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EPIDEMIOLOGY OF HUMAN AND BOVINE TUBERCULOSIS IN THE FEDERAL CAPITAL TERRITORY AND KADUNA STATE OF NIGERIA

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EPIDEMIOLOGY OF HUMAN AND BOVINE TUBERCULOSIS IN THE FEDERAL CAPITAL TERRITORY AND KADUNA STATE OF NIGERIA

BY

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Dedication

This piece of work is dedicated to my daughter Binta for having to cope with my absence when she needed me most, to my son Nurudeen for his love and support, and to the loving memory of my grandmother Binta who taught me all I have learnt about life, for her love and support. May her gentle soul rest in perfect peace-AMEN

Epidemiology of Human and Bovine Tuberculosis in the Federal Capital Territory (FCT) and Kaduna state of Nigeria

By

Aishatu Abubakar

Abstract

The epidemiology of bovine and human tuberculosis (TB) was studied in the Federal Capital Territory and Kaduna state of Nigeria using four diagnostic methods; tuberculin test, culture and acid-fast stain of milk, animal (cattle) tissue and human sputum. Two PCR-based molecular techniques (Spoligotyping and Variable Number Tandem Repeat) were used to identify the species and strains of the isolates, while IS6110-RFLP molecular method was optimised and applied on few samples to determine the efficacy of the method. Of the 967 lactating cows from 57 herds tested for TB, 14.6%, 4% and 81.4% were positive, inconclusive and negative reactors respectively. Tuberculin test also showed that mycobacterial infection was prevalent in the two management systems studied (nomadic and semi-nomadic), but the effect of management on the prevalence of infection was not significant. However, age was found to play a significant role in the prevalence infection where more positive cases were observed among the older age groups. It was also observed that control policy is either not in place or inadequately implemented in the study area. Of the 156 milk samples collected, 12.6% and 23% were culture and acid-fast positive respectively, while out of the 250 tissue samples 17.3% and 20% were culture and acid-fast positive respectively. This finding confirmed a definite relationship between the disease in live and slaughtered cattle. Comparing the three diagnostic methods in

detecting mycobacterial infection in cattle, the smear method was found to have detected more positive cases than the tuberculin and culture tests. Of the 900 suspected human TB patients investigated, 27% and 21.1% were culture and acid-fast positive respectively. This trend of high prevalence of TB among human patients in the area is similar to the trend observed among cattle populations; thus indicating a relationship between the disease in human and infection in cattle. In addition, a significant difference in the prevalence of the disease was observed between male and female patients with more positive cases observed among male patients. The prevalence of the disease was aslso found to be significantly higher in patients who did not have BCG vaccination in the past than those who had. It was also observed that the disease was higher in patients who consume raw milk and milk products. The supporting questionnaire survey among herdsmen, abattoir managers and patients further points that there is high possibility of transmitting the disease from cattle to humans. By DNA fingerprinting, strains of M. *bovis, M. tuberculosis* and *M. africanum* were identified in cattle and humans respectively; thus indicating a typical animal-to-human and human-to-animal transmissions respectively. Combining the two molecular *techniques* in this study has vastly improved the level of discrimination of the isolates where of the 71 isolates typed, 49 patterns were produced by the two methods combined together, instead of only 23 and 41 types by spoligotyping and VNTR typing respectively. Of the 21 strains of *M. tuberculosis* obtained in this study, only 5 strains have been described previously in the international databases searched, out of which only 2 of them have been described previously in Nigeria. The result in this study has valuable epidemiological and public health significance and calls for prompt and decisive action from the government of Nigeria towards controlling this deadly disease in both humans and animals.

List of Contents

Chapter 4

Chapter 4

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AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without the prior agreement of the Graduate Committee.

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Presentations and Conferences attended:

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Abbreviations

%	Percent
χ^2	Chi-square
A	Adenine
AIDS	Acquired immunodeficiency syndrome
ATP	Adenosine triphosphate
BCG	Bacillus Calmette Guerin
Bp	Base pair
C	Cytosine
CDC	Centre for Disease Control and Prevention
CTAB	Hexadecyltrimethyl-ammonium bromide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DOTs	Directly Observed Therapy-Short Course Strategy'
DR	Direct repeat
DVR	Direct variant repeat
ETR	Exact tandem repeat
FCT	Federal Capital Territory
G	Guanine
HCL	Hydrochloric acid
Kb	Kilobase
$MgCL_2$	Magnesium chloride
MIRU	Mycobacterial interspersed repetitive units

Ml	Millilitre
mM	Millimolar
NTBLCP	National Tuberculosis and Leprosy Control program
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NPI	National Program on Immunisation
PCR	Polymerase chain reaction
PFGE	Pulse field gel electrophoresis
PGRS	Polymorphic GC-rich repetitive sequence
Pmol	Picomoles
PPD	Purified proyein derivative
REA	Restriction endonuclease analysis
RFLP	Restriction fragment length polymorphism
RNAse	Ribonuclease
SDS	Sodium dodecyl sulphate
T	Thymine
TB	Tuberculosis
TE buffer	Tris (hydroxymethyl) aminomethane ethylene diamine tetraacetic acid buffer
UV	Ultra violet
VNTR	Variable number tandem repeats
WHO	World Health Organisation
λ	Lambda
µg	Microgram

µl	Microlitre
µM	Micromolar

CHAPTER 1

GENERAL INTRODUCTION

1.1. Definition of Problem

In 1993 the World Health Organization (WHO) declared tuberculosis a 'global emergency' (WHO 2002a) the first to be declared as such. More than a decade after such a declaration, the disease still remains one of the world's leading causes of death among adults (WHO 2003a). More than 8 million people develop active TB annually, and approximately 2-3 million die from the disease each year (WHO 2003a). More than 80% of all TB patients live in sub-Saharan Africa and Asia (WHO 2006). It has been estimated that if the present trend continues, 200 million people are at risk of developing the disease in the next 20 years (van Soolingen 2001).

Tuberculosis is also recognized worldwide as a significant animal health risk, primarily in domestic cattle. Despite considerable success in controlling this disease in cattle populations in many developed countries, tuberculosis remains an important economic and health problem in others, especially Africa, mostly due to socio-political reasons (Morris, Pfeiffer and Jackson 1994). Bovine TB is a zoonotic disease with potential public health and socio-economic significance (Ayele, Neill, Zinsstag, Weiss and Pavlik 2004). However, unlike developed regions of the world, the extent of involvement of *Mycobacterium bovis* in human tuberculosis is not known and its impact on the high-risk group with concomitant HIV/AIDs infection is a growing

concern (Neill, Skuce and Pollock 2005). This is a complication in the recent global resurgence of human tuberculosis, which has precipitated renewed research interest.

Tuberculosis (TB) a chronic debilitating disease, occasionally takes an acute rapidly progressive course (Radostits, Blood and Gay 1994). It is known by several names including 'wasting disease' 'consumptives', 'white plague', and "Pearl disease". In the Hausa speaking region of Nigeria it is also known as 'Mashekari" or 'Tarin fuka'.

Tuberculosis is caused by pathogenic members of the genus *Mycobacterium* which is commonly known as *Mycobacterium tuberculosis* complex (*Mycobacterium tuberculosis, Mycobacterium bovis, Mycobacterium africanum and Mycobacterium microti*). (Collins and Grange 1983; Pfeiffer 2003).

Strain identification of the various species of *Mycobacterium* responsible for causing the disease has been used for epidemiological investigation of tuberculosis and also for identification of potential sources of infection both in the general population and the nosocomial settings to help in developing better control measures. Molecular techniques are now a valuable tool in achieving this (van Soolingen 1993: Durr, Hewinson and Clifton-Hadley 2000; Gori, Espoti, Bandera, Mezzetti, Sola, Marchetti, Ferrario, Salerno, Goyal, Diaz, Gazzola, Codecasa, Penati, Rastogi, Maroni and Franzetti 2005)

1.2. Situation in Nigeria

Nigeria has a population of over 130 million people and ranks 4th among the world's 22 countries with a high TB burden (WHO 2006). Among African countries, Nigeria has the highest estimated number of new TB cases with nearly 368,000 new cases and an estimated 30,000 deaths annually (WHO 2005b). The current annual growth rate of the country is 2.0% (2003 – 2015) and the per capita GNP of Nigeria for 2002 was estimated at US$ 320; expenditure for health services was 5% of central government expenditure and the adult literacy rate is 56%. Infectious and parasitic diseases, malnutrition and respiratory diseases are common and tuberculosis and HIV/AIDS case detection is increasing constituting serious public health problems (Wim, Van, Abdullahi and Mohammed 2004).

Table: 1.1. Estimated Global TB burden Among High-Burden Countries 2004 (WHO 2005a) NB: Showing Nigeria 4th on the list

Estimated Global TB Burden Among High-Burden Countries, 2004

		Population 1,000s	TB Incidence (all forms) number 1,000s*	TB Incidence (all forms) per 100,000 population	TB Mortality (all forms) per 100,000 population	HIV Prevalence in Incident TB Cases %
1	India	1,087,124	1,824	168	30	5.2
2	China	1,307,989	1,325	101	17	0.9
3	Indonesia	220,077	539	245	46	0.9
4	Nigeria	128,709	374	290	82	27
5	South Africa	47,208	339	718	135	60
6	Bangladesh	139,215	319	229	51	0.1
7	Pakistan	154,794	281	181	40	0.6
8	Ethiopia	75,600	267	353	79	21
9	Philippines	81,617	239	293	48	0.1
10	Kenya	33,467	207	619	133	29
11	DR Congo	55,853	204	366	79	21
12	Russian Federation	143,899	166	115	21	6.8
13	Viet Nam	83,123	147	176	22	3.0
14	Tanzania	37,627	131	347	78	36
15	Uganda	27,821	112	402	92	19
16	Brazil	183,913	110	60	7.8	17
17	Afghanistan	28,574	95	333	92	0.0
18	Thailand	63,694	91	142	19	8.5
19	Mozambique	19,424	89	460	129	48
20	Zimbabwe	12,936	87	674	151	68
21	Myanmar	50,004	85	171	21	7.1
22	Cambodia	13,798	70	510	94	13

* The WHO ranks the high-burden countries by the absolute number of new TB cases in each country and is not adjusted due to population size.

Source: "Table 6: Estimated TB burden, 2004," in WHO, *Global Tuberculosis Control: Surveillance, Planning, Financing*, WHO, Geneva 2005, p. 28.

As in most developing countries, in Nigeria, BCG is the first vaccine given in the routine vaccination schedule of the National Programme of Immunizations (NPI), and most children receive BCG at birth or as soon as possible after birth. The programme was first initiated in 1979 when it was known as the Expanded Programme on Immunization (EPI) (Odusanya, Alufohai, Meurice, Clemens and Ahonkhai 2003). The funds for acquisition of vaccines come from both the government and donor agencies such as the World Health Organization (WHO) and other charitable organizations.

The Nigerian government formally launched its National TB and Leprosy Control Programme (NTBLCP) in 1991 and adopted the World Health Organization (WHO)-recommended 'Directly Observed Therapy-Short Course Strategy' (DOTS) in 1993. The DOTS programme was established as the control method for tuberculosis in humans and is integrated into the Primary Health Care of the country through the Federal Ministry of Health (Wim *et al.* 2004).

Despite this expression of political will to control human TB in Nigeria, and a clearly articulated national TB policy, it has not resulted in allocation of sufficient financial support necessary for the effective implementation of this policy (Anon 2006). Effective control is also affected by inadequate trained personnel and political instability. Most importantly also, diagnosis of tuberculosis stops at the smear level, hence the species involved in causing the disease are not known thereby making it difficult to study outbreaks, trace the routes of transmission and also know the species and strains of mycobacterium involved. Another major setback is the inability of the national TB control program to recognize the significance of *M. bovis*, which is a

major public health problem and the general lack of collaboration between human and veterinary medics in this regard. Generally, there has been total lack of synergy and harmony of laws regulating the control of human and bovine tuberculosis in the country.

Nigeria has an estimated cattle population of over 20 million, with the Zebu breed constituting over 90% of the total national herd (Ariyo 2002). The first report of the existence of *Mycobacterium* in West Africa as a causative agent of bovine tuberculosis was in 1913 by Ziemann in Cameroon (Alhaji 1976). But the first report of the existence of bovine tuberculosis in Nigeria based on tuberculin test results and then followed by post mortem and laboratory examination was in 1929, cited by Alhaji 1976. Other studies have further confirmed the existence of bovine tuberculosis in the west African region and Nigeria in particular (Alhaji 1976; Ayanwale 1984; Abubakar 1994; Du-Sai and Abdullahi 1994; Ankugah 2002; Cadmus, Atsanda, Oni and Akang 2004; Awah-Ndukum, Tchoumboue and Aziwo 2005). Some other studies in humans have also shown the involvement of *M. bovis* in causing tuberculosis, for instance, in a study of 102 *M. tuberculosis* complex from patients with pulmonary TB, 4 (3.9%) were *M. bovis* (Idigbe, Anyiwo and Onwujekwe 1986). Cadmus *et al* (2006) have also isolated 3 *M. bovis* strains in a study of 55 human sputum samples in Ibadan, Nigeria.

The control of bovine tuberculosis in Nigeria is regulated by the Federal Ministry of Agriculture. However, this control policy as stated in the Animal Diseases (Control) Decree of 1988 is poorly or inadequately implemented in recent years (Anon 1988). This is largely due to politico-economic reasons, such as the high cost of sustainable

testing and slaughter of infected animals and the subsequent compensation to farmers. Added to this is the problem of social unrest due to political instability and ethnic wars especially between the Fulani herders and local farmers, resulting in the displacement of large numbers of human and animal populations (Ayele *et al.* 2004). Also, socially, the test and slaughter method of controlling bovine tuberculosis is not accepted by the Fulani nomads who own the largest cattle population in Nigeria. There is also the lack of adequate veterinary personnel to implement control measures such as rigorous meat inspection at abattoirs. Poor communication networks, insufficient collaboration with neighbouring countries and the lack of quarantine, and smuggling of live animals across state boundaries have also been identified to cause problem in controlling the disease in cattle (Ayele *et al.* 2004).

From the public health point of view and based on the reports of the prevalence of both human and bovine tuberculosis in Nigeria, it can be concluded that *Mycobacterium* is prevalent in Nigeria and indeed a good percentage of the public stand the risk of getting infected. Also the species of *Mycobacterium* involved in causing disease in both humans and animals in Nigeria is not known. All these need further investigation and are to be made a research priority if effective control of TB is to be achieved.

1.3. Justification for the study

In Nigeria, information on all aspects of bovine and human tuberculosis is limited hence an objective assessment of its epidemiology is a major undertaking. The epidemiology and public health significance of bovine tuberculosis in Nigeria remain unknown. What is known about bovine tuberculosis (BTB) mostly comes from the granulomatous lesions found at slaughter houses/abattoirs and extremely sporadic tuberculin tests conducted on some government and few privately owned herds.

There is also failure or inadequate implementation of control policies for bovine tuberculosis, such as whole herd tuberculin testing and slaughter and rigorous meat inspection to control and monitor epidemiology of the disease.

On the aspect of human tuberculosis, as a routine, identification of the organism stops mostly at the direct smear examination level in hospitals. There is also general underestimation of the importance of zoonotic TB by the public health sector of the national government agency.

The use of molecular techniques for epidemiological studies has not been fully exploited in Nigeria; as such there is no data on the strains of mycobacterium responsible for both human and bovine tuberculosis.

These problems make epidemiological studies possible only on the basis of the limited field survey readily carried out and the scanty records of cases from abattoirs and hospitals.

The effectiveness of the various intervention/control methods for the control TB has not been assessed. Generally, there is lack of synergy and harmony of laws and regulations between veterinary and medical policies for the control of tuberculosis in Nigeria. In view of this, it is therefore justified that this study be embarked upon.

1.4. Aim

The aim of this study is therefore, to improve on the level of information on the epidemiology of human and bovine tuberculosis, to identify risk factors associated with the disease and to determine the involvement of *M. bovis* in human TB in Nigeria. It is also aimed to assess the intervention methods in place and make recommendation for better intervention methods. This information could assist the government in re-designing its control policies for both human and bovine tuberculosis in the Nigeria.

1.5. Objectives

The study was designed with the following objectives: -

1. Determine the Prevalence of human and bovine tuberculosis in the study area through tuberculin testing of lactating cows, culture and isolation of mycobacteria from milk, cattle tissue and human sputum from suspected TB patients
2. Identify species and strains of *mycobacteria* from human and bovine isolates in 1 above through the application of molecular techniques
3. Identify risk factors associated with tuberculosis
4. Assess current intervention methods for bovine and human tuberculosis, and recommend a more effective intervention method.

CHAPTER 2

LITERATURE REVIEW

2.1. Historical background

A familiarity with the history of tuberculosis alerts one to put in perspective mans` struggle against the white plague, which has caused and is still causing a mighty burden of illness and deaths of human beings and animals (WHO 2003b). 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 1994).

Tuberculosis (TB) 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 1994). The danger of eating meat from tuberculosis-infected cattle existed in mosaic laws and the German regulations banning the sale of tuberculosis meat (Collins *et al.* 1983)

Generally, the turning point in the history of mycobacteria and tuberculosis in particular, occurred in 1852 when a German Scientist, Robert Koch, publicly announced that he had observed and cultured the bacillus responsible for tuberculosis (Groschel 1982).

The theory of infectiousness of tuberculosis was placed upon firm foundation and research work since then has been directed towards understanding the epidemiology of the disease and the means by which it can be controlled and ultimately eradicated.

2.2. Epidemiology

Epidemiology is the study of the causes, distribution and control of disease in populations and of factors that determine its occurrence (Thrusfield 1995). *Mycobacterium,* the causative agent of tuberculosis has a worldwide distribution and controlling the worldwide resurgence of TB requires mapping the routes of transmission of the disease. Molecular epidemiology of *Mycobacterium spp* combines molecular techniques such as DNA fingerprinting with conventional epidemiological methods such as the tuberculin test and contact tracing (Kato-Maeda, Bifani, Kreiswirth and Small 2001).

2.2.1. Prevalence and Distribution of Bovine Tuberculosis

Large numbers of cattle and other animals, including wildlife populations, have been known to harbour *M. bovis*, which contributes to the transmission of the disease and also causes difficulty in its control (Morris and Pfeiffer 1995). In industrialised countries, bovine tuberculosis is controlled in farmed animals, as a result of which the disease is rare. These countries are conscious of local and international implications of the disease for trading in animal and animal products.

In Africa and Asia, where animals constantly live in the open, bovine TB had been rare but, the introduction of European breeds of cattle and the subsequent development of intensive agriculture rapidly changed the distribution of the disease in these areas (Alhaji 1976). The disease is now present in almost all African countries, (Anon 1994; Ayele *et al.* 2004) affecting both domestic and wildlife as well as humans. The disease was reported to be prevalent in 33 (80%) of 43 African member countries of the regional commission of the Office International des Epizooties (World Organization for Animal Health, OIE) (Daborn and Grange 1993). In tuberculin testing of cattle in Burkina Faso, Vekemans *et al* (1999) reported a 13% positive reactions and isolation of *Mycobacterium* in 26% of 60 retailed milk samples collected in markets. Also Jiwa and colleagues (1997) reported a 0.2% prevalence of bovine tuberculosis in the Lake Victoria area of Tanzania, while Kazwala *et al* (1998) also isolated mycobacterial species from raw milk of pastoral cattle in the Southern Highlands of Tanzania. Seventeen percent prevalence has also been reported in another study in Chad using purified protein derivative (PPD) tuberculin testing (Schelling, Diguimbaye and Daoud 2000) while 6% isolation of *M. bovis* from apparently healthy milk cows was reported in another study in Nigeria (Okolo 1992).

Prevalence rates of bovine tuberculosis ranging from 0.89-25% have also been reported from several studies in Nigeria and other parts of Africa (Ellwood 1975; Alhaji 1976; Eid 1976; Shehu 1988; Abubakar 1994; Du-Sai *et al.* 1994; Ankugah 2002; Shirima 2003; Awah-Ndukum *et al.* 2005).

Of the 36 Asian nations, 16 reported sporadic low occurrence of bovine TB, and one (Bahrain) described the disease as enzootic; ten did not report bovine TB; and the

remaining nine did not have data (Cosivi, Grange, Darbon, Raviglione, Fujikura, Cousins, Robinson, Huchzermeyer, de Kantor and Meslin 1998). In Latin America and the Caribbean on the other hand, the regional prevalence of bovine TB has been estimated at 1% (de Kantor and Rittaco 1994).

2.2.2. Prevalence and Distribution of Humans Tuberculosis

In humans, tuberculosis continues to be a leading cause of morbidity and mortality worldwide, killing more than 2 million people per year, making it the single leading microbial killer of adults (WHO 2004). According to WHO, if this trend continues one third of the world's population might be infected and mostly from the developing world, particularly Asia (Pace 1999; WHO 2003b). It has also been estimated that approximately 40% of people living in the Indian subcontinent, China and the Pacific are infected with TB (Dye, Watt, Bleed and Williams 2003; Chakraborty 2004). Out of all TB cases notified to the WHO in 2000, 10% were from Europe, with an average incidence of 42/100 000 which is an increase from that reported in 1991 (WHO 2002b). Fifteen countries (Australia, Canada, Greece, Iceland, Israel, Italy, Malta, Monaco, The Netherlands, New Zealand, Norway, San Marino, Sweden, Switzerland, and the USA), were said to have fulfilled the low-incidenced country of WHO with crude case notification rate of below 10/100 000 (WHO 2002a)

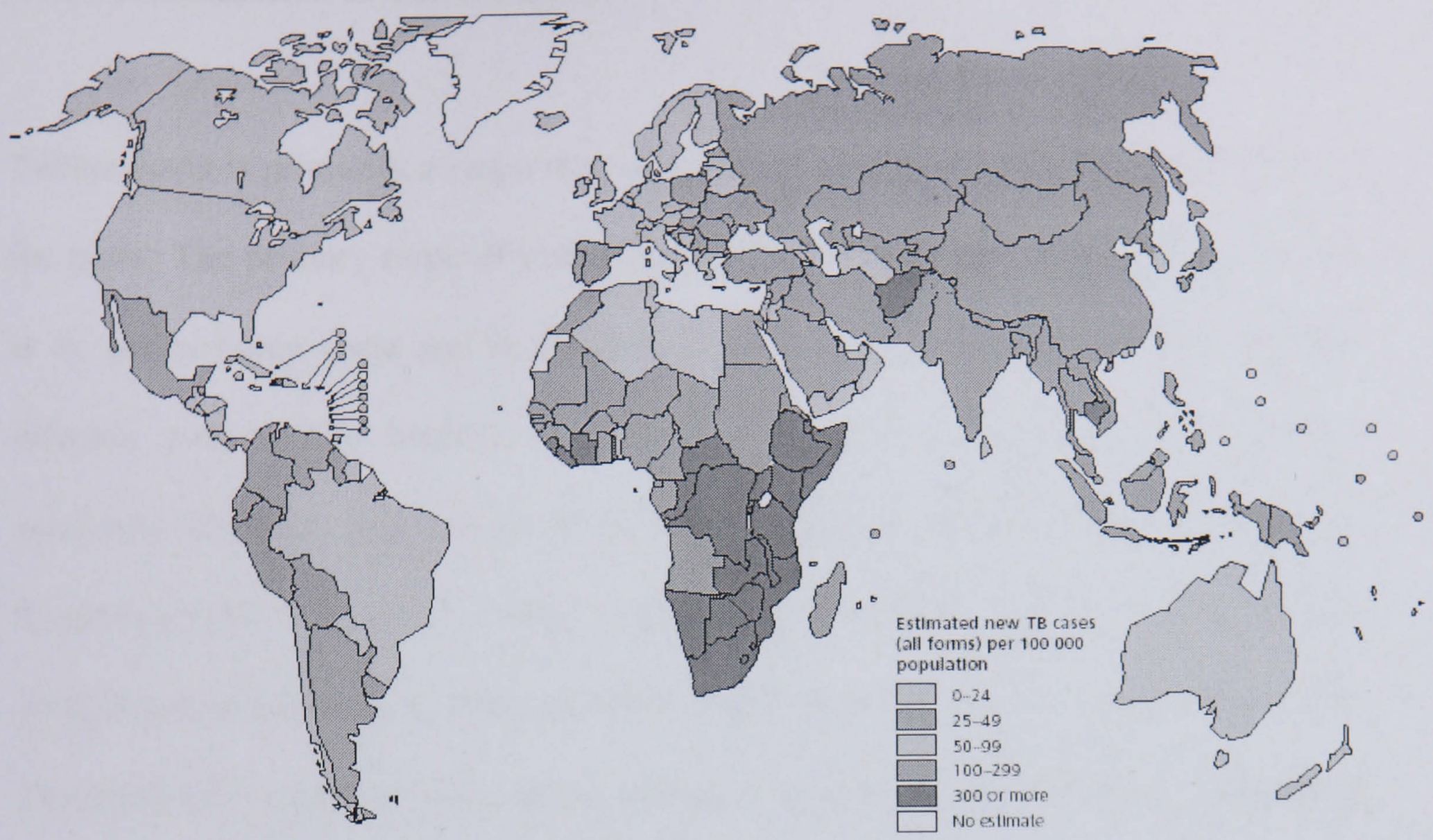

Figure 2.1: Estimated TB incidence rates, 2004 (WHO 2006)

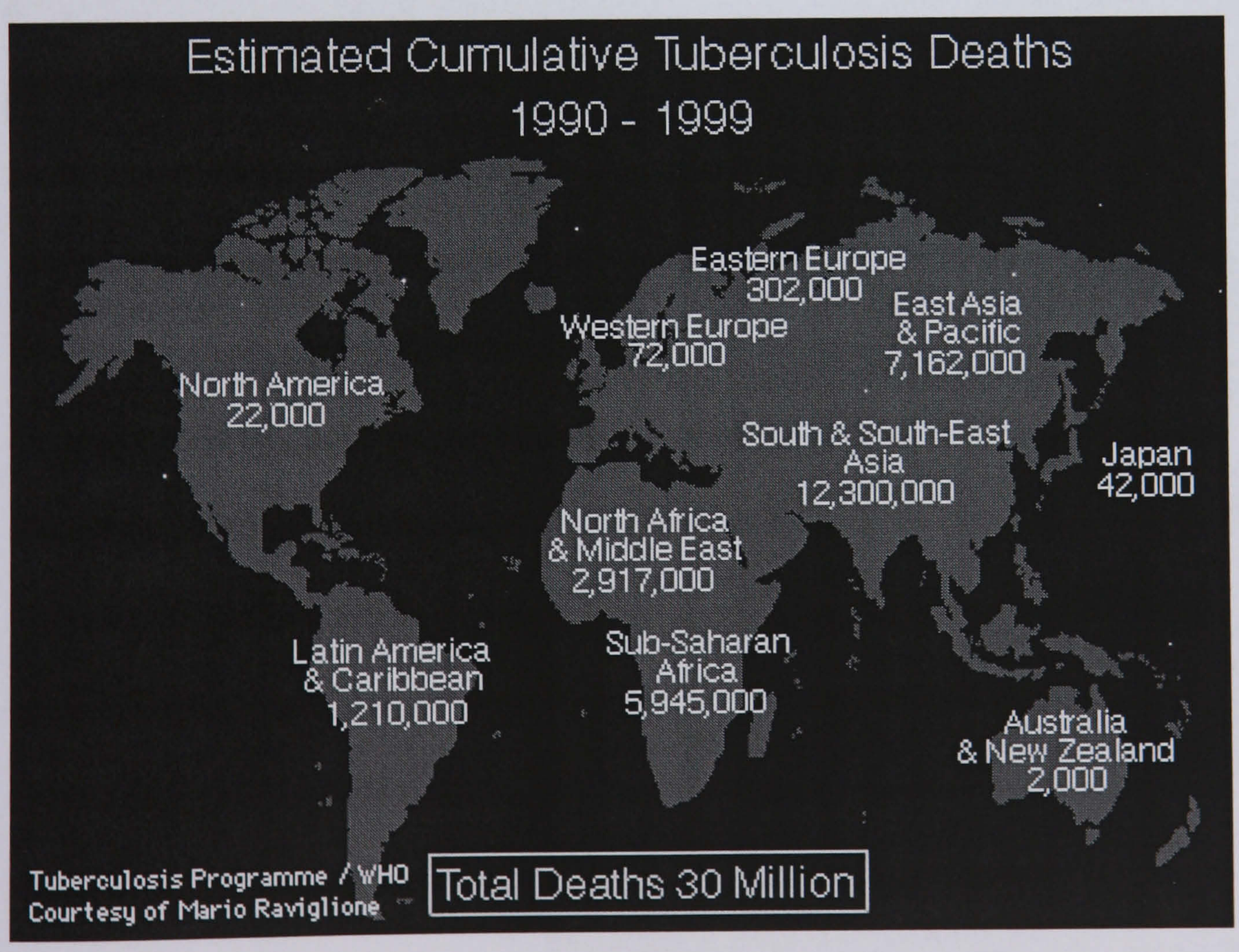

Figure: 2.2. Estimated cumulative tuberculosis deaths 1990-1999 (WHO 2002a)

2.2.3. Transmission of 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 and prolonged contact between infected and healthy humans or animals through the exchange of respiratory secretions (O'Reiley and Daborn 1995; Kempf, Dunlap, Lok, Benjamin, Keenan and Kimerling 2005). However, other routes of transmission such as congenital and vertical transmission have been recorded (Neill, Pollock, Bryson and Hanna 1994). Transmission of tuberculosis can be animal-to-animal, animal-to-human, human-to-animal as well as human-to-human (Collins and Grange 1987).

