PREVALENCE OF MULTIDRUG RESISTANCE TUBERCULOSIS IDENTIFIED BY BACTEC AND PCR TECHNIQUES USING RIFAMPICIN AS A SURROGATE MARKER IN NAIROBI HOSPITAL

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A thesis submitted in partial fulfilment for the award of the degree of Master of Science in Infectious Disease Diagnosis in the School of Pure and Applied Science of Kenyatta University

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DECLARATION

I declare that the work presented in this thesis is my original work and has not been presented for a degree in any other university or any other award name.

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This thesis is dedicated to my husband Mr. James Kamau Kagwi and my children Moses Kagwi, Sarah Wangari and Magdalene Wangu for their total support and encouragement.

To my parents, Josephat Gathua Kiuna and Magdaline Wangu Gathua for the wonderful foundation they accorded me that resulted to my success. To my brothers and sisters for their support.
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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

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ABSTRACT

The emergence of multidrug-resistant strains of M.tuberculosis threatens tuberculosis (TB) control and prevention efforts. It is so far the most severe form of bacterial resistance with a high mortality rate. Lack of proper TB control treatment programs often leads to improper and incomplete chemotherapy, which is one of the major factors contributing to MDRTB outbreaks. The aim of the study was to determine the prevalence of MDRTB by both radiometric Bactec 460 TB system and polymerase chain reaction (PCR) amplification technique using rifampcin as a surrogate marker. The study also determined the prevalence of MTB complex by both smear microscopy and culture by Bactec. The study also looked into some of the factors associated with the development of resistance. Information on patients was obtained through a questioner, which was given to the clinicians to fill. Seven hundred samples, both pulmonary and extra-pulmonary, were collected prospectively over a period of six months. They were all processed and cultured by Bactec radiometric technique. Smears were made, stained by Zielh-Neelsen (ZN) technique and examined microscopically. Identification and drug sensitivity testing was done by the same method on all MTB isolates. Out of 700 samples processed, 91 (13%) were positive for tuberculosis by culture and 46 (6.6%) were positive by smear microscopy. On identification, 86 (94.5%) isolates were MTB complex and 5 (5.5%) were Mycobacterium other than tuberculosis (MOTT). Eight (8) out of 81 MTB isolates (9.3%) were identified as MDRTB by both techniques. This shows an increase in prevalence of MDRTB from 3% to 9.3%, while that of MTB complex declined from 23% to 13%. These figures may not represent the national figures TB prevalence in Kenya as the hospital takes care of able patients. Smear microscopy detection technique is
insensitive and is likely to miss half of TB cases (smear negative TB cases). It is very difficulty to detect TB from extra-pulmonary sites by smear microscopy technique. Eighty seven (87.5%) of the MDRTB was secondary infection and were HIV positive. HIV and incorrect treatment plan were the factors mostly associated with development of drug resistance TB. Twice the men were more affected than women.
CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Background information

Tuberculosis (TB) is a contagious disease that kills around 2 million people each year. In the 1960’s, the incidence of TB was on the decline due to effective drug therapy (Bloom et al., 1992). Re-emergence of the disease occurred during the 1980’s because of; increased immigration from TB endemic countries, increased transmission in institutions settings and increased number of HIV infected or other immuno-compromised conditions. It is estimated that 8.8 million new cases arise annually, which correspond to 52,000 deaths per week or more than 7,000 each day. This translates into more than 1,000 new cases every hour (WHO, 1995). This death rate, however, only partially depict the global TB threats; more than 80% of TB patients are in the economically productive age of 15 to 49 years (WHO, 1997).

The World Health Organization (WHO) has identified 22 high burden TB countries that combined, contribute 80% of the global burden of TB. These include (In order from the highest burden of TB): India, China, Indonasasia, Nigeria, Bangladesh, Parkistan, Ethiopia, Philippines, South Africa, Democratic Republic of congo, Russia, Kenya, Vietnam, Tanzania, Brazil, Uganda, Zimbabwe, Mozambique, Thailand, Afghanistan, Cambondia and Mynmar. India has the largest number of TB cases in the world, accounting for nearly 1/3 of the global burden (WHO, 2002).
In Africa the number of TB cases is still rising at 3 to 5% per year. Sub-Saharan Africa has among the highest rates in the world. Among the 22 highest-burden TB countries, nine are in sub-Saharan Africa.

Kenya ranks among 12th among the 22 countries with a high tuberculosis burden. According to the WHO report (2004), in 2002 Kenya had more than 170,000 TB cases and an incident rate of 223 new sputum smear positive (SS+) cases per 100,000 people. In Kenya, new cases of TB are notified through the National Leprosy and Tuberculosis program (NLTP), which was established in 1956. Case detection rates for all forms of TB rose from 88 per 100,000 populations in 1994 to 103 per 100,000 in 1995 representing a 15% increase (Cauthen et al., 1993). This increase has been attributed primarily to the effect of the human immuno-deficiency virus (NLTP, 1996). The incidence of TB has steadily been declining in most industrialized countries until the trend was reversed by HIV pandemic and poor living standards.

The emergence of AIDS and decline in social economic standards contribute to the disease’s resurgence in industrialized countries (Barnes et al., 1991). In most developing countries, although the disease has always been endemic, its severity has increased because of the global HIV pandemic and extensive social restructuring due to rapid industrialization and conflicts. As a major public health problem world wide, TB is now a global emergency (Kochi et al., 1993). HIV and TB form lethal combination, each speeding the others progress. HIV weakens the immune system making someone infected by both HIV and TB more likely to develop TB disease than someone infected with TB only. Although the currently available drugs kill most isolates of *M. tuberculosis*, strains resistant to each of these drugs have emerged, and multiple resistant strains are increasingly wide spread. The
growing problem of drug resistance combined with a global incidence of 8.8 million new cases per year underscore the urgent need for new anti-tuberculosis therapies (Chiew et al., 1998).

Multidrug resistance refers to the ability of some strains of TB to grow and multiply even in the presence of certain anti-tubercular drugs, which would otherwise kill them. People, who have been exposed to a case of MDRTB, especially if they are immuno-compromised, are at risk for developing MDRTB. Other people who may develop resistance tuberculosis include TB patients who have failed to take anti-tubercular drugs as prescribed, TB patients who have been prescribed an ineffective treatment plan and people who have been treated previously for TB (Bloom et al., 1992)

Short-course chemotherapy forms the backbone of anti-tubercular chemotherapy (Kochi et al., 1993). Proper prescriptions and patient compliance almost always cure the disease. Multidrug resistance TB has been associated with death rates of 50 to 80% and spans a relatively short time (4 to 16 weeks from diagnosis to death) (Dooley et al., 1992). Delayed recognition of drug resistance, which results in delayed initiation of effective treatment, is one of the major factors contributing to MDRTB outbreaks, especially in health care facilities (Crawford (a) et al., 1992). In most countries, MDRTB has increased in incidence and interferes with TB control programs, particularly in developing countries where prevalence rates are as high as 48% (Iseman et al., 1992). The high infection and death rates pose an urgent challenge to rapidly detect cases. In the past few years, genetic and molecular insights have unraveled the mechanisms involved in the acquisition of drug resistance, concomitant with the development of various molecular strategies to rapidly detect MDRTB (Cohn et al., 1997).
1.2 Literature review

1.2.1 Etiology of tuberculosis

Tuberculosis is a lower respiratory tract infection, which is caused by TB bacilli. Usually, it involves the lungs (pulmonary) but may also affect other organs (e.g. secondary site infection). The TB bacillus is the Mycobacterium tuberculosis. Infection usually begins in the mid or lower lung where the organism is deposited. The bacteria are ingested by alveolar macrophage but multiply nevertheless, eventually destroying the phagocytes. Other macrophages and lymphocytes accumulate in indolent form, ingesting bacilli that are released from degenerating cells. Infected macrophages spread to regional lymph nodes and in case of absent specific immunity the infected macrophages may spread through out the body. Distribution is random according to blood flow but the most important areas are those that favour growth, e.g the lymph node, kidneys, CNS and most important apical posterior areas of the lungs (CDC, 1990).

TB bacillus has a hardy and protective waxy coat or capsule that enables it to live outside the body for a long period of time (Koneman et al., 1988). The bacilli are naturally killed in the body in a healthy person by the immune system and only 10% of those infected with TB will develop the disease. In immuno-compromised individuals the percentage of those who develop TB disease is higher. Unlike many other bacteria, specialized disinfectants such as those registered for hospital use, applied over a period of time can kill the bacilli on contaminated objects. M.tuberculosis complex can cause disease by preying on macrophages, i.e the immune system warriors that usually consume bacteria, when the immune system is compromised or overwhelmed. The bacteria enter the macrophage and
apparently multiply until the cell raptures, releasing more bacteria to attack other macrophages (Koneman et al., 1988).

