infected: 3-6% (11/281)	Scott *et al.*, 2000

	who presented with acute pneumonia		pneumonia (272 patients)	lung aspiration	patients with pos mycobacterial cultures		*M. kansasii* (2), *M. terrae* (1), other NTM (3))		
Kenya, Nairobi	Cohort study of HIV-pos and HIV-neg patients with suspected tuberculosis	1989-1990	355	Mainly sputum	342 sputum results: 288 smear pos	320	No NTM	No NTM	Githui *et al.*, 1992
Nigeria, Lagos	Random sample of patients suspected for pulmonary tuberculosis	1983	668 (of the 2,784 patients suspected for tuberculosis)	Repeated sputum samples	Not mentioned (of 102 mycobacterial Isolates)	87 (and 4 *M. bovis*)	11 (6 *M. avium*, 4 *M. kansasii*, 1 *M. fortuitum*)	Proportion patients with disease: 2% (11/668)	Idigbe *et al.*, 1986
Zambia, Lusaka	Patients suspected for tuberculosis at the University Teaching Hospital	1989	249	Sputum, pleural fluid, lymph node, pericardial fluid, lung biopsy	101	137	No NTM	No NTM	Elliott *et al.*, 1995
Rwanda, Kigali	HIV-1 infected adults with pulmonary disease of undetermined	1990	111	Bronchoalveolar lavage (BAL) and transbronchial	Only patients with smear negative sputum for AFB were included	12 (and in 9 diagnosis by histopathologic	No NTM	No NTM	Batungwanayo *et al.*, 1994

	aetiology			biopsy (TBB).		examination and in 4 AFB positive but culture negative)			
Cote d'Ivoire, Abidjan	Prospective study: HIVinfected adults with unexplained fever and fewer than 15% of CD4 Cells	1996-2002	721	Blood and sputum, urine, faeces, pleural fluid	Not mentioned	Not mentioned	37 NTM identified as *M. intracellulare* and *M. avium*	Incidence of NTM disease 1.8/100 person-years overall and 12.2/100 person-years in patients with CD4 cell counts < 100 cell/mm3	Bonard *et al.*, 2004
Uganda, Kampala	Patients with AIDS (blood cultures)	1990	45 patients with blood cultures	Blood and clinical specimens	Not mentioned	Blood: 4	Blood: no *M. avium* (in former study also no *M. avium* in 50 blood cultures)	None of the NTM were thought to be clinically significant	Morrissey *et al.*, 1992

Definition of abbreviations: ref = reference, neg = negative, pos = positive. Source: Buijtels, 2007

2.13.2Burden of NTM in Nigeria

In sub-Saharan Africa, information on the extent of the burden of pulmonary disease from non-tuberculous mycobacteria (NTM) is lacking due to limitations in tools for mycobacterial species identification. However, studies conducted as far back as the late 1950s and early 1960s using traditional tools for identifying mycobacterial groups based on certain characteristics like speed of growth and morphology, have reported the isolation of NTM from both tuberculosis patients and the general public in some African countries including Nigeria (Zykov *et al*., 1967a; Zykov *et al*., 1967b). Failure to characterize acid fast bacilli (AFB) positive NTM lung infections has led to their misclassification and to treatment errors for pulmonary tuberculosis in developing countries. A report from Nigeria found that 12.4% (12/97) of AFB positive patients receiving treatment for pulmonary tuberculosis (TB) had infections with organisms other than Mycobacteria while 4.1% (4/97) had NTM infections (Pokam *et al*., 2012). The implication is that NTM is inappropriately managed with first-line antituberculous drug (Koh and Kwon, 2004; Yim and Hans, 2005), worsening the patient's condition and raising the risk of drug resistance. Although it is known that most sputum smear positive patients are truly TB patients (Idigbe *et al*., 1986), the continued increase in TB drug resistance raises the question on the impact of this indiscriminate use of TB drugs to treat all diagnosed sputum smear positive patients.

2.14. Transmission and risk factors of NTM infection.

Unlike many MTC which affect only mammals, NTM form an integral part of the natural environment and may also prevail in certain man made environments, such as hot water tanks and tap water, thereby infecting and causing disease in vulnerable individuals (Buijtels 2007). Transmission of infection occurs by inhalation or ingestion of aerosolized organisms from soil, dust and water spray and less frequently from instrumentation such as bronchoscopy or by inoculation (Falkinham, 2002). Damaged skin tissues favour infections by particular NTM species including *M. abscessus* (Petrini, 2006).

Direct transmission from animals is not important for human infections (Meissner and Anz, 1977), and human-to-human transmission has not been demonstrated so far. Therefore, patients with disease are not generally isolated. Disease in humans almost

certainly arises as a result of contact with bacilli in the environment. Certain types of conditions including congenital- or acquired immune deficiency of T-cells or macrophages, cystic fibrosis (CF) or bronchiectasis, tend to facilitate NTM infection (Gentry, 2005). Trauma that is often associated with introduction of foreign material such as wood splinters or sand particles, unsterile injections, or surgically implanted cosmetic or medical devices in tissue, may promote disease with different NTM (Reichenbach *et al*., 2001;Aksamit *et al*., 2002; Jones and Havlir, 2002; Tiwari *et al*., 2003; Olivier, 2004). The course of infection depends on the characteristics of the NTM species, the presence of predisposing host factors and the clinical setting (Raju and Schluger, 2000; Thomsen *et al*., 2002). Risk factors may be divided, in decreasing order of importance, into coexisting medical conditions, living and work environment, and patient demographics.

Coexisting medical conditions likely provide the most powerful risk factor for NTM infection. These risk factors may be divided into impairment of local pulmonary defences and generalized immune defects, with examples including cystic fibrosis (CF) and HIV, respectively. Preexisting lung disease, including silicosis and other pneumoconiosis (Mark, 1969; Kaustova *et al*., 1995; Corbett *et al*., 1999b), chronic obstructive pulmonary disease (France *et al*., 1987; Lortholary *et al.*, 1999), bronchiectasis (France *et al*., 1987; Lortholary *et al*., 1999), and other structural changes in the anatomy of the lung consistent with previous tuberculosis (Corbett *et al*., 1999b) have been identified as important risk factors. Alcohol abuse, diabetes mellitus, malignancy (France *et al.*, 1987), and smoking (O'Brien *et al*., 2000) also have been associated with NTM infection.

Living and work environment has consistently been identified as an important risk factor for NTM colonization and infection (Buijtels, 2007). Studies covering large geographic areas have generally found an increased risk of NTM colonization in people living in warmer regions (Edwards, 1970). Living in urban versus rural settings has been associated with altered rates and patterns of NTM colonization in several studies with the urban setting more likely to have higher prevalence (Ahn *et al.*, 1979; O'Brien *et al.*, 1987; Isaac-Renton *et al*., 1985; Bloch *et al*., 1998). The most commonly cited environmental risk factor for NTM is the work environment, specifically mining, and other heavy

industries such as smelting. Also, residence in areas where these industries dominate may be a risk factor (Engel *et al*., 1981; Corbett *et al*., 1999a; Corbett *et al*., 1999b). Certain demographic features have been identified as risk factors for NTM infection, including age, sex, or combination of the two. Increasing age has almost universally been identified as a risk factor for NTM (Edwards, 1970; Robakiewicz and Grzybowski, 1974) as well as male sex (Robakiewicz and Grzybowski, 1974; Ahn *et al*., 1979; Bloch *et al*., 1998). However, exposure to NTM in the work environment during cumulative years could be a confounding factor in that respect (Buijtels, 2007).

There are documented observations regarding the patient profiles of 'elderly females' and 'middle-aged males' being at increased risk for MAC (Ahn *et al*., 1979; O'Brien *et al*., 1987) and *M. kansasii* (Chobot *et al*., 1997). Immune competent patients infected by NTM can be stratified into two groups: the first comprises patients, usually men of more than 50 years old, primarily Caucasian with preexisting lung disease such as chronic airways disease, fibrocavitary diseases and bronchiectasis and often alcoholics and/or smokers (Ahn *et al*., 1982; Teirstein *et al*., 1990; Kilby *et al*., 1992). An additional recognized group of patients comprises predominantly elderly (over age 50) non-smoking women with no pre-existing pulmonary disease and who have interstitial patterns on chest radiography (Prince *et al*., 1989; Reich *et al*., 1992; Swensen *et al*., 1994).

2.15. Diagnosis of non tuberculous mycobacteria

The symptoms of NTM pulmonary disease are variable and nonspecific. However, virtually all patients have chronic or recurring cough. Other symptoms variably include sputum production, fatigue, malaise, dyspnea, fever, hemoptysis, chest pain, and weight loss (Griffith*et al*., 2007). Evaluation is often complicated by symptoms caused by coexisting lung diseases, such as bronchiectasis, chronic obstructive airway disease associated with smoking, CF, and pneumoconiosis (Griffith*et al*., 2007). Physical findings are nonspecific and reflect underlying pulmonary pathology, such as bronchiectasis and chronic obstructive lung disease. On chest auscultation, findings may include rhonchi, crackles, wheezes, and squeaks (Griffith*et al*., 2007). Patients with nodular/ bronchiectatic MAC disease tend to be postmenopausal women, many of whom also have a characteristic

morphotype with a thin body habitus and may also have scoliosis, pectus excavatum, and mitral valve prolapse (Iseman *et al*., 1991**).** The diagnosis of NTM disease is highly complicated. Prior to the 1950s, it was generallyaccepted that NTM do not cause human disease.

Unlike *M. tuberculosis*, NTM were found of low virulence in the guinea pig model; as a result, when they were found in sputum of humans, it was difficult to know whether their presence represented disease or simply colonization (Maoz*et al*., 2008). Finding any *M. tuberculosis* in sputum is regarded as abnormal and indicative of disease, but such is not the case with NTM however;healthy people may expectorate NTM without having evidence of this disease (Buijtels, 2007). The isolation of a few colonies of NTM from the sputum of a healthy person may have no clinical significance,if these organisms are isolated from a person with an abnormal chest X-ray film, further evaluation is necessary (Buijtels, 2007). When NTM are isolated from a usually sterile site (e.g., blood, bone marrow, lymph nodes, synovial fluid), the diagnosis of true disease is generally straight forward. However, when NTM are isolated from non-sterile sites, such as sputum or broncho-alveolar lavage (BAL) fluid, the diagnosis is less definitive, especially when the colony numbers are low or NTM are isolated from only one cultured specimen (Buijtels 2007). A diagnosis of infectionin such a case depends upon other clinical findings and the presence or absence of other pathogens. Organisms that in immune competent persons are considered 'commensals', can be opportunistic pathogens in patients with advanced HIV disease and immunodeficiency(Benator and Gordin, 1996).

Microscopy

Microscopic examination of sputum smears after staining, culture on specific (liquid and solid) media, and molecular detection are nowadays the cornerstones of the laboratory diagnosis of mycobacterioses, including tuberculosis (Parsons *et al*., 2011). Usually, in poor resource settings, microscopy is still the only tool available for the diagnosis of tuberculosis (Muwonge*et al*., 2014). All mycobacteria share the characteristic of 'acid-fastness', i.e.; after staining with carbol-fuchsin or auramine-rhodamine, they do not decolorize with acidified alcohol. Thus, the common term acid-fast bacilli (AFB) are essentially synonymous with mycobacteria. Nocardia, the main exception, is weakly or

variably acid-fast (Buijtels, 2007). Specimens may be stained with the Ziehl-Neelsen stain or one of its modifications, such as the Kinyoun stain, and examined by routine light microscopy (Pandey *et al*., 2009). However, microscopy is relatively insensitive, since at least 10,000 organisms per millilitre of sputum are required for smear positivity (Allen *et al*., 1992). Thus, other procedures are often performed in order to increase the sensitivity of direct microscopy of clinical specimens. Most laboratories in the Western world use a fluorochrome stain such as auramine-O or auramine-rhodamine and examine specimens by fluorescence microscopy (Somoskovi*et al*., 2001). In order to increase the sensitivity, it is also recommended to use *N*-acetyl l-cysteine (NALC) for liquefaction of the sputum together with concentration (Farnia *et al*., 2002, Morcillo *et al*., 2008). Furthermore, liquid specimens may be centrifuged first before the sediment is stained (Buijtels 2007).

Culture

Confirmation of the presence of mycobacteria in clinical specimens has traditionally required culture, because of the relative insensitivity of direct microscopy (Palomino, 2005; Parsons *et al*., 2011). In general, clinical specimens that are normally sterile, such as blood, cerebrospinal fluid, or serous fluids, can be inoculated directly onto media (Lakshmi, 2001). In contrast, non-sterile specimens, such as sputum or pus, must first be chemically decontaminated, in order to eliminate common bacteria and fungi that would overgrow the culture (Buijtels, 2007; Parsons *et al*., 2011). However, decontamination procedures inhibit the growth of mycobacteria as well. Clinical specimens for mycobacterial cultures should be inoculated onto one or more solid media (Lowenstein-Jensen or Middlebrook 7H11 media) and into a liquid medium such as BACTEC 12B broth or MGIT broth (both manufactured by Becton Dickinson). Growth of visible colonies on solid media typically requires three to eight weeks. Primary cultures in liquid media, such as in the MGIT system, usually produce results within 10 to 14 days (Williams-Bouyer et al., 2000). However, this method is not hundred percent sensitive; as a result, this culture method supplements but not replaces traditional solid media (Buijtels, 2007).

Molecular methods

Traditional methods of speciating mycobacterial isolates were based upon growth characteristics on solid media and subsequent biochemical tests, requiring additional weeks for subcultures (Cook *et al*., 2003). These time-consuming methods are being replaced with more rapid techniques. Commercial DNA probes (AMPLICOR nucleic acid amplification test (AccuProbe) Roche Diagnostic Systems, Inc., Branchburg, New Jersey) and Gen- Probe Amplified *Mycobacterium tuberculosis* Direct Test (MTD) (Gen-Probe, San Diego, California) have been available for some time for identification of clinically important mycobacterial species, including *M. tuberculosis* complex,*M. intracellulare*, *M. avium* complex, *M. kansasii*, and *M. gordonae* (Buijtels, 2007). The tests are based on species-specific DNA probes that hybridize with ribosomal RNA released from bacteria (Soini and Musser, 2001). Other methods (INNO-LiPA Mycobacteria; Innogenetics, Ghent, Belgium and GenoType Mycobacterium CM/AS, Hain Lifescience GmbH, Nehren, Germany) are based on reverse hybridization, in which the mycobacterial 16S-23S internal transcribed spacer region or the 23S gene region are amplified by polymerase chain reaction (PCR), and amplicons are subsequently hybridized with probes for several mycobacterial species on paper strips (Buijtels, 2007; Somoskovi and Salfinger, 2014). Polymerase chain reaction (PCR)-based sequencing consists of amplification of mycobacterial DNA with genus-specific primers and sequencing of the amplicons. The micro-organism is identified by comparison of the nucleotide sequence with reference sequences. The target most commonly used is the gene coding for the 16S ribosomal RNA (Clarridge, 2004). Several other target genes have been characterized for this purpose: genes coding for the 32 kDa protein (Soini *et al.*, 1994), the 65 kDa heat shock protein (Kapur *et al*., 1995), and the 16S-23S ribosomal RNA internal transcribed spacer (Roth *et al*., 1998).

2.16. Molecular Epidemiology of Tuberculosis

Molecular epidemiology as a branch of science is a field that has emerged largely from the integration of molecular biology, clinical medicine, statistics, and epidemiology (Mathema*et al*., 2006). In a nutshell, molecular epidemiology focuses on the role of genetic and environmental risk factors, at the molecular/cellular or biochemical level, in

disease etiology and distribution among populations (Mathema*et al*., 2006). In the area of infectious diseases, molecular epidemiology has been employed in a multidisciplinary manner to identify factors that determine the causes, propagation/ dissemination, and distribution (in time and space) of diseases (Eisen and MacCallum, 2009). This is primarily achieved by associating epidemiologic characteristics with the biologic properties of clinical isolates recovered from symptomatic individuals (Mathema*et al*., 2006). The mid-1980s saw the first integration of molecular methods to discriminate between clinical isolates of *M. tuberculosis*. While previous methods, such as colony morphology, comparative growth rates, susceptibility to select antibiotics, and phage typing, were useful, they did not provide sufficient discrimination,thus limiting their utility in TB epidemiology (Mathema*et al*., 2006). That is, prior to molecular methods, understanding the spread of TB was imprecise and relied on observational data or anecdotal correlations (Mathema*et al*., 2006). However, given the abundance of molecular tools available, it is critical to choose an appropriate method(s) to address a particular study question, e.g., transmission dynamics, outbreaks, or phylogenetics. In general, the key aspects in choosing an adequate molecular approach for studying TB epidemiology are the observed rate of polymorphism (stability of biomarker) and the genetic diversity of strains in the population (Mathema*et al*., 2006). Thus, the rate of change of a biomarker must be adequate to distinguish nonepidemiologically related strains and yet sufficiently "slow" to reliably link related cases (Mathema*et al*., 2006). This issue, coupled with general background TB prevalence, should be taken into consideration when choosing molecular epidemiologic methods or in evaluating data (Mathema*et al*., 2006).

2.17. Genotyping in the epidemiology of tuberculosis

The TB research community entered the genomic era in 1998 with the publication of the complete annotated genome of *M. tuberculosis* laboratory strain H37Rv (Cole *at al*., 1998). Since then, *M.tuberculosis* clinical strain CDC1551 and six related mycobacteria, *M. leprae*, *M. ulcerans*, *M. avium*, *M. avium paratuberculosis*, *M. smegmatis*, and *M. bovis*, have been fully sequenced; others, including *M. microti*, *M. marinum*, *M. tuberculosis* strain 210, and *M. bovis* BCG (bacillus Calmette-Gue´rin), are nearing completion. Studies show that the *M. tuberculosis* complex (i.e., *M. tuberculosis*, *M.*

bovis, *M. microti*, *M. africanum*, *M. canettii*, and, more recently, *M. pinnipedii* and *M. caprae* (Frothingham *et al*., 1994; Aranaz *et al*., 1999; Cousins *et al*., 2003) genomes are highly conserved: comparative sequence analysis of the 275-bp internal transcribed spacer (ITS) region, an otherwise highly polymorphic region which separates the 16S rRNA and the 23S rRNA, revealed complete conservation between members of the *M. tuberculosis* complex.

Furthermore, sequence analysis of 56 structural genes in several hundred phylogenetically and geographically diverse *M. tuberculosis* complex (MTC) isolates suggested that allelic polymorphisms are extremely rare (Kapur *et al*., 1994;Sreevatsan *et al*., 1997;Musser and Ramaswamy, 2000). While the members of the MTC display diverse phenotypic characteristic and host ranges, they represent an extreme example of interspecies genetic homogeneity, with an estimated rate of synonymousnucleotide polymorphisms of 0.01% to 0.03% (Sreevatsan *et al*., 1997; Cole *et al*., 1998; Fleischmann *et al*., 2002;Gutacker *et al*., 2002) and no significant evidence for horizontal genetic transfer between genomes, unlike most bacterial pathogens (Brosch *et al*., 2002;Gutacker *et al*., 2002;Alland *et al*., 2003; Supply *et al*., 2003). While the MTCgenome is highly restricted (conserved) in relation to other bacterial pathogens, this monomorphic species does have polymorphic genomic regions. Much like eukaryotic genomes, those of prokaryotes (such as *M. tuberculosis*) are characteristically punctuated by monomeric sequences repeated periodically (repeated units).There are two types of repetitive units, interspersed repeats (IR) (direct repeats and insertion sequence-like repeats) and tandem repeats (TR) (head-to-tail direct uninterrupted repeats). Prokaryotic microsatellites (1- to 10-bp repeats) and minisatellites (10- to 100-bp repeats), commonly referred to as variable-number tandem repeats (VNTR) are located in intergenic regions, in regulatory regions, or within open reading frames and are abundant throughout most bacterial genomes.

Genetically, all the members of *M. tuberculosis* complex are extremely similar, with about 99.9% similarity at the level of nucleotide and they also share identical 16S rRNA sequence (Brosch *et al*., 2002) but differ widely in terms of their host tropisms, phenotypes, and pathogenicity. The evolutionary pathway of *M. tuberculosis*complex is best delineated in the genetic study proposed by Brosch *et al*. (2002) (Figure 2.1). This is

based on the loss of chromosomal region and on sequence polymorphism of selected genes. The population structure of thisgroup of organisms is apparently highly clonal, with no recombination of chromosomal sequences between strains (Cole *et al*., 1998). In the absence of inter-strain recombination, once a non-repetitive chromosomal region has been deleted it cannot be replaced and the deletion is a marker of a single cell and all its descendants (Smith *et al*., 2006).According to Brosch *et al*. (2002), the use of deletion analysis in conjunction with molecular typing and analysis of specific mutation was shown to represent a very powerful approach for the study of the evolutionof the tubercle bacilli and for the identification of evolutionary markers. Furthermore, they proposed that for practical purpose, these regions, primarily RD9 and TbD1 but also RD1, RD2 RD4, RD7, RD8, RD10, RD12 and RD13 represent very interesting candidate for the development of powerful diagnostics tools for the rapid and unambiguous identification of members of the *M. tuberculosis* complex.

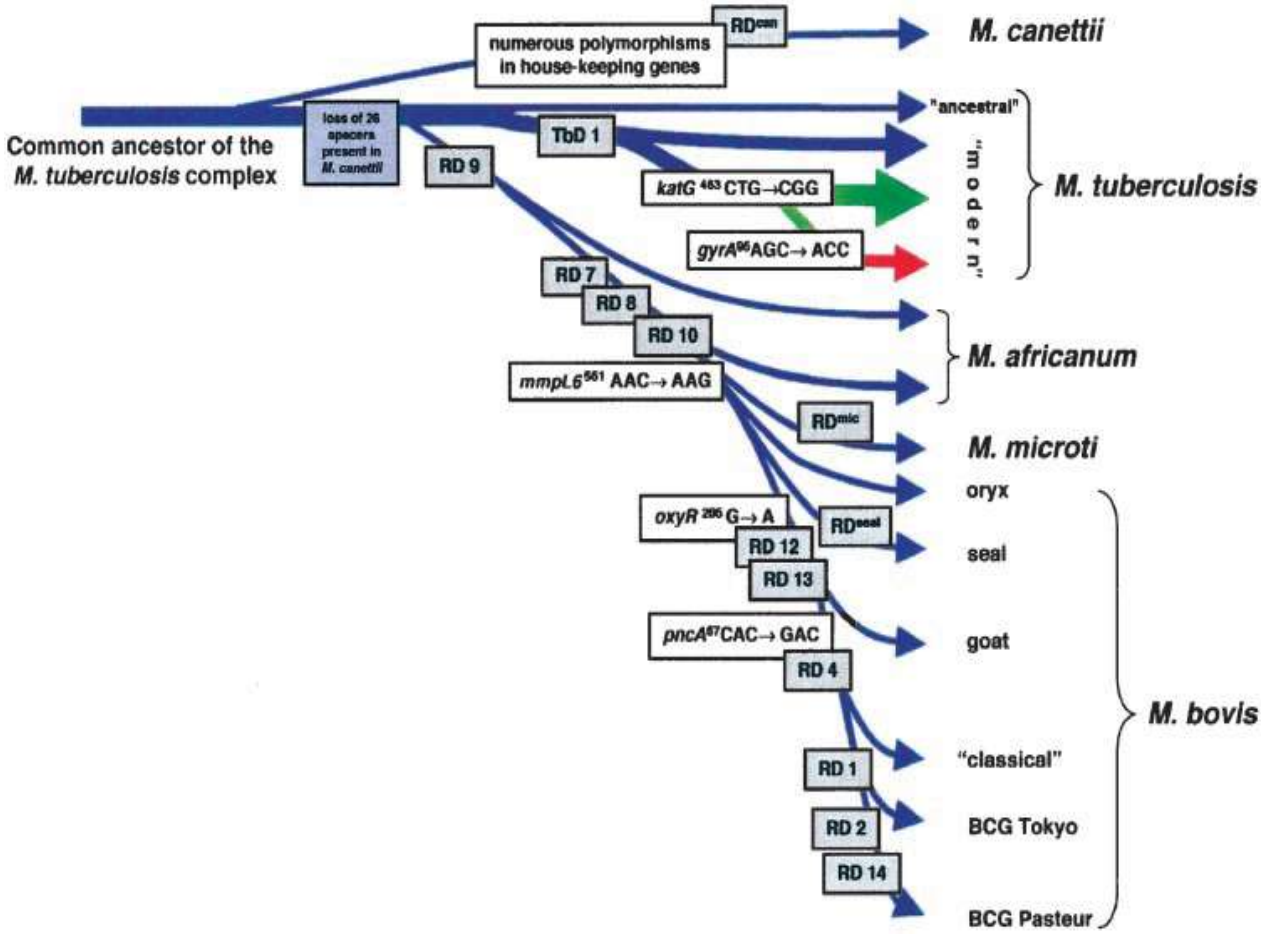

Figure 2.1: Scheme of the proposed evolutionary trend of the tubercle bacilli illustrating successive loss of DNA in certain lineages.

