MOLECULAR CHARACTERISATION OF *MYCOBACTERIUM TUBERCULOSIS* COMPLEX AND RISK FACTORS AMONG PATIENTS ATTENDING A NATIONAL TUBERCULOSIS CENTRE IN ZARIA, NIGERIA

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DEDICATION

To my father who showed me that the best legacy a father can give his child is education. Thank you ADA for you gave me much more. Looking forward to seeing you at the sound of the trumpet.

ABSTRACT

Tuberculosis is one of the most widespread infectious diseases and a leading cause of death among adults worldwide. Globally, Nigeria ranks fourth among high tuberculosis countries and first in Africa with an incidence of 300/100,000. *Mycobacterium tuberculosis* is the most common species causing human tuberculosis but other members of the *Mycobacterium tuberculosis* complex (MTC) are increasingly recognized as a cause of human infections. However, there is paucity of information on species of MTC causing tuberculosis in Nigeria. This study was aimed at characterising MTC species from sputum of tuberculosis patients and identifying risk factors for infection.

The study was conducted in the National Tuberculosis and Leprosy Training Centre, Zaria, Nigeria from April 2010 to June 2010. Case control study design involving consecutive selection of 102 smear-positive tuberculosis patients as cases and simple random selection of 102 smear-negative patients as controls was conducted. A structured interviewer administered questionnaire was used to collect demographics, document *HIV* status, clinical information and risk factors for tuberculosis such as previous contact with tuberculosis patients and cattle, and consumption of unpasteurized milk from 204 respondents. Species of MTC were identified using a multiplex Polymerase Chain Reaction (PCR) method based on genomic regions of difference (RD1, RD4, RD9 and RD12) and Insertion sequence 6110 targeting for 123bp and 245bp fragments. Data was analysed using descriptive statistics, Chi square test and logistic regression at 5% level of significance.

The mean age of the cases and controls were 36.2 ±9.0 and 35.8 ±9.7 years respectively. Majority, 70.6% of cases and 70.6% of controls were males. Fifty eight percent of cases

and 66 % of controls had at least secondary school education. Seventy seven percent of the cases and 81% of the controls reported receiving BCG immunization. Twenty one percent of the cases and 20% of the controls were *HIV* positive. All sputa from the smear positive respondents were positive for MTC. Ninety one (89.2%) of the sputa were indentified as *M. tuberculosis* while 11 (10.8%) were *M africanum*. Fifty percent and 17% of cases and controls respectively had history of contact with TB patients. Many of the cases (59.8%) and 53.9% of controls thought tuberculosis was air borne. Sixty four percent of the cases and 28% of the controls have lived in close contact with cattle. There was no statistical difference in knowledge of transmission of tuberculosis between cases and controls. Those who usually consume unpasteurized milk (AOR: 8.33, 95%CI=4.28-16.2), have had close contact with cattle (AOR: 4.42, 95%CI=2.45-7.98), and had previous contact with tuberculosis patients (AOR: 5.0, 95%CI= 2.61-9.57) were more likely to have tuberculosis.

Mycobacterium tuberculosis remains the major cause of human tuberculosis in Nigeria however, *Mycobacterium africanum* plays a significant role in the aetiology of tuberculosis. Previous contact with patients with tuberculosis, cattle or pets poses risk for infectious tuberculosis. Specific health messages tailored towards these risk factors are needed. Further studies to elucidate the transmission of tuberculosis between pets and humans are recommended.

Key words: Molecular characterisation, Mycobacterium Tuberculosis Complex, *Mycobacterium africanum*

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CERTIFICATION

We certify that this work was done by **Ndadilnasiya Endie Waziri** in the Department of Epidemiology, Medical Statistics and Environmental Health, University of Ibadan.

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CHAPTER ONE

INTRODUCTION

1.1 Background

Tuberculosis (TB) is an infectious and granulomatous disease caused by the acid-fast bacilli of the genus Mycobacterium (Clarke, 1998). It is one of the most widespread infectious diseases and a leading cause of death among adults worldwide with about 9 million new cases and 3 million deaths yearly (WHO, 2002). This chronic disease is characterised by progressive development of tubercles in tissues and organs of the body (Clarke, 1998). *Mycobacterium tuberculosis* is the most common cause of human tuberculosis but other members of the *Mycobacterium tuberculosis* complex (MTC) are increasingly recognised as a cause of human infections (Defra 2006, Cosivi *et. al.*, 1998, Grange 1996, O'Reily & Daborn 1995, Guillespie & Timoney 1983).

In Europe and the United States, improvement in public health led to a reduction in the burden of tuberculosis even before the arrival of specific drugs. Tuberculosis control program such as vaccination and milk pasteurization, reinforced by successful chemotherapy, resulted in a pronounced reduction of infection and death rates. Although, the disease became greatly controlled, it began to rise again from the mid 80s. Even though several inter-related forces drove this resurgence, the increase in population density in suburban areas including increase in prison populations, poverty (Gutierrez *et. al.*, 1998), homelessness, injection drug use, crowded housing and increased immigration from countries where tuberculosis continued to be endemic are some of the factors responsible for this increase (Martinez *et. al.*, 2007). Above all, the decline in tuberculosis control activities and the *human immunodeficiency virus* acquired

immunodeficiency syndrome (*HIV*/AIDS) epidemic. In resource poor nations, TB has remained a significant cause of morbidity and mortality even though it preventable and curable (Corbett *et al.*, 2003). Based on tuberculin reactivity, one- third of the world's population has been infected with MTC and is at risk of having the clinical disease later in life due to ageing or *HIV* co-infection (Lillebaek *et al.*, 2002).

The MTC comprises seven members which include *M. tuberculosis*, *M. bovis* which is the agent of bovine tuberculosis and also includes *M. bovis* bacillus Calmette-Guérin (BCG), *M. africanum* (Kallenius *et al.*, 1999), *M. caprae* primarily isolated from goats (Aranaz *et al.*, 2003), *M. pinnipedii* known as the seal bacillus (Cousins *et al.*, 2003, Zumarraga *et al.*, 1999), *M. microti* which is a pathogen of voles and rarely infects humans (Rastogi *et al.*, 2001, van Sooligen *et al.*, 1997) and *M. canettii*, a rare strain of MTC isolated from humans that have visited the horn of Africa (Pfyffer *et* al., 1998, van Sooligen *et al.*, 1997).

1.2 Statement of Research Problem

Tuberculosis is a major opportunistic disease in individuals living with human immunodeficiency virus infection. About 40% of people infected with AIDS have TB (Raviglione *et. al.*, 1995, Chretien 1990). The epidemic of *HIV* in developing countries, particularly countries in which the condition favour zoonotic transmission of TB could make TB a serious public health threat (Grange *et. al.*, 1994). The incidence of tuberculosis has been observed to be rising in many developing countries of the world especially in Asia and Africa (Asiak *et. al.*, 2007, Ameni *et. al.*, 2003, Cadmus *et. al.*, 1999, Collins 1993). The effect of high prevalence rates of *HIV*/AIDS in developing countries on the occurrence of tuberculosis in humans needs to be further researched.

1.3 Justification

Tuberculosis is occurring in increasing incidence in many parts of the world especially in Africa and Asia, where the standard of living is low, (Ameni *et. al.*, 2003, Cadmus *et. al.*, 1999, Collin 1993, Kochi, 1991). The increase in population density in suburban areas, poverty (Gutierrez *et. al.*, 1998), and the presence of *HIV* are some of the factors responsible for this increase (Martinez *et. al.*, 2007).

Nigeria has the fourth highest burden of human tuberculosis in the world. In 2007, incidence was 300 cases per 100,000 and tuberculosis-*HIV* co-infection rate was 27% (WHO, 2009). Cultural practices that could facilitate transmission of tuberculosis between cattle and humans such as pastoral communities living in close contact with their cattle and the lack of pasteurization of milk exist in Nigeria. In addition, there is little or no surveillance in Nigeria for the control of zoonotic tuberculosis (Cadmus *et.al*, 2006). There are also only a few studies which aimed at characterising the members of the MTC causing tuberculosis infection in Nigeria or the risk factors associated with tuberculosis. It is also important to identify species of strains of MTC for epidemiologic and public health considerations for prevention and control and also to optimize treatment especially in immunocompromised individuals (O'Reilly & Daborn, 1995). This will enable health providers plan and implement effective control measures in this environment.

1.4 Aim

To identify species of *Mycobacterium tuberculosis* complex causing tuberculosis and to determine the risk factors associated with tuberculosis in a referral Hospital in Zaria, Kaduna State.

1.5 Objectives

- To identify the species of MTC from the sputum of smear-positive tuberculosis patients in Zaria using polymerase chain reaction.
- To determine the risk factors for tuberculosis infection in sputum smear-positive patients.
- To determine the TB and *HIV* co-infection rate in the smear-positive patients.

1.6 Research question

What is the association between commonly known social and behavioural risk factors and being sputum smear-positive for persons attending National Tuberculosis and Leprosy Training Centre Hospital in Zaria?

1.7 Hypotheses

Having previous contact with a tuberculosis patient; consumption of unpasteurized milk and milk products; and living in close contact with livestock increases a person's risk of acquiring tuberculosis.

CHAPTER TWO

LITERATURE REVIEW

2.1 Tuberculosis

Tuberculosis is a chronic debilitating disease of man and animals caused by members of the MTC, a group of closely related species. Although the disease is preventable and curable, it has remained a significant cause of morbidity and mortality in resource poor nations (Corbett, *et al*., 2003). It is currently threatening to re-emerge in developed nations as well due to its synergy with *HIV*/AIDS, demographic changes and subsequent immigrations (Davies, 2003). Tuberculosis is one of the most widespread infectious diseases and is a leading cause of death among adults in the world (Cosivi *et* al., 1998). The disease is a major health problem with 8-9 million new cases occurring each year in the world and about 3 million deaths (WHO, 2002). The majority of these are in developing nations. Bases on tuberculin reactivity it is estimated that one third of the world's population has been infected with *Mycobacterium tuberculosis* complex (subspecies that includes *M. tuberculosis, M.bovis, M. africanum, M. microti and the attenuated M.bovis* bacillus Calmette-Guérin (BCG)). Infected individuals are at risk of presenting with disease later in life as their immunity wanes due to ageing or as a result of *HIV* co-infection (Huard *et al*., 2003). In some individuals, initial infection may progress rapidly to active tuberculosis. This is more common among infants, where the disease is often disseminated (e.g.miliary) or meningeal, and in the immunosuppressed, such as *HIV*-positive individuals (Heymann, 2004).

2.2 *Mycobacterium tuberculosis* complex (MTC)

Mycobacteria are gram-positive, slow growing, acid-fast and non-spore forming organisms. The MTC comprises seven members which include *M. tuberculosis*, the primary causative agent of human TB; *M. bovis*, which is responsible for bovine TB and includes the vaccine strain *M. bovis* bacillus Calmette-Guérin (BCG); *M. africanum*, the main causative agent of TB in West Africa (Kallenius *et al.*, 1999); *M. microti* which affects voles and rarely infects humans (Rastogi *et al.*, 2001); the seal bacillus *M. pinnipedii* (Zumarraga *et al.*, 1999; Cousins *et al.*, 1993); and *M. caprae*, primarily isolated from goats (Aranaz *et al.*, 1999). Members of the MTC are highly related mycobacteria exhibiting remarkable nucleotide sequence level homogeneity despite varying in pathogenicity, geographic range, certain physiological features (such as colony morphology as well as profiles of resistance and susceptibility to inhibitors), epidemiology and host preference (Huard *et al.*, 2003, Sreevatsan *et al.*, 1997). However, each of the MTC subspecies is known to infect humans (Horstkotte *et al.*, 2001, Viana-Niero *et al.*, 2001; O'Reilly & Daborn, 1995), and since most laboratories do not fully characterize species of MTC isolates, the true cause of tuberculosis in these patients and its source often remain undiscovered. Of important public health concern is the zoonotic transmission of some MTC strains from animals to humans and vice versa. In particular is the direct transmission of *M. bovis* from cattle to humans through the consumption of unpasteurized milk and *M. bovis* BCG infection of immunocompromised individuals (Huard *et al.*, 2003; O'Reilly & Daborn, 1995).

2.2.1 *Mycobacterium tuberculosis*

This is the predominant cause of human tuberculosis and thought to be the most successful human bacterial pathogen, yet the most poorly understood (Mustapha & Al-Attiyah, 2009; Mahairas *et al.*, 1996). Humans are the only reservoirs of *M. tuberculosis*, but it causes tuberculosis in cattle. This bacillus was first described by Robert Koch in 1888.

2.2.2 *Mycobacterium bovis*

Mycobacterium bovis is the agent of bovine tuberculosis, a mycobacterium very similar to *Mycobacterium tuberculosis*. Cattle is the main reservoir for *M. bovis*, though it affects a wide range of domestic and wild animals including, non-human primates, goats, sheep, cats, badgers, pigs, buffalos, deer and bison (O'Reily & Daborn, 1995). It has been observed that the risk factors for *M. bovis* infections in both animals and humans especially in African settings where domestic animals are an integral part of human social life are close contact with livestock, food hygiene practices like consumption of contaminated dairy and meat products and *HIV*/AIDS infection (Cosivi *et al.*, 1998; Grange, 1996; Guillespie & Timoney., 1983). In man, it is the most frequent cause of zoonotic tuberculosis, which is clinically indistinguishable from tuberculosis caused by *M. tuberculosis*. In many developed countries, bovine tuberculosis was eradicated 30-40 years ago by strong campaigns based tuberculin skin testing (TST) and mandatory slaughter of animals at the abattoirs/slaughterhouse. In these countries, human tuberculosis caused by *M. bovis* accounted for about 1 % of all TB cases. Some of these developed countries, including England and New Zealand, could not completely eliminate bovine tuberculosis, or worse, there is a re-emergence of the disease (Thoen *et*

al., 2006). On the other hand, in many low-income countries, bovine tuberculosis continues to be an important animal health problem (Palomino *et al.*, 2007).