1.2.2 Spread of TB

TB transmission occurs when an individual come into contact with infected airborne droplets of respiratory sections or sputum (1-5 microns). A person can have active or inactive TB. Active TB or TB disease means the bacteria are active in the body and the immune system is unable to stop them from causing illness. In people with active TB, poor ventilation and increased duration of exposure increases transmission (Koneman et al., 1988). Airborne infected droplets are inhaled then become lodged in the alveoli of the lungs causing an infection. In addition to the lungs (pulmonary), other organs including the mouth may also be infected. Once exposed to the infected droplets, incubation can be as long as 12 weeks. This extended period of time can cause difficulty in tracing the origin of the infection (WHO, 2005). Species of atypical mycobacterium ie mycobacterium other than tuberculosis (MOTT) includes M. avium, M. fortuitum, M. gordonae, M. kansasi, and M. xenopi. These organisms may present with symptoms suggestive of pulmonary TB. They are non-contagious and are frequently found as environmental contaminants. MOTT presents as an opportunistic infection in immuno-compromised individual. The disease process may be self-limiting, chronic or life threatening depending on the immune status of the infected. Therefore it is important to identify any TB isolate to differentiate this strain from the MTB complex in order to determine the proper treatment strategy as well as the HIV status of the patient. Decision to treat the patient is at the discretion of the physician. (WHO, 2005). Drugs for the treatment of MOTT are not supplied through the Public Health TB control program and are not provided free of charge.
1.2.3 Epidemiology and Emergence

Overall, almost 3.8 million cases of tuberculosis were reported annually around 1990. The global notification rates (per 100,000 population) increased by 20.8% from the 1984 to 1991. Increases occurred in four WHO regions; the African, the Eastern Mediterranean, the South East Asian, and the Western Pacific. In contrast, the average number of cases decreased in the American and European regions (WHO, 1997).

In developing countries, the impact of TB has intensified with the spread of HIV infection. AIDS and TB have reached epidemic proportions in industrialised countries. Changes in immigration patterns, greater rate of homelessness, reduced funding for public health programs and frequent overseas travel, have led to a reversal in previously well established declines in TB notifications (WHO, 1997). According to WHO figures released in 1997 (WHO, 1997), the reported regional incidence rate of TB ranged from 33.0 per 100,000 in the Americas to 985 per 100,000 in South East Asia.

In Kenya 15% of the population is living with HIV/AIDS, a number that represents 6% of the global total. Kenya has experienced a sharp increase in the incidence of TB. The county annual increase in incidence of 12% is among the highest in sub-Saharan Africa. Kenya ranks 12th among the world’s 22 countries with high TB burden. According WHO, approximately half of new estimated new cases were detected in 2002 and 80% of the cases were detected in 2001. Kenya National Leprosy and Tuberculosis Program (NLTP) began to implement the WHO recommended Directly Observed Short cause Therapy, (DOTS) in 1993 and reported 80% coverage by 2001 (WHO, 2002).
1.2.4 Diagnosis of TB infection

1.2.4.1 Tuberculin skin test (TST)
The tuberculin skin test (TST) involves examining a person who is not ill but may be infected with *M. tuberculosis* due to exposure to someone who has TB. The tuberculin skin test is the only way to diagnose TB infection before the infection has progressed to TB disease. In Mantoux test, or TST, 5 TU of purified protein derivative is used. A TST reaction size of greater than or equal to 5mm of induration is considered positive. Persons with a TST reaction size of less than 5 mm but with a history of exposure to TB could also be infected with *M. tuberculosis* (Sprat, 1994).

1.2.4.2 Chest x-rays
The typical signs and symptoms of pulmonary TB are cough with or without fever, night sweats, weight loss, and upper lobe infiltrates with or without cavitations on chest x-rays. The diagnosis of TB in immuno-compromised host can be associated with typical symptoms, a lack of typical symptoms, and a pancyt of findings in chest x-ray (Kenneth, 2003).

1.2.4.3 Microscopy by staining
Patient’s specimen smear can be stained with Zieehl-Neelsen, kinyonni, or fluorescent dye and examined microscopically for the presence of TB bacilli. Direct microscopy smear is the most applied method of diagnosis in countries with low resources but has low sensitivity (Cheesbrough, 1985)
1.2.4.4 Culture
Patient's samples suspected of TB infection are cultured in egg rich media which could either be in agar or broth form. Some agar media takes up to 8 weeks while some broth media are faster and can give results as from 48hrs to 4 weeks. Culture methods are more sensitive and specific as they can identify the bacilli as either MTB or MOTT and give the anti-tuberculous drug susceptibility results (Middlebrook, 1954).

1.2.5 The impact of HIV/AIDS on tuberculosis in developing countries.
HIV infection weakens the immune system and thus makes active TB disease more likely in an individual infected with the TB bacillus. As a result of the spread of the HIV, an estimated 1.4 million cases of active TB worldwide are expected to occur annually with people co-infected with HIV by the end of the century. Already TB is the leading cause of death worldwide among individuals who are HIV positive (Gross et al., 1988). Pandemic of human immuno-deficiency virus (HIV) infection and acquired immuno-deficiency syndrome has caused marked increases in tuberculosis notifications in some countries. By virtue of its ability to destroy the immune system, HIV has emerged as the most important factor for progression of dormant tuberculosis infection to clinical disease (Gross et al., 1988). In 1990, 4.6% of all tuberculosis deaths were attributable to HIV infection (Imboden et al., 1993). The global WHO program on AIDS (Imboden et al., 1993) estimated that by mid 1994, more than 16 million adults and more than 1million children had been infected with HIV worldwide since the beginning of the HIV epidemic. Nearly 90% of HIV infections have occurred in developing countries and the majority of the patients are between 15 to 49 years of age. The impact of HIV infection on tuberculosis is greatest in
populations where the prevalence of tuberculosis infection in young adults (who are at greatest risk of HIV infection) is relatively high (Roper et al., 1992).

1.2.6 The basis of bacterial resistance to antibiotics

1.2.6.1 Inherent (Natural) Resistance
Bacteria may be inherently resistant to an antibiotic. For example, *Streptomyces* has a gene that is responsible for resisting its own antibiotic; a gram negative bacterium has an outer membrane that establishes a permeability barrier against antibiotics; or an organism lacks a transport system for the antibiotic; or it lacks the target or reaction that is targeted by the antibiotic (Sprat, 1994).

1.2.6.2 Acquired Resistance
Bacteria can develop resistance to antibiotics e.g. bacteria population previously sensitive to antibiotics becomes resistant. This type of resistance results from changes in the bacterial genome. Two genetic processes in the bacteria drive acquired resistance; namely mutation and selection (sometimes referred to as vertical evolution) and exchange of genes between strains and species (sometimes called horizontal evolution). In vertical evolution spontaneous mutation in the bacterial chromosome imparts resistance to a member of bacteria population. In the selective environment of the antibiotic, the wild type (non-mutants) is killed while the mutants are allowed to grow and flourish. The mutation rate for most bacterial genes is approximately 10−8 (Kenneth, 2003). Horizontal evolution occurs often after acquisition of resistant genes from another organism. For example *streptomycetes* has a resistant gene that is resistant to its own antibiotic i.e. Streptomycin but some how that gene escape and gets into E. coli or *Shigella* spp. (Kenneth, 2003).
Bacteria are able to exchange genes in nature by three processes: Conjugation, transduction and transformation. Conjugation refers to cell-to-cell contact where the DNA passes via sex pilli from the donor to the recipient. During transduction, a virus transfers the genes between the mating bacteria while in transformation, DNA is directly obtained from the environment, having been released from another cell (Grange, 1990).

1.2.7 The medical problem of bacterial drug resistance
Obviously, if a bacterial pathogen is able to develop or acquire resistance to an antibiotic, that antibiotic becomes useless in the treatment of infectious disease caused by that pathogen. So as pathogens develop resistance, a new (different) antibiotic to fill the place of the old ones requires to be developed (Iseman et al., 1992). Natural Penicillins have become useless against Staphylococci and must be replaced by other antibiotics. Tetracyclines having been so widely used and misused for decades, have become worthless for many of the infections that once designated it as a wonder drug (Sprat, 1994). Not only is there a problem in finding new antibiotics to fight old diseases (because resistant stains have emerged) there is a parallel problem to find new anti-biotis to fight new diseases (Koneman et al., 1988). In the past two decades “new” bacterial diseases have been discovered e.g. legionsnaire’s disease, gastric ulcer, lyme disease, toxic shock syndrome, skin eating streptococci (Iseman et al., 1992). Broad patterns of resistance exist in these pathogens and it seems likely that we will soon need antibiotics to replace the handful that are effective against these bacteria especially as resistance begins to emerge among them in the selective environment of anti-biotic chemotherapy (Iseman et al., 1992).
1.2.8 Tuberculosis chemotherapy

In 1991 the WHO Assembly adopted resolution WHO 44.8, recognizing "effective case management as the central intervention for tuberculosis control" and recommending the strengthening of national tuberculosis program by short course chemotherapy and improving the treatment management system (WHO, 1998).