2.2.3.1. Animal-to-Animal Transmission

Infectious animals may shed the disease in a number of ways such as faeces, milk, discharging lesion, saliva and urine (Neill, O'Brien and Hanna 1991). Infection has been reported to occur through ingestion of feeds contaminated with *M. bovis* while carnivores became infected by eating infected carcasses (Cosivi *et al.* 1998).

Intensive livestock farming promotes close contact between animals, favouring the spread of *M.* bovis (Alhaji 1976; O'Reiley *et al.* 1995; Shirima 2003) . However, extensive livestock farming, especially the nomadic system with no housing, raises the question how TB transmission can take place. Grazing and watering points, vaccination centres, market places and transportation are the commonest gathering places where transmission of infection can easily occur. This is because, *Mycobacterium* is a robust pathogen and may survive in the environment, in

buildings, on transport vehicles, on pasture and in slurry which implies pasture and vegetable contamination, representing a potential source of infection and re-infection to animals (Ayele *et al.* 2004).

2.2.3.2. Animal-to-Human Transmission

In industrialized countries, the incidence of TB due to *M. bovis* in humans is almost at zero level as a result of pasteurization of milk and milk products and good control measures of bovine tuberculosis in cattle populations, although the potential risk remains (Collins *et al.* 1983; Ayele *et al.* 2004). However, in developing countries the story is analogous to conditions in the 1930s and 1940s in Europe, where more than 50% of cervical lymphadenitis cases in children was caused by *M. bovis* infection (Cosivi, Meslin, Daborn and Grange 1995).

Various routes of infection from animals to humans infection, such as from drinking or handling contaminated milk and milk products, and also infected carcass, has been documented (Wigle, Ashley, Killough and Cozens 1972; Collins *et al.* 1983; Lepper and Corner 1983; Collins *et al.* 1987; Neill *et al.* 1994; Griffin and Dolan 1995; Kazwala, Daborn, Sharp, Kambarage, Jiwa and Mbembati 2001). Agricultural workers and rural dwellers have been reported to have developed pulmonary TB due to *M. bovis* as result of inhalation of cough spray from infected cattle and dust particles, or aerosols, shed by infected animals, while urban dwellers can acquire the infection via the gastrointestinal route and develop extra-pulmonary TB (Daborn, Grange and Kazwala 1996). In countries with relatively high prevalence of bovine TB in cattle, abattoir and farm workers are the groups most exposed to infection.

2.2.3.3. Human-to-Animal Transmission

The role of humans in infecting cattle with bovine TB was reviewed by Torning (1965) while Sjogren and Hillerdal (1978) cited several examples of human-to-cattle transmission, and stressed the potential danger that patients with smear-positive pulmonary TB may pose to animals. High rate of infection in cattle due to workers with renal tuberculosis urinating in cow sheds has also been reported (Collins *et al.* 1983; Grange and Yates 1994). Grange and Yates (1994) have reported that man is also able to infect cattle with both the bovine and the human strains of the tubercle bacillus. An analogous situation is thought to occur in rural Africa, where infected herders may urinate on pasture; then animals craving salt preferentially graze on this grass and may succumb to infection (Ayele *et al.* 2004). However, generally; reports of human infection of cattle are rare (O'Reiley *et al.* 1995)

2.2.3.4. Human-to-Human Transmission

In man, human TB is spread from person to person through TB bacilli (bacteria), which are found in the mucus of infected individuals. Tuberculosis in humans has been associated with poor environmental conditions, age, nutrition, cigarette smoking, alcoholism and overcrowding (Springett 1972; WHO 1994; Derek 1999). Human TB, caused by *M. bovis* as a result of human-to-human transmission, was reported in the Netherlands in 1994 (van Soolingen, de Haas, Haagsma, Eger, Hermans, Ritacco, Alito and van Embden 1994b). Evidence of transmission of *M. bovis* between humans

is considered rare and largely anecdotal, and the rate of transmission seems insignificant compared with animal-to-animal or animal-to-human infection (O'Reiley *et al.* 1995). Human-to-human transmission of *M. bovis* is considered less efficient than that of *M. tuberculosis* (van Soolingen 2001); however, transmission among HIV-infected individuals may be different due to immuno-suppression (Ayele *et al.* 2004).

2.2.4. Control and Eradication of Tuberculosis

Today's intimate and rapid global interconnections mean that an uncontrolled infectious disease in one part of the world could result in a global threat to animal and public health.

In the 1940s, the advent of antibiotics capable of defeating the disease created optimism that TB could soon be controlled and eradicated. In the mid-1980s, that optimism proved to be premature, as a progressive worldwide increase of TB incidence dashed hopes of eradication in the near future (Pace 1999; WHO 2004)

This resurgence has made an effective control strategy indispensable. Many authors have thrown some light on factors that could militate against the total eradication of tuberculosis. The ubiquitous nature of mycobacteria, their ease of interchange between man and animals and the wide range of reservoir hosts are just a few examples of such factors (Collins *et al.* 1983; Pfeiffer 2003; Griffin, More, Clegg, Collins, O'Boyle, Williams, Kelly, Costello, Sleeman, O'Shea, Duggan, Murphy and Lavin 2005). Other factors are mycobacteria's ability to withstand fermentation,

Pasteurization, and resistance to pH changes (Chapman 1968; Draper 1981; Shehu 1988). Other obstacles to tuberculosis control include, the slow-growing and thick wall of *Mycobacterium* and its ability to develop resistance to TB drugs (Springett 1972; Brennan and Nikaido 1995; Dooley 1998; Jayant 2003)

2.2.4.1. Control and Eradication of Bovine Tuberculosis

Despite the problems stated above, most developed countries of the world have made significant progress in the control and eradication of bovine tuberculosis which Clifton-Hadley and Wilesmith (1995) summarized in table 2.1.

Whole herd tuberculin test-and-slaughter programs have been used world-wide for the control of bovine tuberculosis (Cousins 2001). The history of the development of tuberculin has been the subject of reviews by many scholars (Stenius 1938; Francis 1968; Francis, Choi and Frost 1973; Snider 1982; Monaghan, Doherty, Collins, Kazda and Quinn 1994). This test has been used to eradicate, or greatly reduce, the prevalence of bovine tuberculosis in many countries. However, tuberculin test has been reported to have low sensitivity, hence multiple tests at prescribed time intervals have been performed to increase its confidence to detect all infected animals (Wood, Corner, Rothel, Baldock, Jones, Cousins, McCormicks, Francis, Creeper and Tweddle 1991). The application of the test-and-slaughter method at a national scale, in countries where the prevalence of the disease is high, would mean the elimination of a significant number of animals in a short period of time (de Kantor *et al.* 1994). A statement made by Bang in 1892 which is widely quoted states that, "the tuberculin test is no more perfect than most things in this world. Sometimes it fails, but it would

be the greatest folly to reject this method because it is not able to give everything we desire" (Monaghan *et al.* 1994).

Abattoir surveillance based on post mortem inspection of carcass is used as a control measure in many countries like USA, New Zealand, and Australia but the sensitivity has been reported to be less than 50% (Corner, Melville, McCubbin, Small, McCormick, Wood and Rothel 1990)

Trials to evaluate the effectiveness of BCG vaccination for the control of tuberculosis in cattle have been undertaken since 1920 (Francis 1947; Francis 1958; Waddington and Ellwood 1972). But it has been reported that vaccination can induce reactivity to tuberculin, hence causing a major problem (Moodie 1977). The possibility of using a vaccine against bovine tuberculosis warrants serious consideration (Buddle, Keen, Thomso, Jowett, McCarthy, Heslop and De Lisle 1995).

Morris *et al* (1994) considered one area which is undoubtedly crucial to effective TB control but receiving little attention; the influence of farmers attitude and behavior, livestock trading practices and herd management in determining how well TB is controlled in individual herds. However, attempts to eradicate tuberculosis from cattle herds in some developed countries have been frustrated by the presence of reservoirs of *M. bovis* infection in wildlife particularly the possums in New Zealand (1994; Aldwell, Pfeiffer, de Lisle, Jowett, Heslop, Keen, Thomson and Buddle 1995; Pfeiffer 1995), badgers in the UK (Barrow and Gallagher 1981; de Lisle, Mackintosh and Bengis 2001) and white tailed deer in Michigan (Clifton-Hadley and Wilesmith 1991).

Table 2.1: Progress in control/eradication of bovine tuberculosis in some developed countries (Group 2 has been subdivided to highlight countries in which re-infection in farmed deer has been perceived as a threat to control in the national cattle herd at risk)

Group	Control/eradication Progress	Impediments to completion	Risk of reinfection	Examples
1	+++	-	-	Australia
2a	+++	-	+	Canada, Denmark, Sweden
2b	+++	-	+	Austria, Belgium, Finland, France, Germany, Iceland, Italy, Japan, Luxembourg, Norway, Portugal, Spain, Switzerland, The Netherlands
3	++	+	++	Hungary, United States of America
4	+	+++	++	Great Britain, New Zealand
5	+	+++	+++	Northern Ireland, Republic of Ireland

NB: +, ++, +++ degree of relative progress, number of impediments/risk

\- minimal or low risk, Source: (Clifton-Hadley *et al.* 1995)

Though developed countries have developed many strategies to detect and control bovine tuberculosis, most of these strategies are not transferable to developing countries especially the sub-Saharan Africa like Nigeria. This is hampered by many factors; most of them politico-economic. Added to the high costs of a sustainable test

and slaughter program (insufficient funds to compensate farmers for culled animals) is the absence of strict Government policies. Although the traditional 'test and slaughter' approach to control of bovine tuberculosis is economically not viable, it is also socially unacceptable by the herders in many African countries. Problems of social unrest due to political instability and ethnic wars, which results in the displacement of large numbers of humans and animal population, is another major factor affecting control of TB in developing countries (Ayele *et al.* 2004). Other problems affecting control are lack of promotion of heat treatment of milk (pasteurization programs) and inadequate trained veterinary personnel to implement control measures (Siddiqi, Stauffer, Ali and Middlebrook 1976; Cousins 2001; Shirima 2003; Awah-Ndukum *et al.* 2005) . Free movement of cattle both nationally and internationally due to insufficient collaboration with bordering countries and the lack of quarantine measures in terms of animal trading and subsequent smuggling of live animals across borders has not helped in the control and eradication of the disease in developing countries (Caffrey 1994; Du-Sai *et al.* 1994; Ayele *et al.* 2004). These deficiencies have resulted in the emergence and reemergence of infectious diseases such as bovine tuberculosis in the developing world like Nigeria. Since, the above mentioned circumstances prevent the use of measures employed by developed countries for the control of bovine tuberculosis, alternative strategies need to be considered.

2.2.4.2. Control and Eradication of Human Tuberculosis

In humans, in addition to the factors affecting control caused by the organism itself, three main factors have threatened the control of tuberculosis. These are increased global migrations, the rise and spread of HIV/AIDS, and poorly managed TB control programs, which result in multi-drug resistant strains especially in sub-Saharan Africa, the Indian sub-continent, south East Asia, Russia and to a lesser extent other countries of the world (Derek 1999; Chakraborty 2004; Anon 2005).

The World Health Organization's Directly Observed Therapy-Short course (DOTS) strategy to support control of tuberculosis arose from the work of Styblo and others initially in Africa in the 1970s, emerging as a 'brand' in 1995 and has since been promoted to national and local governments (Rouillon 1991; Ogden, Walt and Lush 2003). DOTS is a systematic strategy for TB control and declared by the Director General of WHO in 1997 as the most important public health breakthrough of the decade, in terms of lives that will be saved (Atun, Baeza, Drobniewski, Levicheva and Coker 2005). The strategy comprises of five components which include; government involvement, constant supply of good drugs, microscopy, observed therapy, and monitoring of patients. By 2004, the programme had been applied in 183 countries with an increase in cure rates of up to 90% in some countries (WHO 1997; Bassey, Momoh, Imadiyi, Udofia, Miri, Anukam, Epoke, Benka-Coker and Aluyi 2005; WHO 2006). Peru and Vietnam have been reported to have achieved the WHO's target of 70% detection rate and 85% completion (Jayant 2003; WHO 2003b). Population coverage was reported to be complete in 9 of the 22 high-burden countries (HBCs), and almost complete in 5 (WHO 2006). BCG vaccination at birth is currently

employed in most countries of the world to control tuberculosis. Areas of particular weakness in the control of TB are laboratory services, human resources development and the monitoring control programme (WHO 2006)

2.2.5. Molecular Epidemiology of Tuberculosis

Strain identification of the various species of *Mycobacterium* responsible for causing disease has been used for epidemiological investigation of tuberculosis and also for identification of potential sources of infection both, in the general population and the nosocomial settings, to help in developing better control measures. Recently, molecular techniques have replaced the phenotypic methods previously used and these are now a valuable tool in achieving this (van Soolingen 1993; Durr *et al.* 2000; Gori *et al.* 2005). This has been in development since the mid-1980s leading to the availability of a number of genetic typing systems based upon the bacterial genome (genotyping) and has been actively explored. Considerable progress has so far been made in developing techniques that discriminate between isolates of *M. tuberculosis* complex (Durr *et al.* 2000). Polymorphisms have been found to be present in a number of locations throughout the mycobacterial genome, which are being studied and characterized in order to exploit them in epidemiological studies. For example, the insertion element IS6110 (Van Embden, Cave, Crawford, Dale, Eisenach, Gicquel, Hermans, Martin, McAdam, Shinnick and Small 1993), the polymorphic guanine cytocine-rich (GC-rich) repetitive sequences (PGRS) (Chaves, Yang, Hajj, Alonso, Burman, Eisenach, Dronda, Bates and Cave 1996), the direct repeat region (DR) (Van Embden *et al.* 1993; Van Embden, van Gorkam, Kremer, Jansen, van Der Zeijst and Schouls 2000), major polymorphic tandem repeats (MPTR) and

minisatelites, including the mycobacterial interspersed repetitive units (MIRUs) (Supply, Mazars, Lesjean, Vincent, Gicquel and Locht 2000) and the variable number of tandem repeats (VNTRs) (Frothingham and Meeker-O'Connell 1988) are widely used for epidemiological studies.

However, detailed epidemiological investigation of bovine tuberculosis has been hampered by the lack of an established typing system for *M. bovis* (Durr *et al.* 2000). This has been an impediment to understanding sophisticated epidemiological studies to assist in the control and eradication of tuberculosis in domestic animals. Though a lack of consensus on the best protocol still remains, progress is being made to develop standard protocols (Van Embden *et al.* 1993). Researchers using the different techniques available for epidemiological studies of *Mycobacterium* have been undertaken. The advantages and limitations of various molecular techniques used in the study of the epidemiology of tuberculosis have been reported by many scholars.

2.2.5.1. IS6110-Restriction Fragment Length Polymorphism (IS6110-RFLP)

The most important insertion sequence identified in the *M. tuberculosis* complex is the IS6110. IS6110-RFLP is the most widely used and standardised molecular method for differentiating between *M. tuberculosis* isolates (Van Embden *et al.* 1993).

The IS6110 insertion sites in the chromosome and the copy number varies for different strains of *M. tuberculosis* from zero up to 20 copies present in the genome (figure 2.3) (Brosch, Gordon, Eigglmeier, Garnier, Tekaia, Yeramian and Cole 2000). For instance, there are 16 copies present in the genome of *M. tuberculosis* H37Rv and only four copies in the CDC1551 strain. This resulting degree of polymorphism has

enabled IS6110-RFLP to be used as a genetic marker for epidemiological studies, thus making it the 'gold standard' for molecular epidemiological studies of human tuberculosis (Van Embden *et al.* 1993; Gordon, Heym, Parkhill, Barrel and Cole 1999). Generally, strains from outbreaks or patients with common transmission route will display identical banding patterns.

However, fewer copies of IS6110 are present in *M. bovis* than M. tuberculosis with the majority of bovine isolates having only a single copy, which is located at the direct repeat (DR) region (Collins, Erasmuson, Stephens, Yates and de Lisle 1993; Van Soolingen, dar Haas, Hermans and van Embden 1994a; Cousins, Williams, Liebana, Aranaz, Bunschoten, Van Embden and Ellis 1998). Nonetheless, the use of IS6110-RFLP is recommended to fingerprint *M. bovis* isolates for epidemiological purposes, where the population of the *M. bovis* contains multiple copies of IS6110 (i.e. more than 3 copies) due to the good discrimination offered by the technique. However, if a majority of the isolates contain 3 or fewer copies of IS6110, additional sub-typing methods are required (Durr *et al.* 2000).

The transmission of TB can be investigated and the results compared between different laboratories both locally and internationally through the use of IS6110-RFLP. Van Soolingen *et al* (1995) discovered the prevalence of a single *M. tuberculosis* genotype in various East Asian countries known as the "Beijing family" when they used IS6110-RFLP to investigate the population structure of *M. tuberculosis* strains in China and Mongolia.

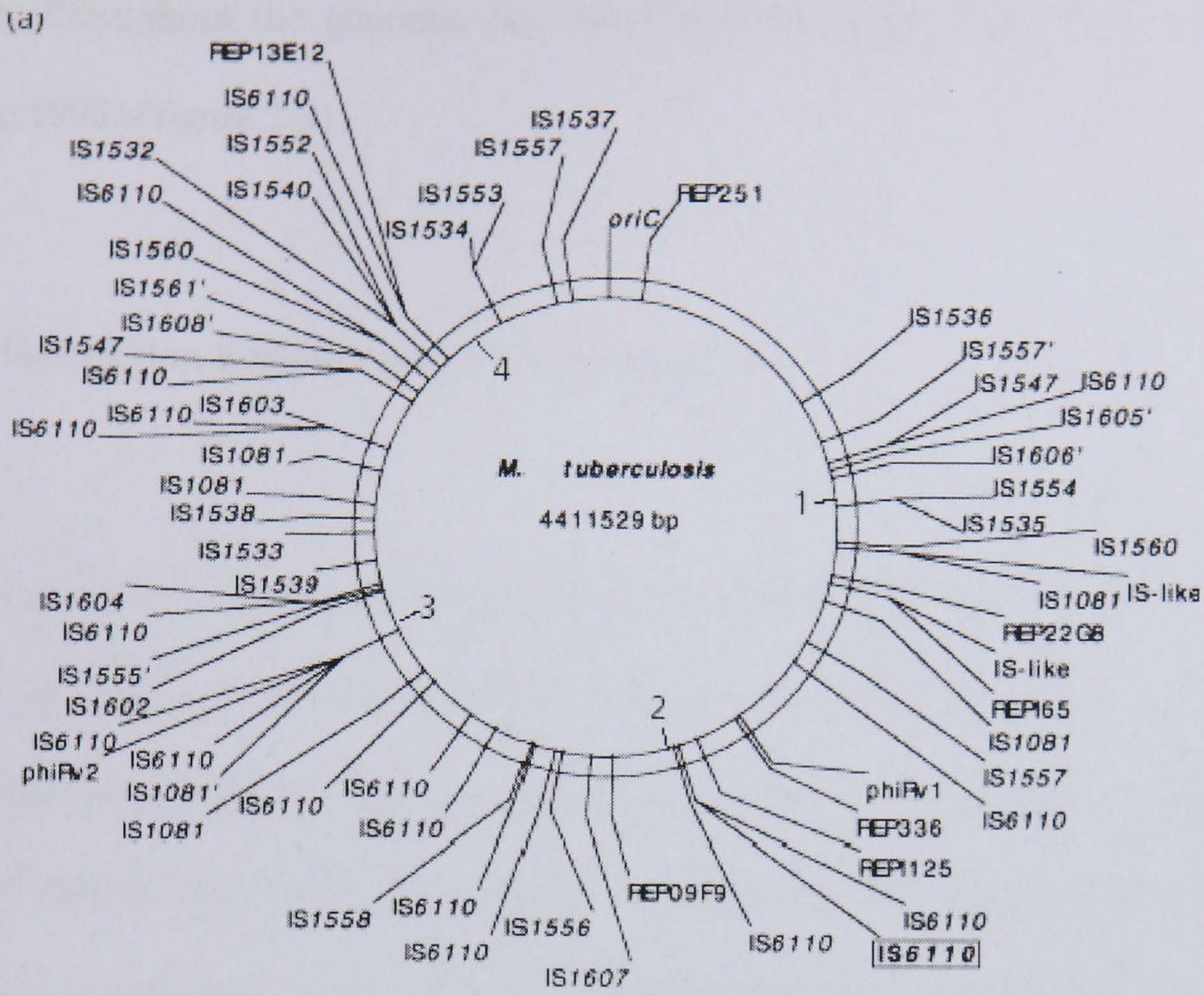

Figure: 2.3. Distribution of the IS6110 insertion sequence on the *M. tuberculosis* (H37Rv) chromosome (Gordon *et al.* 1999)

However, the method has some disadvantages, which include the need for large quantity of good quality DNA; it is a technically demanding and expensive method. The method also requires a longer period to get results; it is less discriminatory for isolates with low copy numbers and less reliable (Cave, Eisenach, Templeton, Salfinger, Mazurek, Bates and Crawford 1994)

In such case a second genotyping technique such as spoligotyping is often used to establish polymorphisms between strains of isolates with low copy number (van Soolingen 2001). It is also possible for isolates to have identical DNA fingerprints

without being epidemiologically linked, as the IS6110 copies are not distributed randomly throughout the genome, but insert themselves at hotspots (McHugh and Gillespie 1998) (figure 2.3)

2.2.5.2. Restriction Endonuclease Analysis (REA)

This method was the first to be developed for intra-specie typing of *M. bovis* (Collins and de Lisle 1985). As the only typing technique available in the late 1980s and early 1990s, REA was used for molecular epidemiological studies in New Zealand and Ireland (Collins and de Lisle 1986). However, Collins and his colleagues reported that the method is technically demanding and interpretation of the complex REA pattern is difficult (Collins *et al.* 1993; Durr *et al.* 2000). But, it plays a good role in interspecies typing and also has great potential for classifying closely related specie (Collins *et al.* 1985). The method has no method for numerically cataloguing types, which makes comparison between laboratories difficult. However, in New Zealand, REA still remains the technique of choice due to familiarity and superior discrimination as compared with new techniques (Collins 1999)

2.2.5.3. Pulse Field Gel Electrophoresis (PFGE)

The problem of excessive number of small DNA fragments encountered with REA was solved by the development of PFGE (Durr *et al.* 2000). The steps are basically the same with only difference in the use of endonulease enzymes to generate small number of large DNA fragments. Zhang *et al* (1995) published one of the earliest

reports on the use of this method for the typing of M. tuberculosis, and later for differentiation of *M. bovis* Bacillus Calmette-Guerin (BCG) substrains. Feizabadi *et al* (1996) also reported a good description between types for isolates of *M. bovis*. unfortunately the application of the technique on *M. tuberculosis* complex is difficult and labour intensive.

2.2.5.4. Polymorphic Guanine and Cytosine-rich Repetitive Sequences (PGRS)

Mycobacterium has very high guanine-cytosine content and within the genome, short repeated sequences are present which have a G-C composition in excess of 80% (Poulet and Cole 1995; Cole, Brosch, Parkhill, Garnier, Churcher, Harris, Gordon, Eigglmeier, Gas, Barry III, Tekaia, Badcock, Basham, Brown, Chillingworth, Connor, Davies, Devlin, Feltwell, Gentles, Hamlin, Holroyd, Hornsby, Jagels, Krogh, McLean, Moule, Murphy, Oliver, Osbourne, Quail, Rajandream, Rogers, Rutters, Seeger, Skelton, Squares, Squares, Sulston, Taylor, Whitehead and Barrel 1998). These polymorphic sequences are present in multiple clusters scattered throughout the genome. The use of PGRS-RFLP has been demonstrated to have a good degree of strain differentiation of *M. bovis* (Cousins, Williams, Ross and Ellis 1993) . It is recommended that PGRS-RFLP is the method of choice for maximum sensitivity in subdividing strains of *M. bovis* that have a single or low copy numbers of IS6110 (Cousins *et al.* 1998). The PGRS elements appear to be quiet stable in *M. tuberculosis*, because an identical PGRS-RFLP fingerprint was reported to be identical over a 3.5 year period from a single patient (Chaves *et al.* 1996).

However, this technique is found to be time consuming and technically demanding and cumbersome; consequently, variable results can be obtained even by the most experienced technician (Durr *et al.* 2000). The method also requires large amount of DNA as hence the need for culturing samples which can cause delay and inconvenient when investigating the epidemiology of an outbreak when results are required quickly.

2.2.5.5. Spoligotyping

This method is known as `spacer oligonucleotide typing` and represents the first PCR based technique to be widely accepted. It is based on DNA polymorphism present at one particular chromosomal locus, the "Direct Repeat" (DR) region, which is uniquely present in *Mycobacterium tuberculosis* complex bacteria (Figure 2.4). It is found between the 36bp direct repeats (DR) in the genomic DR region of the *M. tuberculosis* complex strains (Goyal, Saunders, Van Embden, Young and Shaw 1997; Kamerbeek, Schouls, Kolk, van Agterveld, Van Soolingen, Kuijper, Bunschoten, Molhuizen, Shaw, Goyal and Van Embden 1997). This locus was first described by Hermans and colleagues (1991) who sequenced this region in *Mycobacterium bovis* BCG, the strain used worldwide to vaccinate against tuberculosis. The DR region in *M. bovis* BCG consists of directly repeated sequences of 36 base pairs, which are interspersed by non-repetitive DNA spacers, each 35 to 41 base pairs in length. The spacer sequences of the DR region are amplified by PCR using labelled primers that anneal to the end of the DRs and then hybridise to the oligonucleotides on the membrane, which correspond to various spacer sequences bound to it (figure 2.4). By

spoligotyping one can detect the presence or absence of spacers of known sequence. The 43 spacer sequences used are derived from 37 *M. tuberculosis* H37Rv and six *M. bovis* BCG P3 strains. Individual *M. tuberculosis* complex isolates contain various numbers of these sequences and these can be used to differentiate them by the patterns of positive and negative hybridisation signals (Goyal *et al.* 1997). The function of these DR loci is however unknown.