2.2.3 The Bacille Calmette-Guérin vaccine

The Bacille Calmette-Guérin (BCG) is a live, attenuated vaccine derived from a virulent strain of *M. bovis*. Calmette and Guérin performed 230 in vitro passages of *M. bovis* until the organism lost its virulence (Calmette, 1927). While this strain has been used as a live attenuated vaccine, it may cause disease but only after vaccination with BCG. BCG is known to cause local reactions consistent with primary infection with an attenuated strain (i.e. a small localized ulcer and possible regional lymphadenopathy), and more severe reactions are thought to be rare. Deep ulcers, prolonged drainage, lymphadenitis (1%), abscess (2%) (Turnbull *et al.*, 2002), osteitis (0.04%) (Kroger *et al.*, 1995), and rarely disseminated infection have all been reported (Albot *et al.*, 1997). The age of the recipient and the dose of vaccine affect the incidence of local complications. Disseminated disease is thought to be rare, in the order of 1/1,000,000 doses and directly related to immune dysfunction (Turnbull *et al.*, 2002). The major worldwide concern about the risk of disseminated infection has been connected to the risk of *HIV*-related immunosuppression in the recipient. BCG is given routinely to newborns in many countries. However, this practice is under active review because of concerns that the vaccine's problems may outweigh its efficacy. Some authors recommend that BCG vaccination should be confined to groups of infants with a high risk of tuberculosis infection, and should be given at six months of age, in order to reduce severe disease and deaths among infants with immunodeficiency disorders (Romanus *et al.*, 1993). Because

of the significant risk of dissemination in immunocompromised patients, the place of BCG vaccination in tuberculosis control programs is being carefully re-assessed.

2.2.4 *Mycobacterium africanum*

This species is heterogeneous and has characteristics that appear to lie between *M. bovis* and *M. tuberculosis*. It was first isolated from a Senegalese patient suffering from pulmonary tuberculosis in 1968 (Castets *et al.*, 1968). *M. africanum* is predominantly isolated in Africa and, in certain areas of the continent. It is thought to produce a significant proportion of the cases of pulmonary tuberculosis (Frothingham 1999, Haas 1997). Reports on the sporadic isolation of *M. africanum* in Europe and the United States (Desmond *et al.*, 2004) have also been made, including one outbreak of multidrug-resistant (MDR) *M. africanum* (Schilke 1999). Based on biochemical characteristics, two major subgroups of *M. africanum* have been described, corresponding to their geographic origin in Western (subtype I) or Eastern (subtype II) Africa. Numerical analyses of biochemical characteristics revealed that *M. africanum* subtype I is more closely related to *M. bovis*, whereas subtype II more closely resembles *M. tuberculosis* (Niemann 2002, Sola *et al.*, 2003). *M. africanum* subtype II was classified by its resistance to thiophen-2-carboxylic acid hydrazide (TCH). It is the main cause of human tuberculosis in Kampala, Uganda (East Africa). Spoligotyping does not lead to a clear differentiation of *M. tuberculosis* and *M. africanum*, but all *M. africanum* subtype II isolates lack spacers 33 to 36, differentiating them from *M. africanum* subtype I (Brudey 2004, Mostowy *et al.*, 2004).

2.2.5 *Mycobacterium microti*

Mycobacterium microti was first isolated in1937 as the causative agent of pulmonary TB in the wild vole (*Microtus agrestis*) (Wells 1937). It was considered to be a-virulent for humans, cattle and laboratory animals and was therefore proposed as a live vaccine against tuberculosis. However, *M. microti* has been recently identified as the causative agent of pulmonary TB in both immunocompromised and immunocompetent humans (van Soolingen *et al.*, 1998, Horstkotte *et al.*, 2001). Genotypic analysis of *M. microti* showed the existence of two different variants of *M. microti*: vole and llama types. Three out of the first four *M. microti* isolates from humans were obtained from immunocompromised patients (two had undergone kidney transplantation; one was *HIV* infected). Two of the patients with *M. microti* infection had a history of contact with mice, which was found to be suggestive of zoonotic transmission (van Soolingen *et al.*, 1998, Brodin 2002). The first case of human infection with *M. microti* of the llama-type was reported in Germany: the patient was *HIV*-infected and presented with pulmonary tuberculosis (Horstkotte *et al.*, 2001). Recent data demonstrated that *M. microti* can cause severe pulmonary tuberculosis in immunocompetent patients (Niemann 2000a). *M. microti* has been isolated in Germany from two *HIV*-negative immunocompetent patients with pulmonary tuberculosis. According to spoligotype patterns, one of the isolates belonged to the llama type and the other to the vole type. These findings emphasize the relevance of *M. microti* as a pathogen in immunocompromised as well as immunocompetent patients. The prevalence and clinical importance of the different types of *M. microti* may have been underestimated so far because of difficulties with primary isolation and differentiation. Hence, further studies applying molecular methods are necessary to analyze the epidemiology of *M. microti* more thoroughly.

2.2.6 *Mycobacterium caprae*

This species was originally described as preferring goats to cattle as hosts (Aranaz 1996, Gutierrez *et al.*, 1998) and has been found in Spain, Austria (Prodinger *et al.*, 2002), France (Haddad 2001), Germany, Hungary, Italy and Slovenia (Erler *et al.*, 2004) and the Czech Republic (Pavlik *et al.*, 2003). In addition, *M. caprae* was isolated from humans and wildlife species such as red deer (Prodinger *et al.*, 2002) or wild boar (Erler *et al.*, 2004, Machackova *et al.*, 2003). In Central European regions, where *M. caprae* is the major cause of tuberculosis in cattle, it is also the predominant agent of tuberculosis in humans (Kubica *et al.*, 2003, Prodinger *et al.*, 2002). The major phenotypic difference between the caprine mycobacterial isolates and *M. bovis* is the sensitivity to pyrazinamide (PZA), which has been used as a major criterion for separation of *M. bovis* from the other members of *the M. tuberculosis* complex. Growth of *M. bovis* is not inhibited by PZA, while other *M. tuberculosis* complex species are susceptible to this antimycobacterial drug. However, *M. caprae* is similar to *M. bovis* in its preference for pyruvate for growth, which differentiates both species from other members of the MTC. *M. caprae* also has specific fingerprinting patterns obtained by IS*6110* RFLP, as well as a spoligotype pattern that is very different from those obtained for other members of the complex.

2.2.6 *Mycobacterium pinnipedii*

Mycobacterium pinnipedii was first isolated from captive and wild sea lions and fur seals from New Zealand and Australia (Cousins *et al.*, 2003, Cousins *et al.*, 1993). Similar organisms were subsequently recovered from the same mammal species in South America (Bastida 1999, Bernardelli 1996, Romano *et al.*, 1995) as well as from a Brazilian tapir (Cousins *et al.*, 2003). Recently, their ability to cause disease in guinea

pigs and rabbits has been demonstrated by experimental inoculation (Cousins *et al.*, 2003). This fact, together with the finding of a human isolate from a seal trainer, who worked in an affected colony in Australia (Thompson *et al.*, 1993), and a bovine isolate in New Zealand (Cousins *et al.*, 2003), suggests that *M. pinnipedii* can cause infection across a wide host range. Many of the isolates obtained in Australia, Uruguay, and Argentina have been well characterized (Alito 1999, Zumarraga *et al.*, 1999, Romano *et al.*, 1995, Bernardelli 1996, Romano *et al.*, 1995, Cousins *et al.*, 1993,). This information, together with preliminary tests on seal isolates from Great Britain and New Zealand, suggested that the seal bacillus (Cousins *et al.*, 1993), isolated from pinnipeds from all continents, might be a unique member of the *M. tuberculosis* complex. The results of biochemical tests clearly confirmed that the seal isolates belong to the *Mycobacterium tuberculosis* complex. Most seal isolates grow preferentially on media that contained sodium pyruvate, although some also grew on Löwenstein–Jensen medium containing glycerol. Isolates inoculated into guinea pigs produced significant lesions or death within six weeks and those inoculated into rabbits caused death within six weeks, confirming that the isolates were fully virulent for both laboratory animals.

2.2.7 *Mycobacterium canettii*

In an attempt to characterize an unusual mycobacterial strain isolated from a 2-year old Somali patient with lymphandenitis, van Soolingen *et al.* (1997) applied various molecular methods to a bacillus that produces smooth and glossy colonies. In a different study Milkgen *et al.* (2002) also indentified an unusual strain of mycobacteria from two patients with pulmonary tuberculosis by its smooth, glossy appearance and primarily its genotypic characteristics. This was later typed as *M. canettii* by spoligotyping and

IS6110. All known cases of tuberculosis caused by *M. canettii* have been contracted from the horn of Africa (Pfyffer *et al., 1998*). The prevalence of *M. canettii* isolates may be underestimated in routine microbiology laboratory due to their close similarities with *M. tuberculosis* particularly as the smooth colony type easily reverts to give a stable rough colony type in *M. canettii.*

2.3 Identification of species within the *M. tuberculosis* complex

The high degree of sequence conservation among members of the MTC makes differentiation of species in the clinical mycobacteriology laboratory a difficult task. Routine differentiation is still based on phenotypic characteristics, such as oxygen preference, niacin accumulation, nitrate reductase activity and colony morphology. *Mycobacterium tuberculosis* is the most frequent cause of human tuberculosis, but some cases are caused by *M. bovis*. It is necessary to differentiate between *M. bovis* and *M. tuberculosis* in order to know the prevalence and distribution of human tuberculosis due to *M. bovis* (Palomino *et al.*, 2007). This may contribute to knowledge about the risk factors associated with the transmission of *M. bovis* to the human population. *M. bovis* differs from *M. tuberculosis* in having a low growth rate on egg media supplemented with glycerol, but a faster growth on egg media supplemented with pyruvate (Stonebrink medium). Several molecular techniques were designed to differentiate *M. tuberculosis* complex, including methods to detect mutations in *pncA* and *oxyR* genes (Scorpio *et al*., 1997), *mtp40*-PCR (Liebana *et al.*, 1997, Del Portillo 1991), and PCR-amplification of regions of difference (RD) (Huard *et al.*, 2003, Parsons *et al.*, 2002), among others. Some techniques are useful for the differentiation of *M. tuberculosis* and *M. bovis*, such as *pncA* and *oxyR*. A species specific mycobacterial DNA element in the *M. tuberculosis*

complex has been described by Del Portillo (1991), the *M. tuberculosis mpt40* protein fragment which was originally described as being produced only by *M. tuberculosis*. Now, it is well know that this protein is encoded by the *plcA* gene, contained in RD5. This region is present in most, but not all, isolates of *M. tuberculosis*, *M. africanum*, *M. pinnipedii*, and *M. microti*, and is consistently absent from *M. bovis* and *M. bovis* BCG isolates. Given the high polymorphism in this region, the use of the *mpt40* sequence as a genetic marker for *M. tuberculosis* is very restricted (Viana-Niero *et al.*, 2004).

Spoligotyping is also used for differentiation of members of the *M. tuberculosis* complex (Kamerbeek 1997). For instance, the spoligotypes of "modern" *M. tuberculosis* strains typically lack spacer sequences 33-36 in the direct repeat (DR) region. Similarly, *M. bovis* and *M. caprae* strains are known to lack spacers 3, 9, and 16. All *M. bovis*, *M. caprae*, and *M. microti* strains are known to lack spacers 39 to 43 in their spoligotypes (Zumarraga *et al.*, 1999). It should also be noted that all MTC organisms along the *M. africanum* type I to *M. bovis* evolutionary track lack spacers 9 and 39. Therefore, spacers 9 and 39 are potential markers for the differentiation of *M. tuberculosis* from the remaining *M. tuberculosis* complex species by spoligotyping. Although their absence has been noted in *M. africanum* subtype I isolates, they are present in *M. africanum* subtype II. RD analysis is currently used for differentiation between species of the *M. tuberculosis* complex. TbD1 is a deletion found only in *M. tuberculosis*, all other *M. tuberculosis* complex strains, including some *M. tuberculosis* have TbD1. Based on the presence or absence of this *M. tuberculosis*-specific deletion 1 (TbD1). TbD1 is always absent in *M. africanum* type II strains. Previously, based on *katG* codon 463 (*katG*463) and *gyrA* codon 95 (*gyrA*95) sequence polymorphisms, Sreevatsan *et al.* (1996 and

1997) defined three groups among the tubercle bacilli: group 1 with *katG*463 CTG (Leu), *gyrA*95 ACC (Thr); group 2 with *katG*463 CGG (Arg), *gyrA*95 ACC (Thr); and group 3 with *katG*463 CGG (Arg), *gyrA*95 AGC (Ser). *M. tuberculosis* organisms belonging to group 1 have *katG* and *gyrA* sequences indistinguishable from those of *M. microti*, *M. africanum* and *M. bovis*.

Mycobacterium tuberculosis strains containing the TbD1 region belong to group 1, and are considered ancestral strains. However, *M. tuberculosis* with TbD1 deletion can also be in group 1, although most strains presenting TbD1 deletion belong to groups 2 and 3, groups 2 and 3 lack TbD1. Furthermore, a subsequent loss of DNA, reflected by the deletion of DR9 was identified for an evolutionary lineage that diverged from the progenitor *M. tuberculosis* strains. It is represented by *M. africanum*, *M. microti*, *M. caprae*, *M. pinnipedii*, and *M. bovis* (Brosh 2002). Thus, RD9 allows differentiation between *M. tuberculosis* and the other strains of the *M. tuberculosis* complex. Other regions of difference, such as RD7, also allow differentiation between *M. tuberculosis* and the other species. RD7 deletion was observed in *M. bovis*, *M. microti*, some *M. africanum*, and *M. pinnipedii*.

M. bovis BCG strains possess a specific polymorphism – the RD1 deletion. This deletion allows the differentiation between BCG and all the other species of the *M. tuberculosis* complex. *M. pinnipedii* and *M. microti* are very closely related microorganisms.

Human beings can be infected by *M. caprae* or *M. bovis* from infected livestock, and infection with both species remains a serious public health problem in some countries. Differentiation of these species is important for epidemiological reasons (Brosh 2002).

2.4 Epidemiology of Tuberculosis

Worldwide, one person out of three is infected with *Mycobacterium tuberculosis* – two billion people in total. Tuberculosis accounts for 2.5 % of the global burden of disease and is the commonest cause of death in young women, killing more women than all causes of maternal mortality combined. Despite projected decline in disease burden due to infectious agents, tuberculosis ranks seventh among the global causes of death in adults and is likely to maintain that position through to 2020 unless intensive efforts are made to prevent and control the disease (Smith *et al.*, 2004; Dye *et al.*, 1999). Every 15 seconds, someone in the world dies from tuberculosis despite availability of effective drugs to treat and cure the disease for over 50 years. More alarming is the fact that a person is newly infected with tuberculosis every second of every day and a person with active tuberculosis can infect an average of 10 to 15 other persons every year (Dye *et al.*, 2005).

In most countries, more cases of TB are reported among men than women. This is partly due to the fact that women have less access to diagnostic facilities in some settings, even though it also reflects real epidemiological differences both in exposure to infection and in susceptibility to disease between males and females (Ernesto & Rodriguez, 2007). In regions where transmission of tuberculosis has been stable or on the increase, the incidence rate is highest among young adults and most cases are caused by recent infection or re-infection. As transmission falls, the case-load shifts to the older age groups and a higher proportion of cases come from the reactivation of latent infection (Borgdoff 2000).