DOTS are the brand name of WHO recommended tuberculosis control strategy. Tuberculosis control requires effective, inexpensive, simple and largely standardized technology, and the managerial skill to implement them as a large-scale intervention in each country (WHO, 2000). When tuberculosis cases are treated, poor drug prescription and poor case management is creating more tuberculosis patient excreting resistant tubercle bacilli. Primary resistance is defined as the presence of a drug strain to one or more anti-tuberculous drugs in a TB patient who has never received prior treatment. Acquired resistance is defined as resistance to one or more anti-tuberculous drugs, which occurs during the course of treatment usually as a result of non-adherence to the recommended regimen or incorrect prescription (Stead et al., 1987). Multidrug resistant strains of M. tuberculosis seriously threaten tuberculosis control and prevention efforts. Molecular studies of the mechanism of action of anti-tubercular drugs have elucidated the genetic basis of drug resistance in M. tuberculosis. Drug resistance in M. tuberculosis is attributed to accumulation of mutants in the drug target genes. These mutations lead either to an altered target e.g. RNA polymerase and catalase peroxidase in Rifampicin and Isoniazide resistance, respectively or to a change in titration of the drug (e.g. in Isoniazide resistance) (Middlebrook, 1954). From the limited information available, initial drug resistance rate range from 0-16% for isoniazide (I), 0.1 – 23.5% for streptomycin (S), 0-0.3% for
rifampicin (R), and 0 – 4.2% for ethambutol (E). Rates of acquired drug resistance range from 4.0 – 53.7%, 0 – 19.4%, 0 – 14.5% and 0 – 13.7% for I, S, R and E respectively (Githui et al., 1992). Previous resistance surveys performed in 1964, 1974 and 1984, indicated that, initial resistance ranged from 7.1% - 8.9% for I, 0.5% - 1.4% for S, and 1.3% - 1.4% for both I and S. Acquired resistance ranged between 16% and 17% for I and between 2% and 16% for both I and S. There was no resistance to rifampcin (Imboden et al., 1993). In addition, data from cohort study of patients with TB carried out at the infectious Disease Hospital in Nairobi showed that resistance to I and S were 9% among patients with HIV 1 infection compared with 5% among non-HIV infected patients (Githui et al., 1992). While this difference was not statistically significant, it does suggest an increasing trend in the HIV-infected population and is a course of concern. Several changes in the management of TB, as well as the increasing prevalence of HIV infection highlighted the importance of assessing ant-tuberculosis drug resistance at Nairobi Hospital environment.

1.2.8.1 Mechanism of drug resistance
Currently, TB is treated with an initial intensive 2 months regime comprising multiple antibiotics – rifampicin (R), isoniazide (I), pyrazinamide (PZA) and ethambutol (E) or streptomycin (S) to ensure that mutants resistant to a single anti-tuberculous drug do not emerge. In the next 4 months only R and I are administered to eliminate any persisting tuberculosis bacilli. Isoniazide and Rifampcin, the two most potent anti-tubercular drugs kill more than 99% of tuberculosis bacilli within 2 months of initiation of effective therapy (Mitchison, 1985). Along with these two drugs, PZA, which has a very high sterilizing effect, appears to act on semi-dominant bacilli not affected by any other anti-tubercular drugs (CDC, 1992). Using these drugs in combination reduces anti-tubercular therapy from
18 months to 6 months. Therefore, the emergence of strains resistant to either of these drugs causes major concern, as it leaves only drugs that are far less effective and have more toxic side effects (Iseman et al., 1989).

The phrase "MDR state" in mycobacteriology refers to simultaneous resistance to at least Rifampicin and Isoniazide (Crofton et al., 1994) with or without resistance to other drugs. Genetic and molecular analysis to drug resistance in MTB suggest that, resistance is usually acquired by the bacilli either by alteration of the drug target through mutation (Spratt, 1994), or by titration of the drugs through over production of the target (Davis, 1994). MDRTB results primarily from accumulation of mutations in individual drug target genes. The probability is very high for less effective anti-tuberculous drugs such as thiacetazone, ethionomide, cepromycin, cyclocerine and viomycin, intermediate for drugs such as isoniazide, streptomycin, ethambutol, kanamycin and P-amino salicylic acid and lowest for rifampicin (Shimao, 1987). Consequently, the probability of mutation is directly proportional to the bacterial load. A bacillary load of 10^9/ml will contain several mutants resistant to any one of anti-tubercular drugs (Grange, 1990).

1.2.8.2 Resistance to Isoniazide

Isoniazide (isonicotinic acid hydrazide, 4-pyridinecarboxylic acid hydrazine), is highly active against the MTB complex (M. tuberculosis, M. bovis, M. africanum and M. microti), with very low MIC of (0.02 ug/ml to 0.06 ug/m (Youatt, 1969). The mechanism of action of isoniazide as well as the mechanism conferring resistance to isoniazide is complex and not completely understood. However, evidence suggest that isoniazide inhibit the biosynthesis of cell wall mycolic acid (long-chain -branched (-hydroxylated fatty acid) thereby making the Mycobacteria susceptible to reactive oxygen radicals and other
environmental factors. Activation of the drug to unstable electrophilic intermediate requires the enzyme system catalase-peroxidase (kat G, encoded by kat G gene) and an electron sink (hydrogen peroxidase) (Bowman et al., 1985), although the hydrazine formed after isoniazide spontaneously decomposes may mediate activation of isoniazide (CDC, 1992). Nevertheless, kat G is the only enzyme capable of activating isoniazide and consequently, kat mutant strains are invariably isoniazide resistant.

Early studies by Middlebrook (1954) demonstrated that isoniazide resistance was associated with loss of catalase activity. Genetic studies demonstrated that transformation of isoniazide resistant M. smegmatis and MTB strains with functional kat G restored susceptibility and put forth the hypothesis that kat G deletion may cause isoniazide resistance in MTB (Allen et al., 1992; Garbe et al., 1993).

1.2.8.3 Resistance to Rifampicin

Rifampicin, first introduced in 1972, as an anti-tubercular drug, is extremely effective against MTB. It has an MIC of 0.1ug to 0.2ug/ml (David et al., 1972). Because of its high bactericidal action, rifampicin along with isoniazide forms the backbone of short-course chemotherapy (Kochi, 1993). However, rare resistance to Rifampicin is increasing because of wide spread application resulting in selection of mutants resistant to other components of short-course chemotherapy. In this study rifampicin is considered as a surrogate marker for the first line anti TB drug. Rifampicin had long been believed to target the mycobacterial RNA polymerase enzyme and thereby kill the organism by interfering with the transcription process (Gubanov et al., 1981). Using purified RNA polymerase from M. smegmatis strain MC2 155, Levin and Hatful demonstrated that rifampicin specifically
inhibited the elongation of full length transcript and had no effect on the initiation of transcription (Hatfull et al., 1993).

RNA polymerase, a complex oligomer composed of four different subunits encoded by rpoA, rpoB, rpoC, and rpoD is highly conserved among bacterial species (Gubanov et al., 1981). Characterisation of the rpoB gene in E.coli demonstrated that, rifampicin specifically interacted with the (-subunits of RNA polymerase, thereby hindering transcription, and that mutations in the rpoB locus conferred conformational changes leading to defective binding of the drug and consequently resistance (Gross et al., 1988). Most mutations were found to be restricted to an 81-bp core region and are dominated by single nucleotide changes, resulting in single amino acid substitutions although inframe deletions and insertions also occur at lower frequencies. Changes in the codons at Ser 531 and His 526 have been documented in more than 70% of the rifampicin resistant isolates (Cooksey et al., 1997). A very small number of mutations in rifampicin-resistant isolates do not map in this 81-bp core region: it is speculated that additional mechanisms, including rifampicin permeability and mutations in sub units of RNA polymerase, may also be involved in conferring the resistance phenotype. The consistency in the rpoB locus and the rifampicin resistant phenotype (>95%) has marked clinical implications (Ahmad et al., 1997). Because it may act as a surrogate marker for anti-TB drugs, rifampicin resistance has prompted development of various diagnostic tests to improve the sensitivity of mutation detection. Although automated sequencing has been unambiguously applied to characterize mutations associated with rifampicin resistance, a number of other techniques such as PCR-SSCP (Eisenach et al., 1995) dideoxy finger printing (Felmlee et al., 1995), heminested PCR,
PCR heteroduplex analysis (Crawford (b) et al., 1992) and line probe hybridization (Benveinster et al., 1973) are used to detect these mutations (Banerjee et al., 1994).