Adapted from Brosch *et al*., 2002

2.18. Molecular typing techniques

There is a wide variety of genotyping markers for *Mycobacterium tuberculosis.* However a few of these exhibitenough discrimination and reproducibility for wide scaleadaptation. Thus a number of DNA fingerprinting methods are based onanalysis of the degree of similarity and distribution of thesevariable elements between isolates, which are used as geneticmarkerelements, usually less than 2.5 kb in size that are widely distributedin most bacterial genomes (Chandler and Mahillion, 2002).

2.18.1. IS6110-RFLP restriction fragment length polymorphism

IS elements are commonlydefined as carrying only the genetic information relatedto their transposition and regulation, unlike transposons,which can also carry genes that encode phenotypic markers(e.g., antibiotic resistance) (Mathema *et al*., 2006). Transposition of IS elements oftencauses gene disruptions that can have strong polar effects andin other cases can lead to the activation or alteration of expressionof adjacent genes due to the regulatory sequences,including promoters and protein-binding sequences (Chandler and Mahillion, 2002;Safi *et al*., 2004).From an evolutionary perspective, there are at least two distincthypotheses explaining the role of IS elements in genomes.One regards the elements as genomic parasites that, on balance, harm their hosts (i.e., bacteria) (Charlesworth *et al*., 1994). In contrast, otherspostulate that IS elements are important to their hosts for adaptive evolution, which is maintained by selection of occasional advantageous IS-derived mutations (Blot, 1994).

IS elements in bacterial species are present in varying numbers of copies: IS*1* in *Escherichia coli* strains is present in 2 to 17 copies, whereas the *Shigella* species contain from 2 to 40 copies (Chandler and Mahillion, 2002). Thierry *et al*. first described IS*6110*, a 1,355-bp member of the IS*3* family that, when intact, is unique to the MTC(Thierry *et al*., 1990). IS*6110* has an imperfect 28-bp inverted repeat at its ends and generates a 3- to 4-bp target duplication on insertion. Although “hot spots” have been noted (regions in the *M. tuberculosis* chromosome where IS*6110* seems to preferentially insert), IS*6110* elements aremore or less randomly distributed throughout the genome,with copy numbers ranging from rare clones lacking any IS*6110* elements to those with 26 copies (Kurepina *et al*., 1998;McHugh and Gillespie, 1998). In 1993,van Embden and colleagues proposed

a standardized method for performing IS*6110*-based Southern blot hybridization analysis(van Embden *et al*., 1993). The recommendation was based on the use of a common restriction endonuclease (PvuII, which cleaves IS*6110*at a single asymmetric site and yields reasonable-size *M. tuberculosis* chromosomal fragments), a hybridization probe (specificto the right side of IS*6110*, whereby each hybridizing band corresponds to a PvuII-PvuII chromosomal fragment with a single IS*6110* insertion), and standardized molecular weight markers (Hermans *et al*., 1990). The concurrent development of software applications that assist in the analysis of the resulting IS*6110*-basedrestriction fragment length polymorphism (RFLP) patterns has allowed for intra- and inter-laboratory comparisons of clinical isolates and the establishment of large national and international strain (and genotype) archives (e.g., Centers for DiseaseControl and Prevention, Atlanta, GA; Public Health Research Institute, Newark, NJ; National Institute of Public Health and Environment, Bilthoven, The Netherlands) (van Soolingen *et al*., 1991; Heersma*et al*., 1998; Kremer *et al*., 1999, Suffys *et al*., 2000).

Initially, the dynamics of IS*6110* transposition juxtaposed with the stability required for use in epidemiologic investigations was a cause for concern. However, when strains were cultured in vitro (liquid media) for 6 months, in macrophages over a 4-week period, and in a guinea pig model for more than 2 months, their IS*6110*-based RFLP patterns remained stable (van Soolingen *et al*., 1991; Cave *et al*., 1994). These studies attest to the stability of IS*6110* over short time periods while transposing over longer time intervals. The IS*6110* transposition half-life (*t*1/2) (the period over which the IS-specific hybridization pattern does not change), taken from sequentially positive culture with sampling intervals ranging from days to months, was estimated to be between 3 and 4 years (Yeh *et al*., 1998;de Boer *et al*., 1999). Warren *et al*., (2002) investigated the stability of IS*6110* banding patterns in serial *M. tuberculosis* isolates collected from patients living in areas of high TB incidence and noted a half-life of 8.74 years when a constant rate of change was assumed (Warren *et al*., 2002). The authors note that the rate may be composed of the high rate of change seen during the early disease phase (*t*1/2 = 0.57 years), when the mycobacterial replication rate is presumably high, and the lower rate in the late disease phase (*t*1/2 = 10.69 years), when bacterial doubling times are longer during or after

treatment. Therefore, they conclude that the observed IS*6110* stability is strongly influenced by the time between onset of disease and sample collection. Another investigation of serial patient isolates used deterministic and stochastic simulation models to estimate an IS half-life of 2.4 years for a strain that has 10 IS*6110* copies (Rosenberg *et al*., 2003). Indeed, IS*6110* transposition, which is a replicative processand half-life, may be heavily dependent on strain-specific in vivo replication rates, host-pathogen interactions, or anatomical properties. Nonetheless, IS*6110*-based RFLP patterns seem to be sufficiently stable (and polymorphic) for studying TB transmission dynamics at the local or population level and over time. For instance, Lillebaek *et al*. used IS*6110* genotyping to demonstrate endogenous reactivation of TB after over 30 years of latency (Lillebaek *et al*., 2002).

The utility of any molecular epidemiologic method in population analysis, in addition to adequate stability/polymorphism, is reliant on sufficient biomarker-specific diversity of isolates. Assignment of a genotype is strengthened when there is adequate background strain diversity. In a population-based study in New Jersey, Bifani *et al*. (1999) noted that approximately one-third of the 1,207 clinical isolates subjected to IS*6110*- based RFLP analysis were unique (or "orphans") to the sample, while a third of the isolates were categorized into 11 major strain groups that consisted of isolates from 10 or more patients (Bifani *et al*., 1999). Presumably there is a discrete number of distinct strain types circulating within any given population; classifying a genotype as rare or unique is heavily dependent on the isolate sampling schemes and the size and diversity of the reference database.

2.18.2. Polymorphic GC-Rich Repetitive Sequence(PGRS)

Like IS*6110*-based RFLP analysis, polymorphic GC-rich repetitive sequence (PGRS) genotyping, first described by Ross *et al*., is a Southern blot hybridization technique that utilizes the PGRS-specific probe (a 3.4-kb fragment of the PGRS sequence) cloned in plasmid pTBN12 (Ross *et al*., 1992). When pTBN12 is used on AluI-digested DNA, it can distinguish strains from unrelated cases of TB and demonstrate identical banding patterns for isolates from epidemiologically related cases (Ross *et al*., 1992; Yang *et al*., 1996). In fact, isolates clustered by IS*6110*-based RFLP analysis were further discriminated by

PGRS typing (Chaves *et al*., 1996). This is particularly the case when IS*6110* low-copy-number strains are further analysed by PGRS genotyping (Rhee *et al*., 2000; Yang *et al*., 2001). This method, like IS*6110* genotyping, is resource intensive, but unlike the IS*6110* system, the hybridization patterns generated by PGRS typing are often too complex to computerize for standardization and analysis.

2.18.3.Spacer oligonucleotide typing (spoligotying).

After IS*6110*-based RFLP analysis, spacer oligonucleotide typing (spoligotyping) is the most commonly used PCR-based technique for sub-speciating *M. tuberculosis* strains (Groenen *et al*., 1993). *M. tuberculosis* complex strains contain a distinct chromosomal region consisting of multiple 36-bp direct repeats (DRs) interspersed by unique spacer DNA sequences (35 to 41 bp).Two forms of genetic rearrangements have been observed: one type consists of variation in one or a few discrete, contiguous repeats plus spacer sequences (DVRs), which is probably driven by homologous recombination between adjacent or distant chromosomal DRs; the other is driven by transposition of IS*6110*, which is almost invariably present in the DR locus of *M. tuberculosis* complex strains (van Embden *et al*., 2000). As a result of these events, some spacers may be deleted from the genome. Spoligotyping is based on the detection of 43 interspersed spacer sequences (originally identified in laboratory strain H37Rv and *M. bovis* BCG vaccine strain P3) in the genomic DR region of *M. tuberculosis* complex strains. Additional spacers in this region have been reported (van Embden *et al*., 2000). Membranes spotted with 43 synthetic oligonucleotides are hybridized with labeled PCR-amplified DR locus of the tested strain, resulting in a pattern that can be detected by chemi-luminescence (Kamerbeek *et al*., 1997). The results are highly reproducible, and the binary (present/ absent) data generated can be easily interpreted and computerized and are amenable to intra-laboratory comparisons.

Anedition of the international spoligotyping database, SpolDB4, contains 1,939 different spoligotypes (ST) identified worldwide that are organized into large ST families (Brudey *et al*., 2006). Spoligotypefamilies are nominated based on the common motif of deletedspacers. A web-based program has been developed toplace spoligotypes into ST families (Vitol *et al*., 2006). Spoligotyping, unlike IS*6110* genotyping, which requires

approximately 2 g of bacterial DNA, can be performed with considerably less DNA and in a fraction of the time; it also allows genotyping of boiling prepared or impure DNA, nonviable specimens, paraffin-embedded material, and material from slides of Ziehl-Neelsen stainings (van der Zanden *et al*., 1998; Driscoll *et al*., 1999; Qian *et al*., 1999). In some instances, spoligotyping can distinguish among members of the *M. tuberculosis* complex based on the species-specific presence/absence of spacers (Kamerbeek *et al*., 1997; Heyderman *etal.*, 1998). It is thought that DR regions irreversibly lose spacers due to homologous recombination or IS*6110* transposition events and cannot gain additional DNA fragments. Of note, deletions of DRs and spacers can occur multiple times and independentlyin unrelated strains, leading to convergent evolution, i.e., the appearance of identical spoligopatterns in phylogenetically unrelated *M. tuberculosis* strains (Warren *et al*., 2002). Although spoligotyping can be a powerful method to study the molecular epidemiology of *M. tuberculosis*, its discriminatory power in general is inferior to that afforded by IS*6110*- based RFLP analysis (Kremer *et al*., 1999). Strains having identical spoligotype patterns yet distinct IS*6110* fingerprint profiles are often encountered (van Embden *et al*., 2000;Bifani *et al*., 2001;Mathema *et al*., 2002).

2.18.4. Variable Number Tandem Repeat (VNTR) and Mycobacterial Interspersed Repetitive Units (MIRU) analysis

Frothingham and Meeker- O'Connell (1998) performed a systematic analysis of VNTR loci in *M.tuberculosis* complex strains and found 11 loci comprising five major polymorphic tandem repeats (MPTR) (A to E) and six exact tandem repeats (ETR) (A to F) ranging in size from 53 to 79 bp (104). Since then, additional VNTR loci have been reported (Hermans *et al*., 1992; Goyal *et al*., 1994; Magdalena *et al*., 1998; Namwat *et al*., 1998;Smittipat and Palittapongarnpim, 2000; Skuce *et al*., 2002; Kovalev *et al*., 2005). Supply *et al* (2000)identified 41 VNTR of mycobacterial interspersed repetitive units (MIRU) (tandem repeats of 40 to 100 bp) located in mammalian-like minisatellite regions scattered around the chromosome of H37Rv, CDC1551, and AF2122/97 (Supply *et al*., 2000; Mazars *et al*., 2001), including loci 4 (VNTR0580) and 31 (VNTR3192), which correspond to ETR D and E, respectively (Frothingham *et al*., 1998). Twelve of the 41 MIRU loci were selected for genotyping of *M. tuberculosis* clinical isolates and were

reported in a 12-digit format correspondingto the number of repeats at each chromosomal locus(Supply *et al*., 2000; Mazars *et al*., 2001).

The digitized data generated by MIRU-VNTR profilingis highly amenable to inter- and intra-laboratory comparisons (Mathema *et al*., 2006).As additional *M. tuberculosis* VNTR loci have been included,the various nomenclatures from one laboratory toanother have created some confusion (Mathema *et al*., 2006). As such standardization of the VNTR nomenclature based on the four digits of the locus position on the H37Rv genome has been proposed (Smittipat *et al*., 2005). The discriminatory power of MIRU-VNTR analysis is typically proportional to the number of loci evaluated; in general, when only the 12 loci are used, it is less discriminating relative to IS*6110* RFLP genotyping for isolates with high-copy-number IS*6110* insertions but more discriminating than IS*6110*RFLP genotyping for isolates with low-copy-number IS*6110*(Mathema *et al*., 2006). When more than 12 loci are used, or MIRU analysis is combined with spoligotyping, the discriminatory power approximates that of IS*6110* RFLP analysis (Mathema *et al*., 2006). A comparative study of genotyping methods aimed at evaluating novel PCRbased typing techniques found VNTR analysis to have the greatest discriminatory power among amplification-based approaches (Kremer *et al*., 2005). MIRU-VNTR genotyping has been used in a number of molecular epidemiologic studies, as well as to elucidate the phylogenetic relationships of clinical isolates (Sola *et al*., 2003; Supply *et al*., 2003;Sun *et al*., 2004; Warren *et al*., 2004; Kremer *et al*., 2005b). VNTR analysis has also been used to evaluate *M. bovis* transmission (Roring *et al*., 2004).

A high-resolution MIRU-VNTR genotyping system using an automated sequencer and PCR primers tagged with one of four fluorescent dyes (FAM, NED, VIC, and HEX) has been developed, allowing amplification of four different loci simultaneously by multiplex PCR.VNTR loci have a variable range of alleles; for example,within the 12 MIRUs, MIRU loci 2 (VNTR0154) and 24 (VNTR2687) have mostly 1 or 2 copies, while VNTR3820 can have from 3 to 32 copies (Cowan *et al*., 2002;Sola *et al*., 2003; Sun *et al*., 2004;Smittipat *et al*., 2005). Likewise, the discriminating capacity of a given locus, the molecular clock, or variability in alleles also varies extensively among the loci. For example MIRU10 (VNTR0960) has been found to be the most polymorphic, having

mostly 1 to 7 copies or up to 12 alleles in the *M. tuberculosis* collections analyzed (Cowan *et al*., 2002;Sun *et al*., 2004).

Variability at specific MIRU loci often depends on the sample collection (e.g., nationwide, population based, or convenience sampling), geographic origin, and inherent genetic diversity of the strains. For example VNTR2059 has been found to be polymorphic in some studies but not in others (Cowan *et al*., 2002;Smittipat *et al*., 2005). An alternative selection of VNTRs should consider the intrinsic differences and variability within different genetic groups and the endemicity or predominance of clones in specific geographic and demographic populations. The use of different sets of VNTR from one collection to another would hamper the ease of inter-laboratory analysis, one of the advantages of VNTR analysis (Mathema *et al*., 2006). Broadly increasing the overall number of loci for genotyping would increase the cost and labour required for analysis and complicatesanalysis and interpretation, not to mention reducing enthusiasm for routine epidemiological investigations. Presently, there is a concerted effort to select a better combination of VNTR for genotyping (Supply *et al*., 2006). Fifteen of 29 MIRU-VNTR were selected, and 800 clinical isolates of diverse origin were analyzed for discriminatory power relative to IS*6110* genotyping (Mathema *et al*., 2006).

2.18.5. Single-Nucleotide Polymorphisms SNP

As extensive comparative genomic analysis of *M. tuberculosis*has revealed remarkable DNA conservation between chromosomes, noted genetic polymorphisms at the nucleotide level have provided researchers with markers to differentiate clinical isolates as well as to study the phylogenetic relatedness of clinical strains (Mathema *et al*., 2006). Both non-synonymous single-nucleotide polymorphisms (nsSNP) and synonymous SNP (sSNP) provide useful genetic information that can be applied to differentiate *M.tuberculosis* strains; however, they address different biologic questions (Mathema *et al*., 2006). In general, non-synonymous polymorphisms create an amino acid change that might be subject to internal or external selection pressure (Mathema *et al*., 2006). As such, non-synonymous changes in drug resistance-determining genetic loci can result in phenotypic drug resistance. Accordingly, *M. tuberculosis* resistance to anti-tuberculosis agents nearly always correlates with genetic alterations (non-synonymous point mutations, small

duplications, or deletions)in resistance-conferring chromosomal regions (Table 2.6)(Ramaswamy *et al*., 1998; Rengarajan *et al.*, 2004;Maus *et al*., 2005; Zhang *et al*., 2005). nsSNP in genes that confer drug resistance can aid in understanding the nature and spread of resistance between and within populations. In contrast, synonymous changes, which are considered functionally neutral, do not alter the amino acid profile (Mathema *et al*., 2006). These neutral alterations, when in structural or housekeeping genes, can provide the basis to study genetic drift and evolutionary relationships among mycobacterial strains (Mathema *et al*., 2006).

2.18.6. Genomic deletion analysis

Comparative genomic analysis of strains H37Rv and CDC1551 has revealed large-sequence polymorphisms (LSP) in addition to SNP (Fleischmann *et al*., 2002). LSP are thought to mainly occur as a result of genomic deletions and rearrangements rather than through recombination following horizontal transfer (Brosch *et al*., 2001). In the absence of horizontal gene transfer, deletions are irreversible and often unique events and therefore have been proposed for genotyping as well as for constructing phylogenies (Brosch *et al*., 2002; Goguet et al., 2004;Tsolaki *et al*., 2004). It was found that up to 4.2% of the entire genome can be deleted in clinical isolates compared to the genome of laboratory strain H37Rv (Tsolaki *et al*., 2004). Brosch and colleagues were able to discern the *M. tuberculosis*complex by deletion analysis by showing that the majority of deletions are not the outcome of independent events but rather are scars of successive deletions (Brosch *et al*., 2002). Once a deletion occurs in the progenitor strain, the specific deletion can serve as a genetic marker for the genotyping progenies of this strain. For instance, deletion of TbD1 (for "*M. tuberculosis* specific deletion 1," a 2,153-bp fragment) was identified in all modern *M. tuberculosis* strains; in contrast, ancestral strains tested have this locus present (Sun *et al*., 2004).

Studies of genomic LSP have indicated that deletions are not always randomly distributed in the chromosome but tend to be aggregated (Kato-Meada *et al*., 2001; Tsolaki *et al*., 2004). Some loci are "hot spots" for DNA deletions and can occur independently in unrelated strains or lineages. Some chromosomal deletions are associated with IS transposition; this is particularly true of loci which are hot spots for IS*6110* insertions,

such as in the RvD5 and DR regions (Brosch *et al*., 2002; Sampson *et al*., 2003). For other deletions (such as TbD1), the correlation with IS elements has not been determined. Deleted sequences can include putative open reading frames as well as intergenic regions and housekeeping genes (Kato-Meada *et al*., 2001; Brosch *et al*., 2002). Using deleted fragments as genetic markers, this analysis can be performed by a simple PCR-based method or by automated GeneChip techniques (Tsolaki *et al*., 2004).

A summary of the methods currently used to study the molecular epidemiology of TB is presented in table 2.6.

Table 2.7: Evaluation of methods currently used to study the molecular epidemiology of TB adapted from Mathema *et al*., 2006

Typing technique	Advantages	Limitations
IS*6110*	Gold standard for the molecularepidemiology of MTC*a* strains Patterns can be computerized with specialized software. Widely utilized; hence, much data available for comparison Biological clock (biomarker stability) has proven to be very adequate for the study of transmission Extensive diversity in patterns for isolates with IS*6110* insertions. Membranes can be rehybridized with other probes, e.g., for IS mapping or deletion analysis. Mixed infection readily detected by varying intensity of the hybridization bands Applications include molecularepidemiology, evolutionary andphylogeny studies, and detectionof laboratory error/crosscontamination.	Requires subculturing and DNA isolation Slow turnaround time (30–40 days) Process is laborious. Cannot be used to reliably type isolates with ≤ 6 IS*6110* insertions Poor portability: interlaboratory comparative analysis of RFLP patterns can be tedious. Strains with no IS*6110* insertion (rare)
Spoligotyping	Simplest technique for MTCstrain genotypingData are presented in binary format,allowing inter- and intralaboratorycomparisons.Commercial hybridizationmembranes available for thesimultaneous analysis of 45samples. Standardized analysis for 43 spacers. Can be performed directly on celllysate; no DNA purificationrequired. Can be performed on nonviablebacteria. Two large databases available forcomparative analysis (seecomments). Applications: ideal for a first-stepanalysis of *M. tuberculosis*,particularly in regions with diversepopulations; molecularepidemiology; and detection oflaboratory error/crosscontamination	Less discriminatory than IS*6110* RFLPanalysis and MIRU-VNTR (12 and15 loci). Cannot recognize mixed infections Less informative in regions with predominant or endemic strains; e.g.,W-Beijing in China, Southeast Asia,and Russia
MIRU-VNTR (12 loci)	Rapid, high-throughput techniquefor MTC strain genotyping.Better resolution than spoligotyping. Digitized results (number of copiesof each repeat) are very portable. Well suited for large-scale genotyping. Can be performed directly on celllysate; no DNA purificationrequired. Manual analysis possible by 12individual PCR amplificationsfollowed by gel electrophoresis. Automated analysis possible withfluorescence-tagged PCR primersand capillary separation(sequencer) or nondenaturinghigh-performance liquidchromatography. Labelled primers allow for multiplexPCRs: 4 reactions of 3multiplex each.Can be used to identify mixedinfections. Applications include molecularepidemiology, the	Less discriminatory than IS*6110*RFLP genotyping. Combined biological clock of 12-locusMIRU-VNTR too slow for the studyof endemic strains. Similar patterns may be found indistinct lineages

	potentialfor real-time genotyping, andhigh-throughput typing.	
Deletion mapping and deligotyping	Irreversible genetic marker usedHigh throughput with microarrayanalysis. Reverse line probe withhybridization membrane possible. Results can bedigitalized. Multiplex PCR for 43 loci available. Single-deletion analysis can identify*M. bovis*BCG. Applications include phylogenetic/evolutionary studies, facilitation ofgenome structure-function studiesand host-pathogen interactionsbased on specific genomicdeletions, and molecularepidemiology	Not yet standardized Representative target deletions need tobe determined. Technique has yet to be evaluated indifferent settings
Insertion site mapping and insertion site typing (Insite)	Very precise determination ofstrain relatednessStrain-specific markers can be usedfor rapid identification of aparticular strain or strain family. Amplification-based investigation forrapid detection. Highly informative when studyingstrain relatedness and clonality. Applications: best suited forconfirmation of clustering ofstrains; also phylogenetic studiesand molecular epidemiology.	Need to predetermine IS*6110*flanking regions. For Insite, need to amplify andimmobilize target DNA onmembrane firstLaborious
SNP analysis	Most-precise information on strainsbased on sequencing ofpolymorphic lociHigh resolution. Some selected SNP can behighly informative. Technique can be automated forlarge-scalegenotyping.Applications include phylogeneticand population geneticinvestigations, molecularepidemiology, studies of drugresistance, and research onhost-pathogen interactions	Requires extensive genomic sequencingof multiple chromosome targets

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CHAPTER THREE

ISOLATION OF *MYCOBACTERIUM SPECIES* IN MILK AND MILK PRODUCTS CONSUMED IN PARTS OF OYO STATE

3.1 **Introduction**

The increasing demand for protein in the developing countries as a result of malnutrition and among the immune-compromised individuals dictate that animal protein especially from cattle such as milk and meat should be safe and wholesome for human consumption. Nevertheless, zoonotic diseases such as BTB caused by *M. bovis* are still being transmitted from cattle to humans through the ingestion of infected animal products such as milk (Cosivi *et al*., 1998; Zinsstag *et al*., 2006; Abubakar *et al*., 2011)in the developing countries mainly because pasteurization of milk is rarely observed and/or not enforced.

In most African countries as well as in Nigeria, consumption of unpasteurized milk is a regular practice in urban, peri-urban and rural areas where milk form part of the daily meals for most individuals (Ayele *et a*l., 2004). Milk is seldom pasteurised in pastoral societies, and, even if soured, it can still contain infective levels of mycobacteria (Kazwala *et al*., 1998; Coetzer and Tustin, 2005).

Traditionally in Africa, cows suckle their calves and are milked at the same time. Usually the cow is separated from the calf for about 12 hours, either at night or, more usually, during the day and milked as the calf is returned to her. An infected cow could produce milk containing mycobacteria, or cough infected droplets in the direction of the milker (Coetzer and Tustin, 2005). Animals in traditional African farming systems are seldom culled and there is a greater chance for chronic tuberculosis in old cows, particularly those subjected to stress (Michel *et al*., 2004) and therefore there is the risk of humans contracting the disease through the consumption of contaminated milk.

Early researches carried out in Northern Nigeria have confirmed the isolation of *M. bovis* in one (9.1%) out of 11 milk samples screened and atypical mycobacteria from 11 (4.2%) out of 262 market-based locally fermented milk "nono" sampled (Idrisu and Schnurrenberger 1977; Shehu, 1992). While recent studies conducted in Ibadan on milk from cows brought for sale at the cattle market and those awaiting slaughter in Bodija Municipal abattoir isolated mycobacterial species from 11 and 6 samples revealing the prevalence of 11.3% (n=53) and 5.7% (n=105) respectively (Cadmus and Adesokan, 2007; Cadmus *et al.*, 2008). The species isolated were *M. bovis*, *M. tuberculosis* and *M. africanum*; these were also isolated some decades ago by Idrisu and Schnurrenberger (1977).