While the human immunodeficiency virus (*HIV*) infection has clearly had a profound effect on the epidemiology of tuberculosis, other potentially important risk factors have

been somewhat neglected. In the coming years, more attention needs to be given to the interaction between chronic diseases and tuberculosis, including diabetes, under-nutrition, and respiratory illnesses caused by tobacco and air pollution (Corbett *et al.*, 2003; World Health Organization 2004).

Global efforts to control tuberculosis were reinvigorated in 1991, when a World Health Assembly resolution recognized tuberculosis as a major global public health problem. Two targets for tuberculosis control were established as part of this resolution – detection of 70 % of new AFB smear-positive cases, and cure of 85 % of such cases by the year 2000. Despite intensified efforts, these targets were not met; more than 80% of known cases are successfully treated, but only 45 % of cases are detected (WHO 2006a, WHO 1994, WHO 1993).

Tuberculosis accounts for 2.5% of the global burden of disease and is the most common cause of death in young women. Despite projected decline in disease burden due to infectious agents, tuberculosis ranks seventh among the global causes of death and is likely to maintain that position through to 2020 unless intensive efforts are made (Smith *et al.*, 2004; Dye *et al.*, 1999).

The World Health Organization (WHO) reported 8.9 million new cases of tuberculosis worldwide in 2004 (140/100,000 population), about 3.9 million of these cases (62/100,000) were acid fast bacilli (AFB) sputum smear-positive, the most infectious form of the disease. There were 14.6 million prevalent cases (229/100,000), of which 6.1 million were AFB sputum smear-positive (95/100,000). An estimated 1.7 million people (27/100,000) died from tuberculosis in 2004, including those co-infected with *HIV* (248,000). The WHO African region has the highest estimated incidence rate

(356/100,000), but the majority of patients with tuberculosis live in the most populous countries of Asia; Bangladesh, China, India, Indonesia, and Pakistan which together account for half (48 %) of the new cases that arise every year. About 80 percent of new cases of tuberculosis occur in the 22 top-ranking countries (Dye 2006, WHO 2006a).

In 2004, the estimated tuberculosis incidence per capita was stable or falling in five out of six WHO regions, even though it was still growing at 0.6 % per year globally. The African region is the only exception African region, where the incidence of tuberculosis was still rising, due to the spread of *HIV*. However, the rate of increase in the number of cases notified from the African region is slowly decreasing each year, probably because the *HIV* epidemic in African countries is being contained. In Eastern Europe (mostly countries of the former Soviet Union), the incidence per capita of tuberculosis increased during the '90s, peaked around 2001, and has since declined. The average downturn in case notifications in Eastern Europe is mainly due to data from Russia and the Baltic States of Estonia, Latvia, and Lithuania; however, incidence rates might still be increasing in the central Asian republics of Tajikistan and Uzbekistan (Dye 2006, WHO 2006a). In all other regions, the incidence rate was stable or decreasing between 1990 and 2003. The decline was relatively quick in Latin America, Central Europe and the established market economies. In summary, the global trend in incidence rate was increasing most quickly at 1.5 % per year in 1995 but has since been decelerating. If the trends suggested by the case notifications are correct, and if these trends persist, the global incidence rate will reach about 150 per 100,000 in 2015, resulting in more than 10 million new cases in that year (Dye 2006, WHO 2006b, WHO 2006a).

There are 22 high-burden countries, which account for approximately 80% of the estimated number of new tuberculosis cases (all forms) arising worldwide each year. These countries (Table 1) are the focus of intensified efforts in Directly Observed Treatment, Short-course (DOTS) expansion. The high-burden countries are not necessarily those with the highest incidence rates per capita; many of the latter are medium-sized African countries with high rates of TB/*HIV* co-infection (Dye 2006, WHO 2006a; WHO 2006 b). Tuberculosis death rates in these high-burden countries varied from 9 per 100,000 populations in Brazil to 139 per 100,000 in South Africa. In these two countries, the overall case fatality rates for tuberculosis were 13% and 27%, respectively, and the difference was due largely to the difference in *HIV* infection rates (Dye 2006, WHO 2006a).

Since 2000, the United Nations Millennium Development Goals have provided a framework for evaluating implementation and impact under target 8 (among 18), which is to "*have halted by 2015 and begun to reverse the incidence of malaria and other major diseases*" (including tuberculosis). Although the objective is expressed in terms of incidence, it also specifies that progress be measured in terms of the reduction in tuberculosis prevalence and deaths. The target for these two indicators, based on a resolution passed at the 2000 Okinawa (Japan) summit of Great Eight (G8) industrialized nations, and subsequently adopted by the Stop TB Partnership, is to halve tuberculosis prevalence and death rates between 1990 and 2015 (evolution TB control). These additional targets are much more of a challenge in Africa and Eastern Europe where prevalence of the disease is high (United Nations Statistics Division 2006, WHO 2005b, WHO 2000).

able 2.1 Estimated TB burden in High TB in High TB countries

igh burden untries	Incidence		Prevalence, all forms per 100,000 pop	Mortality, all forms per 100,000 pop	*HIV* prevalence in incident TB cases %
	All forms per 100,000 pop	Smear positive per 100,000 pop			
ia	168	75	312	30	5.2
na	101	46	221	17	0.9
nesia	245	110	275	46	0.9
eria	290	125	531	82	27
h Africa	718	293	670	135	60
ladesh	229	103	435	51	0.1
tan	181	81	329	40	0.6
pia	353	154	533	79	21
pines	293	132	463	48	0.1
	619	266	888	133	29
ngo	366	159	551	79	21
n Fed.	115	51	160	21	6.8
am	176	79	232	22	3.0
zania	347	147	479	78	36
	402	175	646	92	19
	60	26	77	7.8	17
lan	333	150	661	92	0.0
	142	63	208	19	8.5
ue	460	191	635	129	48
	674	271	673	151	68
	171	76	180	21	7.1
	510	226	709	94	13

Report, 2004

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High burden countries	Incidence		Prevalence, all forms per 100,000 pop	Mortality, all forms per 100,000 pop	*HIV* prevalence in incident TB cases %
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India	168	75	312	30	5.2
China	101	46	221	17	0.9
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Nigeria	290	125	531	82	27
South Africa	718	293	670	135	60
Bangladesh	229	103	435	51	0.1
Pakistan	181	81	329	40	0.6
Ethiopia	353	154	533	79	21
Philippines	293	132	463	48	0.1
Kenya	619	266	888	133	29
DR Congo	366	159	551	79	21
Russian Fed.	115	51	160	21	6.8
Viet Nam	176	79	232	22	3.0
UR Tanzania	347	147	479	78	36
Uganda	402	175	646	92	19
Brazil	60	26	77	7.8	17
Afghanistan	333	150	661	92	0.0
Thailand	142	63	208	19	8.5
Mozambique	460	191	635	129	48
Zimbabwe	674	271	673	151	68
Myanmar	171	76	180	21	7.1
Cambodia	510	226	709	94	13

Source: WHO Report. 2004

2.5 Epidemiology of Tuberculosis and *HIV* co-infection

Tuberculosis is a major opportunistic infection in *HIV*-infected persons, the two diseases forming a lethal combination and each speeding the other's progress (Raviglione *et* al., 1995). *HIV* infection is a potent risk factor for tuberculosis; not only does *HIV* increase the risk of reactivating latent tuberculosis infection, it also increases the risk of rapid tuberculosis progression soon after infection or re-infection. In persons infected with the *Mycobacteria* only, the lifetime risk of developing tuberculosis ranges between 10 % and 20 %. In persons co-infected with *Mycobacteria* and *HIV*, however, the annual risk can rise up to 50% (Ernesto & Rodriguez 2007). According to WHO global estimates, of the 9.4 million people infected with both *HIV* and tuberculosis in mid-1996, 6.6 million (70%) live in sub-Saharan Africa (WHO 2004). The greatest impact of *HIV* infection on tuberculosis is in among young adults. The occurrence of both infections in an individual makes tuberculosis infection very likely to progress to active disease.

HIV pandemic presents a massive challenge for global tuberculosis control. The prevention of *HIV* and tuberculosis, the extension of WHO DOTS programs, and a focused effort to control *HIV*-related tuberculosis in areas of high *HIV* prevalence are matters of great urgency (WHO 2006a, Aaron 2004, WHO 2002a,b). *HIV* infected patients contract less of pulmonary tuberculosis and thus harder to detect by sputum microscopy which is one of the cornerstones for diagnosis in resource limited settings, where *HIV*-TB co-infection are actually high (Godfrey-Faussett & Ayles 2003).

The tuberculosis burden in countries with a generalized *HIV*/AIDS epidemic has increased rapidly over the past decade, especially in the severely affected countries of

eastern and southern Africa due to high *HIV*/TB co infection rates. Tuberculosis is one of the most common causes of morbidity and the most common cause of death in *HIV*-positive adults living in less-developed countries, yet it is a preventable and treatable disease (WHO 2006b, Aaron *et al.*, 2004, Corbett *et al.*, 2003).

In addition to increasing individual susceptibility to tuberculosis, a high burden of *HIV*-associated tuberculosis cases also expands transmission rates of tuberculosis at the community level, threatening the health and survival of *HIV*-negative individuals as well. In several countries, *HIV* has been associated with epidemic outbreaks of tuberculosis. Many of the reported outbreaks involved multidrug-resistant (MDR) strains, which respond poorly to standard therapy (WHO 2006b; WHO 2006a, Aaron *et al.*, 2004, Corbett *et al.*, 2003).

According to Corbett *et al.*, (2003), an estimated 8.3 million new tuberculosis cases were reported in 2000 worldwide, and 9% of all new tuberculosis cases in adults (aged 15-49 years) were attributable to *HIV* infection. In the WHO African Region and the United States of America, 31% and 26% respectively of all new tuberculosis cases in adults were attributable to *HIV* infection. In these regions, there was an estimated 1.8 million deaths from tuberculosis, of which 12 % were attributable to *HIV*. In turn, tuberculosis was the cause of 11 % of all adult AIDS deaths. The worldwide prevalence of TB-*HIV* co-infection in adults was 0.36 % (11 million people). Co-infection prevalence rates equaled or exceeded 5 % in eight African countries. In South Africa alone, there were 2 million co-infected adults (Corbett *et al.*, 2004; Corbett *et al.*, 2003).

Other studies have reported that much of the observed increase in the incidence of global tuberculosis since 1980 is attributable to the spread of *HIV* in Africa. Globally, an

estimated 13% of adults with newly diagnosed tuberculosis patient were infected with *HIV* in 2004, but there was great variation among regions — from 34% in the African region to 1.4% in the Western Pacific region. Rates of *HIV* infection in patients with tuberculosis have so far remained below 1% in Bangladesh, China, Indonesia, and Pakistan. In African populations with high rates of *HIV* infection, a relatively high proportion of patients with tuberculosis are women aged between 15 and 24 years. *HIV* has probably had a smaller effect on prevalence of tuberculosis than on incidence because the virus significantly reduces the life expectancy of patients with tuberculosis (Dye 2006, Dye *et al.*, 2005, Asamoah- Odei *et al.*, 2004). In regions where *HIV* infection rates are high in the general population, they are also high among patients with tuberculosis; estimates for 2004 exceeded 50 % in Botswana, South Africa, Zambia, and Zimbabwe, among other countries (WHO 2006a, Dye 2006, Dye *et al.*, 2005, Corbett *et al.*, 2003).

The survival rate of *HIV*-positive tuberculosis patients varies according to acid fast bacilli (AFB) smear status and treatment regimen. Survival is generally higher for AFB smear-positive than for smear-negative patients, and it is lowest with rifampicin-based treatment regimens (WHO 2006a, Dye 2006, Dye *et al.*, 2005, Corbett *et al.*, 2003).

Mycobacterium bovis has been isolated from *HIV*-infected persons in industrialized countries. In France, *M. bovis* infection accounted for 1.6% of tuberculosis cases in *HIV*-positive patients. All isolated strains were resistant to isoniazid (Dupon & Ragnaud 1992). Taking into consideration the intrinsic resistance of *M. bovis* to pyrazinamide, two of the first-line anti-tuberculosis drugs were not effective. WHO-recommended standard treatment for new tuberculosis cases includes, in the initial phase, isoniazid,

rifampicin, pyrazinamide, and streptomycin or ethambutol. In situations of high primary resistance to isoniazid and streptomycin, the intrinsic resistance of *M. bovis* to pyrazinamide may severely limit the efficacy of treatment of tuberculosis caused by *M. bovis*.

2.6 Economic Impact of Tuberculosis

Tuberculosis hinders socioeconomic development of many countries as over 75% of people with tuberculosis are within the economically productive age group of 15-54 years (WHO 2006a, Dye 2006, Ernesto & Rodriguez 2007). Although the direct costs of diagnosis and treatment are significant for poor families, the greatest economic loss occurs as a result of indirect cost, such as loss of employment, travel to health facilities, sale of assets to pay for treatment- related costs, and in particular, loss of productivity from illness and premature death, the later being an intangible cost (WHO 2005a, Smith *et al.*, 2004, Floyd 2003). Zoonotic tuberculosis can cause severe economic losses due to loss of productivity of farm animals and deaths of livestock. In addition, the presence of infection in wildlife populations poses a threat to the survival of endangered wildlife species.

2.7 Treatment of Tuberculosis

The history of tuberculosis changed dramatically after the introduction of anti-mycobacterial agents. Drug treatment is fundamental for controlling tuberculosis, promoting the cure of the patients and breaking the chain of transmission when the anti-tuberculosis drug regimen is completely and correctly followed (da Silva & Ainsa 2007). Antituberculosis drug treatment started in 1944, when streptomycin (SM) and paraaminosalicylic acid (PAS) were discovered. In 1950, the first trial was performed

which compared the efficacy of SM and PAS both as monotherapy or combined. The study demonstrated that combined therapy was more effective and resulted in the first multidrug antituberculosis treatment that consisted of a long course of both drugs. In 1952, a third drug, isoniazid (INH), was added to the previous combination, greatly improving the efficacy of treatment, but which still had to be administered for 18-24 months. In 1960, ethambutol (EMB) substituted PAS, and the treatment course was reduced to 18 months. In the '70s, with the introduction of rifampicin (RIF) into the combination, treatment was shortened to just nine months (da Silva & Ainsa 2007). Finally, in 1980, pyrazinamide (PZA) was introduced into the antituberculosis treatment, which could be reduced further to only six months. Two biological features explain why combined drug therapy is more effective at curing tuberculosis than monotherapy. One is that treatment of active tuberculosis with a single drug results in the selection of drug resistant bacilli and failure to eliminate the disease. The other is that different populations of tubercle bacilli – each of them showing a distinct pattern of susceptibility for anti-tuberculosis drugs – may co-exist in a tuberculosis patient (Shamputa *et al.*, 2006).