1.2.8.4. Resistance to Ethambutol

Ethambutol [dextro –2, 2’ – (ethylidimino) –di-ionol] is a synthetic compound with profound antimycobacterial effects (Baughan et al., 1961). It is a first line anti-MTB drug with a broad spectrum of activity. Unlike isoniazide, ethambutol is also advocated in disseminated M. avium complex infections, particularly in HIV infected persons (Masur, 1993). Specificity of ethambutol for mycobacterial species indicated that, it targets the construction of outer cell wall. Synergy resulting from co-administration of ethambutol and other drugs gave further evidence for involvement of ethambutol in obstructing the formation of the cell (Goh et al., 1992). The synergistic effect was explained as a consequence of increased permeability of the Mycobacterial cell wall leading to increased drug intake (Goh et al., 1990;). Earlier studies demonstrated that administration of Ethambutol led to rapid cessation of mycolic acid transfer to the cell wall and equally rapid accumulation of trehalose mono-and di-mycolteles (Armstrong et al., 1979).

1.2.8.5 Resistance to Pyrazinamide

Pyrazinamide, a structural analog of nicotinamide, was shown to have considerable anti-MTB activity in 1952 but it became an important component of short-course chemotherapy only in the mid-1980s. Pyrazinamide is active against semi- dominant bacilli not affected by any other drug. It has a strong synergy with isoniazide and rifampicin and shortens the chemotherapeutic schedule for anti-tubercular treatment from 9 to 12 months to 6 months (Kilburn et al., 1989). Depending on the assay system and conditions applied, MIC of pyrazinamide varies from 8ug/ml to 60ug/ml. However, at very high MIC, pyrazinamide
has no significant bactericidal effect and is primarily considered as a “sterilizing drug”. Activity of pyrazinamide is highly specific for MTB and has scant or no effect on other mycobacteria including M. bovis which demonstrate high-level intrinsic resistance to it (Konno et al., 1959). Naturally occurring resistance strains of M. bovis lack the enzyme Pzase that hydrolyses pyrazinamide to pyrizinoic acid, the presumed active form of pyrazinamide (Feldman et al., 1967).

1.2.8.6 Resistance to streptomycin and other inhibitors of protein synthesis
Various drugs exert their antibacterial effects by inhibiting the protein transitional machinery. Among these, aminoglycosides, macrolides, tetracyclines and basic peptides like viomycin and capreomycin are active against mycobacteria (Benveinster, 1973). Streptomycin, one of the oldest drugs known to be active against MTB disrupts the decoding of the aminoacyl-tRNA and thus inhibits mRNA translation or causes inefficient translation (Davies, 1997). One of the most common mechanisms for acquisition of resistance to Streptomycin is acetylation of the drug by aminoglycoside-modifying enzyme (Davies, 1997). However, this mechanism is not found in MTB. Instead, resistance to streptomycin is attributed at least to two distinct classes of mutations including point mutations in S12 ribosomal protein, encoded by rpsL gene (Bottger et al., 1993) and mutations in the riS operon encoding the 16S rRNA (Bange et al., 1994).

1.3 Statement of the problem
Kenya is one of the 22 countries of the world termed as “heavy burden countries” with tuberculosis. About 2.4 million people in Kenya are already infected with HIV/AIDS which is the most known factor fuelling TB disease. Proper preventive and treatment strategies must be put in place in order to reverse the increasing trend of TB. Early
detection is mandatory for early initiation of TB therapy. This therefore requires a quick and effective method of diagnosis, which will also increase the detection rate. Proper treatment strategies increase the cure rate by reducing defaulters. This will also prevent development of resistance.

1.4 Hypothesis

Prevalence of MDTB has increased.

1.5 General objectives

To determine the prevalence of MDRTB using rifampcin as a surrogate marker

1.5.1 Specific objectives

(i) To determine the prevalence of TB by smear microscopy.

(ii) To isolate, and determine the prevalence of TB by Bactec (culture).

(iii) To differentiate between MOTT and MTB by Bactec.

(iii) To determine drug susceptibility on all identified MTB isolate by Bactec.

(iv) To determine the prevalence of MDRTB by PCR and Bactec techniques.

(v) To determine the MDRTB sub-types by PCR.

(vi) To determine some of the risk factors associated with development of drug resistance TB.
1.6 Justification

Multidrug resistant TB is currently the most severe form of bacterial infection. It is associated with a high death rate of 50% to 80%. Studies have shown that the disease is on the increase thereby requiring urgent attention globally. Proper and effective TB control guidelines must be installed as a joint program if the trend of the disease is to be reversed. Update on the prevalence rate and risk factors associated with the development of resistance must be established if proper control measures are to be instituted.
CHAPTER TWO

MATERIALS AND METHODS

2.1 Research Design
The study was conducted at Nairobi Hospital in the department of microbiology, tuberculosis unit and targeted both pulmonary and extra-pulmonary samples for diagnosis of *M. tuberculosis*.

2.2 Study population
The study included both in-patients and out-patients. Out-patient were sent to the laboratory by clinicians where they were given instructions on how to collect the samples and were issued with specimen containers. For in-patients, specimens were collected in the wards and sent to the laboratory. Extra-pulmonary specimens, except urine and blood were collected by the clinicians.

2.3 Sample size
Prevalence of all forms of tuberculosis cases from clinical specimens at Nairobi Hospital record is 23%. The minimum sample size of 272 was calculated by the formula of (Fisher et al, 1998) as follows:

\[ N = Z^2 \times P \times q / D^2 \]

Where *N* = desired minimum sample size; *Z* = Standard normal deviation = 1.96 (from the tailed normal table); *P* = Prevalence of condition under study and *q* =1-\(P\); *D* = Precision required for the study at 95% confidence level =0.05
2.4 Microscopic examination

All samples received in the TB section underwent microscopic examination after staining with Ziehl-Neelsen (ZN) technique (Cheesbrough, 1985). The smears were made on glass slides inside a biological safety cabinet and air dried completely. The smears were then fixed by passing the slide over the flame several times and then flooded with carbolfuchsin stain (see appendix). The slides were heated underneath until fumes come out of the preparation and left to stain for 5 – 10 minutes. The slides were then washed and decolorized with 3% acid alcohol (3ml of concentrated HCl + 97mls absolute alcohol) until no color came out of the preparation. This was followed by counter staining with methylene blue stain, washing with tap water, air-drying and examined under 100x objective for acid alcohol-fast bacilli (AAFB) (Chesbrough, 1985).

2.5 Preparation of specimens for culture by bactec system

The specimens were decontaminated and digested by addition of 4% sodium hydroxide solution (see appendix). All procedures involved in detection of TB were carried out in lamina flow safety cabinet. A portion of specimen was obtained by use of sterile swab and added into a sterile plastic graduated tube. All tubes were labeled according to the identity of the patient. An equal volume of 4% Sodium hydroxide was added, cap tightened, and the tube inverted several times, vortexed and left to stand for 15 minutes. After decontamination and digestion, phosphate buffer pH 6.8 was added to fill the tube. This was to neutralize the sodium hydroxide and stop further reaction. The mixture was then inverted to mix and then centrifuged at 3,000 rpm for 15 minutes. The supernatant was discarded and a small quantity of phosphate buffer added to the sediments to resuspend it.
The concentrate was then used to make the smear for staining and for inoculation into the culture media.

**Principle**

Bactec TB medium (12B) is an enriched middlebrook 7H9-broth base broth. Mycobacterium utilizes a $^{14}$C-labeled substrate (fatty acid) present in the media and release $^{14}$CO2 gas into the atmosphere above the broth medium (Becton and Dickinson Bactec TB system procedure manual). When the broth medium containing the released carbon dioxide is tested on the Bactec 460 instrument, the $^{14}$CO2 released is aspirated from the vial and its radioactivity will be determined quantitatively in terms of a number on a scale from 0 - 999. These numbers are designated as the growth index (GI). The GI numbers are displayed by the Bactec 460 instrument and printed out along with identifying rack and bottle numbers. The daily increase in the GI output is directly proportional to the rate and amount of growth in the medium.