Related studies in Kaduna State reported a prevalence rate of 1.7%, even as 1.4% of freshly drawn milk and 2.2% of "nono" samples in Makurdi were positive for *M. bovis* (Okaiyeto *et al*., 2008; Ofukwu *et al*., 2008) while a prevalence of 1.25% was observed in milk of pastoral cattle in Niger State. Meanwhile, Abubarkar (2007) reported that out of 20 milk samples collected from tuberculin positive animals screened in the FCT Abuja, 18 were positive by Ziehl-Neelsen (ZN) and 15 by culture on Lowenstein Jesen medium (LJ). These have shown that the human population of this country who relish the consumption of milk and its products are at risk of contracting the disease.

This study was therefore aimed to:

i. Isolate mycobacteria species from the milk of pastoral cattle, fermented milkand ready-to-eat cheese in parts of Oyo State.
ii. Characterize the species of the mycobacteria isolated from (i) above.

3.2 Materials and methods

3.2.1. Study design:

A cross-sectional study was used for the study of the milk and milk products.

3.2.2. Study Area: Oyo State Nigeria

Oyo State is located in southwestern Nigeria and is bounded by Ogun, Kwara, Osun States and the Republic of Benin.The climate is equatorial notably with dry and wet seasons and relatively high humidity. The dry season lasts from November to March while the wet

season starts from April and ends in October. Average daily temperature ranges between 25 °C (77.0 °F) and 35 °C (95.0 °F), almost throughout the year and it is well drained with two grazing reserves in Wasimi and Igangan where cattle graze throughout the year. The climate in the state favours the cultivation of crops like maize, yam, cassava, millet, rice, plantain, cocoa tree, palm tree and cashew. There are a number of Government Farm Settlements in Ipapo, Ilora, Sepeteri, Eruwa, Ogbomosho, Iresaadu, Ijaiye, Akufo and Lalupon (Oyo State Government, 2014).The state which is homogeneous has a population of about 4.5million(NPC, 2006) and predominantly occupied by Yoruba people. The state has the third highest TB prevalence in Nigeria, with about 6000 TB cases per annum.The TB prevalence in the state has increased by 46.5% from 2008 to 2010 and there has been seven MDR-TB cases on treatment as at 2010 (Nigeria Tuberculosis Fact Sheet, 2012).

3.2.3. Sample size

Based on the expected BTB prevalence for milk and cheese as previously sited (Cadmus and Adesokan, 2007 = 11.3%, we arrived at a minimum of 154 samples.

$$n=Z_{\alpha}^{2}\times p \times (1-p)/d^{2}$$

Where:

For the fresh milk samples

n= sample size

Z_{α}= value of the standard distribution corresponding to a significance level of α (1.96 for a 2-sided test at 0.05 level).

P= expected prevalence in the population = 0.113

d= absolute precision = 0.037

$n= 1.96^{2}\times 0.113 \times (1-0.113)/0.037^{2}$ $n= 3.8416\times 0.113 \times 0.887/0.0014$

n= 269

(Thrusfield, 1997).

For the cheese and sour milk samples

n= sample size

Z_{α}= value of the standard distribution corresponding to a significance level of α (1.96 for a 2-sided test at 0.05 level).

P= expected prevalence in the population = 0.113

d= absolute precision = 0.03618

n= 1.96^2× 0.113 × (1-0.113)/0.03618^2 n= 3.8416× 0.113 × 0.887/0.001309

n= 294

For the sour *"nono"* milk samples

n= sample size

Z_α= value of the standard distribution corresponding to a significance level of α (1.96 for a 2-sided test at 0.05 level).

P= expected prevalence in the population = 0.113

d= absolute precision = 0.0506

n= 1.96^2× 0.113 × (1-0.113)/0.0506^2 n= 3.8416× 0.113 × 0.887/0.002506

n= 150.406

3.2.4. Sample collection

Fresh milk

Milk samples were collected from 269 pastoral cattle in the following areas in Oyo State: Wasinmi (89 from 16 herds) in Iwajowa Local Government Area (LGA), Igangan (48 from 4 herds) in Kajola LGA, Igana (80 from 10 herds) in Ibarapa North LGA and Ijaye (52 from 8 herds) in Akinyele LGA where there is high population of livestock and most of the cheese (wara) consumed by the residents of Ibadan, the state capital are produced there (Figure 3.1). The choice of herds from which milk was collected was none random.

Milk samples were collected into 20ml sterile universal sample bottles and placed in cool boxes. The samples were transported at 4ºC to the laboratory and stored at 4ºC until processing at the Tuberculosis and Brucellosis Research Laboratory of the Department of Veterinary Public Health and Preventive Medicine, University of Ibadan.

Cheese

One hundred and fifty (150) cheese samples were collected from cheese processors. That is;five (5) cheese samples were obtained five (5) processors in each of the following rural areas: Igbora, Wasinmi, Ijaye, Igangan, Oyo and Igana.

Another 144 samples were obtained from sellers in 18 market locations within Ibadan Metropolis; comprising (8) cheese samples from each market (Figure 3.2).

Thus; a total of 294 cheese samples (approximately 60g)obtained and placed in sterile polythene sample bags in cool boxes at 4ºC. The same was applicable to those sold at the different market points. These were transported to the laboratory in a cool box and stored at 4ºC until processing.

Sour milk

Sour milk "nunu" samples (30ml) were obtained from 150 vendors from the Akinyele International Cattle Market and Bodija Market both in Ibadan at different occasions. These markets have high population of pastoral females selling the products and buyers often come from different areas of Ibadan to buy and sell to the public.

The "nono" samples were collected from the vendors into 20ml sterile universal sample bottles and placed in cool boxes at 4ºC. The samples were stored in the refrigerator at 4ºC until processing.

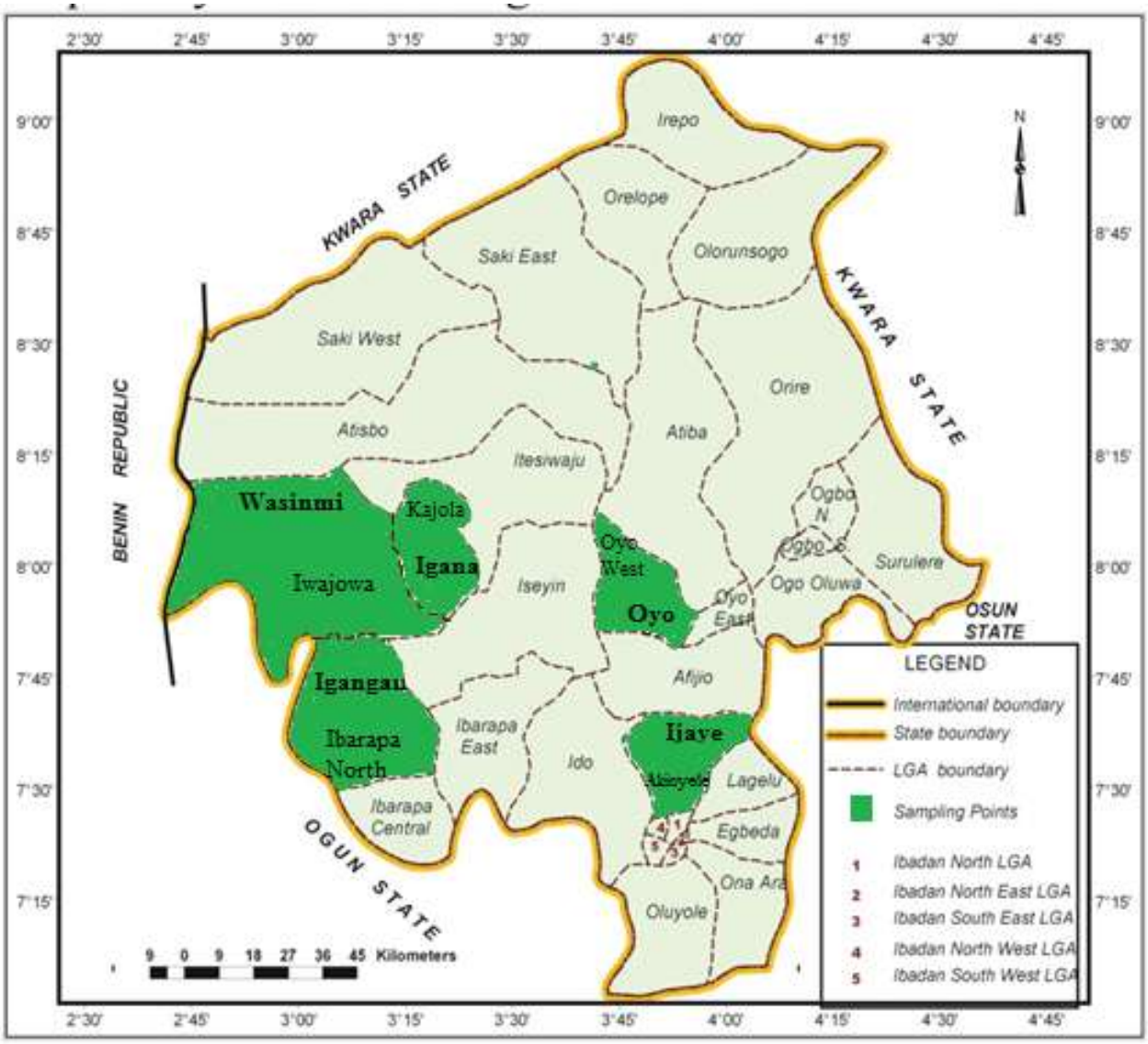

Fig. 3.1: Map of Oyo State showing the location of Pastoral herds

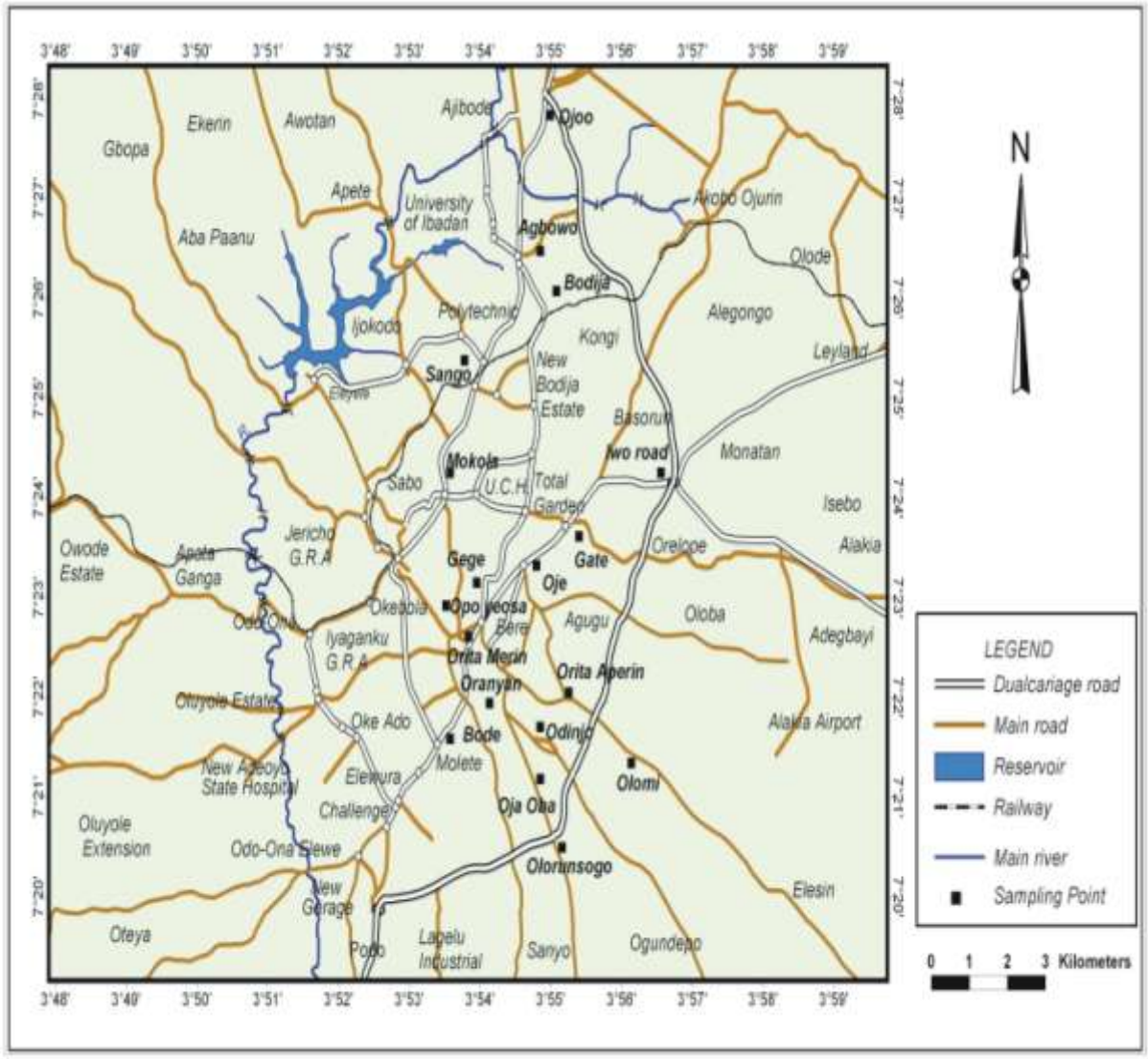

Fig. 3.2 Map of Ibadan Metropolis showing market locations used for sampling wara

3.2.5. Laboratory processing

3.2.6. Processing of samples

The samples were processed using the BD BBL™ Mycoprep™ N-Acetyl L-Cysteine-sodium hydroxide (NALC-NaOH) decontamination method (Kent and Kubica, 1985; BD BBL Mycoprep, 2000) to decontaminate and concentrate the samples to get pellets.

3.2.7. Preparation of the buffer and NALC reagent

BBL™ Mycoprep™ phosphate buffer was prepared by pouring one packet of the buffer powder into a 500ml volumentric flask. The flask was filled to the 500ml line with sterile distilled water. The solution was transfered to a screw capped container and with the cap loosened, it was autoclaved at 121^0C for 15 minutes, cooled at room temperature and the cap tightened. With caution, the screw-cap on the Mycoprep Reagent bottle was loosened and the ampoule containing NALC was located, excess air from the bottle was released and the cap tightened. With the bottle held in an upright position, the bottle was squeezed until the ampoule broke. The bottle was shaken gently to dissolve the NALC, exessive agitation was avoided.

3.2.8. Decontamination test procedure

In a biological safety cabinet, equal volume of activated NALC was added to milk sample in aerosol-free 15ml centrifuge tube with screw cap, the cetrifuge tube was capped and rocked gently until the specimen liquefied. If the sample remained viscous, more NALC reagent was added and the mixing repeated. The mixture was allowed to stand at room temperature for 15 minutes with occassional gentle shaking. Buffer of three times (3x) equivalent of the sample was added to the mixture, mixed by rocking gently and centrifuged, for 15 minutes at 300xg. All the supernatant were carefully decanted, small quantity of phosphate buffer was added and the sediment resuspended. The suspension was used for smear preparation and perfomance of mycobacteriological procedures.

3.2.9. Microscopy (Ziehl-Neelsen staining method)

Loopful smears of final deposits were made on clean, grease-free, duly labelled dry slides. The smear was approximately 20mm by 10mm, corresponding to about 100 oil immerssion fields. Care was taken to ensure that the smears were not thick. The smears

were left to dry naturally in the air before fixing over bunsen flame. The slides were stained by the ZN technique (Appendix 3.0).

The classical ZN method depends on the ability of mycobacteria to retain basic dye even when treated with mineral acid or an acid-alcohol solution (Shresth*a et al*., 2005).

The slides with fixed smears were arranged on staining rack over a sink. Freshly prepared carbol fuchsin was poured over the slides so that the smears were completely covered. The slides were then gently heated from below with a bunsen flame until steam rose and allowed to stay for about five minutes. The stained slides were washed with water under running tap and excess water on the slide was drained by tilting the slides. The slides were then replaced on the rack and the decolorizer (acid-alcohol) was poured over the slide to cover the smears and allowed to act for about three minutes. The slide were washed under the running tap. The counter-stain (methylene-blue) was poured on the slides and left to stay for about one minute before washing with water under running tap. The slides were drained, arranged vertically on a slide rack and allowed to dry naturally. Dried slides were examined under the oil-immerssion objective of a binocular microscope for the presence of acid fast bacilli, which appeared brick red against a blue background (WHO, 2009b).

3.2.10. Isolation of Mycobacteria

Lowenstein-Jensen medium which is an egg-based medium was used for the isolation of *Mycobacterium* species. The media were prepared in duplicate – one containing 0.5% pyruvate for the isolation of *M. bovis* and the other containing glycerol for the isolation of *M. tuberculosis.* The egg medium was prepared by dissolving 37.2g of the L-J medium base powder in 600ml of sterile distilled water, autoclaved and allowed to cool to about 45^0C - 60^0C. Homogenised eggs (thoroughly washed in water and then cleansed with methylated spirit before breaking) was aseptically added, thoroughly mixed and distributed in 10-15 ml volumes in sterile MacCartney bottles and the caps were securely fastened. The medium was inspissated in a slanted position to coagulate the already sterile medium.

The two media types were properly labelled, dated and inoculated in duplicate (one on pyruvate containing medium and the other on glycerol containing medium) with the final

sediment spread evenly on the surfaces of pairs of slopes of L-J mediun using Pasteur pipettes (two drops). All innoculated slants were incubated first in a slanting position for 24 hours to allow for even distribution of inoculum at 37^0C. The bottles were then re-arranged in an upright position to increase incubator space. Bottles were arranged in chronological order to make for easy examination. The bottles were incubated for 6 to 8 weeks

3.2.11. **Examination of mycobacterial culture slants**

All cultures were examined daily for the first seven days after incubation to detect rapidly growing mycobacteria and also to detect contamination. Thereafter, the cultures were examined once a week for eight weeks to detect positive cultures of mycobacteria before adjudging the culture to be negative if there was no growth. Cultures with completeley contaminated surfaces, liquified or discoloured were removed from the incubator, sterilised, discarded.

Colonies from all resultant growths were examined for morphological appearanceand acid-fast properties. Cultures with acid-fast bacilli properties were harvested into broth of 7H9 Middlebrook medium in a microcentrifuge tube and stored at -20^0C until needed for further investigation.

3.2.12. **Identification**

Smears of the colonies were taken and then stained by the ZN technique (Grange, 1988) for examination of acid fast bacilli (AFB).

The presence of AFB and cording of bacilli were indicative of *Mycobacterium* species.

3.2.13. **Molecular identification**

All strains of the mycobacteria obtained were subjected to molecular characterisation using genus and deletion typingbased on polymerase chain reaction (PCR) as well as PCR-restriction enzyme pattern analysis (PRA-*hsp65*) for the confirmation of their identity.

***Mycobacterium* genus typing**

The genus typing was carried out according to the methods of Wilton and Cousins (1992) and SOP CBU0247 (2005). The materials used for the PCR reaction include: HotStarTaq DNA polymerase (Qiagen, Hilden, Germany) (10 µl), Primers including Mycgen-R

100um (0.3 µl), Mycgen –F 100um (0.3 µl), Mycar –R 100um (0.3 µl), Mycint –F 100um (0.3 µl), TB1-F 100um (0.3 µl), TB1-R 100um (0.3 µl) (Table 3.1), sterile water (6.2 µl) and DNA (isolate) (2.0 µl); while DNA Ladder, loading dye, Agarose, 10x TAE runing buffer and ethidium bromide were used for the gel electrophoresis.

The master mix was prepared on the day of use in a sterile eppendorf tube. Into a PCR tube, 18 µl of the mix was aliquoted and in a different area of the laboratory, the DNA templates were added to the respective tubes. The tubes were placed in the PCR machine (Mygene Series Peltier Thermal Cycler Model M696) and amplification was initiated by incubation at 95^{O}C for 10 minutes followed by 45 cycles at 95^{O}C for 1 minute, 61^{O}C for 0.5 minute and 72^{O}C for 2 minutes. After the last cycle, the samples were incubated at 72^{O}C for 10 minutes. Into a weighing boat, 3.0% agarose was measured into 1xTAE buffer, heated in the microwave oven for 1 minute/100ml or until boiling but was not to boiling out till all crystals were dissolved. About 5-10 µl ethidium bromide solution per 150ml agarose gel solution was added. The agarose was allowed to cool to 50^{0}Cbefore the gel was casted. The gel was placed in the tank and the samples together with the DNA ladder were loaded and the electrophoresis was run at 100V.

Table 3.1: Primer used for Genus typing

Primer name	Target gene	Sequence
MYCGEN-F	16S rRNA	5 ’— AGA GGT TGA TCC TGG CTC AG—3 ’
MYCGEN-R	16S rRNA	5 ’— TGC ACA CAG GCC ACA AGG GA—3 ’
MYCINT-F	16S rRNA	5 ’— CCT TTA GGC GCA TGT CTT TA— 3’
MYCAV-R	16S rRNA	5 ’— ACC AGA AGA CAT GCG TCT TG—3 ’
TB1-F	MPB70	5 ’— GAA CAA TCC GGA GTT GAC AA—3 ’
TB1-R	MPB70	5 ’— AGC ACG CTG TCA ATC ATG TA—3’

Key: F= Forward, R=Reverse

Deletion typing

This was carried out as described by Warren *et al*. (2006). The reagents used for the PCR reaction include Q-Buffer, 10xBuffer, 25mMgcl_2, 2.5mMdNTPs, and the primers which include RD1A, RD1B, RD1C, RD4A, RD4B, RD4C, RD9A, RD9B, RD9C,RD12A,RD12B and RD12C. All these with HotStarTag, isolate DNA and distilled water were added together and mixed for the running of the PCR reaction.

Primer design

Primers were designed in silico, according to the previously described DNA sequence of the region of difference (Brosch *et al.*, 2002, Marimiesse *et al.*, 2004 and Mostowy *et al*., 2004). Primer set 1 included RD1, RD4, RD9 and RD12 primers and primer set 2 included $RD1^{mic}$ and $RD2^{seal}$ primers (Table 3.2).

Table 3.2: PCR primer sequence and corresponding amplification product sizes indicating the presence or absence of genomic regions of difference in different members of *Mycobacterium tuberculosis* complex

Primer sequence	RD	*M. canetii*	*M. tuberculosis*	*M. africanum*	*M. microti*	*M. pinnipedi*	*M. caprae*	*M. bovis*	*M. bovis BCG*
AAGCGGTTGCCGCCGACCGAC **CTGGCTATATTCCTGGGCCCGG** **GAGGCGATCTGGCGGTTTGGGG**	1 1 1	RD1 present (146 bp)	RD1 present (146 bp)	RD1 present (146 bp)	RD1 present (146 bp)	RD1 present (146 bp)	RD1 present (146 bp)	RD1 present (146 bp)	RD1 absent (196 bp)
ATGTGCGAGCTGAGCGATG **TGTACTATGCTGACCCATGCG** **AAAGGAGCACCATCGTCCAC**	4 4 4	RD4 Present (172 bp)	RD4 Present (172 bp)	RD4 Present (172 bp)	RD4 Present (172 bp)	RD4 Present (172 bp)	RD4 Present (172 bp)	RD4 absent (268 bp)	RD4 absent (268 bp)
CAAGTTGCCGTTTCGAGCC **CAATGTTTGTTGCGCTGC** **GCTACCCTCGACCAAGTGTT**	9 9 9	RD9 Present (235 bp)	RD9 Present (235 bp)	RD9 absent (108 bp)	RD9 absent (108 bp)	RD9 absent (108 bp)	RD9 absent (108 bp)	RD9 absent (108 bp)	RD9 absent (108 bp)
GGGAGCCCAGCATTTACCTC **GTGTTGCGGGAATTACTCGG** **AGCAGGAGCGGTTGGATATTC**	12 12 12	RD12 absent	RD12 present (369 bp)	RD12 present (369 bp)	RD12 present (369 bp)	RD12 present (369 bp)	RD12 absent (306 bp)	RD12 absent (306 bp)	RD12 absent (306 bp)
CGGTTCGTCGCTGTTCAAAC **CGCGTATCGGAGACGTATTTG** **CAATCAGCCAAGACGAGGTTTG**	1^{mic} 1^{mic} 1^{mic}			$RD1^{mic}$ present (195 bp)	$RD1^{mic}$ absent (127 bp)	$RD1^{mic}$ present (195 bp)			
TCAGCGGTCTCATAGCATTGC **CGGGTTGGGAATGTCAGAAAC** **GCGGCAAGGTACGTCAGAAC**	2^{seal} 2^{seal} 2^{seal}			$RD2^{seal}$ absent	$RD2^{seal}$ present (293 bp)	$RD2^{seal}$ absent (168 bp)			

Warren *et al*., 2006

PCR amplification

Each PCR reaction contained 1µl DNA template, 5 µl Q-buffer, 2.5 µl 10 Xbuffer, 2 µl 25 mM $MgCl_2$, 4 µl 10 mM dNTPs, 0.5 µl of each primer (50 pmol/µl), 0.125 µl HotStarTag DNA polymerase (Qiagen, Hilden, Germany) and was made up to 25 with water. Amplification was initiated by incubation at 95^0C for 15 minutes followed by 45 cycles at 94^0C for 1 minute, 62^0C for 1 minute and 72^0C for 1 minute. After the last cycle, the samples were incubated at 72^0C for 10 minutes. PCR amplification products were electrophoretically fractionated in 3.0% agarose in 1Xtbe pH 8.3 at 6V/cm for 4 hours and visualised by staining with ethidium bromide.