Antituberculosis treatment has two main objectives (Onyebujoh *et al.*, 2005). First, to rapidly kill those bacilli living extracellularly in lung cavities, which are metabolically active and are dividing continuously; this is required in order to attain the negativization of sputum and therefore to prevent further transmission of the disease. Second, to achieve complete sterilization and elimination of those bacilli replicating less actively in acidic and anoxic closed lesions, and to kill semi-dormant bacilli living intracellularly in other host tissues, otherwise these bacilli may persist and will be responsible for

subsequent tuberculosis relapses. INH is the drug with the highest activity against rapidly dividing bacilli, whereas RIF and PZA have the greatest sterilizing activity against bacteria that are not dividing. These reasons, along with the prevention of drug resistance, support the use of a combination therapy for the treatment of tuberculosis. Drugs for treating tuberculosis are usually classified as first- and second-line drugs. Traditionally, there are five first-line drugs: INH, RIF, PZA, EMB, and SM. Second-line drugs include the aminoglycosides kanamycin and amikacin, the polypeptide capreomycin, PAS, cycloserine, the thioamides ethionamide and prothionamide and several fluoroquinolones such as moxifloxacin, levofloxacin and gatifloxacin. Some reports, however, include SM among the second-line drugs, since its use has declined in recent years, due to the high rates of resistance, and also, because other more effective drugs have been incorporated into the anti-tuberculosis treatment (da Silva & Ainsa 2007; Onyebujoh *et* al. 2005).

Similarly, new drugs such as the rifamycin derivatives rifapentine and rifabutin can be considered among the first-line drugs, and in the near future, it is quite likely that some fluoroquinolones could be incorporated into the standard antituberculosis treatment, thus being considered as first-line drugs. The current short-course treatment for the complete elimination of active and dormant bacilli involves two phases (da Silva & Ainsa 2007):

2.7.1 Initial phase: three or more drugs (usually isoniazid, rifampicin, pyrazinamide and ethambutol or streptomycin) are used for two months, and allow a rapid killing of actively dividing bacteria, resulting in the negativization of sputum.

2.7.2 Continuation phase: fewer drugs (usually isoniazid and rifampicin) are used for 4 to 7 months, aimed at killing any remaining or dormant bacilli and preventing recurrence.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

The study was conducted in the National Tuberculosis and Leprosy Training Centre, Zaria, Kaduna State. Zaria is the second largest city in Kaduna State. It is located in the northern part of the State. The hospital is a referral hospital for tuberculosis. Most referrals are from the North-West geopolitical zone of the country. The outpatient unit see approximately 200 patients per hospital day which is usually twice a week and patients can be admitted directly to an onsite ward for hospital care.

3.2 Study Design

The study design was a hospital based case control study. Cases were defined as any newly diagnosed sputum smear-positive patient diagnosed for the first time at the hospital from 1st April 2010 to 3rd July 2010. Cases were confirmed by two consecutive sputum smears positive for acid-fast bacilli. Cases diagnosed before 1st of April 2010, whether partially treated or relapsed were excluded. This was done to ensure that cases were not on any anti tuberculosis treatment which might mask the laboratory results. Cases were recruited consecutively. Controls were both clinically negative and sputum smear-negative patients seen at the hospital for other reasons during the same period of time. For each case, one control was recruited.

Figure 3.1 Map of Nigeria showing location of Kaduna State

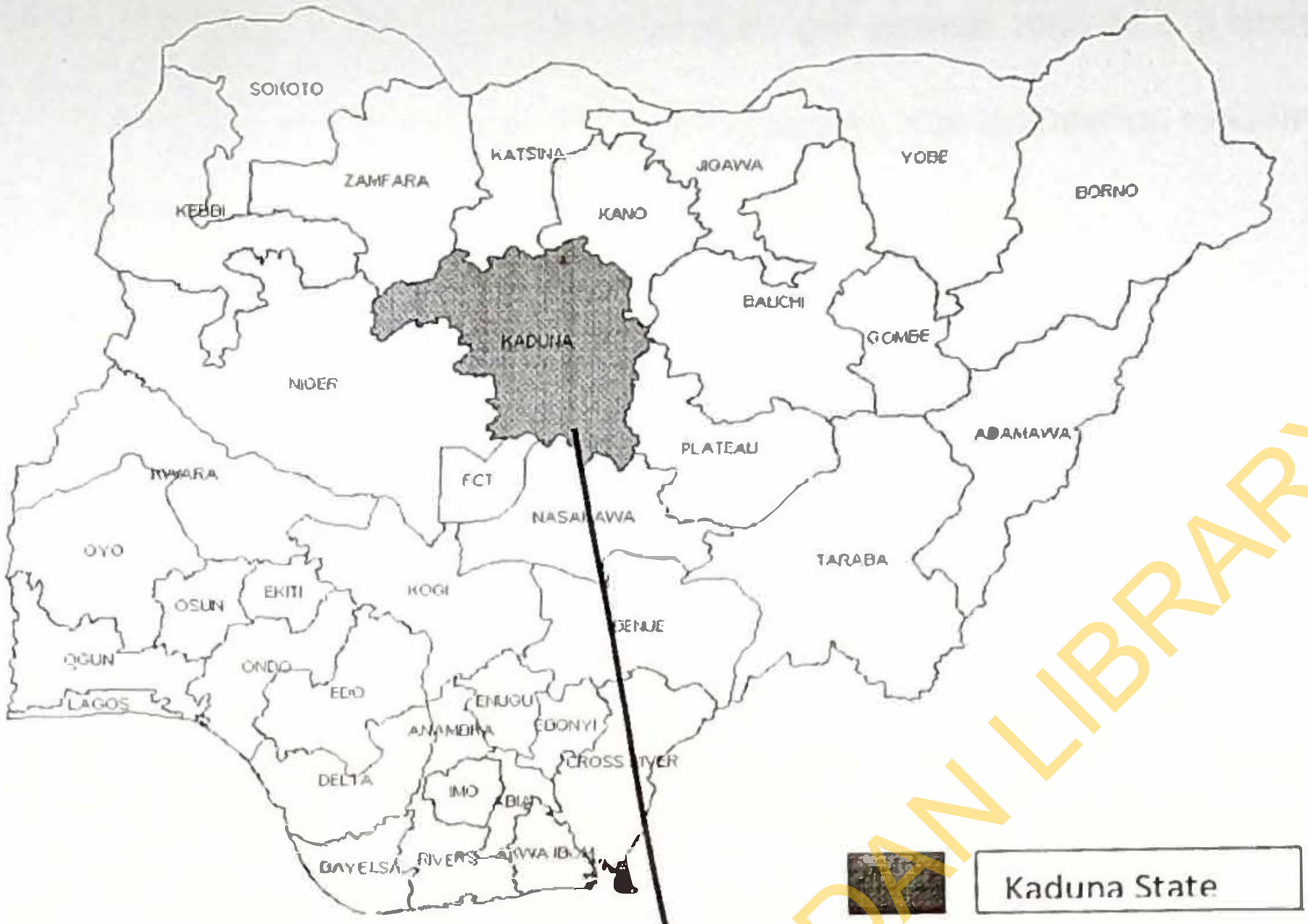


Figure 3.2 Map of Kaduna State showing location of Zaria

3.3 Sample Size

At 95.00% confidence interval, a power of 80.00% and an odds ratio of 3, a sample size of 112 cases and 112 controls was arrived at using sample size calculation module of Epi Info 3.3.1 software.

3.4 Ethical Issues

A consent form was included in the questionnaire. Only patients who gave informed consent were included in the study. Confidentiality of collected data is maintained and data collected is stored in a password protected computer.

3.5 Sputum collection

All sputum specimens collected were processed in the Molecular biology laboratory of National Veterinary Research Institute, Vom, Nigeria, to confirm for presence of MTC members using a Polymerase Chain Reaction (PCR) method based on primers that amplify segments of the IS*6110* element, particularly targeting for the 123 bp and 245 bp fragments, and JB21/JB22 focused on the amplification of a 500 bp DNA fragment in *M. bovis* (Figueiredo *et al.*, 2009), and PCR amplification of genomic region of difference (Warren *et al.*, 2006). All patients from whom sputum was taken were interviewed to obtain information on relevant risk factors (such as history of contact with cattle, consumption of unpasteurized milk, having previous contact with a tuberculosis patient and having a pet at home) for tuberculosis.

3.6 Sputum Preparation

Specimens were homogenized by digestion for one minute at room temperature with 1ml of 25mg/ml NALC (*N*-acetyl-L-cysteine) in phosphate buffer (pH 6.8), vortexed with several 4mm glass beads for 30 seconds and then decontaminated using 1% NaOH and

concentrated at 4000xg for 15 minutes. Sediments were reconstituted to 5mls, irrespective of the original sample volume using phosphate buffer pH 6.8 (Abbadi *et al.* 2009).

3.7 DNA Purification

DNA from sputa was purified as described by Huard *et al.*, (2003). Twenty microlitres (20µl) of Proteinase K was put into a 1.5 ml micro centrifuge tube and 200µl of sputum each were added in separate tubes and spun down, re-suspended in 1 ml of Tris-EDTA buffer (10 mM Tris-Cl [pH 8.0], 1 mM EDTA), and transferred to a 2-ml Eppendorf tube. Lysozyme solution (100 µl; 10 mg/ml in Tris-EDTA buffer) and five 3-mm-diameter glass beads were then added, vortexed, sonicated for 10 minutes, vortexed again, and then incubated at 37°C for 2 hours with brief vortexing every 30 minutes. To the resultant suspension, after water bath sonication for 10 minutes and division into two 1.5-ml Eppendorf tubes, was added 70 µl of 10% sodium dodecyl sulfate and 10 µl of proteinase K (10 mg/ml). The mixture was then vortexed and incubated for 2 hours at 65°C, with brief vortexing every 30 minutes. Afterwards, 100 µl of 5 M NaCl was added and vortexed, and following the addition of 80 µl of 10% hexadecyltrimethyl ammonium bromide (Sigma, St. Louis, Mo.) in pure water, the mixture was incubated at 65°C for 30 minutes. For DNA extraction 750 µl of chloroform was added, mixed well, and centrifuged at 14,000 rpm for 5 minutes in a Microfuge. The resultant upper phase was transferred to a clean tube with 420 µl of isopropanol and mixed gently. The tubes were then cooled on ice and spun in a Microfuge for 30 minutes at 14,000 rpm and 4°C. Following removal of the supernatant, the DNA pellet was washed with 75% ethanol

and air dried. The DNA was then re-suspended in RNase- and DNase-free water, quantified, diluted to 50 to 500µg/ml, and used for PCR (Huard *et al.* 2003).

3.8 PCR Amplification

3.8.1 Multiplex

Purified genomic DNA from all the samples were subject to a PCR using a mixture of four primers, aiming to identify bacteria as MTC members (INS1 and INS2) as well as to distinguish *M. bovis* isolates (JB 21 and JB 22) from other members of this complex. The multiplex PCR was performed in a 50 µl reaction mix containing 5 µl of 10 X PCR buffer (Qiagen®), 200 µM dNTPs (AppliedBiosystems®), 2.5 U of recombinant *Taq* polymerase (Qiagen®), 0.2 µM of each primer JB21, JB22, INS1 and INS2 (Inqaba®) (Table 2) (Figueiredo *et al* 2009), 2.0 mM $MgCl_2$, 5 µl of purified DNA template and the reaction made up to 50µl with nuclease free water. Amplification was carried out in a GeneAmp PCR System 9600 (Applied Biosystems®) using the following cycling parameters: initial denaturation at 94°C for 5 min. followed by 30 cycles of 94°C for 1 min, 68°C for 1 min and 72°C for 1 min, with a final extension step at 72°C for 7 min. PCR products were analyzed by electrophoresis on 1.5% agarose gels stained with ethidium bromide (10 µg/ml). DNA bands were visualized and captured by ChemiGenius BioImaging System (Syngene®).

3.8.2 MTB Genomic Region of Difference

Each PCR reaction contained 1µl DNA template, 5µl Q-buffer (Qiagen®), 2.5µl 10x PCR buffer (Qiagen®), 2µl of 25 mM $MgCl_2$, 4µl 10 mM dNTPs, 0.5µl of each primer (50 pmol/µl) (Inqaba®) (Warren et al. 2006), 0.25 µl HotStarTaq DNA polymerase (Qiagen®, Hilden, Germany) and was made up to 25µl with Nuclease free water (Promega®). Amplification was initiated by incubation at 95°C for 15 minutes, followed by 45 cycles at 94°C for 1 minute, 62°C for 1 minute, and 72°C for 1 minute. After the last cycle, the samples were incubated at 72°C for 10 minutes. PCR amplicons were electrophoresed through 2.0% agarose in 1xTBE pH 8.3 supplemented with 50µg ethidium bromide, at 120V for 90minutes. DNA bands were visualized and captured by ChemiGenius BioImaging System (Syngene®). Primer sequence used is shown in table 3.

