BIOCHEMICAL CHARACTERIZATION OF ANTIMICROBIAL SENSITIVITIES OF MYCOBACTERIA CAUSING PULMONARY TUBERCULOSIS IN HIV AND NON-HIV POSITIVE PATIENTS

BY

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DECLARATION

I declare that this study was carried out by me at the Department of Microbiology, under the joint supervision of Prof. Mercy J. Newman, head, Department of Microbiology, University of Ghana Medical School, and Dr. Kwasi K. Addo, Research Fellow, Noguchi Memorial Institute for Medical Research.

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DEDICATION

This work is dedicated to all tuberculosis patients in Ghana. May you be helped by all the efforts being made.

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ABBREVIATIONS

AFB	Acid Fast Bacilli
AIDS	Acquired Immune Deficiency Syndrome
CFU	Colony Forming Units
DOTS	Directly Observed Treatment, Short Course
HIV	Human Immunodeficiency Virus
INH	Isoniazid
KOH	Potassium Hydroxide
LJ	Lowestein Jensen
MAC	Mycobacterium Avium Complex
MDR	Multi-drug Resistance
MOTT	Mycobacteria Other Than Tuberculosis
MTB	*Mycobacterium tuberculosis*
NACP	National Aids Control Programme
NALC	N-acetyl-L-cysteine
NTM	Non-tuberculous mycobacteria
NTP	National Tuberculosis Control Programme
PAS	Para-amino salicylic acid
PNB	Para-nitrobenzoic acid
TB	Tuberculosis
TCH	Thiophene-2-carboxylic acid Hydrazide
WHO	World Health Organization

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ABSTRACT

The purpose of this study was to find out the species of mycobacteria causing pulmonary tuberculosis in HIV and non-HIV seropositive patients in Accra, Ghana, and to determine the drug susceptibility patterns in the two groups of patients.

From November 2002 to March 2003, new patients attending the Chest Clinic of the Korle-Bu Teaching Hospital and diagnosed with tuberculosis by sputum smear microscopy were enrolled. Patient sputum was cultured and drug susceptibility testing done for Isoniazid (I), Rifampicin (R), Ethambutol (E) and Streptomycin (S).

HIV/AIDS testing was also carried out for consenting patients. In all 96 patients were enrolled.

Of the 92 patients who were tested for HIV, 22 (23.9%) were seropositive for the virus.

Sixty-six isolates were obtained, out of which 61 (92.4%) were *Mycobacterium tuberculosis* complex strains while the remaining were environmental mycobacteria. For the *Mycobacterium tuberculosis* complex strains, overall drug resistance (resistance to one or more drugs) was 50.8% (95% CI 38.3-63.3), with an initial multi-drug resistance rate of 4.9%. Percentage resistances to the various drugs were Isoniazid (4.9%), Ethambutol (24.6%), Rifampicin (26.2%) and Streptomycin (34.4%).

There was no significant association between HIV seropositivity and sputum smear results, drug susceptibility or species of mycobacteria isolated. It was however found that environmental mycobacteria were more likely to be resistant to streptomycin ($p=0.04$) and rifampicin ($p=0.01$).

The study found high initial resistance to the first line drugs for treating tuberculosis in Accra and its surrounding towns.

CHAPTER ONE

INTRODUCTION AND BACKGROUND

1.1 INTRODUCTION

Tuberculosis, an infectious disease caused by a number of species of mycobacteria, is a disease of public health importance. Each year, there are 8 million new cases of tuberculosis and nearly 3 million deaths worldwide.(WHO 2002) The advent of HIV-AIDS has worsened the situation due to the low cellular immunity in such patients. Human tuberculosis is traditionally caused by organisms in the *Mycobacterium tuberculosis* complex namely, *Mycobacterium tuberculosis*, *Mycobacterium bovis* and *Mycobacterium africanum*.

In the United States, up to 95% of cases were due to *M. tuberculosis* before the advent of HIV. With the advent of HIV-AIDS, a number of other *Mycobacterium* species (called atypical mycobacteria) had emerged as important pathogens each with different disease causing potential and often unique antimycobacterial drug susceptibility patterns, which must be determined by laboratory tests. Notables among these organisms are *Mycobacterium avium intracellulare* complex (MAC) and *Mycobacterium kansasii.* Currently in the US, only *M. tuberculosis* is being isolated more than *Mycobacterium avium intracellulare* complex (MAC)(Koneman 1992)

Multi-drug resistance (MDR) in tuberculosis is a common phenomenon. Studies have shown it to be 1.6% in the USA, 1.1% in the UK, 4.6% in Argentina and

14.4% in Latvia. Other studies done in France, and the Netherlands show significant drug resistance.(Robert 2000; Geerlings 2000). Data from African countries including Ghana on biochemical characterization of mycobacteria are very much limited. In sub-Saharan Africa, 17-55% of proven tuberculosis patients are HIV positive. (Aris 1999; Torrens 2001)

Postmortem studies done on 93 HIV patients in Abidjan, Ivory Coast found 41 having disseminated tuberculosis.(Lucas 1994) Although this study did not characterize the species causing disease, studies in the United States have shown that such disseminated tuberculosis in HIV patients are typical of MAC.(Krance 1996).So far as drug susceptibility patterns are concerned, recent studies have been carried out in many West, Eastern and Southern African countries.(Kuaban2000; Warndorff 2000; Toloba 2000; Glyn 1995; Yang 1995) The drug resistance vary in all these countries with multi-drug resistance ranging from 4.7% in Kampala, Uganda (Joloba 2000) to 27.6% in Yaounde, Cameroon(Kuaban, 2000)

In Ghana the National Tuberculosis Programme estimates 55,000 new cases every year with over 25,000 deaths.(Bonsu, 1997). In many developing countries including Ghana, patients with tuberculosis are generally treated with a combination of drugs after diagnosis based on history, physical examination, positive sputum smears showing acid fast bacilli and (or) characteristic chest radiograph.

Mycobacteria cultures are not done prior to treatment. Current data on sensitivity patterns for mycobacteria is lacking in Ghana. In a study done in 1985-87, Van der Werf and associates isolated 57 *Mycobacterium tuberculosis* and 42 *Mycobacterium africanum*. The situation could be very different now. They also found high primary resistance to isoniazid, (27%), streptomycin,(23%) and to thiacetazole(29%) Only 45% of the isolates were sensitive to all three drugs. (van der Werf 1989). Primary resistance to rifampicin, pyrazinamide or ethambutol was not observed. This study will find out the current primary resistance situation in Accra, Ghana.

Patients who are diagnosed with tuberculosis and who harbour organisms resistant to one or more anti-tuberculosis drugs but have never been previously treated for tuberculosis or have been treated for less than one month are said to have primary resistance. On the other hand patients with a history of previous anti-tuberculosis treatment lasting at least one month and have developed resistance are said to have secondary or acquired resistance.

It must be stated that Van de Werf and associates carried out their study in the Agogo district of Ghana and this might not be representative of the whole country. It is however a fact that physicians treating tuberculosis in Ghana have been reporting of treatment failures in a number of patients. The National Tuberculosis Programme (NTP) has been concerned about this development

and has now directed that culture and sensitivity be done for all treatment failures before considering the use of second line drugs.

The relationship between HIV sero-positivity and multi-drug resistance is yet to be confirmed. While studies done in the United States suggest that HIV patients are more likely to have multi-drug resistance tuberculosis, similar studies in Africa have failed to show such a relationship (Joloba 2000; Glyn 1995). This could be due to differences in species in the two regions. This study will find out the current situation in Ghana.

1.2 IMPORTANCE OF THE STUDY

From the foregoing, this study is long overdue and important because it will determine:

- the species of mycobacteria causing tuberculosis in Ghana especially in HIV seropositive patients. When the percentage of atypical mycobateria causing tuberculosis in this country is known, it will add to epidemiological knowledge on the tuberculosis in Ghana and help in control efforts.
- current drug susceptibility patterns, which is crucial for any tuberculosis control programme. Without such knowledge one will always have to treat tuberculosis for several months before realizing that a patient is not responding and therefore a different drug combination is necessary.

- whether there is significant difference in drug susceptibility profiles of mycobacteria isolated in HIV and non-HIV seropositive patients. A difference may mean different drug combinations for the treatment of the two groups.

1.3 HYPOTHESIS

The hypothesis for this study is that mycobacteria species causing pulmonary disease in HIV patients in Ghana are significantly different from those causing disease in non-HIV patients; and the two groups have different antimicrobial sensitivity patterns.

1.4 AIM

The main aim of the study is to determine and compare the species and antimicrobial sensitivity patterns of mycobacteria causing pulmonary tuberculosis in HIV and non-HIV positive patients

1.5 OBJECTIVES

The objectives of this research are:

1. To isolate mycobacteria from HIV positive and non-HIV tuberculosis patients
2. To do biochemical characterization on the isolates to find the prevalence of various mycobacteria species causing pulmonary tuberculosis in Ghana.

3. To determine the sensitivities of the isolates to current conventional anti-tuberculous drugs using conventional methods
4. To find out whether there is a significant difference in species causing pulmonary tuberculosis in HIV and non-HIV patients.
5. To find out if there are significant differences between the antibiograms of mycobacterial isolates from HIV and non-HIV seropositive patients.
6. To make appropriate recommendations to clinicians and policy makers.

CHAPTER TWO

LITERATURE REVIEW

2.1 HISTORY OF TUBERCULOSIS

2.1.1 Origins

Tuberculosis was a common disease in ancient Egypt even as early as 4000BC. Changes of ulcerating spines have been found in Egyptian mummies. One famous mummy of an Egyptian high priest still has the track of pus discharging from a spinal abscess along the course of the psoas muscle, a finding which only occurs in tuberculosis. (Ryan 1992). Some authorities speculate that the epidemic form of tuberculosis really began in the first cities of the ancient world, which means that the ancient Egyptians may in addition to civilization, bequeathed the world this disease.

2.1.2 Modern History

About the turn of the nineteenth century, the death rate worldwide due to pulmonary tuberculosis was estimated at seven million people per year with fifty million openly infected, with London and New York being two of the world's most affected cities. During this period, there was fear that the disease might destroy European civilization. (Ryan 1992)

The cause of tuberculosis was not known till 24th March 1882, when Robert Koch announced the discovery of the bacillus. The occasion for this

announcement was a meeting of the Physiological Society of Germany with famous scientists like Rudolf Virchow and Paul Earlich in attendance. When Koch died in 1910, the search for a cure for tuberculosis fell into a decline for more than 30 years. (Ryan 1992)

2.2 MICROBIOLOGY OF TUBERCULOSIS

2.2.1. Taxonomy

The name mycobacterium means "fungus-like bacterium" and arose from the characteristic fungus-like pellicle produced by the tubercle bacillus when grown on liquid media. (Collins 1985). The genus Mycobacteria is the only genus in the family Mycobacteriaceae. The genus includes a number of species; some are pathogenic for man or animals, some are opportunistic and others are essentially saprophytic. Currently there are over 71 recognized species of mycobacteria. The minimal standards for including a species in this genus are:

1. acid-alcohol fastness (i.e. resist decolorization by acidified alcohol after being stained with a basic fuschin dye).
2. the presence of mycolic acids containing 60-90 carbon atoms which are cleaved to C22-C26 fatty acid methyl esters by pyrolysis and
3. a G+C content of the DNA of 61-71mol%. (Good and Schinnick 1998)

A natural division occurs between slowly and relatively rapidly growing species. Slow growers generally require more than seven (7) days to produce easily seen colonies on solid media while fast growers grow in less than

seven days. Most fast growers will only cause disease in severely immunocompromised individuals.

2.2.2. Morphology and Staining

The tubercle bacilli are slender, sometimes slightly curved rods, 0.2 to 0.6 um in diameter and 1 to 4 um in length. They are non-motile and non-sporing. The granular structure of the individual cells is marked. Vacuoles often occur in abundance and may even give the stained cell the appearance of a chain of cocci. The significance of the small, deeply staining bodies sometimes observed within the cells has been variously interpreted but they are believed to be granules of polyphosphate. Lipid vacuoles, which stain with lipophylic dyes, are also observed.

The tubercle bacilli cannot be stained by the Gram stain method. The cells may be stained in two or three minutes by steaming strong carbol fuchsin or by 18 hours exposure to the dye at room temperature. Once stained the bacilli are difficult to decolorize and resist the action of alcohol and dilute solutions of mineral acids and for that reason are termed "acid fast" The bacilli may be demonstrated by Ziehl-Neelsen staining in which the smear is stained with hot carbol fuchsin, decolorized with acid alcohol (3% H_2SO_4 in 95% ethanol), and counterstained with a dye of contrasting colour. Methylene blue is commonly used and the bacilli are seen as red in a blue background. This is the method recommended by the WHO and the International Union against /Tuberculosis

and Lung Disease (IUATLD) for low income countries. This method, unlike the fluoresescence microscopy with auramine O, provide consistently good results without need for special equipment, and the required binocular microscope can be used for other purposes as well. (Rieder 1998).