PCR-restriction enzyme pattern analysis (PRA-*hsp65*)

This was carried out as earlier described by Telenti *et al*. (1993) with slight modification.

Amplification

Five microliters of lysate was added to each reaction tube. The composition of the PCR mixture (25, µl) was 50mM (1.25µl) KCl, 5mM (2.5µl) Tris-HCl (pH 8.3), 0.75 mM (2.0 µl) $MgCl_2$, 200uM (0.2 µl) (each) deoxynucleoside triphosphate, 0.25uM (1.0 µl) (each) primer, and 1.25 U (4.0 µl) of Taq polymerase (Inqaba biotec™, South Africa). The reaction was subjected to 45 cycles of amplification (1 min at 94°C, 1 min at 60°C, 1 min at 72°C); this was followed by 10 min of extension at 72°C. Primers Tb 11 (5'-ACCAACGATGGTGTGTCCAT) and Tb12 (5'-CTTGTCGAACCGCATACCCT) amplified a 439-bp fragment between positions 398 and 836 of the published gene sequence.

Restriction analysis

For BstEII digestion, 10.0 µl RI of PCR product was added directly to a mixture containing 0.5 pl (=5 U) of enzyme, 2.5, ul of restriction buffer (5 x buffer B), and 12.0µl of water, and the mixture was incubated for 60 min at 60°C. Similarly, 10.0 µl of product was digested at 37°C in a solution containing HaeIII enzyme, the corresponding buffer (5 x buffer M), and water. Enzymes and buffers were purchased from Fermentas™**.**

Evaluation of restriction patterns

After digestion, 4.0µl of gel loading buffer (0.25% bromophenol blue, 40% sucrose in water) was added, and 10.0µl of the mixture was loaded onto a NuSieve 3:1 agarose gel

(FMC Bioproducts^{TM}). Fragments were visualized by ethidium bromide staining and UV light.

The analyzed strainswere identified by comparing the restriction enzyme fragments patterns with that of the open access PRASITE data base (PRASITE, 1999).

3.3. RESULT

The study reports the isolation of *Mycobacterial* species from fresh milk of pastoral cattle and ready to eat cheese in parts of Oyo State. The individual and herd prevalence of 8.2% (out of 269 cattle) and 42.1% (out of 38 herds) were recorded respectively for fresh milk while 0.3% was observed for cheese.

3.3.1. Results of Ziehl-Neelsen stain and Culture of fresh milk, sour milk (nono) and Cheese (wara).

The result of acid-fast staining of the fresh milk, sour milk (*nono*) and soft cheese (*wara*) collected from pastoral cattle, cheese processors and market locations in parts of Oyo State is presented in Table 3.3.

Of the 269 fresh milk collected from the pastoral cattle, 15.6% (n=42) were acid-fast positive and 69.5% (n=187) were negative. The result for sour milk shows that 11 (7.3%) were acid-fast positive while 139 (92.7%) were negative. Only 5 (1.1%) of the 295 cheese samples were acid-fast positive.

For culture, of the 269 fresh milk sampled, 22 (8.2%) were positive on LJ media (Plate 3.1 and 3.2), 157 (59.3%) were negative; 49 (18.2%) of the culture were overgrown by contaminants. The sour had 5 (3.3%) of the samples showing growth on Lowenstein Jensen media even as 35 (23.3%) of the culture were overgrown by contaminants. While only one (0.3%) of the 295 cheese sample was positive on culture and 47 (15.9%) of the culture were over grown by contaminants Table 3.4.

Table 3.3. Results of Ziehl-Neelsen (ZN) of fresh milk, sour milk and cheese

Type of Sample	**Total Number Sampled**	**Number (%) Positive**	**Number (%) Negative**
Fresh Milk	269	42 (15.6)	187 69.5)
Sour milk (*Nono*)	150	11 (7.3)	139 (92.7)
Cheese (*Wara*)	295	5 (1.7)	290 (98.3)

Table 3.4. Results of culture of fresh milk, sour milk and cheese

Type of Sample	Total Number Sampled	Number (%) Positive	Number (%) Negative	Number (%) contaminated
Fresh Milk	269	22 (8.2)	157 (59.3)	49 (18.2)
Sour milk (Nono)	150	5 (3.3)	110 (73.3)	35 (23.3)
Cheese (Wara)	295	1 (0.3)	247 (83.7)	47 (15.9)

Table 3.5 Factors associated with the isolation of Mycobacterial species from fresh milk and milk product

	Variable	Number sampled	Number positive*	OR (95% CI)	$\chi 2$	
Type of Sample	**Cheese**	**295**	**1 (0.3)**	**1**		
	Fresh milk	269	22 (8.2)	26.2 (3.5 – 195.7)		P < 0.0001
	Sour milk	150	0 (0)			
Herd size	X ≤ 20	52	2 (3.8)	1		
	X ≥ 21	217	20 (9.2)	2.5 (0.6 -11.2)		P = 0.1616
Breeds	Cross	29	2 (6.9)	1		
	White Fulani	161	13 (8.1)	1.2 (0.3-5.6)		
	Red Bororo	26	2 (8.3)	1.3 (0.2-8.6)		P = 0.3140
	Sokoto Gudali	54	5 (9.3)	1.4 (0.3-7.6)		
Location	Igangan	48	0			
	Ijaye	52	2 (0.3)	1		
	Igana	80	8 (10.0)	2.8 (0.6 -13.6)		
	Wasimi	89	12 (13.5)	3.8 (0.8 – 18.2)		P= 0.51 00
	Igangan	48	0			

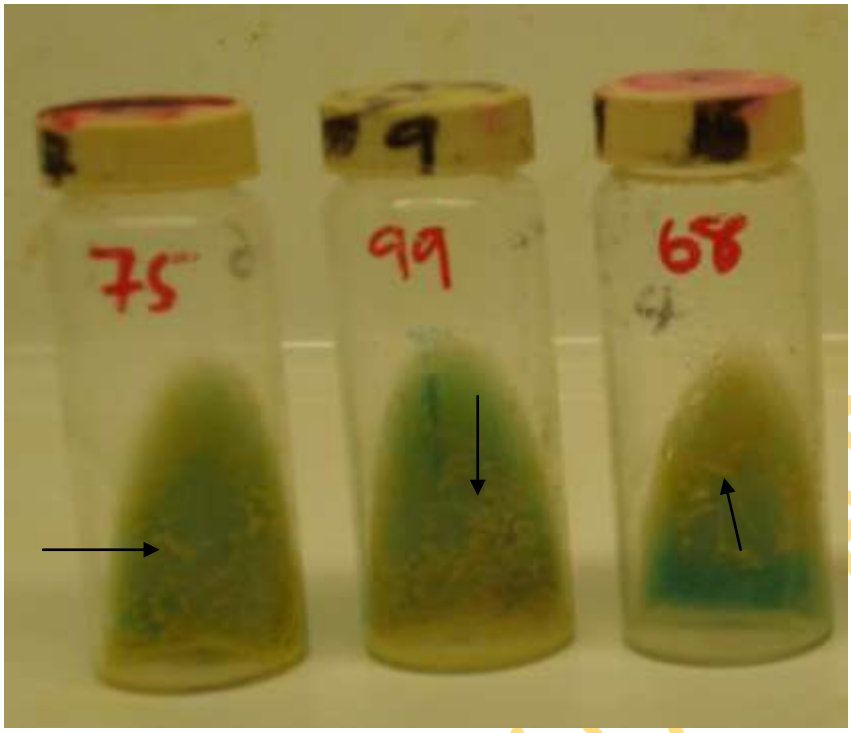

Plate 3.1 Mycobacterial growth on Lowestein-Jenseen medium

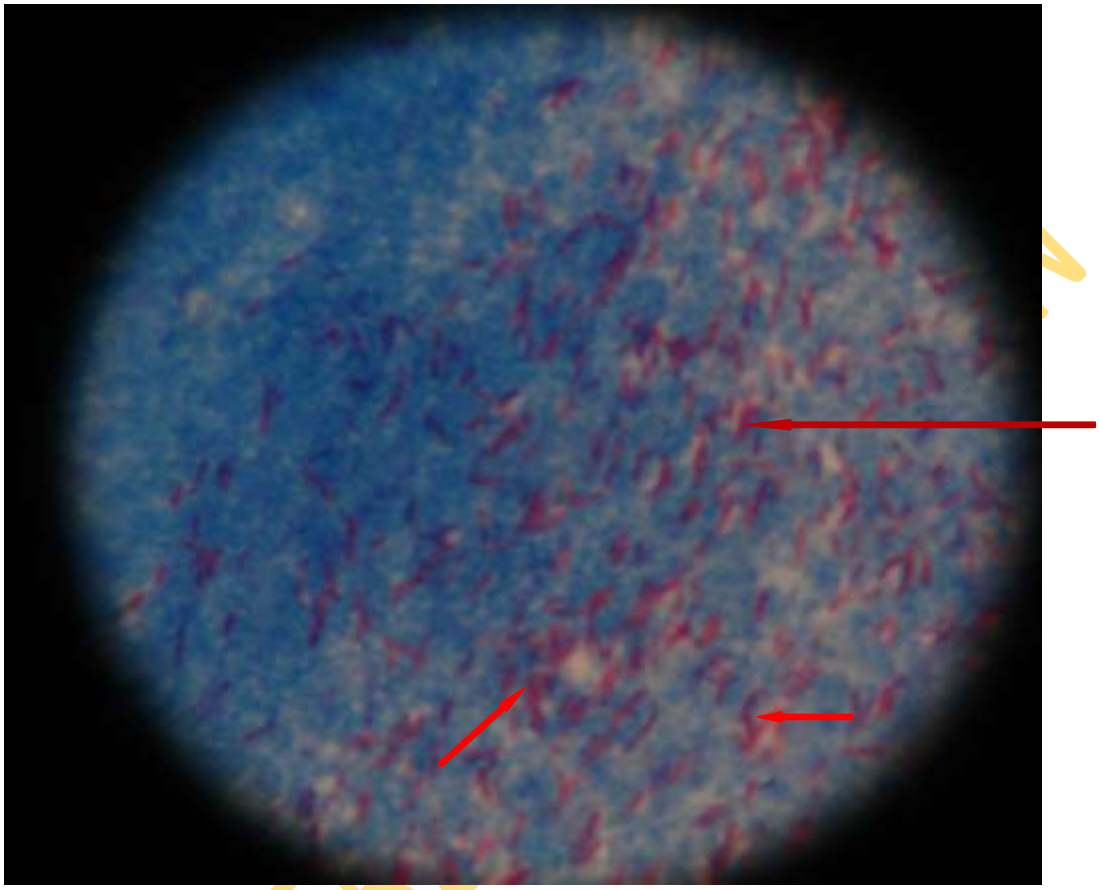

Plate 3.2. Arrows showing acid fast positive bacilli of Ziehl-Neelsen stain of mycobacteria colony on Lowestein-Jenseen medium. At x100 magnification.

3.3.2. Result of Genus and Deletion typing of the strains isolated from fresh milk, sour milk and cheese

Of the 22 fresh milk contaminated with the mycobacteria seven (7) had isolates on both LJ with glycerol and pyruvate; as a result, 29 isolates were obtained. When characterized by Genus typing for confirmation of *Mycobacterium* specie, all the 29 were identified as *Mycobacterium* species of which three (10.3%) were identified as members of the MTC (Plate 3.3 and 3.4). Meanwhile, none of the five strains obtained from the culture of the sour milk samples was identified as *Mycobacterium* species when characterized by Genus typing. While the only strain isolated by culture from the cheese was confirmed to be member of the genus *Mycobacterium* well as when characterized by Genus typing.

On further characterization of the four MTC strains from the fresh milk and cheese 'wara' for species identification by the Deletion typing; two of the strains from milk and the only strain isolated from wara were identified to be all *Mycobacterium africanum*(Table 3.5 and Plate 3.5). This gives the prevalence of 0.8% and 0.3% of *M africanum* in fresh milk of pastoral cattle and wararespectively.

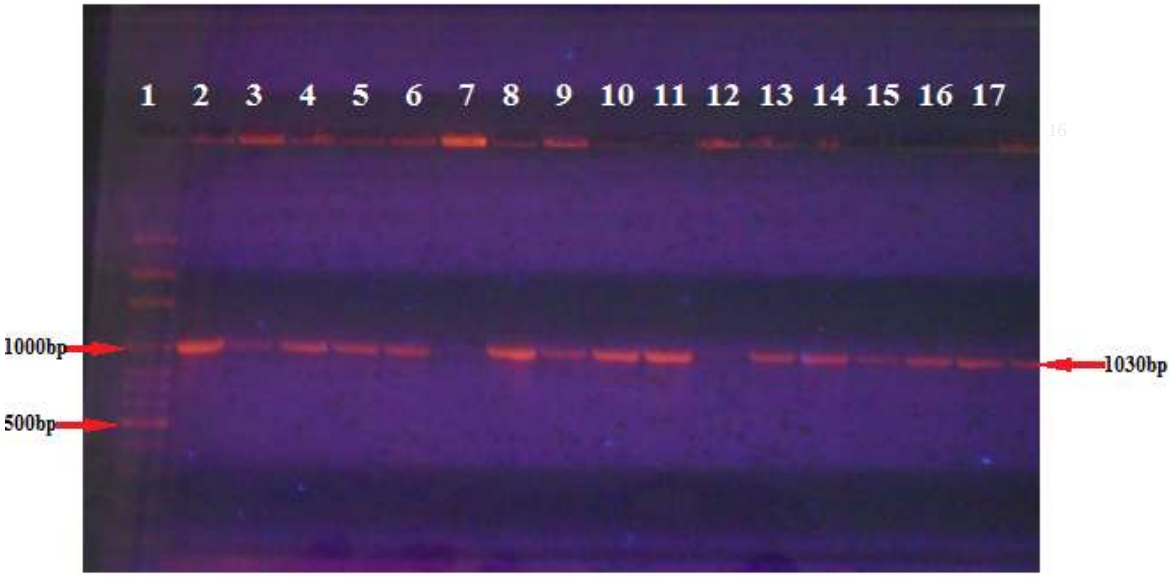

Plate 3.3 Gel electrophoresis separations of PCR products by multiplex PCR genus typing of mycobacteria isolated from fresh milk in Oyo State. Lane 1= 100bp ladder; Lane 2-16 were isolates from fresh milk of pastoral cattle, Lane 7 was negative for genus *Mycobacterium* while all others were positive for *Mycobacterium*.

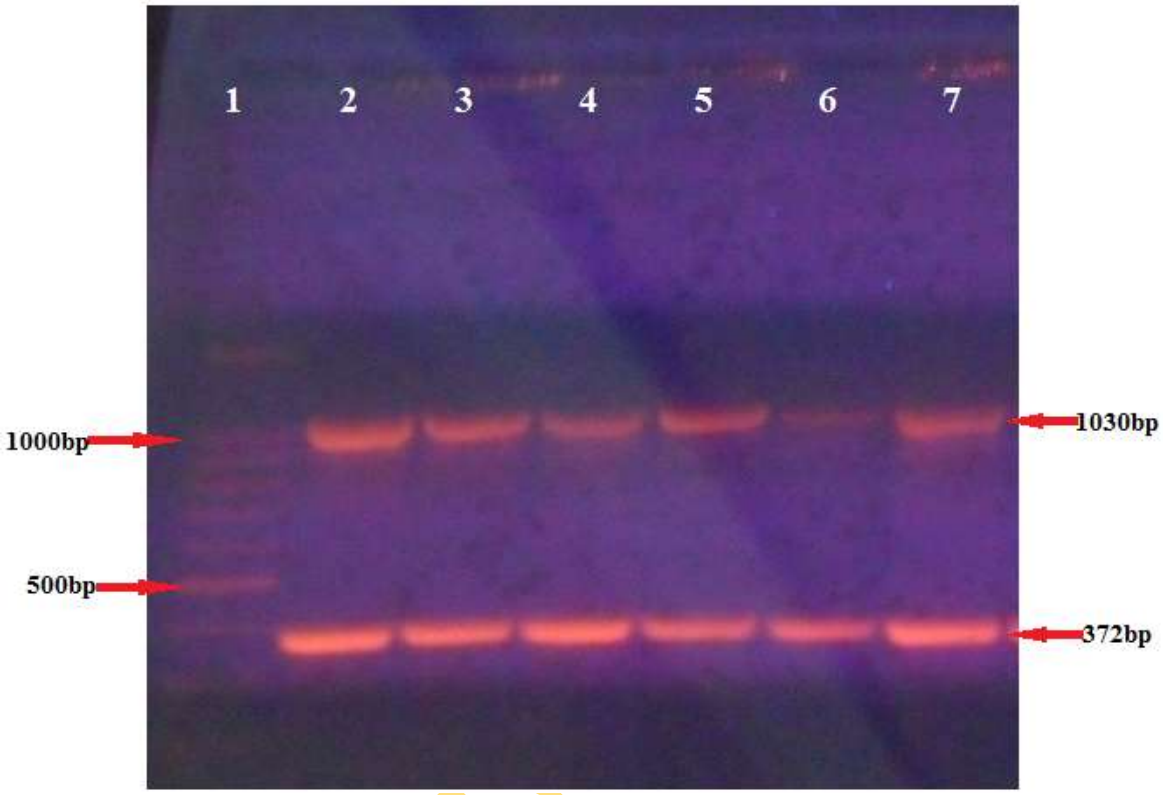

Plate3.4 Gel electrophoresis separations of PCR products by multiplex PCR genus typing of mycobacteria isolated from fresh milk, cheese and humans in Oyo State.Lane 1= 100bp ladder; Lane 2= *Mycobacterium tuberculosis* H37Rv (positive control); Lane 3 and 4 were isolates from fresh milk, Lane 5 was isolate from cheese, Lane 6 and 7 were isolates from butchers.

Table 3.6 Result of Deletion Typing of mycobacteria isolated from fresh milk, cheese and humans in Oyo State

Region of Difference	**Isolate 1**	**Isolate 2**	**Isolate 3**	**Isolate 4**	**Isolate 5**
Sample	Fresh milk	Fresh milk	Cheese (*wara*)	Humans	Humans
RD 1	Present	Present	Present	Present	Present
RD 4	Present	Present	Present	Present	Present
RD 9	Absent	Absent	Absent	Absent	Absent
RD 12	Present	Present	Present	Present	Present
***Mycobacterium* Spp**	*M. africanum*	*M. africanum*	*M. africanum*	*M. africanum*	*M. africanum*

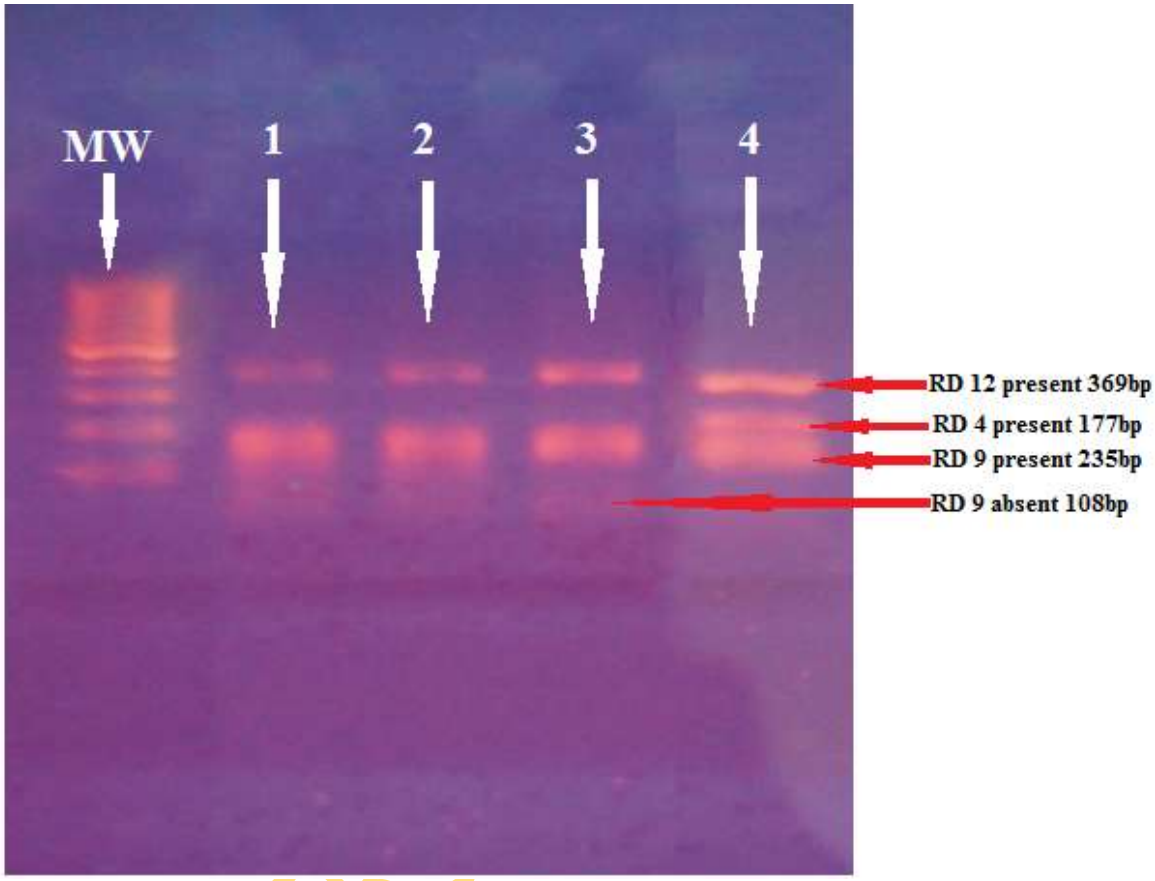

Plate 3.5 Gel picture of PCR products in agarose gel for the Deletion Typing.Showing MW= 100bp molecular weight marker, 1, 2, *M. africanum* from milk, 3 *M. africanum* from wara, 4 = *M. tuberculosis* (H37Rv) control

3.3.3. Result of PCR-restriction enzyme pattern analysis (PRA-*hsp65*)

The result of the PRA-*hsp*65 of the 26 non-tuberculous mycobacteria (NTM) or mycobacteria other than mycobacterium tuberculosis (MOTT) indicated the following *Mycobacterium gordonae* 12 (n=26, 46.2%), *M. senegalense*seven (26.9%), *M.* fortuitumfour (15.4%) and *M. avium* 1 (3.9%); while one of the isolates could not be classified. The fragment sizes of mycobacterial 439bp *hsp*65 PCR products after digestion by Bst EII and HaeIII are presented in Table 3.7, Plates 3.6, 3.7 and 3.8.

Table 3.7 Fragment sizes of mycobacterial 439bp *hsp*65 PCR products after digestion by Bst EII and HaeIII

BstEII Digestion	**HaeIII Digestion**	**Mycobacterial isolates**	**PRAsite Database Result Score**	**Number of isolates**
240/125/85	170/120/70	*Mycobacterium gordonae* type 1	17	12
		Mycobacterium gordonae type 9	22	
230/140/85	200/140/90	*Mycobacterium senegalense* type 4	49	7
230/125/85	140/115/60	*Mycobacterium fortuitum* type 2	9	4
		Mycobacterium avium		1

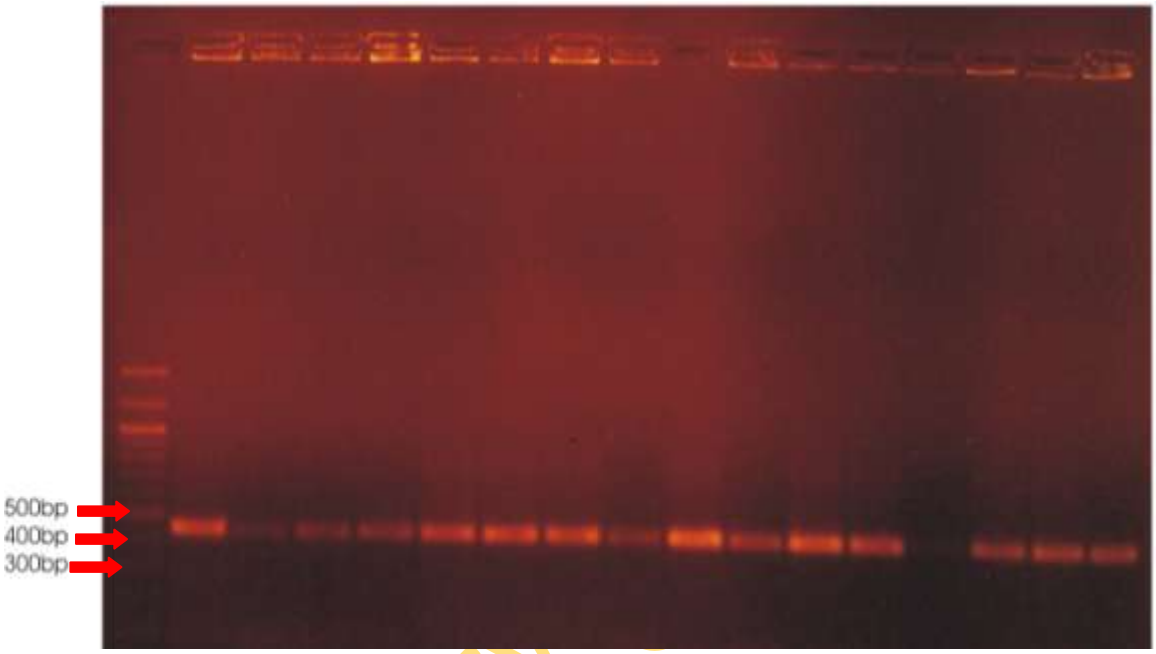

Plate 3.6. Gel picture showing the amplified 437bp fragment of *hsp*65 of the strains isolated.