Table 3.1 Oligonucleotide primers for multiplex PCR

Primer	Sequence (5'-3')
JB21	5'-TCGTCCGCTGATGCAAGTGC- 3'
JB22	5'-CGTCCGCTGACCTC AAGAAAG-3'
INS1	5'-CGTGAGGGCATCGAGGTGGC-3'
INS2	5'- GCGTAGGCGTCGGTGACAAA-3'

Table 3.2 PCR primer sequence & amplification product sizes in different MTC members

Primer sequence	RD	*M. canettii*	*M. tuberculosis*	*M. africanum*	*M. bovis*	*M. bovis* BCG
AAGCGGTTGCCG CCGACCGACC	1	RD1 present (146 bp)	RD1 present (146 bp)	RD1 present (146 bp)	RD1 present (146 bp)	RD1 absent (196 bp)
CTGGCTATATTCC TGGGCCCGG	1					
GAGGCGATCTGG CGGTTTGGGG*	1					
ATGTGCGAGCTG AGCGATG	4	RD4 present (172 bp)	RD4 present (172 bp)	RD4 present (172 bp)	RD4 absent (268 bp)	RD4 absent (268 bp)
TGTACTATGCTGA CCCATGCG	4					
AAAGGAGCACCA TCGTCCAC	4					
CAAGTTGCCGTTT CGAGCC	9	RD9 present (235 bp)	RD9 present (235 bp)	RD9 absent (108 bp)	RD9 absent (108 bp)	RD9 absent (108 bp)
CAATGTTTGTTGC GCTGC	9					
GCTACCCTCGACC AAGTGTT	9					
GGGAGCCCAGCA TTTACCTC	12	RD12 absent†	RD12 present (369 bp)	RD12 present (369 bp)	RD12 absent (306 bp)	RD12 absent (306 bp)
GTGTTGCGGGAA TTACTCGG	12					
AGCAGGAGCGGT TGGATATTC	12					

Source: Warren *et al.*, 2006 RD: region of difference

3.9 Data collection

It was determined statistically that 112 cases (with an equal number of controls) would be sufficient to detect an odds ratio (OR) of 3.0 with a 95% significance level (two-sided) and 80% power, given a prevalence of exposure (risk factor) of 10% in controls. However, only 102 new smear-positive cases were seen at the hospital during the study period and all were recruited. Controls were randomly selected among the smear-negative individuals attending the hospital. A structured interviewer's administered questionnaire was administered to all subjects to obtain information on demographics, previous clinical history, *HIV* status, knowledge of modes of transmission of tuberculosis and exposure to various risk factors such as consumption of unpasteurized milk and milk products, living in close contact with livestock, overcrowding characteristics and having previous contact with a tuberculosis patients. All questionnaires were piloted to remove ambiguity in the translation process and administered by trained interviewers. Range and consistency checks were performed to validate the accuracy of data entry. All data were crosschecked a second time against the questionnaires. Clinical records of patients were also reviewed.

3.10 Data Analysis

Data were entered and analyzed using Epi-info version 3.3 statistical software and Microsoft Excel 2003. Descriptive statistics such as means, standard deviations and proportions were determined. Responses on questions assessing knowledge of transmission of tuberculosis were ranked and scored according to importance in transmission of tuberculosis. Maximum score obtainable was 3 while minimum was 0. Chi square test was used to determine associations between categorical variables (e.g. gender, marital status, education level, occupation,) and the outcome variable (smear-

positive). Univariate analysis was performed to examine the effect of each variable of interest such as having close contact with livestock, consumption of unpasteurized milk and previous exposure to a tuberculosis patient on being smear-positive. Level of significance was set at 5%. Bivariate analysis was used to compare various risk factors between smear-positive and smear-negative. Odds ratios (OR) and their 95% confidence intervals (CI) were estimated using conditional logistic regression, with tuberculosis as an outcome. Multivariate models were then constructed, including variables that showed a significant statistical effect in the prediction of tuberculosis in univariate analysis ($P < 0.05$)

CHAPTER FOUR

RESULTS

4.1 Laboratory Results

4.1.1 Multiplex PCRs

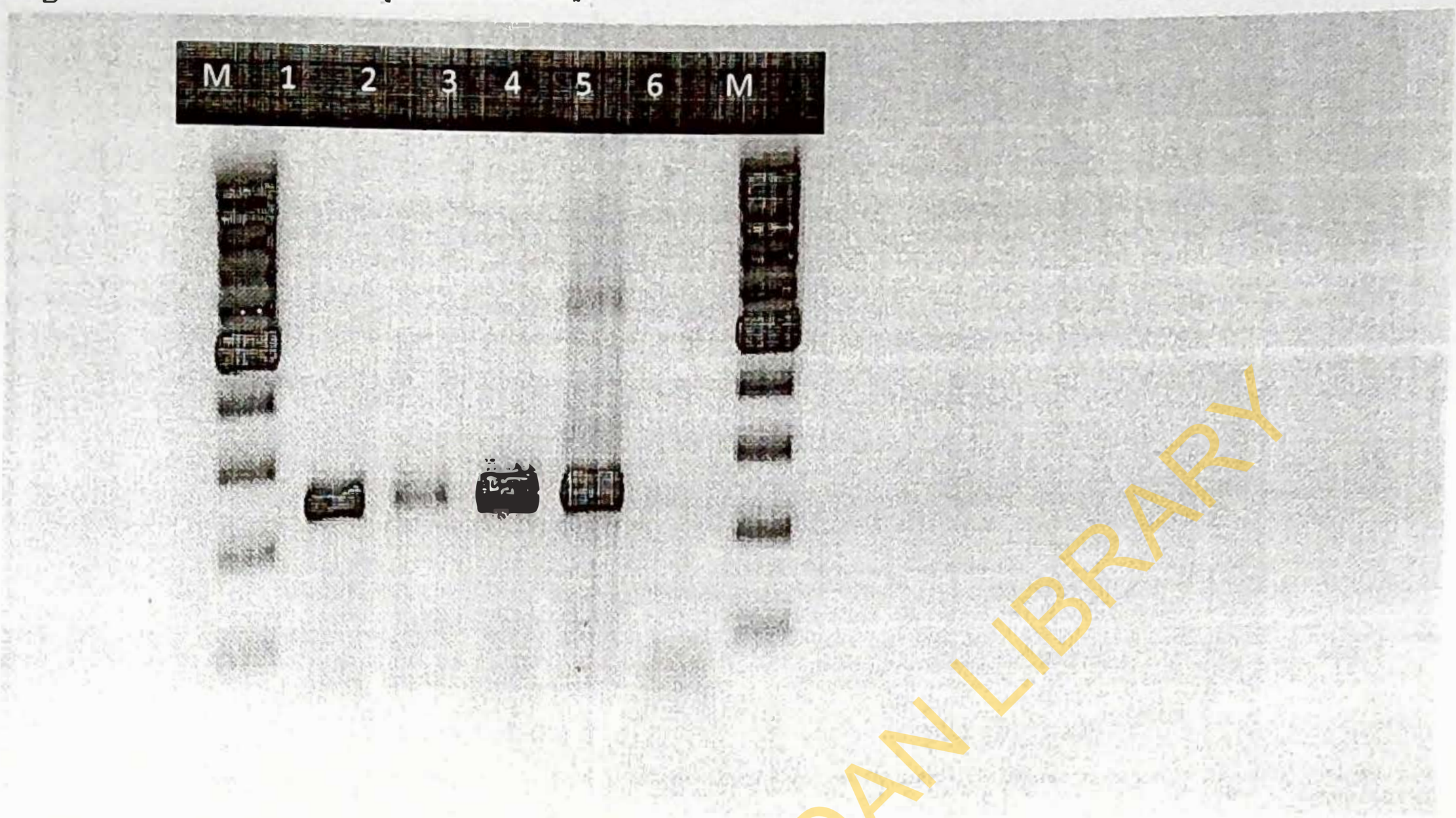
In all the 102 sputum specimens, multiplex-PCR successfully amplified the target region, 245-bp fragment typical and diagnostic for *Mycobacterium tuberculosis* complex (MTBC). However, the 500-bp fragment specific for *M. bovis* was not amplified in any of the specimens Figures 1 and 2.

4.1.2 Genomic Region of Difference

Ninety one (89.2%) of the sputa were identified as *M. tuberculosis* while 11(10.8%) were *M africanum*. *M. tuberculosis* and *M. africanum* species were detected based on the confirmation of fragment sizes for the four regions of difference (RD) depicting presence or absence (Figures 3, 4, and 5). All *M. tuberculosis* samples displayed 4 distinct bands, each band representing a specific RD. The 146 bp represents RD1 (present), the 172 bp represents the RD4 (present), 235 bp is the target fragment for RD9 (present) and 369 bp targets the RD12 (present) 9.

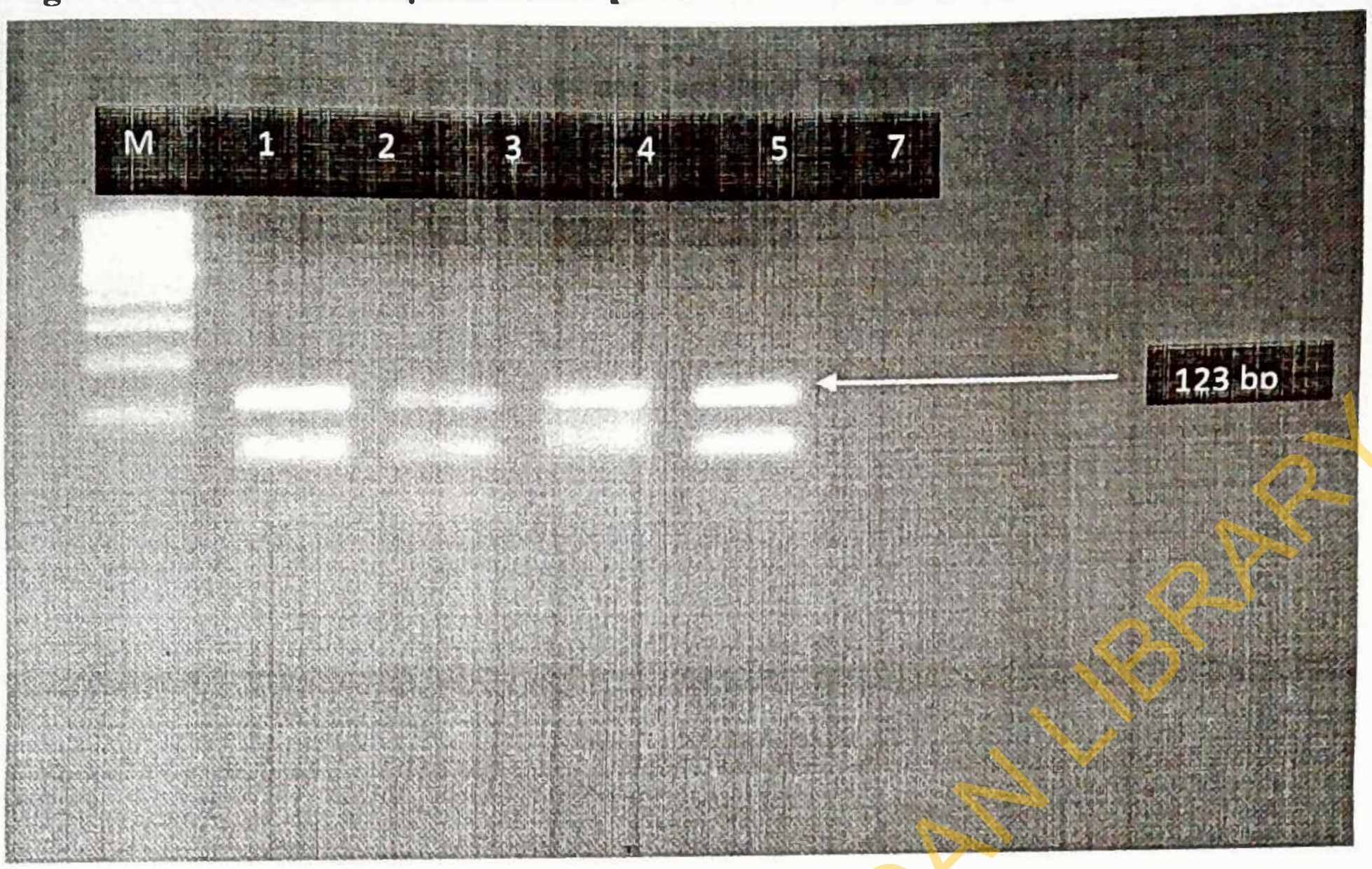
M. africanum was identified by the amplification of a 108 bp targeting the RD9 (absent), 146 bp which targets RD1 (present), 172 bp targeting the RD4 (present) and finally a 369 bp fragment which targets the RD12 (present).

Figure 4.1 Insertion sequence 245bp



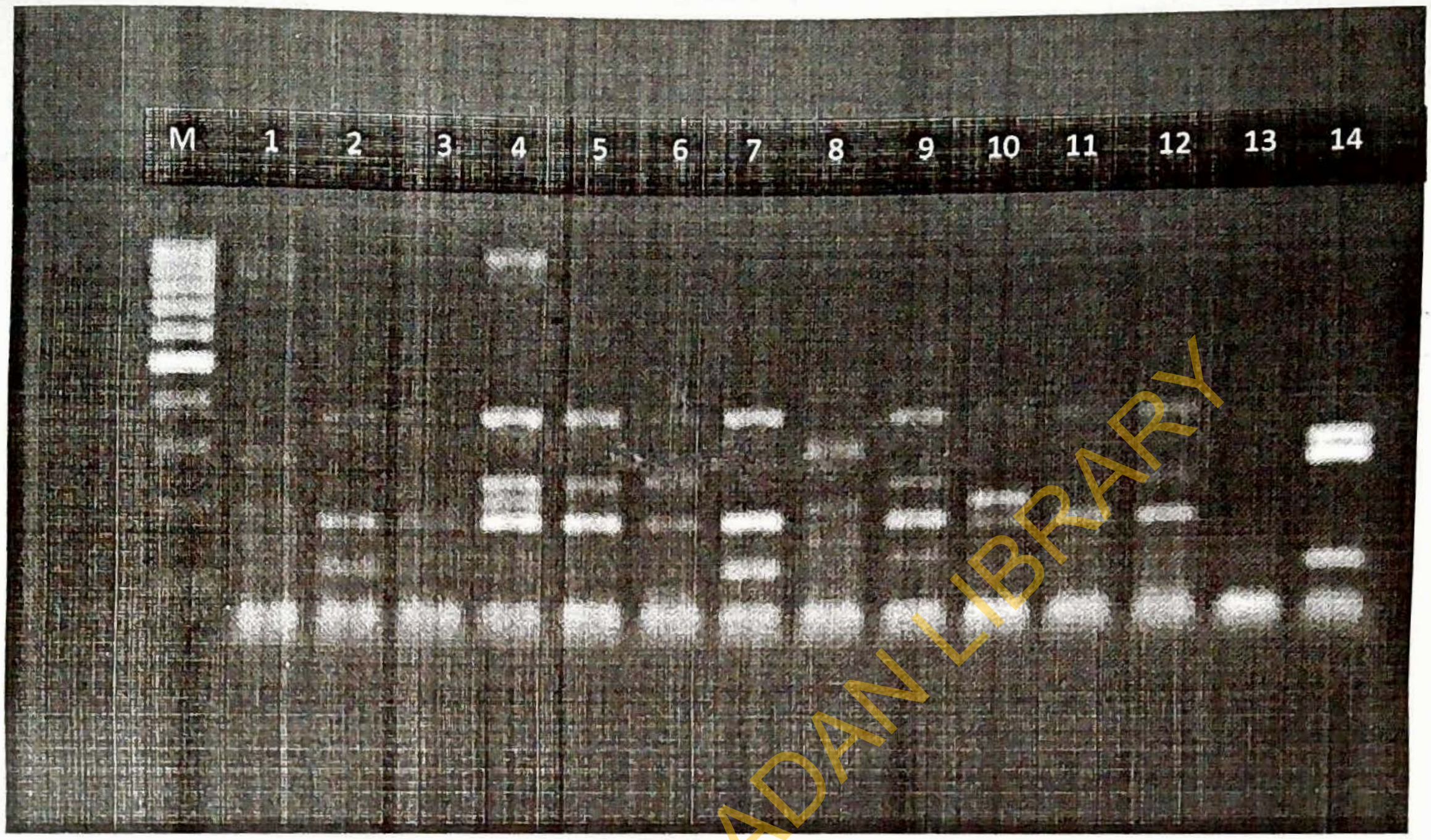
Lane: M: 100-bp DNA ladder. Lanes: 1, 2, 3, 4 - *M. Tuberculosis*: Lane 5 – Positive control: Lane 6 – Negative control