The bacilli may also be stained with carbol-auramine, a dye that fluoresces as brilliant yellow in weak ultraviolet light. (Freeman 1979). However the sensitivity and specificity of acid-fast stains are not optimal. Approximately 10^4 bacilli per ml of specimen are required for a positive result; therefore, smear-negative, culture-positive results often occur.(Sepkowitz 1995).

The sensitivity (proportion of actual cases identified) of sputum smear microscopy in the diagnosis of pulmonary tuberculosis is far from perfect. With diligent technique, approximately 60% of all adults with pulmonary tuberculosis can be identified this way and the proportion is perhaps even lower among patients co-infected with the HIV virus and very low among children. (Rieder 1998, ASM 2002).

2.2.3 Physiology

Mycobacteria are obligate aerobes though M. *bovis* may be microaerophilic. Growth is slow or very slow. Visible colonies appear in 2-60 days at optimum temperature. *Mycobacterium tuberculosis* for instance has a generation time of 16 hours at 37°C and need incubation for 4-6 weeks to see visible colonies while

fast growers like *M. fortuitum* or *M. chelonei* have a generation time of 2 hours and require less than one week to see visible growth. (Kingsbury 1985).

2.2.4. Media

Recovery of mycobacteria from their normal habitats is dependent on providing the conditions that are necessary for optimal growth. Currently three media are routinely used in most laboratories for primary isolation, drug susceptibility testing, biochemical tests and determination of colonial morphology. Lowenstein-Jensen (LJ) medium is most commonly used for growth of all mycobacteria. The medium is prepared with glycerol for the cultivation of M. *tuberculosis* or with sodium pyruvate for the cultivation of M. *bovis,* which grows poorly or not at all on the medium prepared with glycerol. Middlebrook 7H10 and 7H11 medial are agar based and used in many laboratories for the general colonial morphology. Other available media include Ogawa, American Thoracic Society (ATS) medium and Petragnani medium. All the media mentioned are egg based except Middlebrook which is agar based. (Good, 1989). In addition to these, there are also liquid media for culturing mycobacteria, these include Dubos and Davis medium and Selective Kirchner medium (Laidlaw 1989). These liquid media give uniform dispersed cultures but could easily produce infective aerosols.

Colonies on solid media may appear as either smooth or rough transparent or opaque. Colonial morphology is often suggested as a feature for identification; however, too much variation occurs in this characteristic for it to be used except

as an indication of what the species might be. Characteristically, strains of *M. tuberculosis* form cords when growing on solid media.

When colonies are suspended in liquid or when they are grown in liquid media, the cording characteristic can be seen clearly in stained preparations. Colonies of tubercle bacilli on solid medium typically appears as intertwined cords that are heaped up and dry and that may often have a smoother veil of growth surrounding the central formation. This eugonic colony form has often been described to have the appearance of bread crumbs and is in contrast to the dysgonic growth of *M. bovis* on a medium that contains glycerol. When grown on pyruvate containing medium, *M. bovis* growth improves. Colonies of *Mycobacterium avian* complex strains are smooth and transparent in primary cultures of clinical specimens but opaque and rough in subcultures.

The BACTEC radiometric broth system has been an important addition to culture methods. The system uses Middlebrook 7H12 broth with [^{14}C]palmitic acid, and growth of mycobacteria is detected by an instrument that measures radio-labeled CO_2 which is released when the organism metabolizes the palmitic acid. The system includes a decontaminating solution, containing polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin, (PANTA) and a growth factor, polyoxyethylene stearate (POES). In an early evaluation of smear-positive specimens, the BACTEC system detected M. *tuberculosis* in an average of 8.3 days compared with 19.4 days for conventional agar or egg-based media. The

sensitivity of the BACTEC system was 96.4%, compared with 91.3% for conventional media. (Roberts et al 1983)

The Septi-Chek AFB system (Becton-Dickinson Microbiology Systems, Cockeysville, Md.) is a biphasic single-bottle system that combines a paddle containing 7H11, modified egg based, and chocolate solid media with a bottle containing 20 ml of 7H9 broth and an internal CO_2 source. Solutions with antimicrobial agents and growth factors are also provided. In two studies, Septi-Chek was as sensitive as BACTEC for the recovery of *M. tuberculosis* (95.7 and 95.9% for Septi-Chek compared with 93.3 and 95.0% for BACTEC) but the organisms were detected later by Septi-Chek (21.8 and 19.1 days with Septi-Chek versus 18.5 and 13.4 days with BACTEC) (Abe 1992, Isenberg 1991)

Blood culture systems used primarily for detection of *Mycobacterium avium intracellulare* bacteremia also recovers *M. tuberculosis*. The Isolator lytic system (Wampole Laboratories, Cranbury, N.J.) can be used to quantitate mycobacteremia. Lysed blood from the Isolator collection tube is inoculated onto agar plates (e.g., 7H11); the colonies that develop can be counted; and the number of CFU per milliliter of blood can be calculated. Quantitative changes can be used to monitor the effect of antimycobacterial therapy. Radiometric 13A broth blood culture bottles can be used as part of the BACTEC TB system. The Isolator and BACTEC systems were shown to be approximately equal in sensitivity and time to detection of mycobacteremia (Kiehn 1988).

2.2.5 Biochemical methods for identification of Mycobacteria

The identification of mycobacteria depends on a battery of biochemical tests. Some of these include

1. Pigmentation. Determining whether a *Mycobacterium* specie isolate is capable of producing colony pigmentation in the dark (scotochromogens) or only after exposure to light (photochromogens) or not at all are helpful characteristics in making a final species identification. Members of the *mycobacterium tuberculosis* complex fail to produce any pigment. *M. kansasii* is photochromogen (yellow color) while *M. xenopi* is a scotochromogen.
2. Niacin Accumulation. Niacin is formed as a metabolic by product of all mycobacteria but most species possess an enzyme that converts free niacin to niacin ribonucleotide. *M. tuberculosis, M. simiae* and occasional strains of *M. marinum/M.chelonei/M. bovis* lack this enzyme and accumulate niacin as a water soluble by-product in the culture medium. The amount of niacin present in culture slant is in part a reflection of the number of colonies on the slant and the age of the culture. Reagent impregnation paper strips are used. The development of a yellow colour in the test medium incubated with reagent strip is indicative of niacin accumulation and positive test.
3. Reduction of Nitrate to Nitrite. Only few mycobacteria notably *M. tuberculosis* produce nitroreductase, which catalyses the reduction of nitrate to nitrite. The development of a red colour on addition of sulfanilic

acid and n-naphthylethylene-diamine to an extract of the unknown culture is indicative of t he presence of nitrite and a positive test. *M. kansasii, M. szulgai* and *M. fortuitum* are also nitrite positive.

4. Growth on Thiophene-2-carboxylic acid Hydrazide (TCH). This test is very useful in distinguishing *Mycobacterium bovis* and *M. tuberculosis*. TCH has the ability to inhibit growth of *M. bovis* while M tuberculosis grows on it. It has been found however that some variants of *M. tuberculosis* (Asian Human Variant) is sensitive to TCH. (Collins 1985).
5. Pyrazinamide Reaction. The deamination of pyrazinamide to pyrazinoic acid in 4 days is a useful physiologic characteristic by which *M. marinum* (positive) can be differentiated from *M. kansasii*(negative) and by which weakly niacin positive strains of *M. bovis* (negative) can be distinguished from M. tuberculosis and *Mycobacteria avium* complex (both positive). The deamination of pyrazinamide to form pyrazinoic acid produces a red band in the culture medium.

2.2.6. Identification of Mycobacteria using modern methods

1. Nucleic Acid Probes

Cultures of mycobacteria can also be identified with DNA probes on the basis of the principle that complementary nucleic acid strands bind to form stable double-stranded (hybrid) complexes. Methods in which whole chromosomal DNA probes or DNA probes that detect a complementary sequence on the 16S rRNA are used are highly specific. The AccuProbe system (GenProbe, San Diego, Calif.)

uses a DNA probe labeled with an acridinium ester directed at the rRNA of the *Mycobacterium*.

After the mixture of probe and organism is briefly incubated, unhybridized probe is chemically degraded, and the esterified acridinium on the hybridized probe is hydrolyzed by the addition of an alkaline hydrogen peroxide (H_2O_2) solution, resulting in the production of visible light, which is measured with a luminometer. The AccuProbe *M. tuberculosis* probe was shown to be 100% specific for the *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, and *M. africanum*), but the probes have a detection limit of approximately 10^6 CFU/ml and hence require actively growing cultures for use. Probes are also available for *M. avium, M. intracellulare*, *Mycobacteria avium complex*, *M. gordonae* and *M. kansasii*. (Goto et al 1991).

2. High Performance Liquid Chromatography (HPLC):

Cultures of *M. tuberculosis* containing at least 10^7 mycobacteria can be identified by reverse-phase high-performance liquid chromatography (HPLC). Most species of mycobacteria have unique patterns of mycolic acid esters. A sample of the organism is prepared, saponified overnight, and analyzed by an instrument with a detector linked to a computer that stores data from control organisms. The equipment is rather expensive, but sample preparation is inexpensive. This method allows rapid identification of all clinically significant mycobacteria in a few hours (once grown on culture). It also allows identification of species for which

biochemical patterns have either not been defined or cannot be identified only by biochemical testing. HPLC is used primarily by reference laboratories.

3. Polymerase Chain Reaction (PCR)

Definitive laboratory diagnosis of tuberculosis relies on the production of a sufficient number of mycobacteria or amount of product that can be identified. Accumulation of the slowly growing organism by culture takes several days or weeks. Methods that shorten the accumulation time to hours by rapidly amplifying nucleic acids of the organism with the use of primers have been developed. Primers used in the amplification process can be either species specific or genus specific. Once amplified, the characteristic nucleic acids of mycobacteria can be rapidly identified. Assays in which PCR is used for amplification with species-specific primers for *M. tuberculosis* are the most thoroughly studied of the new techniques.

4. Species-specific primers. PCR is part of the diagnostic process in which mycobacterial DNA in a clinical sample is extracted, amplified, and identified. The double-stranded target DNA of the organism is first denatured by heating and depends on several factors, including the guanine-cytosine content of the DNA fragment. Next, a pair of oligonucleotide primers is annealed to the complementary (usually 5') ends of each of the target single strands. These primers determine the specificity of the reaction.

(Friedman 1994, Sepkowitz 1995)

The sensitivity of the PCR assay for *M. tuberculosis* varies from approximately 50 to 100%, and the specificity is uniformly high at 95 to 100%. A seven-laboratory blinded study (Noordhoek 1994) confirms earlier observations that there is considerable laboratory-to-laboratory variability in the sensitivity and specificity of the PCR assay for detecting *M. tuberculosis* in clinical specimens. PCR sensitivity is dependent on the DNA extraction procedure, cycling parameters, and methods used to detect and identify the amplified DNA product. Because of their waxy coat, *M. tuberculosis* bacilli are quite resistant to simple disruption procedures. Sensitivity can be enhanced by extraction procedures that involve protease digestion, phenol-chloroform extraction, and ethanol precipitation of the extracted DNA; however, these laborious procedures may not be conducive to use in the busy clinical laboratory. A simpler preparation method, in which samples are heated in lysis buffer containing nonionic detergents, is less sensitive (Shawar 1993).

2.2.7. Susceptibility Testing

Susceptibility of mycobacteria to various antibiotics used is very important especially when there are resistant strains. In Ghana susceptibility testing is done when treatment failure is encountered. Currently, the most popular and acceptable methods are the reference proportion method and the radiometric method. The reference proportion method is described under Materials and Methods. The radiometric method used uses the Bactec Method described above with daily measurement in the Bacted 460 instrument from day four up to

day 12. The advantage of this method over the traditional proportion method is that it takes less than 2 weeks while the proportion method takes up to 4-6 weeks. However because radioactivity is involved it requires special conditions and careful disposal of radioactive materials. (Lorian, 1995)

Other methods worth mentioning are the absolute concentration and the resistance ratio methods. A new method, called the E-test which can potentially perform sensitivity testing in 4-10 days is currently under a lot of review. (Hazbon, 2000).

2.3. EPIDEMIOLOGY

2.3.1. Basic Epidemiologic Model.

A population may be divided into three groups so far as infection with tuberculosis is concerned. The first is the unexposed group. These are people who have never been exposed to the bacillus. The second group is those who are exposed and infected with the *Mycobacterium tuberculosis* but not having active disease. It is estimated that one third of the world's population are in the second category. The third group is made up of those who have active tuberculosis.

2.3.2 Transmission

Tuberculosis is transmitted in three ways: (i) inhalation of infectious droplet nuclei containing *M. tuberculosis* bacteria; (ii) ingestion of contaminated material,

usually milk; and (iii) direct inoculation, usually occurring among health care workers.