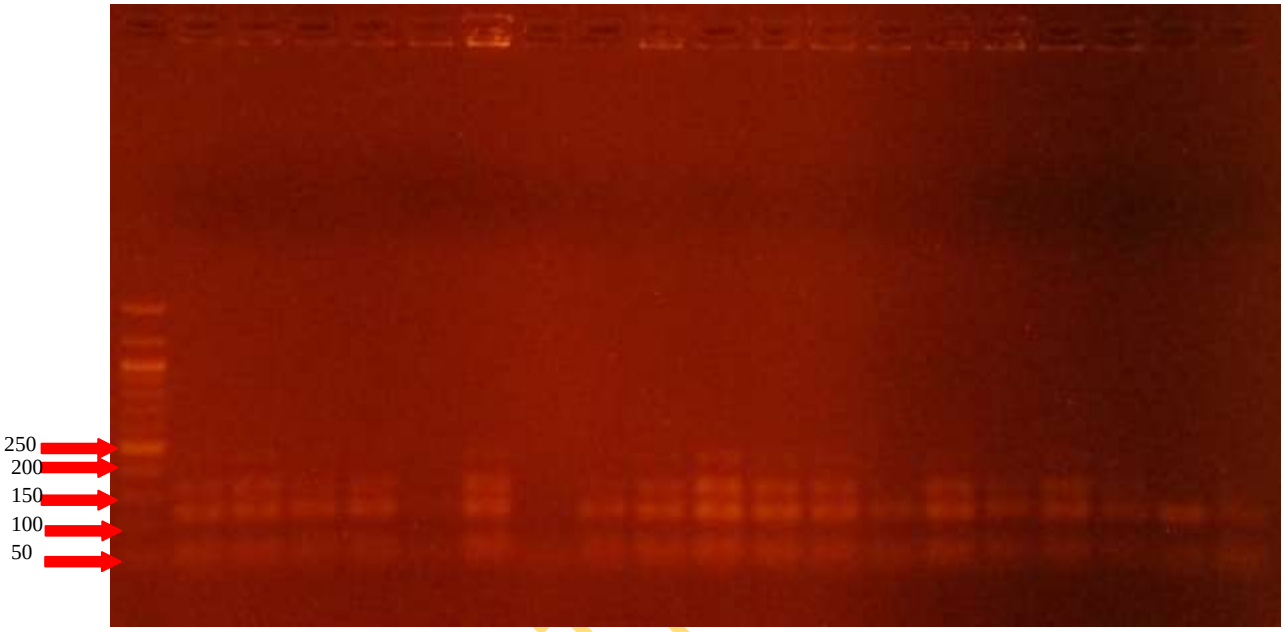

Plate 3.7. Gel picture of PCR products in agarose gel for the PCR-restriction enzyme pattern analysis showing the HaeIII Enzyme Digestion.

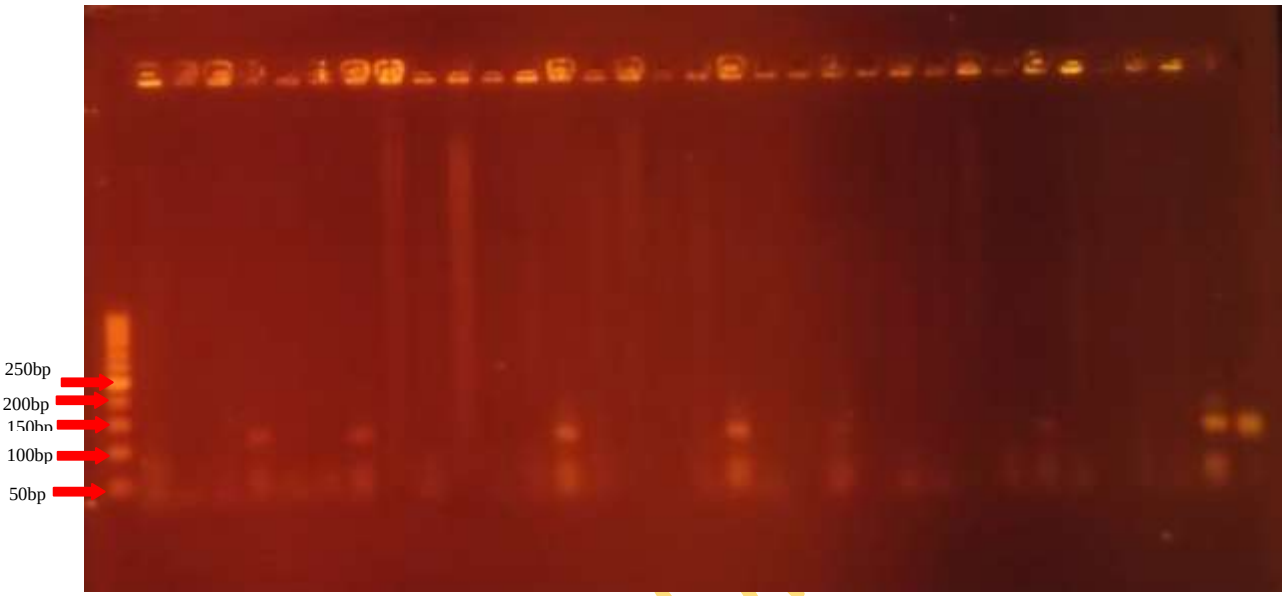

Plate 3.8. Gel picture of PCR products in agarose gel for the PCR-restriction enzyme pattern analysis showing the Bst Enzyme Digestion

3.4 Discussion

This study provides the first data on the individual and herd prevalence, with molecular characterization of *Mycobacterial* species isolated from fresh milk of pastoral cattle and ready to eat cheese in Oyo State. This indicates that mycobacteria infection is present in the cattle population screened and in the country as reported by other workers (Cadmus *et al.,* 2010b; Ibrahim *et al.*, 2010).

A predominance of NTM (89.7%) was observed with only two isolates (10.3%) being MTC (*M. africanum*) which was similar to what Kazwala *et al.,* (1998) observed in Tanzania and Leite *et al.,* (2003) reported in Brazil, the only variation is the species of the MTC isolated which is *M. africanum*. This underscores the importance of NTM in the epidemiology of tuberculosis in cattle and the public health implication of consuming their products.

However, the prevalence of 0.8% of MTC in this study is lower than 5.7% and 11.3% reported on milk from trade cattle in Ibadan (Cadmus and Adesokan, 2007 and Cadmus *et al.,* 2008) as well as 1.25% and 1.70% observed in pastoral cattle in northern Nigeria (Okaiyeto *et al.,* 2008; Ofukwu *et al.,* 2008; Cadmus *et al.*, 2010a). The low prevalence in the present study could, among other, be due to the fact that the study animals were kept in small herds and widely exercise free grazing in the field, which minimises the transmission of mycobacteria contrary to the trade cattleand large size herds that are present in northern Nigeriawhich favours the spread of mycobacteria because of overcrowding and confinement.

In this study, we observed that herd size and location were not significantly associated with the isolation of the mycobacteria. There is a significant association between isolation of mycobacteria and type of sample. Fresh unpastuerized milk is over twenty times more likely to have mycobacteria isolated (OR: 26.2; 95%CI 3.5 – 195.7).

This study also reports the isolation of *M. africanum* (pathogenic mycobacteria) from fresh ready to eat cheese ("wara") in Oyo State. The prevalence observed (0.3%) was lower than 1.67% determined in fresh cheese expended at markets in Mexico (Martinez-Herrera *et al* ., 2013); 4.9% reported in the U.S. (Harris *et al.,* 2007) in fresh cheese from Mexican origin and 3.6% from different cheeses from Greece and the Czech Republic by

bacterial culture (Ikonomopoulos *et al.*, 2005). The reason for this may be that wara undergoes some heating or boiling in its processing while some of the cheese obtained in the US and Mexico were from unpasteurized milk; hence heating may have killed some of the *Mycobacterium* in our samples.

Mycobacterium africanum was the only strain of MTC isolated in this study. This finding supports the premise that cattle are reservoirs of *M. africanum with* fresh milk and wara being a vehicle for its transmission to humans in Nigeria (Cadmus *et al*., 2010b).

The additional *Mycobacterium* species identified are considered potentially pathogenic and cause a variety of clinical manifestations in humans (Leite *et al.*, 2003, Katoch, 2004; Daniel *et al.*, 2011). *M. gordonae* is the least pathogenic and ubiquitous but has been implicated in infections in both immunocompetent and immuno-compromised individuals (Weinberger *et al*., 1992; Maslo *et al*., 1995; Foti *et al*., 2009; Asija *et al*., 2011). *Mycobacterium avium and M. fortuitum* have been isolated from humans with pulmonary infections (Idigbe *et al.*, 1989; Mawak *et al*., 2006; Daniel *et al*., 2011).

So many NTM have been reported in Nigeria (Idigbe *et al.*, 1989; Mawak *et al.*, 2006, Cadmus *et al*., 2010), however, this work to our knowledge is one of the first to report the isolation of *M. senegalense* from fresh milk of cattle in Nigeria. It is the causative agent of bovine farcy, a chronic suppurative granulomatous inflammation of the skin and lymphatics of cattle seen mostly in sub-Saharan Africa (Hamid, 2012) and have been associated with catheter-related hematological infection in a 49-year old woman with non-Hodgkin's lymphoma in Korea and tissue infection in a child after fish tank exposure in Canada (Oh *et al*., 2005; Talavlikar *et al*., 2011).

The fact that no mycobacteria were isolated from sour milk could be due to reduced sensitivity of culture as a result of poor storage quality due to the epileptic power supply and contamination of the samples among others. Another factor the author observed was the adulteration of the sour milk with homemade yoghurts prepared from powdered milk by the sour milk "nunu" vendors in Ibadan.

The predominance of NTM in this study signifies the importance of these species in the epidemiology of tuberculosis in this region and should be considered to be a danger to animal as well as human health as causes of opportunistic infections.

CHAPTER FOUR

ISOLATION OF *MYCOBACTERIAL* SPECIES FROM NASAL SECRETIONS AND FAECES OF SLAUGHTERED CATTLE WITH POSITIVE TUBERCULOUS LESIONS AT BODIJA MUNICIPAL ABATTOIR

4.1. Introduction

Bovine tuberculosis caused by *M. bovis,* an obligate intracellular pathogen is an important chronic infectious respiratory disease of animals and man (Allen *et al*., 2010; Skuce *et al*., 2012). The disease is a chronic contagious respiratory disease of cattle which spreads horizontally within and between species, by aerosol and ingestion (O'Reilly and Daborn, 1996) and is characterized by formation of nodular granulomas known as tubercles in any organ in most species. Cattle is the main reservoir host, while others include pigs, goats, dogs, camels, foxes, deer, badgers, oppossums lions, baboons, African buffalo and man (Beit *et al*., 2005).

The primary route of transmission of infection within and between species is by the airborne route and is facilitated by close, prolonged contact between infected and healthy humans or animals through the exchange of respiratory secretions (O'Reilly and Daborn 1995; Kempf *et al*., 2005) in crowded and less ventilated settings. However, other routes of transmission such as congenital and vertical transmission have been recorded (Collins and Grange 1983; Neil *et al.*, 1994). The distribution of pathological lesions in cattle, which are concentrated at the respiratory system and associated lymph nodes (Phillip *et al*., 2003), in addition to a very low dose of *M. bovis* needed to initiate infection from a respiratory tract challenge (Wells *et al*., 1948; Dean *et al*., 2005) suggest that the disease is spread mostly by airborne transmission.

Occasionally, infection may be acquired orally (VerCauteren*et al*., 2008). When generalized tuberculosis occurs in cattle, *M. bovis* may be shed in mucus, milk, urine and faeces.

The faecal shedding of *M. bovis* by tuberculous cattle was first reported by Schroedr and Cotton (1907) and by Reynolds and Beebe (1907). Others have also confirmed the presence of the pathogen in the feaces of reactor cattle (Williams and Hoy 1927; Maddock 1936; Reuss1955; Neil *et al*., 1988). *M. bovis*, shed in the faeces of tuberculous cattle or in other excretions, may be capable of surviving for long periods in stored cattle slurry or in the environment on pastures (Walter *et al*., 2012). The prolonged survival of pathogenic bacteria increases the risk of animals as well as farmers acquiring the infection from environmental sources (Scanlon and Quinn, 2000; Humblet *et al*., 2009).

Acid-fast bacteria such as *M. bovis* are well known for their ability to survive dehydration, fluctuations in temperature, moderate pH changes and exposure to sunlight. In addition, the mycobacterial cell wall has a high lipid and wax content which confers hydrophobicity, rendering these bacterial cells less susceptible to many chemical disinfectants than other conventional bacteria (Russell, 1996). When such contaminated dung is used to fertilize farmlands, it would results to source of infection to farmers that cultivate such lands.

This study was therefore aimed at:

i. Determining if *M. bovis* is being excreted in the nasal secretions and faeces of slaughtered cattle with positive tuberculous lesions at Bodija abattoir.
ii. Identifying the risk factors for the excretion of *M. bovis* by the cattle slaughtered at Bodija abattoir.

4.2 Materials and Methods

4.2.1. Study Area

The Bodija Municipal abattoir, the largest abattoir in Oyo State was the study site. The abattoir services almost the entire Ibadan city which comprises eight Local Government Areas; in addition, large numbers of cattle are slaughtered daily to service the need of the human population of the city. Like every other abattoir in the country, it has little or no

facilities and is compounded by overcrowding, deplorable water supply system and lack of proper effluent disposal system (Nwanta *et al*., 2008). There is a high probability of zoonotic transmission of TB in the abattoir due close contact between humans and cattle as a result of the high level of human activity and the volume of animal slaughter going on in the abattoir.

4.2.2 Study design:

An abattoir based cross-sectional study was carried out to determine the prevalence and risk factors associated with the excretion of mycobacteria in nasal secretions and fecal samples of slaughtered cattle with positive lesions of TB in the abattoir.

4.2.3. Sample size:

Based on the bovine TB prevalence for slaughtered cattle = 8.8% (Cadmus, 2006).

$n=Z_{\alpha}^{2}\times p \times (1-p)/d^{2}$

Where: -

n= sample size

Z_{α}= value of the standard distribution corresponding to a significance level of α (1.96 for a 2-sided test at 0.05 level)

P= expected prevalence in the population= 0.088

d= absolute precision = 0.0498

$n= 1.96^{2}\times 0.088 \times (1-0.088)/0.0498^{2}$ $n= 3.8416\times 0.088 \times 0.912/0.00248$

n= 124.35

(Thrusfield, 1997).

Nasal and faecal samples were collected from a total of 124 cattle with suspected tuberculous lesions.

4.2.5. Sampling technique

Inclusion/exclusion criteria

Cattle sluaghtered at the Bodija Municipal Abattoir with granulomatous lesions suspected to be infected with BTB were included in the study while those without granulomatous lesions were excluded.

Sample Collection

Visits were made to the abattoir between 8.00 am and 12.00 pm on Mondays, Wednesdays and Fridays weekly for 10 months to inspect cattle slaughtered in the abattoir and any carcass with suspected lesions of tuberculosis were noted and samples were taken for culture in the laboratory. Information such as breed, sex and age of the animal were recorded. Nasal secretions as well as faecal samples were collected from suspected diseased cattle using sterile swab and sterile universal bottles respectively. The slaughtered cattle with suspected lesions of TB were sampled consecutively until the desired sample size was attained; furthermore, the total number of cattle slaughtered on the days of sampling was noted.

To collect nasal samples, sterile swab was inserted into the nostrils of the suspected animal, rubbed against the nasal cavity, and then placed back into its container. Equally,about 10 grams of fecal sample was collected from thesame animal into sterile universal bottle and transported in a cool box at 4^{O}Cto the Tuberculosis and Brucellosis Research Laboratory of the Department of Veterinary Public Health and Preventive Medicine, University of Ibadanfor further processing.

4.2.6. Sample Processing

The samples were processed based on the Becton Dickinson digestion and decontamination procedure. Using a sterile, 20ml centrifuge tube with a screw cap, the swab stick containing the nasal secretion was placed in the test tube containing 2ml of sterile distilled water and equal amounts of activated NALC (N-acetyl-L-cysteine)-NaOH was added and shaken thoroughly to mix. The mixture was allowed to stand at room temperature for 15 min with occasional gentle shaking. Prepared phosphate buffer was then added to the 10 ml mark on the centrifuge tube and mixed, which was followed by centrifugation for15 min at 3000 x g. The supernatant was carefully decanted. The suspension was smeared on the slide for Zeihl Nelsen staining and microscopy while some were inoculated onto two Lowenstein-Jensen (L-J) slopes (one with pyruvate and the other with glycerol) and incubated at 37ºC for at least 6 weeks with weekly observation for signs of growth.

The faecal samples were processed by adding about 100mg of the faeces to the centrifuge tube containing sterile distilled water to which equal amount of NALC-NaOH was added and shaken thoroughly to mix. The samples were further processed as that of the nasal secretion above.

4.2.7. Identification

Identification was done by observation of growth on the L-J with glycerol and pyruvate media based on the criteria for distinguishing *M. tuberculosis* and *M. bovis*(Marks, 1976). Smears of the colonies were taken and then stained by the ZN method (Grange, 1988) for examination of AFB. The presence of AFB and cording of bacilli were indicative of *Mycobacterium* species.

4.2.8. Molecular identification

All isolates indicative of the mycobacteria obtained from culture were subjected to further characterization by genus typing using polymerase chain reaction (PCR) for the confirmation of their identity.

Genus typing

The genus typing was carried out according to the methods of Wilton and Cousins (1992) and SOP CBU0247 (2005). The materials used for the PCR reaction include: Hotstar Tagmastermix (10 µl), Mycgen-R 100um (0.3 µl), Mycgen –F 100um (0.3 µl), Mycar –R 100um (0.3 µl), Myint –F 100um (0.3 µl), TB1-F 100um (0.3 µl), TB1-R 100um (0.3 µl), sterile water (6.2 µl) and DNA (isolate) (2.0 µl); while DNA Ladder, loading dye, Agarose, 10Xtae Runing buffer and Ethinidium Bromide were used for the Gel Electrophoresis.

The master mix was prepared on the day of use in a sterile eppendorf tube. Into a PCR tube, 18 µl of the mix was aliquoted and in a different area of the laboratory, the DNA templates were added to the respective tubes. The tubes were placed in the PCR machine and amplification was initiated by incubation at 95^0C for 10 minutes followed by 45 cycles at 95^0C for 1 minute, 61^0C for 0.5 minute and 72^0C for 2 minutes. After the last cycle, the samples were incubated at 72^0C for 10 minutes. Into a weighing boat, 3.0% agarose was measured into 1xTAE buffer, heated in the microwave oven for about 1

minute/100ml or until boiling but was not allow toboil out. Heating of the agarose continued until all crystals are dissolved. About 5-10 µl Ethidium Bromide solution per 150ml agarose gel solution was added. The agarose was allowed to cool to 50^0C before the gel was casted. The gel was placed in the tank and the samples together with the DNA ladder were loaded and the electrophoresis was run at about 100V.

PCR-restriction enzyme pattern analysis (PRA-*hsp65*)

This was carried out as described by Telenti *et al*., 1993 with slight modification.

Amplification

Five microliters of lysate was added to each reaction tube. The composition of the PCR mixture (25, µl) was 50mM (1.25µl) KCl, 5mM (2.5µl) Tris-HCl (pH 8.3), 0.75 mM (2.0 µl) $MgCl_2$, 200uM (0.2 µl) (each) deoxynucleoside triphosphate, 0.25uM (1.0 µl) (each) primer, and 1.25 U (4.0 µl) of Taq polymerase (Inqaba biotec™, South Africa). The reaction was subjected to 45 cycles of amplification (1 min at 94°C, 1 min at 60°C, 1 min at 72°C); this was followed by 10 min of extension at 72°C. Primers, Tbll (5'-ACCAACGATGGTGTGTCCAT) and Tb12 (5'-CTTGTCGAACCGCATACCCT), amplified a 439-bp fragment between positions 398 and 836 of the published gene sequence.

Restriction analysis

For BstEII digestion, 10.0 µl RI of PCR product was added directly to a mixture containing 0.5 pl (=5 U) of enzyme, 2.5, ul of restriction buffer (5 x buffer B), and 12.0µl of water, and the mixture was incubated for 60 min at 60°C. Similarly, 10.0 µl of product was digested at 37°C in a solution containing HaeIII enzyme, the corresponding buffer (5 x buffer M), and water. Enzymes and buffers were purchased from Fermentas™ (Telenti *et al*., 1993).

Evaluation of restriction patterns

After digestion, 4.0µl of gel loading buffer (0.25% bromophenol blue, 40% sucrose in water) was added, and 10.0µl of the mixture was loaded onto a NuSieve 3:1 agarose gel (FMC Bioproducts™). Fragments were visualized by ethidium bromide staining and UV light.

4.3. RESULT

4.3.1 Result of Ziehl-Neelsen ZN, culture and genus typing of nasal secretions and faeces of slaughtered cattle with positive tuberculous lesions

Of the 124 of the nasal secretions collected from cattle with lesions of tuberculosis slaughtered in Bodija Municipal abattoir; 11.3% (n= 14) were acid fast positivein the same vein; of the 124 faecal sample collected, 3.2% (n= 4) were acid fast positive. Interestingly; 1.61% (n= 2) of the animals sampled turned out to be acid fast positive on both nasal secretions and faecal samples.

For culture, of the 124 nasal secretions collected, 5.7% (n=7) were positive on culture, while only1.6% (n= 2) of faecal sample were positive (Table 4.2).

The risk factors identified to be associated with the excretion of mycobacteria were age and predilection sites (Tables 4.3, 4.4); though there was no significant association with the secretion of mycobacteria and sex, age, breed as well as organs condemned.

The result of molecular characterization revealed the seven isolates of of mycobacteria from the nasal secretion to be four *M. gordonae*, two *M. fortuitum* and one *M. senegalense*, while the two from faecal sample were *M. fortuitum*.

Table 4.1 Result of Ziehl-Neelsenstain of nasal secretions and faecal samples

Type of sample	Total number (n)	No (%) positive	No (%) negative
Nasal secretions	124	14 (11.3)	110 (88.7)
Faecal samples	124	4 (3.2)	120 (96.8)

Table 4.2 Results of culture of nasal secretions and faecal samples

Type of sample	Total No (n)	No (%) positive	No (%) negative	No (%) contaminated
Nasal secretions	124	7 (5.7)	107 (82.3)	15 (12.1)
Faecal samples	124	2 (1.6)	98 (79.0)	24 (19.4)

Table 4.3 Risk factors associated with acid-fast stain of nasal secretions and faeces of slaughtered cattle with positive tuberculous lesions

Variable	Total (%)	No. positive NS (%)	OR (95% CI)	$\chi 2$ (P value)	No. positive FS (%)	OR (95% CI)	$\chi 2$ (P value)
Sex							
Female	114 (91.9)	12 (10.5)	1	0.058	3 (2.6)	1	1.330
Male	10 (8.1)	2 (20.0)	2.3	(0.809)	1 (10.0)	4.1	(0.249)
Age							
Young adult	48 (38.7)	2 (4.2)	1	1.820	0		2.523
Adult	76 (61.3)	12 (15.8)	4.3	(0.177)	4 (5.3)		(0.112)
Breed							
White Fulani	91 (73.4)	10 (11.0)	1		3 (3.3)	1	1.139
Red Bororo	27(21.8)	4 (14.8)	1.4	3.105	1 (3.7)	1.3	(0.987)
Kuri	2 (1.6)	0		(0.376)	0		
Cross	4 (3.2)	0			0		
Organs Condemned							
Lungs	90 (72.6)	8 (8.9)	1	2.042	2 (2.2)	1	0.526
Liver	5 (4.0)	1 (20.0)	2.6	(0.728)	0		(0.971)
Lungs & liver	9 (7.3)	3 (33.3)	5.1		2 (22.2)	12.6	
Lungs & heart	3 (2.4)	2 (66.7)	20.5		0		
Lungs, spleen, diaphragm & mesenteric lymph node	8 (6.5)	0			0		
Others	9 (7.3)	0			0		

NS= Nasal secretion, FS= faecal sample

Table 4.4 Risk factors associated with isolation of Mycobacterial species from nasal secretions and faeces of slaughtered cattle with positive tuberculous lesions

Variable	No. tested	No. positive NS (%)	OR (95% CI)	χ2 (P value)	No tested FS	No. positive FS (%)	OR (95% CI)	χ2 (P value)
	Nasal secretions				**Feacal samples**			
Sex								
Female	99	6 (6.1)	1	0.613	92	2 (2.3)		**3.361**
Male	10	1 (10.0)	1.7	(0.736)	8	0		**(0.3)**
Age								
Young adult	42	1 (2.4)	1	2.346	40	0		**1.254**
Adult	67	6 (9.0)	4.0	(0.310)	60	2 (3.3)		**(0.534)**
Breed								
White Fulani	79	6 (7.6)	1.9	9.41	71	1 (1.3)	1	**2.123**
Red Bororo	24	1 (4.2)	1	(0.152)	23	1 (4.2)	3.2	**(0.9)**
Kuri	2	0				0		
Cross	4	0				0		
Organs Condemned								
Lungs	80	3 (3.8)	1	**8.343**		77	2 (2.6)	**1.956**
Lungs & liver	9	1 (11.1)	2.8	**(0.401)**		9	0	**(0.982)**
Liver	5	1 (20.0)	6.4			5	0	
Lungs & heart	3	2 (66.7)	51.3			3	0	
Lungs, spleen, diaphragm & mesenteric lymph node	6	0					0	
Others	7	0				6	0	

NS= Nasal secretion, FS= faecal sample

4.4.**Discussion**

Our findings revealed that among the cattle screened, no MTC was isolated from the nasal secretions and faeces, however,NTM were isolated from the nasal secretions and feces of these cattle. These findings are in consonance with an earlier study (Ayanwale *et al*., 1989) which also reported the recovery of mycobacterial species (*M. bovis*) from nasal exudates of naturally infected cattle in Nigeria. In relation to the findings of this study, previous studies in Asia (Srivastava *et al*., 2008), South America (Jalil *et al*., 2003) and Europe (Mcllroy *et al*., 1986) have also reported evidence of the excretion of mycobacterial species (*M. bovis*) in the nasal exudates of naturally infected cattle.