Figure 4.2 Insertion sequence 123bp



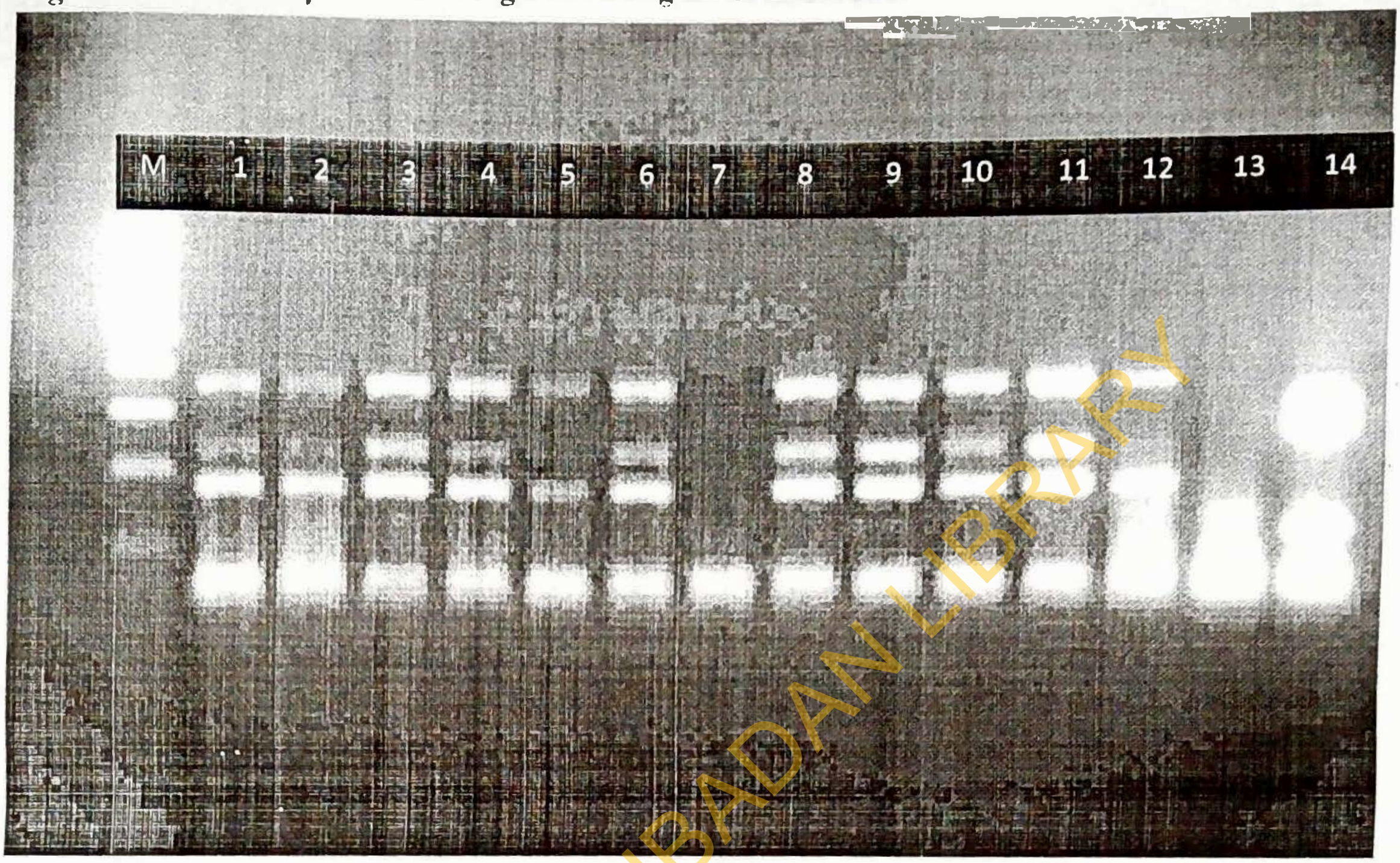
Lane: M: 100-bpDNA ladder. Lanes: 1, 2, 3, 4 - *M. Tuberculosis*: Lane 5 – Positive control: Lane 6 – Negative control

Figure 4.3 PCR amplification of genomic region of difference



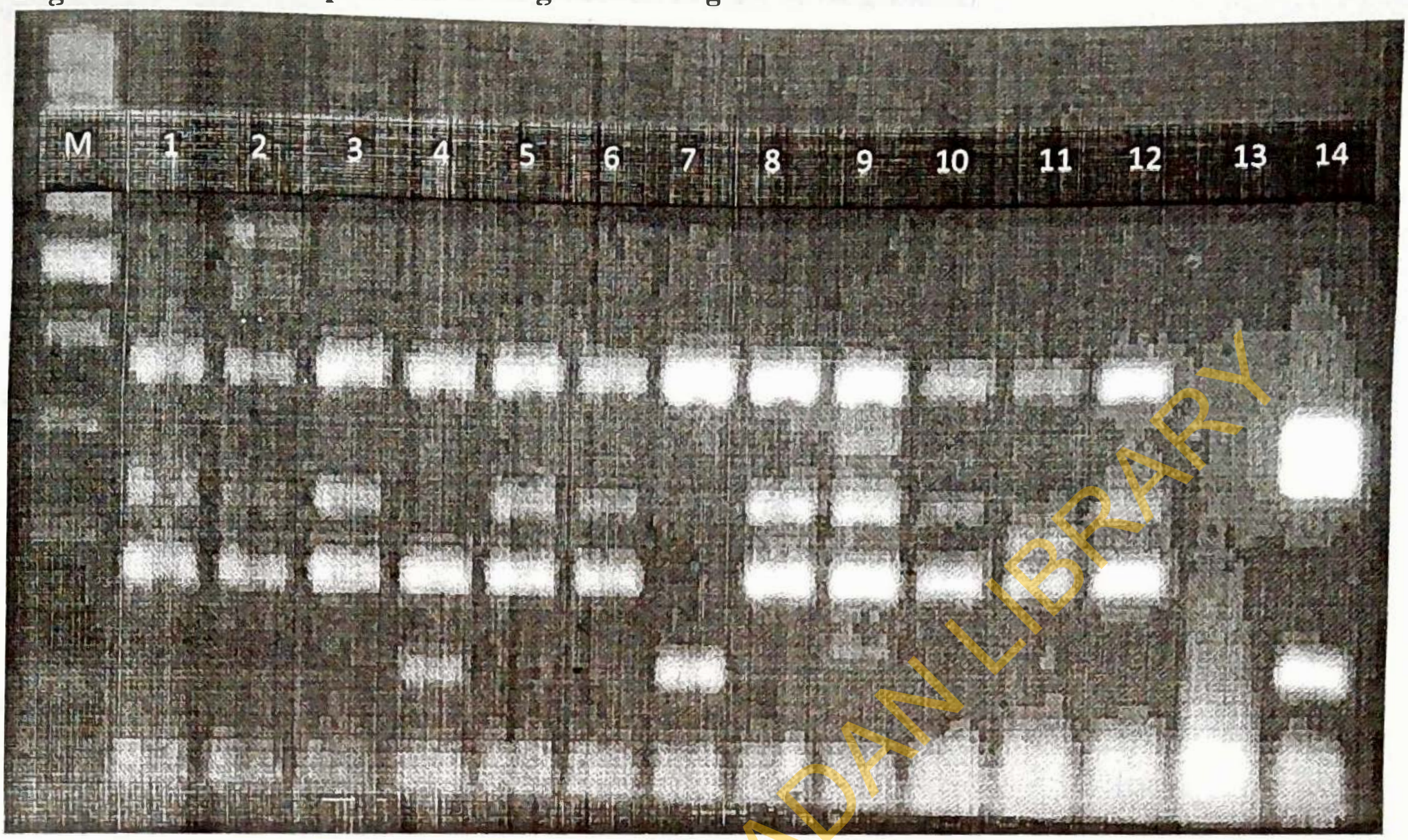
Lanes: M: 100-bp DNA ladder, 1, 3, 4, 5, 6, 8, 9, 11, 12 - *M. Tuberculosis*: Lanes: 2, 7,10 - *M africanum*, 13 Negative control, 14 - Positive control (Previously typed *M bovis*.)

Figure 4.4 PCR amplification of genomic region of difference



Lane: M: 100-bp DNA ladder. Lanes: 1, 2, 3, 4, 6, 7, 9, 10, 11, 12 - *M. Tuberculosis*, 5 - *M.africanum*, 13 – Negative control, 14 – Positive control

Figure 4.5 PCR amplification of genomic region of difference



Lane: M: 100-bp DNA ladder. Lanes: 1, 2, 3, 5, 6, 8, 9, 10, 11, 12 13- *M. Tuberculosis*; Lanes: 4, 7, - *M africanum*, 13 – Negative control, 14 - Positive control

4.2 Case control

4.2.1 Descriptive Statistics

Mean ages of the cases and controls were 36.2 ±9.0 and 35.8 ±9.7 years respectively with majority (24.5% and 21.6% respectively) of both cases and controls belonging to age group 36-40 years. Table 4.1 and Figure 4.6. Equal number of males 72(70.6%) were recruited among both cases and control. Majority of the cases and controls were married (81.4% and 78.4 %) and informally employed (63.7% and 56.9 % respectively). Fifty eight percent of cases and 66 % of controls had at least secondary school education Prevalence of *HIV* among cases and controls was 20.6% and 19.6% respectively. Table 4.2 and Figure 4.7.

4.2.2 Age of oldest and youngest persons in households of cases and controls

There was no statistical difference in ages of oldest and youngest persons in households of both cases and controls ($P>0.05$). Mean ages of oldest person in households of cases was 48 ± 12.5 years while that of controls was 47.4 ± 12 years. Median age of oldest persons in households of cases was 45 years (Range: 25 years – 90 years) and 45.5 years (Range; 26 year – 73 years) in households of controls. Mean ages of youngest person in households of cases was 3.9 ± 4.5.5 years while that of controls was 5 ±6.8 years. Median age of youngest persons in households of cases was 2 years (Range; 1year – 23 years) and 3 years (Range; 1year – 47 years) in households of controls.

Table 4.1 Demographic characteristics, vaccination and *HIV* status of cases and controls

Variables	Cases (n = 102)	Controls (n = 102)
Age, years		
Mean (std dev)	36.2 (±9)	35.8(±9.7)
Median (range)	37 (18–61)	37 (19–62)
Gender, N (%)		
Male	72 (70.6%)	72 (70.6%)
Female	30 (29.4%)	30 (29.4)
BCG, N (%)		
Yes	71 (70%)	83 (81%)
N0	31 (30%)	19 (19%)
***HIV* Status**		
Positive	21 (20.6%)	20 (19.6%)
Negative	78 (76.5%)	82 (80.4%)
Missing record	3 (2.9%)	0 (0)