The respiratory route accounts for the overwhelming number of cases worldwide, especially in countries that routinely pasteurize milk, thereby removing *M. bovis*, which is part of the "*M. tuberculosis* complex" and causes human tuberculosis.

2.3.3. Natural History of Tuberculosis

The outcome of untreated tuberculosis has global importance. Only 46% of all persons in the world who have tuberculosis are in any way reached by control programmes and not all those who are reached will receive effective treatment.(Friedman 1994). The rest of the people will become chronically disabled (and continue to spread the infection), die or sometimes recover.

2.3.4. Factors Affecting Tuberculosis Infection

Age: the annual risk of infection (ARI) is weakly related to age. For ascending age cohorts in a given year, the ARI increases slightly up to the age of about 20 years. For recently infected persons, the risk of disease has a complex association with age. Children younger than 4 years old are at high risk with disease developing in as many as 60% of newly infected. Children of school going age are at a lower risk of 5-10%. The estimated average risk of disease in an adult infected with *mycobacterium tuberculosis* is 5-15% distributed over a

lifetime and about half of the risk is incurred within the first 2 years following infection. The remaining risk is distributed over the rest of a lifetime.

Gender: The difference in tuberculosis indices by gender vary with age and are not the same from population to population. Among adults, males are affected more than females with a ratio of approximately 2:1.

Race: Wherever different races coexist within an area reporting tuberculosis, incidence rates differ by race. After tuberculosis was found to be an infectious disease, debates arose about possible links between race and inherited susceptibility to infection or disease. Such studies usually have many confounding factors due to the many socio-economic factors which may be peculiar to particular races in the community. Higher incidence rates have been reported for indigenous peoples such as American Indians and Alaskan natives in the U.S. (MMWR 1987).

Occupation: Three independent sources for the association between tuberculosis and occupation can be postulated. First, workers may have come from populations where rates of tuberculosis are high. Second, there may be transmission in the workplace and thirdly a toxic contaminant in the workplace may alter the host response to tuberculosis infection. Silicosis is a confirmed example of an association between a harmful contaminant and tuberculosis. There is increased rate and severity of tuberculosis among miners who have

pulmonary silicosis and potters whose exposure to silica dust is high. (Edwards 1991). Laboratory workers especially, those in Microbiology laboratories had been found to have an increased risk of infection with mycobacteria (Kao 1997).

Special Risk Groups: The elderly in nursing homes, inmates of correctional facilities, migrant farm workers and the homeless are people with high risk of infection. For migrant farm workers, it is difficult to administer a complete course of tuberculosis drugs to them due to their itinerant nature. Tuberculosis is endemic in homeless population of major urban arrears. Rates of latent infections are as high as 42.7% among the homeless in New York. (Torres, 1990).

Ghana, with a population of 18.8 million people is estimated to have about 55000 cases per year with over 25000 deaths. (Bonsu, 1997). In a large cohort of patients, incidence of HI V among tuberculosis patients was found to be 23% in Kumasi. (Frimpong 1997, Lawn, 2001).

2.3.5. Molecular Epidemiology

The use of laboratory techniques to support studies of the epidemiology of tuberculosis has been few because of limited ability of the laboratory to differentiate between specific strains of *M. tuberculosis*. In the past, investigators were forces to depend on comparisons of drug resistance patterns and later on phage typing data. These methods provided only limited assistance since most

strains showed identical drug susceptibility patterns and only a few phage types were known – most strains showed the same phage type.

2.4. PATHOGENESIS OF TUBERCULOSIS

2.4.1 Pathogenesis

The interplay between *M. tuberculosis* and the human host determines the outcome after infection. With respect to the human host, other innate and adaptive defense mechanisms are involved. After uptake of *M. tuberculosis* in alveolar macrophages, several possible scenarios may be envisaged. *M. tuberculosis* may be destroyed immediately, in which case no adaptive T-cell response is developed. When infection is established, however, a focal non-specific inflammatory response follows. This initial response determines the local outgrowth of *M. tuberculosis* (sometimes dissemination) or containment of infection. Phagocytic cells also play a key role in antigen presentation and the initiation of T-cell immunity which follow. At many stages in the host response, *M. tuberculosis* has developed mechanisms to circumvent or antagonize protective immunity. (van Crevel 2002)

M. tuberculosis pathogenicity is related to its ability to escape killing by macrophages and induce delayed type hypersensitivity. (Quinn 1996) This has been attributed to several components of the *M. tuberculosis* cell wall. First is cord factor surface glycolipid that causes *M. tuberculosis* to grow in serpentine cords in vitro. Virulent strains of *M. tuberculosis* have cord factor on their surface,

whereas avirulent strains do not, and injection of purified cord factor into mice induces characteristic granulomas.

Second, lipoarabinomannan (LAM), a major heteropolysaccharide similar in structure to the endotoxin of gram negative bacteria, inhibits macrophage activation by interferon-gamma. LAM also induces macrophage to secrete TNF-α, which causes fever, weight loss and tissue damage, and IL-10, which suppresses mycobacteria-induced T-cell proliferation. Third, complement activated on the surface of mycobacteria may opsonize the organism and facilitate its uptake by the macrophage complement receptor CR3 (Mac-1 integrin) without triggering the respiratory burst necessary to kill the organisms. Fourth, a highly immunogenic 65-kD M. tuberculosis heat-shock protein is similar to human heat-shock protein and may have a role in autoimmune reactions induced by M. tuberculosis.

M. tuberculosis resides in phagosomes, which are not acidified into lysosomes. (Clemens 1996) Inhibition of acidification has been associated with urease secreted by mycobacteria and with uptake of mycobacteria by complement or mannose binding receptors

Primary Infection. The primary phase of *M. tuberculosis* infection begins with inhalation of the mycobacteria and ends with T cell-mediated immune response that induces hypersensitivity to the organisms and control 95% of the infection.

Most often in the periphery of one lung, inhaled *M. tuberculosis* is first phagocytosed by alveolar macrophages and transported by these cells to hilar lymph nodes. Naïve macrophages are unable to kill the mycobacteria which multiply, lyse the host cell, infect other macrophages, and sometimes disseminate through the blood to other parts of the lung and elsewhere in the body. After a few weeks, T cell-mediated immunity demonstrable by a positive purified protein derivative (PPD) test reaction develops. Mycobacteria-activated T cells interact with macrophages in three ways.

First CD4+ helper T cells secrete interferon gamma, which activates macrophages to kill intracellular mycobacteria through reactive nitrogen intermediates including NO, NO_2, and HNO_3. This is associated with the formation of epitheloid cell granulomas and clearance of the mycobacteria. Second, CD8+ suppressor T cells lyse macrophages infected with mycobacteria. Third, CD4-CD8-(double negative) T cells lyse macrophages in a, without killing mycobacteria. Lyses of macrophages results in the formation of caseating granulomas. Direct toxicity of the mycobacteria to the macrophages may contribute to the necrotic caseous centers. Mycobacteria cannot grow in this acidic, extracellular environment lacking in oxygen and so the mycobacterial infection is controlled. The ultimate residuum of the primary infection is a calcified scar in the lung parynchyma and in the hilar lymph node, together referred to as the Ghon complex. (Cotran 1999)

Secondary and Disseminated Tuberculosis. Some individuals become re-infected with mycobacteria, reactivate dormant disease, or progress directly from the primary mycobacterial lesions into disseminated disease. This may be because the infecting strain may be particularly virulent, or the host is particularly susceptible. In mice, susceptibility to mycobacterial infection is determined by an autosomal dominant gene called Bcg, which encodes a membrane transport protein.

Whether the protein acts at the level of the plasma membrane or interferes with the bacterial killing in the phagolysosome is unclear (Vidal 1993). Granulomas of secondary tuberculosis most often occur in the apex of the lungs but may be widely disseminated in the lungs, kidneys, meninges, marrow and other organs. These granulomas, which fail to contain the spread of the mycobacterial infection, are the major cause of tissue damage in tuberculosis and are a reflection of delayed type hypersensitivity. Two special features of secondary tuberculosis are caseous necrosis and cavities; necrosis may cause rupture into blood vessels, spreading mycobacteria throughout the body, and break into airways, releasing infectious mycobacteria in aerosols. (Cotran, 1999).

Influence of HIV Infection on Pathogenesis

HIV infection exerts a pronounced influence on the natural history of tuberculosis in several ways; in addition, infection with *M. tuberculosis* appears to affect the course of HIV disease, as follows :

(i) Among persons latently infected with tuberculosis who become HIV infected, active tuberculosis develops at a rate of 7 to 10% per year rather than 8% per lifetime (Allen 1992) (ii) Persons with HIV (regardless of CD4 cell counts) who are newly infected with *M. tuberculosis* progress to active tuberculosis at a rate as high as 37% in the first 6 months rather than 2 to 5% in the first 2 years. (iii) HIV confers anergy upon a large number of persons with HIV infection, thus confounding TST interpretation The prevalence of anergy increases as the CD4 cell count decreases (Jones 1993) . In many areas where tuberculosis is endemic, prophylactic INH therapy is recommended for anergic persons with HIV infection (Selwyn 1992). (iv) HIV-infected persons may malabsorb drugs perhaps because of HIV-related enteropathy, which may further complicate the treatment of tuberculosis.

Tuberculosis may influence the natural history of HIV infection by activating macrophages that harbor HIV. The result of activation is expression of HIV, rather than prolonged latency without expression of HIV. In one study, progression to AIDS occurred sooner among Mantoux-positive persons not treated with INH than those treated with INH, even when tuberculosis was excluded as AIDS indicator disease. This suggests that treatment of latent tuberculosis can help preserve the immune function of HIV-infected individuals. These influences have conspired to create an epidemic of tuberculosis within the HIV epidemic that is particularly threatening to the public health in areas such as

Africa, where large numbers of individuals are dually infected with *M. tuberculosis* and HIV. (Sepkowitz 1995)

2.5 CLINICAL FEATURES

Active tuberculosis may develop in two settings: first, persons with latent infection may, under certain conditions, reactivate and develop clinical disease. This classically has been described with immune senescence but also may occur as a result of immunosuppressive therapy, HIV infection, or other immunocompromising conditions. In many persons, no obvious cause of immunocompromise is apparent. Second, acute infection may progress immediately to active disease, especially among infants and HIV-infected persons. The calculated risk of developing active disease according to underlying condition is shown below. (Sepkowitz 1995)

TABLE 2.5 Risk factors for the development of active tuberculosis [a] among persons infected with *M. tuberculosis*

Risk Factor	Increased risk (fold) compared with persons with no known risk factor
AIDS	170
HIV Positive	113
Other Immunocompromising[a] condition	4-16
Recentness of infection(<2years)	15
Age of contact (<5yrs and >60yrs)	2-5

[a] Includes diabetes mellitus, renal failure, carcinoma of the head and neck, and iatrogenic immunosuppression.

2.5.1 Normal Host

The many manifestations of tuberculosis have been well appreciated for centuries. Classical symptoms and signs include, fever, productive cough, and/or hemoptysis, with an abnormal chest radiograph. Tuberculosis has dermatologic, hematologic, neurologic, gastrointestinal, urologic, and other manifestations. This is well documented by the continued prevalence of tuberculosis as the final diagnosis in 5 to 10% of cases of fever of unknown origin reported over the last 40 years. (Knockaert 1992).

2.5.2 Influence of HIV Infection on Clinical Presentation

The presentations of tuberculosis in HIV patients are not clear cut. (Barnes 1991) Because of this, many cases are diagnosed late or not at all, resulting in persons remaining infectious longer and thereby potentially spreading tuberculosis to hospital staff and other patients. Overall, persons with higher CD4 cell counts often present in "classic" fashion whereas persons with low CD4 cell counts are more likely to present atypically: brain abscess, meningitis, bacteremia, skin lesions, breast abscess, and visceral abscess have been described.

The sputum smears of approximately 50% of HIV-infected patients with pulmonary tuberculosis are negative by acid-fast stain, a rate possibly higher than that encountered in HIV negative patients. There is debate whether bronchoscopy increases the incidence of positive smear and culture. (Schluger 1994)

Extra-pulmonary tuberculosis is seen commonly in patients with HIV-associated tuberculosis. Such patients should be placed in respiratory isolation pending evaluation of sputum specimens, since many have concurrent pulmonary infection, even in the absence of respiratory symptoms. In addition, extra-pulmonary tuberculosis may be infectious Patients with HIV infection and pulmonary tuberculosis may present with an atypical chest radiograph. Lobar infiltrates with or without hilar adenopathy or diffuse infiltrates resembling the interstitial pattern seen in *P. carinii* pneumonia are seen. The chest radiograph may be normal patients with AIDS, whereas cavitary disease is much less common, possibly reflecting the overall immune dysfunction of patients with advanced HIV infection.

