TUBERCULOSIS IN AMBULATORY HIV-INFECTED PATIENTS IN DAR ES SALAAM, TANZANIA: PREVALENCE, ETIOLOGICAL MYCOBACTERIAL SPECIES AND THEIR SUSCEPTIBILITY TO FIRST-LINE ANTI-TB DRUGS

By

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A dissertation submitted in partial fulfilment of the requirements for the degree of Master of Medicine in Microbiology and Immunology of the University of Dar es Salaam.

University of Dar es Salaam

July 2005



CERTIFICATION

The undersigned certify that he has read and hereby recommend for acceptance by the University of Dar es Salaam a dissertation entitled: Tuberculosis in ambulatory HIV infected patients in Dar es Salaam, Tanzania: prevalence, etiological mycobacterial species and their susceptibility to first-line anti-TB drugs in partial fulfilment of the requirements for the degree of Master of Medicine (Microbiology/Immunology).

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I, Joel Bazira, hereby declare that this dissertation is original work and that it has not been presented and will not be presented to any other University for a similar or any other degree award.

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DEDICATION

I dedicate this dissertation to my parents Mr George William Kiddu and Mrs Dezi Nambi Kiddu, my wife Prosy, our daughter Faraja Tendo and to our late kids Joshua and Jedi.

ABSTRACT

Background: The HIV pandemic has led to a resurgence of tuberculosis, and with it, increased need for prompt and appropriate diagnosis, treatment and management of tuberculosis in HIV-infected individuals. **Objectives:** To determine, among ambulant HIV infected individuals in Dar es Salaam, the prevalence of tuberculosis, aetiological mycobacterial species and their susceptibility to first-line anti-TB drugs.

Methodology: During the study period, January to June 2004, a total of 5064 sputum specimens from 1688 known HIV infected patients, 473 (28%) males and 1215 (72%) females, aged between 18 and 70 years (mean 44 years) were examined by microscopy and growth on Lowenstein Jensen medium. Positive cultures were identified to species level by growth and biochemical characteristics. Drug susceptibility was performed by the conventional proportional method to four first line TB drugs- rifampicin, ethambutol, isoniazid and streptomycin. **Results:** The prevalence of tuberculosis was 5.2%. Majority (53.8%) of the isolates were *M. tuberculosis*, most of which (89.3%) were fully sensitive to the first line anti-tuberculous drugs. *M. fortitum* and other NTM were isolated from ten (19.2%) and nine (17.3%) patients respectively. Five (9.7%) isolates were untypable .MDR was 3.6 %, 66.7%, 90%, and 20% for MTB, NTM, *M. fortitum* and untypable isolates. **Conclusion:** In this population of ambulant HIV patients, with CD 4 counts ≥ 200 cells/ml, the prevalence of tuberculosis was low (5.2%) and was not correlated with CD4 counts. Majority (53.8%) of the isolates were *M. tuberculosis* and were fully sensitive to the first line drugs with only one isolate (3.6%) being multi drug resistant. **Recommendations**: There is a need for simple and rapid techniques for identification and speciation of mycobacteria and for anti-TB drug sensitivity surveillance. The high isolation rate of NTM needs to be investigated further.

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LIST OF ABBREVIATIONS

AFB	Acid fast bacilli
AIDS	Acquired immunodeficiency syndrome
BCG	Bacille Calmette Guerin
CDC	Centers for Disease Control
ddF	Dideoxy finger printing
DNA	Deoxyribonucleic acid.
DOTS	Directly observed therapy short course
HIV	Human immunodeficiency virus
IS	Insertion sequence
IUATLD	International Union Against Tuberculosis and Lung Disease
LJ	Lowenstein Jensen
MB	Middlebrook
MDR-TB	Multidrug resistant Tuberculosis
MGIT	Mycobacterial growth indicator tube
MIC	Minimum inhibitory concentration
MOH	Ministry of Health
MOTT	Mycobacterium other than tuberculosis
MTBC	Mycobacterium tuberculosis complex
MTT	3-(4, 5-dimethylthiazol-2yl)-2-5-diphenyltetrazolium bromide
NTCP	National Tuberculosis Control Programme
NTM	Non-tuberculous mycobacteria

PCR	Polymerase chain reaction
PNB	Para-nitrobenzoic acid
PTB	Pulmonary Tuberculosis
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
SSCP	Single-strand conformation polymorphism
TB	Tuberculosis
TCH	Thiophenochloroacetic acid Hydrazide
WHO	World Health Organization.
ZN	Ziehl-Neelsen

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 General introduction

Worldwide, tuberculosis is the most common opportunistic infection affecting HIV-seropositive individuals (1, 2) and the commonest cause of death in patients with AIDS killing 1 of every 3 patients (3). The World Health Organization (WHO) estimates that one-third of the world's population is infected with *Mycobacterium tuberculosis*, resulting in an estimated 8 million new cases of tuberculosis and nearly 2 million deaths each year (4). Approximately 10 million people are estimated to be coinfected with *M tuberculosis* and HIV, and over 90% of these dually infected individuals reside in developing nations. In some areas of sub-Saharan Africa, the rates of coinfection exceed 1,000 per 100,000 population (5). Because of the relentless spread of tuberculosis throughout the world, the World Health Organisation (WHO), in 1993, took the unprecedented step of declaring tuberculosis a global emergency (6).