However, no strain of MTC was isolated from the nasal secretions and feacal samples of cattle screened in this study. This finding is in contrast to previous studies carried out in the same study location (Cadmus *et al*., 2008; Cadmus *et al*., 2010; Jenkins *et al*., 2011), which reported the isolation of *M. tuberculosis* and *M. bovis* from livestock. A plausible explanation for this is the fact that cattle sampled in this study were at the chronic state of the disease and had developed granulomatous lesions which limit the dissemination of the bacteria (Kao *et al.*, 2007; Menin *et al.*, 2013). In addition, the unhygienic condition of the study site is another possible factor that can be implicated. Most abattoirs in Nigeria are usually unkempt, making them to remain perpetually in a dirty state (Nwanta *et al*., 2008). This, coupled with the nature of the sample collected (nasal secretion and faecal sample) is a likely source of introduction of contaminants into the samples, which in the long run interfere with the growth of the desirable organisms in culture.

In addition, our findings reported the isolation of seven NTM species from nasal secretions and faecal samples of cattle. This is consistent with previous studies in other parts of Africa (Diguimbaye-Djaibe *et al*., 2006; Oloya *et al*., 2007; Shihun, 2008; Berg *et al.*, 2009) which also reported the isolation of different species of NTM from nasal exudates and faecal samples. The isolation of NTM from cattle in this study is a cause for public health concern owing to the fact that these NTMs have been implicated in the presence of granulomatous lesions in cattle. Going by the fact that these cattle are in close contact with humans, livestock workers are at higher risk of cattle-to-human transmission

considering the zoonotic importance of the disease. Incidentally, this could eventually negatively affect the precise diagnosis of TB in humans, due to the similiarity in clinical manifestations which NTM and classical TB infections elicit.

Furthermore, this study recorded the highest number of isolates being recovered from the nasal secretion, implicating lungs as the organ that is most affected. This suggests that the major route of exposure to mycobacterial infection in the cattle examined might be aerogenous. The fact that mycobacterial infection on the lungs is mainly via the intranasal route has been supported by various evidences (Dean *et al.*, 2005; Cassidy, 2006; Menin *et al.*, 2013). These reports demonstrated that bovine infection with *M. bovis* via the intranasal route result in pathology confined to the respiratory pathway. Our findings further revealed no significant association between isolation of mycobacterial species and sex, age, breed as well as organs of cattle.

Finally, the fact that no MTC was isolated from nasal exudates and feaces of cattle screened in this study should not negate the importance of the NTM isolated. Non tuberculous mycobacteria have been implicated in pathology as these mycobacteria have been reported to be recovered from cattle with granulomatous lesions (Berg *et al*., 2009). The public health implication of these findings is that NTM are very important in the epidemiology of tuberculosis in both cattle and human populations in the study area. In most developing countries where diagnosis of TB is basically via smear microscopy, species identification of the mycobacteria that causes disease is very important in optimizing robust therapeutic package geared towards effective control of the disease.

CHAPTER FIVE

ZOONOTIC TUBERCULOSIS AND ANALYSIS OF RISK FACTORS ASSOCIATED WITH TRANSMISSION AMONG THE OCCUPATIONALLY EXPOSED IN OYO STATE, NIGERIA.

5.1 Introduction

Tuberculosis caused by *M. tuberculosis* complex affects about 8.4 million people and has caused over 1.5 million deaths annually; hence an important global health concern (WHO, 2010). Tuberculosis remains a major public health concern due to its high risk of person-to-person transfer as well as high level of morbidity and mortality (Ortu *et al*., 2006). Most of cases of TB transmitted from man-to-man are due to *M. tuberculosis,* the human tubercle bacillus; however, the proportion due to *M. bovis* is unknown (Acha and Szyfres, 1987).

Historically, TB caused by*M. bovis* in humans is associatedwith consumption of unpasteurized milk andthis is still the most important route of exposurein developing countries (Wilkins *et al*., 2008). In addition, inhalation of airborne droplets containing mycobacteria from animals with pulmonary TB especially in crowded and less ventilated settings can be the potential route of transmission from animals to man and vice versa (Challu, 2007). In countries with a relatively high prevalence of bovine tuberculosis (BTB) in cattle, abattoir and farm workers are thegroups most exposed to infection (Ayele *et al*., 2004). Furthermore, it has been observed that a high rate of *M. bovis* infection is commonly associated with occupational exposure (Challu, 2007).

Nigeria, with a population of about 170 million, is 13th among the countries with the highest burden of TB (WHO, 2013).

Cultural habits and practices which facilitate transmission from cattle to humans abound and these include: close association between farmers and animals; fattening of cattle in close proximity to the homes; wearing of minimal protective clothing and the use of bare hands by butchers to process carcass and offal; drinking raw unpasteurized milk; consumption of milk products such as 'wara' and 'nono' and the crowding of cattle and humans in the cattle market (Ayele, *et al*., 2004; Cadmus *et al*., 2006; Abubakar, 2007; Rodwell *et al*., 2008).

Bovine tuberculosis is endemic in the cattle population (Cadmus *et al*., 2010). Furthermore,*M. bovis* have been isolated from nasal secretions of tuberculin positive cattle and unpasteurised cow milk (Ayanwale, 1989; Cadmus and Adesokan 2007) and from 3.9-10% of sputum samples of patients with pulmonary infections in Lagos and Jos (Idigbe *et al*., 1986; Mawak *et al*., 2006) as well as from cases of extra-pulmonary TB in Sokoto (Garba *et al.*, 2004).

Many people are not aware of the risk factors associated with the transmission of BTB; and lack of knowledge about the disease have been documented to result in delay in care seeking and could consequently result in the further spread of the disease (Enwuru *et al*., 2002; McGeary, 2008; Falodun *et al*., 2014).

Various studies have been carried out to assess the degree of awareness/knowledge about TBglobally among patients presenting at different health centers but few (Ameni *et al*., 2003; Gele *et al*., 2009; Desalu *et al*., 2013) have been documented on BTB especially in developing country like Nigeria. This necessitates the need to determine the prevalence of BTB amongst the most occupationally exposed groups comprising: the producers (i.e. herdsmen), the marketers and processors (butchers) as well as to investigate the degree of their awareness, knowledge and practices with regards to BTB. Precisely, this study was aimed to determine the risk associated with the transmission of zoonotic TB in occupationally exposed individuals in Oyo State.

Study Objectives

The objectives of this study include:

1. Determine the prevalence of BTB among occupationally exposed groups comprising the producers (herdsmen), marketers and processors (butchers).
2. Investigate the knowledge, attitude and practices of the occupationally exposed groups with regards to BTB.

5.2. Materials and Methods

5.2.1. Study population and sample collection

This study was conducted in Akinyele International Cattle Market, Bodija Municipal Abattoir as well as some herds' locations at Wasimi in Iwajowa, Igangan in Ibarapa North, Igana in Kajola and Ijaye in Akinyele LGAs of Oyo State.

Akinyele International Cattle Market is the main trading point for cattle brought from northern Nigeria and other parts of Africa to Oyo State. The site is a center of livestock market activity; characterized by overcrowding which can aid the transmission of zoonotic BTB from cattle to humans by means of aerosol.

Bodija Municipal abattoir is a major abattoir that services Ibadan Municipality where an average of 250 cattle is slaughtered daily. Again, due to the unregulated crowd control, the abattoir is often overcrowded by both butchers and the general public. In addition, the butchers wear minimal protective clothing while dressing carcasses as well as use bear hands to process offals from carcasses including diseased ones. Resulting from these, the opportunities for infection with zoonotic BTB therefore abound through aerosol spread, skin infection and in some cases by ingestion due to the habit of eating while processing infected carcasses.

5.2.2. Study design

This was a cross-sectional study.

5.2.3. Sampling technique

Multi-stage sampling was used. Purposive sampling was used to determine the sampling site while systematic random sampling was used to sample the livestock workers.

5.2.4 Eligibility criteria

Livestock workers aged 18years and above working in Bodija Municipal Abattoir, Akinyele International Cattle Market and in herds in Wasimi, Igana, Igangan and Ijaye were recruited for the study. This is in order to obtain informed consent directly from the respondent.

5.2.5 Sample size

Based on an earlier report of 5% prevalence of *M. bovis* infection amongst humans in Nigeria by Ofukwu, (2006) and an absolute precision of 0.0443%; the estimated sample size was 93 individuals. Sputum samples were collected from individuals willing to participate in the study, 31 samples were collected from each group comprising butchers traders at the cattle market and herdsmen. The study objective was explained to them and their due consent was fully taken.

5.2.6 Ethical consent and approval

Informed consent were sought from adult participants who volunteered to participate in the research following adequate information on the objectives and implications of the study. Furthermore,ethical approval was obtained from the Oyo State Research Ethical Review Committee, Ministry of Health, Oyo State and the University of Ibadan/University College Hospital Ethical committee (refer to the Ethical Approval Letter in the Appendix 12).

5.2.7 Sputum sample collection and preservation

Sputum samples were collected in leak-proof and clean sputum containers, properly labelled and transported to the laboratory for analysis in ice-packs. The containers used were firm to avoid crushing in transit and possessed a water-tight wide-mouthed screw cover to prevent leakage and contamination. Also, the containers were made of transluscent material in order to observe specimen volume and quality without necessarily opening the containers.

Sputum collection procedures

With the help of the health officials, clear instructions were given to participants who agreed to participate in the study. They were instructed to remove the sputum container cap, and be careful not to place mouth on the rim of the sputum container. They were

further instructed to take three good, deep breaths and cough deeply enough to bring up secretions (NOT saliva) from their chest. These secretions were spat into the tube. They were instructed to place the cap straight and tight on the sputum tube avoiding a crooked position. In order to avoid generating infective aerosols, participants were told to cough up in the open air or away from other people and not in confined spaces such as toilets. Early morning specimens were collected before breakfast to avoid getting food particles into them. Overall, on-the-spot samples were collected early morning anddate of sputum collection and identification number was written on each sputum container.

5.2.8 Transportation and storage of samples

Properly labelled sputum containers were transportedin a cooler with ice-packs to the laboratory on the day of collection, stored in a refrigerator and processed within 48 hours.

5.2.9 Laboratory Processing

Processing of sputum samples

The sputum samples were processed using the BD BBL™ Mycoprep™ N-Acetyl L-Cysteine-sodium hydroxide (NALC-NaOH) decontamination method (Kent and Kubica, 1985; BD BBL Mycoprep, 2000) to decontaminate and concentrate sputum to get deposits.

Preparation of the buffer and NALC reagent

Preparation of buffer and NACL reagent was done as earlier carried out in Chapter 3 and 4

Decontamination test procedure

As earlier carried out in Chapters 3 and 4 for milk, nasal secretions and faeces

Microscopy (Ziehl-Neelsen staining method)

Loopful smears of final sputum deposits were made on clean, grease-free, duly labelled dry slides. The smear was approximately 20mm by 10mm, corresponding to about 100 oil immerssion fields. Care was taken to ensure that the smears were not thick. The smears were left to dry naturally in the air before fixing over bunsen flame. The slides were stained by the Ziehl-Neelsen (Z-N) technique (Appendix 3.0).

The classical Z-N method depends on the ability of mycobacteria to retain basic dye even when treated with mineral acid or acid-alcohol solution (Shresth*a et al*., 2005).

The slides with fixed smears were arranged on staining rack over a sink. Freshly prepared carbol fuchsin was poured over the slides so that the smears were completely covered. The slides were then gently heated from below with a bunsen flame until steam rose and allowed to stay for five minutes. The stained slides were washed with water under running tap and excess water on the slide was drained by tilting the slides. The slides were then replaced on the rack and the decolorizer (acid-alcohol) was poured over the slide to cover the smears and allowed to act for three minutes. The slide were washed under the running tap. The counter-stain (methylene-blue) was poured on the slides and left to stay for one minute before washing with water under running tap. The slides were drained, arranged vertically on a slide rack and allowed to dry naturally. Dried slides were examined under the oil-immerssion objective of a binocular microscope for the presence of acid fast bacilli, which appeared brick red against a blue background.

5.2.10 Isolation of Mycobacteria

Lowenstein-Jensen (L-J) medium which is an egg-based medium was used for the isolation of *Mycobacterium* species. The media were prepared in duplicate – one containing 0.5% pyruvate for the isolation of *M. bovis* and the other containing glycerol for the isolation of *M. tuberculosis*. The egg medium was prepared by dissolving 37.2g of the L-J medium base powder in 600ml of sterile distilled water, autoclaved and allowed to cool to about 45^0C - 60^0C. Homogenised eggs (thoroughly washed in water and then cleansed with methylated spirit before breaking) was aseptically added, thoroughly mixed and distributed in 10-15 ml volumes in sterile MacCartney bottles and the caps were securely fastened. The medium was inspissated in a slanted position to coagulate the already sterile medium.

The two medium types were properly labelled, dated and inoculated in duplicate with the final sendiments obtained from the sputum spread evenly on the surfaces of pairs of slopes of L-J mediun using Pasteur pipettes (two drops). All innoculated slants were incubated first in a slanting position for 24 hours to allow for even distribution of inoculum at 37^0C. The bottles were then re-arranged in an upright position to increase incubator space. Bottles were arranged in chronological order to make for easy examination. The bottles were incubated for 6 to 8 weeks.

5.2.11 **Examination of mycobacterial culture slants**

All cultures were examined daily for the first seven days after incubation to detect rapidly growing mycobacteria and also to detect contamination. Thereafter, the cultures were examined once in a week for eight weeks to detect positive cultures of mycobacteria before adjudging the culture to be negative if there was no growth. Cultures with completeley contaminated surfaces, liquified or discoloured were removed from the incubator, sterilised, discarded.

Colonies from all resultant growths were examined for morphological appearance, pigmentation and acid-fast properties. Cultures with acid-fast bacilli properties were harvested into broth of 7H9 Middlebrook medium in a microcentrifuge tube and stored in a deep-freezer at -20^{0}C until needed for further investigation.

The bacterial suspension of each isolate was stained by the Z-N method as earlier described (section 3.6.4). This was done to confirm the presence of acid-fast bacilli in the resultant growth.

5.2.12 **Molecular identification**

All strains of the mycobacteria obtained were subjected to further characterization using genus typing and deletiontyping using polymerase chain reaction (PCR) for the confirmation of their identity.

5.3 Questionnaire administration

The traders in the cattle market, butchers, and herdsmen were interviewed using a pre-tested structured questionnaire (Appendix 13). The questions were focused on determining the respondents' awareness about the transmission of TB from cattle to humans and vice versa, habits of milk and meat consumption, whether or not they had BCG vaccination, history of immunosuppressive diseases (especially HIV/AIDS), alcoholism and drug abuse; recent history of TB cases in the family, and, if present, the type of TB. Questions related to general livestock husbandry in the area were also included. Local names were used for all scientific terms during the interview.

5.3.1 Data Analysis

Data were analysed with SPSS version 16, variables were tested with Pearson's chi-square; however, Fisher's chi-square was reported when 50% of cells or more have expected counts less than 5.

5.4. Results

5.4.1. Results of the sputum analysis

For the ZN stain, of the 93 sputum samples processed, only eight (8, 8.6%) were positive, 6.5% (2) from the herders, 9.7% (3) of the samples were from the cattle traders, and another 9.7% (3) from butchers (Table 5.1).

The result of culture of the sputum samples collected from humans occupationally at risk is presented in Table 5.1. Of the 93 sputa collected, only two (2.2%) were positive by culture while 68 (73.1%) were negative.

The bacilli were isolated from two butchers; one of whom had the habit of eating raw meat and cherish wara (cheese). In addition to this he had a history of ill health for one month with the symptoms of cough, night sweats, weakness and loss of appetite.

The two acid fast bacilli isolated were characterized by genus typing and confirmed as *Mycobacterium* and also belonging to the MTC. Further characterization using deletion typing identified them to be *M. africanum*; thus giving a prevalence of 2.2% (Plate 3.4 and 5.1).

Table 5.1: Results of Zeihl Neelsen stain and culture of the sputum samples collected from herdsmen, cattle traders and butchers.

Occupation	Total No	Result of ZN		Result of culture	
		No (%) Positive	No (%) Negative	No (%) positive	No (%) negative
Herdsmen	31	2 (6.5)	29 (93.5)	0	31 (100)
Cattle traders	31	3 (9.7)	28 (90.3)	0	31 (100)
Butchers	31	3 (9.7)	28 (90.3)	2(6.5)	28 (90.3)
Total	93	8 (8.6)	85 (91.4)	2 (2.2)	91 (97.8)

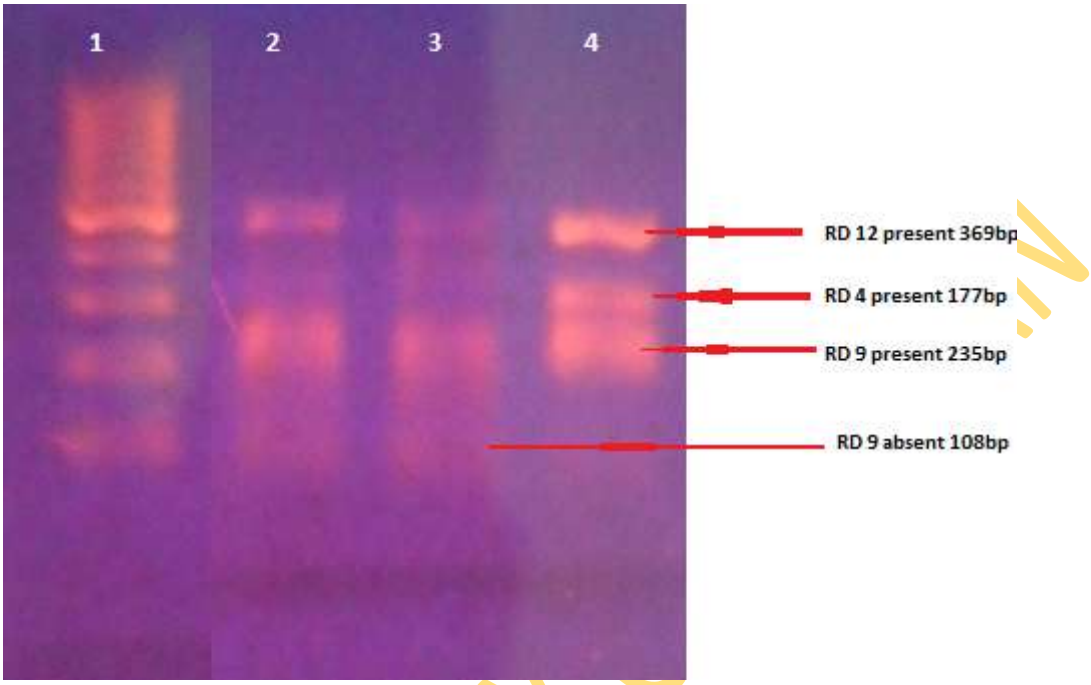

Plate 5.1. Gel picture of PCR products in agarose gel for the Deletion Typing. Showing Lane 1= 100bp molecular weight marker, Lane =1-2, *M. africanum* from humans, Lane 4= *M. tuberculosis* (H37Rv) control

5.4.2 Result of the questionnaire survey

Overall,92 (4.2%) of 124 questionnaires were administered through face to face interview in the six areas where animals especially cattle were being raised, slaughtered or sold. Ten (10.9%) were from Igangan, six (6.5%) from Ijaye, 29 (31.5%) from Akinyele, 22 (23.9%) from Bodija, 10 (10.9%) from Iganna, and 15 (16.3%) from Wasimi Grazing Reserve. Of all the respondentsinterviewed41 (44.6%) were herdsmen while cattle traders and butchers made up 29 (31.5%) and 22 (23.9%) respectively. The responses showed that the cattle business in Oyo State is carried out by men (93.5%) aged between 20-70 years and have been in the business for more than 3 years (92.9); 6.5% of them were single and 93.5% were married (monogamy: 31.0%; polygamy: 69.0%). Less than 10% of them had post-primary education.

The primary occupations of the respondents were mainly related to cattle rearing, cattle production and handling of fresh cattle products (Table 5.2).Exactly half were Fulani, 24 (26.1%) were Yoruba while others were Hausa, Kanuri, and Igbo. Over 90% of the livestock workers interviewed for this study practiced Islam and were married (monogamy: 31.1%; polygamy 68.9%) and of those that were married, only 1 (1.1%) was not living with his spouse as at time of study. More than half the total number of respondents (56.2%) live in low density area while 17(20.2%) and 21(23.6%) were resident in medium and high density areas. More than 90% had been in livestock business for more than 3 years and had experienced disease outbreaks in their flock within the last few years. The major diseases incriminated in the outbreaks were Foot and Mouth Disease and Contagious Bovine Pleura Pneumonia.

Overall, the age, primary occupation, education and duration in cattle business of respondents were considered to be important determining risks factors for potential exposure and transmission of BTB.Fresh milk consumption habits of livestock workers, their level of awareness and knowledge of the modes of transmission of TB including zoonotic BTB, their attitude and practices as well as Chi square test of significance between variables with are summarized in Tables 5.3- 5.5.

Table 5.2 Demographic and socioeconomic characteristics of respondents.