2.5.1 **Inoculation of the decontaminated concentrate sample**

Ten percent (10%) CO2 gas was first added into broth base media culture bottles automatically by the Bactec machine followed by addition of 0.1ml PANTA which is an antimicrobial mixture which contains polymyxin B, Amphotericin B, azlocillin and trimethoprim. PANTA eliminates any contamination that may have by-passed decontamination process. 0.5 ml of the concentrates was inoculated into the bottle base medium, and the bottles were then incubated at 37oC. After incubation the bottles were read after every 4 days for the first four weeks and weekly thereafter for another 4 weeks using Bactec 460 TB.
A GI of above 10 was considered potentially positive and Cultures showing GI of more than 10 were separated from the negative ones for frequent monitoring. Negative cultures (GI less than 10) were re-incubated and reading continued as mentioned above. The GI index of every culture was recorded in a work sheet every time the bottles were read for effective monitoring. Uninoculated control medium bottle was included in every batch of the culture to rule out cross-contamination from one bottle to another during reading (Bactec TB system product and procedure manual).

2.5.2 Screening of cultures with high GI
Once a culture attained a GI above 50, it was read daily until the GI reached 100 or more. A ZN smear was prepared and one drop of culture broth inoculated on to a blood agar (BA) plate and incubated at 37°C to rule out bacterial contamimation. A culture bottle was considered positive if the ZN smear was positive and no growth in the BA plate after 24 hours of incubation. If the ZN smear was positive and growth appeared on the BA plate, culture was considered positive but contaminated with other ordinary bacteria. This type of culture was reprocessed again to eliminate the contaminating bacteria. This was achieved by aspirating all the content of the culture bottle and decontamination performed again followed by inoculation and incubation at 37°C.

2.5.3 Identification of MTB from MOTT
Bactec identification test, help in differentiation of the MTB complex from other atypical *Mycobacterium*. NAP (-nitro-acetylamino-hydroxy-propiophenone), an intermediate compound in the synthesis of chloramphenicol, inhibit *Mycobacterium* belonging to the MTB complex (*M. tuberculosis, M. bovis, M. africanum and M. microti*) almost completely while non-tuberculous TB strains show either slight or no inhibition.
When MTB complex is inhibited in the presence of NAP, the evolution of \(^{14}\)CO\(_2\) is also inhibited, as indicated either by no increase or a decrease in the GI output. This effect on the GI is used as a tool for identification *Mycobacterium* species. The Bactec NAP test vial contains a disc impregnated with 5 \(\mu\)g/ml of NAP.

The test uses non-contaminated positive culture. The procedure was carried out in a biosafety cabinet. Positive cultures were homogenized by vortexing for few seconds to mix. 1ml of the positive culture was aspirated and transferred into the NAP bottle. The culture bottle acted as the control. The two bottles i.e, the test and the control were run through the Bactec machine to introduce 10% CO\(_2\) gas. The bottles were then incubated at 37°C for 2 to 6 days. The GI of both the test and the control were read and recorded daily.

If the GI of the cultures was more than 100, the cultures were diluted as:

<table>
<thead>
<tr>
<th>GI</th>
<th>Dilution Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 – 100</td>
<td>No dilution</td>
</tr>
<tr>
<td>101 – 200</td>
<td>0.8 ml into a fresh 12B vial</td>
</tr>
<tr>
<td>201 – 400</td>
<td>0.6 ml into a fresh 12B vial</td>
</tr>
<tr>
<td>401 – 600</td>
<td>0.4 ml into a fresh 12B vial</td>
</tr>
<tr>
<td>601 – 800</td>
<td>0.3 ml into a fresh 12B vial</td>
</tr>
<tr>
<td>801 – 999</td>
<td>0.2 ml into a fresh 12B vial</td>
</tr>
</tbody>
</table>

After the dilution, 1ml of the diluted culture was then transferred into the NAP test bottle. Before incubation, both the GI of the test and control bottles were read to obtain the base readings. This applied to the undiluted bottles with a GI of between 50-100.
2.5.4 TB drug susceptibility testing by Bactec

The Bactec procedure for drug susceptibility testing of Mycobacterium is based on the same principle employed in the conventional method. The only difference is that a liquid medium is used and instead of counting of colonies after about 3 weeks, the growth is monitored radiometrically and the results are reported within 4 to 5 days. The principle of the radiometric susceptibility assay for Mycobacterium, is similar to the one utilized in the primary isolation procedure. When Mycobacterium grows in 7H12 media containing $^{14}$C-labeled substrate, they utilise the substrate and $^{14}$CO$_2$ is produced. The amount of $^{14}$CO$_2$ detected reflects the rate and the amount of growth occurring in the vial and is expressed in terms of GI. When an anti-tuberculosis drug is added into the broth medium, suppression of growth occurs if the test organism is susceptible. This suppression is detected by either decline or a very small increase of the GI output as compared to the control. However, if the organism is resistant, little or no suppression occurs. The drugs used were streptomycin, isoniazide, ethambutol and rifampicin.

A. Uniform inoculum was prepared by mixing a positive and non-contaminated broth culture thoroughly by vortexing. 12B vials are arranged and labeled accordingly with the names of the drugs used which included, S, I, R, and E. One 12B vial was used as control. 0.1ml of each drug suspension was added into each of labeled Bactec 12B vials. No drug was added to the control vial. 0.1ml of well-mixed positive culture was then added to each of the labeled vial. The control was prepared by first diluting the positive culture 100 times in diluting fluid. This was achieved by taking 0.1ml of the positive culture into a vial of diluting fluid. This was then mixed and 0.1ml of the diluted culture was transferred into a 12B vial. The top of each inoculated bottle was swabbed with cotton gauze pad soaked with 5% phenol followed by cleaning with 70% alcohol. The culture bottles were then incubated at 37°C. The first reading was done after
48 hours and thereafter daily. The final concentrations of the drugs were as follows; streptomycin 6.0 µg/ml, isoniazide 0.1µg/ml, rifampicin 2.0 µg/ml and ethambutol 7.5 µg/ml.

2.6 Detection of rifampicin resistance strain of MTB complex by PCR
The amplification was based on polymerase chain reaction (PCR). The primers used were to amplify the rpoB gene in M. tuberculosis. By heating, the two strands of DNA helix were separated (denaturation) to expose the target sequence to the biotinylated outer primers. These oligonucleotide primers are complimentary to the very conserved regions flanking the target sequence. Therefore, upon cooling to well-defined temperatures, the primers were bound to their specific sequence (annealing). At another temperature, and using the dNTPs, the thermostable DNA polymerase extended the annealed primers along the target template (extension). This way, a biotinylated exact copy of the template sequence was produced after one cycle of denaturation, annealing and extension. This process was repeated for 30 cycles, thus yielding a multi-fold amplified biotinylated target sequence. For detection, the INNO-LIPA Rif. TB technique, a line probe assay for in vitro use was used. This test allows for the detection of Mycobacterium tuberculosis complex and its resistance to rifampicin after amplification of material from clinical specimens. In this study, Mycobacterium tuberculosis complex isolate was used. The INNO-LIPA Rif TB typing test is based on the reverse hybridization principle.

Amplified biotinylated DNA material was hybridized with specific oligonucleotide probes immobilized as parallel lines on membrane-based strip. After hybridization, streptavidin labeled with alkaline phosphate was added and bound to any biotinylated hybrid previously formed. Incubation with Bcip/NBT chromogen resulted in a purple brown precipitate. This
uses INNO-LIPA Rif. TB system manufactured by Innogenetics. 0.5 ml of the culture broth was added into a sterile screw-capped micro centrifuge tube and equal volume of TE (INNo-LiPA Rif. TB procedure manual) buffer added. The mixture was then boiled for 15 minutes at 95°C in order to lyse the cells and expose the DNA. After boiling, the content was cooled and centrifuged for 5 minutes at 13000 rpm. Two microlitres of the supernatant was then used for amplification in a total reaction volume of 50 ml. The following was added in an autoclaved 1.5ml sterile pipette tube.

N X 10μl Amplification Buffer (AB)

N X 10μl Primer Mix (PM)

N X 10μl MgCl2 solution

N X 1 unit of Taq polymerase.

N = number of samples to be amplified + one blank + one control (No DNA added).

Autoclaved distilled water was then added to a total volume of (NX48) μl and 48 μl of this prepared amplification mix added into each labeled autoclaved amplification tube. This was followed by adding 2 μl of the DNA preparation into each amplification tube except the blank tube and 2 drops of mineral oil added. 2 μl of TE buffer was added to the blank tube instead of the DNA, and 2 μl of the amplified DNA from the known positive MTB complex added to the control tube. The DNA preparation in the amplification tubes was put to an initial denaturation step for 5 minutes at 95°C to activate Taq DNA polymerase,
followed by 30 cycles of 1 minute denaturation at 95°C, 1 minute primer annealing at 55°C, 1 minute of primer extension at 72°C and a final elongation step at 72°C for 10 minutes.