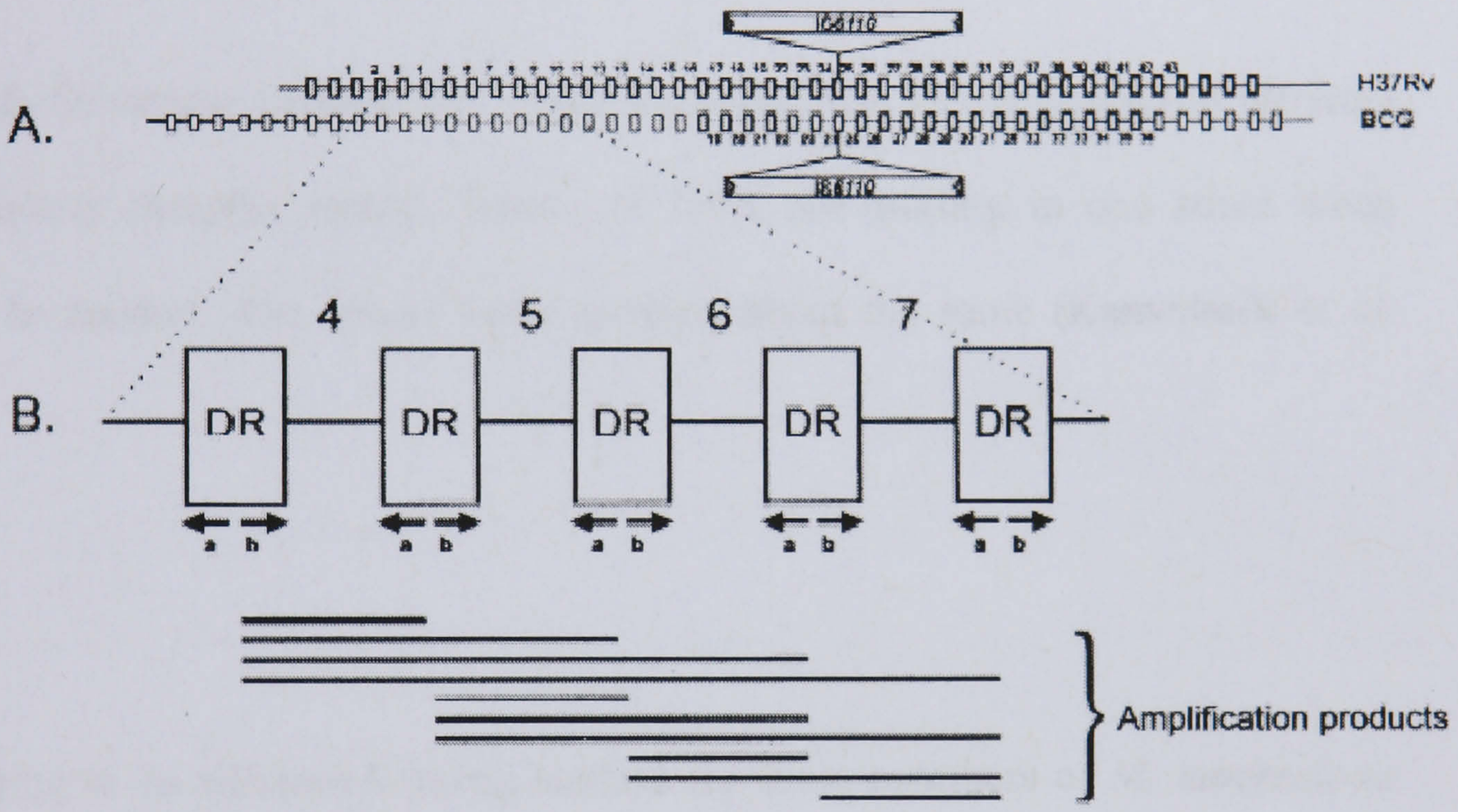

Figure: 2.4. Structure of Direct Repeat loci in the mycobacterium genome (Kamerbeek *et al.* 1997). NB- B is the magnification of the DRs

The chromosomes of *M. tuberculosis and M. bovis* contain 48 and 41 DRs respectively which are interspaced with unique spacers varying in length from 35 to 41 bp (Kamerbeek *et al.* 1997). The polymorphism seen between clinical isolates at this locus is mainly due to the presence or absence of one or more direct repeats plus the adjacent spacer, known as the direct variant repeat (DVR) (Figure 2.5.)

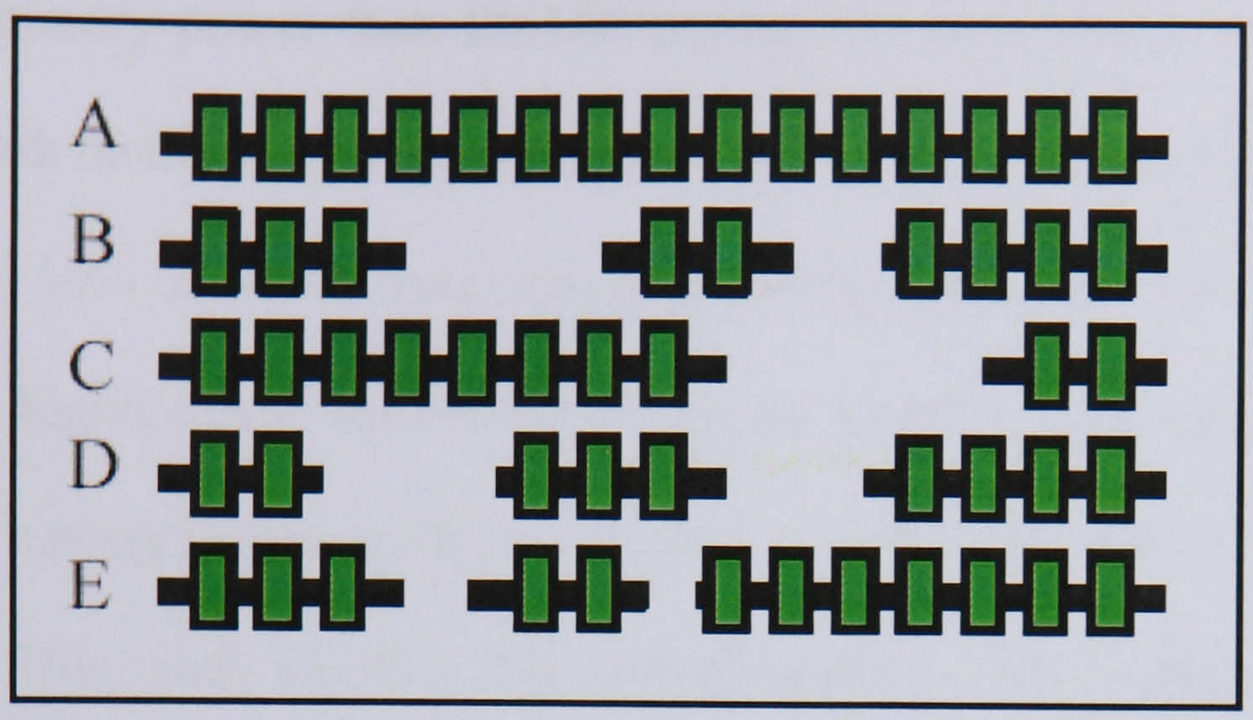

Figure: 2.5. Schematic presentation of the polymorphism in DR regions of different *M. tuberculosis* complex strains. Blocks of DVR are missing in one strain when compared to another. The spacer order remains about the same (Kamerbeek *et al.* 1997).

Spoligotyping is an additional typing method for those members of *M. tuberculosis* complex isolates with fewer copy numbers of IS6110 (Goyal *et al.* 1997). The value of this method for strain differentiation of has been assessed in several studies (Durr *et al.* 2000). Aranaz *et al* (1996) have shown that the technique did enable the grouping of the affected host specie, which presumably reflected an underlying difference in epidemiology of the disease but differentiation of isolates was poor compared with IS6110-RFLP. Similarly, it has been demonstrated that spoligotyping is particularly useful in eliminating the suggestion of transmission between patients who are epidemiologically linked, but was also found to be less discriminatory than RFLP (Goyal *et al.* 1997; Goyal, Lawn, Afful, Acheampong, Griffin and Shaw 1999).

Bauer (1999) used spoligotyping as an additional method to type *M. tuberculosis* strains with low IS6110 copy numbers and they found that spoligotyping has higher discriminatory power than IS6110 typing. For their study, 249 isolates were typed using both methods (spoligotyping and IS6110-RFLP) and IS6110-RFLP produced 56 patterns, 16% of which were unique while the remaining 83% were clustered into 16 groups. Eighty-seven different spoligotype patterns were observed with 25% of all isolates having unique spoligotypes whilst the remaining 75% were clustered into 24 groups. This study showed that spoligotyping is very useful as a secondary typing method especially for isolates with low IS6110 copy numbers. Recently, Gori *et al* (2005) also compared spoligotyping and IS6110-RFLP and demonstrated spoligotyping sensitivity of 97.6% with specificity of 47%.

Spoligotyping is good at getting a first good picture of strain identity in a new and more localized or confined setting and it's useful in case of epidemic spread. It is recommended as a valuable first step in analysing single or low copy number of *M. bovis* strains (Cousins *et al.* 1998). The method is particularly useful to simultaneously detect and type *M. tuberculosis* complex bacteria directly from clinical samples; therefore there is no delay since the time wasted for culture is not there (Goyal *et al.* 1999). It is also an easy and quick method compared to IS6110 RFLP and the patterns are easy to read and compare via computer software.

However, some spoligotype patterns may be indistinguishable and difficult to interpret, which could be due to chains of recent transmission causing isolates to cluster, or it could also represent broad genetic similarities between isolates which

share a more distant common ancestor (Hayward and Watson 1998). The main disadvantage of spoligotyping is that all genetic polymorphism is restricted to a single genomic locus, which is the DR cluster. It has also been reported that the method can result in overestimation of recently transmitted disease (Gori *et al.* 2005).

2.2.5.6. Mycobacterial Interspersed Repetitive Units (MIRUs)

MIRUs are DNA elements ranging from 40-100bp in size dispersed throughout the intergenic regions of the *M. tuberculosis* complex genomes. MIRUs are thought to be involved with regulation of gene expression, differential translation of genes within a polycistrotic operon and some may function as structural components for chromosome organisation (Supply, Magdalena, Himpens and Locht 1997). Supply and colleagues (2000) have identified 41 of such loci in the *Mycobacterium tuberculosis* H37Rv genome and have termed them mycobacterial interspersed repetitive units. They are also shown to contain 65 copies of these repeats in the 41 loci; twelve of which display differences in tandem repeat copy numbers and sometimes in the sequences of the repeat units (Brosch *et al.* 2000), this make them similar to human minisatelite variable number tandem repeat (VNTR) regions.

MIRU-VNTR PCR based method is based on the variable number tandem repeats of MIRUs. The isolates are typed by the number of copies of repeat units found at the loci; which is determined by the size of the fragment produced by amplifying the entire locus using primers that anneal to the flanking DNA (Mazars, Lesjean, Banuls,

Gilbert, Vincent, Gicquel, Tibayrene, Locht and Supply 2001). The repeat units are known to be between 52 to 77 base pairs (bp) in length.

MIRU-VNTR typing produced more distinct patterns when compared with IS6110 RFLP and spoligotyping (Barlow, Gascoyne-Binzi, Gillespie, Dickens, Shanam and Hawkey 2001; Cowan, Mosher, Diem, Massey and Crawford 2002). In a study of 180 *M. tuberculosis* and *M. bovis* isolates with low IS6110 copy number between six and zero, Cowan and colleagues (2002), found that MIRU-VNTR method produced 80 distinct MIRU patterns with 6 isolates possessing unique MIRU patterns and 120 grouped into 20 clusters; while IS6110-RFLP typing produced 58 patterns in total. Thirty-eight of the isolates had unique patterns, whilst 142 were grouped into 20 clusters. On the other application of spoligotyping on the same samples produced 59 distinct spoligotype patterns with 43 unique patterns and 137 isolates grouped into 16 clusters. Combining the three methods led to maximum specificity in typing.

MIRU-VNTR has the advantage of rapid turn round hence can be automated for large-scale typing projects and it does not requires large quantity of DNA (Supply *et al.* 2000; Sola, Filliol, Legrand, Lesjean, Locht, Supply and Rastogi 2003)

2.2.5.7. Variable Number Tandem Repeat (VNTR)

Genetic loci containing variable numbers of tandem repeats (VNTR loci) form the basis for human genetic mapping. The technique is also used for human forensic and paternity testing (Goyal, Young, Zhang, Jenkins and Shaw 1994; Frothingham 1995).

VNTRs are hypervariable loci within the genome of tandemly repeated DNA sequences, which vary in copy number at a given site between different individuals of a population resulting in allelic variation. This variability is thought to be generated by slippage during replication, which results in the newly synthesized strand having extra or missing repeat units in comparison with the original template (van Belkum. Scherer, van Alphen and Verbrugh 1998).

The technique is also PCR based and primers are designed to anneal to the DNA flanking the VNTR locus, so that after amplification the polymorphism in the number of repeats can be examined by gel electrophoresis. Conservation of flanking DNA is generally good between strains, and can sometimes be observed among different species (van Belkum *et al.* 1998). This allows the analysis of the polymorphisms at the VNTR loci to be investigated across genera.

Many possible VNTR loci have been studied in isolates of *M. tuberculosis* and *M. bovis* (Frothingham *et al.* 1988; Goyal *et al.* 1994; Skuce, McCorry, McCarroll, Roring, Scott, Brittain, Hughes, Hewinson and Neill 2002). Almost 42 VNTRs have been identified that could be used for differentiation of *M. tuberculosis* complex strains. These include the exact tandem repeat (ETRs) (Goyal *et al.* 1994), the major polymorphic tandem repeat (MPTR) (Supply *et al.* 2000) and also the sets of Queen's University Belfast (QUB) VNTRs (Roring, Scott, Brittain, Walker, Hewinson, Neill and Skuce 2002; Skuce *et al.* 2002). ETR-A, ETR-B, ETR-C, ETR-D, ETR-E are the most common ETR for strain typing with ETR-A being the most discriminatory compared with the others (Sola *et al.* 2003).

In a study conducted by Skuce and colleagues (2002), on 100 *M. bovis* isolates, the technique identified 33 allele profiles for the six novel VNTRs in comparison to 29 profiles produced by spoligotyping on the same isolates. VNTR typing was also reported to be more discriminatory than IS6110-RFLP in another study conducted by (Spurgiesz, Quitugua, Smith, Schupp, palmer, Cox and Keimi 2003). In another study of 461 isolates of *M. bovis* from Northern Ireland, Skuce and colleagues (2005) reported that VNTR assay discriminated 40 different profiles, with the most prevalent constituting 21 per cent of the total while spoligotyping discriminated only 14 profiles.

VNTR typing has the advantage of being highly discriminatory and reproducible among laboratories, and the data can be displayed in a simple numerical format that is easy to manage (Skuce *et al.* 2005). However, deletions and insertions within the repeat are also observed at some VNTR loci, this causes ambiguities in the definition of the size coding between studies (Le Fleche, Fabre, Denoeud, Koeck and Vergnaud 2002)

Though there are various techniques for genotyping, the choice for an epidemiological study depends on the particular circumstances such as study of global, national or local transmission patterns. It also depends on cross contamination, or investigation, of local outbreaks. Each of these require a different combination of techniques (Durr *et al.* 2000; Kaduma, McHugh and Gillespie 2003). However, cost and availability of infrastructure may be important in selecting techniques in developing countries like Nigeria. Nonetheless, a choice of one or more relatively cheap and less technically

demanding techniques might be incorporated to achieve maximum strains discrimination in these areas.

2.3. Mycobacterium infection in other Animals

There is some degree of host susceptibility to the various species of *Mycobacterium*, although cross infections are known to occur, with animal species being more susceptible to bovine tubercle bacillus, while humans on the other hand is more susceptible to *M. tuberculosis* (Alhaji 1976; Pfeiffer 2003). Susceptibility to *Mycobacterium* by other species of animals has been reported by several authors. For instance, it has been demonstrated that the deer (Beatson 1985; de Lisle *et al.* 2001), small ruminants (Lall 1969; Cordes, Bullians, Lake and Carter 1981; Davidson, Alley and Beatson 1981; Lu, Tsai, Tsung, Chen, Lee, Hung and Lee 1992; Niemann, Richter and Rusch-Gerdes 2000 ; Cousins 2001) and pigs (Snider 1971; de Lisle, Collins, Loveday, Young and Julian 1990; Pfeiffer 1995; Pfeiffer 2003) to be susceptible to mycobacterium. Equally it has been shown that cats and dogs (Myers and Steel 1969; De Lisle 1994; Radostits *et al.* 1994) as well the horse have been shown to be susceptible (Bywaters 1949; Francis 1958; Morris *et al.* 1994). However, species are not equally susceptible and are often grouped into spill over (end) hosts and maintenance hosts (Ayele *et al.* 2004).

CHAPTER 3

MATERIAL AND METHODS

3.1. Safety

All fieldwork carried out in this study had safety assessment undertaken and necessary precautions taken to avoid injury to the researcher, assistants and the animals. Where reagents were used, safety assessment was performed in order to comply with the Control of Substances Hazardous to Health Regulations.

3.2. Study Area

All fieldwork was carried out in the Federal Capital Territory, Abuja, and Kaduna State of Nigeria. The initial laboratory work (staining, culture and isolation) were carried out at the tuberculosis and HIV laboratory of Zankli Medical Center, Abuja, Nigeria. Molecular techniques were conducted at the School of life sciences, University of Hertfordshire and the Veterinary Laboratory Agencies in the UK.

3.2.1. Federal Capital Territory (FCT) of Nigeria

Abuja is the new capital city of Nigeria and has witnessed a high influx of people from all states of the federation, either due to movement of government ministries, or to find new jobs. The Federal Capital Territory (FCT) has six area councils with an estimated population of over 3 million people and over 100,000 head of cattle. It

occupies an area of 3000-square meters (7,770 km²) and it is located at 9° 10` north and 7° 10` East. It is located north of the confluence of the Niger and Benue rivers and situated at the geographical heartland of the nation between the northern ecological zone, with its characteristic savannah vegetation, and the southern ecological zone, dominated by forest (Ariyo 2002) This may be why it attracts large a population of nomads with their cattle in search of a greener pasture especially in the dry season.

3.2.2. Kaduna State of Nigeria

Kaduna state is located in the central area of what used to be called the Northern region of Nigeria as well as the regional headquarters. Kaduna city (the headquarters of Kaduna state) is an important industrial centre in the North and also houses a large number of the cattle population in the north. Kaduna state has 23 local Government Areas and has an estimated population of over 5.5 million inhabitants making it the second most populated in Northern Nigeria (Gwani 2002). The State has an estimated population of over 200,000 head of cattle (Ariyo 2002).

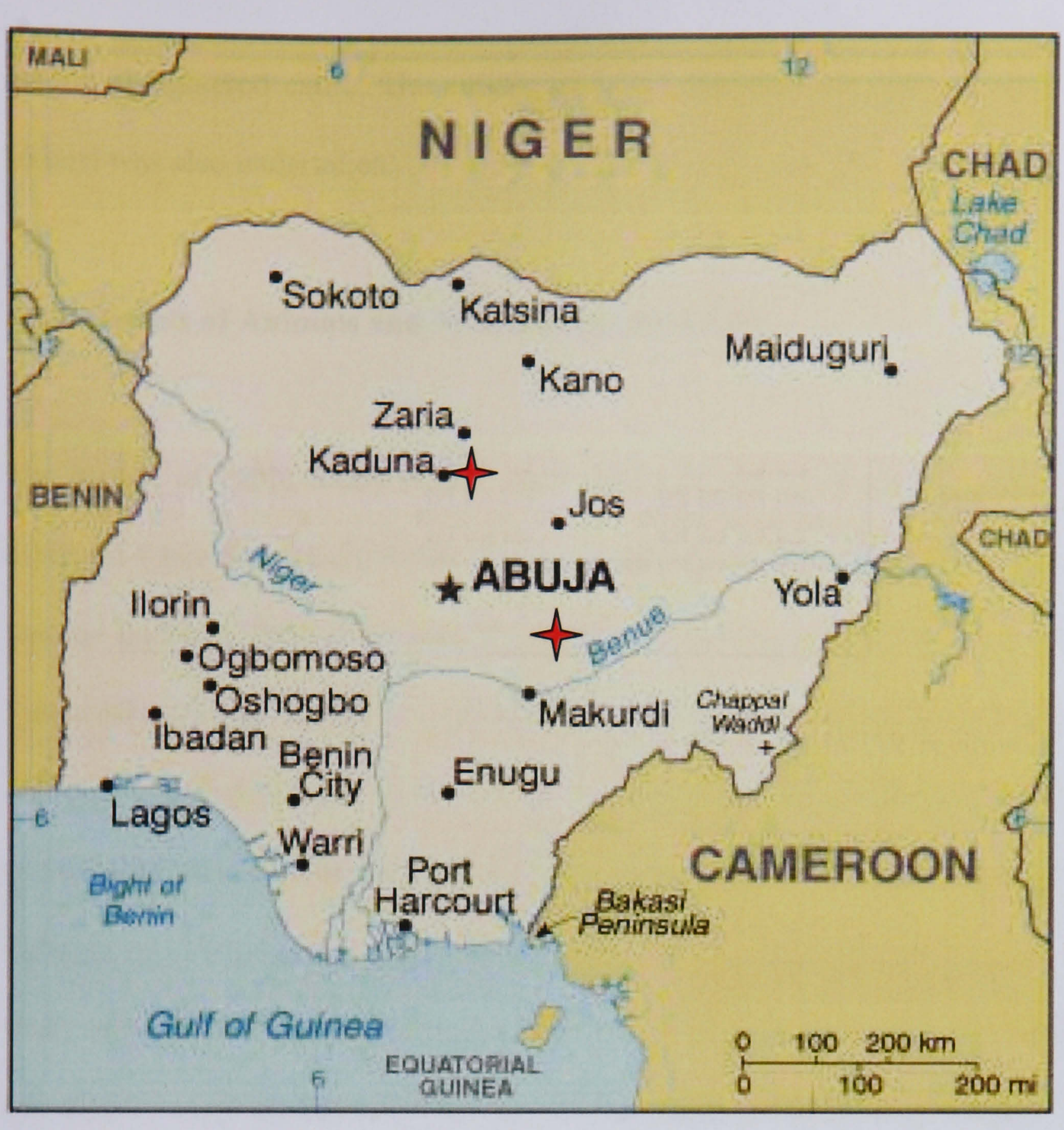

Figure: 3.1. Map of Nigeria showing the Federal Capital Territory, Abuja, and Kaduna state (study area represented by ✦)

3.3. Prevalence of Bovine Tuberculosis in the Federal Capital Territory (FCT) and Kaduna State of Nigeria

This study investigates the current prevalence of bovine tuberculosis in the study area; to highlight its potential dangers to domestic and wild animals as well as the emerging public health threat of this disease for humans and the need to implement control measures. The study was undertaken through tuberculin testing of Fulani lactating cows which are the main source of milk and milk products and also the survey of TB

among slaughtered cattle. The culture and acid-fast stain of milk and suspected TB lesions was also undertaken.

3.3.1. Breeds of Animals and Management practices

The breeds of cattle found in the study area are mainly the Zebu, which has four different types (Bunaji or White Fulani, Rahaji or Red Bororo, Adamawa gudali & Sokoto gudali). They constitute about 90% of the total national herd. The White Fulani (Bunaji) are the most numerous and widespread of all Nigerian cattle breeds and they account for over 37.2% of the national herd and are the most common type in the study area. It is believed by the Fulani pastoralist to be superior to all breeds of Zebu in resisting diseases. Few Rahaji breed of the Zebu type are also found in the study area. Animals selected for the study receive little or no veterinary attention, are not usually supplemented and are owned mainly by the Fulani pastoralist. They are kept on free-range grazing system either on constant move (nomadic) or limited movement (semi-nomadic), and using communal grazing grounds and watering points. The pastoralists respond to the various constraints on production such as disease, pasture, pests and political developments extremely rapidly, moving their stock and mostly ignoring international borders, control posts and Veterinary regulations.

3.3.2. Sampling procedures and Animals selected

Only cows that had calved at least once and lactating were included in the study because, the milk and milk product from these animals is used to produce 'Nono' and

'Man-shanu' which are a local, naturally fermented milk and local butter respectively. These are local delicacies in Nigeria especially in the northern region. Cows were grouped into four age groups (2-3yrs, >3-4yrs, >4-5yrs and >5yrs). Herds were randomly selected from all the Area councils or local Governments of the FCT and Kaduna state respectively based on contact with their society called the 'Miyetti Allah Cattle Rearers Association'. Free treatment for minor illnesses, de-wormers and acaricides were provided as an incentive after taking the final reading of the tuberculin test (after 72hrs). Animals selected for the study were grouped into two, based on management systems, the nomadic (those always on the move i.e. migratory pastoralist) and the semi-nomadic (non migratory and partly kept indoors) systems. This is because they are the main sources of milk and milk products to the public. Conservative age of individual cows was determined based on history from the herdsmen and/or cornual ring method. Cows were restrained using various physical restraint methods such as ear twitch, casting and so on depending on the temperament of individual animal. Cows were also properly identified by their names and/or colour markings. Epidemiological data was also taken from each animal sampled as well as the herd owner in the form of questionnaires to help in the investigation and determination of risk factors.

3.3.3. Tuberculin Test

Tuberculin testing was conducted using purified protein derivative (PPD) obtained from Veterinary Laboratory Agency (VLA) UK, to screen cows for tuberculosis using the single intra-dermal comparative tuberculin test (SICTT) by means of standard method (Lesslie and Herbert 1975; Monaghan *et al.* 1994; Shirima 2003). Both sites

on the neck (left and right) were shaved and washed with soap to avoid contamination. Skin thickness was measured using the Vernier calliper and recorded prior to injecting the antigen. Then 0.1ml equivalent to 2,500 IU of avian tuberculin was injected intra-dermally on the right side and an equivalent dose of bovine tuberculin was injected into the left side. The tuberculin was injected using 2ml McLintock pre-set automatic syringe (no. 622848) each identified by the colour of its cap (red for avian and blue for bovine) (Fig. 4). Effort was made to avoid flow back of the antigen by avoiding over-stretching of the skin around the neck region. Skin-fold thicknesses of both sites were measure and recorded again 72hrs post-injection.

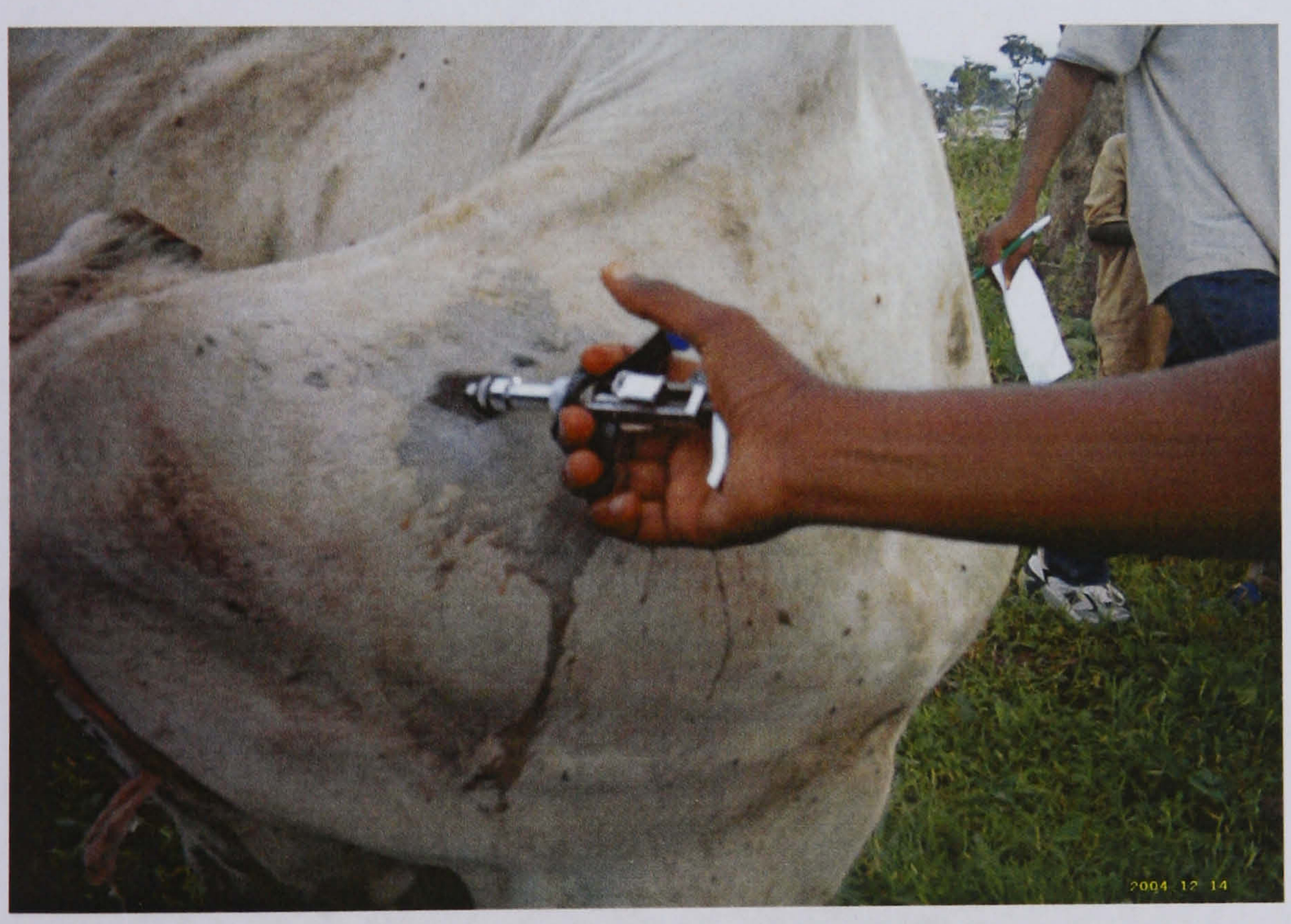

Figure: 3.2. Administration of Purified Protein Derivative (PPD) with McLintock syringe

The tuberculin result was interpreted using the New Zealand standard interpretation of the comparative intradermal test for cattle where a positive result is recorded when the increase in skin thickness to the bovine PPD is ≥4 mm more than the response to avian PPD 72hrs post injection. An inconclusive result is recorded when the thickness is 3 mm and negative when it is <3 (Buddle *et al.* 1995).