Variables		Frequency	Percentage
Age	20-25 years.	11	12.1
	26-30 years.	10	11.0
	31-35 years.	19	20.9
	36-40 years.	19	20.9
	>40 years.	32	35.2
Gender	Male.	86	93.5
	Female.	6	6.5
Religion	Christianity	8	9.0
	Islam	81	91.0
Marital Status	Never Married	6	6.5
	Married not Living with	1	1.1
	Spouse.	85	92.4
	Married Living with spouse.		
How many wives do you have?	1	26	31.0
	2	45	53.6
	3	10	11.9
	>3	3	3.6
Highest Level of Education.	None	68	76.4
	Primary	12	13.5
	Secondary	9	10.1
Tribe	Fulani	46	50.0
	Yoruba	24	26.1
	Hausa	1	1.1
	Kanuri	19	20.7
	Igbo	2	2.2
Sampling Site	Igangan	10	10.9
	Ijaye	6	6.5
	Akinyele	29	31.5
	Bodija	22	23.9
	Iganna	10	10.9
	Wasimi Grazing Reserve	15	16.3
Which area of the city do you live?	High Density Area	21	23.6
	Medium Density Area	17	20.2
	Low Density Area.	50	56.2
How long have you been in business?	1-3 years	6	7.1
	>3 years	79	92.9
When last did you experience disease outbreak in your flock?	<1 year	3	8.8
	1-3 years	6	17.6
	>3 years	23	67.6
	Never	2	5.9
What was your diagnosis?	FMD & CBPP	3	20.0
	FMD alone	9	60.0
	FMD & Trypanosomosis.	3	20.0
Do vets or health Officers check your animals?	Yes	4	11.4
	No	31	88.6

Table 5.3: Awareness and knowledge of tb among livestock workers in Oyo State

Variable	Know diseases can be transmitted from animals to humans			Know that humans can contact TB from animals			Know how is TB transmitted				Know the signs of TB		Know TB can be cured			Best treatment for TB		
	Yes	No	Don't know	Yes	No	Don't know	Direct Contact	Meat	Milk n milk products	Inhalation of cough sprays	Yes	No	Yes	No	Don't know	Modern	Traditional	Faith healing
Occupation.	Ø			Ø			Ø				#		#			Ø		
Herdsmen.	24.2	36.4	39.4	21.2	39.4	39.4	24.2	75.8	0.0	0.0	53.1	46.9	45.5	21.2	33.3	100	0.0	0.0
Traders.	32.4	48.6	18.9	21.6	59.5	18.9	2.7	2.7	48.6	45.9	45.5	54.5	52.8	5.6	41.7	19.4	66.7	13.9
Butchers.	50.0	45..5	4.5	36.4	59.1	4.5	54.5	40.9	0.0	4.5	52.6	47.4	75.0	10.0	15.0	95.5	0.0	4.5
Academic Status.	#			#			Ø				#		#			Ø		
None.	27.9	45.6	26.5	22.1	52.9	25.0	26.5	29.4	26.5	17.6	43.5	56.5	52.2	10.4	37.3	64.2	35.8	0.0
Primary.	33.3	41.7	25.0	25.0	50.0	25.0	8.3	91.7	0.0	0.0	63.6	36.4	45.5	36.4	18.2	100.0	0.0	0.0
Secondary.	66.7	33.3	0.0	55.6	44.4	0.0	11.1	22.2	0.0	66.7	75.0	25.0	75.0	0.0	25.0	33.3	0.0	66.7
Duration in business.	#				#		#				#		#			#		
≤3 years.	50.0	33.3	16.7	33.3	50.0	16.7	0.0	33.3	33.3	33.3	50.0	50.0	50.0	0.0	50.0	33.3	66.7	0.0
>3 years.	29.1	45.6	25.3	24.1	50.6	25.3	25.3	39.2	20.3	15.2	46.6	53.4	55.8	14.3	29.9	71.8	24.4	3.8
Age. Years	#			#			Ø				#		#			Ø		
≤25	36.4	36.4	27.3	18.2	54.5	27.3	18.2	45.5	0.0	36.4	62.5	37.5	54.5	9.1	36.4	63.6	36.4	0.0
25-30	40.0	60.0	0.0	10.0	90.0	0.0	20.0	20.0	20.0	40.0	66.7	33.3	55.6	0.0	44.4	40.0	60.0	0.0
31-35	47.4	42.1	10.5	31.6	52.6	15.8	15.8	15.8	31.6	36.8	47.1	52.9	63.2	0.0	36.8	31.6	42.1	26.3
36-40	10.5	52.6	36.8	10.5	57.9	31.6	31.6	15.8	42.1	10.5	36.8	63.2	52.6	15.8	31.6	68.4	31.6	0.0
>40	37.5	34.4	28.1	37.5	34.4	28.1	21.9	68.8	6.3	3.1	53.3	46.7	53.3	20.0	26.7	96.8	0.0	3.2

Ø= chi square test is significant, P value is < 0.05,

#= chi square test is not significant, P value is >0.05

Table 5.4: Attitude of livestock workers to tuberculosis

Variable	Is it important to protect yourself while handling animals?			Have you ever had BCG vaccination?		
	Yes	**No**	**Don't know**	**Yes**	**No**	**Don't know**
Occupation.	#			#		
Herdsmen.	66.7	15.2	18.2	12.1	69.7	18.2
Cattle traders.	41.7	27.8	30.6	8.3	77.8	13.9
Butchers.	75.0	20.0	5.0	10.0	75.0	15.0
Academic Status.	#			#		
None.	49.3	26.9	23.9	6.0	77.6	16.4
Primary.	90.9	0.0	9.1	27.3	54.5	18.2
Secondary.	75.0	12.5	12.5	25.0	75.0	0.0
Duration in business.	#			#		
≤3 years.	83.3	16.7	0.0	0.0	100.0	0.0
>3 years.	57.1	23.4	19.5	11.7	70.1	18.2
Age. Years	Ø			#		
≤25	63.6	18.2	18.2	18.2	81.8	0.0
25-30	55.6	22.2	22.2	0.0	55.6	44.4
31-35	52.6	21.1	26.3	10.5	73.7	15.8
36-40	26.3	36.8	36.8	0.0	84.2	15.8
>40	80.0	13.3	6.7	16.7	70.0	13.3

Ø= chi square test is significant, P value is < 0.05,

#= chi square test is not significant, P value is >0.05

Table 5.5: Practices of the Livestock workers about Tuberculosis

Variable	Kind of milk consumed			Kind of milk sold			Ways to protect against animal infections.			Action taken if animal is suspected of TB		
	Fresh	Sour	Boiled	Fresh	Sour	Boiled	Protective material	Limited contact	Hand washing	Allow to enter food chain	Slaughter & dispose carcass	Personal consumption
Occupation.	Ø	Ø	Ø	Ø			Ø			Ø		
Herdsmen.	100.0	100.0	100.0	12.1	87.9	0.0	19.0	19.0	61.9	100.0	0.0	0.0
Cattle traders.	5.9	5.9	5.9	3.6	67.9	28.6	0.0	100.0	0.0	64.9	21.6	13.5
Butchers.	100.0	100.0	100.0	28.6	71.4	0.0	0.0	57.1	42.9	95.5	0.0	4.5
Academic Status.	Ø	Ø	Ø	#			#			Ø		
None.	55.9	55.9	55.9	17.2	70.3	12.5	13.8	48.3	37.9	88.2	11.8	0.0
Primary.	100.0	100.0	100.0	0.0	100.0	0.0	0.0	12.5	87.5	100.0	0.0	0.0
Secondary.	60.0	60.0	60.0	0.0	100.0	0.0	0.0	33.3	66.7	33.3	0.0	66.7
Duration in business.	#	#	#	#			#			#		
≤3 years.	33.3	33.3	33.3	0.0	100.0	0.0	0.0	0.0	100.0	66.7	33.3	0.0
>3 years.	65.4	65.4	65.4	14.9	75.7	9.5	10.3	41.0	48.7	88.6	7.6	3.8
Age. Years	Ø	Ø	Ø	Ø			#					
≤25	63.6	63.6	63.6	28.6	71.4	0.0	28.6	0.0	71.4	63.6	36.4	0.0
25-30	40.0	40.0	40.0	10.0	50.0	40.0	0.0	50.0	50.0	100.0	0.0	0.0
31-35	37.5	37.5	37.5	0.0	85.7	14.3	0.0	50.0	50.0	63.2	10.5	26.3
36-40	47.4	47.4	47.4	10.5	78.9	10.5	11.1	55.6	33.3	89.5	10.5	0.0
>40	93.5	93.5	93.5	16.1	83.9	0.0	6.3	37.5	56.3	96.9	0.0	3.1

Ø= chi square test is significant, P value is < 0.05,

#= chi square test is not significant, P value is >0.05

5.4.4. Awareness and knowledge of BTB among livestock workers in Oyo State

More butchers than cattle traders and herdsmen agreed that diseases most especially TB can be transmitted to humans. Similarly, knowledge and awareness of TB transmission among these cattle professionals increase with their academic status. The age, academic status, and the period spent in business did not significantly affect their knowledge of TB transmission while occupation did (Table 5.3).Many livestock workers knew at least one of the common vehicles of transmitting bovine TB to humans (Table 5.3).The butchers (54.5%) agreed that direct contact with infectious agent is the most common route, the herdsmen (75.8%) preferred meat consumption while cattle traders regarded consumption of milk and milk products and inhalation of cough sprays as the common vehicles of TB transmission. No significant relationship exists between occupation, academic status, and duration in business, age as well as the respondents' knowledge of TB signs and symptoms. More butchers compared to cattle traders and herdsmen also agreed that TB can be cured. More respondents with secondary education also agreed that TB can be cured (Table 5.3).

5.4.5. Attitude and Practices of livestock workers to BTB

Our respondents also agreed that it is important that they protect themselves as they handle animals and their products(Table 5.4). Most of them would do this through limited contact with animals and animal products, frequent hand washing, and the use of protective materials such as overall and hand gloves(Table 5.5). However a small proportion of them(less than 30%) had history of BCG vaccination (Table 5.4). About 30% of the butchers agreed to have consumed raw meat. As for action taken on sick animals due to BTB (Table 5.5), many respondents (<20%) reported consuming meat from sick animals while more than 60% will sell the meat to the general public. Moreover most of our respondents reported that they frequently drank fresh, sour, and boiled milk which they also sell at the markets (Table 5.5).

Discussion

This study was undertaken to determine the prevalence of zoonotic TB among major stakeholders in the beef industry in Nigeria; which comprises of the producers (herdsmen), marketers (cattle traders) and processors (butchers) and to investigate the degree of their awareness, knowledge and practices with regards to BTB. Most of the earlier studies conducted in Nigeria regarding zoonotic TB were limited to a unit of the occupationally exposed. For example, Adesokan *et al*. (2012) worked on cattle traders while Ibrahim *et al*. (2012) and Damina *et al*. (2011) focused on pastoralist.

The result of this study indicate a prevalence of 2.2% among the occupationally exposed group in Oyo State and it does confirm the report by Adesokan *et al*. (2012) of the prevalence of TB in livestock workers. In contrast to other findings (Kiros, 1998; Pavlik *et al*., 2003; Ayele *et al*., 2004; Adesokan *et al*., 2012) neither *M. tuberculosis* nor *M. bovis* was isolated but *M. africanum*. The isolation of *M. africanum* from butchers in this study may indicate occupational exposure since it has been isolated from slaughtered cattle and raw milk from cows awaiting slaughter at the same Bodija Abattoir (Cadmus *et al*., 2006; Cadmus and Adesokan 2007; Cadmus *et al*., 2008). Though the strains isolated were not genotyped to discover transmission relationship, the habit of consuming uncooked meat and wara which characterizes these butchers have been documented to be potential risk of mycobacterial transmission (Mfinanga *et al*., 2003; Ayele *et al*., 2004; Cadmus *et al*., 2008).

In this study, mycobacterial species were not isolated from sputum samples from cattle traders as it was observed by Adesokan *et al*. (2012) neither was it isolated from the sputum of herdsmen. This is similar to other findings (Damina *et al*., 2011), where no *Mycobacterium* species was detected by acid fast test as well as by deletion analysis from the sputum of abattoir workers, butchers and herd owners.

Regarding the degree of livestock professionals' awareness, knowledge and the practices ofBTB, the findings of this study is consistent with those of Ameni *et al*. (2003) who reported that the livestock workers had knowledge of BTB and how the disease can be transmitted. However, more butchers than cattle traders and herdsmen agreed that diseases

especially TB can be transmitted from animals to humans.This coroborates the findings of Munyeme *et al*. (2010) in Zambia who reported that cattle owners had low level of awareness about BTB. The reason why butchers were more aware could be that they often see veterinarians condemn carcasses or organs affected by TB. Their knowledge and awareness of BTB transmission increases with their academic status, this is consistent with what others have documented (Ameni *et al*., 2003; Gele *et al*., 2009; Desalu *et al*., 2013). However; age, academic status and the period spent in business did not significantly affect their knowledge of BTB transmission but occupation did. Also, we found out that most of the livestock workers were aware that TB can be cured; similar to findings by Mfinanga *et al*. (2003) in Tanzania; even though more butchers than cattle traders and herdsmen agreed that TB can be cured.

Most of the human cases of infection of *M. bovis* are associated by close contact withinfected animals, occurring among veterinarians, zoo workers,hunters, abattoir workers, and infection among dairy farm workers havebeen reported in developed countries (Torres-Gonzalez*et al*., 2013). Our respondents also agreed that it is important that they protect themselves while handling animals and animal products by frequent hand washing and the use of protective materials such as overall and hand gloves. However, only a few of them (less than 30%) have been vaccinated against TB in the past; this could be as a result of the fact that most births were out of health facility. Thus, unlike what is obtainable in other countries like Mexico where BCG vaccination is administered to all new born (Torres-Gonzalet *et al*., 2013). In Nigeria most births occur outside the health facility and studies have shown that the place of birth and mother's ownership of immunization card significantly and independently increase the chance of obtaining BCG (Waters *et al*., 2004; Wammanda *et al*., 2004; Chahabral *et al*., 2007; Babalola and Lawan, 2009).

The habit of consuming milk and milk products is common amongst all the groups; with butchers and herdsmen prefering all the three types namely, fresh, sour and boiled. The consumption of unpasteurized cows' milk poses a public health risk that is not only related to *M. bovis* but to other zoonotic infections (Holsinger *et al*., 1997; Casemore 2001; Kavanagh 2002; Anon 2003). Mycobacteria are reported to be the most heat resistant of

the pathogens likely to be present in milk but pasteurization (the heating of milk to 63.5^{o}C for 30 min or to 72^{o}C for 15 s) completely inactivates *M. bovis* (Grant *et al*., 1996; Holsinger *et al*., 1997; de la Rua-Domenech 2006). Most livestock workers particularly the butchers do not consider BTB to be a serious health threat and believe that they are not susceptible to BTB. According to the Health Belief Model (HBM), a meat trader in an abattoir is likely to uphold health-related precautionary measures, such as avoiding eating raw meat or not selling contaminated meat, if he/she considers BTB to be a serious health threat and believes himself/herself to be susceptible to BTB (Hambolu *et al*., 2013). That is why the responses of more than 60% of our respondent that they would sell or share suspected TB infected animal to the public, 20% of butchers reporting to have consumed meat from diseased cattle as well as 30% of butchers accepting to eating raw meat is of serious public health concern.This is because most of the animals slaughtered for human consumption do not undergo proper inspection in Nigeria as TB lesions in skeletal muscles and offals are common in our abattoirs and most times the suspected animals with tuberculous lesions are confirmed positive by culture (Cadmus *et al*., 2008) and humans are at risk of getting infected as a result of consuming undercooked infected meat.

This study has shown that livestock workers; particularly butchers were infected with *M. africanum*,and are also involved in practices that expose them to the risk of infection with zoonotic TB through the consumption of raw unpasteurized milk and raw meat. The unhygienic handling and processing of meat by these butchers may lead to the contamination of the meat and zoonotic transmission to the public.In addition, the livestock workers are knowledgeable about TB however the knowledge increases with increase in their educational status.

Finally, a number of important limitations need to be considered. First, *M. africanum* isolated from the butchers were not spoligotyped to determine if the strains have been isolated from cattle to indicate zoonotic transmission. Secondly, the samples collected were for the diagnosis of active pulmonary infection, clinical parameters that could indicate suspicion of ongoing extra-pulmonary infection were not obtained. And thirdly, the population sampled was small especially those of the herdsmen, this is due to their reluctance to participate in the study.

CHAPTER SIX

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

6.1. Discussion (General).

The zoonotic transmissions of *Mycobacteria tuberculosis* complex and non-tuberculous mycobacteria in Nigeria have received very little attention in-spiteof the fact that cultural practices that facilitate the transmission of these bacteria between cattle and humans abound. Practices such as close association between farmers; cattle traders as well as butchers and cattle, unhealthy meat processing practices by butchers, consumption of unpasteurized milk and milk products are common. The limited knowledge of the rural dwellers, herders, farmersas well asthe general public about the epidemiology of BTB, makes prevention and control programmes difficult and often impossible to implement (Shitaye *et al*., 2007).

The circulating *Mycobacterium tuberculosis* complex and non-tuberculous mycobacteria in the human population especially among livestock workers and animals are largely unknown. Similarly the contribution of NTM to diseases in humans in most instances remains unknown, and the precise epidemiology of bovine tuberculosis in animal and human populations in developing countries has not been established (Neil *et. al*., 2005; Buijtels 2007). Also, the basis for tuberculosis diagnosis in developing countries has continuedto be microscopy of stained smear of expectorated sputum to visualize acid-fast bacteria (AFB) (Buijtels *et al* 2009). Unfortunately, this technique is not able to differentiate between MTC and NTM, as the involvement of acid-fast NTM in tuberculosis-like syndromes mightresult in the misdiagnosis of tuberculosis (Buijtels, 2007).

The primary sources of infection for humans are consumption of unpasteurised milk and close association with animals (Coetzer and Tustin, 2005).

Rural inhabitants and some urban dwellers in Africa still consume unpasteurised and soured milk potentially infected with mycobacteria. Milk-borne infection is the main cause of non-pulmonary TB in areas where BTB is common and uncontrolled (Ayele *et al*., 2004). The agro-pastoral system of farming in Africa also exposes the farmer to the mycobacteria which may be present in the faeces excreted by infected animal often used as manure to fertilize the farmlands (Ayele *et al*., 2004).Confirming the sources of exposure in humans will therefore help to guide the direction of prevention and control of the diseases.

This study in Chapter 3 was able to provide data on the prevalence as well as molecular characterisation of mycobacterial species from fresh milk of pastoral cattle and ready-to eat cheese "wara" in Oyo State, an indication that the milk and milk products consumed in the state are contaminated by mycobacterial species. *Mycobacterium africanum* was the specie of MTC isolated from the fresh milk and cheese while diverse strains of NTM (*M. gordonae*; *M. fortuitum*; *M. senegalense*; *M. avium*)were also obtained. This study has shown the importance of these fresh milk and cheese in the epidemiology of tuberculosis and mycobacterial diseases in this region.

The prolonged survival of pathogenic bacteria in the environment increases the risk of animals as well as farmers acquiring the infection from environmental sources (Scanlon and Quinn, 2000; Humblet *et al*., 2009). *M. bovis*, shed in the faeces of tuberculous cattle or other excretions may be capable of surviving for long periods in stored cattle slurry or in the environment on pasture (Walter *et al*., 2012). Chapter 4 focused on determiningwhether *M. bovis* is being excreted in the nasal secretions and faeces of slaughtered cattle with positive tuberculous lesions at Bodija abattoir in addition to identifying the risk factors for the excretion of *M. bovis* by the cattle slaughtered at Bodija abattoir.

No MTC was isolated from the nasal secretions and faeces, however, NTM were isolated. The result of molecular characterization revealed the seven isolates of mycobacteria from the nasal secretion to be four *M. gordonae*, two *M. fortuitum* and one *M. senegalense*, while the two from faecal sample were *M. fortuitum*. Even though these bacteria are NTM, they have been identified as potentially pathogenic and cause a range of clinical

manifestations in humans. *M. gordonae* which is considered to be the least pathogenic and ubiquitous has been implicated in infections in both immunocompetent and immunocompromised individuals (Weinberger *et al*., 1992; Foti *et al*., 2009). While *M. fortuitum* have been isolated from humans with pulmonary infections in Nigeria (Idigbe *et al.,* 1989; Mawak *et al*., 2006; Daniel *et al*., 2011); *M. senegalense*have been associated with catheter-related hematological infection in a 49-year old woman with non-Hodgkin's lymphoma in Korea and tissue infection in a child after fish tank exposure in Canada (Oh *et al*., 2005; Talavlikar *et al*., 2011).

Furthermore, the study implicated the lungs as the organ that is most affected with the highest number of isolates being recovered from the nasal secretion. This suggests that the major route of exposure to mycobacterial infection in the cattle examined might be aerogenous. The fact that mycobacterial infection on the lungs is mainly via the intranasal route has been supported by various evidences (Dean *et al*., 2005; Cassidy, 2006; Menin *et al.,* 2013).

It has been observed that a high rate of zoonotic tuberculosis is commonly associated with occupational exposure (Challu, 2007), especially in crowded and less ventilated settings which can be the potential route of transmission from animals to man and vice versa (Challu, 2007). Many people are not educated about BTB; and lack of knowledge about the disease have been documented to result in delay in care seeking and could consequently result in the further spread of the disease (Enwuru *et al*., 2002; McGeary, 2008; Falodun *et al*., 2014). This necessitates the need to determine the prevalence of BTB amongst the occupationally exposed groups comprising: the producers (i.e. herdsmen), the marketers and processors (butchers) as well as to investigate the degree of their awareness, knowledge and practices with regards to BTB.

The result of this study in Chapter 5 indicated a prevalence of 2.2% among the occupationally exposed group in Oyo State, which confirms that prevalence of TB in livestock workers with *M. africanum* being the specie of mycobacteria implicated. Also livestock workers had knowledge of BTB and how the disease can be transmitted. However, more butchers than cattle traders and herdsmen agreed that diseases especially TB can be transmitted from animals to humans. Nevertheless, most livestock workers

(70%) consume unpasteurised milk, 30% of butchers consume raw meat, and all agreed to consumption, selling or sharing of suspected infected animal products to the public.

6.2. Conclusion and recommendations

This study reports the isolation of *M. africanum*, a natural human pathogen, from fresh milk of pastoral cattle and soft cheese. Its presence in cattle and subsequently in the samples may have been caused by direct transmission from infected humans, particularly traditional cattle owners and herdsmen as they are known to have close and repeated contacts with their cattle. The isolation of *M. africanum* from butchers in this study may indicate occupational exposure since it has been isolated from slaughtered cattle and raw milk from cows awaiting slaughter at the Bodija Abattoir (Cadmus *et al.*, 2006; Cadmus and Adesokan 2007; Cadmus *et al.*, 2008). However, the strains isolated were not further characterized by spoligotyping or MIRU-VNTR to discover transmission chain. The habit of eating while dressing carcasses; consumption of uncooked meat and wara which are common practices of these butchers have been documented to be potential risk of mycobacterial transmission (Mfinanga*et al.*, 2003; Ayele*et al.*, 2004; Cadmus *et al.*, 2008).

The predominance of NTM in this study highlights the significance of these species of mycobacteria in the epidemiology of TB among pastoral cattle in Nigeria. This is of great public health concern with underlining grave implications for the treatment and control of multi-drug resistant TB in humans; considering the practice of consumption of unpasteurized milk as well as close contact between livestock workers and cattle. Bearing in mind the fact that laboratory diagnosis of TB in Nigeria relies mainly on acid-fast staining and X-ray, although inexpensive, they do not provide information on the species of mycobacteria causing the disease.

So many NTM have been reported in Nigeria (Idigbe*et al.*, 1989; Mawak*et al.*, 2006; Daniel *et al.*, 2013), however, this work to our knowledge is one of the first to report the isolation and molecular characterization of *M. senegalense*, an emerging zoonotic infectious agent from fresh milk and nasal secretion of cattle in Nigeria.The organism is arecognized cause of human disease, including skin and softtissue, post-traumatic or

postsurgical osteomyelitis,catheter-related and possibly pulmonary infections (Oh *et al.*, 2005; Wallace *et al* 2005; Talavlikar*et al.*, 2011).

The study also shows that the livestock workers are knowledgeable about BTB but are involved in practices that would enhance the spread of the disease to the public. With majority of the workers reported consuming meat of suspected tuberculous cattle, more than 60% will pass such meat into the human/public food chain in addition to 30% of the butchers consenting to have consumed raw meat. This is of grave consequences in areas where HIV and other immunosuppressive diseases are prevalent in humans, the cycle oftransmission between cattle and humans, as well as within humans may possibly be easily established.

There is therefore the need for the following:

- Education of the general populace on the signs and symptoms of TB in animals and humans; the public health implication of consuming unpasteurized milk and milk products; improperly processed meat and meat products as well as close association with infected animals. As well as regular appraisal of livestock workers' level of knowledge about the disease in the bid to reduce the risk of transmission of the disease from cattle to humans and humans to cattle thereby breaking the cycle of transmission.
- Concerted veterinary, medical and biomedical efforts to increase TB detection rate, active involvement of the population at risk of exposure and transmission.
- In addition, hospitals shouldbe encouraged to include culture and molecular characterization in the diagnosis of TB to confirm the strains of the mycobacteria causing the disease in humans, in order to effectively tackle the incidence/cases misdiagnosed as multi-drug resistance.
- A nationwide molecular epidemiological survey in the indigenous livestock population and affected humans should be conducted in order to have a better insight into the transmission scenario between humans and animals.

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APPENDICES

Appendix1.Lowestein-Jenseen medium Base

Reagents

L-Asparagine	3.6g
Monopotassium Phosphate	2.4g
Magnessium Sulfate	0.24g
Magnessium citrate	0.6g
Potato flour	30g
Malachite Green	0.4g

Procedure

Suspend 37.2g of the powder in 600ml of purified water containing glycerol. Do not add glycerol if bovine tubercle bacilli or other glycerophobic organisms are to be cultivated. Mix thoroughly, heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Autoclave at 121^0C for 15 minutes, cool to 45-60^{o}C. Aseptically add 1 litre of fresh, uniform egg supplement prepared under aseptic conditions. Mix well, dispense into sterile tubes, slant and coagulate at 85^0C for 45 minutes.

Appendix2. 7H9 Broth

Reagents

Ammonium sulphate	0.5g
L-Glutamic Acid	0.5g
Sodium citrate	0.1g
Pyridoxine	0.001g
Biotin	0.0005g
Disodium phosphate	2.5g
Monopotassium phosphate	1.0g
Ferric Ammonium citrate	0.04g

Magnesium sulphate	0.05g
Calcium chloride	0.0005g
Zinc sulphate	0.001g
Copper sulphate	0.001g

Procedure

Suspend 4.7g of the powder in 900ml of purified water containing 2ml glycerol or 0.5g polysorbate 80, if desired. Autoclave at 121^{o}C for 10 minutes. Aseptically add 100ml Middlebrook ADC Enrichment to the medium when cooled to 45^{o}C.

Appendix 3. Ziehl-Neelsen (ZN) Technique

Stock Reagents were manufactured by GCC Diagnostics, Sandycroft. Deeside. UK.

Carbol Fuschin

Basic fuschin	5.0g
Phenol crystals	25.0g
95% Ethanol	50.0g
Distilled water	500.0g

Acid alcohol (Decolouriser)

85% Ethanol	970.0ml
Concentrated H_2S0_4	30.0ml

Counterstain

Methylene blue	2.5g
Distilled water	500.0g

Method

- A smear of about 1cm x 2cm was made on a clean grease free new slide. The slide was allowed to dry.
-The slide was heat fixed using Bunsen flame.
-The slides were arranged on slide rack over a sink and smear was flooded with carbol fuschin stain.
-The slide was allowed to stand at room temperature for about 5 minutes.
-The slides were heated gently until steam arouses but stain was not allowed to dry or boil.