After the amplification process, the amplified DNA product was used for the test performance. By use of tweezers the required numbers of strips were removed from the tube and patient identification number indicated with a pencil. A strip for the blank and positive control samples were always included. The required number of test trough (to carry the strips), were placed in the tray after labeling accordingly. This was followed by addition of 10μl of denaturation solution into the upper corner of each tray, 10μl of the amplified DNA product added to the denaturation solution and the mixture mixed by pipetting up and down. Denaturation was left to proceed for 5 min at room temperature (RT). 1ml pre-warmed ready for use hybridization Solution (HS) was added to the denatured amplified product in to each trough and mixed by gentle shaking and immediately the strips were placed on to the trough with the marked side up making sure that they were completely sub-merged in the solution. The strips were hybridized by placing them in a shaking water bath heated at 62°C with the lid closed for 30 min. After hybridization the strips were washed for 20 seconds. This brief washing was repeated once. After washing, the strips were rinsed and 1ml of diluted conjugate solution was added to each trough and incubated 30 min while agitating the tray on the shaker. The strips were then washed twice, using 1 ml diluted rinse solution and once more using 1 ml substrate buffer (SB) followed by 1 ml of prepared substrate solution added to each trough and incubated for 30 minutes while agitating the tray on the shaker. The strips were then removed from the substrate for reading.
2.6.1 Interpretation of PCR results

The uppermost red line is the marker line. The first positive line is the conjugate control line and the second positive line controls for the addition of amplified material for hybridization. This line is positive if the amplified DNA contains MTB complex isolate. That is MOTT reacts negative and therefore were used as negative controls. When all the S-probes (S1, S2, S3, S4 and S5) gave a positive result and when all the r- probes reacted negatively, *M. tuberculosis* was sensitive to rifampicin. When at least one negative signal with the S - probe is obtained M. tuberculosis strain is resistant to rifampicin.

2.7 Determination of risk factors associated with development of drug resistance TB

This was done by face to face discussions with the doctors who saw the patients that tested MDRTB positive. A short questionnaire was prepared that provided the baseline information (see appendix IV).

2.8 Data analysis

Raw data was entered in Excel to determine the prevalence of MTB-complex and that of MDRTB strains.
A total of 700 specimens received for diagnoses of TB were processed for a period of six months. The specimens were both pulmonary and extra-pulmonary. The type of specimens (Table 1) processed were sputum 558 (79.7%), body fluids 62 (8.9%), blood 25 (3.6%), bronchial aspirate lavage (BAL) 21 (3%), urine 16 (2.3%), pus 9 (1.3%), and biopsies 9 (1.3%). Sputum the most frequent specimens had an MTB prevalence of 81 (11.6%) (Table 1). By smear microscopy, the overall prevalence of MTB was 46 (6.6%) (Figure 1a) while culture by Bactec had a prevalence of 91 (13.0%) (Figure 1b).

Of the 91 positive TB cases isolated, one was below 10 years, two were between 10 and 20 years, 44 were between 20 and 45 years, 9 were above 45 years and 35 were identified as adults; gender wise 57 were males, 24 were females and 10 had no gender identity; 36 were hospitalized, 42 were outpatients and 13 were immigrants (Table 2). Out of the 91 positive TB cases, 86 (94.5%) were MTB and 5 (5.5%) were mycobacterium other than tuberculosis (MOTT) (Table 2; Figure 2). Of the 86 MTB, 6 (7%) were resistant to streptomycin, 10 (11.6%) to isoniazide, 8 (9.3%) to rifampicin and 12 (14%) to ethambutol. All the five MOTT isolates were 100% resistant to the four anti-TB drugs (Table 3).

Out of the 86 identified MTB, 8 of them were identified as MDRTB of which 5 were of sub-type R5, 2 of sub-type R4a and 1 of sub-type R2. Seven (87.5%) had previous exposure to TB treatment (secondary resistance) while only 1 (12.5%) had primary resistance (Figure 4). Eighty seven (87.5%) of the MDRTB were HIV positive (Figure 5).
Assessment of the factors associated with multi-drug resistance in 8 patients through follow up and discussions with their physicians revealed that, seven had a previous treatment for TB; five had a history of traveling to various parts of Kenya and outside Kenya and seven were HIV positive (Table 6). The Age distribution of MDRTB were 1 in the age bracket 20-29.5, 6 in the age bracket 30-39.5 and 1 in the age bracket 40-49.5 (Table 5).
<table>
<thead>
<tr>
<th>Types of Specimen</th>
<th>Number of Specimen</th>
<th>Positive ZN smear</th>
<th>Positive Culture by Bactec</th>
<th>Rate of MTB infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum</td>
<td>558</td>
<td>45</td>
<td>81</td>
<td>11.6%</td>
</tr>
<tr>
<td>BAL</td>
<td>21</td>
<td>0</td>
<td>1</td>
<td>0.1%</td>
</tr>
<tr>
<td>Biopsies</td>
<td>9</td>
<td>0</td>
<td>2</td>
<td>0.3%</td>
</tr>
<tr>
<td>Blood</td>
<td>25</td>
<td>0</td>
<td>2</td>
<td>0.3</td>
</tr>
<tr>
<td>Urines</td>
<td>16</td>
<td>0</td>
<td>1</td>
<td>0.1%</td>
</tr>
<tr>
<td>Pus from lymph node</td>
<td>9</td>
<td>1</td>
<td>2</td>
<td>0.3%</td>
</tr>
<tr>
<td>Body fluids (CSF, Pericardial fluids, Effusions)</td>
<td>62</td>
<td>0</td>
<td>2</td>
<td>0.3%</td>
</tr>
<tr>
<td>Total</td>
<td>700</td>
<td>46</td>
<td>91</td>
<td>13.0%</td>
</tr>
</tbody>
</table>

Table I: Prevalence of TB infection by specimen type
Figure 1: Prevalence of TB infection diagnosed by Microscopy and Culture
<table>
<thead>
<tr>
<th>Age (Years)</th>
<th>Total number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Below 10 years</td>
<td>1</td>
</tr>
<tr>
<td>10 – 20 years</td>
<td>2</td>
</tr>
<tr>
<td>20 – 45 years</td>
<td>44</td>
</tr>
<tr>
<td>Above 45 years</td>
<td>9</td>
</tr>
<tr>
<td>Stated as adults</td>
<td>35</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gender</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>57</td>
</tr>
<tr>
<td>Female</td>
<td>24</td>
</tr>
<tr>
<td>Gender not stated</td>
<td>10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Out/In Patients</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Out patients</td>
<td>55</td>
</tr>
<tr>
<td>In patients</td>
<td>36</td>
</tr>
</tbody>
</table>

Table 2: Demographic characteristics of TB positive patients at Nairobi Hospital
Figure 2: Types of TB infections after identification by both PCR and Bactec
<table>
<thead>
<tr>
<th>Isolate type</th>
<th>TB cases</th>
<th>Streptomycin</th>
<th>Isoniazide</th>
<th>Rifampicin</th>
<th>Ethambutol</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTB complex</td>
<td>86</td>
<td>6 (7%)</td>
<td>10 (11.6%)</td>
<td>8 (9.3%)</td>
<td>12 (14%)</td>
</tr>
<tr>
<td>MOTT</td>
<td>5</td>
<td>5 (100%)</td>
<td>5 (100%)</td>
<td>5 (100%)</td>
<td>5 (100%)</td>
</tr>
</tbody>
</table>

**Table 3:** Percentage resistance of TB to individual anti-TB drugs by Bactec technique

**Figure 3:** Prevalence of MDRTB and individual Anti-TB drugs.
Figure 4: Prevalence of MDRTB strains by PCR and Bactec techniques
Figure 5: Types of MDRTB Subtypes by PCR technique
<table>
<thead>
<tr>
<th>Identification profile</th>
<th>Previously treated patients</th>
<th>Untreated patients</th>
<th>Total patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTB wild type</td>
<td>26 (14%)</td>
<td>52 (28%)</td>
<td>78 (42%)</td>
</tr>
<tr>
<td>R5 resistant strain</td>
<td>5 (5.8%)</td>
<td>0 (0%)</td>
<td>5 (5.8%)</td>
</tr>
<tr>
<td>R4a resistant strain</td>
<td>1 (1.2%)</td>
<td>1 (1.2%)</td>
<td>2 (2.4%)</td>
</tr>
<tr>
<td>R2 resistant strain</td>
<td>1 (1.2%)</td>
<td>0 (0%)</td>
<td>1 (1.2%)</td>
</tr>
</tbody>
</table>