3.3.4. Collection and Handling of Milk samples

Milk samples were collected from positive or inconclusive tuberculin reactors. However, milk could not be collected from some positive or inconclusive reactors because some cows escaped and could not be restrained for milking while others were milked very early in the morning and their udders were dried before the arrival of the researcher. Prior to collection of milk the udders were cleaned to reduce contamination.

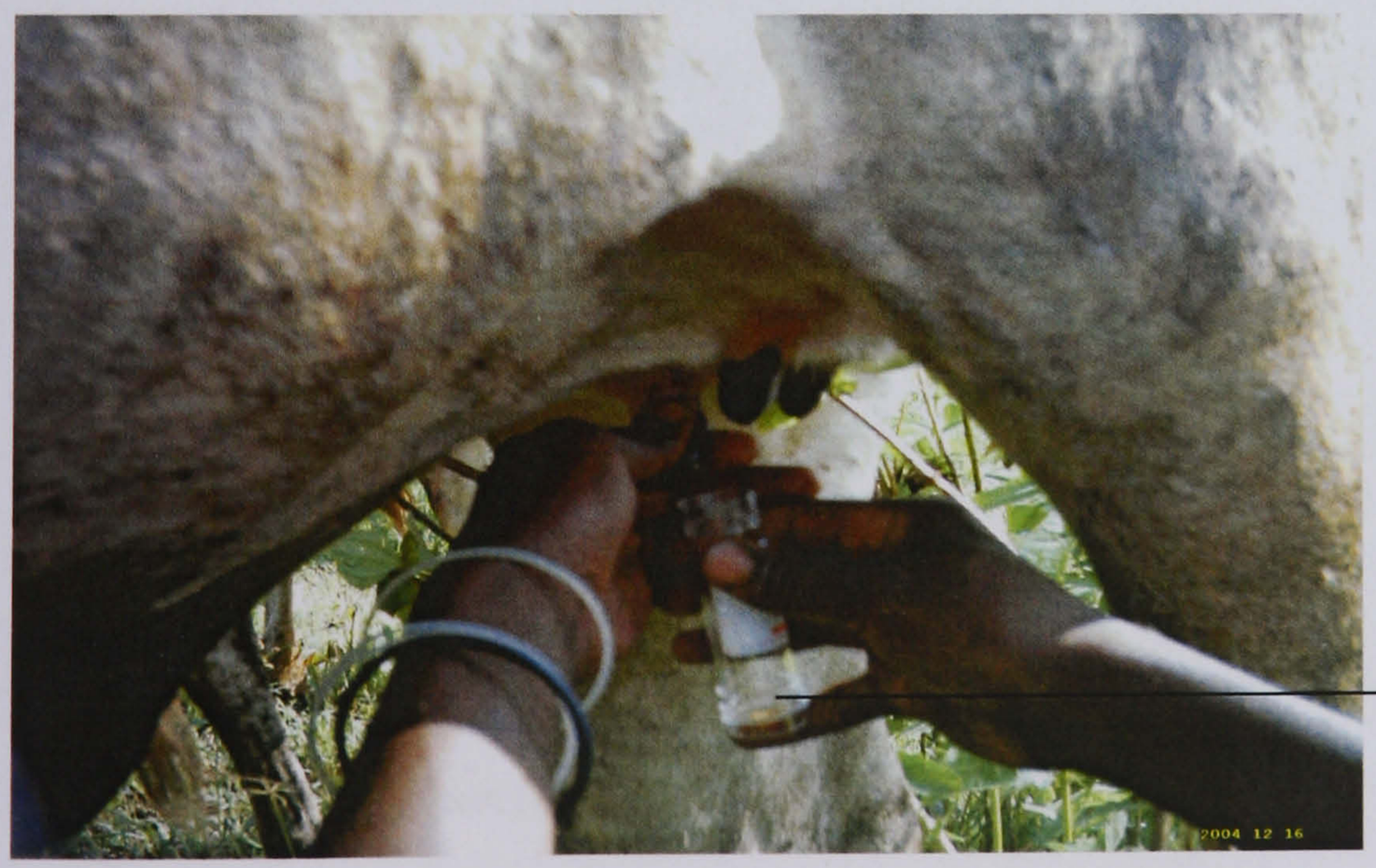

Figure: 3.3. Collection of milk sample from a tuberculin positive cow

Milk samples were collected into sterile screw-capped universal containers (one tube per cow), which were properly identified and dated. Samples were conveyed to the laboratory within 24 hrs of collection iced cooled and stored in a freezer until processed.

3.3.5. Laboratory Analysis of Milk Samples

3.3.5.1. Culture

Milk obtained from individual cows with positive or inconclusive PPD result, were processed separately. Primary mycobacterial isolation was undertaken using Lowenstein-Jensen media (Becton-Dickenson, UK) and culture procedures were carried out according to standard methods as described in the CRC manual of clinical laboratory procedures (Anonymous 1970). About ml of milk sample was placed in a centrifuge tube and centrifuged for 15mins. The supernatant was discarded and the residue re-suspended with 20ml 2% NaOH containing phenol red indicator. The suspension was allowed to stand for 30mins before neutralizing by drop-wise addition of 6NHCL until the colour changed from purple to pink. It was then centrifuged again for 20mins and the supernatant was discarded. The sediment was inoculated on two Lowenstein-Jensen media (glycerol and pyruvate enriched slants), using sterile swabs. The slants were incubated at 37°c for a minimum of 8 weeks.

3.3.5.2. Acid-Fast /Zeihl-Neelsen (ZN) Stain

Ziehl-Neelsen staining was carried using standard protocol ((Kazwala *et al.* 1998) to detect acid-fast bacilli. A drop of the processed milk sample above was placed on a new, clean and labeled grease-free slide and spread with a sterile pipette to make a thin smear (1cm x 2cm). The slide was air-dried and heat fixed by passing it through a flame with the specimen side up, this was to fix the specimen to the slide and preserve the bacterial morphology. The slide was then flooded with carbol fuschin and then steamed gently with flame from the underside for 1min. It was then rinsed off with water and decolorized with 5% alcohol for 1-2mins until the red colour was gone. The slide was rinsed again with water and counter stained with methylene blue for 1min. Additional rinsing with water was used to remove excess colour and dried. It was then examined under a microscope with oil immersion lens x 100 to look for acid-fast bacilli (AFB). The bacilli (positive) were stained red, straight or slightly curved rods occurring either singly or in small groups while non acid-fast microorganisms (negative) stained blue (figure 3.4.).

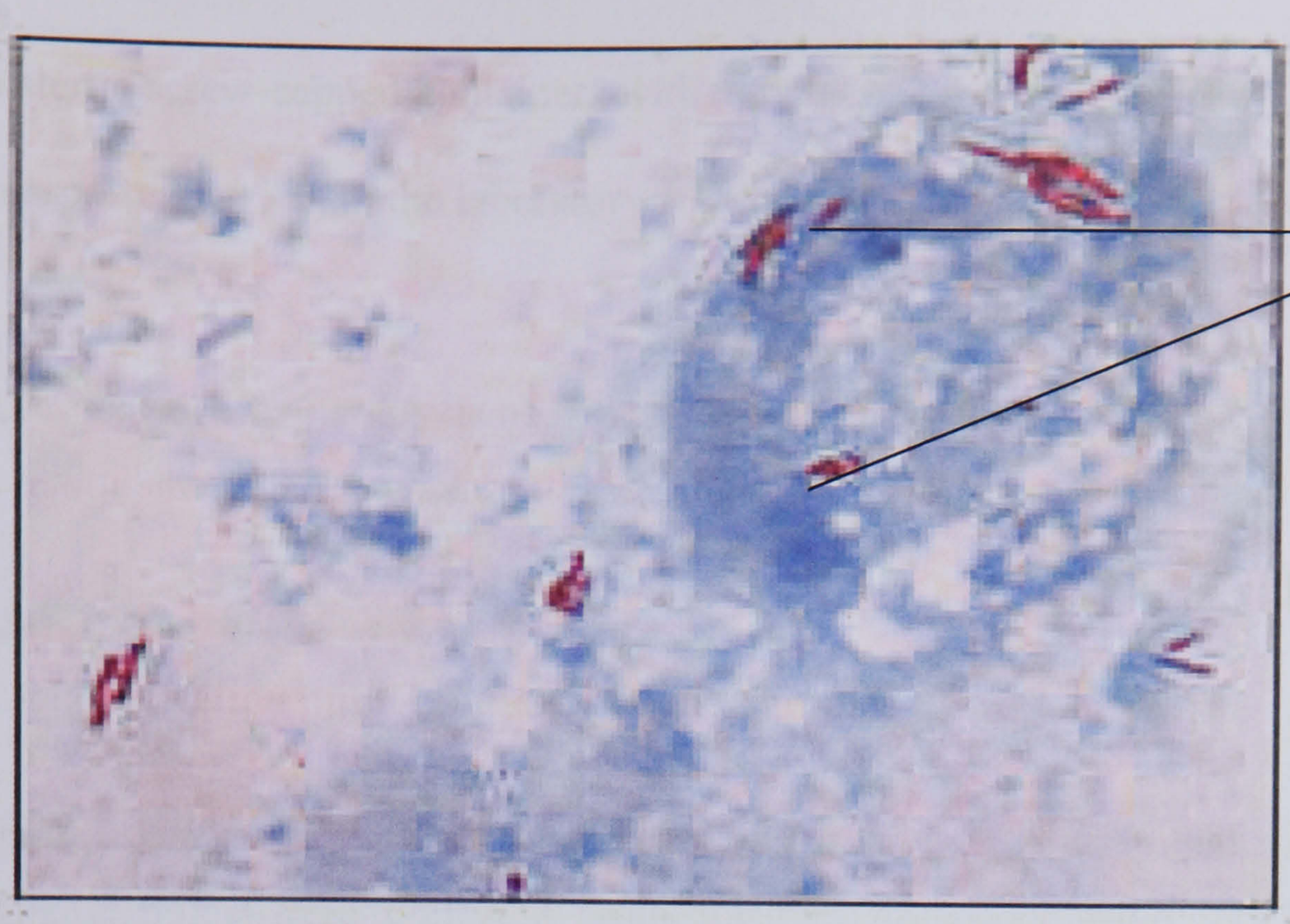

Figure: 3.4. *Mycobacteria spp* (acid-fast positive) under the microscope at x100 lens magnification

3.3.6. Collection and Handling of Bovine Tissue samples

Tissue Samples were taken from slaughtered animals with lesions, which were suspected to have or be compatible with tuberculosis. The samples were taken from lymph nodes (Retropharyngeal, and Mediasternal), lung, liver or any organ with lesions such as the heart, spleen and mammary gland. Epidemiological data such as age, sex and location of lesion were taken from each carcass sampled. The samples were collected from the major abattoirs that supply meat to the public within the Federal Capital Territory and Kaduna state between 5.00-9.00am. These organs were examined visually for changes in colour, (pale yellow or gray) or morphology and then palpated before dissecting from the surrounding tissue. Samples were collected

in sterile screw-capped containers with normal saline solution to keep them moist and transported, on ice, to the laboratory where they were frozen until processed.

3.3.7. Laboratory analysis of Bovine Tissue samples

3.3.7.1. Decontamination

Tissue samples were decontaminated prior to culture as described in the veterinary laboratory manual (Anon 1970; Corner and Trajstman 1988) to reduce contamination. The sample was removed from the freezer and submerged in diluted (100ppm) locally produced household bleach (Jik, 3.5% NaOCl) (Reckitt Benkiser. Nigeria Ltd). It was left overnight at room temperature before rinsing with freshly diluted bleach the next day for culture.

3.3.7.2. Culture

Tissue samples were homogenized (individually) in a sterile blender for 2mins in 50ml nutrient broth containing phenol red. Five milliliters of the aliquot homogenate was transferred into 50ml screw capped centrifuge tube and 20ml of 2% NaOH was added and shaken for few seconds. The sample was allowed to stand for 40mins at room temperature before adding 6NHCL drop-wise to neutralize until first colour change and then centrifuged for 30mins. The supernatant was discarded leaving about 2mls, which was mixed with the pellets and inoculated on two Lowenstein-Jensen slants, glycerol and pyruvate enriched (Becton-Dickenson), using sterile swabs. The

tubes were then incubated at 37°C for a minimum of 8 weeks (Anonymous 1970; Parra, Larrassa, Garcia, Alonso and Hermoso de Mendoza 2005).

3.3.7.3. Acid-fast (ZN) stain was done as earlier described in 3.3.5.2. above.

3.3.8. Questionnaires

Epidemiological data relating to tuberculin-tested cows and slaughtered cattle such as age, sex, breed and location of suspected TB lesions were obtained from herd during tuberculin testing and also at the abattoir during sample collection. The information obtained was clearly recorded for analysis. Furthermore herd owners and abattoir managers/vets were also interviewed during tuberculin testing and sample collection respectively. Interview was conducted in the form of questionnaires to get information relating to tuberculosis control and the handling and processing of meat (managers) and milk and milk products (herders) (appendix 2, & 3). All data gathered were analysed statistically using MINITAB 13 for windows.

3.4. Prevalence of Human Tuberculosis in the Federal Capital Territory, and Kaduna Sate of Nigeria

This study was aimed at determining the prevalence of human tuberculosis among suspected TB patients in the study area through culture and acid-fast stain of sputum samples. Possible risk factors associated with the disease and possible sources of infection especially animal-to-human transmission as well as effectiveness of control measures currently in place were also investigated.

3.4.1. Ethical Consideration

Ethical approval was obtained from the ethics committees of all participating hospitals and TB centres in the FCT, Abuja and Kaduna state prior to the study.

3.4.2. Demographic Details of Patients

Patients were enrolled with their informed consent and the following details were recorded: name, age, sex, occupational history, present and previous addresses and dates; history of BCG vaccine in the past, and history of contact with animals and consumption of locally produced milk 'nono' and milk products. This information was obtained in the form of questionnaires session with trained nurses who fill in the form for the patients (Appendix 4). These questionnaires were analysed statistically using MINITAB 13 for windows to identify risk factors associated with TB as well as to know if control measures are working effectively or not. They were also meant to know the prevalence of TB between various age groups, sex and history of contact with animals and consumption of milk and milk products.

3.4.3. Collection and Handling of Human Sputum Samples

Sputum samples were collected from 900 (500 from FCT and 400 from Kaduna State) patients with clinical suspicion of TB that are attending hospitals involved with the

WHO's Directly Observed Therapy, short-course strategy (DOTS), as well as designated TB centres within the study area between September 2003 to April 2005. The sputum of herders and butchers who consented and have cough for more than 3 weeks was also collected and analysed. Three sputum samples were collected from each patient i.e. 1st spot, overnight and 2nd spot as recommended by the International Union Against Tuberculosis and Lung Diseases (IUATLD) (Ipuge, Riedaer and Enarson 1996). Samples were collected in sterile screw capped containers, transported ice cool to the laboratory and processed within 24 hours.

3.4.4. Laboratory Analysis of sputum samples

3.4.4.1. Culture

Sputum samples were decontaminated using the modified Petroff decontamination method before inoculating on to LJ-slopes using standard method (Van Embden *et al.* 1993). To an Xml of sputum (depending on the quantity available), an equal volume of 4% NaOH was added and shaken properly to digest the sputum. The mixture was then allowed to stand for 15mins at room temperature with occasional shaking before centrifuging at 3000g for 15mins. The supernatant was poured off and 15ml sterile saline was added to re-suspend the sediment before centrifuging again for another 15mins. The supernatant was decanted and the sediments inoculated onto 2 slants of Lowenstein-Jensen media (glycerol and pyruvate enriched) before incubating at 37°c for a minimum of 8 weeks (figure 3.5).

3.4.4.2. Acid-Fast/Zeihl Nelsen stain Zeihl Neelsen stain was done as earlier described

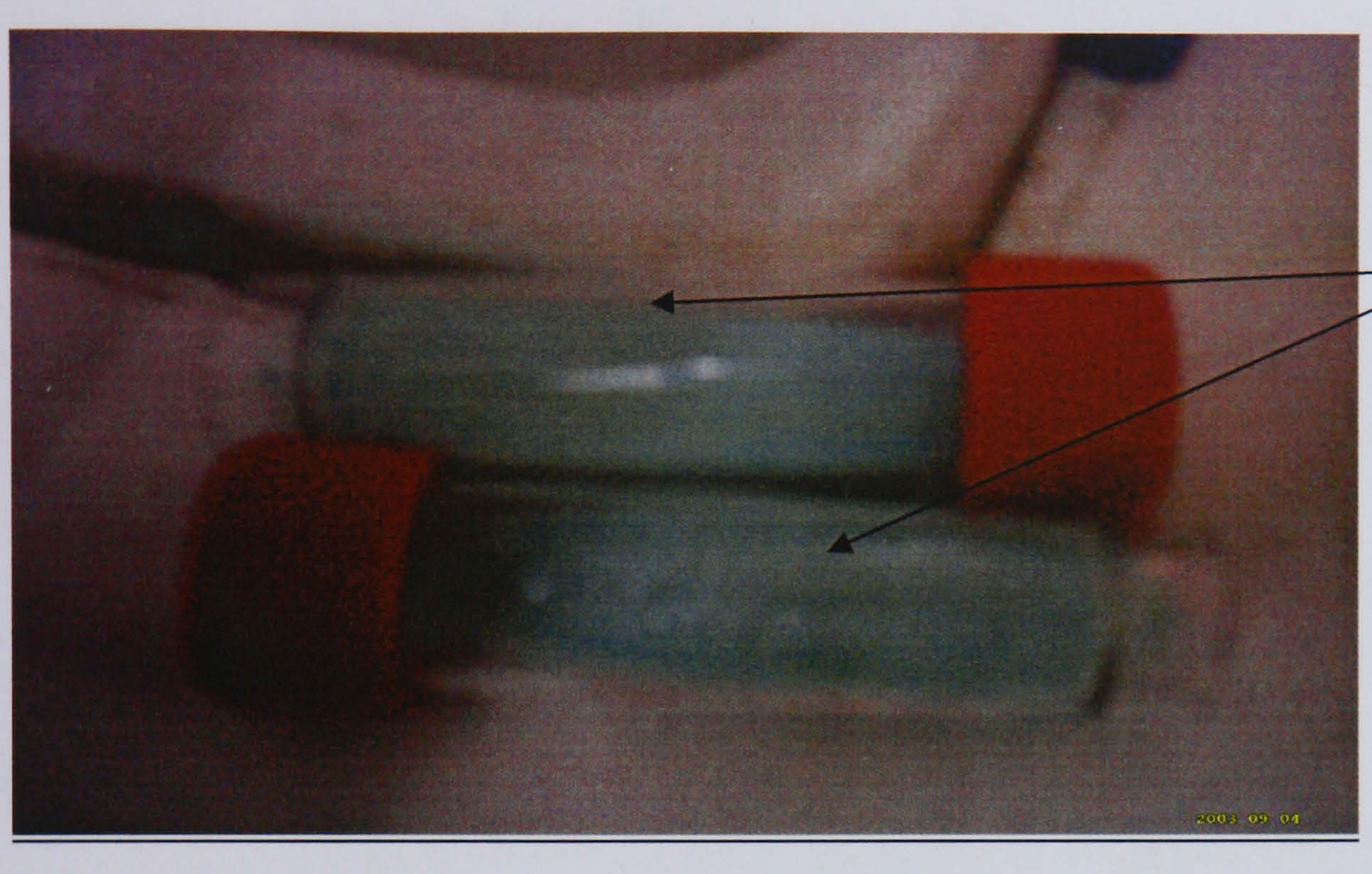

Figure: 3.5. *Mycobacteria spp* growing on LJ-media

3.5. DNA Fingerprinting of Human and Bovine Isolates

This study was designed to determine the species and strains of *Mycobacterium* in humans and cattle using two PCR based molecular techniques; the spoligotyping and VNTR and IS6110-RFLP methods. The typing patterns obtained were compared with the patterns held at the international databases at Pasteur in France and the Veterinary Laboratory Agency, UK.

3.5.1. Handling and Processing of Mycobacterium Isolates

3.5.1.1. Heat killing

Seventy-one clinical isolates obtained from culture of tissue, milk and sputum samples were heat killed before transporting to the United Kingdom (using renowned courier services) for safety reasons. *Mycobacterium* colonies were scraped from LJ media and transferred into eppendorf tubes containing 1ml of sterile distilled water using sterile swab. They were then incubated at 80˚C for 1hr to kill the cells. The heat-killed cells were transported to the United Kingdom via a renowned courier services at 4˚C.

3.5.1.2. DNA Extraction

The samples were divided into two parts; one part for spoligotyping and Variable Number of Tandem Repeats (VNTR) which are PCR based DNA fingerprinting which do not require high quality DNA and the other part for Restriction Fragment Length Polymorphism (IS6110-RFLP), which requires a large quantity of high quality DNA.

DNA extraction for Spoligotyping and VNTR Fingerprinting

For spoligotyping and VNTR methods, the heat-killed samples were centrifuged at 12,000g for 5mins. After centrifugation the supernatant was harvested and stored at –

20 °C until further use (Parra, Fernandez-Llario, Tato, Larrassa, Garcia, Alonso, Hermoso de Mendoza and Hermoso de Mendoza 2003)

DNA Extraction for RFLP-IS6110 Fingerprinting

Mycobacterial DNA was extracted and subjected to restriction fragment analysis as described by (Collins *et al.* 1985; Van Soolingen *et al.* 1994a). To the heat killed sample 50µl of 10mg/ml lysozyme (Sigma) was added and the mixture was incubated at 37°C for at least 1hr. Then 75µl of 10mg/ml proteinase K and10% sodium dodecyl sulfate (SDS) (5µl proteinase K and 70µl 10% SDS) were added to the lysozyme-treated samples. After vortexing and incubating for 10mins at 65°C, 100µl of 5M NaCl was added and subsequently pre-warmed at 65°C before adding 100µl of N-cetly-N,N,N,-trimethyl ammonium bromide (CTAB)/NaCl (Sigma) solution (4.1g NaCl and 10g CTAB) with the aim of binding cell wall debris, denatured proteins and polysaccharides. The mixture was vortexed until the liquid content became white (milky) before incubating for 10mins at 65°C. Seventy five micro-litres of chloroform/isoamyl alcohol (24:1) was added to the mixture and vortexed for 10secs and then centrifuged at room temperature for 5mins at 12,000rpm. The supernatant was harvested into a fresh microcentrifuge tube and 450µl of isopropanol was added and incubated for 10mins on ice before centrifuging for 15mins at room temperature at 12,000g. The supernatant was discarded and the pellet was washed with 1ml of 70% ethanol and then centrifuging for 5mins at room temperature. The supernatant was discarded and the pellet dried and re-dissolved in 20µl of 1xTE buffer. Five microliters of the extracted DNA was run on 0.8% agarose gel to confirm the presence of DNA and 2µl was also taken to determine the purity and concentration

using a spectrophotometer. The remaining DNA sample was stored at -20°C until further use.

3.5.1.3. DNA Quantification

The concentration and quality of the extracted DNA was quantified using spectrophotometer and λ-DNA of different concentrations.

Spectrophotometery

The concentration of the DNA was checked by measuring the absorbance at 260nM (A_{260}) in a spectrophotometer using a quartz cuvette and the purity was determined by the ratio of the readings at 260nM and 280nM (A_{260}/A_{280}). Protocol was according to manufacturer's specification (BioRad). Two hundred microlitres of TE buffer was taken in a quartz cuvette and used as blank before the sample. Then 2µl of the extracted DNA was diluted with 198µl of TE buffer in a cuvette and placed in the spectrophotometer. Three readings of both concentration (OD) and purity (A_{260}/A_{280}) were taken and the average was calculated to get the DNA concentration and purity respectively. An absorbance of 1unit at 260nM corresponds to 50µg of genomic DNA per ml (A_{260} = 1 = 50µg/ml). Good DNA should be between 0.1 and 1.0 while pure DNA should have A_{260}/A_{280} ratio of between 1.7 and 1.9.

Use of Lambda λ- DNA

Different concentrations of λ-DNA (Invitrogene) were prepared by serial dilution of an initial stock of 500ng/µl with TE buffer. Different concentrations of λ-DNA were then run on agarose gel along with the DNA samples as markers to determine the concentration of the extracted DNA.

3.5.2. Agarose Gel Electrophoresis

A 0.8% gel was prepared as described by Sambrook and Russel (2006). The mouth of the conical flask was loosely covered with a small plastic beaker and the mixture was microwaved for 2 or 3 second periods, until the agarose was completely dissolved. The agarose solution was allowed to cool for at least 10mins before pouring into the gel former with the comb placed about 3cm to one end and the edges covered with masking tape. The agarose was allowed to set for about 15-20mins then the comb was removed gently before placing it in the gel running tank with the wells nearest to the positive (black) terminal. Enough gel running buffer was added to cover the gel avoiding bubbles. Five micro-liters of the sample with 2µl of mini gel stop mix was loaded into the wells (making note of what was loaded in each well) using micropipette. Four wells were loaded with the different concentrations lambda (λ) DNA above as markers. The gel was run at 100 volts for 45mins or until 2/3rd of the gel was run. The gel was then removed from the running tank and placed in 250ml of gel running buffer containing 50µl of 5mg/ml ethidium bromide to stain. After 20-30mins the gel was transferred into another buffer without ethidium bromide to

distain. The gel was viewed and photographed using UV transilluminator (UVP gel documentation system, Syngene) (figure 3.6)

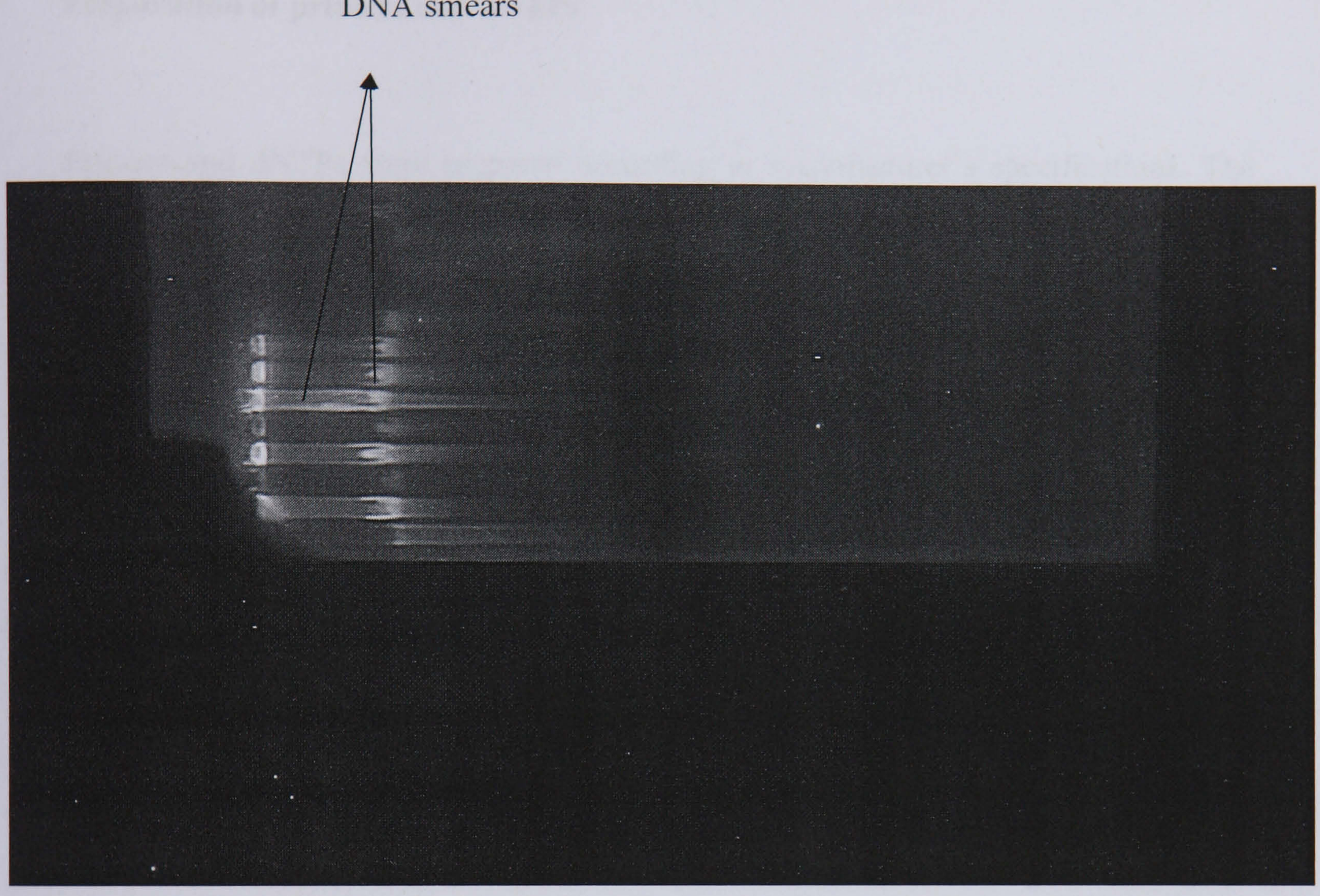

Figure: 3.6. Extracted DNA on agarose gel

3.5.3. Spoligotyping

Spoligotyping was performed as described by Kamerbeek (1997) with minor modifications. The direct repeat (DR) region was amplified by polymerase chain reaction (PCR) with oligonucleotide primers derived from the DR sequence. Two primers (forward and reverse) were used with the following DR sequences (Invitrogen).