The burden of HIV related tuberculosis is not evenly distributed as it is determined by the overall prevalence of infection by the tubercle bacillus and HIV in the community (7). From the global perspective, the region most affected is sub-Saharan Africa, where 83% of all AIDS related deaths have occurred (8-10). Since the early 1980s, HIV infection has emerged as by far the most important of all the predisposing factors for tuberculosis (11). In persons co-infected with the tubercle bacillus and HIV, the overall annual risk of developing active tuberculosis rises 20 times (12). This risk however, depends on the degree of immunosuppression, with AIDS patients having the greatest risk, therefore HIV and *M tuberculosis* "have been dubbed the cursed duet" (13).

Tuberculosis in HIV positive persons could be due to reactivation, primary infection or reinfection with tubercle bacillus (14). The risk of primary infection and reinfection with tuberculosis is related to the prevalence of tuberculosis in a community (15). Infection with *M tuberculosis* occurs after inhaling particles (<5 μm in size) containing the tubercle bacilli (16). If the bacilli reach the pulmonary alveoli, they may be ingested by alveolar macrophages, which are the first line of defence against *M tuberculosis*. Surviving tubercle bacilli multiply within the macrophage and eventually undergo haematogenous spread to other areas of the body. Although the immune response to *M tuberculosis* is important in controlling disease, immune activation may also be associated with increased HIV viral load and accelerated progression of HIV infection (17). Thus, it is likely that tuberculosis acts to accelerate the clinical course of HIV infection.

1.2 Clinical presentation of HIV related tuberculosis

The nature, presentation, clinical and radiological features of tuberculosis depend on the degree of immunosuppression. In individuals with high CD4+ cell counts, and a low viral load, the manifestations and presenting symptoms of tuberculosis are essentially similar to those in HIV negative persons. As HIV infection progresses, the CD4 + T-lymphocytes decline in number and function. Thus, the immune system becomes less able to prevent the growth and local spread of *M.tuberculosis*, characterized with disseminated and extra pulmonary forms of the disease (18). There is an increasing incidence of atypical presentations of tuberculosis, and diagnostic difficulties are posed

by the rather non-specific presenting features, which may be confused with those of other HIV related infections (19-21). These atypical forms of tuberculosis include rapid progression of clinical disease after infection and a high proportion of extrapulmonary, multisite, and widely disseminated tuberculosis.

The commonest forms of extrapulmonary tuberculosis include pleural effusion, lymphadenopathy, pericardial disease, miliary disease, meningitis and disseminated tuberculosis (with mycobacteraemia). For reasons that are not clear, involvement of the central nervous and genitourinary systems is, relative to the industrialised countries, uncommon in Africa (22).

The table 1 summarizes the clinical picture, sputum smear result and CXR appearance in early and late HIV infection.

Table I: Clinical picture, sputum smear results and CXR appearance in early and late HIV infection

Features of PTB	Stage of HIV infection	
	Early	Late
Clinical picture	Often resembles post-primary PTB	Often resembles primary PTB
Sputum smear result	Often positive	Often negative
CXR	Often cavities	Often infiltrates with no cavities.

1.3 Diagnosis of tuberculosis

Traditionally, the diagnosis of tuberculosis has been made on the basis of clinical findings, chest radiographs and confirmed by microscopic demonstration of acid-fast bacilli in biological specimens and by in vitro cultivation of tubercle bacilli. These methods remain the "gold standard" for diagnosis. However, neither of the above approaches is completely satisfactory and attention has recently turned to molecular methods of diagnosis which are more sensitive. An overview of the TB diagnostic methods is presented below.

1.3.1 Clinical Diagnosis

The clinical manifestations of tuberculosis largely depend on the site of infection and degree of immunosuppression. Irrespective of this, the pulmonary form of tuberculosis is the most common type of tuberculosis and is the most important one for control programmes. Patients with pulmonary tuberculosis usually present with weight loss and productive cough for more than three weeks (23). These defining symptoms and others such as haemoptysis, night sweats, chest pain, dyspnoea, fever and anorexia have been shown to be common presenting symptoms of patients with pulmonary form of TB (23-25). A history of contact with a known case of tuberculosis is also considered as one of the main criteria for the diagnosis of tuberculosis especially in children less than five years of age (26, 27). It is important to note that many patients with extra pulmonary

tuberculosis also have pulmonary tuberculosis (28, 29). The clinical manifestations of extra pulmonary TB, in addition to the constitutional features i.e. fever, night sweats and weight loss, include symptoms specific to the affected body site (23).