Table 4: Prevalence of resistant and wild type strains in previously treated and untreated patients (primary and secondary infection)
<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Residence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1069</td>
<td>23</td>
<td>M</td>
<td>Kisumu</td>
</tr>
<tr>
<td>1151</td>
<td>30</td>
<td>F</td>
<td>Kiambu</td>
</tr>
<tr>
<td>924</td>
<td>32</td>
<td>M</td>
<td>Somalia</td>
</tr>
<tr>
<td>1277</td>
<td>33</td>
<td>M</td>
<td>Machakos</td>
</tr>
<tr>
<td>1305</td>
<td>35</td>
<td>M</td>
<td>Kakuma Refugee Camp</td>
</tr>
<tr>
<td>789</td>
<td>32</td>
<td>M</td>
<td>Somalia</td>
</tr>
<tr>
<td>674</td>
<td>22</td>
<td>F</td>
<td>No history</td>
</tr>
<tr>
<td>1260</td>
<td>45</td>
<td>M</td>
<td>No history</td>
</tr>
</tbody>
</table>

Table 5: Prevalence of MDRTB in relation to age, sex and area of residence
<table>
<thead>
<tr>
<th>Patients</th>
<th>Treatment</th>
<th>Travel History</th>
<th>Present Status</th>
<th>HIV Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1069</td>
<td>Patient Started on treatment but abandoned before completing the course. No reasons for abandoning.</td>
<td>Not Travelled</td>
<td>Died</td>
<td>+ve</td>
</tr>
<tr>
<td>1151</td>
<td>Previously treated. Had financial difficulties in Meeting the cost of the drugs. Abandoned treatment after feeling better.</td>
<td>Has been to India for 3yrs, Mombasa 2yrs</td>
<td>Dead</td>
<td>+ve</td>
</tr>
<tr>
<td>924</td>
<td>Previously treated. Had completed the course of treatment.</td>
<td>Has been to Somalia, Kenya and Uganda.</td>
<td>No Contact</td>
<td>+ve</td>
</tr>
<tr>
<td>1277</td>
<td>No previous treatment</td>
<td>Has been to Kisumu, Mombasa, And Tanzania Lorry driver</td>
<td>Died</td>
<td>+ve</td>
</tr>
<tr>
<td>1305</td>
<td>Previously treated</td>
<td>Refugee at Kakuma Camp</td>
<td>No Contact</td>
<td>-ve</td>
</tr>
<tr>
<td>787</td>
<td>Previously treated – completed treatment</td>
<td>Somalia, Kenya</td>
<td>Died</td>
<td>+ve</td>
</tr>
<tr>
<td>674</td>
<td>Previously treated -No reasons</td>
<td>No History</td>
<td>Died</td>
<td>+ve</td>
</tr>
<tr>
<td>1260</td>
<td>Previously treated – No reasons.</td>
<td>No History</td>
<td>No Contact</td>
<td>+ve</td>
</tr>
</tbody>
</table>

Table 6: Factors associated with drug resistance in patients diagnosed with MDRTB at Nairobi Hospital
Acquired Resistance Primary Resistance

Figure 6: Rate of Acquired and primary Resistance
Figure 7: Rate of MDRTB in relation to HIV status of a patient
<table>
<thead>
<tr>
<th></th>
<th>POSITIVE CONTROL</th>
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<th>NEGATIVE CONTROL</th>
<th></th>
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</thead>
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<tr>
<td></td>
<td>R4a</td>
<td></td>
<td>R4a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R2</td>
<td></td>
<td>R5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R4a</td>
<td></td>
<td></td>
<td></td>
</tr>
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</tr>
</tbody>
</table>
CHAPTER FOUR

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

4.1 DISCUSSION

The high prevalence of 11.6% of MTB in the sputum specimen by radiometric Bactec culture is a reflection of the greater number of patients in the age bracket 15 - 49 years involved in the study. Adults can produce sputum while the production of sputum by children is rare (Lim, 1999). This MTB peak age group compares with the MTB peak age of 25 - 44 years observed by CDCP, (Center for Disease control and Prevention) (1998) in a study done in USA in the period 1993 - 1994. The lower number of MTB positive cases between 5 - 15 years could be due to a form of immunity which could be related to hormonal regulation of the immune response in this "golden age group" (Lim, 1999). The elderly and the very young are more susceptible to TB infections. The 1.3% prevalence of extra pulmonary MTB was within the expected prevalence of less than 15% (Lim, 1999).

The study indicated that the ratio of men to women infected with TB is 1:2.4. That more men were infected with TB than women in this study is in agreement with the data from the national morbidity survey in Bangladesh (1994-1995) which showed a male: female sex ratio of 2:1 (Holmes et al., 1988). In Sri Lanka and Thailand a similar male: female sex ratio of 2:1 was also observed (Murray et al., 1996). In Bhutan however, equal number of males and females were reported to be affected by tuberculosis (Murray et al., 1996). Under notification may contribute to the low reported prevalence of TB in women.

This may be attributed to women having poorer access than men to TB diagnostic services and hence effective treatment. Among the reasons which may lead to this, are lack of time
and resources and lack of decision making power (Casseles et al., 1993). Factors like occupation such as travelling and interactions which take place more in men than women may contribute to high prevalence of TB in men (WHO, 1998).

Sex differences in the prevalence of tuberculosis may vary across age groups. The study indicates that the highest burden of TB falls in the age group 20-45 years which comprises 51% of the cases. According to a population-based survey conducted by (Gathio et al., 1974) in 119 randomly selected villages in Bangalore district in India between 1961 and 1968, there was no sex difference in TB prevalence rates for study subjects below 14 years. Beyond this age (>14 years), the prevalence rates for males were 20% to 70% higher than for females. However, age and sex-specific data on reported cases of TB in 28 hospitals in Bhutan showed a higher proportion of female than male in the age group 0-14 (1 male to 1.3 females) and a higher proportion of males than females in the age group 15 years and above.

The 13% overall prevalence of MTB in this study in both pulmonary and extrapulmonary specimen is higher than the one reported by (Githui et al., 1993) of 0.1 - 0.3%. The lower prevalence (6.6%) of MTB by microscopy relative to that of radiometric Bactec culture could be explained by reduced sensitivity of microscopy especially if the bacterial load is low in specimens.

Microscopy is only able to pick the MTB bacillus if a specimen contains more than 5000 organisms / ml (Lim, 1999) while the radiometric Bactec culture can detect as few as 10 organisms / ml (CDC, 1990). Considering the fact that, more than 95% of our TB diagnostic delivery services rely on microscopy smear techniques we therefore say that half
of TB cases are missed out. These patients go untreated and they continue to spread the disease to the community. Other patients are put unnecessarily on TB treatment if the clinician has to go by the clinical presentation of the patients where the patient is treated empirically. Systems and approaches to improve smear microscopy technique need to be established. The low sensitivity is reflected by the high rates of smear negative pulmonary TB cases and not having the targeted suspects (patients) for diagnosis of TB (CDC, 1990).

The diagnosis of MTB by microscopy is made more difficult than ever before especially among the HIV / MTB cases with low concentrations of bacilli in their pretreated sputum specimen and who may therefore have negative sputum smear (Brindle et al., 1993). Smear negative TB patients are capable of spreading 40-60% cases of TB (Lim, 1999).

The radiometric Bactec culture proved to be more sensitive than smear microscopy by picking twice the number of cases that of smear microscopy. Although it is a fairly sensitive method its advantage may not be enjoyed by many because the cost of the test is beyond the reach of many.

The observed prevalence of monoresistance to isoniazide (11.6%), rifampicin (9.3%), streptomycin (7%) and ethambutol (14%) is higher than that reported by Migliori et al. (2004) in Italy during the period 1998 - 2000 of 3.5%, 0.8%, 4.3%, and 0.5%, respectively for patients with no history of previous TB treatment (primary) and 5.3%, 4.3%, 4.3% and 0.3% respectively for TB patients with history of previous treatment (CDC, 1998). However, the MTB monoresistance to these four drugs was lower than that reported by Deodha et al. (1999) in India of 30.4%, 58.6%, 47%, and 3.7%, respectively. These investigators also observed MTB monoresistance to second line anti-TB drugs (cycloserine (24.3%), kanamycin (14.4%), ethionamide (60.7%), amikacin (15.6%) and ciprofloxacin
(7.5%). (Githui et al., 1993) reported primary monoresistance prevalence of isoniazide of 10.2% and that of streptomycin of 1.8% and no resistance to rifampcin. The same investigators reported resistance to both isoniazide and streptomycin of 2.4%. (Githui et al., 1999) reported a decreased primary isoniazide monoresistance prevalence of 5.3% and that of both isoniazide and streptomycin of 1%. They also reported acquired isoniazide monoresistance of 30.4% and that of both isoniazide and streptomycin of 6.5% but observed no resistance to ethambutol and rifampicin. Resistance surveys performed in Kenya in 1964, 1974, and 1984 revealed primary monoresistance to isoniazide of 7.1 - 8.9 %, streptomycin of 0.5 - 1.4 % and for both isoniazide and streptomycin of 1.3-1.4% and acquired monoresistance to isoniazide of 16 - 17%, both isoniazide and streptomycin of 2 - 16% but no resistance to rifampicin (Githui et al., 1999).