DR (forward) 5'-CCA AGA GGG GAC GGA AA-3' (35.4nmoles) (Invitrogene)

DR (reverse) 5'-GGT TTG GGT CTG ACG AC-3' (30.2nmoles) (Invitrogene)

The reverse primer was biotin labeled

Preparation of primers and dNTPs

Primers and dNTPs were prepared according to manufacturer's specifications. The tubes with lyophilized primers were centrifuged for 1min to collect the DNA at the bottom of the tube before adding 100µl of TE buffer and left at room temperature for 2mins. The tubes were vortexed for 15mins to mix and then centrifuged for 1min to collect the DNA solution at the base of the tube (these are the primer stock solutions). The concentrations for the two primers were calculated accordingly (appendix 5)

Polymerase chain reaction (PCR) protocol

To 10µl of the DNA sample, 20pmoles of each of the primers working stock solutions equivalent to 4.13µl and 3.53µl of the reverse and the forward primers were added respectively. One milliliter of dNTP mix equivalent to 2.5mM each dNTP, final concentration of 0.2mM each dNTP was added to the tube followed by 5µl of 10X concentrated super reaction buffer (Invitrogen). To the tube 0.1µl of Taq DNA polymerase (5units/µl) and 3µl of 25mM $MgCl_2$ (Invitrogen) were added. Two tubes were included as positive and negative controls. To the negative control no DNA template was added and to the positive control, 2µl of H37Rv DNA (Veterinary Laboratory Agency, UK) was added in place of the sample DNA. Finally all the tubes were made up to 50µl by adding 23.24µl of RNAnase free water to tubes with sample DNA and 33.24µl and 31.24µl to the negative and the positive control tubes

respectively. When many samples were involved a PCR mix was prepared for all the tubes without the DNA template, which is added at the last minute, and the tubes made up to 50µl each. The tubes were placed in the PCR machine for amplification, and the cycling temperatures were 1min at 96°C, 1min at 55°C, 30sec at 72°C for 20 cycles while the first denaturation was 3min at 96°C and the final extension was 5min at 72°C. The holding temperature after end of programme was 4°C. Ten microliters of the PCR product was run on 0.8% agarose gel using 1kb ready-load DNA ladder (Invitrogen, Canada) as a marker while the remaining was stored at -20°C until use.

Hybridization with Oligonucleotides

The amplified products were hybridized to a set of 43 immobilized oligonucleotides (Amersham), each corresponding to one of the unique spacer DNA sequence within the DR locus. Prior to hybridization all buffers to be used were pre-warmed to 60°C. Twenty microliters of the PCR products including positive and negative controls (H37R and *M. bovis* BCG were used as positive control) were added to 150µl of 2xSSPE (0.36M NaCl, 20mM NaH_2PO_4 and 2mM EDTA) /0.1% SDS and the mixture was incubated at 99°C for 10min and cooled immediately on ice to denature the PCR product. The membrane was washed for 5min at 60°C in 250ml of 2xSSPE/0.1% SDS and subsequently the membrane and a support cushion were placed into the miniblotter, in such a way that the slots were perpendicular to the line of pattern of the oligonucleotides. Residual fluid was removed from the slots of the miniblotter by aspiration. The slots were filled with 150µl of the diluted PCR products using micropipettes (avoiding bubbles) and then hybridized for 60min at 60°C on a horizontal surface in a hybridization oven without shaking to avoid

contamination of neighboring slots. The samples were removed from the miniblotter by aspiration and the membrane taken out of the miniblotter using forceps. The membrane was washed twice in 250ml 2xSSPE/0.5% SDS for 10min at 60°C each washing and then placed in a rolling bottle and allowed to cool down to prevent inactivation of the peroxidase in the next step. The membrane was incubated in 1:4,000-diluted streptavidin-peroxidase-conjugate (Roche, Germany), (2.5ml Streptavidin + 10ml secondary buffer) for 45min at 42°C in the rolling bottle. After incubation the membrane was washed twice in 250ml of 2xSSPE/0.5% SDS for 10min each at 42°C and then rinsed twice with 250ml of 2xSSPE for 5min at room temperature. The hybridized DNA was detected using enhanced chemiluminiscent detection (ECL) method (Amersham).

Chemiluminiscent Detection

In the dark room, the membrane was incubated for 1min in ECL detection liquid (Amersham) and then covered with a cling film before exposing it to light sensitive X-ray film (Hyperfilm ECL; Amersham) for 20min under safe light. After exposure the film was developed and the spoligotype patterns were read using standard method. The patterns were numbered and prefixed with 'HM' if they were isolated from humans and 'BV' if isolated from bovine.

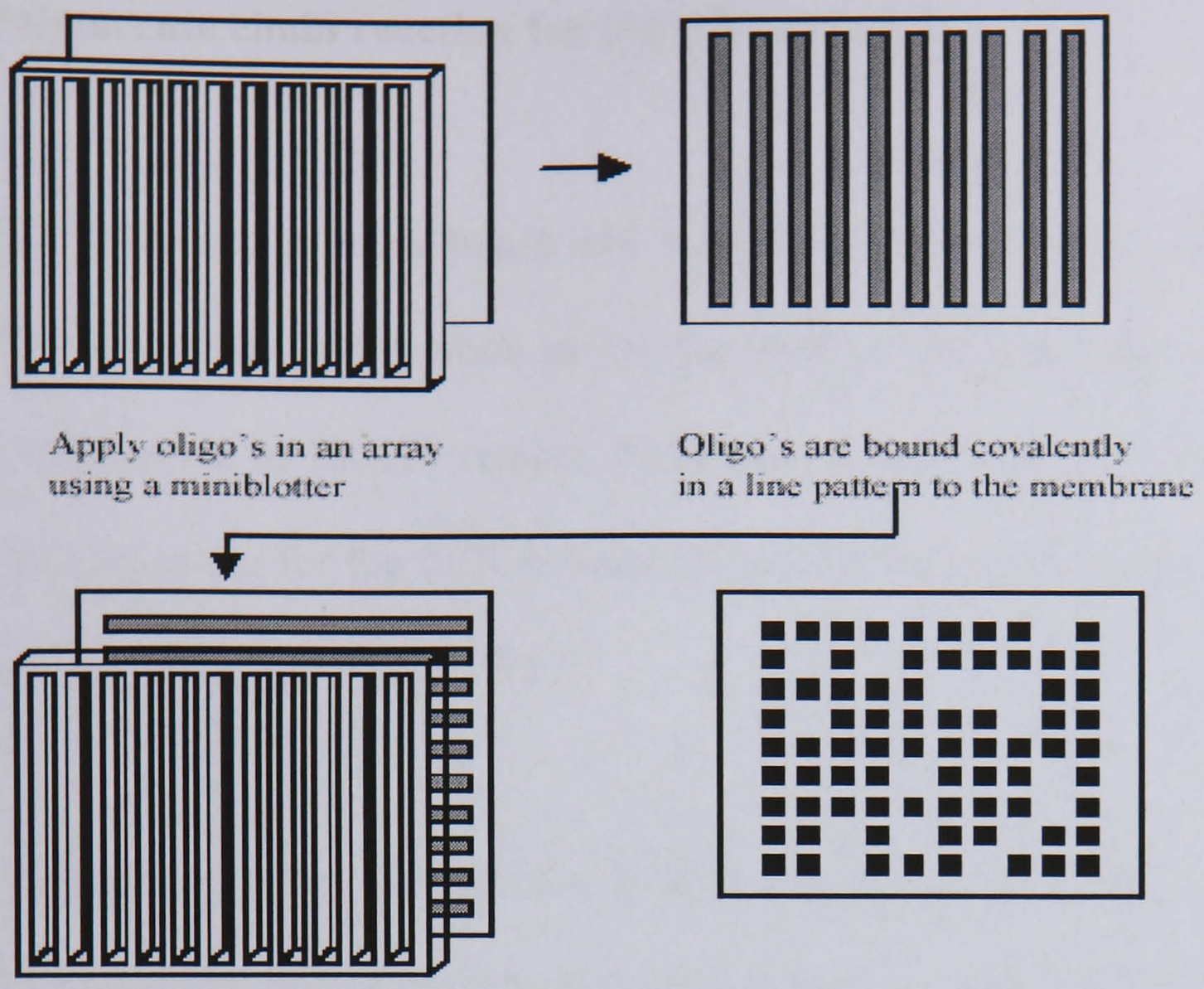

Figure: 3.7. Diagrammatic overview of spoligotyping method (Spoligotype protocol manual)

3.5.4. IS6110-Restriction Fragment Length Polymorphism (IS6110-RFLP)

RFLP using IS6110 as the insertion sequence was done as described by van Embden et al (1993). IS probe was prepared by peroxidase labeling of a 245-bp fragment obtained from purified chromosomal *M. bovis* DNA. DNA s digested with Pvu II enzyme and the DNA fragments were separated by electrophoresis on 1% agarose gel run at 100V for 3hrs. The fragments were then transferred onto nylon membrane using southern blotting (vacuum transfer) and the DNA bands were viewed using enhance chemilumuniscent detection system (ECL, Amersham).

Polymerase chain reaction for IS6110 (probe)

Preparation of primers (main and working stock solutions), dNTPs and setting up PCR was done as described earlier for spoligotyping, but the primers used were for amplification of IS6110 region. Three microliters of *M. bovis* DNA was used as the DNA template for the PCR to amplify the IS6110 region using the primers. This was used to prepare probe for RFLP.

IS (forward) primer 5'-CGT GAG GGC ATC GAG GTG GC-3'

IS (reverse) primer 5'-GCG TAG GCG TCG GTG ACA AA-3'

(Invitrogene)

The cycling temperatures were 1min at 94°C, 1min at 57°C and 1min at 72°C for 35 cycles while the initial denaturation was 1min at 94°C and final extension was 10min at 72°C. The PCR product was run on 1% agarose gel to purify the DNA for preparation of IS6110 probe using the GenElute Extraction kit

Agarose gel electrophoresis

A 1% agarose gel was prepared as described earlier and all the PCR products were loaded including 1kb ready-load DNA marker to separate, excise and purify DNA band of approximately 264bp for preparation of probe (figure 3.8).

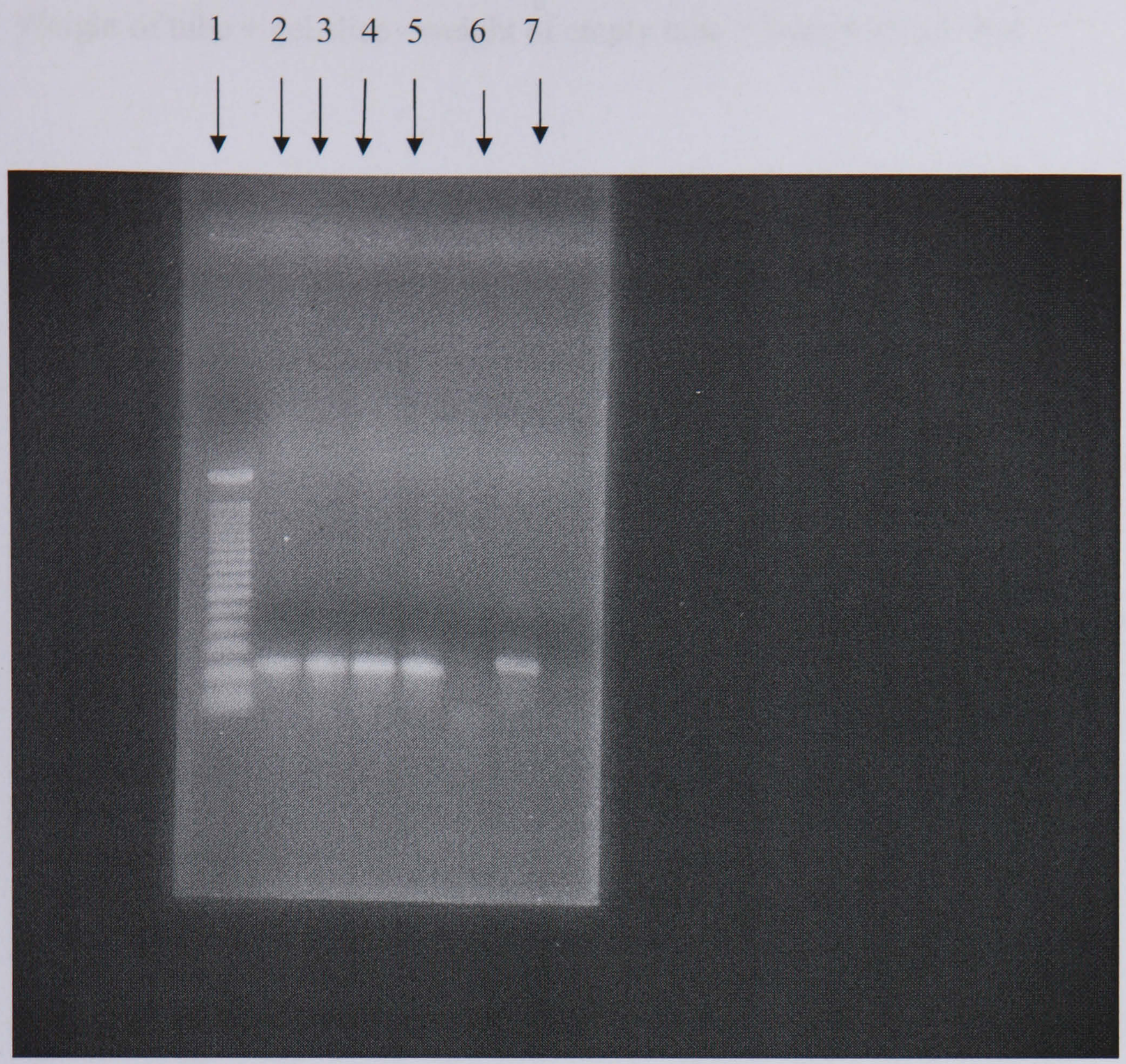

Figure: 3.8. Syngene GeneSnap image of a 2% agarose gel visualizing PCR products with 100-bp ladder size marker lane. Lane 1: 100-bp DNA marker, Lane 2-5 PCR products, Lane 6: negative control, Lane 7: H37Rv positive control

DNA purification from Gel

DNA band of 264bp was excised from the gel and purified using the GelElute gel extraction kit (Amersham) as specified by the manufacture. The DNA band (264bp) was excised under UV light with a very sharp scalpel blade and excess gel trimmed off. An empty eppendorf tube was weighed and the gel was placed in the tube and weighed again to know the weight of the gel.

Weight of tube + gel slice - weight of empty tube = weight of gel slice

To the gel slice 3x the gel volumes of solubilization solution was added (for every 100mg of agarose gel 300ml of solubilization solution was added) to solubilize the gel. For the gel to dissolve completely, the gel mix was incubated at 50-60°C for 10min with vortexing every 2-3min. While incubating the gel mix, binding column was prepared by placing GenElute binding column G (supplied in the kit) into one 2ml collection tube. Then 500µl of column preparation solution was added in the binding column and centrifuged for 1min and the flow-through liquid discarded.

Labeling of probe

The purified DNA from gel was diluted to a concentration of 10ng/µl and a final volume of 10µl using the water supplied in the kit and denatured by heating for 5min in a boiling water bath to make it a single stranded DNA for good labeling efficiency. The DNA was cooled immediatedly on ice for 5min and spinned briefly to collect the contents at the bottom of the tube. Then an equal amount of the DNA labeling reagent (10µl) was added to the cooled DNA and mixed gently but thoroughly before adding an equal amount of glutaraldehyde solution to the mixture. The mixture was mixed thoroughly and spinned and then incubated for 20min at 37°C before using it for hybridization.

Restriction Fragment analysis

To 2-µg amount of the DNA sample, 2U of PvuII (Invitrogene) was added and mixed well. It was spun briefly before incubating at 37°C for at least 1hr. The digested DNA fragments were separated by electrophoresis on 1% agarose gel to which Gelstar nucleic acid gel stain was added at 5µl /50ml of agarose gel to aid viewing with viewing with uv light. The gel was then run at 100v for 3hrs and then exposed to UV light for at least 30 before southern blotting.

Southern Blotting (Vaccum transfer)

For southern blotting the vacuum transfer was used instead of capillary method using the vacuum blotter (Bio-Rad, Canada) following the manufacturer's instructions. Nylon membrane and filter paper (about 0.5cm bigger than each boarder of the pre-cut window on the casket) were cut. The nylon membrane was immersed in double distilled water by slowly lowering it at 45° angle to the water and then both the nylon membrane and the filter paper were transferred into the transfer solution (10xSSC) before placing them onto the porous vacuum plate with the filter paper uppermost. The window casket was then placed on top of the membrane and the filter paper making sure it covers the entire o-ring on the vacuum stage. Bubbles were removed and the reservoir seal was also wet with water to hold tight and create a good vacuum. The UV exposed gel was placed into 0.5N NaOH for 30min with gentle shaking for denaturation before rinsing it with de-ionized water. The gel was then immediately transferred on to the vacuum blotter with the well side up (over lapping the window). Bubbles were removed by rolling with a pipette before placing the sealing frame on

top of the vacuum stage and the four latch posts locked. The vacuum regulator bleeder valve was then unscrewed the prevent strong initial vacuum and the vacuum source was started and the bleeder valve turned slowly until the gauge reading was 5¨Hg. Pressure was applied gently with the finger on top of the gel along the window boarder to form a tight vacuum between the gel and the window gasket. About 1000-1500ml of the transfer solution (10xSSC) was gently poured into the upper reservoir and the lid was placed on top of the vacuum blotter. The transfer was run for 90min checking occasionally for buffer level and pressure gauge.

At the end of 90min the gel was removed from the blotter and viewed under uv to confirm if the transfer was successful. The membrane was removed and soaked in 2xSSC for 5min and air dried between 2 sheets of filter paper before drying in a vacuum oven at 80°C for 30min. The blot was then ready for hybridization.

Hybridization

Hybridization was done using the ECL Direct Nucleic Acid Labeling and Detection System kit (Amersham) following the manufacturer's protocol. Hybridization buffer was prepared by taking the liquid hybridization buffer in the kit was at 0.25ml/cm^2 of the blot size and dissolving 0.5M NaCl in it, before adding 5% weight/volume blocking agent and then mixing them thoroughly at room temperature for 1hr on a magnetic mixer to get the blocking agent into free suspension. The mixture was heated to 42°C for 0.5-1hr with occasional mixing before placing the blot in the buffer and pre-hybrdized for 15min at 42°C with gentle agitation. The labeled probe was then added and mixed gently and avoiding placing it directly on the membrane and incubation at 42°C with gentle agitation was continued overnight.

Chemiluminiscent Detection

The hybridized membrane was exposed to X-ray film for signal detection (bands). An equal volume of ECL detection reagent 1 and 2 were mixed to give sufficient solution to cover the blot (0.125ml/cm^2). In the dark room, the membrane was incubated for 1min in the ECL detection liquid and then covered with a cling film before exposing it to light sensitive X-ray film (Hyperfilm ECL) for 30min under safe light. The film was then developed and fixed and bands were viewed under light for the presence of bands.

3.5.5. Variable Number Tandem Repeat (VNTR) Typing

Polymerase Chain Reaction (PCR) Protocol

VNTR fingerprinting was done as previously described by Frothingnam and Meeker-O'Connell (1988). Seven loci consisting of exact tandem repeat loci A to E and the Queens University Belfast (QUB) 11a and 11b were amplified from the isolated DNA from samples to determine the number of tandem repeats at each locus in each strain. *M. tuberculosis* H37Rv or *M. bovis* and RNAase free water were used as positive and negative controls respectively. PCR amplifications were performed in 50µl volumes in 0.2ml PCR tubes. To avoid waste and for accuracy, a PCR mix without DNA template and RNAase free water for each PCR set was prepared and then the mixture was dispensed into each PCR tube. To each PCR reaction, 25µM of both forward and reverse primers (for a single VNTR primer set), 50mM of $MgCL_2$ and 5µl of 10x

PCR buffer were added. This is followed by adding 2U of *Taq* DNA polymerase and 200μM each of the four dNTPs. To the positive control 2μl of *M. tuberculosis* H37Rv or *M. bovis* strains was added and no DNA was added to the negative control. All PCR tubes with the PCR mixture was made up to 50μl by adding RNAase free water. PCR was performed on an Eppendorf Mastercycler Gradient (Eppendorf) and the thermal cycle used for amplification was an initial denaturation at 95°C for 12mins, followed by 30 cycles of denaturation at 94°C for 1min, annealing at 57°C (for QUB-11a, QUB-11b), 65°C for (ETR-B and C) and 66°C for (ETR-A, D, E and F) for 1min and extension at 72°C for 2min. This was followed by a final extension at 72°C for 10min then a holding step at 4°C.

The size of each exact tandem repeat and Queen University Belfast repeat at each locus was determined by running the PCR product on a 2% w/v agarose gel at 100v and 400mA for 90min with 100-bp and 50-bp ready-load DNA ladder (Invitrogene) used as a DNA marker to determine the sizes of the bands. After electrophoresis the gel was stained with ethidium bromide for 30min, destained in gel running buffer for 5min before visualizing under UV light using UV transillumination system (GeneGenius, Syngene, UK). Bands were measured using GeneRuler 100-bp and 50-bp to determine the sizes of the bands.

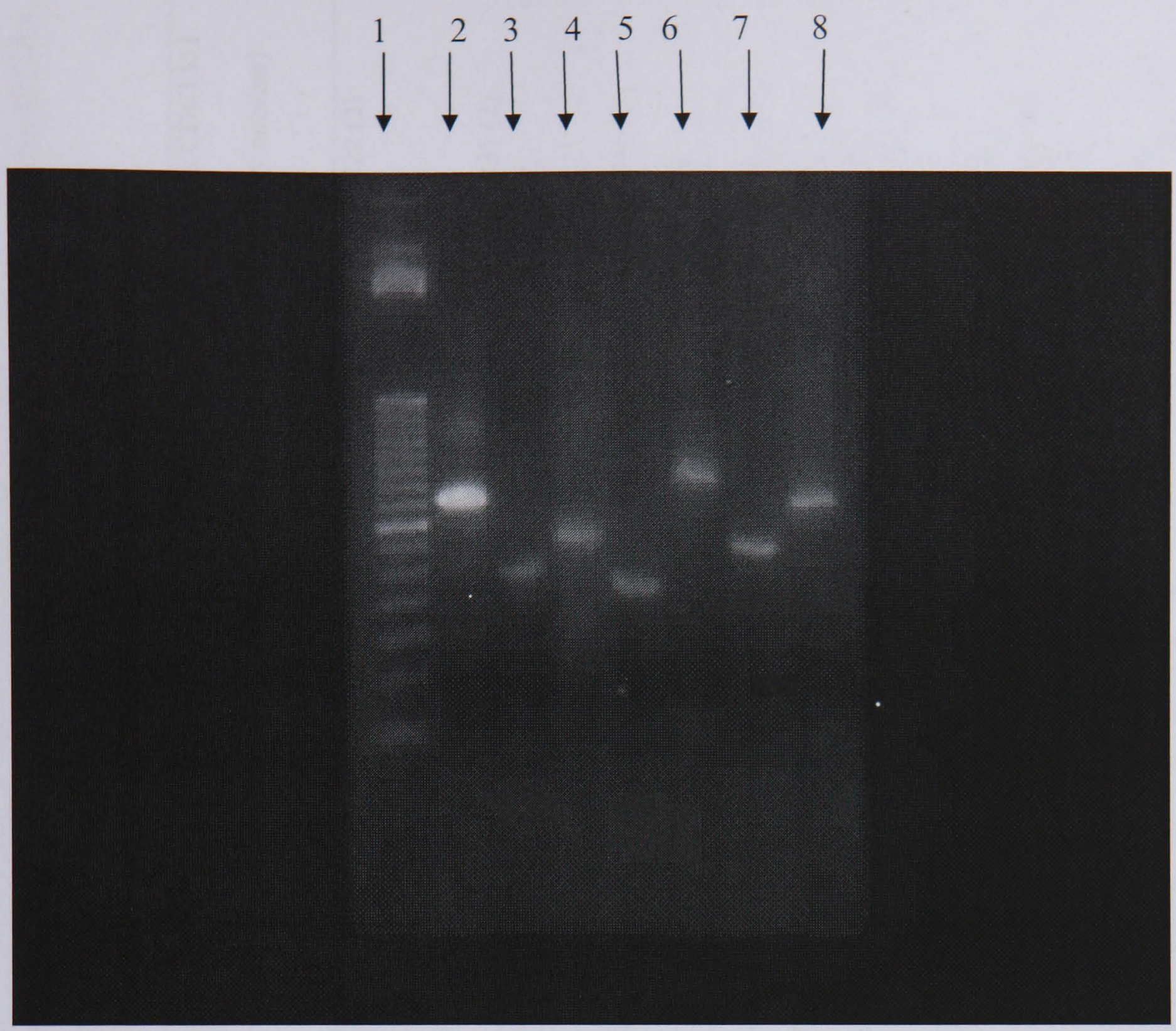

Figure: 3.9. Syngene GeneSnap image of a 2% agarose gel visualizing sizes of tandem repeats at each locus with a 100-bp ladder size marker lane. Lane 1: 50-bp DNA ladder, Lane: 2: ETR-A, Lane 3: ETR-B, Lane 4: ETR-D, Lane 5: ETR-E, Lane 6: ETR-F, Lane 7: QUB-11a and Lane 8: QUB-11b

Table 3.1. List of PCR primers and their sequences used in this study and the predicted PCR product sizes (bp) for M. tuberculosis H37Rv, CDC1551 and M. bovis (Frothingham *et al.* 1988; Goyal *et al.* 1994; Le Fleche *et al.* 2002)

VNTR Primers	Forward primer sequence (5' to 3')	Reverse primer sequence (5'-3')	Size in H37Rv (Copy number)	Size in *M. bovis* (copy number	Size in CDC1551 (copy number)
ETR-A	AAATCGGTCCCATCACCTTCTTAT	CGAAGCCTGGGGTGCCCGCGATTT	420 (3)	847(9)	322 (2)
ETR-B	GCGAACACCAGGACAGCATCATG	GGCATGCCGGTGATCGAGTGG	292 (3)	406 (5)	235 (2)
QUB-11a	CCCATCCCGCTTAGCACATTCGTA	TTCAGGGGGGATCCGGGA	305 (3)	788 (10)	581 (7)
QUB-11b	CGTAAGGGGGATGCGGGAAATAGG	CGAAGTGAATGGTGGCAT	412 (5)	343 (4)	274 (3)
ETR-D	CAGGTCACAACGAGAGGAAGAGC	GCGGATCGGCCAGCGACTCCTTC	353 (3.3)	483 (5)	330 (3)
ETR-E	CTTCGGCGTCGAAGAGAGCCTC	CGGAACGCTGGTCACCACCTAAG	651 (3)	651 (3)	651 (3)
ETR-F	CTCGGTGATGGTCCGGCCGGTCAC	GGAAGTGCTCGACAACGCCATGCC	476 (2.8)	421 (2.1)	476 (2.8)

3.6. Statistical Analysis

The data in this study are non parametric categorical. In order to compare classes of this sort of data, chi-square test (χ^2) test of significance with their appropriate degrees of freedom (df) was adopted, assuming a null hypothesis to calculate the expected values. The calculated chi-square was compared with the tabulated chi-square values to specify the level of significance or association in case such exists. Comparison between observed and expected values was used to reflect on any association or discrepancy. All statistical manipulations were carried according to Bland (2003).

CHAPTER 4

RESULTS

4.1. Prevalence of Bovine Tuberculosis in the Federal Capital Territory (FCT) and Kaduna state of Nigeria

4.1.1. Questionnaire

A total of 57 herdsmen whose herds were part of this study were interviewed by way of questionnaire (appendix 2). The data obtained showed that all of them have been herding for over 10 years. The response also showed that 46 (80.7%) out of the 57 herdsmen do not boil their milk before selling to the public while all herdsmen and their families consume the milk and products from their cows as a staple food. The data also showed that over 90% of the herders claimed that they are able to recognize an animal with tuberculosis out of which 93% of them said that they sell milk from these TB suspected cows.