The symptoms of tuberculosis in this population are particularly nonspecific. Signs and symptoms such as fever, weight loss, and fatigue may be due to tuberculosis but also may be due to *Mycobacterium avium intracellulare* infection, lymphoma, AIDS-wasting syndrome, cytomegalovirus infection, or other diseases. Because of this and because of the contagious nature of tuberculosis, the treating physicians may be uncertain about when to institute a trial of anti-tuberculosis therapy in a patient with AIDS who has nonspecific but significant constitutional symptoms. Such decisions should be guided by the prevalence of tuberculosis in a given community, any special risks for tuberculosis that a specific patient might have, and the results of various tests.

2.6. TREATMENT OF TUBERCULOSIS

2.6.1 First Line Drugs

There are six essential drugs that are active against M. tuberculosis: isoniazid, rifampicin, pyrazinamide, ethambutol, streptomycin and thioacetazone. (Rieder 2002).

ISONIAZID was synthesized in 1912 at the German University of Prague by Meyer and Mally. In 1952 it was independently re-discovered by the Bayer Laboratories in Germany, Hoffman-La Roche in Switzerland/United States and Squibb Laboratories in the United States without the knowledge of the other groups working on the drug.

Isoniazid is only active against mycobacteria. Isoniazid has the most potent early bactericidal activity of all the anti-tuberculosis drugs. Adding other drugs will not increase this activity. Thus the rapid reduction in infectiousness observed following initiation of chemotherapy is most likely attributable to a considerable extent to the bactericidal activity of isoniazid.

INH has been the mainstay of therapy since its introduction in 1953. The usual dose is 300 mg daily. The most common side effect is liver toxicity, which correlates with increasing age and dose and is generally reversible with cessation of the drug. Rare fatal cases of fulminant hepatitis have been described, particularly among postpartum women.

The current concept classifies isoniazid as a pro-drug which requires the katG gene product for activation by the catalase, targeting the last steps in mycolic acid synthesis. Several mutations have been identified which confer resistance in *M. tuberculosis*. Important mutations are located on the katG gene and the inhA gene, of which the later is responsible for approximately 25% of clinical isolates that demonstrate resistance, generally associated with low-levels of resistance.

Susceptibility to isoniazid is dependent on the presence of the catalase-peroxidase enzyme encoded by the katG gene. Mutations in catalase-peroxidase lead to high level isoniazid resistance. Approximately 60% to 70% of isoniazid resistant strains carry mutations in one of several genes involved in its activation from pro-drug or in drug target. However, the mechanism of resistance for one third of isoniazid-resistant strains remains to be elucidated. The maximum proportion of isoniazid resistant mutants able to grow during isoniazid monotherapy of isoniazid susceptible strain is estimated to be approximately 1 in 10^6. (Rieder 2002).

RIFAMPICIN

Isolated and semi-synthesized by Maggi and collaborators in 1966, rifampicin is active against a wide range of micro-organisms including *M. leprae, S. aureus, N. meningitides.* It acts by interfering with the synthesis of mRNA by binding to the RNA polymerase. Mycobacteria develop resistance to rifampicin by mutations in a defined region for the RNA polymerase subunit beta. Mutations in the rpoB

gene of *M. tuberculosis* are responsible for most of the resistance. Mutations have been found in more than 97% of resistant isolates. The maximum proportion of rifampicin-resistant mutants able to grow during rifampicin montherapy of an isoniazid susceptible strain is estimated to be approximately 1 in 10^8. The usual dose is 600 mg daily.

ETHAMBUTOL

The synthesis of ethambutol was reported in 1961. It is bactericidal and only active against mycobacteria. Ethambutol specifically inhibits biosynthesis of mycobacterial cell wall. It acts on the biosynthesis of arabinogalactan, the major polysaccharide of the mycobacterial cell wall. It inhibits the polymerization of cell wall arabinogalactan and of lipoarabinomannan. The maximum proportion of ethambutol-resistant mutants able to grow during ethambutol monotherapy of isoniazid-susceptible strain is estimated to be approximately 1 in 10^8 The absorption of ethambutol is rapid. The drug is not extensively metabolized. Up to 80% is eliminated through the kidney unchanged; hence dose adjustment for those with renal failure is mandatory. The recommended dosage range is 15 to 20mg/kg. The most important adverse event of ethambutol is ocular toxicity (optic neuritis) which tends to be dose dependent. When the dosage above is used optic neuritis is rare. Ethambutol may rarely cause aplastic anaemia.

STREPTOMYCIN

Streptomycin was isolated from the bacteria *Streptomyces griseus* in 1944 and was the first antimicrobial to be used for tuberculosis treatment. It has a broad spectrum of activity against many gram positive and gram negative bacteria and against various species of mycobacteria. Streptomycin inhibits protein synthesis of *M. tuberculosis* by acting on ribosomes to cause misreading of the genetic code, inhibit translation of mRNA and aberrant proofreading. The maximum proportion of streptomycin resistant mutants able to grow during streptomycin monotherapy of an isoniazid susceptible strain is estimated to be approximately 1 in 10^8.

Like all aminoglycosides, streptomycin is excreted by glomerular filtration and thus it required dosage adjustment in renal impairment. The recommended dose is 15mg/kg body weight with a usual maximum dose for adults being one gram. It has to be administered parenterally, usually by intramuscular injection, but intravenous application is preferred by some because of higher peak but lower trough levels. The main adverse effect of streptomycin is vestibule-cochlear toxicity, which is usually but not always dose-dependent. This ototoxic effect is increased by diuretics such as frusemide and ethacrynic acid. Hypersensitivity reactions are also relatively frequent and important, not only in patients, but also in health care personnel administering the medication. Because of penetration into amniotic fluid and its ototoxic effect on the fetus, streptomycin should never be administered to pregnant women. Streptomycin may cause neuromuscular

blockade not reversed by neostigmine but this is rare (less than one per thousand). (Rieder 2002)

THIOACETAZONE

Thiosemicarbazones including thioacetazone, are only active against mycobacteria, and favorable in vitro and in vivo results against *M. tuberculosis* were published in 1949. The mode of action of thioacetaone has not been elucidated, although it has been shown that thioacetazone forms copper complex salts and it has been postulated that these might represent the effective compound. The drug is rapidly absorbed and the maximum serum concentration is achieved about four hours after ingestion and is eliminated from serum almost completely within 24 hours. The current recommended dosage of thioacetazone is 2.5mg/kg body weight per day.

Once daily treatment is recommended. Thioacetazone frequently causes adverse events which occur in up to 40% of patients. The most frequent are gastrointestinal (weight loss, nausea, vomiting), central nervous effect (headache, blurred vision, numbness, mental and peripheral nerve symptoms), and cutatneous necrolysis which can be fatal. Fatal toxic epidermal necrolysis occur in up to 30% of HIV patients on thioacetazone. Hence thioacetazone should never be given to patients known to be HIV infected. It is also sensible to relinquish its use in countries where HIV prevalence among tuberculosis patients is known to be high.

2.6.2. Second Line Drugs

The second-line agents are a group of antimicrobial agents which could be used for treatment of TB, but these are seldom used except in areas with high rates of drug resistance or in treatment of atypical mycobacterial infections. They include paraaminosalycylic acid (PAS), cycloserine, ethionamide, capreomycin, kanamycin and amikacin (Lester 1986). Others are the fluoroquinolones, such as ciprofloxacin ofloxacin, and its L-isomer, levofloxacin, are active in vitro and are being actively investigated (Yew 1990, Kohno 1992) for their uses against mycobacteria. The usual dose of ofloxacin is 400 to 800 mg/day. Ciprofloxacin is usually used in doses of 500 to 750 mg twice daily. Fever, rash, agitation, and gastrointestinal disturbance are the most common side effects.

Cycloserine commonly causes central nervous system side effects ranging from somnolence to irritability and seizure. Addition of quinolones may increase the rate of serious central nervous system side effects. The usual dose is 500 to 750mg/day. Pyridoxine (vitamin B6) administration may decrease some central nervous system side effects.

Capreomycin is an injectable polypeptide that causes renal toxicity. It is given at 1 g daily intramuscularly, although there is growing experience with intravenous dosing that suggests that this route is also safe.

Ethionamide causes gastrointestinal upset. The usual dose is 500 to 750 mg/day, beginning at 250 mg and increasing slowly, according to patient tolerance. PAS

can cause hepatitis, fluid retention due to an obligate sodium load, and profound gastrointestinal upset. The usual dose is 8 to 12 g/day.

Rifabutin is a rifamycin similar to rifampin. Most experts do not use rifabutin if a mycobacterial strain is resistant to rifampin, even if in vitro tests show that rifabutin may be active. However, levels of rifabutin in serum are 7 to 10 times lower than those achieved with rifampin, and this may be insufficient.

2.6.3. Treatment Regimens

First line regimens of six to eight months duration are the most efficacious available. All are based on a four-drug initial intensive phase. Whether a four-month (with rifampicin) or a six-month continuation phase without rifampicin is selected depends on the availability of resources for drugs and personnel. Many developing countries including Ghana use the eight month regimen. The regimen used in Ghana consists of 2 months intensive phase with streptomycin, isoniazid, rifampicin and pyrazinamide, followed by 6 months continuation phase with isoniazid and thiacetazone. Patients who are smear negative or having extra-pulmonary tuberculosis are treated with a 12-month regimen. This consist of 2 months of streptomycin, isoniazid and thioacetazone followed by a 10 month regiment of isoniazid and thioacetazone. (Bonsu 1997). Most developed countries use the 6 month course consisting of initial 2-month intensive phase with ethambutol or streptomycin plus isoniazid plus rifampicin plus pyrazinamide; with four-month continuation phase of isoniazid and rifampicin.

2.6.4. Influence of HIV on choice of Regimen

Among tuberculosis patients with HIV infection, two major issues need to be addressed. The first concerns the initial observation made by clinicians when treating HIV-infected patients with anti-tuberculosis drugs: tolerance of the medications was poorer than in patients without HIV infection. A second issue concerns the efficacy of the regimens usually prescribed. Patients with HIV infection may suffer from diarrhea, which may , through its lowering of drug serum concentrations, adversely compromise the efficacy of the regimen, favoring the emergence of resistance and subsequent relapse.

Adverse drug events: these occur much more frequently among HIV infected tuberculosis patients. In particular, cutaneous hypersensitivity reactions are frequent. These have mostly been attributable to thioacetazone. And to a lesser extent to streptomycin rifampicin and isoniazid. The frequent and sometimes fatal cutaneous adverse drug events among HIV infected patients due to thioacetazone preclude its use in these patients. An increased frequency of non-cutaneous adverse drug events (hepatotoxicity, gastrointestinal disturbances, thrombocytopenia) to isoniazid and rifampicin has been reported. Anti-retroviral therapy poses particular problems because of interactions with rifampicin that preclude simultaneous use of the two regimens.

Treatment Efficacy: As enteropathy is a frequent occurrence in HIV infected patients, anti-tuberculosis medications might be less well absorbed, thus leading

to treatment failure, relapse or acquisition of drug resistance. However, mal-absorption of anti-tuberculosis drugs does not seen to be a major issue in most HIV infected patients. Sputum conversion is rapid, and even faster among HIV positive than HIV-negative patients. Regimens of six to nine months duration containing rifampicin throughout have been highly efficacious in terms of both low frequency of bacteriologic failure and relapse.

Eight month regimens give acceptable results in the field. In contrast, 12-month regimens that do not incorporate rifampicin have shown a high frequency of failures and relapse. (Hawken et al 1993). Bwire et all (1999) reporting from Uganda demonstrated that HIV-seropositive status is not a principal factor in delaying sputum conversion among patients receiving intensive phase tuberculosis treatment.

2.6.5. THE DOTS STRATEGY

The WHO Global Programme on tuberculosis (GTB) has used DOTS as a brand name for the WHO TB control strategy (DOTS – Directly Observed Therapy, Short-course). (Bonsu 1997). DOTS is a strategy that provides the most effective medicines to TB patients, ensures that they regularly take these medicines as prescribed and monitors their progress towards cure. Elements of DOTS include: case finding through sputum smear microscopy, standardized short course chemotherapy administered under direct observation by a health worker, regular drug supply, rigorous supervision and political commitment to TB control.

With DOTS, an observer dispenses medicine and then watches a specific patient take the pills. This method is easiest when patients keep scheduled clinic visits. However, field workers may be asked to keep track of the whereabouts of 15 to 20 patients who cannot or will not come to the clinic. The whereabouts of these patients may be difficult to ascertain, and patients are occasionally found in drug "shooting galleries" or other dangerous places. Weis et al. showed that DOTS was more effective than traditional unsupervised therapy. Of 407 patients on traditional therapy, 85 (21%) relapsed on therapy (25 [6%] had MDR organisms), while only 32 (5.5%) of 581 on DOT relapsed. No patients with relapses who were treated on the DOT regimen had drug-resistant organisms on repeat cultures.