Figure 4.6 Age groups of cases and controls

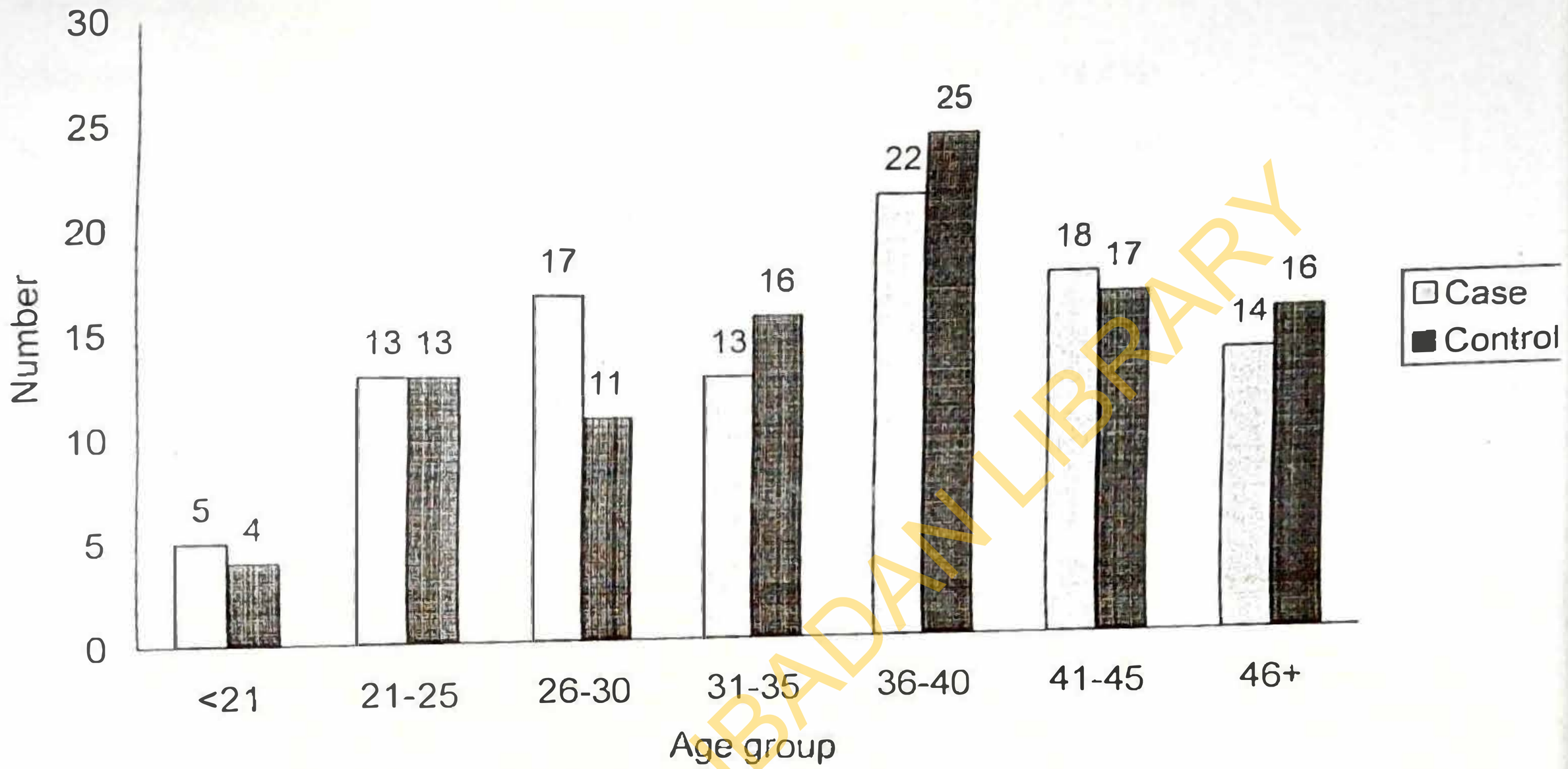
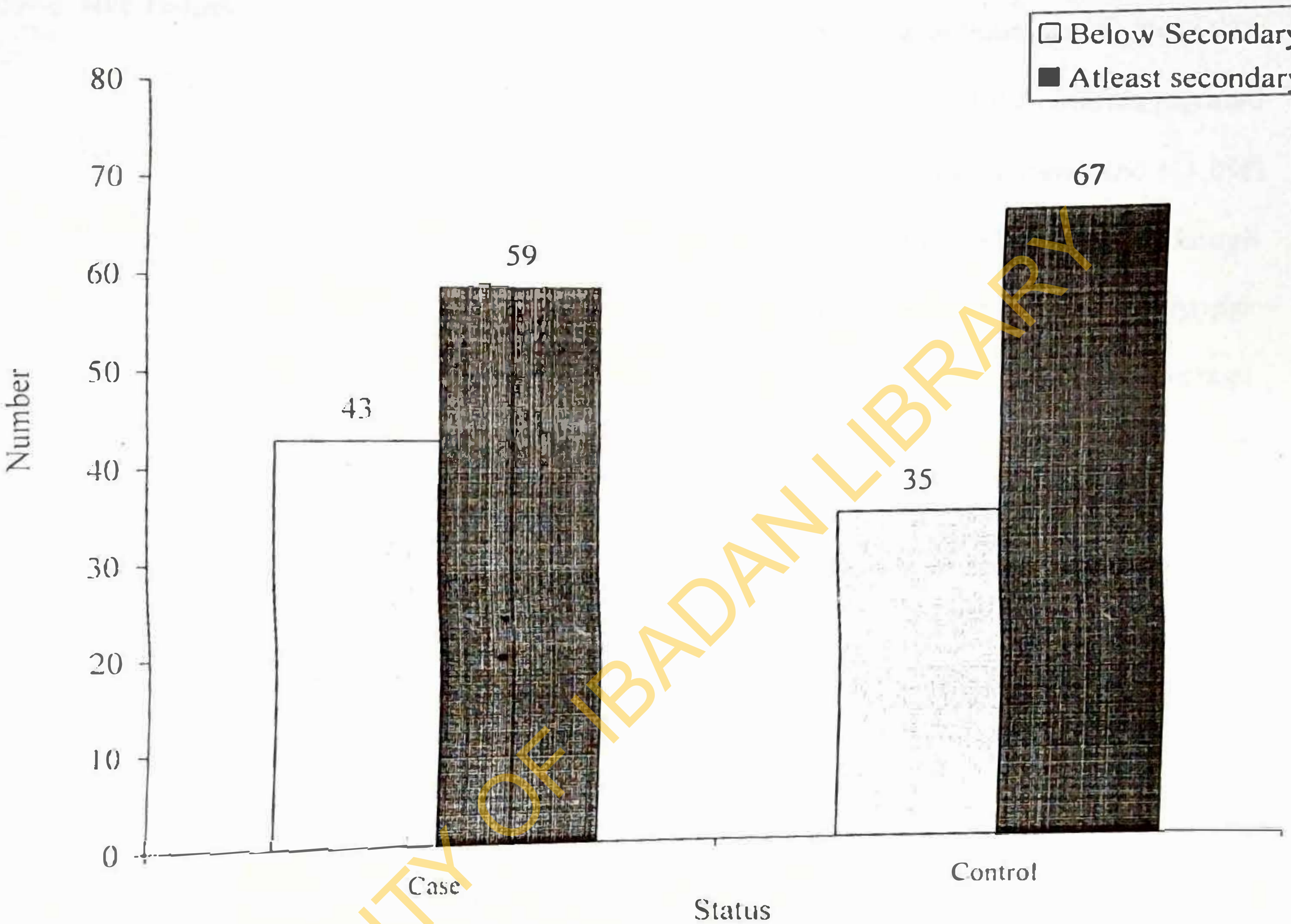


Table 4.2 Marital status, occupation and level of education for cases and controls

Variable	Cases		Control	
	No.	(%)	No.	(%)
Marital Status				
Married	83	(81.4%)	80	(78.4%)
Single	19	(18.6%)	22	(21.6%0
Occupation				
Unemployed	3	(2.9%)	3	(2.9%)
Informal	65	(63.7%)	58	(56.9%)
Formal	28	(27.5%)	31	(30.4%)
Student	6	(5.9%)	10	(9.8%)
Level of Education				
No Formal Education	17	(16.7%)	10	(9.8%)
Primary	26	(25.5%)	25	(24.5%)
Secondary	26	(25.5%)	30	(29.4%)
Tech/NCE/OND	22	(21.6%)	26	(25.5%)
HND/BSc.	10	(9.8%)	11	(10.8%)
Postgraduate	1	(1%)	0	(0.0%)

Figure 4.7 Education levels of cases and controls



4.3 Housing conditions of cases and controls

Most of the cases (81.4%) and controls (60.8%) lived in houses that had between one to five rooms, however, four (3.9%) of the cases reported living in houses with more than ten rooms (Table 4.3). Seven (6.9%) of the cases and 16 (15.7%) of the controls reported having less than 6 persons living in their homes, while 4 (3.9%) of the cases and 1(1.0%) of the controls reported having 26 to 30 persons in their homes (Table 4.4). Though majority of both cases and controls reported having on the average 2 to 3 persons per room, it was observed that only 24 (23.5%) and 31 (30.4%) of cases had on the average 2 and 3 persons per room respectively as against 43 (42.1%) and 47 (46.1%) of the controls that had on the average 2 and 3 persons per room respectively (Figure 4.8).

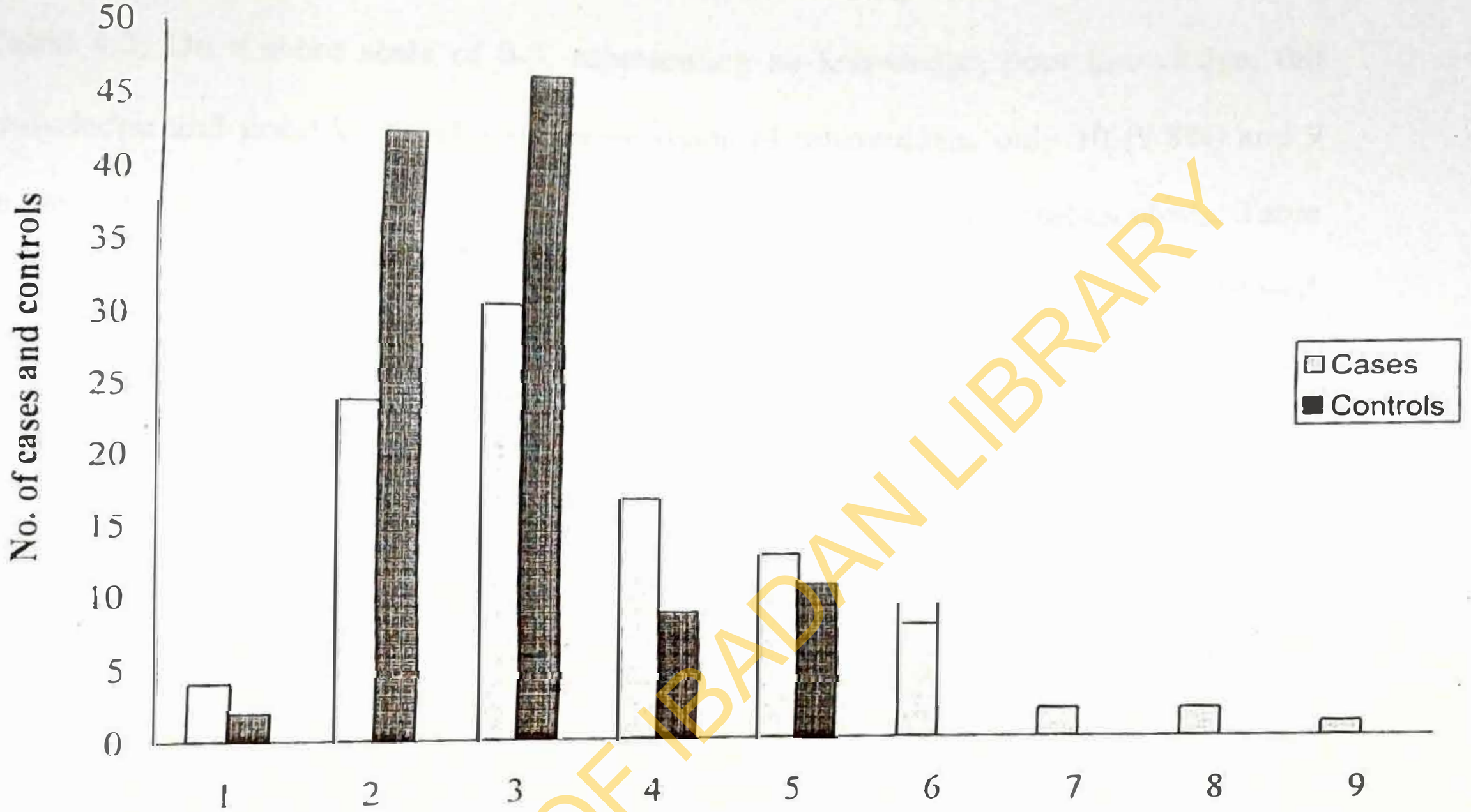
Table 4.3 Number of rooms in homes of cases and controls

No. of rooms	Cases No. (%)	Control No. (%)
1-5	83 (81.4%)	62 (60.8%)
6-10	16 (15.7%)	40(39.2%)
11-15	3(2.9%)	0 (0.0%)
15	1 (1.0%)	0 (0.0%)
Total	**102 (100%)**	**102 (100%)**

Table 4.4 Number of persons in households of cases and controls

No of persons	Cases No. (%)	Control No. (%)
<6	7 (6.9%)	16 (15.7%)
6-10	36 (35.3%)	56 (54.9%)
11-15	27 (26.5%)	24 (23.5%)
16-20	20 (19.6%)	4 (3.9%)
21-25	5 (4.9%)	1 (1.0%)
26-30	4 (3.9%)	1 (1.0%)
36+persons	3 (2.9%)	0 (0.0%)
Total	102 (100%)	102 (100.0%)

Figure 4.8 Average numbers of persons per room in homes of cases and controls



4.4 Knowledge of transmission of tuberculosis

Many, 59.8% of cases and 53.9% of the controls said tuberculosis was spread by air while few, 6.9% of the cases and 2% of the controls thought it was spread by insects, Table 4.5. On a score scale of 0-3, representing no knowledge, poor knowledge, fair knowledge and good knowledge of transmission of tuberculosis, only 10 (9.8%) and 9 (8.8%) of cases and controls had good knowledge of transmission of tuberculosis, Table 4.6. Rating for knowledge of at least one mode of transmission of tuberculosis showed that majority 72 (70.6%) and 60 (58.8%) of cases and control had knowledge of at least one mode of transmission of tuberculosis. This was statistical different between cases and controls ($P<0.05$).

Table 4.5 Modes of transmission of tuberculosis as perceived by cases and controls

Variable	Cases No (%)	Control No (%)
Air	61 (59.8%)	55 (53.9%)
Blood	2 (2%)	2 (2%)
Contaminated objects	23 (22.5%)	33 (32.4%)
Food	6 (5.9%)	1 (1%)
Insects	7 (6.9%)	2 (2%)
Saliva	42 (41.2%)	17 (16.7%)
Sexual intercourse	2 (2%)	0 (0%)

Table 4.6 Knowledge scores of modes of transmission of tuberculosis in cases and controls

Score	Cases	Control
	No (%)	No (%)
0	30 (29.4%)	42 (41.2%)
1	29 (28.4%)	24 (23.5%)
2	33 (32.4%)	27 (26.5%)
3	10 (9.8%)	9 (8.8%)

Table 4.7 Level of education of cases and controls that knew at least one mode of transmission of TB

Education Level	Case No. (%)	Control No. (%)	Total No. (%)
Tertiary	33 (45.8%)	37 (61.7%)	70 (53%)
Secondary	19 (26.4%)	22 (36.7%)	41 (31.1%)
Primary/ No formal education	20 (27.8%)	1 (1.7%)	21 (15.9%)
TOTAL	**72 (100%)**	**60 (100%)**	**132 (100%)**

Chi square = 18.02 P= 0.003

4.5 Analysis for host related factors for tuberculosis

Results from univariate analysis showed that marital status [Odds Ratio (OR) 1.20, 95% Confidence Interval (CI) =0.61-2.39], education level (OR: 1.40, 95%CI= 0.79-2.46) *HIV* (OR: 1.1, 95% CI=0.53-2.32) infection and having received BCG vaccination (OR: 0.79, 95%CI=0.40-1.56) did not increase the risk of tuberculosis (Table 4.8) as these did not differ significantly between cases and controls (P>0.05).

4.6 Analysis for risk factors for tuberculosis

Results from bivariate analysis carried out are displayed in Table 4.9. Consumption of unpasteurized milk and milk products was found to increase the risk of tuberculosis (OR: 8.33, 95%CI=4.28-16.21). Having previous contact with tuberculosis patients and living in close contact with cattle also increased the risk of having tuberculosis [(OR: 5.0, 95%CI= 2.61-9.57) and (OR: 4.42, 95%CI=2.45-7.98) respectively. However, having less than three persons in a room was found to reduce the risk of developing tuberculosis (OR: 0.15, 95%CI= 0.06-0.34).