A total of 16 abattoir managers (vets and livestock officers) were also interviewed by questionnaires (appendix 3). The response obtained showed that all managers are aware of legislature governing meat inspection but, 98% of them confirmed that strict control measures are not applied. This, they said, was mainly due to lack of funds to compensate butchers and herders; thus, only partial condemnation of infected organs

is done without compensation; and even in this case, it is only implemented if the butchers/animal owners agree.

4.1.2. Tuberculin Test

4.1.2.1. Geographical Distribution of mycobacterial infection based on tuberculin test

The result of tuberculin test carried out in the 57 Fulani herds within the study area is presented in table 4.1 and figure 4.1. A total of 967 lactating cows were tested, (336 from the FCT and 631 from Kaduna state) out of which 20 (2%) cows were missed due to inability to take a reading 75hrs after the tuberculin test. Of the remaining 947 cows, 139 (14.6%) tested positive to tuberculin, 37 (4%) were inconclusive reactors while 771 (81.4%) were negative reactors.

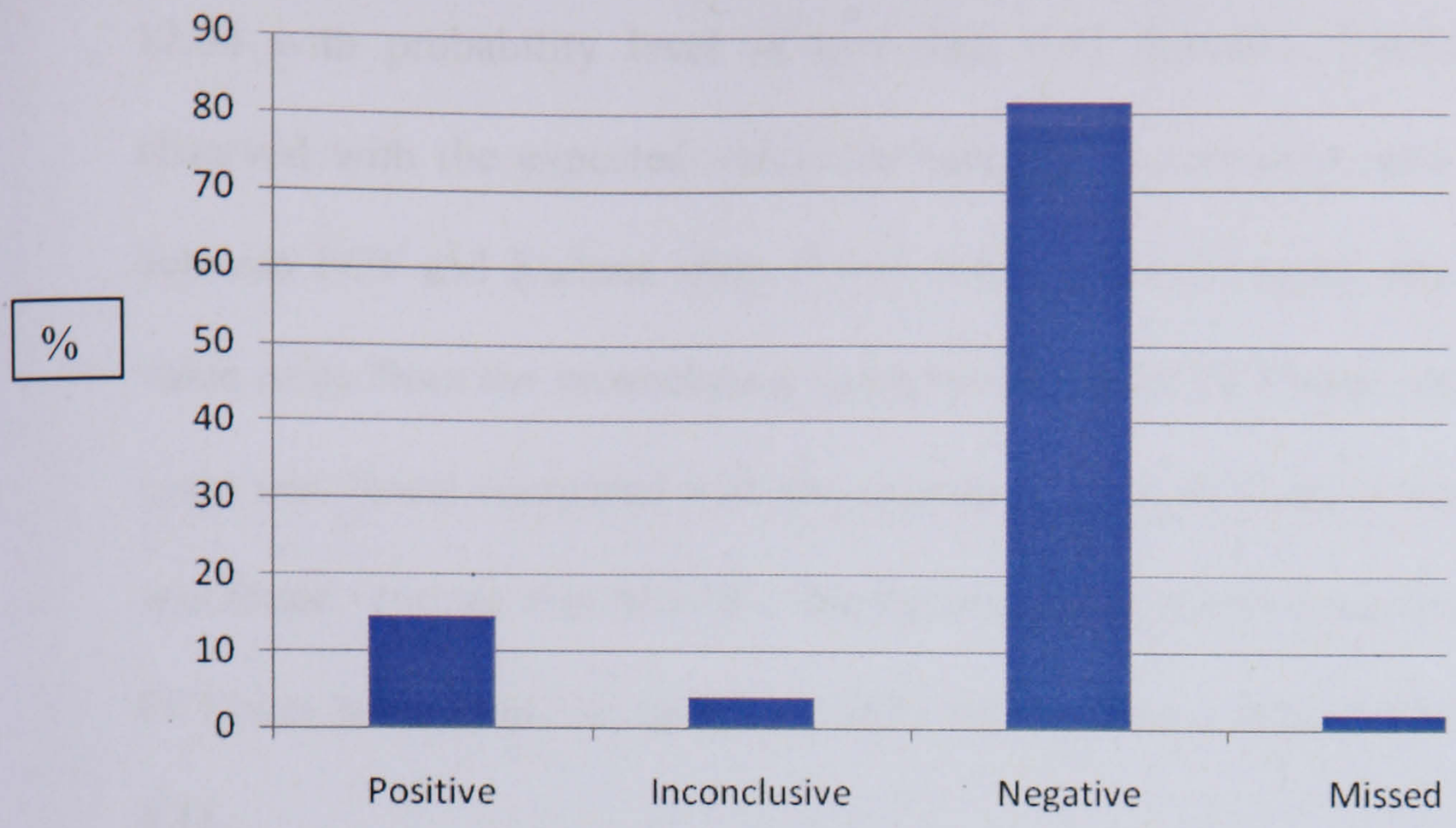

Figure: 4.1. Histogram of Prevalence of mycobacterial infection among lactating cows in the Federal Capital Territory (FCT) and Kaduna state of Nigeria based on tuberculin test.

Out of the 336 cows tested in the Federal Capital Territory, 10 were recorded as missed due to inability to take a reading 72hrs after the testing. Of the 326 cows left, 54 (16.6%) were positive reactors, 22 (6.7%) were inconclusive/doubtful reactors while 250 (76.7%) tested negative (table 4.1). In Kaduna state on the other hand, of the 631 cows tested, 10 were also missed and out of the remaining 621 cows left, 85 (13.7%) tested positive, 15 (2.4%) were inconclusive/doubtful reactors while 521 (83.9%) were negative (table 4.1).

The number of samples that tested positive, inconclusive and negative was compared between the FCT and Kaduna state using chi-square (table 4.1). This comparison was to investigate the level of mycobacterial infection in cattle in the two different areas and also the incidence of TB among the human population using milk and milk products. The chi-square (χ^2) test of significance with 2 degrees of freedom (df) was

12.84 with probability level of less than 0.01 (actually 0.002). Comparing the observed with the expected values for positive, inconclusive and negative reactors between FCT and Kaduna state, it was noticed that the major contribution to the χ^2 value came from the inconclusive cases where, in the FCT more inconclusive reactor cases was found compared with the expected, while in Kaduna state a reverse trend was found. It must also be noted that the number of positive reactors observed for the FCT was more than the expected while the reverse is noted in Kaduna state (table 4.1).

Table: 4.1. Chi-square table of comparison of tuberculin test between the Federal Capital Territory and Kaduna State of Nigeria

Location		No. Positive	No. Inconclusive	No. Negative	Total
FCT, Abuja	O:	54	22	250	326
	E:	47.85	12.74	265.41	
Kaduna state	O:	85	15	521	621
	E:	91.15	24.26	505.59	
	Total	139	37	771	947

O: Observed values, E: Expected values

χ^2 = with 2df = 12.84, P<0.01

The highest number of positive reactors was recorded from a herd in Zango area of Kaduna State, where amongst the 46 cows tested in the herd, 26 (56.5%) of them were positive to tuberculin test. Ten out of the total herds tested in the FCT recorded no positive reactors at all. A relatively low number of inconclusive reactors also came from these herds. Of all the herds tested within the study area, 15 (26.3%) herds (three herds from FCT and twelve herds from Kaduna state) were found to be free of demonstrable exposure to mycobacterial infection, i.e. none of the cows tested either positive, or had inconclusive reactors which could be due to good management practices observed in the farm.

4.1.2.2. Effect of Management on the prevalence of mycobacterial infection in cows

The prevalence of mycobacterial infection in lactating cows by management systems (nomadic and semi-nomadic) is presented in table 4.2 and figure 4.2. Out of the 947 cows from 57 herds screened for mycobacterial infection, 663 (70%) of the cows were from the nomadic pastoral system while the remaining animals were from the semi-nomadic pastoral system. Of the 663 cows in the nomadic system, 104 (15.7%) (which constitutes 75% of the total positive reactors recorded in the study), were positive, 26 (4%) were inconclusive while 533 (80.3%) were negative reactors. In the semi-nomadic system on the other hand, out of the 284 cows tested, 35 (12.3%) were positive, 11 (3.9%) were inconclusive/doubtful reactors while 238 (83.8%) were negative reactors. Of the 15 (26.3%) herds, which were found to be one hundred percent free of demonstrable exposure to mycobacterial infection as indicated earlier,

60% were from the semi-nomadic pastoral system while the rest were from the nomadic pastoral system. The prevalence of infection in the two management systems was compared using the chi-square test of significance and it reflected no significant effect ($P>0.05$).

Table 4.2: Prevalence of mycobacterial infection in the nomadic and semi-nomadic management systems in the Federal Capital Territory and Kaduna state of Nigeria based on tuberculin tests

Management system	N	Positive reactors (%)	Inconclusive (%)	Non reactors (%)
Nomadic	663	104 (15.7)	26 (4)	533 (80.3)
S/Nomadic	284	35 (12.3)	11 (3.9)	238 (83.8)
Total	947	139 (14.6)	37 (4)	771 (81.4)

NB: Those missed are not included in this table, N = number tested

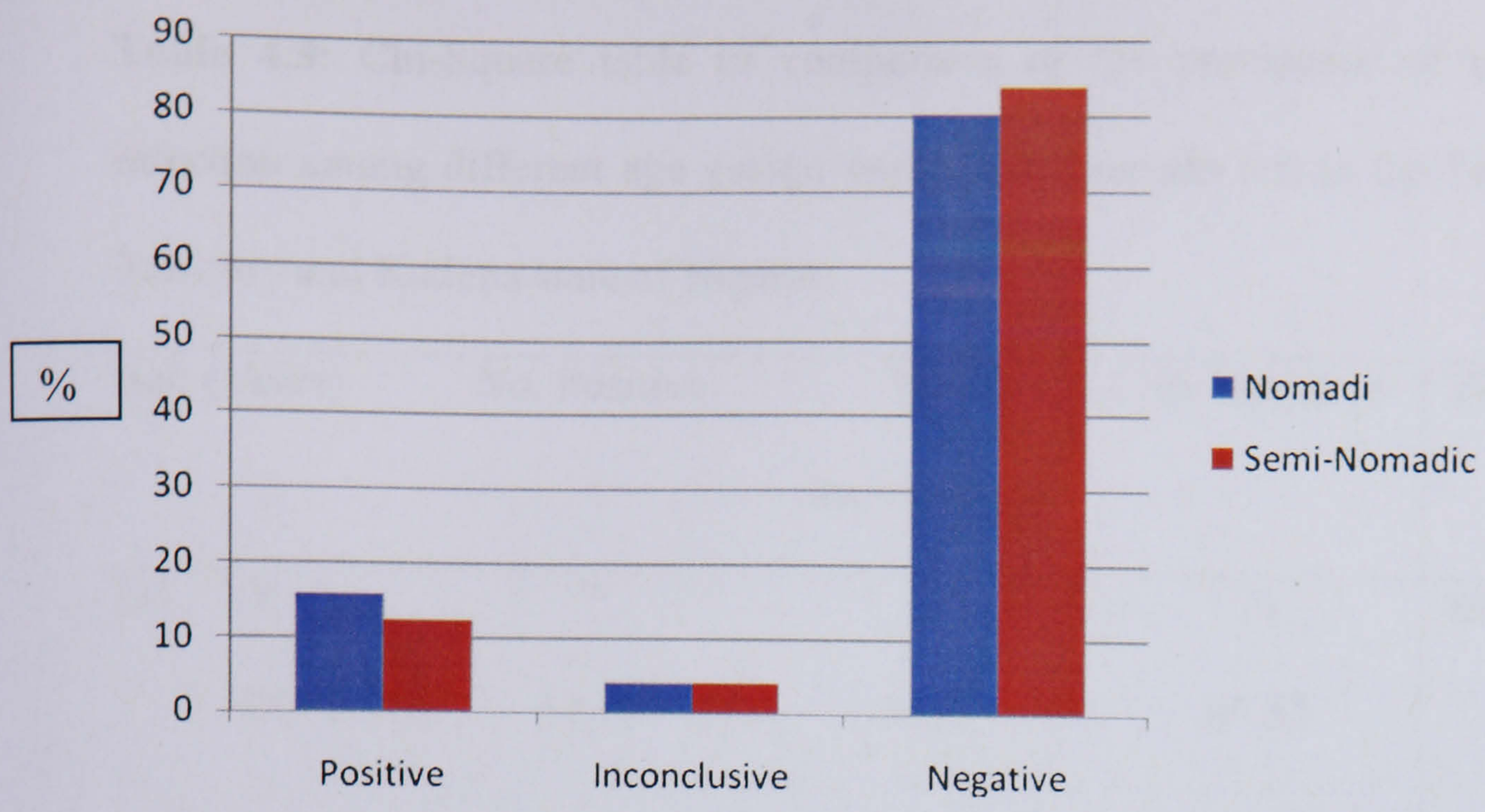

Figure: 4.2. Histogram of Prevalence of mycobacterial infection in the nomadic and semi-nomadic pastoral systems in the Federal Capital Territory (FCT) and Kaduna state of Nigeria as indicated by tuberculin test

4.1.2.3. Prevalence of mycobacterial Infection among different Age groups

The age of cows sampled was distributed into four age groups as described in the materials and methods. In order to verify the age effect, the chi-square test of significance was used to compare the prevalence of infection among the four different age groups. The chi-square with 6 degrees of freedom was 13.78 giving a probability level of significance of less than 0.05. The number of positive reactors observed for age group 2-3 and >3-4 yrs was less than the expected value while that of the >5 years old is higher than the expected (table 4.3) because older animals have more chance of being infected.

Table 4.3: Chi-Square table of comparison of the prevalence of mycobacterial infection among different age groups based on tuberculin test in the Federal Capital Territory and Kaduna state of Nigeria;

Age (Years)		No. Positive	No. Inconclusive	No. Negative	Total
2-3	O:	7	5	71	83
	E:	12.18	3.24	67.57	
>3-4	O:	27	8	217	252
	E:	36.99	9.85	205.17	
>4-5	O:	36	10	212	258
	E:	37.87	10.08	210.05	
>5	O:	69	14	271	354
	E:	51.96	13.83	288.21	
Total		139	37	771	947

O: Observed values, E: Expected values

χ^2 with 6 df = 13.78, P<0.05

4.1.3. Culture on Lowenstein-Jensen media and Acid-Fast (Zeihl-Neelsen) stain of Milk samples

The result of culture of milk samples collected from tuberculin positive and inconclusive reactors is presented in table 4.4. Of the 156 milk samples collected, 71 and 85 were from the FCT and Kaduna state respectively out of which 45 samples were lost from power failure, collapse of media and contamination during protocol. Out of the remaining 111 samples processed, 14 (12.6%) were positive on Lowenstein-Jensen (LJ) media (6 from FCT and 8 from Kaduna) while 97 (87.4%) were negative.

For acid-fast stain, 130 samples were acid-fast stained out of which 30 (23 %) were acid-fast positive while 100 (77%) were negative. All the 14 samples that were found to be culture positive and 3 and 4 of those that collapsed and got contaminated during protocol respectively were among the ones found to be acid-fast positives.

It is worthy of note that 2 (5%) of the 37 milk samples collected from cows with inconclusive tuberculin reactors turned out to be positive for both culture and acid-fast stain.

Table 4.4: Prevalence of tubercle bacilli in milk as indicated by culture on Lowenstein-Jensen media

Location	N	Culture Pos. (%)	Culture Neg. (%)
FCT, Abuja	56	6 (10.7)	50 (89.3)
Kaduna Sate	55	8 (14.5)	47 (85.5)
Total	111	14 (12.6)	97 (87.4)

N = number of samples

4.1.4. Efficiency of diagnostic methods used in detecting mycobacterial infection among cows: Tuberculin Test, the Culture and Acid-Fast (ZN) stain of Milk samples

The efficiency of tuberculin testing of cows, culture and acid-fast stain of milk samples in the level of detection of positive cases was compared using the chi-square and is shown in table 4.5. The χ^2 with 2 degrees of freedom is 6.17 with a probability level of significance of less than 0.05. Comparing the observed and the expected values showed that tuberculin and culture detected less positive cases than the expected while the smear test detected more than the expected.

Table 4.5: Chi-Square table of comparison between the efficiency of the different diagnostic methods used in detecting mycobacterial infection among cows: tuberculin test, culture and acid-fast stain of milk

Tests		No. Positive	No. Negative	Total
Tuberculin test	O:	139	771	910
	E:	144.68	765.32	
Culture	O:	14	97	111
	E:	17.65	93.35	
Smear	O:	30	100	130
	E:	20.67	109.33	

O: observed values, E: expected values; N = number tested, NB: those cows with inconclusive tuberculin test result are not reflected in this table

χ^2 with 2df = 6.17, P<0.05

4.1.5. Level of mycobacterial infection among slaughtered cattle

A total of 250 (150 from FCT and 100 from Kaduna state) suspected TB lesions were collected from slaughtered cattle during meat inspection in abattoirs. The samples were cultured on Lowenstein-Jensen media and acid-fast (ZN) stained (table 4.6). Sixty five of the total samples collected were lost due to power failure, contamination and collapse during protocol. Of the remaining 185 samples processed 32 (17.3%) were culture positive while the remaining 153 (82.7%) samples were culture negative. Out of the 32 culture positive tissue samples, 27 (85%) were from cows and the remaining 5 (25%) were from bulls. Also 200 out of the total of 250 samples were

acid-fast stained where 40 (20%) were found to be acid-fast positive while 160 (80%) were negative (table 4.6).

In the Federal Capital Territory, of the 99 samples cultured (table 4.6), 20 (20.2%) were positive while 79 (79.8%) were negative. Also of the 116 samples acid-fast stained, 26 (22.4%) samples were acid-fast positive while 90 (77.6%) were negative.

In Kaduna state on the other hand, of the 86 samples cultured (table 4.6), 12 (14%) were culture positive while 74 (86%) were negative. With the acid-fast stain, 14 (16.7%) of the 84 samples acid-fast stained were negative while 70 (83.3%) were acid-fast stained.

Table: 4.6. Level of mycobacterial infection among slaughtered cattle in the FCT and Kaduna state based on culture and acid-fast stain of suspected TB lesions

Location	N	Culture		N	Acid-fast stain	
		Positive (%)	Negative (%)		Positive (%)	Negative (%)
FCT	99	20 (20)	79 (79.8)	116	26 (22.4)	90(77.6)
Kaduna state	86	12 (14)	74 (86)	74	14 (16.7)	70(83.3)
Total	185	32 (17.3)	153 (82.8)	190	40 (20)	160 (80)

N = number of samples

4.1.5.1. Distribution of suspected TB lesions in different organs of the body of infected cattle

The distribution of TB lesions in various organs of the body showed that the lung has the highest number of TB lesions with 25 (78.1%) out of the 32 positives recorded.. This is followed by the lymph nodes (submandibular and thoracic) with 5 (15.6%) positives while the liver and the spleen had 1 (3%) positive each (Figure 4. 3).

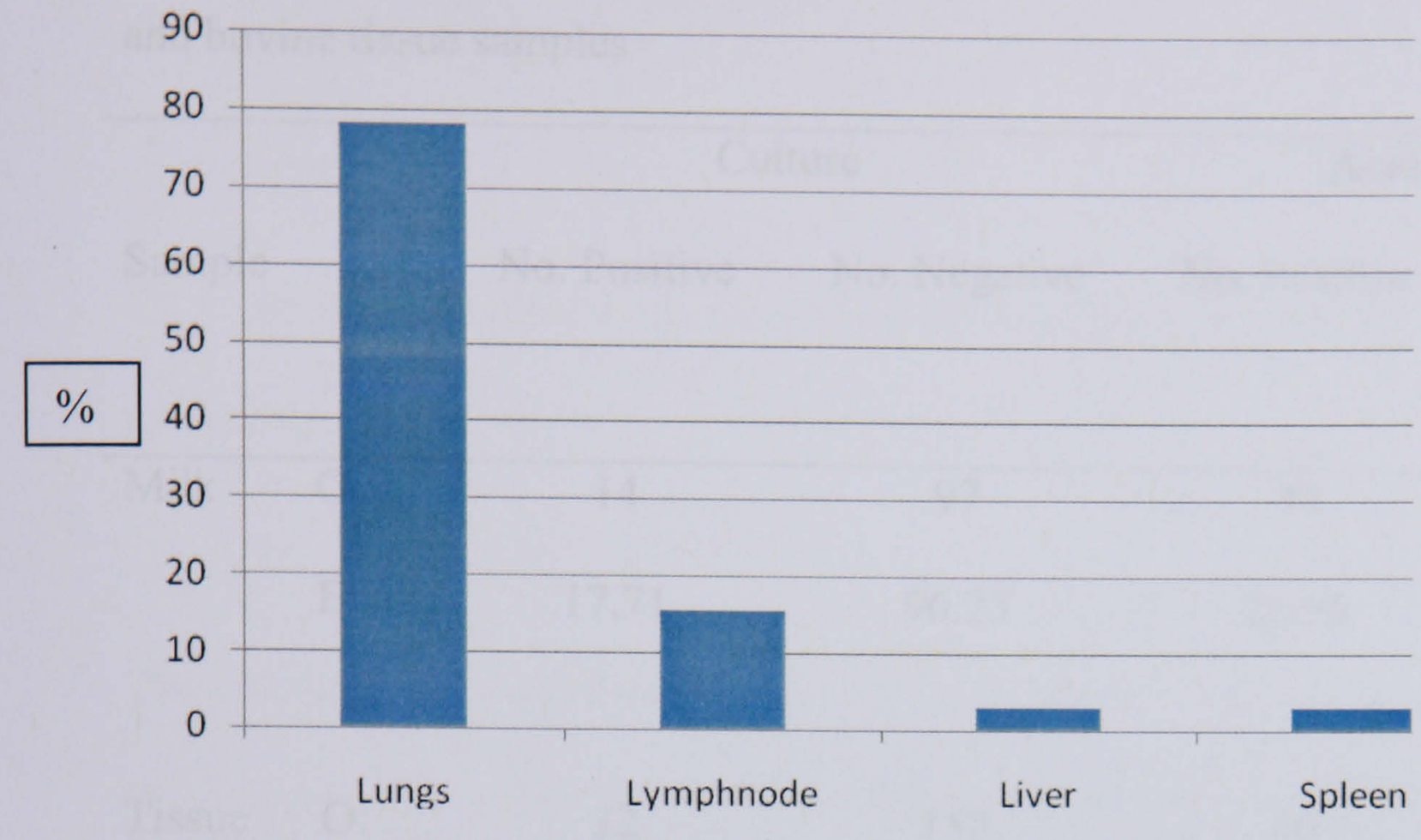

Figure 4.3: Histogram of Distribution of TB lesions in different organs of infected cattle

4.1.6. Efficiency of culture and acid-fast stain methods in detecting *Mycobacterium* in tissue and milk samples

A comparison of the efficiency of culture and acid-fast stain methods in detecting positive cases among tissues with suspected TB lesions using chi-square test revealed a non significant difference ($P>0.05$). However, a comparison between observed and expected values indicated, to an acceptable degree, that the culture method detected slightly more positive cases than the expected. Also, the chi-square test value with 3 degrees of freedom was 3.15 reflected no significant association, or difference, in detecting positive cases whether the samples originated from milk or tissue (table 4.7).

Table 4.7: Chi-Square table of comparison between culture and acid-fast stain of milk and bovine tissue samples

		Culture		Acid-fast stain	
Sample		No. Positive	No. Negative	No. Positive	No. Negative
Milk	O:	14	97	30	100
	E:	17.71	96.25	26.95	100.10
Tissue	O:	32	153	40	160
	E:	28.29	153.75	43.05	159.90

O: observed values, E: expected values

χ^2 with 3df = 3.15, P>0.05

4.1.7. Relationship between mycobacterial infection among Live and Slaughtered Cattle

Comparing the prevalence of mycobacterial infection among lactating cows (live animals) as indicated by the number of positive cases diagnosed through tuberculin testing and by culture among slaughtered cattle, the χ^2 test reflected no significant difference (P>0.05). Though comparing the observed and the expected cases of the proven positive ones, the number observed within the tissue culture is more than the expected while the reverse was noticed for tuberculin testing of live cows

4.2. Prevalence of Human Tuberculosis in the Federal Capital Territory and Kaduna state of Nigeria

4.2.1. Epidemiological Characteristics of suspected human TB Patients based on questionnaire

The characteristics of suspected human TB patients obtained from the questionnaire are presented in table 4.8. The mean (±SD) age of the 900 patients included in this study was 34 ±12 years with 35 ±11 years for males and 33 ±12 years for females. Of the 900 patients in the study, 556 (61.7%) were males while 344 (38.3%) were females (table 4.1). Three hundred and ninety (43.3%) of the patients were between the age of 25 and 34 years old. Of all patients screened in this study, 615 (68.3%) had BCG immunization in the past while the rest had not.

On the history of contact with cattle (living, working or staying in a farm or occupational contact), 380 (42.2%) of the patients had such exposure history with cattle at one time of their lives or another and 85.5% percent of the patients consumed the locally produced milk `fura da nono` (unpasteurized dairy product).

Table: 4.8. Some epidemiological Characteristics of suspected TB Patients based on questionnaires

Patient Characteristics	No. (%)
Sex	
- Males	556 (61.7)
- Females	344 (38.3)
Contact with cattle	
- Yes	380 (42.2)
- No	520 (57.8)
Consumption of unpasteurized dairy products	
- Yes	770 (85.5)
- No	130 (14.5)
BCG in the past	
- Yes	615 (68.3)
- No	385 (31.7)

4.2.2. Culture and Acid-Fast (ZN) stain of Sputum samples

Results of culture and acid-fast stain are presented in table 4.8. Out of the 900 patients screened for TB (500 from FCT and 400 from Kaduna state, (table 4.10), 138 (15%) samples were lost due to contamination and collapse of media during protocol. Of the

remaining samples that were cultured (762 samples), 210 (27.6%) and 190 (24.9%) were culture and smear positive respectively while the rest were negative.

In the FCT, out of the 428 samples cultured, 120 (28%) were positive, while the remaining 308 (72%) were negative. One hundred and ten (22%) of the 500 patients were found to be smear positive while 390 (78%) were smear negative (table 4.10.). In Kaduna state on the other hand, of the 400 patients screened, 334 sputum samples were cultured, out of which 90 (26.9%) were found to be positive and 244 (73.1%) were culture negative. All 400 samples were acid-fast stained out of which 80 (20%) were positive while the rest were negative.

4.2.3. Efficiency of test: culture and acid-fast

The efficiency of the two diagnostic methods (culture and acid-fast stain) were compared using chi-square and no significant difference was found between the two methods ($P>0.05$). However, it is worthy of note that, about 15% of the samples were lost during culture.

Table: 4.9. Prevalence of human TB among patients in the Federal Capital Territory (FCT) and Kaduna state of Nigeria based on culture and acid-fast stain of sputum samples

Location	N	Culture		N	Acid-fast stain	
		Positive (%)	Negative (%)		Positive (%)	Negative (%)
FCT	428	120 (28)	308 (72)	500	110(22)	390 (78)
Kaduna	334	90 (26.9)	244 (73.1)	400	80 (20)	320 (80)
Total	762	210 (27.6)	552 (72.4)	900	190 (21.1)	710 (78.9)

N: number of patients screened

4.2.4. Prevalence of Tuberculosis among male and female Patients

Of the 210 culture positive patients, 145, which constitute 69% of the total positive cases recorded in this study, were males while, 65 (31%) were females (table 4.10). Number diagnosed as positives within the sexes were tested for any difference or association as diagnosed by culture using chi-square. The difference was found to be significant at the 5% level which reflects that there are more positive cases within male patients compared with females patients screened in this study.

Table 4.10: Chi-square table of comparison between the prevalence of Tuberculosis among male and female patients based on culture

Sex		Culture Positive	Culture Negative	Total
- Male	O:	145	331	476
	E:	131.18	334.82	
- Female	O:	65	221	286
	E:	78.82	205.19	
Total		210	552	762

O: observed values, E: expected values

χ^2 with 1df = 5.35, P <0.05

4.2.5. Relationship between BCG vaccination and the Prevalence of Human Tuberculosis

Out of 525 sputum samples cultured from patients who had BCG vaccination in the past, 110 (20.9) were found to be culture positive, which constitutes over 52% of the total culture positive patients observed in this study (table 4.11). Of the 235 sputum

samples from patients who had no BCG vaccination in the past, 100 (42.5%) were culture positive. Comparing the incidence of the disease between those who previously had BCG vaccination in the past and those who had not using chi-square of significance with 1 degree of freedom, indicated a highly significant difference ($P<0.00001$). The BCG group showed a lower incidence than the non-BCG group.