In a DOTS program in Denver, Colorado, fewer than 10% of patients were lost to follow-up in a cost analysis study; Iseman et al. (1993) determined in this study that there is no significant cost difference between running a DOTS program and having patients take self-administered regimens. However, when one takes into account the decreased costs from better infection control and less disease spread when compliance is improved, the cost benefit of DOT is apparent.

2.6.6 DRUG RESISTANCE IN TUBERCULOSIS

The following terms must first be defined:

1. Primary Resistance: Patients who are diagnosed with TB and who harbour organisms resistant to one or more anti-TB drugs but have never been

previously treated for TB or have been treated for less than one month are said to have primary resistance.

2. Secondary Resistance/Acquired Resistance: Patients with a history of previous anti-TB treatment lasting at least one month and have developed resistance are said to have secondary or acquired resistance.
3. Multi-drug Resistance (MDR): This is defined as resistance to at least isoniazid and rifampicin.

Patient noncompliance, acute infection with already resistant strains, and 40 years of ineffective administration of effective medicines has conspired to create a growing number of persons with resistant tuberculosis. Therapy of resistant tuberculosis is slower, more toxic, and more expensive than therapy of susceptible disease, with lower cure rates and an increased likelihood that the patient will remain infectious for an extended period (Dooley 1992). Research to identify genes coding for different antibiotic resistances appears promising and may lead to diagnostic and therapeutic innovations

Current WHO recommendations are for persons with suspected tuberculosis to receive at least four drugs initially, including three drugs to which the patient's organism is likely to be susceptible. The best "empirical therapy" for patients with possible resistant disease cannot be generalized, since resistance patterns vary from hospital to hospital and city to city.

In general, there are two key drugs in the treatment of tuberculosis: INH and rifampin. Susceptibility to both allows 6- to 9-month regimens; susceptibility to rifampin but not INH allows 9- to 12-month regimens; and susceptibility to INH but not rifampin allows 12- to 18-month effective regimens. However, when an isolate is resistant to both INH and rifampin, the outcome is uncertain.

CHAPTER THREE

MATERIALS AND METHODS

3.1 STUDY SITE AND PATIENT SELECTION

3.1.1 Study Sites

The study was carried out at the following places:

1. Chest Clinic of the Korle-Bu Teaching Hospital, Accra. The Korle-Bu Teaching Hospital is the foremost teaching hospital in Ghana and takes care of patients from all over the country especially the southern half. The chest clinic diagnoses and treats cases of tuberculosis referred from within the hospital and also several places closer to Korle-Bu. The Laboratory of the clinic uses sputum smear microscopy to diagnose tuberculosis. Sputum samples were therefore collected from this laboratory and sputum smears done there
2. Public Health Reference Laboratory (PHRL), Accra. This is the most well equipped reference laboratory for the Ministry of Health in Ghana. The laboratory is well equipped to do work on mycobacteria. Media preparation, inoculation, sensitivity testing were all done in this laboratory.

3.1.2. Patient Selection and Specimen Collection

Starting 1st November 2002 to 31st March 2003, all patients newly diagnosed by sputum smear microscopy were recruited for the study. Patients who had received previous anti-tuberculosis therapy for more than one month were

excluded. Patients were asked whether they have received daily injections for one month or more(possibly streptomycin) or have had to take tablets to treat a cough for more than 2 months. All who answered in the affirmative were excluded. The laboratory routinely collects three specimens from patients within 24 hours. Sputa which were acid fast bacilli positive were kept for the study. Patient demographic information like age, sex, and location were obtained from the Chest Clinic Laboratory records.

Consent was then sought from the patients as follows: they were told that their sputum was going to be used for a study to determine current drug susceptibility patterns among mycobacteria to help in better treatment of patients. A request for 5mls of blood was also made. They were made aware that this is voluntary and totally confidential. They were also counseled that HIV test may be done on blood as part of the study. Patients who were interested in knowing the test results could come by the Fevers Unit by the end of May 2003 for their results. Before results would be released patients would be required to undergo counseling from the HIV counselor at the Fevers Unit. Most Patients consented while a few objected to the taking of blood. Once taken both blood (serum) and sputum samples were kept frozen in a -20^{o}C freezer till the time they were worked on.

3.2 CULTURE OF SPUTUM

All culture and sensitivity work was done in a Biosafety cabinet class IIA present both at the Chest Clinic laboratory and the PHRL.

3.2.1 Acid Fast Bacilli Smears.

This was done using the Ziehl-Neelsen method as follows:

- Sputum smears were prepared on slides
- The whole slide was flooded with Ziehl's carbol fuchsin and gently heated with a gas burner to steaming. Care was taken to avoid boiling. The warm stain was allowed to stand for 5 minutes and gently washed under tap water until all macroscopically visible stain had been washed away.
- The slide was then decolorized with acid alcohol for up to 3 minutes and rinsed gently under tap water till all visible stain is removed.
- The smear was finally flooded with methylene blue solution for one minute. It was then rinsed under tap water until all excess stain has been washed away and allowed to dry in open air
- The slide was then examined under oil immersion (x100). Mycobacteria are stained red with a blue background. Smears were reported as scanty, 1+,2+, and 3+ according to the following criteria:

Microscopic Finding	**Report**
No acid fast bacilli in over 100 fields	Negative
1 to 9 AFB/100 fields	Scanty/exact figure/100 fields

10 to 99 AFBs/100 fields	1+
1 to 10 AFBs/50 fields	2+
More than 10 AFBs in at least 20 fields	3+

3.2.2 Media Preparation

Lowestein-Jensen (LJ) medium slants in universal bottles (28mls) containing either glycerol or pyruvate were used for primary isolation throughout the study. The medium was prepared as follows:

LJ base powder obtained from Merck (Darmstadt, Germany) was used and manufacturer's instructions followed as:

Exactly 37.5g of LJ base powder was mixed with 0.6 liter of distilled water and 12mls of glycerol (or 12g of pyruvate), stirred to mix well, autoclaved for 15 minutes at 121°C and allowed to cool. Fresh, antibiotic-free eggs were scrubbed with 70% ethanol and broken into a sterile blender and homogenized. To the autoclaved solution, one litre of homogenized egg was added and the solution mixed and dispensed aseptically into the tubes. The media was then put on an inspissator slant set at 85°C for 45minutes.

3.2.3 Decontamination and Digestion of Sputum

The N-acetyl-L-cystein-Alkali (NALC-NaOH) decontamination method was used. The working reagent was prepared as follows: 50mls of 2.94% trisodium citrate

(0.1M) was added to 50mls of 4% NaOH. To this solution was added 0.5g of NALC just before use. This formed the "working decontamination solution" The decontamination procedure was as follows:

- Equal volume of the decontamination solution was added to the same volume of the sputum in a sterile 20ml screw cap centrifuge tube, thus making the final concentration of NaOH to be 1%.
- Sterile water was added as top up for purposes of balance in centrifugation and to dilute alkali and increase liquefaction.
- The tube was then tightly stoppered and the content was mixed on a vortex mixer for about 20 seconds or until liquefied.
- The mixture was then allowed to stand for about 5 minutes to allow for complete liquefaction and centrifuged for 15 minutes at 4000 rpm.

The supernatant was then decanted into 5% phenol solution. Inoculation onto Lowestein Jensen slants was done using disposable pipette tips delivering 3 drops to each medium. Two media were inoculated per sample, one L-J slant containing glycerol and other containing pyruvate. Pyruvate-containing LJ medium improve the growth of M. *bovis* and *M. africanum*

The NaOH-NALC method is not only considered to be one of the most satisfactory digestion decontamination method, but also the most widely recommended and used technique for the isolation of mycobacteria from clinical specimens. (Ratman 1987).

3.2.4 Incubation and Reading of Cultures

Inoculated media were put on slants for one week in an incubator at 37°C and made to stand upright after one week. The caps were not tightened though well closed. Cultures were read weekly after incubation and growth recorded accordingly. Cultures which did not grow were discarded after 12 weeks of incubation

3.3 DRUG SENSITIVITY TESTING – PROPORTION METHOD

This is the most common method used for tuberculosis sensitivity testing. The drugs tested were streptomycin, rifampicin, isoniazid and ethambutol.

3.3.1 Drug Media Preparation

Glycerol based LJ media were prepared as in 3.2.3 above with the appropriate concentration of the respective drugs added at the stage when the egg is added. The following recommended drug concentrations for LJ medium were used (Rieder 1998):

Drug	**Concentration (µg/ml)**
Isoniazid	0.2
Rifampicin	40.0
Streptomycin	4.0
Ethambutol	2.0

The appropriate concentrations were added to one liter of prepared LJ media with glycerol and could be stored at 4°C for not more than 30 days.

3.3.2 Inoculum Preparation and Inoculation

Four to six week old colonies were used for sensitivity testing. Colonies were scraped using a sterile loop touching many colonies or scraping different parts of confluent growths. This was put in 2-4mls of Middlebrook 7H9 broth and homogenized with vortex mixture to form a uniform solution. This was then adjusted to McFarland standard number one using the same broth. Two dilutions of the original solution namely 10^{-2} and 10^{-4} (100% dilution) was then made.

One slope of control (drug free) media and two slopes of each drug containing media were inoculated with 0 .01mls of each dilution and incubated at 37°C for 6 weeks. The drug free media was inoculated with the 10^{-2} dilution.

The sensitivity was read at 4 and 6 weeks. For the proportion method, an organism is taken as resistant if number of countable colonies on any of the drug media is up to one percent or more of growth on the drug free media. That is, resistance is expressed as the percentage of colonies on drug containing media in comparison to the growth on the drug free medium at the critical concentration of the drugs. The usual criterion for resistance is 1% of growth for all the drugs. (Murray 1995).

Control Strain Used: M. tuberculosis $H_{37}Rv$

At present, the most widely used technique is the proportion method. Unfortunately, this method has limitations, such as standardization for only the for first line antituberculosis drugs (streptomycin, isoniazid, ethambutol and rifampicin). Also this method has the disadvantage of taking up to 6 weeks for sensitivity results to be known (Sanchez 1999).

3.4. BIOCHEMICAL TESTS

The following biochemical tests were used for preliminary identification of the mycobacteria isolated:

Growth on para-nitrobenzoic acid (PNB). All members of the *Mycobacterium tuberculosis* complex are sensitive to PNB. Hence all isolates that grew on PNB were designated as Mycobateria other than tuberculosis (MOTT) or "Atypical Mycobacteria".

Growth on thiophene-2-carboxylic acid hydrazide (TCH). Only *M. tuberculosis* is resistant to this agent. Hence it was used to distinguish *M. tuberculosis* from *M. africanum* and *M. bovis* which do not grow on TCH.

Nitrate Test

This was done to confirm *M. tuberculosis* (positive test) and *M. africanum* (negative test). The basis of the test is described in 2.2.5 and the test itself is described below.

3.4.1 Test for Growth on PNB

This was done together with the drug susceptibility testing for expediency. Glycerol containing LJ slants were prepared as in 3.2.2 and 500mg/L of PNB added at the stage where the egg is added. The media was stored at 4°C for not more than one month. Preparation of inoculum and inoculation of the media was done as in 3.3.2 with the 10^{-2} dilution. Reading was done at 6 weeks and recorded as "growth" or "no growth".

3.4.2 Test for Growth on TCH

This was done together with the drug susceptibility testing for expediency. Glycerol containing LJ slants were prepared as in 3.2.2 and 5mg/L of TCH added at the stage where the egg is added. The media was stored at 4°C for not more than one month. Preparation of inoculum and inoculation of the media was done as in 3.3.2 with the 10^{-2} dilution. Reading was done at 6 weeks and recorded as "growth" or "no growth".

3.4.3 Nitrate Test

BBL Taxo Nitrate Test Strips were obtained from Becton Dickinson (BD, France S.A) and stored at 4°C.

Three to four week old cultures on LJ media were used for the test and the manufacturer's procedure followed as below:

- Two to three colonies or clumps of growth were removed with sterile spatula and emulsified in 0.5mls of distilled water in a 20x110mm screw cap tube

- Using a flamed forceps, BBL Taxo Nitrate Strip was carefully added to the tube. The orientation of the strip was such that the bottom of the strip with the arrow was inserted into the tube first. The tube was kept vertical.
- The tube was capped and incubated at 37°C for 2 hours. The tube was shaken gently at the end of the first and second hours of incubation. After 2 hours of incubation the tube was carefully tilted back and forth 6 times to wet the entire strip. The tubes were then slanted at room temperature to cover the strip with the liquid and allowed to remain in position for 10 minutes.

Positive Results: Strip changed colour to light blue or dark blue

Negative Results: No colour change

Positive Control: *Mycobacterium tuberculosis* H37Ra, ATCC 25177

Negative Control: *Mycobacterium intracellulare* ATCC 13950

3.5 Identification Scheme

The following identification scheme which is a modification of Collin, Yates and Grange (1985) was used.