Both the 8.1% acquired prevalence and 1.2% primary prevalence of MDRTB in this study is higher than that reported by (Githui et al., 1993) of 0.3%. This increase in MDRTB prevalence in this study could be due to increased prevalence of HIV infection which has been picked as an important risk factor for development of TB which itself is a risk factor for MDRTB. Another factor is improper use of antibiotics in chemotherapy of drug-susceptible TB patient. This improper use is as a result of a number of actions, including administration of improper treatment regimens by health care workers and lack of direct observation of patients during treatment. Essentially, drug resistance arises in areas with poor TB control and treatment programs (Afrain et al., 1997). That HIV is a major contributor of MDRTB is supported by the observation in this study that 87.5% of the MDRTB cases were both HIV and TB positive and had previously been on anti-TB drugs. HIV may lead to MDRTB partly because of the circulation of MDR-TB strains in HIV.
sero-positive communities although the association may occur because the contribution of the immune system in eradicating bacteria during chemotherapy is crucial in preventing the emergence of resistance. Also patients with tuberculosis and HIV may be unable to absorb their drugs due to concomitant gastrointestinal disease, exposing the organisms to subtherapeutic concentrations. Extra pulmonary disease is more common in case of HIV infection and this may provide the opportunity for the growth of organisms in protected compartments (Gillespie, 2002). That these patients were immuno-compromised is also supported by the 5.5% prevalence of MOTT which are indicators of AIDS opportunistic infections (Richter et al., 2002). That HIV increases the number of MTB cases is supported by the report of CDC (1998) of a high prevalence of MTB cases (11.3%) in HIV infected MTB patients than in HIV negative MTB cases (5.5%). HIV seropositive patients are more likely to develop acquired MTB resistance than seronegative cases (Nolan et al., 1995; Bishai et al., 1996; Bradford et al., 1996; Gooze and Daley, 2003). In a case control study involving 16 cases of acquired drug resistance in San Francisco between 1990 and 1994, AIDS, non-adherence to tuberculosis treatment regimen, and gastrointestinal symptoms were each independently associated with the acquisition of drug resistance (Bradford et al., 1996). During the study period one of every 16 AIDS patients with tuberculosis had either gastrointestinal symptoms or nonadherence developed acquired drug resistance; mostly rifampicin monoresistance tuberculosis.

Infections with non-tuberculous mycobacteria such as *M. avium*, *M. xenopi*, *M. kansaii*, *M. gordonae*, *M. fortuitum*, *M. chelonae*, *M. malmoense*, *M. marinum* and *M. celatum* occur in patients who have low CD4+ counts (less than 50) or high HIV virus counts. *Mycobacterium avium complex includes the species M. avium and M. intracellulare*, with
M. avium consisting of M. avium subspecies avium, M. avium subspecies sylvaticum, and M. avium subspecies paratuberculosis (Richter et al., 2002). Inadequate therapy may lead to selection for drug resistant mutants of M. tuberculosis in susceptible population, prolonged infectiousness of patients and increased transmission of drug resistance strains and further resistance among patients already sick with drug resistant TB. MDRTB is caused by accumulation of mutations in individual drug target genes including rpoB gene for rifampicin, katG and /or oxyR-ahpC gene for isoniazide, rpsL gene for streptomycin and emb CAB gene for ethambutol or to a change in titration of the drug through overproduction of the target such as InhA in isoniazide resistance.

In addition to accumulation of mutations in the individual drug target genes, the permeability barrier imposed by the MTB cell wall can also contribute to the development of low-level drug resistance. As a result of the spread of HIV, an estimated 1.4 million cases of active TB are expected to occur annually in people infected with HIV worldwide (Bottger et al., 1992). In this study PCR identified rifampicin MDR-TB at S2, R4a, and S5 regions of the MTB genome. MDRTB can be prevented by the use of directly observed therapy (DOT), which ensures patient compliance and cure as well as preventing the development of drug resistance (WHO, 2000). DOT consists of closely supervised treatment to make sure that every dose is taken to the end of the treatment period.

4.2 CONCLUSION

- The study indicated TB decline from 23% to 13% while that of MDRTB has increased from 2% to 9.3% at Nairobi Hospital.
• The 87.5% of MDR-TB infection observed in this study is man-made (secondary infection) and therefore could be avoided.

• HIV, and inadequate treatment are major risk factors for fuelling TB and MDRTB

• Smear microscopy diagnostic technique is insensitive and inadequate.

4.3 RECOMMENDATIONS

• Smear microscopy technique need to be improved to increase its efficiency.

• WHO recommended treatment strategy of DOTS (direct observed treatment) should be adopted in all Health care facilities on all patients with active TB to ensure treatment completion.
REFERENCES


APPENDICES

Appendix I

ZN Reagents and staining procedures

(Solution 1)

Basic fuschin ................................................... 3gm

95% ethanol ................................................... 100mls

Dissolve 3 g of basic fuschin in 100ml technical grade ethanol.

ZN Reagents and staining procedures

(Solution 2)

Phenol crystals ................................................. 5gm

Distilled water ...................................................... 1000ml

Dissolve 5g phenol crystals in 1000 ml distilled water and heat gently.

Working solution.

Solution 1 ......................................................... 10mls

Solution 2 ......................................................... 90mls

Decolorizing agent: 3% acid alcohol

Concentrated HCl (technical grade) ......................... 3mls
95% ethanol.................................................................97mls

**Counter stain : Methylene blue**

Methylene blue..........................................................0.3g

Distilled water..............................................................100mls

**ZN staining procedure**

Clean, dry grease free slides were marked with identity of the patient using a diamond pencil.

Smears were made on the slide over an area of approximately 2.0 cm by 1.0 cm. Two smears per patient were made.

The smears were allowed to air dry for about 15 minutes.

The smears were then fixed on the slide by passing them through a flame three or four times with the smear facing uppermost.

The slides were then placed on staining rack then flooded with Ziehl-Neelson carbol fuchsin that was filtered prior to use.

The slides were then heated underneath unstill the stain steamed. The steaming was maintained for 3-5 minutes by using slow heat.

Each slide was then rinsed individually in a gentle steam of running tap water until all free stain was washed away.
The slides were then decolorized with 3% acid alcohol for a maximum of 3 minutes.

This was followed by rinsing the slides with running water.

The slides were then flooded with methylene blue counter strain for 60 seconds, rinsed with water and allowed to air dry.

After drying the slides were then examined using 100-x objective.

Minimums of 300 fields were examined before a smear was reported negative.

The report of the smear was then recorded against the patient’s identity (Middlebrook, 1954)

Appendix II

Slides scoring format by WHO method of reporting

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 -</td>
<td>in 100 fields no AAFBs seen (negative)</td>
</tr>
<tr>
<td>1 - 9</td>
<td>in 100 fields exact number of AAFBs were reported</td>
</tr>
<tr>
<td>10 - 99</td>
<td>for 100fields +</td>
</tr>
<tr>
<td>1 - 9</td>
<td>per fields ++</td>
</tr>
<tr>
<td>Over 10</td>
<td>per field +++</td>
</tr>
</tbody>
</table>

Storage of slides after examination

After examination the slides were the put in xylene for 5 minutes to remove oil and then kept in a safety cabinet for future reference.
Reagents for the decontamination of specimens before culture by Bactec

Sodium hydroxide (NaOH)................................. 4%

Phosphate buffer.............................................. pH 6.

Appendix III
Bactec set up and procedure

10 ml plastic centrifuge tubes were used and equal volumes of NaOH and the specimen added.

The content was then vortexed and left for 15 minutes for decontamination to take place.

After the digestion and decontamination step, phosphate buffer pH 6.8 was added to top up the centrifuge tube.

The content was the concentrated by centrifuging at a speed of 4000 RCF for 15- minutes.

After centrifugation, the supernatant fluid was decanted and a small quantity of phosphate buffer pH 6.8 (1-2 ml) added to resuspend the sediment with Pasteur pipettes.

0.5 µl of the concentrate was then inoculated into the culture media and incubated.

Appendix IV
Questionnaire

As per our telephone conversation regarding the study on prevalence of MDRTB, i will be grateful if you could provide me with some information of the below mentioned patient
Doctor’s name

Patient’s name

Date: 

A: Age of the patients

B: Patients occupation

C: Treatment compliance: if not compliant please indicate reasons

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