Table 4.11: Chi-square table of comparison between the Prevalence of TB among patients who had BCG vaccination in the past and those who did not based on culture

BCG in the past		Culture positive	Culture Negative	Total
- Yes	O:	110	415	525
	E:	144.69	380.31	
- No	O	100	137	235
	E:	65	171.69	
Total		210	552	762

O: observed values, E: expected values,

χ^2 with 1df =36.90, $P<0.0001$

4.2.6. Relationship between TB in Human and the Consumption of Milk and Milk Products

Of the 770 patients who usually consume locally produced milk, 'nono', 23.3% were culture positive while 23% of the 130 patients who do not consume nono were found to be culture positive (table 4.12). However, in this present study chi-square test showed no significant association between consuming and not consuming milk and milk products (P>0.05).

Table 4.12: Prevalence of tuberculosis among patients who consumed and those who do not consume raw milk and milk products in the Federal Capital Territory and Kaduna state of Nigeria based on culture

Consumption of Milk/ products	N	Culture positive (%)	Culture Negative (%)
- Yes	670	180 (23.3)	490(76.7)
- No	92	30 (23)	62 (77)
Total	762	210	552

N = number of patients

4.2.7. Relationship between Bovine and Human Tuberculosis in the Federal Capital Territory and Kaduan state of Nigeria

The number of positive cases within the suspected human TB patients screened and the number of positive cases within cattle population in this study was compared using chi-square test of significance and is presented in table 4.13. The diagnostic tool used for human patients in this comparison was the culture while that for the cattle was the PPD. The χ^2 with 1 degree of freedom showed a highly significant difference and association in the occurrence of mycobacterial infection among cattle and human populations. This observed value within human patients (210) compared with the expected on the null hypothesis (159.7) and the reverse trend within the cattle population; where, the observed (139) was less than the expected (189.3) might be taken as an indication that the prevalence of TB among the human population is higher than that among cattle population.

Table 4.13: Chi-square table of the comparison between Bovine and Human Tuberculosis in the Federal Capital Territory and Kaduna state of Nigeria based on culture

Test		Positive	Negative	Total
PPD (Cows)	O:	139	771	910
	E:	189.3	720.73	
Culture (Human)	O:	210	552	762
	E:	159.7	608.27	

O: observed values, E: expected value

NB: Inconclusive reactors are not shown in this table

χ^2 with 2df = 6.17, P<0.0001

4.3. DNA Fingerprinting

4.3.1. Spoligotyping and VNTR Typing

Of the 71 *Mycobacterium* isolates obtained from DNA fingerprinting, 57 (80.2%) were from human sputum samples, 6 (8.5%) from milk and 8 (11.3%) from cattle tissue. DNA patterns were successfully produced from 56 (78.8%) and 51 (71.8%) of the isolates by Spoligotyping and VNTR methods respectively. No patterns were produced by 13 (21.2%) and 18 (28%) isolates by the two methods respectively, which could probably be due to lack of DNA or test limitations. The spoligotype and

VNTR patterns of the 56 isolates are shown in table 4.15. DNA fingerprinting identified 46 (82.1%) of the isolates as *M. tuberculosis,* 8 (14.3%) as *M. bovis* and 2 (3.6%) as *M. africanum* type 1.

Using the spoligotyping method, a total of 23 spoligotype patterns were identified. Of these, 16 (28.6%) of the isolates showed unique patterns while the remaining 40 (71.4%) isolates were clustered in 7 different clusters.

The VNTR typing method on the other hand separated the 51 isolates into 36 allele profiles. Of these isolates, 27 showed unique patterns while the remaining 24 isolates were clustered into 9 different clusters.

However, when the two methods were combined, 42 different patterns were obtained. Out of these, 37 of the isolates showed unique patterns while the remaining 14 isolates were clustered in 5 different clusters (table 4.14 and 4.15).

Table 4.14. The efficiency of discrimination of mycobacterium isolates by spoligotyping and VNTR methods used alone and in association

Typing method	No. of different patterns	No. of clusters	No. of clustered isolates	No. of unique isolates
Spoligotyping.	23	7	40	16
VNTR	36	9	24	27
Spoligotyping + VNTR	42	5	14	37

The most common spoligotype pattern identified in this study involves 19 out of the 56 isolates (C8, C9, C55, C48, C40, C35, C32, C11, C12, C13, C15, C21, C23, C26, C27, C29, C53, C46). This spoligotype pattern has not been previously described in the international spoligotype database hence; and does not have a Shared International Type number (SIT). The VNTR typing method on the other hand has further sub-divided this spoligotype pattern into 10 different VNTR profiles with 14 of the isolates placed in 5 different clusters, while the other 5 isolates having unique VNTR profiles (table 4.15). Of these 19 isolates, 1 isolate was from a milk sample (C55) and the rest were from human sputum. Four (21%) of the isolates from humans were from Kaduna state (C48, C49, C53 and C46) while 15 (79%) of them were from the

Federal Capital Territory (FCT). The only milk sample in the group was from a herd in the FCT belonging to a herder (C40) whose sputum had a similar spoligotype pattern but different VNTR profile with the milk isolate. No epidemiological link was found between the other patients in this cluster except that from history, 2 of the patients (C21 and C8) from FCT were living in Kaduna before moving to Abuja a year earlier and they share the same spoligotype and VNTR patterns with a patient from Kaduna state (C48).

The second spoligotype pattern involved 6 isolates all from Kaduna state with 5 (83.3%) of the isolates (C56, C62, C52, C44 and C45) from human patients and 1 isolate (C54) from milk. This type has been previously described in the international database (www.pausteur-guadeloupe.fr) with SIT 200. They were described in studies in Australia, Great Britain, Mexico, Malawi, Norway, USA, Côte d'Ivoire and Gambia. It was also described in India, Liberia and Nigeria (Abuja); and identified as *M. tuberculosis*. However, by VNTR typing method, all the 6 isolates with this spoligotype pattern have different VNTR profiles.

The third spoligotype pattern involves 3 isolates (C22, C16, and C69), but all had different VNTR profiles. Two of these isolates were from human patients and 1 isolate from milk samples. This spoligotype has been previously described in the International Database with SIT number 253 and belongs to the *M. tuberculosis* T1 family. The type has been described in many countries in the Europe, in Taiwan, the USA, Agentina, Brazil and Russia, but not in Nigeria.

The fourth spoligotype pattern had 2 isolates (C36 and C39) from patients in the Federal Capital Territory who had no link at all but both isolates have different VNTR profiles. This type has also not been previously described in the international database. However, the spoligotype pattern observed, is associated with strains of *M. africanum* (absence of spacers 8, 9 and 39) and appear to be typical "subtype 1" West African *M. africanum*.

The fifth spoligotype pattern has 3 isolates (C2, C51 and C38) which the VNTR method further separated into different VNTR patterns. Two of the isolates were from patients in FCT who are husband and wife, while the other isolate was from a patient in Kaduna state who has no epidemiological link with the other two. This type has been described previously in the international database with SIT number 53 which, belongs to the *M. tuberculosis* U family.

The 6th spoligotype pattern involved 3 isolates with 2 of them from cattle (C67, C59 & C57) (tissue and milk) and one from human sputum belonging to a Fulani herder. However, by VNTR all isolates were separated into different VNTR profile. This type has been previously described in the international database earlier mentioned as well as at VLA *M. bovis* database (www.*Mbovis*.org) with SIT number 663 and SB426 respectively belonging to Bovis 1 label. The pattern was identified as *M. bovis* (absence of spacers 3-5, 9, 16, 21 and 39 through 43) and has been previously described in Canada, Spain, Italy, Netherlands, France, Russia, Argentina but not Nigeria.

The 7th spoligotype pattern identified in this study involves 4 isolates, 3 from cattle with 2 tissues and 1 milk samples (C60, C61 & C64) and 1 from human sputum sample (C1). It was identified as *M. bovis* and has been previously described in the VLA *M. bovis* and Pasteur databases as SB0944 and SIT 1037 respectively based on their characteristic spoligotype pattern (absence of spacers 3, 9, 16 and 39 through 43). However, VNTR analysis on type was inconclusive, since repeat testing of one or more of the alleles gave variable repeat numbers; therefore, no definitive VNTR score could be given and was scored "repeat fail" (RF). The spoligotype has been previously described in France, USA, and Cameroon as well as Nigeria

Of the 16 isolates with unique spoligotype patterns, only 3 isolates (C33, C18, and C68) had been previously described in the international database while the others were not. Two of the types previously described were both from human patients in FCT, Abuja with SIT numbers 52 (C33) and 393 (C18). SIT 52 belongs to *M. tuberculosis* T2 family which is a very popular strain with 339 profiles worldwide, but not previously described in Nigeria. SIT 393 has also not been previously described in Nigeria but was described in Argentina, Denmark, Spain, Zambia, USA, Italy, Egypt, Sweden and Italy. The 3rd type with a unique spoligotype pattern was identified as *M. bovis* and had been previously described in both international databases (Pasteur and VLA databases) with SIT number 665 and SB134 respectively. It belongs to the Bovis 1 family and was described previously in Belgium but not in Nigeria. The type was isolated from a milk sample in FCT, Abuja. However, all the isolates have different VNTR profiles.

A distinct group of another 2 isolates though with unique pattern but very slight difference with cluster 4 (C30 and C20) (absence of spacers 8, 9. and 39) also appeared to be typical "subtype 1" West African *M. africanum*. One of these isolates was from human (C20) while the other (C30) was from cattle, but all isolates have different VNTR profiles.

All the other 11 types with unique spologotype and VNTR patterns have not been previously described and were identified as *M.* tuberculosis (presence of spacers 39 through43)

Table 4.15: Spoligotype patterns and VNTR profiles of *Mycobacterium* isolates from humans and cattle

Sample No	Source of sample	Spoligotype descriptive binary No.	SIT No.	Spoligotype descriptive octal No.	VNTR profile	VNTR group
C8	Sputum	777777763760771	none	●●●●●●●●●●●●●●●●●●●●●●●●--●●●●●●●----●●●●●●●	3233255	1
C21	Sputum	777777763760771	none	●●●●●●●●●●●●●●●●●●●●●●●●--●●●●●●●----●●●●●●●	3233255	1
C48	Sputum	777777763760771	none	●●●●●●●●●●●●●●●●●●●●●●●●--●●●●●●●----●●●●●●●	3233255	1
C11	Sputum	777777763760771	none	●●●●●●●●●●●●●●●●●●●●●●●●--●●●●●●●----●●●●●●●	3233214	2
C15	Sputum	777777763760771	none	●●●●●●●●●●●●●●●●●●●●●●●●--●●●●●●●----●●●●●●●	3233214	2
C35	Sputum	777777763760771	none	●●●●●●●●●●●●●●●●●●●●●●●●--●●●●●●●----●●●●●●●	42331*53	3
C49	Sputum	777777763760771	none	●●●●●●●●●●●●●●●●●●●●●●●●--●●●●●●●----●●●●●●●	42331*53	3
C13	Sputum	777777763760771	none	●●●●●●●●●●●●●●●●●●●●●●●●--●●●●●●●----●●●●●●●	4233255	4
C32	Sputum	777777763760771	none	●●●●●●●●●●●●●●●●●●●●●●●●--●●●●●●●----●●●●●●●	4233255	4
C40	Sputum	777777763760771	none	●●●●●●●●●●●●●●●●●●●●●●●●--●●●●●●●----●●●●●●●	4233255	4
C46	Sputum	777777763760771	none	●●●●●●●●●●●●●●●●●●●●●●●●--●●●●●●●----●●●●●●●	4233255	4
C26	Sputum	777777763760771	none	●●●●●●●●●●●●●●●●●●●●●●●●--●●●●●●●----●●●●●●●	4233255	4
C29	Sputum	777777763760771	none	●●●●●●●●●●●●●●●●●●●●●●●●--●●●●●●●----●●●●●●●	4233253	5
C12	Sputum	777777763760771	none	●●●●●●●●●●●●●●●●●●●●●●●●--●●●●●●●----●●●●●●●	4233253	5
C27	Sputum	777777763760771	none	●●●●●●●●●●●●●●●●●●●●●●●●--●●●●●●●----●●●●●●●	4233273	9
C23	Sputum	777777763760771	none	●●●●●●●●●●●●●●●●●●●●●●●●--●●●●●●●----●●●●●●●	42331*52	U
C53	Sputum	777777763760771	none	●●●●●●●●●●●●●●●●●●●●●●●●--●●●●●●●----●●●●●●●	415331*5	U
C55	Milk	777777763760771	none	●●●●●●●●●●●●●●●●●●●●●●●●--●●●●●●●----●●●●●●●	42331*55	U
C9	Sputum	777777763760771	none	●●●●●●●●●●●●●●●●●●●●●●●●--●●●●●●●----●●●●●●●	4233267	6
C56	Sputum	700076777760700	200	●●●---------●●●●●-●●●●●●●●●●●●●----●●●----	3233362	U
C62	Sputum	700076777760700	200	●●●---------●●●●●-●●●●●●●●●●●●●----●●●----	32331*53	7
C54	Milk	700076777760700	200	●●●---------●●●●●-●●●●●●●●●●●●●----●●●----	32331*62	U
C52	Sputum	700076777760700	200	●●●---------●●●●●-●●●●●●●●●●●●●----●●●----	3233252	U
C44	Sputum	700076777760700	200	●●●---------●●●●●-●●●●●●●●●●●●●----●●●----	3233362	U
C45	Sputum	700076777760700	200	●●●---------●●●●●-●●●●●●●●●●●●●----●●●----	3233352	U
C22	Sputum	777777663760771	253	●●●●●●●●●●●●●●●●●●-●●--●●●●●●●----●●●●●●●	4333355	U
C16	Sputum	777777663760771	253	●●●●●●●●●●●●●●●●●●-●●--●●●●●●●----●●●●●●●	4233267	6
C69	Milk	777777663760771	253	●●●●●●●●●●●●●●●●●●-●●--●●●●●●●----●●●●●●●	42332?2	U
C36	Sputum	774077627777071	none	●●●●●●●-----●●●●●●●--●-●●●●●●●●●●●---●●●●	4233273	9
C39	Sputum	774077627777071	none	●●●●●●●-----●●●●●●●--●-●●●●●●●●●●●---●●●●	42331*72	U

C51	Sputum	777777777760771	53	●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●----●●●●●●●	3233354	U
C38	Sputum	777777777760771	53	●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●----●●●●●●●	3233162	U
C2	Sputum	777777777760771	53	●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●----●●●●●●●	4?3**?3	U
C41	Sputum	777777763760751	none	●●●●●●●●●●●●●●●●●●●●●●●--●●●●●●●----●●●●-●●	4233255	4
C50	Sputum	777767763760771	none	●●●●●●●●●●●●●●-●●●●●●●●--●●●●●●●----●●●●●●●	42331*65	8
C5	Sputum	777767627760771	none	●●●●●●●●●●●●●●-●●●●●--●-●●●●●●●●----●●●●●●●	3?33*?3	U
C7	Sputum	777767663760771	none	●●●●●●●●●●●●●●-●●●●●-●●--●●●●●●●----●●●●●●●	3233244	U
C47	Sputum	700000000000071	none	●●●------------------------------------●●●●	32331*73	U
C10	Sputum	776377700000001	none	●●●●●●●●--●●●●●●●●●●●---------------------●	3233153	U
C14	Sputum	774077627777060	none	●●●●●●●-----●●●●●●●●--●-●●●●●●●●●●●●---●●--	4333373	U
C30	Tissue	774057633777071	none	●●●●●●●-----●-●●●●●●--●●-●●●●●●●●●●●---●●●●	42321*73	U
C20	Sputum	770077626077071	none	●●●●●●------●●●●●●●●--●-●●----●●●●●●---●●●●	4233273	9
C33	Sputum	777777777760731	52	●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●----●●●-●●●	4233352	U
C42	Sputum	777777763760771	none	●●●●●●●●●●●●●●●●●●●●●●●--●●●●●●●----●●●●●●●	3233245	U
C18	Sputum	777757777760777	393	●●●●●●●●●●●●●-●●●●●●●●●●●●●●●●●●----●●●●●●●	4?331*63	U
C17	Sputum	077777770020731	none	---●●●●●●●●●●●●●●●●●●●●●-------●----●●●-●●●	3233312	U
C24	Sputum	674077627736071	none	●●-●●●●-----●●●●●●●●--●-●●●●●●-●●●●----●●●●	2233373	U
C43	Sputum	400003763760777	none	●---------------●●●●●●●--●●●●●●●----●●●●●●●	42331*65	8
C68	Milk	616773777777600	665/SB134	●●---●●●-●●●●●●-●●●●●●●●●●●●●●●●●●●●●●-----	4?331*53	U
C67	Tissue	616773677777600	663/SB426	●●---●●●-●●●●●●-●●●●-●●●●●●●●●●●●●●●●●-----	3233363	U
C59	Milk	616773677777600	663/SB426	●●---●●●-●●●●●●-●●●●-●●●●●●●●●●●●●●●●●-----	32331*53	7
C57	Sputum	616773677777600	663/SB426	●●---●●●-●●●●●●-●●●●-●●●●●●●●●●●●●●●●●-----	RF	-
C60	Tissue	676773777677600	1037/SB0944	●●-●●●●●-●●●●●●-●●●●●●●●●●●●●-●●●●●●●●-----	RF	-
C61	Tissue	676773777677600	1037/SB0944	●●-●●●●●-●●●●●●-●●●●●●●●●●●●●-●●●●●●●●-----	RF	-
C64	Milk	676773777677600	1037/SB0944	●●-●●●●●-●●●●●●-●●●●●●●●●●●●●-●●●●●●●●-----	RF	-
C1	Sputum	676773777677600	1037/SB0944	●●-●●●●●-●●●●●●-●●●●●●●●●●●●●-●●●●●●●●-----	RF	-
						-

RF: Repeat fail, U: Unique pattern, *: Not complete repeat, ?: No VNTR locus

4.3.2. IS6110-RFLP

RFLP-IS6110 protocol was optimised using few of the samples and some DNA from VLA but could not be applied on all the samples due to non availability of a large quantity of high quality DNA samples required to undertake this technique. The method was also found to be technically demanding, time consuming and expensive. This made it impossible to do RFLP-IS6110 method leaving us with the option of doing the spoligotyping and VNTR typing methods which do not need large quantity of high quality DNA.

CHAPTER 5

DISCUSSION

5.1. Bovine Tuberculosis

The tuberculin test result obtained in this study showing over 14% tuberculin reactor rate among lactating cows whose milk and milk products are the major source to the public is of great epidemiological and public health significance. This is important because it has been documented that humans can contract the disease from their cattle as a result of close association (Cosivi *et al.* 1998). This is a major problem especially where cultural practices exist; such as exemplified by the Fulani herdsmen, who live their entire lives with their animals, offering ample opportunity for zoonotic transmission of infection. Also, *M. bovis* infection has been recognized as a potential occupational risk for farm and abattoir workers (Torning 1965; Sjogren *et al.* 1978; Cosivi *et al.* 1995; Daborn *et al.* 1996; Ayele *et al.* 2004). However, it is realised that other microorganisms like *Norcadia sp* could also trigger delayed type of hypersensitivity reactions, but the swelling may not be up to 4mm in diameter. The result obtained from this study is slightly higher than the 11.8% recorded in a similar study conducted by Shehu (1988), but does not differ much from other studies conducted in other parts of the country (Du-Sai *et al.* 1994; Cadmus *et al.* 2004). This indicates that mycobacterial infection is prevalent in all parts of Nigeria and might suggest the existence of foci of infection in the different parts of the country. The reason for these foci are unknown, but might be attributed to management practices such as migration of nomads between the north and southern regions in search of

greener pasture during the dry and the raining seasons and the presence and build up of mycobacterial infections in the environment. It could also be due to lack of implementation of control measures in the country.

The total absence of tuberculin positive cows in 15 herds observed in this study with over 60% of them from the semi-nomadic pastoral system could be attributable to good management practices observed in these herds such as; non indiscriminate introduction of animals into the herd and controlling grazing and watering of animals. It might also be that the infected cows were not tested as a result of sampling limitations

5.1.1. Management effect

Although management has been demonstrated to influence the prevalence of tuberculosis among cattle populations where, the disease was found to be more prevalent among intensively managed cattle than semi-intensive cattle population (O'Reiley *et al.* 1995; Shirima 2003; Kempf *et al.* 2005); this study did not show any significant effect of management system on the prevalence of infection. This might be explained by the fact that cattle under the nomadic system are always on the move and are more exposed to cattle of unknown health status during grazing and also at watering points, particularly during the dry season. It could also be as a result of the animals grazing on contaminated pasture as they move/migrate. Nevertheless, this study has shown that mycobacterial infection occurs in both nomadic and semi-nomadic pastoral farming systems and this invariably means that the cultural habit of

the pastoralists living indoors with their animals and the public who consume raw milk from these infected animals equally stand the risk of getting infected. It might also be a potential danger to wildlife and other animals, which are susceptible to *Mycobacteria* species. A similar observation was made in a study conducted by Shehu (1988) in Zaria, Nigeria where a 13% reactor rate was reported among 315 head of cattle in the nomadic herds tested and 10% among 155 head of cattle in the semi-intensive (semi-nomadic) herds.

5.1.2. Age effect

This study has shown that the effect of age on the prevalence of mycobacterial infection as reflected by PPD is statistically significant (table 4.3). Infection was found to be more prevalent among cows of older age (>5years) where over 19% prevalence rate was observed. The outcome of this finding might be attributed to the chronic nature and long incubation period TB and the fact that animals of older age are more at risk of getting infected. This finding has both epidemiological and public health importance because Fulani herders normally sell very old cows after their productive years to other livestock owners for fattening and later sale for slaughter. The implication of this is that, it could facilitate the spread of infection to other herds both at the cattle market and also when introduced into new herd. If sold for slaughter in abattoirs or private slaughter on the other hand, the risk of transmission from infected carcass to meat handlers which has been documented (Collins *et al.* 1987) is possible, especially where butchers and meat inspectors process and inspect meat and meat products with minimal protective clothing and process offal from infected

carcasses with bare hands. It could also be a source of danger to consumers of undercooked or raw meat. A similar observation was also made by the study conducted by Shehu (1988) where he reported 20.9% tuberculin positive reactors among cattle of older age group.

5.1.3. Prevalence in Nigeria

Interestingly, the prevalence of mycobacterial infection as observed in this study based on tuberculin test (14.6%) and the results of other studies conducted in Nigeria have indicated a steady increase in infection rate over the last 30 years (1976 and 2006). For instance, Alhaji (1976) reported a 0.2% prevalence rate in a study of 529 cattle in the northern region of Nigeria while in the same year Eid (1976) reported a 1.5% reaction rate for cattle in the north western part of Nigeria. Similarly, Ayanwale (1984) reported 7.8% infection rate in a study in some Southern states of Nigeria while Shehu (1988) reported 11.8% infection rate in Kaduna state. Other such studies both within Nigeria and other African countries have shown a similar trend over the years (Ellwood 1975; Abubakar 1994; Du-Sai *et al.* 1994; Jiwa *et al.* 1997; Vekemans *et al.* 1999; Shirima 2003; Cadmus *et al.* 2004). This might be due to the total lack of, or inadequate implementation of a control policy in the region, mainly for socio-economic reasons. Added to the high costs of a sustainable control programme are problems of social unrest due to political instability and ethnic wars; especially among the local farmers and the Fulani herdsmen. It could also be as a result of intercontinental cattle movement due to lack of proper border control as a result of insufficient collaboration with bordering countries and the smuggling of live animals

and hence lack of quarantine. This poor or lack of, implementation of control policy was further corroborated by the interview session held with abattoir managers/vets.

5.1.4. Milk as a source of infection for humans

The result obtained from screening of milk from tuberculin positive and inconclusive reactors where it indicated that over 12% and 23% were culture and acid-fast positive respectively is alarming. This result has serious epidemiological and public health implications especially in a society where milk and milk products are consumed raw as a local delicacy ('Fura da nono' and `man shanu`); and where the major source of milk and milk products is from these Fulani herds. This is also a risk for those working and/or living on farms with infected cattle and to the Fulani people whose stable food is the milk and its products from their animals. This finding is similar to several other studies conducted in other parts of Nigeria where infection rate ranged between 2% to 10% (Alhaji 1976; Shehu 1988; Okolo 1992). Another major issue is that over 80% of the Fulani herders involved in this study do not boil milk before selling to the public as indicated by the response from interview held with them. This further shows that both consumers and the herders are at risk of contracting tuberculosis; as it has been recorded that TB can be transmitted through ingestion or handling of unpasteurized infected milk and milk products (Wigle *et al.* 1972; Collins *et al.* 1983; Lepper *et al.* 1983; Collins *et al.* 1987; Neill *et al.* 1994; Kazwala *et al.* 2001). Also the risk of spread of *M. bovis* in the herd and outside by herdsmen exposed to infection through handling and consumption of infected milk and milk products is high. It is worthy of note that the number of infected lactating cows in a

herd does not really matter because milk is usually pooled from all lactating cows into one container in a herd, so one infected cow can contaminate all the milk collected from that herd and still pose great danger to consumers.

5.1.5. Epidemiological importance of inconclusive/doubtful reactors

Of the 967 cows tested, 4% were shown to be doubtful/inconclusive reactors and they were supposed to be re-tested after 45-60 days for final judgement of whether to retain them as negative or positive. This is important because inconclusive reactors in other studies have shown lesions which yielded mycobacterium on culture (Griffin *et al.* 1995). This is further reconfirmed in this study where 5% of the milk samples collected from cows with an inconclusive reaction turned out to be culture positive. However, in this study, the cows were not re-tested because the herders who are mainly nomads could not be traced to repeat the test. However, they were advised when results were taken to mark these animals as dangerous until proved negative.

5.1.6. Slaughtered cattle as a source of infection

The detection of about 13% of TB infection rate among slaughtered cattle in an area where a similar observation was made among live animals through tuberculin test (14.3%) and where these herds are the main source of animals for slaughter has serious epidemiological and public health importance. This becomes even more serious when milk samples collected from these herds indicated high number of acid-fast and culture positive cases. The result obtained also showed that out of the 32

culture positive tissue samples, 27 (85%) of these samples were from females, which further shows the public health importance of this finding considering the fact that the tuberculin testing focused mainly on lactating cows. This finding further indicates that there is a definite relationship between prevalence of mycobacterial infection among slaughtered cattle and the prevalence of infection among live cattle population. This poses great danger of contracting the disease by the public especially abattoir workers and herdsmen. It further confirms that control measures are either not in place or inadequately applied; because in countries where control of bovine TB is in place, detection of lesions at the abattoir during meat inspection is usually very minimal. This finding also agrees with an earlier suggestion that, abattoir monitoring could be an essential element in the national bovine tuberculosis campaign and the most effective means of detecting residual infection in herds especially in countries that have achieved control of the disease (Corner *et al.* 1990).

Results from this study showing 25 (78.1%) of the 32 culture positive tissue (figure 4.3) from the lungs further confirms that the most common route of transmission is through aerosol. This finding agrees with an earlier study where it was found that 70% to 90% of TB lesions were found in the lungs and lymph nodes of the head or thoracic cavity (Lepper 1973).

5.2. Human tuberculosis

Another major finding in this study is the high prevalence of human tuberculosis (23.3% and 21.1% culture and acid-fast positive respectively) observed among

suspected TB patients in the same region where a similar observation was made among cattle populations (both live and slaughtered). The association between the two was found to be significant ($P<0.05$). It should be noted that this study was conducted among patients who were already suspected of having TB on the basis of clinical symptoms and not from the open population. However, with the recent increase in the prevalence of TB worldwide especially in Africa and Asia, which is mainly as a result of the high prevalence of HIV/Aids in these regions (Derek, 1993), this result is not surprising. Nonetheless, this finding does not differ much from the 30% smear positive recorded by Lawson (Lawson 2006) in a similar study in the Federal Capital Territory, Abuja among 1185 patients. Also the 22% sputum smear positive recorded in this study does not differ much from that reported in the NTBLCP tuberculosis case finding reports of 2003 and 2004 (Wim *et al.* 2004; NTBLCP 2005). Since culture is not routinely used as a diagnostic tool in Nigeria, as in most economically constrained countries, not many reports of the prevalence of TB by culture are available.

5.2.1. Sex effect

This study has shown a significant difference in the prevalence of TB between males and female, where it reflected that there are more positive TB cases among male than female patients (table 4.10). Other authors have reported similar findings in their studies (Holmes, Hausler and Nunn 1998; Borgdorff, Nagel, Dye and Nunn 2000; Lawson 2006). It could be argued that males might be more at risk of having TB than females due to the nature of their work, which exposes them to more risks; especially in Northern Nigeria where the women are mostly at home unemployed and thereby

hardly exposed to the risk of infection from contact with infected people. Although, most studies agree with this finding, Nwachokor (2000), in a 30 year review of tuberculosis in Ibadan, Nigeria reported that more females were affected with TB which reached twice the rate of males. It could be because Ibadan being which is in the Southern part of Nigeria, more women are working than the North where the reverse is the case. Also the incidence of the disease was higher among age groups 25 and 34 years old, which is the age group often associated with TB and also HIV (Lawson 2006).