Organism	**Biochemical Reactions**		
	PNB	TCH	NITRATE
Mycobacteium tuberculosis	No growth	Growth	Positive
Mycobacterium africanum	No growth	No growth	Negative
Non-tuberculous Mycobacteria (atypical)	Growth	No growth	Positive/Negative

3.6 HIV SCREENING

Ninety two patients were screened for their HIV status. Blood samples were not available for four patients. The Determine HIV – 1/2 kit from ABBOTT Laboratories (100 Abbott Park Road, IL 60064, USA) and the Biotec HIV 1 and 2 Rapid device (Biotec Laboratories, Ipswich, UK) were used for the screening. The WHO recommend that two rapid test based on different antibodies could be used to diagnose HIV without the need for ELISA or Western blot (WHO 1992). Samples which were positive by both tests were deemed "positive"

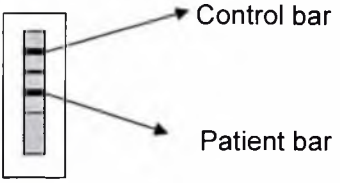
7.1 Biological Principles – Determine Kit

The Abbott Determine HIV -1/2 is an in vitro, visually read, qualitative immunoassay for the detection of antibodies to HIV-1 and HIV-2 in human serum, plasma or whole blood. Determine HIV -1/2 is an immunochromatographic test for the qualitative detection of antibodies to HIV 1 and 2. Sample is added to t he sample pad. As the sample migrates through the conjugate pad, it reconstitutes and mixes with the selenium colloid-antigen conjugate. This mixture continues to migrate through the solid phase to the immobilized recombinant antigens and synthetic peptides at the patient window site.

If antibodies to HV-1 and/or HIV-2 are present in the sample, the antibodies bind to the antigen-selenium colloid and to the antigen at the patient window, forming a red line at the patient window site. If antibodies to HIV-1 and/or HIV-2 are

absent, the antigen-selenium colloid flow past the patient window and no red line is formed at the patient window site. To insure assay validity, a procedural control bar is incorporated in the assay device.

Red bars appear in both the control window and the the patient window of the strip (Positive case)



One red bar appears in the control window of the strip and no red bar appears in the patient window of the strip. (Negative case)

Test Procedure

Using a precision pipette, 50ul of serum sample was applied to the sample pad of the device. The results were read after 15 minutes and not more than one hour as follows:

Positive: red bars appear in both the control window and the patient window.

Negative: One red bar appears in the control window of the strip and no red bar appears in the patient window.

Invalid: No red bar in the control window of the strip irrespective of whether there is a red bar in the patient window or not. There was only one invalid result which was repeated and found to be negative.

3.7.2 Biotec HIV Kit

The Biotec HIV 1 and 2 Rapid device (serum/plasma) is a rapid test for qualitative detection of the presence of antibody to HIV-1 and or HIV-2, in serum or plasma specimens. The test utilizes a combination of protein-A coated particles and multiple recombinant HIV antigens on the membrane in the test line region.

Procedure

The manufacturer's instructions were followed. The device, its buffer, serum samples were allowed to come to room temperature.

The device was removed and placed on a flat surface. 5ul of serum together with 2 drops of buffer (80ul) were placed in the specimen well. The results were read at 10-20 minutes. The results were interpreted as follows:

Positive: Two distinct red lines appeared, one in control region and the other in the test region.

Negative: One red line appeared in the control region.

Invalid: No red line appeared in the control region.

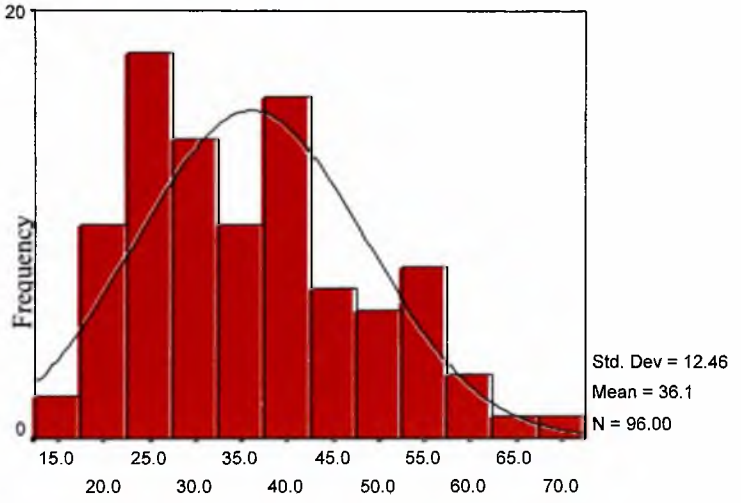
CHAPTER FOUR

RESULTS

4.1 AGE OF PATIENTS

The age of the 96 patients whose sputum were cultured ranged from 17 to 72. The mean, mode and median ages were 36.08, 38.00 and 35.00 respectively. The standard deviation and standard error for the mean age were 12.46 and 1.27 respectively. Up to 83.3% of the patients were 50 years or below. The histogram below gives a distribution of the patients by age.

Fig.4.1 Histogram showing age distribution



4.2. SEX

Seventy (72.9%) of the patients were male while 26 (27.1%) were females.

4.3 RESIDENCE

Eighty-three out of the 96 (86.5%) patients reside in Accra while the remaining were outside Accra. Places outside Accra with two or more patients included Gomoa Buduburam (2), Nsawam (2) and Kasoa(4).

4.4. SPUTUM SMEARS

The majority (46.9%) of the sputum smears were three plusses (3+), 31.3% were 2+ and 19.6% were 1+ as shown in the table below.

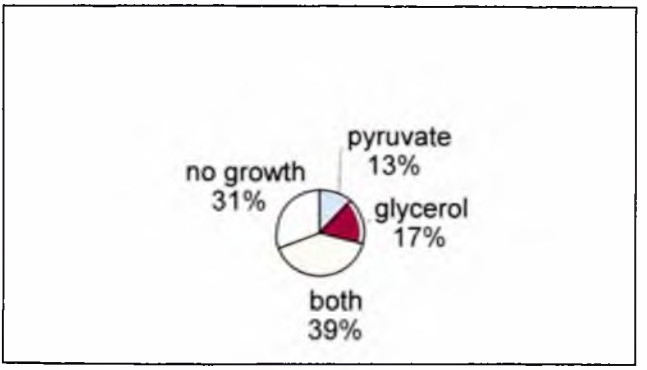
Table 4.4. Sputum smear results of patients

SPUTUM RESULTS	FREQUENCY	PERCENTAGE
3+	45	46.9
2+	30	31.3
1+	19	19.6
Scanty	2	2.1
Total	96	100

4.5 PRIMARY CULTURE GROWTH

Of the 96 primary cultures done, 66 (68.8%) showed growth for further analysis giving culture recovery rate of about 69%. Of these, 38 (60%) grew on both pyruvate and glycerol based LJ media, 16 grew on only glycerol while 12 grew only on pyruvate as the pie chart below illustrates.

Figure 4.5. Bar chart showing growth of cultures



Growth on glycerol based media was mostly confluent in nature while growths on pyruvate based media were in countable colonies.

4.6. SPUTUM SMEAR AND GROWTH IN CULTURE

A cross-tabulation of sputum smear and growth in culture is shown below. It has a Pearson's Chi-Square value of 3.82 with 3 degrees of freedom and significance level of 0.28 ($p>0.05$). Hence the amount of bacilli as found by sputum smear microscopy did not significantly correlate with growth in culture. However, it is notable that the 1+ had the highest percentage (84.2%) of positive cultures.

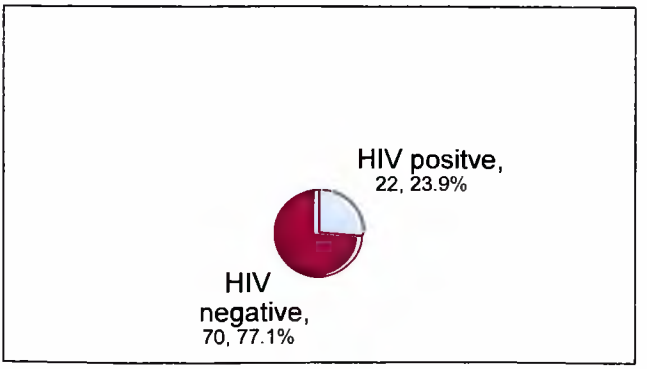
Table 4.6 Sputum smear versus growth in culture

Sputum smear	Growth		Total	Percent Growth
	Growth	No Growth		
3+	29	16	45	64.4
2+	19	11	30	63.3
1+	16	3	19	84.2
Scanty	2	0	2	100
Total	66	30	96	

4.7. HIV STATUS

Serum samples were available for 92 of the 96 patients for HIV testing. Out of the 92 patients tested, 22 were positive while 70 were negative for HIV. Hence 23.9% of the active tuberculosis patients were HIV positive.

Figure 4.7. Pie chart showing HIV status of patients



4.8. HIV STATUS AND GROWTH IN CULTURE

Analysis of HIV status and growth of sputum during culture showed that 62.5% of the HIV positive patients had their sputum showing growth as against 69.1% for the HIV negative patients. A chi square analysis gives a value of 2.3 with a significance level of 0.323 ($p>.05$). Hence there are is no significant association between HIV positivity and growth in culture.

Table 4.8 HIV status and results of culture

HIV status	Growth		Total	% Growth
	Growth	No Growth		
Positive	13	9	22	59.1
Negative	49	21	70	70.0
Total	62	30	92	

4.9. HIV STATUS AND AGE

Table 4.9 shows that the age group with the highest number of HIV positive patients(29.1%) is the youngest age group of 15-24. Also 95.5% (21 out of 22) of the HIV positive patients are below 55 years. However, the 45-54 age group have the greatest percentage (66.7%) of HIV patients.

Table 4.9 Distribution of HIV status and age of patients.

AGE HIV STATUS	15-24	25-34	35-44	45-54	55-64	65-74
Positive	7	4	4	6	1	0
Negative	13	21	20	9	7	1

4.10. HIV STATUS AND SEX

Table 4.10 below shows the distribution of sex and HIV status of patients. Of the 22 HIV positive patients, 9 (40.9%) were females while the remaining were males. Hence the male sex predominate HIV positive status in the study though this result was not statistically significant (p=.19)

Table 4.10 Sex and HIV status

SEX	HIV STATUS		Total	Percent HIV Positive
	Positive	Negative		
Female	9	15	24	37.5
Male	13	53	68	19.1
Total	22	70	92	

4.11. HIV STATUS AND SPUTUM SMEAR RESULTS

From the table below, more than half of the HIV positive patients (54.2%) showed 3+ on sputum smear as against 44.1% of the HIV negative patients. There is not much difference in the percentage of HIV positive patients for the various sputum smears as shown below.

Table 4.11 HIV status and sputum smear results

Sputum Smear	HIV Status		Total	% HIV Positive
	Positive	Negative		
3+	11	32	43	34.3
2+	7	22	29	31.8
1+	4	15	19	26.7
Scanty	0	1	1	0
Total	22	70	92	

4.12 MYCOBACTERIA SPECIES

Growth on TCH, PNB and nitrite test were used to classify the isolates as *Mycobacteria tuberculosis* (Mtb), *Mycobacterium africanum* (*M. africanum*) and Non-tuberculous mycobacteria/environmental mycobacteria (NTM). Fifty four isolates grew on TCH, 5 on PNB and only one isolate grew on both. The following criteria were used for identification

M. tuberculosis – Growth on TCH, No growth on PNB, Nitrite positive

M. africanum – No growth on TCH, No growth on PNB, Nitrite Negative

NTM – No growth/growth on TCH, growth on PNB, Nitrite positive/negative.

All the M. *africanum* were nitrite negative while all the M. *tuberculosis* isolates were nitrite positive. Table 4.12 below gives the various species as identified. Since all the cultures grew after 2 weeks, it indicated that the NTM isolated are all slow growers. One of the 5 NTM isolates was nitrite positive giving an indication that it could be *M. kansasii.*

Table 4.12 Species of Mycobacteria isolated

SPECIES	Frequency	Percent
M. tuberculosis	51	77.3
M. africanum	10	15.2
NTM	5	7.6
Total	66	100.0

4.13 HIV STATUS AND MYCOBACTERIA SPECIES

As shown in tables 4.13a&b below, no *M. africanum* or NTM were isolated from HIV patients. A Chi square analysis of the data below give a value of 6.315 with p value of .043 ($p<.05$). Hence HIV patients were more likely to be infected by *M. tuberculosis.*

Table 4.13a Association of species of mycobacteria with HIV status

	HIV positive	HIV negative	Total	% HIV positive
M. tuberculosis	15	32	47	31.9
M. africanum	0	10	10	0
NTM	0	5	5	0
Total	15	47	62	

However when *M.tuberculosis* and *M. africanum* are combined as *Mycobacterium tuberculosis* complex, there is no significant association between HIV status and mycobacteria species as shown in the table below. There is Chi Square value of 1.736 with a p value of 0.188. Hence species affecting HIV positive patients are not significantly different from those affecting non-HIV patients.