Table 4.8 Comparison of host related factors between cases and controls

Factor	Cases No. (%)	Control No. (%)	OR(95% CI)	P value
Marital status				
Married	83 (50.9%)	80 (49.1%)		
Single	19 (46.3%)	22(53.7%)	1.20 (0.61-2.39)	0.73
Education level				
Below secondary	43 (55.9%)	35 (44.1%)		
At least secondary	59 (46.8%)	67 (53.2%)	1.40 (0.79-2.46)	0.31
HIV				
Positive	21 (20.6%)	20 (19.6%)		
Negative	78 (76.5%)	82 (80.4%)	1.1(0.53-2.32)*	0.92*
BCG				
Yes	79 (48.8%)	83 (51.2%)		
No	23 (54.8%)	19 (45.2%)	0.79 (0.40-1.56)	0.60

*3 Cases didn't not have records for *HIV* status and were excluded in the analysis

Table 4.9 Comparison of risk factors for tuberculosis in cases and controls

Variable	Cases No. (%)	Control No. (%)	OR(95% CI)	P value
Consumption of under-cooked meat				
Yes	101 (99%)	102 (100%)	0	1.000
No	1 (1%)	0 (0%)		
Consumption of unpasteurized milk & milk products (Nono, Wara)				
Yes	86 (84.3%)	40 (39.2%)	8.33 (4.28-16.21)	0.0000
No	16 (15.7%)	62 (60.8%)		
Associate closely with cattle				
Yes	65 (63.7%)	29 (28.4%)	4.42 (2.45-7.98)	0.000002
No	37 (36.3%)	73 (71.6%)		
Previous contact of TB patient				
Yes	51 (50%)	17 (16.7%)	5 (2.61-9.57)	0.000002
No	51 (50%)	85 (83.3%)		
Housing condition				
≤ 3Persons per room	59 (57.8%)	92 (90.2%)	0.15 (0.06-0.34)	0.0000003
>3 Persons per room	43 (42.2%)	10 (9.8%)		

4.7 Predictors of tuberculosis by logistic regression analysis

Each factor that showed a statistically significant difference between cases and controls in univariate and bivariate analysis was incorporated into a logistic regression model. The inclusion of age-group as a categorical variable in the model did not change the effect of the other variables, indicating that there was no residual confounding of age. Results showed that consumption of unpasteurized milk and milk products (Nono, Wara), having had contact with a TB patient, associating closely with cattle and having three (3) or less persons staying in a room where important predictors for developing tuberculosis, Table 4.10.

Table 4.10 Multivariable analysis: unconditional logistic regression model for risk factors for tuberculosis

Variables	Adjusted Odds Ratio (AOR)	95% Confidence Interval	P-value
Consumption of unpasteurized milk and milk products (Nono, Wara)	8.33	4.28-16.2	0.0000
Associating with cattle	4.42	2.45-7.98	0.000002
Have had contact with a TB patient	5	2.61-9.57	0.000002
Having ≤ 3 persons in a room	0.15	(0.06-0.34	0.0000003

CHAPTER FIVE

DISCUSSION

5.1 Molecular characterization of MTC

Analysis by multiplex PCR (m-PCR) showed 100% agreement with the Ziehl-Neelsen (ZN) test. Two distinct and discreet fragments were expected, a 245 bp which confirms bacteria belonging to the *M. tuberculosis* complex and a 500bp which targets *M. bovis*. However, no *M. bovis* band was detected indicating the absence of this species in the samples tested. The 245 bp which detects MTC also detects *M. bovis*, as this region is common to all members of the group. The regions of difference (RD) PCR highlighted in this study possess the discriminatory power to identify multiple species of the MTBC in one reaction. This test allows for the identification of mixed infections in patients with pulmonary tuberculosis. All samples in this study were subsequently submitted to a similar diagnostic PCR to confirm presence of MTC; this procedure detects a 123 bp fragment belonging to the IS6110 element within member of the complex (Eisenach *et al.*, 1990). These results are valid as the validity and sensitivity of the protocols are proven and reported (Figueiredo *et al.*, 2009, Eisenach *et al.*, 1990).

Even though *M. tuberculosis* is still the major cause of tuberculosis in humans in this study, the finding of *M. africanum* suggests that this *Mycobacteria* is playing a significant role in the etiology of infectious tuberculosis in Nigeria. Although human infections with members of the MTC require distinct treatment regimen and public health measures, mycobacteria belonging to the MTC commonly isolated from clinical specimens are not routinely identified to the species level.

5.2 Case control

Cases and controls were similar in both age group and gender. Majority were in the most productive age and thus could have serious economic implications. Because an equal number of males and females were recruited for both cases and controls, this study could not assess the possibility of gender being a risk factor for tuberculosis. However, the high number of males reported in this study agrees with what has been reported in most African countries where notification rates are higher for men than for women (WHO 2001, Holmes *et al.*, 1998). This difference could be attributed to difference in health seeking behavior and access to care between males and females (Harper *et al.*, 2003, Hudelson, 1996).

Cases and controls were also similar in marital status and level of education with most being married. It was also observed that majority of both cases and controls were informally employed although up to a third of each group had formal employment. Even though most of the cases and controls had attended secondary school, only about a third had tertiary education.

About a fifth of both cases and controls were *HIV* positive. Although Tuberculosis-*HIV* co-infection was high, it is less than the 27% reported by WHO (2006b). Prevalence of 8% has been previously reported in sputum smear positive cases in The Gambia (Hill *et al.*, 2004). *HIV* infection was not found to be associated with tuberculosis in this study. This could be attributed to either a small sample size or a real decline in Tuberculosis-*HIV* co-infection rates.

Majority of the cases lived in overcrowded conditions. This finding is of substantial public health importance as increased household size has been found to be important and

overcrowding has been documented as a risk factor for TB from several other studies in a variety of settings (Coker *et al.*, 2006, Gustafson *et al.*, 2004). Overcrowding also increases the risk of infecting healthy individuals and thereby increasing tuberculosis infection rates. It is of note that three quarters of cases and more than 50% of controls in this study were from overcrowded households. This is quite similar to the findings of Hill *et al.*, (2006) were three quarters of cases and 60% of controls were from households that were in the highest crowding category-reflected in Gambia.

The knowledge of transmission of tuberculosis among both cases and controls was poor. Accurate knowledge regarding possible routes of transmission is not only critical for decreasing the infection rate, it is also important to dispel persistent myths. Poor knowledge and misconceptions about tuberculosis are key factors in people's lack of efforts at prevention. Also the fact that less than 10% of cases and less than only 1% of controls thought tuberculosis could be spread by foods such as unpasteurized milk (nono) or under-cooked meat is of serious public health significance as the consumption of unpasteurized milk have been reported as risk factors for tuberculosis (Coker *et al.*, 2006) and both *M. bovis* and *M. tuberculosis* have been found in milk in Nigeria (Cadmus *et al* 2006, Cadmus *et al* 1999, Idrisu & Schnurrenberger 1977). In a study to assess the knowledge about tuberculosis in urban Morocco, Ottmani *et al.*, (2008) reported that most tuberculosis patients had little knowledge of tuberculosis, its causes or its transmission. This study also reveals that knowledge of transmission of tuberculosis increased with increase in level of education.

In this study, marital status, education level and type of employment did not increase the risk of tuberculosis as these did not differ significantly between cases and controls. *HIV*

infection and receiving BCG vaccination also did not have any effect on the risk of developing tuberculosis. This may be due to relatively small sample size used in this study.

Consumption of unpasteurized milk and milk products was a major risk factor indentified in this study. Consumption of unpasteurized or contaminated milk has long been regarded as a principal mode of transmission of zoonotic tuberculosis (Acha and Szyfres 1987). Both *M. bovis* and *M. tuberculosis* have been found in milk samples in Nigeria (Idrisu A and Schnurrenberger 1977) and Egypt (Nafeh 1992). Of recent, *M africanum* was isolated from fresh milk in Nigeria (Cadmus *et al.*, 2010). Thus, the possibility of transmission of zoonotic tuberculosis and other milk-borne pathogens through the consumption of contaminated milk and milk products should not be underestimated. Although proper food hygiene practices could play a major role in controlling this form of transmission, but this may be difficult to enforce in many developing countries.

Previous exposure to a tuberculosis patient was a risk factor for tuberculosis identified and this is consistent with other studies where 24% of West African tuberculosis patients had a family history of tuberculosis compared to controls (AOR: 3.24, 95%CI=2.3-4.6; p<0.001) (Lienhardt *et al.*,2001). This is also similar to findings reported by Tocque *et al.*, (2001) in England where cases of tuberculosis were found to be more likely to have lived with known tuberculosis patients. Exposure to a known tuberculosis case was the an important risk factor for tuberculosis in the study by Hill *et al.*, (2006) and Coker *et al.*, (2006) also found living with a relative who had tuberculosis to be associated with a greater risk of developing the disease. This finding is of substantial public health importance as

most cases lived in overcrowded conditions and active contact tracing is rarely done. Therefore TB patients need to complete treatment and achieve cure in order to reduce the risk of transmission to others.

Living in close contact with livestock was also identified as a risk factor for tuberculosis in this study. Close physical contact between humans and potentially infected animals is present in most African communities, especially in developing regions where cattle form an integral part of human social life (Cosivi *et al.*, 1998). In Nigeria, pastoral communities live in close contact with their cattle. These cultural practices could facilitate transmission of tuberculosis between cattle and humans (Cadmus *et.al*, 2006). *Mycobacterium tuberculosis* and *M. africanum* has been reported from cattle (Cadmus *et al.*, 2006. Ayele *et al.*, 2004) suggesting human to cattle transmission of tuberculosis and *vice versa*. Also *M. bovis* has been reported in humans in Nigeria (Garba & Galadima, 2006, Cadmus *et. al.*, 2006).

Our study shows that having three or less persons in a room was protective against the risk of developing tuberculosis. Increased household size and overcrowding have been documented as a risk factor for tuberculosis from several other studies in a variety of settings (Coker *et al.*, 2006, Mangtani *et al.*, 1995). In a study in Guinea Bissau, adult overcrowding was a risk factor for tuberculosis (Gustafson *et al.*, 2004) while in India, Shetty *et al.*, (2006) did not find overcrowding as a significant risk factor for tuberculosis

5.3 Conclusion

Even though *M. tuberculosis* is the major cause of human tuberculosis in northern Nigeria, a significant proportion is still attributable to *M. africanum*. Majority of tuberculosis cases are young adults in the most productive age group. There is a general low level of knowledge of causes and modes of transmission of tuberculosis in this part of the country and Tuberculosis-*HIV* co-infection rates are high. This study identified key risk factors for tuberculosis in northern Nigeria. These factors are consumption of unpasteurized milk and milk products, previous contact with a tuberculosis patient, close association with cattle and having three (3) or more persons staying in a room.

5.4 Recommendations

With respect to opportunities for intervention, this study has identified several possibilities.

1. Routine characterization of MTC should be done in order to know the prevalent species of MTC in Nigeria and direct appropriate treatment.
2. Improve health education of tuberculosis patients on modes of transmission of tuberculosis.
3. Education of the general public on the importance of pasteurization of milk before consumption should be a public health priority.
4. Pasteurization of milk on small dairy/pastoral farms should be enforced by the Government.
5. Active tracing of tuberculosis case contacts should be routinely done to identify co-prevalent cases and to encourage early attendance at tuberculosis clinics and DOTs centers for those who have symptoms in the long term.
6. While it might be difficult to prevent close association with cattle especially with regards to livestock owners, it is important to educate them on safe measures of animal handling practices.
7. Vigilance and more collaboration between clinicians, veterinarians and laboratory workers in the identification of the possible route of transmission and the responsible agents for the transmission of zoonotic tuberculosis.
8. Possible ways to avoid overcrowding in all settings should be sought and implemented by the Government. Even though there is no easy solution to this problem, as it is inextricably linked to indices of wealth at a population level.

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APPENDICES

1. CONSENT FORM

MOLECULAR CHARACTERIZATION OF MYCOBACTERIUM TUBERCULOSIS COMPLEX AND RISK FACTORS FOR TUBERCULOSIS AMONG PATIENTS ATTENDING A NATIONAL TUBERCULOSIS CENTRE IN ZARIA, NIGERIA

Introduction: We are carrying out a research on Prevalence of *Mycobacterium bovis* Infection in Smear positive patients in a TB referral centre in Zaria, Kaduna State, Nigeria and will like to get some information from you. Participation is optional and be assured that any information you provide will be treated with confidentiality and personal information collected will not appear in any documents or reports based on this study. You are free to withdraw from the study during the course if you wish to without any victimization.

Thank you.

CONSENT: The details of the study have been explained to me and I fully understand. I am hereby willing to participate in the study.

Signature of participant /Date

Signature of interviewer /Date

2. QUESTIONNAIRE

MOLECULAR CHARACTERIZATION OF MYCOBACTERIUM TUBERCULOSIS COMPLEX AND RISK FACTORS FOR TUBERCULOSIS AMONG PATIENTS ATTENDING A NATIONAL TUBERCULOSIS CENTRE IN ZARIA, NIGERIA

Questionnaire No. **Date:**

Name of interviewer:

PERSONAL IDENTIFICATION

Code No.

Age: _____ Sex: _____ Marital Status: _____

Occupation: _____

Educational Level: No formal education ☐

Primary ☐

Secondary ☐

Tech/NCE/OND ☐

HND/BSc ☐

Post graduate ☐

Others (Specify) ☐

Address: _____

_____

Housing Information

Number of people in household ☐

Age of oldest person in the house ☐ Age of youngest person in the house ☐

Number of rooms in the house ☐

Average number of persons in each room ☐

How long have you lived in this house ☐

Knowledge of tuberculosis

Do you know what your illness is? Yes ☐ No ☐

What do you think is the cause of your illness?

_____

Before coming to the hospital did you know what TB is? Yes ☐ No ☐

In your opinion, how is TB spread

Contact with contaminated objects ☐

Sexually ☐

Food ☐

Saliva ☐

Blood ☐

Air ☐

Others (Specify) _____

Risk Factors

1. Do you eat meat and other meat products e.g. lungs/liver/kidney?

Yes ☐ No ☐

2. Do you drink unpasteurized milk and milk products e.g. Fura, nunu, wara?

Yes ☐ No ☐

3. Do you have pets at home? Yes ☐ No ☐

4. Do you associate closely with cattle? Yes ☐ No ☐

5. Have you ever received BCG vaccination?

Yes ☐ No ☐

6. Have you had any previous contact with TB patients?

Yes ☐ No ☐

7. Have you ever had TB before? Yes ☐ No ☐

8. How long have you had TB? ☐

Health Information

***HIV* Status**

Positive ☐

Negative ☐

Is your partner aware of your *HIV* status?

Yes ☐ No ☐

***HIV* Status of Partner**

Positive ☐

Negative ☐

Don't know ☐

Thank you.