5.2.2. Efficiency of BCG vaccination as a control measure

Another important finding in this study is that, the prevalence of TB among patients who had never had BCG vaccine in the past is significantly higher than among those who had been immunized in the past ($P<0.05$). This high probability level observed highlights that BCG immunization, which is currently employed in Nigeria as in most developing countries, as the first vaccine given in the routine vaccination schedule of the National Programme on Immunizations (NPI) seems to be working. It should continue to run concurrently with other TB control measures in the country. Although, this study shows that BCG vaccine seems to be working, its significance has recently been questioned as an efficient method of prevention of TB especially in countries with high disease burden (Smith 1982; Collins 2001), but it is still a subject of continuous debate.

5.2.3. Tuberculosis in humans and the consumption of milk and milk products

Pasteurization of milk is undoubtedly an important public health measure for the prevention, not only of *M. bovis*, but also for other zoonotic infections (Collins *et al.* 1987). Despite the finding in this study that, the prevalence of tuberculosis among people who consume raw milk and those who do not, did not show any statistically significant difference, the expected value among those who consume raw milk is higher than the expected. This non significant value observed could be due to sampling limitations and the results from screening of milk showed evidence of tubercle bacilli in the milk samples should; hence this finding should be taken very seriously and not be ignored. However, the finding might also highlight the need to trace other sources of *M. bovis* infection in humans other than milk.

5.3. Efficiency of diagnostic methods

The efficacies of the various diagnostic methods used were compared and it was observed that the acid-fast test detected more positive cases than the expected, while PPD and culture detected less. Also, comparing the results presented on the effectiveness of the culture and smear test for milk, tissue and sputum samples, it shows that the culture method is only slightly more efficient than the smear method in detecting positive cases, even though the culture is said to be the 'gold standard'. Though this result gave such an impression, it is worthy of note that many samples were lost during culture (collapsed and contamination) and this might have contributed to the lower efficiency of the method. However, this might justify saying that the smear test is either more efficient than, or nearly as efficient as the culture

method. Taking this into consideration might help to suggest that due to the low requirement for smear test compared to the culture method; one can depend on the acid-fast test at least for field and epidemiological purposes. It could also be very useful when conducting some PCR based molecular methods like the spoligotyping and VNTR which do not need high quality DNA and also be conducted directly from clinical samples.

5.4. DNA fingerprinting

The ability to accurately identify and trace strains of mycobacteria causing disease using molecular techniques has been identified as a powerful tool in investigating disease transmission within a population both in developed and developing countries (Cousins *et al.* 1998). Therefore, molecular investigation of the isolates in this study has enhanced our understanding of the strains involved in causing disease in both humans and animals in the study area, as well as helping to identifying outbreaks and trace possible routes of transmission.

This result pointed that spoligotype cluster 1 (with 19 isolates) is the most dominant type of *M. tuberculosis* in the study area. Although this spoligotype has not been previously described in the international database, it has a pattern similar to that of the Cameroon family of *M. tuberculosis* strains (deletion of spacers 24, 25 and 33 through 36 and the presence of spacers 39 through 43); the difference only being the presence of spacer 23 (Njanpop-Lafourcade, Inwald, Ostyn, Durand, Hughes, Thorel, Hewinson and Hadad 2001; Njobe-Eyangoh, Kuaban, Sorlin, Thonnon, Vincent and Gutierreze 2004), thus suggesting a common evolutionary descent. This finding

implies that the majority of tuberculosis cases in humans in the study area are caused by this dominant type. However, by VNTR typing, this cluster was further classified into 11 VNTR allele profiles, which could be due to minor mutation within the strain and VNTR being more discriminatory than spoligotyping method. Most importantly, the dominance of this strain in this region may represent the clonal expansion of strains within this region, which supports the observation of a stable association of specific clones with geographically localized populations (Filliol, Driscoll, Van Soolingen, Kreiswirth, Kremer, Valetudie, Dang, Barlow, Banerjee, Bifani, Brudey, Cataldi, Cooksey, Cousins, Dale, Dellagostin, Drobniewski, Engelmann, Ferdinand, Gascoyne-Binzi, Gordon, Gutierrez, Haas, Heersma, Kassa-Kelembho, Ho, Makristathis, mammina, Martin, Mostrom, Mokrousov, Narbonne, Narvskaya, Nastasi, Niobe-Eyangboh, Pape, Rasolofo-Razanamparany, Ridel, Rossette, Stauffer, Sufys, Takiff, Texier-Maugein, Vincent, de Waard, Sola and Rastogi 2003; Smith, Dale, Inwald, Palmer, Gordon, Hewinson and Smith 2003) . Even though this dominant spoligotype has not been previously described in any of the international databases searched as well as in Nigeria, it could be as a result of the few studies conducted in molecular epidemiology in Nigeria. One of the isolates in this cluster was obtained from the milk sample of a cow belonging to one of the Fulani pastoralist who was infected with the same strain of *M. tuberculosis*. This could be a typical human-to-animal transmission or a case of cross contamination from infected herders during milking or from milk hawker to milk during processing of milk.

The second most common type identified in this study, with 6 isolates, has been described previously in many countries including Nigeria with SIT number 200, but all have different VNTR profiles. In Nigeria, the type was reported in Abuja, while in

this study all the 6 isolates were from Kaduna state. Due to lack of data, an epidemiological link could not be traced between these two studies. Interestingly, a high prevalence of this spoligotype was reported in Liberia in the international database. This finding is very important because, Liberia is a country where the Nigerian military and police are on a peace keeping mission and one of the patients (C45) is military personnel, but there is no information on whether he has been on peace-keeping mission to Liberia or not to prove this argument.

Another important finding is that, of all the 21 strains of *M. tuberculosis* obtained in this study, only 5 strains (SIT, 200, 253, 53, 52 and 393) have been previously described in the international databases searched, out of which only 2 of them have been previously described in Nigeria (SIT 200 and 53). Strain SIT 53, though not the most common strain found in the study area, is the most common and ubiquitous of all *M. tuberculosis* strains with over 2380 profiles worldwide according to the database. However, this might not mean that these strains that have not been previously described in Nigeria are totally absent in the country, but might be attributable to the limited molecular epidemiological studies conducted and also that this study was conducted in a limited area.

Another major finding in this study is the isolation of *M. tuberculosis* from cattle; even though human-to-cattle transmission of *M. tuberculosis* has been reported (Ayele *et al.* 2004), the disease in cattle due to *M. tuberculosis* is less severe than that caused by *M. bovis* (Francis 1958). However, the possibility of cross-contamination from infected herder during milking and processing of milk cannot be ruled out. This

is also of public health importance as consumers of this contaminated milk stand the risk of getting infected.

All the 8 types of *M. bovis* isolated from both humans and cattle in this study have been previously described in the international databases searched in this study (SIT 665/SB134, 663/SB426 and 1037/SB0944). It has also been observed from their spoligotype pattern that, all the types can be generated from the SB0944 spoligotype pattern through a single-step deletion of spacers, for instance, SB426 was generated by the deletion of spacers 4, 5 and 21. van Embden and colleagues (2000) suggested that spoligotype patterns evolve as a result of the deletion of spacer units, which means that pattern SB0944 may represent the spoligotype pattern of the ancestral strain of this group of closely related types. Isolates with spoligotype SB0944 has been previously described in France, USA and Nigeria (Kremer, Van Soolingen, Frothingham, Haas, Hermans, Martin, Palittapongarnpin, Plikaytis, Riley, Yakrus, Musser and Van Embden 1999; Hadad, Ostyn, Karoui, Masselot, Thorel, Hughes, Inwald, Hewinson and Durand 2001; Cadmus *et al.* 2006). It has also been described as being dominant in Cameroon, a country bordering Nigeria (Njanpop-Lafourcade *et al.* 2001; Cadmus *et al.* 2006), which indicates that, the Nigerian and the Cameroon strains seem to share a common evolutionary origin. This could be due to cattle trading links between the two countries and also unrestricted cattle movement between Nigeria and Cameroon as well as other African countries.

The finding of strains of *Mycobacterium* that are common between Nigeria and its neighbouring countries (in human and animals) demonstrates a geographical cross-over of DNA fingerprints in the different parts of the country and neighbouring

countries. This cross-over is to be expected in view of the fact that cattle kept by the Fulani pastoralist are constantly moving in search of grazing areas. In the course of this migration, some animals or their products are sold for slaughter and to local communities respectively, thereby creating a likelihood of infected cattle spreading the disease. Infected humans moving with these animals are also another source of infection across the borders. It could also be as a result of illegal immigrants and tourist bringing infection into the country.

Another significant finding of public health importance in this study is the isolation of *M. bovis* from human sputum. This finding further reveals that there is an association between animal infection and the disease in human which is also shown to be statistically significant in this study. It further demonstrates the zoonotic importance of *M. bovis* in human infection in Nigeria and also shows a typical animal-to-human transmission. Despite the few number of *M. bovis* isolated from humans which could be due to the difficulty in cultivating *M. bovis* in the laboratory encountered, but with the high prevalence of infection observed in the cattle populations (live and slaughtered), it could justify a recommendation that any preventive measure for TB in the human population in Nigeria should lean heavily on and be associated with the same level of measures in the cattle population. A similar finding was made in two other studies conducted in Nigeria (Idigbe *et al.* 1986; Cadmus *et al.* 2006).

The *M. bovis* type obtained from herders and their cattle with the same spoligotype pattern might suggest a common source of infection which further confirms the risk faced by these herdsmen and other individuals working with cattle as well as the general public who consume products from these animals. However, due to lack of

adequate abattoir record, retrospective tracing was difficult to help find out if slaughtered cattle with same spoligotype pattern as milk samples were from the same herd. In addition, no apparent association was observed between the breed of cattle and *M. bovis* through molecular typing in this study.

Similarly, the isolation of *M. africanum* from both humans and cattle is of interest. Although *M. africanum* is said to be virulent in cattle, its rarely isolated from this host (de Kantor, Pereira, Miquet and Rovere 1979; Weber, Reischl and Naumann 1998), nevertheless, the possibility of cross-contamination cannot be ruled out. However, the identification of *M. africanum* in human is of importance especially in immuno-compromised individuals. A similar finding was reported by Cadmus and colleagues (2006) in a study in Ibadan, Nigeria

5.4.1. Efficiency of molecular techniques

When the results of the two methods were compared, VNTR method using the seven VNTR loci (ETR-A, ETR-B, ETR-D, ETR-E, ETR-F and QUB 11a and b) was found to be more discriminatory than spoligotyping method; where a total of 41 strains were identified by VNTR methods compared to only 23 by spoligotyping method. A similar observation was made by other scholars (Le Fleche *et al.* 2002; Gibson, Hewinson, Goodchild, Watt, Story, Inwald and Drobniewski 2004). Nonetheless, the use of spoligotyping will take care of low-copy number *M. tuberculosis* complex species (Goyal *et al.* 1997; Goyal *et al.* 1999). However, when the two methods were combined, the level of discrimination was vastly improved, where 49 patterns were produced by the two methods combined together, instead of only 23 and 41 types by

spoligotyping and VNTR typing respectively (table 4.14). A similar finding was also reported in other studies conducted by other researchers (Sola *et al.* 2003; Gibson *et al.* 2004). Furthermore, the two methods have been shown to have high typeability and reproducibility in identifying the various species and strains of *Mycobacterium* obtained from humans and cattle. The techniques were also found to be good for the simultaneous detection and typing of *M. tuberculosis* complex strains obtained, hence showing great potential for use in economically constrained countries like Nigeria. The two methods were also found to be simple, cheap and highly discriminatory as well as rapid and robust for use directly from clinical samples; without the need for high quantity DNA as required by RFLP-IS6110 typing. Similar findings were also reported by other scholars (Goyal *et al.* 1997; Goyal *et al.* 1999; Durr *et al.* 2000; Gori *et al.* 2005). This is a major advantage when related to the earlier finding, where the smear test was found to be nearly as effective as and cheaper than the culture technique hence molecular typing can be done directly from clinical samples after acid-fast staining.

Although IS6110-RFLP was not used in analysing all the samples in this study, the protocol was optimized using few of the samples and non pathogenic strains of *Mycobacterium* and when compared with spoligotyping and VNTR typing methods, it was found to be highly discriminatory but very tedious, technically demanding and time consuming. It was also found to be more expensive and requires large quantity of high quality DNA. These findings were equally recorded by other scholars (Goyal *et al.* 1997; Gori *et al.* 2005; Skuce *et al.* 2005).

It has been shown that it is important for any eradication or control programme to understand the role of different species in the epidemiology of disease since the design of disease control programmes will differ depending on the strains involved. Molecular epidemiology is said to play a role in identifying whether transmission of infection occurs within or between different species (Cousins *et al.* 1998). It can also be used to identify risk factors, practices and environment where significant transmission is occurring (Parra *et al.* 2005). Generally, the identification of outbreaks and tracing routes of transmission of particular strains of *Mycobacterium tuberculosis* complex are important in the control of tuberculosis and the current diagnostic and control methods in place where diagnosis stops only at the smear level is greatly affecting its success.

This study has further demonstrated that the benefit of molecular typing especially spoligotyping and VNTR methods could be so great that the Nigerian government should be willing to invest considerable resources to achieve these gains.

CHAPTER 6

6.1. Conclusion and Recommendations

The major finding in this study is that a high prevalence of mycobacterial infection was observed in cattle (live and slaughtered) in the study area where a corresponding high prevalence of tuberculosis in humans was also observed. This indicates a possible relationship between the infection in the cattle and the disease in humans. It was also observed that despite the high uptake of the DOTS programme and the BCG given at birth in the most favourable context, (which this study has shown seems to be working), but without the same input for control of bovine TB, incidence and mortality from TB is still enormous in Nigeria (WHO 2006). Also by molecular typing, strains of *M. bovis* and *M. tuberculosis* were identified from human and cattle samples respectively, thus indicating a typical animal-to-human and possibly human-to-animal transmission. It was also found that control of TB in cattle is virtually absent or inadequate in the study area. Furthermore, the integration between epidemiological and microbiological data in this study has helped in obtaining more information on the molecular epidemiology of human and bovine tuberculosis in the study area as well as classifies risk factors associated with the disease.

In view of the above, it could justify a recommendation that any preventive measure for TB in the human population in Nigeria should lean heavily on and be associated with the same level of measures in the cattle populations. This could help significantly to check the high prevalence of the disease in cattle and its subsequent implication in humans which has been shown in the results obtained from this and other similar

studies. This combination will reduce the national incidence and mortality rates from tuberculosis currently observed in the country. For this to happen there must be cooperation among different disciplines involved with health care delivery in the country such as; the human medical practitioners, veterinarians, sociologist, statisticians, epidemiologist and so on. This is because, disease control programmes worldwide have begun to shift perspective from strictly one directional to multidisciplinary with the multi-sectorial collaborations that are required for successful control, particularly in developing countries (Peabody, Shimkhada, Tan and Luck 2005). This multidisciplinary cooperation could have a new value in informing such comprehensive policies for TB control. However, the major factors that may affect the control programme in cattle are the initial high cost due to the high prevalence rate of the disease, which is a consequence of the lack of or poor implementation of control measures observed over the years. Control could also be affected by the initial high compensation paid for the slaughter and disposal of animals testing positive for the disease. Although, initially expensive, the cost would be far outweighed by the magnitude of its benefits in terms of reduced national incidence and mortality rates in humans, cost of treatment of infected people and reduced economic loss in the livestock industry; and investing now could make a substantial difference. Nonetheless, this high initial cost will be drastically reduced once adequate control measures are put in place and maintained over a period of time. The programme will also require an extraordinary mobilization and awareness campaign among the Fulani nomads. However, initiating and sustaining such a large-scale effort would be challenging, but clearly, the first step must be a realistic assessment of the potential benefits of these initiatives. These benefits could be so

enormous that the society and the government should be willing to invest considerable resources to achieve these gains.

Abattoir monitoring has been identified as an essential element in a national bovine tuberculosis campaign and the most effective means of detecting residual infection in herds especially in countries that have achieved control of the disease (Corner *et al.* 1990). Once the test and slaughter method is in place, it should be followed with rigorous meat inspection at abattoirs to help in detecting residual infections in herds. Also meat inspectors must carry out inspection using maximum protective clothing to protect them against infection.

Furthermore, this study indicates that *M. bovis* plays a role in increasing the incidence and mortality from tuberculosis in humans. It also shows that exposure to cattle or un-pasteurized milk is an important risk factor for human infection with *M. bovis*. This means that protecting those working with cattle and ensuring microbiological safety of dairy products to the public would be an important public health measure. This therefore calls for prompt implementation of control measures in the country to safeguard the human population and also to reduce economic losses in the livestock industry. It is also important that the government should make a legislation concerning the sale of un-pasteurized milk to the public to reduce the risk of human infection from infected dairy products. In view of this, development of pasteurization points should be considered to pool milk from Fulani hawkers for pasteurization before selling to the public.

This study has further indicated an epidemiological link between the strains of *Mycobacterium* (from both human and cattle) found in the country and neighbouring countries; which could be linked to the poor border control for cattle movement and their herders. This implies that an effective border control could help in the control and subsequent eradication of tuberculosis in Nigeria. However, this might be very difficult unless there is good cooperation between the neighbouring countries and for the government to develop good grazing reserves for cattle with good all year round water supply.

Molecular typing has been shown to help in tracing routes of transmission and outbreaks (Durr *et al.* 2000) which means that, diagnosis should not stop at smear level only if an effective control measure is to be achieved. Culture or smear (as the case may be) and molecular characterization of clinical isolates must be included as a diagnostic tool to ensure correct estimation of the true burden of infection caused by each species of *Mycobacterium*. This present study has shown that good strain discrimination can be achieved when spoligotyping is combined with VNTR typing. The techniques were found to be fast, practical, reproducible and most importantly relatively cheap when compared with IS6110-RFLP, which means they can be used in economically constrained countries like Nigeria.

In conclusion, it is pertinent to say that, a comprehensive, multi-disciplinary analysis can be a starting point for policy-makers. How many people are dying, how much does this cost the society and how much would it take to 'fix' the problem, are basic questions that health policy makers should try to answer. Equipped with these

answers, policy-makers and civil society may be able to more effectively reduce the clinical and economic burden of tuberculosis in Nigeria.

6.2. Future Work

The high prevalence of both human and bovine tuberculosis observed in this study and the subsequent relationship between human and bovine tuberculosis as shown by molecular typing shows the need for a national survey of the impact of tuberculosis to be conducted. This would be in order to study the national disease burden of both human and bovine tuberculosis in Nigeria. The next step would be to relate this disease burden with its economic costs and also to undertake a costs/benefits analysis of controlling the disease in both humans and cattle. These will help in the complete assessment of the impact of TB in Nigeria, not simply to inform policy-makers of the burden of the disease, but also to provide the bases for targeted interventions in TB control to contain the present situation.

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Appendix 1: Glossary

3' terminus	One of the two ends of a polynucleotide which carries the hydroxyl group that is attached to the 3` position of the sugar.
5` terminus	One of the two ends of a polynucleotide which carries the hydroxyl group that is attached to the 5` position of the sugar.
Acid-fastness	The staining property of *Mycobacterium* species where the cells stained with carbolfuschin do not decolourize with acid-alcohol.
Allele	One of the possible different forms of a gene at a given locus.
Amplification	Increasing the number of copies of a specific DNA molecule.
Anneal	To reform the duplex structure of a nucleic acid that has been dissociated by heating.

Base pair (bp)	A pair of complementary nucleotides in double-stranded DNA.
Chromosome	A self replicating nucleic acid molecule carrying a number of genes.
Deletion	Part of a sequence alignment where one sequence appears to have fewer (deleted) monomers compared with another sequence.
Denaturation	Breaking down of hydrogen bonds (involved in base pairing) of nucleic acid molecules by chemical or physical means.
Disease	Injury to host that impairs with the host`s function.
DNA fingerprinting	A molecular technique which produces a pattern of hybridising bands in a southern blot that can be used to identify an organism.
Electrophoresis	The separation of molecules on the basis of their mass to charge ratio.

Epidemic	Disease occurring in an unusually high number of individuals in a population at the same time
Epidemiology	The study of the incidence and prevalence of disease in populations.
Evolution	Change in a line of decent over time leading to the production of new species or varieties within a species.
Gene	A segment of a DNA that codes for an RNA and/or polypeptide molecule.
Genome	The complete set of genes of an organism.
Genotype	Particular set of alleles that is present in an individual.
Genus	A taxonomic group of related species.
Gram-positive	A prokaryotic cell whose cell wall consists chiefly of peptidoglycan and which lacks the outer membrane of gram-negative cells.

Homology	Similarity of nucleotide sequence between two distinct DNA molecules or it can be said that it's a sequence similarity relating to evolutionary relationship between the two molecules.
Hybridisation	The process of complementary base pairing between two single starnds of nucleic acid.
Insertion	Part of a sequence alignment where two sequences appears to have extra (inserted) monomers compared with another sequence.
Insertion (IS) sequence or element	A short (800-1400 bp) DNA sequence found in bacteria that is capable of transposing to a new genomic location.
Kilobase (kb)	A measure of the length of a nucleic acid molecule. One kilobase is equal to 1000bases or base pairs of nucleic acid.
Locus (pl., loci)	A fixed position on a chromosome that is occupied by a given gene or one of its alleles.

Lowenstein-Jensen medium	Medium used for the isolation and cultivation of *Mycobacteria* and as bases for selective, differential and enriched media for *Mycobacterium*
Lysozyme	An enzyme that weakens the cell walls of certain types of bacteria.
Oligonucleotides	A short nucleic acid molecule either obtained from an organism or synthesized chemically.
Outbreak	The occurrence of a large number of cases of a disease in a short period of time.
P value	A probability used to test the significance of a sequence similarity score
Pathogen	An organism that causes disease.
Polymerase chain reaction (PCR)	A technique that enables the enzymatic amplification of a target DNA sequence to produce multiple copies of DNA molecule.

Polymorphism	A variation in the sequence of DNA. so that two or more versions exist
Primer	A short single stranded oligonucleotide, which when attached by base-pairing to a single stranded template molecule acts as the start point for complementary strand synthesis directed by a DNA polymerase enzyme
Probe	A molecule that is labelled and used to detect another molecule (target) through its specific affinity. Applies especially to nucleic acids used to detect targets by hybridization and antibodies used to detect proteins.
Purified protein derivative (PPD)	An antigen that stimulates the immune system to eliminate or fight foreign substance in the body. PPD is taken from dead tuberculosis bacteria (*Mycobacterium)*
Southern blot	DNA that has been electrophoretically separated and immobilized on a solid support (nylon or nitrocellulose).

Species	A collection of closely related strains sufficiently different from all other strains to be recognised as a distinct unit.

Appendix 2

MOLECULAR EPIDEMIOLOGY OF BOVINE AND HUMAN TUBERCULOSIS IN THE FCT AND KADUNA STATE OF NIGERIA.

INTERVIEW WITH HERD OWNERS

Study No: ☐☐☐

Name _____

Address _____ ___

Tribe_____

Age _____

A. Background

Education: Primary ☐ Secondary ☐ Others (specify) ☐

How long have you been a cattle rearer? 0-5yrs ☐ 6-10yrs ☐ over 10yrs ☐

How many head of cattle do you have? .....

How many cows are lactating? .....

What do you do with the milk? Sell ☐ Consume ☐ both ☐

B. AWARENESS

Can you recognize an animal yes no

with suspected TB infection? ☐ ☐

Do you boil milk before yes no

selling to the public? ☐ ☐

If yes, for how long do you boil? ……

Do you have cough of 3weeks or above ? Yes No

☐ ☐

***If yes, take sputum sample (voluntaryl) sputum taken Not taken

☐ ☐

How many of your family none 1-2 all

members have chronic cough ? ☐ ☐ ☐

*****If yes, take sputum samples(voluntary) Yes No

☐ ☐

Appendix 3

MOLECULAR EPIDEMIOLOGY OF BOVINE & HUMAN TUBERCULOSIS IN FCT AND KADUNA STATE OF NIGERIA

Study No: ☐☐☐

INTERVIEW WITH ABBATOIR MANAGERS

NAME _____
ADDRESS _____
TRIBE _____
AGE _____

A. Background

Education: Primary ☐ Secondary ☐ ND/HND ☐ Univ. ☐

B. Awareness

Are you aware of the legislation governing meat inspection? yes ☐ No ☐

If yes, do you apply them in this abattoir? Yes ☐ No ☐

If not, why?

_____

Where do cattle slaughtered come from? neighbouring local govt ☐ neighbouring state ☐ not sure ☐

C. Activities

In this abattoir?
How is the inspection done? Thoroughly ☐ Superficially ☐

Who inspects meat?

Vet Dr ☐ livestock Supt ☐ livestock Sup &Vet Dr ☐ nobody ☐

How are TB cases diagnosed ?

visually ☐ lab. test ☐ not sure ☐

.Do you condemn suspected TB cases?

totally ☐ only affected part ☐ not at all ☐

APPENDIX 4

MOLECULAR EPIDEMIOLOGY OF BOVINE & HUMAN TUBERCULOSIS IN FCT AND KADUNA STATE OF NIGERIA

INTERVIEW WITH PATIENTS

Date ☐☐☐☐☐☐

Name: _____
Age: ☐☐

Address: _____
Sex:

Occupation: _____

Tribe: _____

A. Background

Education: *(none=0, primary=1, secondary=2, university=4)*

B. Clinical Data

Have you had BCG at birth or at any other time? *(Yes=1, no=2, notkn own=3*
(Years) ☐☐☐

Have you had cough recently? *(Yes=1,no=2)* ☐ ☐☐
(Weeks)

C. Examinations

Presence of BCG scar (yes=1, no=2)

 ☐☐☐

D. Contact with animals/milk consumption

Do you keep animals? Yes ☐ No ☐

If yes to above what type animal? cat ☐ Dog ☐ Cattle ☐ Other pets ☐ other L stock ☐

Do you take fresh unpasteurized milk? Yes ☐ No ☐

E. Laboratory examinations - Taken? *(yes=1, no=2)* ☐

1st sputum (on the spot)? ☐ Morning sputum taken? ☐

3rd sputum (on the spot) ☐

F. Laboratory results

Sputum smear results

1st spot										
Morning										
2nd spot										
Bleach/ ZN										

	Taken		Results		Taken	
Results						
Culture LJ	**Y** ☐	**N** ☐			**Y**	**N**

Appendix 5

Calculation and preparation of primers and dNTPs

Primer (Forward): Initial concentration was 35.4nMoles (lyophilized) and after adding 100µl of TE buffer it becomes 354µM stock solution

= 35.4nMoles / 100µl

= 0.354nMoles / µl

= 354pmoles / µl

= 354µmoles / L

= 354µM solution

Primer (Reverse): Initial concentration given was 30.2nMoles (lyophilized) and after adding 100µl of TE buffer it becomes 302µM stock solution as explained above.

Primer stock solutions were stored at -20°C until use.

A 100µl of 25µM working stock solution was prepared from each of the primer stock solutions above to run PCR using the formula below to determine the volume of primer stock required.

N1V1 = N2V2

Where N1 is the concentration of main primer stock (depends on the primer)

V1 is the volume of main primer stock required to make working stock

N2 is the concentration of the working primer stock (25µM)

V2 is the volume of the working primer stock solution (100ml)

Preparation of 100µl of 25µM of DR (Forward) primer working stock solution

N1V1 =N2V2

Where: N1 = 354µM

V1 = ?

N2 = 25µM

V2 = 100µl

354 x V1 = 25 x 100

V1 = 25 x 100 /354 = 7.06µl

To 7.06µl of primer stock solution 92.94µl of TE buffer was added to make a 100µl of 25µM working stock solution.

Preparation of 100µl of 25µM of DR (Reverse)-biotin-labeled primer working stock solution

N1V1 = N2V2

Where: N1 = 302µM

V1 = ?

N2 = 25µM

V2 = 100µl

302 x V1 = 25 x 100

V1 = 25 x 100 / 302 = 8.27µl

To 8.27µl of primer stock solution, 91.73µl of TE buffer was added to make a 100µl of 25µM working solution.

Preparation of dNTPs

A 100µl of dNTP (Promega, WI) stock mix at a concentration of 10mM for each dNTP was prepared. 10µl of each of the dNTPs were mixed together with 60µl of TE buf

10µl of 100mM dATP

10µl of 100mM dCTP

10µl of 100mM dGTP

10µl of 100mM dTTP

60µl of TE buffer

The dNTP stock mix was stored at 10µl aliquots at -20°C until use.