Table 4.13b Comparison of *Mycobacterium tuberculosis* complex and non-tuberculous mycobacterium with HIV status

	HIV positive	HIV Negative	Total	% HIV positive
Mtb complex	15	42	57	26.3
NTM	0	5	5	0
Total	15	47	62	

4.14. DRUG SENSITIVITY AND RESISTANCE

This was analyzed separately for the *Mycobacterium tuberculosis* complex and the environmental mycobacteria (NTM). This is what is recommended by the WHO. (WHO 2000).

The drugs tested were isoniazid, rifampicin, ethambutol and streptomycin.

Of the 61 *M. tuberculosis* complex isolates, 40(65.6%) were sensitive to streptomycin, 45(73.8%) sensitive to rifampicin, 46 (75.4) sensitive to ethambutol and 58 (93.9%) were sensitive to isoniazid as shown in table 4.14a below.

Table 4.14a. Drug Sensitivity of the mycobacteria isolates

DRUG	No. of sensitive Isolates	No. of Resistant Isolates	% Sensitive	% Resistant
Isoniazid	58	3	95.1	4.9
Ethambutol	46	15	75.4	24.6
Rifampicin	45	16	73.8	26.2
Streptomycin	40	21	65.6	34.4

Thirty of the isolates (49.2%) of the isolates were sensitive to all four drugs while three were resistant to all four drugs giving a multi-drug resistance rate of 4.9%. Multi-drug resistance (MDR) is defined as resistance to at least both isoniazid and rifampicin. Thirty one isolates (50.8%, 95% CI 38.3-63.3) were resistant to one or more drugs.

As shown in Table 4.14b below, resistance to one drug was the most common, followed by resistance to two drugs.

Table 4.14b. Resistance Pattern for the 31 *Mycobacterium tuberculosis* isolates

Resistance To:	One Drug	Total	Two Drugs	Total	Three drugs or more	Total	Overall Total
	S R E I		SR SE RE		SRE SREI		
No. of patients	6 5 5 0	16	5 4 0	9	3 3	6	31
% of total population (n=61)		26.2		14.7		9.8	
% of resistant pop.(n=31)		51.6		29.0		19.4	

S = Streptomycin R = Rifampicin I = Isoniazid E = Ethambutol

4.15. HIV STATUS AND DRUG SUSCEPTIBILITY

Both HIV status and drug sensitivity were done for a total of 62 isolates. This is because there was no blood sample available for four of the isolates. There was no significance between HIV status and sensitivity to any of the four drugs as shown in the table below.

Table 4.15. HIV status and drug susceptibility

	Rifampicin		Isoniazid		Ethambutol		Streptomycin	
HIV STATUS	Se	Re	Se	Re	Se	Re	Se	Re
Positive	11	2	12	1	9	4	9	4
Negative	32	12	43	1	34	10	29	15
Total	43	14	55	2	43	14	38	19
P value (Chi Square)	.382		.351		.554		.789	

Se = sensitive Re = resistant

4.16 MYCOBACTERIA SPECIES AND DRUG SUSCEPTIBILITY

When sensitivity and resistance of the *Mycobacterium tuberculosis* complex (*M. tuberculosis* and *M. africanum*) and the NTM are compared, the following table is obtained.

Table 4.16. Mycobacteria species and drug susceptibility

	Rifampicin		Isoniazid		Ethambutol		Streptomycin	
Species	Se	Re	S e	Re	Se	Re	Se	Re
Mtb complex	45	16	58	3	45	16	40	21
NTM	1	4	4	1	2	3	1	4
Total	46	20	62	4	47	19	41	25
P value (Chi Square)	0.012		0.174		0.109		0.043	

Se = sensitive Re = resistant

Hence atypical mycobacteria were more likely to be resistant to rifampicin and streptomycin ($p<.05$)

4.17 NON-TUBERCULOUS MYCOBACTERIA (ATYPICAL MYCOBACTERIA)

Five (7.6%) out of 66 patients were infected by atypical mycobacteria. All the patients were HIV negative. One isolate was sensitive to all the four drugs. All the isolates were sensitive to isoniazid. Four (80%) of the isolates were resistant to both Streptomycin and rifampicin. Atypical mycobacteria were more likely to be resistant to streptomycin ($p=.043$) and to rifampicin ($p=.012$).

4.18. DRUG RESISTANCE IN GHANA AND OTHER COUNTRIES

Drug resistance rates as seen in Ghana over the years and a comparison of the current study to recent studies in Africa are shown in Table 4.18 below.

Table 4.18. Drug resistance of rates of various studies done in Ghana

Year	Authors	Drugs tested	Location	% Resistance
1961	Bell and Brown	I, PAS, S	Accra	23.3 (n=60)
1965	Sodhi and Salles	I, PAS, S	Accra	24.0 (n=30)
1987	Van der Werf et al	I, R, E, T, S	Agogo	55.0 (n=99)
2003	This study	I, R, E, S	Accra	50.8 (n=61)

I = Isoniazid, PAS = Paramino-salicyllc acid, S=Streptomycin, E=Ethambutol, R=Rifampicin T = Thioacetazone

CHAPTER FIVE

DISCUSSION OF RESULTS

5.1. AGE OF PATIENTS.

The average age of 36.06 years and the fact that 80% of the patients are below the age of 50 years shows that most of the patients with active tuberculosis are in the productive age group; this is a well-known fact as it is on record that more than 80% of TB patients are in the economically productive age of 15 to 49 years (Rattan 1998). It is also this age group who are very mobile due to their economic and social activities. Hence apart from having the effect of reducing productivity for the whole nation, infection in this productive age group also means rapid spread of the disease to many people as they mix with the crowd at work places, buses, churches and other such crowded environments.

In Cameroon, the average age for 346 active tuberculosis patients in 1995 was 32 years with a range of 15 to 84. (Bercion 1997).. Also, the average age for tuberculosis patients in Uganda was 28 years. The age range of 17 to 72 in the current study indicate that tuberculosis affect both the young and the old in Ghana. A recent study in Accra that had a larger sample (n=668) had an average age of 37.4 years. (Hesse and Neequaye 2003).

5.2. SEX

Seventy (72.9%) of the 96 patients were male while only 26 were female giving a male female ratio of 2.7:1. It is well known that males are more affected by

tuberculosis than females. This has been attributed to men engaging in occupations that compromise their lungs like mining and also the high rate of smoking among males. The usually quoted ratio however for males : females is 2:1. (Friedman 1994). The ratio for males in this study is thus on the high side since the ratio of 2:1 has also been found in Ghana. (Hesse and Neequaye 2003, Frimpong et al. 1996)

HIV status, Age and Sex

The youngest age group of 15-24 had the highest number of HIV positive patients (7 out of total of 24, 29.1%) while the elderly age group of 55-74 had one HIV patient out of eight. This trend is not strange since one expects younger people to be more sexually active and also engage in more risky sexual behaviour. However, it was found that 66.7% of patients in the age group 45-54 were HIV positive. This percentage is very high compared to the general rate of 22%. One explanation could be that these patients have been carrying the bacilli for a long period of time and may also have carried the HIV virus for a longer period of time thus succumbing to tuberculosis at this middle age. However it is noted that a larger study will be needed to confirm or validate this finding.

A greater percentage of females (60%) were HIV positive compared to only 22.1% of their male counterparts. However this relationship was not significant statistically and approximates the national ratio where there are more females with HIV but not up to 2:1. The presences of HIV in the sexes in tuberculosis patients thus mirror that of the general population.

5.3. RESIDENCE OF PATIENTS

Most of the patients (86.5%) reside in Accra while the remaining lived outside the city. For those in Accra, it was noted that they came from all parts of the city including Dansoman, Adenta, Madina and Nungua. The policy by the National Tuberculosis Programme to decentralize tuberculosis diagnosis and treatment is thus far from being achieved. A likely reason for all these patients traveling long distances to Korle-Bu Chest Clinic Laboratory could be that, they were specifically told by the referring doctors to send their sputum samples there (in fact most patients say so). This is because in the past this laboratory was deemed to be more reliable for Z-N staining. However, in the last few years, a lot of training has gone on for laboratory personnel in all the polyclinics and hospitals in the city to be able to do sputum smear microscopy properly.

Doctors should therefore be educated about this development to reduce the load on the Korle-Bu Chest Clinic. This will also enable patients to spend less money in seeking diagnosis and treatment for their condition since they would have to spend less money on transport. It is also important to note that the more distance tuberculosis patients travel, and the longer the distance of travel, the more they have opportunity to spread the bacilli through coughing in public transport. Successful decentralization of the tuberculosis diagnosis and treatment is also good for control efforts. It will help to know the districts or sections in the city with the highest incidence and spread and even drug sensitivity patterns could be done for various parts of the city. For instance, in New York, it was found that

more populous boroughs of the city had a higher rate of MDR tuberculosis and thus need different treatment schedules (Sepkowitz 1994).

The spread of the patients throughout the city however indicates that the study is quite representative of Accra as a whole. Thirteen of the patients resided outside Accra mainly from Kasoa, Gomoa Buduburam and Nsawam. These areas are several kilometers from Korle-Bu and thus go to emphasize the fact that decentralization should be strengthened to save patients unnecessary travel cost.

5.4. SPUTUM SMEAR MICROSCOPY

Forty-five out of the 96 patients (46.5%) showed 3+ on microscopy with 88.2% being 2+ or 3+. Infectiousness of patients correlates well with the amount of bacilli found in sputum. This means that most of tuberculosis patients in this study are highly infectious and will be releasing large amounts of bacilli per cough. Since patients who are sputum smear positive are 10 times more likely to transmit tuberculosis than their sputum negative counterparts, (Rieder 1998) all efforts should be made to treat these highly infectious patients to render them non-infectious. This is usually achieved within the first four weeks. Currently passive case finding is used to identify tuberculosis patients. (Bonsu 1997). With the knowledge that most of our patients are highly infectious, it may be cost-effective to identify and screen close contacts for tuberculosis. This is because

people who are regular contacts of active tuberculosis patients are almost certain to contract the disease as ascertained by several studies. (CDC 1991) .

5.5 SPUTUM SMEAR AND CULTURE

Out of the 96 specimens cultured, 66 (68.8%) grew on culture. This recovery rate of almost 69% is low since most studies show a recovery rate of between 70 to 90%. The relatively low recovery rate was probably due to harsh decontamination procedures since most of those that showed "no growth" were not contaminated. In fact out of the 96 cultures only six showed contamination giving a contamination rate of 6.25%.

A similar study in Cameroon yielded 89% on culture (Bercion 1997).

This shows that despite recent improvements, the sensitivity of culture is still not optimal. During specimen digestion, all mycobacteria may not be released from proteinaceous material by the mucolytic agent, thus decreasing the inoculum size; also, the decontaminating agent used to kill non-acid-fast organisms may kill some of the mycobacteria, thus further decreasing the inoculum size. (Good 1989).

When growth on culture was compared to sputum smears, it was found that 84.2% of 1+ as against 64.4% of 3+ grew on culture. One would have expected that a higher proportion of the 3+ will grow on culture, though this result was not statistically significant. This however is not very surprising since even up to 30%

of sputum negative patients will have their specimens growing on culture. (Rieder 1998). It means that sputum smear is not an accurate index of which specimen will grow on culture. Only a small amount of sputum is spread and viewed under the microscope and so this situation is to be expected.

5.6 HIV STATUS

Almost twenty-four percent of the tuberculosis patients were HIV positive. This means that about one in four of tuberculosis patients are HIV positive from this study. This compares well with the 23% found in Kumasi in 1996. (Frimpong et al 2001). This rate of HIV positivity among tuberculosis patients is high considering the fact that the rate of infection among the general population is only 3.6%. The incidence of HIV among tuberculosis patients in other African countries are, South Africa (45%), Botswana (50%), Uganda (61%), (WHO 2000), Nigeria (12.7% Onipede 1999), Kenya (41%, Van Gorkom 1999). The rate for Nigeria was for the Ile-Ife area and not for the whole country. Among Ghana's neighbours prevalence of HIV among tuberculosis patients are Togo 30.4%, Burkina 20-29% and Ivory Coast 45% (Frimpong et al 1996)

Currently in Ghana, tuberculosis patients are not routinely tested for their HIV status. It is however recommended that all tuberculosis patients be screened for HIV after VCT. This is because treatment of people who have tuberculosis and AIDS raises five key issues. Firstly, patients may fail to properly absorb the antituberculosis drugs, which may increase the risk of treatment failure, relapses, and acquired drug resistance. Secondly, drug-drug interactions may compromise

antiretroviral and anti-tuberculosis treatment, as well as increase the risk of acquired drug resistance and toxicity. It is recommended that people who have both disorders are managed by clinicians who have special experience and interest in this patient population. (Chan 2002). Thirdly, since pilot schemes have started for the treatment of HIV patients in Ghana, screening these patients would ensure that they do not miss out in this programme. Fourthly, it is on record that HIV positive tuberculosis patients are more likely to die than their HIV seronegative counterparts.