-Stain was allowed to stand for about 5 minutes on the slide.
-The slides were washed under running water and excess water was drained by tilting the slides.
-The slides were decolorized for about 3 minutes and rinsed with running water and tilted to drain. -The slides was flooded with methylene blue for about a minute.
-The slides were rinsed under running water, arranged on slide rack and allowed to dry.
-The slides were examined under a microscope using oil-immersion objective.

Appendix 4. Material used for the PCR

Laminar flow cabinet, 'DNA away', and filter tips.
Nuclease free water (Qiagen).
HotStarTag Master Mix Kit (Qiagen; prod No 203445). This mix includes DNA polymerase, buffer, $MgCl_2$, and dNTPs.
Stock solutions of oligonucleotide primers:

100µM MYCGEN-F	5′ -AGA GTT TGA TCC TGG CTC AG - 3′
100µM MYCGEN-R	5′-TGC ACA CAG GCC ACA AGG GA - 3′
100µM MYCAV-R	5′ –ACC AGA AGA CAT GCG TCT TG – 3′
100µM MYCINT –F	5′ –CCT TTA GGC GCA TGT CTT TA – 3′
100µM TB1-F	5′ – GAA CAA TCC GGA GTT GAC AA – 3′
100µM TB1-R	5′ –AGC ACG CTG TCA ATC ATG TA - 3′

Materials used for Gel electrophoresis
DNA Ladder and Loading Dye.
Agarose.
10x TAE Running Buffer.
Ethidium Bromide.
Appendix 5. Primer used for Genus typing

Primer name	Target gene	Sequence
MYCGEN-F	16S rRNA	5 '— AGA GGT TGA TCC TGG CTC AG—3 '
MYCGEN-R	16S rRNA	5 '— TGC ACA CAG GCC ACA AGG GA—3 '
MYCINT-F	16S rRNA	5 '— CCT TTA GGC GCA TGT CTT TA— 3'
MYCAV-R	16S rRNA	5 '— ACC AGA AGA CAT GCG TCT TG—3 '
TB1-F	MPB70	5 '— GAA CAA TCC GGA GTT GAC AA—3 '
TB1-R	MPB70	5 '— AGC ACG CTG TCA ATC ATG TA—3'

Appendix 6: Volume of the master mix for PCR amplification

PCR mix	No of samples		
	10	20	30
6.2ul H20 Qiagen	62	124	186
10ul Mastermix	100	200	300
0.3ul MYCGEN-F 100uM	3	6	9
0.3ul MCGEN-R 100uM	3	6	9
0.3ulTB1-F 100uM	3	6	9
0.3ulTB1-R 100Um	3	6	9
0.3ul MYCAV-R 100uM	3	6	9
0.3ul MYCINT-F 100uM	3	6	9
18ul/tube +2ul DNA template			
20ul total volume/reaction			

Appendix 7: Result interpretation for Genus typing

Mycobacteria species	**PCR fragments**		**Primers sequence**
All members of mycobacteria genus	1030bp		5'—AGA GGT TGA TCC TGG CTC AG—3 ' MYCGEN-F 5'—TGC ACA CAG GCC ACA AGG GA—3' MYCGEN-R
M.avium	180bp 1030bp	&	5'—AGA GGT TGA TCC TGG CTC AG—3 ' MYCGEN-F 5'—ACC AGA AGA CAT GCG TCT TG—3' MYCAV-R
M.intracellulare	850bp 1030bp	&	5'—CCT TTA GGC GCA TGT CTT TA —3' MYCINT-F 5'—TGC ACA CAG GCC ACA AGG GA—3' MYCGEN-R
***M.tuberculosis* complex**	372bp 1030bp	&	5 '— GAA CAA TCC GGA GTT GAC AA—3 ' TB1-F 5 '— AGC ACG CTG TCA ATC ATG TA—3' TB1-R

Appendix 8.AboutPRAsite web site

This site provides access to a database of known restriction fragment profiles obtained by the PCR-RFLP analysis (PRA) method. PRA patterns are used for rapid mycobacterial species identification.

Query forms

PRA database is available for searching via 2 methods.

1. Species Name

By entering the name of a mycobacterial species the specific PRA pattern and information are shown.

2. PRA Pattern

This page will allow a comparison of a submitted PRA-pattern with a list of 10 mycobacterial species displaying the closest pattern.

To submit your pattern, you can either select from the list the pattern closest to yours or enter your own PRA-pattern values.

Recent Results

The table contains the list of the strains recently added to the database, or for which the data have been modified.

Submission and Contact

Users of the PRA test are encouraged to provide corrections, new information, for inclusion in the database; unpublished information will be held confidential.

QUERY RESULTS

A) Strain characteristics

The following data are presented for each strain included in the database:

a) ID:	The identification number of the strain
b) Organism:	the name of the strain, including the subtype number (by default the subtype is equal to 1)
c) BstEII pattern:	length of the restriction fragment in base pairs obtained by the restriction enzyme BstEII

d) HaeIII pattern:	length of the restriction fragment in base pairs obtained by the restriction enzyme HaeIII
e) ORIGIN:	the source of the strain used for the PRA database
f) Medline reference:	the number of the reference describing the corresponding PRA pattern - click to link with the reference
g) hsp65 sequence:	the accession number of the hsp65 sequence from the strain corresponding to the PRA pattern - click to link with the reference
h) Phenotype:	growth characteristic

B) Query PRA database results

First table: Submitted query pattern

Second table: results of the query

a) ID:	The identification number of the strain - press to obtain the link with the strain characteristics.
b) Species:	the name of the strain, including the subtype number (by default the subtype is equal to 1).
c) BstEII pattern:	length of the restriction fragment in base pairs obtained by the restriction enzyme BstEII.
d) HaeIII pattern:	length of the restriction fragment in base pairs obtained by the restriction enzyme HaeIII.
e) Score:	the score value represents the Euclidian distance between the strain submitted to analysis and the query. The smaller the distance between two organisms, the greater is the resemblance between them.

Appendix 9. Query result of Strain 1

QUERY PRA DATABASE RESULTS 08/05/2013

Query	BstE II	Hae III
Your Query	240/125/85	170/120/70

ID	Species	BstE II	Hae III	Score
59	Mycobacterium gordonae type 1	235/120/85	160/115/60	17
66	Mycobacterium gordonae type 9	235/120/100	160/115/60	22
170	Mycobacterium xenopi type 1	235/120/85	160/105/60	22
163	Mycobacterium tuberculosis complex type 1	235/120/85	150/130/70	23
70	Mycobacterium heckeshornense type 1	235/120/100	160/105/60	26
51	Mycobacterium fortuitum s. acetamidolyticum type 1	235/120/85	145/120/60	28
100	Mycobacterium mageritense type 1	235/130/85	145/125/60	28
152	Mycobacterium smegmatis type 1	235/130/85	145/125/60	28
48	Mycobacterium fortuitum type 1	235/120/85	145/120/60	28
58	Mycobacterium goodii type 1	235/130/85	145/125/60	28

Appendix 10. Query result of Strain 2

QUERY PRA DATABASE RESULTS 08/05/2013

Query	BstE II	Hae III
Your Query	230/125/85	140/115/60

ID	Species	BstE II	Hae III	Score
49	Mycobacterium fortuitum type 2	235/120/85	140/120/60	9
51	Mycobacterium fortuitum s. acetamidolyticum type 1	235/120/85	145/120/60	10
48	Mycobacterium fortuitum type 1	235/120/85	145/120/60	10
139	Mycobacterium senegalense type 1	235/120/85	140/125/60	12
34	Mycobacterium conceptionense type 1	235/120/85	140/125/60	12
169	Mycobacterium wolinski type 1	235/130/85	140/125/60	12
113	Mycobacterium neworleanense type 1	235/120/85	140/125/60	12
75	Mycobacterium houstonense type 1	235/120/85	140/125/60	12
140	Mycobacterium senegalense type 2	235/130/85	140/125/60	12
44	Mycobacterium farcinogenes type 1	235/120/85	140/125/60	12

Appendix 11. Query result of Strain 3

QUERY PRA DATABASE RESULTS 08/05/2013

Query	BstE II	Hae III
Your Query	230/140/85	200/140/90

ID	Species	BstE II	Hae III	Score
142	Mycobacterium senegalense type 4	235/120/85	180/140/50	49
3	Mycobacterium agri type 1	235/130/85	160/145/60	51
129	Mycobacterium peregrinum type 3	235/130/85	145/140/100	57
177	Mycobacterium fragae type 1	235/130/85	145/125/80	59
163	Mycobacterium tuberculosis complex type 1	235/120/85	150/130/70	59
59	Mycobacterium gordonae type 1	235/120/85	160/115/60	60
66	Mycobacterium gordonae type 9	235/120/100	160/115/60	61
170	Mycobacterium xenopi type 1	235/120/85	160/105/60	64
64	Mycobacterium gordonae type 6	235/130/85	140/120/95	64
100	Mycobacterium mageritense type 1	235/130/85	145/125/60	65

Appendix.12 Ethical Approval

INSTITUTE FOR ADVANCED MEDICAL RESEARCH AND TRAINING (IAMRAT)
COLLEGE OF MEDICINE, UNIVERSITY OF IBADAN. IBADAN, NIGERIA.
Director: **Prof. A. Ogunniyi,** B.Sc(Hons), MBChB, FMCP, FWACP, FRCP (Edin), FRCP (Lond)
Tel: **08023038583, 08038094173**
E-mail: **aogunniyi@comui.edu.ng**

UI/UCH EC Registration Number: **NHREC/05/01/2008a**

NOTICE OF FULL APPROVAL AFTER FULL COMMITTEE REVIEW

Re: Epidemiology of Bovine Tuberculosis, Zoonotic Transmission and risk Factors Associated with the Infection in Humans in Parts of Oyo State.

UI/UCH Ethics Committee assigned number: UI/EC/11/0238

Name of Principal Investigator:	**Dr. Charity A. Agada**
Address of Principal Investigator:	Department of Veterinary Public Health & Preventive Medicine, University of Ibadan, Ibadan

Date of receipt of valid application: 15/09/2011

Date of meeting when final determination on ethical approval was made: **N/A**

This is to inform you that the research described in the submitted protocol, the consent forms, and other participant information materials have been reviewed and *given full approval by the UI/UCH Ethics Committee.*

This approval dates from 15/03/2012 to 14/03/2013. If there is delay in starting the research, please inform the UI/UCH Ethics Committee so that the dates of approval can be adjusted accordingly. Note that no participant accrual or activity related to this research may be conducted outside of these dates. *All informed consent forms used in this study must carry the* UI/UCH EC *assigned number and duration of* UI/UCH EC *approval of the study.* It is expected that you submit your annual report as well as an annual request for the project renewal to the UI/UCH EC early in order to obtain renewal of your approval and avoid disruption of your research.

The National Code for Health Research Ethics requires you to comply with all institutional guidelines, rules and regulations and with the tenets of the Code including ensuring that all adverse events are reported promptly to the UI/UCH EC. *No changes are permitted in the research without prior approval by the* UI/UCH EC *except in circumstances outlined in the Code. The* UI/UCH EC *reserves the right to conduct compliance visit to your research site without previous notification.*

UI/UCH EC
APPROVED
COLLEGE OF MEDICINE, U.I.

Prof. A. Ogunniyi
Director, IAMRAT
Chairman, UI/UCH Ethics Committee
E-mail: uiuchirc@yahoo.com

Appendix.13 Questionnaires

F QIN | | **UI//*TB/2011***

Knowledge, Attitude and Practice of Livestock Workers about Human Health Risks Associated with Bovine Tuberculosis

Site:_____ | | |

Result Code

Completed ---------- 1

Partially completed ---------- 2

Respondent on leave ---------- 3

Respondent refused ---------- 4

SECTION A: DEMOGRAPHIC AND SOCIOECONOMIC CHARACTERISTICS OF RESPONDENTS

QN	QUESTIONS & FILTERS	RESPONSE OPTIONS	CODE
A1	How old were you as at your last birthday?	20 -25…… 26 – 30…….. 31 – 35 …… 36 – 40 …… 41 and above …….. -	01 02 03 04 05
A2	Record sex of the respondent	Male ---------- Female----------	1 2
A3	What is your religion	Christianity …….. Islam …….. Traditional …….. Other (Specify) ……	01 02 03 04
A4	What is your marital status?	Never married (Single) ---------- Currently married, not living with spouse Currently married, living with spouse ---- Widowed ---------- Divorced ---------- Separated ---------- Co-habiting----------	1 2 3 4 5 6 7
A5	If married, how many wives do you have?	One wife---------- Two wives---------- Three wives---------- More than three wives----------	1 2 3 4
A6	If female, are you the only wife? If no what is your position?	Yes---------- No---------- ----------	1 2
A7	What is your highest level of educational attainment?	No formal education ---------- Primary ---------- Secondary ---------- Tertiary ---------- Others (specify) _____	1 2 3 4 5
A8	What area of the city do you live?	High density area…… Medium density area …….. Low density area…… .	1 2 3
A9	How many of you on the average live together in a room?	1…….. 2 – 3…… More than 3 persons……	1 2 3
A10	How many adults? How many children?	Adults---------- Children----------	
A11	How many windows does the room have?	1---------- 2---------- 3---------- 4----------	1 2 3 4

A12	How long have you been in this business?	Less than one year …… 1 – 3 years ……. More than 3 years ……	1 2 3
A13	When last did you have an outbreak of disease in your livestock?	Less than one year …… 1 – 3 years ……. More than 3 years …… None……	1 2 3 3
A14	What was your diagnosis?	TB ……... Others (specify)…….	1 2
A15	Do vet or health officers check your animals?	Yes …… No……	1 2

SECTION B: AWARENESS OF TB

QN	QUESTIONS & FILTERS	RESPONSE OPTIONS	CODE
B1	Are you aware that people can contract any disease from animals?	Yes…….. No…… Don't Know……	1 2 3
B2	If yes, what type of disease can be contracted?	……	
B3	If no, why do you think that man cannot contract disease from animals?	……..	
B4	Do you know that someone can contract TB from animals?	Yes…….. No…… Don't Know……	1 2 3
B5	How do you think man can be infected with TB?	Direct contact…….. Consumption of meat …… Milk & milk products…… Others (specify)……..	1 2 3 4
B6	Do you know the symptoms of the disease in animals?	Yes…….. No……	1 2
B7	What are the symptoms of the disease in animals?	_____ _____	
B8	Have any of the cattle been coughing in the last 12 months?	Yes…….. No……	1 2
B9	Have any of the female cattle had swollen mammary gland in the last 12 months?	Yes…… No……	1 2
B10	Have any of your animals been loosing weight in the last 12 months?	Yes…… No……	1 2

B11	Have any of your animals died in the past 12 months?	Yes…… No……..	1 2

SECTION C: KNOWLEDGE OF TB

QN	QUESTIONS & FILTERS	RESPONSE OPTIONS	CODE
C1	Do you know of anybody with TB now or before now?	Yes…….. No……	1 2
C2	If yes, what are signs/symptoms of TB you observed in the person?	Cough…… Wasting…… Sweat in the night…… Body weakness…… Others……	1 2 1 2 1 2 1 2
C3	Do you think that TB can be cured?	Yes…….. No…… Don't Know……	1 2 3
C4	What type of treatment do you think is best for TB?	Modern medicine…… Traditional medicine……..…….. Faith healing…… Others (Specify)……..	1 2 3 4
C5	Have you observe any member of your household with these symptoms in the past 3 months?	Cough…… Wasting…….. Sweat in the night…… Body weakness…… Others……	1 2 1 2 1 2 1 2

SECTION D: ATTITUDE TO TB

QN	QUESTIONS & FILTERS	RESPONSE OPTIONS	CODE
D1	Do you think diseases can be transferred from animals to man?	Yes…….. No……	1 2
D2	If yes, why do you think so?		

D3	If no, why do you think so?		
D4	Do you think that it is important to protect yourself while handling animals?	Yes…….. No…… Don't Know……	1 2 3
D5	Do you think that you need to go for medical check up for infection periodically?	Yes…….. No…… Don't Know……	1 2 3
D6	Have you been given BCG vaccination, which is the one given at birth?	Yes…….. No…… Don't Know……	1* 2 3

SECTION E: PRACTICES ABOUT TB

QN	QUESTIONS & FILTERS	RESPONSE OPTIONS	CODE
E1	What do you do to protect yourself from animal infections?	Immunization…… Use of preventive medicine…… Good hygiene practices…….. Use of herbs…… Prayer…… Others (specify)……	1 2 3 4 5 6
E2	Have you ever been tested for TB	Yes…….. No……	1 2
E3	If no, why didn't you go for test?	I am not sick……. I cannot have TB…….. I don't know there is treatment for it.....	1 2 3
E4	What kind of milk do you take?	Fresh un-boiled milk…… Boiled milk…… Sour milk……	1 2 3
E5	What kind of milk do you take to the market to sell?	Fresh un-boiled milk…… Boiled milk…… Sour milk……	1 2 3
E6	What would you do if you see your animal having TB symptoms?	I will sell it…….. I will kill it and bury it…… I will eat it personally at home…… Others (specify)……	1 2 3 4
E7	If you have TB where would you go for treatment?	Modern medicine…… Traditional medicine……..…….. Faith healing……	1 2

		Others (Specify)……..	3 4
E8	What type of hygiene practice do you observer to protect yourself from contracting TB from animals?	Putting on proactive material while working…….	1 2
		Limited contact with animals or carcass …….	1 2
		Washing of hands after touching live or processed animals……	1 2
		Others (specify)	
		None	
A7	What disease condition have you experienced recently?	Cut/sores……... Cough lasting more three week …… Wasting ……. Others (specify)……	1 2 3 4

Imo, ise ati ihuwasi awon to nsise eran nipa awon ewu to ro mo kokoro tubakulosisi ara eranko

Abala A: awon ohun to je mo awon olukopa

Q/N	Awon ibeere	Idahun olukopa	Ami fun idahun
A1	Kini ojo ori re ni ojo ibi re to gbehin?	20 -25…… 26 – 30…….. 31 – 35 …… 36 – 40 …… 41 ati siwaju …… -	01 02 03 04 05
A2	Se okunrin ni olukopa tabi obinrin	okunrin ---------- obirin ----------	1 2
A3	Kini esin re?	kristiani ……. islamu …….. esin ibile ……. Omiran (iru esin wo) ……	01 02 03 04
A4	Kini ipo ti o wa nipa igbeyawo?	Nko ti se gbeyawo (odo langba) ---------- Mo gbeyawo sugbon a o gbe papo Mo gbeyawo a si ngbe papo ---- opo ---------- ati kora wa sile ---------- ati pinya pelu ara wa------ Mo kan ngbe pelu enikeji mi ni ---------- ----------	1 2 3 4 5 6 7
A5	Ti o ba ti gbeyawo, Iyawo melo lo ni	Eyo kan---------- meji ---------- meta---------- o ju meta----------	1 2 3 4
A6	Ti o ba je obirin, se iwo nikan ni oko re fe niyawo? Bi beeko, ipo wo no o wa?	Yes---------- No---------- ----------	1 2
A7	Iwe melo lo ka?	Nko kawe rara ---------- alakobere ---------- girama ---------- ile iwe giga ---------- omiran (soo jade) _____	1 2 3 4 5
A8	Iru agbegbe wo lomgbe?	Ibi ti ero ti po…… Ero po die …….. Ero o po…… .	1 2 3

A9	Eyin melo len gbe yara?	Eni kan Meji si meta …… A ju meta lo……	1 2 3
A10	Awon melo ni agbalagba? Awon melo no omode?	Agba---------- Omode ----------	
A11	Ferese melo lo wa ni yara re?	1---------- 2---------- 3---------- 4----------	1 2 3 4
A12	Odun melo lo ti wa ni enu ise yi?	Ko pe odun kan …… Odun kan si meta …… Ole ni odun meta ……	1 2 3
A13	Igba wo ni o ni iriri aisan laarin agbo eran re gbehin?	Ko pe odun kan …… Odun kan si meta …… O le ni odun meta …… Kosi ……	1 2 3 3
A14	Iru ayewo wo lo se?	Iko egbe …… Omiran (daruko won) ……	1 2
A15	Se awon dokita eranko tabi awon alayewo eran se ayewo eran re?	beeni …… beeko……	1 2

ABALA B: IMO NIPA IKO FEE

QN	AWON IBEERE	IDAHUN OLUKOPA	CODE
B1	Nje o mo wipe eniyan lee ko arun lara eranko?	beeni…….. beeko…… nko mo……	1 2 3
B2	Bi beeni, iru arun wo?	……	
B3	Bi beeko, ki lo de to fir o wipe eniyan ko le ko arun lara eranko?	……..	
B4	Nje o mo pe eniyan le ko arun iko fee latara eranko?	beeni…….. beeko…… nko mo……	1 2 3
B5	Bawo lo se ro wipe eniyan lee ko arun iko fee?	Nipa isunmoni…….. Eran jije …… Wara mimu…… Ona miran (daruko won)……	1 2 3 4
B6	Nje o mo ami arun yii lara eranko?	beeni…….. beeko……	1 2

B7	Kini awon ami naa?	_____ _____	
B8	Nje ari lara maalu re to ti nwuko lati bi osu mejila sehin?	beeni…….. beeko……	1 2
B9	Nje a ri lara awon eranko maalu obirin ti inu re wu?	beeni…… beeko……	1 2
B10	Nje ari lara awon eranko re to ti nru lati bi osu mejila sehin?	beeni…… beeko……	1 2
B11	Nje ari lara eranko re to ti ku ni bi osu mejila sehin?	beeni…… beeko……..	1 2

Abala C: Imo iko fee

QN	Awon ibeere	Idahun olukopa	Ami
C1	Nje o mo eniti o ni arun ko fee bayi tabi nigbakan ri?	Beeni …….. Beeko ……	1 2
C2	Bi beeni, kini awon ami ti o ri lara eni naa?	Iko …… Riru…….. Oogun loru…… Rire…… Awon miran……	1 2 1 2 1 2 1 2
C3	Nje o mo pea run ko fee gbo iwosan?	beeni…….. beeko…… ngo mo……	1 2 3
C4	Iru oniwosan wo lo ro wipe o dara ju fun arun ko fee?	Iwosan igbalode…… iwosan ibile……..…….. awon aladura …… awon miran (daruko won……	1 2 3 4
C5	Nje oti ri lara ebi re to ni iru ami yii ni nkan bi osu meta sehin?	Iko …… Riru …….. Oogun oru…… Aare ara …… Omira (daruko won……	1 2 1 2 1 2 1 2

Abala D: isesi nipa iko fee

QN	Awon ibeere	Idahun olukopa	Ami
D1	Nje o mo pe eniyan le ko arun lara eranko?	beeni…….. beeko……	1 2
D2	Bi beeni, ki lo de to fir o be?		
D3	Bi beeko, ki lo de to fir o be?		
D4	Nje o ro wipe o dara lati daabo bo ara re to ba nsise lara eran?	beeni…….. beeko…… nko mo……	1 2 3
D5	Nje o ro wipe o nilo lati lo maa se ayewo ara re lodo oniwaosa lore ko re?	beeni…….. beeko…… nko mo……	1 2 3
D6	Nje o to gba abere ajesara fun iko ri?	beeni…….. beeko…… nko mo……	1* 2 3

abala E: abuda nipa iko fee

QN	Awon ibeere	Idahun olukopa	Ami
E1	Kini o ma nse lat daabo bo ara re ki o ma ba ko aru ara eranko?	Abaer ajesara…..…… Oogun lilo……. imototo…….. agbo lilo…… adura gbigba…… Omiran (daruko)……	1 2 3 4 5 6
E2	Nje won sayewo fun o tori iko fee ri?	beeni…….. beeko……	1 2
E3	Bi beeko, ki lode ti o ko lofun ayewo?	Nko saisan……. Nko le ni iko fee…….. Nko mo pe itoju wa fun.....	1 2 3
E4	Iru wara wo lo maa nmu?	Eyi ti a ko se…… Eyi ti a se…… Eyi to ka……	1 2 3

E5	Iru wawr wo lo maa ngbe lo soja fun tita?	Eyi ti a ko se…… Eyi ti a se…… Eyi to kan……	1 2 3
E6	Ki ni o maa se to bar wipe eran re ni ko fee?	Maa ta…….. Maa pa maa si ri mole…… Maa gbe lo sile fun jije…… Omiran (daruko)……	1 2 3 4
E7	To ba ni iko fee nibo lo ti maa lo gba iwosan?	Iwosan igbalode…… iwosan ibile……..…… awon aladura …… awon miran (daruko won ……..	1 2 3 4
E8	Iwa imototo wo lo maa nfi si ise ki o ma ba ko arun ko fee lara eranko?	Aso idaboobo ti mo ba nsise…. Nko nje ki ara era maa kan mi lara ……. Owo fifo lehin ise…… Omira (daruko) kosi	1 2 1 2 1 2
A7	Iru aisan wo lo se o laipe yi?	Egbo ……... Iko to le ni ose kan …… Riru ……. Omiran (daruko)……	1 2 3 4