In a study done in Kumasi to find out the factors associated with mortality among tuberculosis patients, it was found that AIDS patients were 3.4 times more likely to die than their non-HIV positive counterparts. (Lawn et al 1999). However a study in Uganda it was found that among HIV seronegative patients, conversion to a negative smear status occurred in 76% persons compared to 78% in HIV seropositive patients. This difference was not statistically significant (Bwire et al 1999). This means that such patients should be identified early and monitored closely during treatment so that the issues above could be detected early and addressed.

Finally, thioacetazone is one of the main drugs used in Ghana to treat tuberculosis during the last six months. However it was recognized relatively early that patients with HIV infection have increased susceptibility to developing toxic epidermal necrolysis when given thioacetazone and this causal relationship has been well demonstrated. (Ipuge et al 1995) Since toxic epidermal necrolysis has a case fatality rate of 20 to 30%, it has been recommended that

thioacetazone should never be given to patients who are HIV positive. (Rieder 2002). With the current practice in the NTP where patients are not routinely tested for HIV, it means that approximately one in four patients is being treated with a drug which can easily lead to their demise. This situation should be corrected.

5.6.1 HIV STATUS SPUTUM SMEARS AND GROWTH ON CULTURE

Out of 22 HIV positive patients, more that half (54.2%) showed 3+ on sputum smear microscopy. Since it is known that HIV patients are more likely to be sputum negative (Schluger et al 1994), especially in advanced AIDS. However all the patients in this study were walk-in patients and thus were not advanced AIDS patients and this could account for the difference. In advanced AIDS patients usually do not have cavities and thus are more likely to be sputum negative.

Approximately 59% of the HIV positive patients, had their sputum showing growth on culture as against 70% of the HIV negative patients. Hence the recovery rate on culture for the two groups was similar. This is not unexpected since the recovery of mycobacteria in sputum (once sputum is produced) depends on factors like decontamination and media used rather than one's HIV status.

5.7. DRUG SENSITIVITY AND RESISTANCE

Sensitivity testing was done for 66 isolates with isoniazid, rifampicin ethambutol and streptomycin.

5.7.1 Streptomycin

Forty of the 61 *Mycobacterium tuberculosis* complex isolates were sensitive to streptomycin giving a resistance rate of 34.4%. Resistance to this drug has been quoted to be 23% in Ghana in 1985-87 (Van der Werf et al 1989); 20.5% in Cameroon in 1995 (Bercion et al 1997); and 6.1% in Uganda (Joloba 2000). Hence the rate found for Accra in this study is very high. This is not surprising however since streptomycin was used for several years as monotherapy in Ghana prior to the NTP Streptomycin is no more used in the regimen in developed countries due to its low efficacy and also the fact that it has to be given intramuscularly. (Edwards et al 1991)

5.7.2. Rifampicin

Sixteen out of 61 isolates were resistant to this drug giving a resistance rate of 26.2%. This is a very surprising finding since rifampicin resistance has not been previously reported in Ghana (Van de Werf 1989 and Lawn et al 2001). It must however be noted that the Van der Werf study was in a rural area (Agogo) 17 years ago while the Lawn study in Kumasi examined only 23 isolates. Low rates of rifampicin resistance has been reported in Cameroon in 1995 (0.8%), where the author explained that the drug was recently introduced into that country. (Bercion 1997) In Uganda, the rate was found to be only 1.4%. (Joloba et al 2000). Such high rates of resistance as found in this study have been reported in Thailand 12.6% in 1996 (Riantawan et al 1998); New York 14-16% (Sepkowitz et al 1994) and a very high rate of 36% has been reported in Djibouti.

Enquiries made by the author indicated that rifampicin has been in the country for over 15 years and was being used before the DOTS programme was started. Also because it came in a capsule form, and Ghanaians generally believe that capsules are more efficacious than tablets, some patients were taking only rifampicin without taking other drugs. The combination drug of rifampicin and isoniazid came to save this situation. It was also found that some pharmacies in Accra and Kumasi still stock this drug and sell it for the treatment of infections like genito-urinary tract infections.

Since this drug is central to the success of the tuberculosis control, all efforts must be made to control its usage and restrict it only for the treatment of tuberculosis. One could however say that the national picture may not be the same. Since Accra is a big city people are more likely to get access to drugs from over the counter. This calls for a national survey of availability of rifampicin from drug outlets.

5.7.3 Isoniazid

Only three isolates (4.9%) were resistant to this drug in the study and all these isolates were resistant to all the other three drugs. Isoniazid resistance has been previously found to be 27% in Agogo, Ghana, 12.4% in Yaounde, Cameroon and 7.9% in Uganda. The rate in this study is thus on the low side. This is good especially in the light of the high resistance level found for rifampicin. This relatively low rate of resistance could be due to the fact that it is difficult to abuse

isoniazid since it is only active against mycobacteria and cannot be used for anything else. Again this calls for a national survey to get a true picture.

5.7.4 Ethambutol

Nineteen out of 66 isolates (24.6%) were resistant to ethambutol. This high rate of ethambutol resistance is strange since the drug is not routinely used in Ghana for other purposes. It is used mainly for pregnant women and those who cannot take streptomycin for one reason or another. Perhaps a larger sample will give a better picture. Ethambutol resistance has not been previously reported in Ghana and found to be less than 2% in Cameroon and Uganda. It is however known that isolates resistant to rifampicin are also resistant to other components of short-course chemotherapy like ethambutol. (Rattan 1998,). This association could explain this finding. One other likely explanation is that patients who reported as "new" were actually people who have had treatment failure in other centers in Ghana or failed to complete previous treatments.

5.7.5 Overall Resistance and Sensitivity

Less than half (30, 49.2%) of the isolates were sensitive to all four drugs. This compares well with the 45% reported by van der Werf et al in 1987. This figure is however low compared to 68.2% in Cameroon (Bercion 1995), 80.2% in Uganda, 79.3% in Mozambique 92 % in South Africa and 75.2% in Sierra Leone. (WHO 2000). Hence the overall sensitivity in Ghana is poor compared to other African countries.

Thirty one isolates (50.8%, 95% CI 38.3-63.3) were resistant to one or more drugs. This figure is very high compared to other African countries. One consolation is that over half (51.6%) of the resistance isolates were resistant to only one drug. This means that drug combinations will work for these patients.

The high level of initial resistance could be due to the fact that some patients may have refused to disclose previous tuberculosis treatment. Since Accra is the capital and Korle-Bu Chest Clinic is perceived to be the best place to get tuberculosis treatment, patients who may not have been cured from elsewhere in the country could come as "new patients" and refuse to disclose past history. Hence a national survey on anti-tuberculosis drug susceptibility patterns is urgently needed to make issues certain. It must however be stated that resistance to one or more drugs has not been good in Ghana as shown in Table 4.18a.

5.8. HIV AND DRUG RESISTANCE

As seen in Table 4.15 there was no significant association between HIV seropositivity and resistance or sensitivity to any of the four drugs. This is corroborated from a large scale study (215 patients) in Uganda where they found that there were no significant differences in resistance rates between patients with and without HIV infection (Joloba 2000). However, a study in 1999 in a different district of Uganda found that isoniazid resistance was more likely in HIV seronegative patients with *M. tuberculosis* strains compared to HIV seropositive persons ($p < 0.005$). (Bwire et al 1999). Although it has been suggested that

primary resistance accounts for a large number of drug-resistant adult cases, particularly among HIV-infected persons in the United States, (Sepkowitz et al 1995) this has not been confirmed in Africa.

5.9. MULTI-DRUG RESISTANCE

Multi-drug resistance is defined as resistance to at least both isoniazid and rifampicin. This has been found to be 1.4% in the United States (although it is 14% in New York), 4.6% in Argentina, 14% in Latvia (Manzour 2000), and the current study found it to be 4.9% in Accra, Ghana. It is considered that since Accra is a large city with a lot of slums, it is expected to have high rate of MDR than the country as a whole.

In the current study, only one of the three patients with MDR was HIV positive. It should be noted that this is termed "initial MDR" since these patients have not been treated previously for tuberculosis. Table 4.18b gives information on drug sensitivity and HIV prevalence among tuberculosis patients in some African countries. This was adapted from WHO data and gives a good idea about the situation in Ghana.

There are no figures for Ghana at the WHO. What is quoted for Ghana is the results of the present study which was done in Accra. It should be noted that the WHO requires national surveys every 3 to 5 years with a total sample over 300. Ghana has never done such a survey. It is strongly recommended that such a survey be done as a matter of urgency.

5.10 MYCOBACTERIA SPECIES

With the advent of molecular methods for the detection of species of mycobacteria, biochemical characterization is used for cases without DNA probes and for equivocal cases on PCR. Biochemical characterization was however used for this study. As expected, the study found that 51 (77.3%) of the isolates were *M. tuberculosis* while ten (15.2%) were identified to be *M. africanum*. Only 5 isolates (7.6%) were found to be atypical mycobacteria. The number of *M. africanum* is small in number compared to 42% found in Agogo by van der Werf in 1987. *M. africanum* incidence has been found to be 45% in Mauritania, 27.6% in Sierra Leone and 17.9% in Uganda. (Bercion 1997). It must be stated however that all these studies used biochemical methods for speciation which may not be full proof. It is often difficult to distinguish *M. africanum* and *M. bovis*. In the current study therefore, it is expected that there may be a few *M. bovis* within the 10 isolates identified as *M. africanum*. With the use of clear agar media like Middlebrook 7H10, colonies could be visualized easily to aid in differentiation of *M. africanum* and *M. bovis*.(Master 1992) Molecular identification in future studies with the same isolates is expected to resolve this.

5.11 SPECIES, DRUGS SUSCEPTIBILITY AND HIV

All the five environmental mycobacteria isolated were from patients who were HIV negative. This is not expected, since one would expect that environmental mycobacteria would affect HIV patients due to their low immunity. This finding however is consistent with what is prevailing in Africa where HIV patients are

affected mainly by members of the *Mycobacterium tuberculosis* complex. This is because most of the tuberculosis occurring in Africa is due to reactivation of a preexisting latent infection rather than a new infection. Environmental mycobacteria like *Mycobacterium avium complex* and *M. kansasii* among HIV patients are more common in the developed world. (Koneman, 1992).

It was also noted that the NTM were more likely to show resistance to streptomycin and rifampicin than members of the *M. tuberculosis* complex. (p=.043 and .012 for streptomycin and rifampicin respectively). However there was no such significance where isoniazid and ethambutol was concerned. In fact all the NTM isolates were sensitive to isoniazid while two were sensitive to ethambutol. Three out of five (60%) of the NTM isolates were resistant to three drugs compared with 7.6% for all the isolates combined, confirming what is known already that environmental mycobacteria are more difficult to treat and may need different drug combinations. (Krance et al 1996).

5.12 METHODS OF DIAGNOSIS

The advent of PCR and thus Restriction Fragment Length Polymorphism(RFLP) has eliminated this problem because it detects genotypic variations among members of the species *Mycobacterium tuberculosis*. These studies have shown that RFLP analysis can be used to obtain a "fingerprint" for each isolate of *M. tuberculosis*. Hence person to person transmission will show the same fingerprint. (Friedman 1994, Hermans 1990).

5.13. CONCLUSIONS

- Most of the tuberculosis patients in Accra are highly infectious, and thus efforts should be made to trace regular contacts as much as possible.
- Approximately one in four of tuberculosis patients in Accra are HIV positive.
- There is high initial drug resistance to rifampicin, ethambutol and streptomycin.
- HIV seropositivity does not affect resistance, sputum smear results, or species of mycobacteria isolated. The same drug regimen could therefore be used to treat both HIV positive and HIV negative patients
- Multi-drug resistance rate for new cases of tuberculosis in Accra is 4.9%.
- The presence of environmental mycobacteria among tuberculosis patients in Accra is 7.6% and these species have a resistance level far above the average.

5.14. RECOMMENDATIONS

1. Decentralization of the tuberculosis programme is commended. What is needed now is vigorous public education so that patients will seek treatment in their vicinity instead of traveling long distances and putting the populace at risk.

2. The DOTS programme should be strictly enforced to stem the high resistance levels.

3. There is an urgent need for a national survey of anti-tuberculosis drug susceptibility patterns to get a national picture.

4. A survey of drug outlets in the big cities in Ghana by the Pharmacy Council is required to determine the availability of rifampicin in the open market.

5. There is the need for integration or closer collaboration between the NTP and the National AIDS Control Programme.

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APPENDIX ONE - PICTURES

PICTURE ONE

Typical Growth on LJ Media

MTB Growth on TCH (left) and "no growth" on PNB (right)

One nitrite negative (middle) and two nitrite positive (blue coloration on strip)