1.3.2 Chest radiography

The radiological appearance of pulmonary tuberculosis in both HIV negative and HIV seropositive patients is very variable, but more so in the latter (30, 31). Classical chest radiograph appearances of tuberculosis are seen in approximately one third of HIV infected patients while the others show a range of atypical appearances. In HIV-related tuberculosis there is significantly increased incidence of lymphadenopathy, pleural effusions, parenchymal changes, consolidation and miliary disease, but significantly less cavitary disease and atelectasis (23,24,31). Intrathoracic lymphadenopathy, uncommon in HIV uninfected adults with post-primary tuberculosis, is evident in 25%-50% in HIV infected adults with pulmonary tuberculosis (32). The occurrence of normal radiographs in 7-14% of cases (33, 34) and of atypical radiological manifestations in HIV related pulmonary tuberculosis has implications for the accurate diagnosis of the disease and a high index of suspicion must be maintained in evaluating an HIV-infected patient with symptoms suggestive of tuberculosis.

1.4 Laboratory diagnosis of tuberculosis

For laboratory investigations, usually three sputum specimens are collected, two spot specimens and one morning specimen. The morning specimens are more likely to yield positive results. Approximately 80% of patients ultimately smear positive will be so on the first specimen, an additional 15% on the second and 5% on the third (35). The recommended volume of sputum to be collected should be about 5 mls (36). The qualit of specimen is of utmost importance. A good sputum specimen consists of freshly expectorated material from the bronchial tree, with minimum amounts of oral or nasal material. Satisfactory quality implies the presence of mucoid or mucopurulent material. The type of sputum container is important; it should be wide mouthed, made of break-resistant plastic, with a screw cap to prevent leakage, desiccation and aerosol formation. Specimens should be transported to the laboratory as soon as possible after collection. If a delay is unavoidable the specimens should either be refrigerated or mixed with cetylpyridinium chloride-sodium chloride (CPC-NaCl) to inhibit the growth c contaminants.

1.4.1 Microscopic examination

The use of microscopy to reach a rapid preliminary diagnosis of tuberculosis is of great value especially in the detection of active, infectious cases. Smear microscopy is a rapid diagnostic technique with a turnaround time of one working day from specimen receipt. However, a direct smear has limited sensitivity and specificity (37) since it can only detect 5000-10000 acid-fast bacilli (AFB) per millilitre of specimen. The concentratio:

of decontaminated specimens by centrifugation increases the detection of mycobacteria by smear microscopy by up to 20% without much extra input (38, 39).

The two most commonly used stains are the Ziehl-Neelsen method, which involves staining with a strong carbol fuchsin dye, and decolourised upon washing with acid alcohol and the auramine-rhodamine flourochrome, which requires a fluorescence microscope (40). Fluorescence acid fast staining increases the sensitivity because slides can be examined faster at lower magnifications and shortens the time required for reading smears but is more expensive than conventional Ziehl-Neelsen (ZN). In addition, fluorescence positive slides must also be confirmed by ZN staining (41)

1.4.2 Culture-based-techniques

Definitive diagnosis of tuberculosis can only be done by culturing clinical specimens and further testing the isolates in view of their identification. Culturing of clinical specimens increases the case detection rates because the sensitivity of culture is much higher than that of the smear microscopy since it can detect 10^1 to 10^2 bacilli per ml (42). There are numerous TB culture methods, a few of which are discussed below:

1.4.2.1 Traditional culture methods

Traditional culture for mycobacteria is performed on either solid or liquid media such as Lowenstein Jensen media (LJ) or various Middlebrook formulations (7H9, 7H10, and 7H11). Methods based on a combination of liquid or biphasic (solid and liquid) media together with solid media are used to attain maximum sensitivity of detection and are

considered to be the current gold standard for culture. The mean time for detection of the *M. tuberculosis* complex is about 20 days for currently used solid media (43).

The choice and preparation of specimens by various pre-treatment procedures has tremendous influence on the sensitivity of results. The positivity of culture largely depends on the technique of decontamination used by various laboratories, visa viz the chemicals used for decontamination and the centrifugation method adopted for processing specimens for culturing mycobacteria by inoculating into solid or liquid media (44).

Although a combination of solid and liquid media is currently the gold standard for the primary isolation of mycobacteria, a few modern, rapid methods are also available (45-48). These include micro-colony detection on solid media (including the rapid slide culture technique), septicheck AFB method, and microscopic observation in broth culture (MODS), the BACTEC 460 radiometric system, BACTEC MGIT 960 system (Becton Dickson), MB/BacT system (Organon Teknika), and the ESP II culture system (45-48).

1.4.2.2 Radiometric BACTEC 460 TB method

This technique is specific for mycobacterial growth, wherein ^{14}C labelled palmitic acid in 7H12 medium is used. This system detects the presence of mycobacteria based on their metabolism (49) rather than visible growth. When the ^{14}C labelled substrate present in the medium is metabolised, $^{14}CO_2$ is produced and measured by the BACTEC system instrument and reported in terms of growth index (GI) values. The BACTEC

system is also useful in the identification of *M.tuberculosis* using the specific inhibitor, para-nitro-a-acetylamino-b-hydroxypropiophenone. Using the same system, drug susceptibility tests can also be performed for all the anti-tuberculous drugs when sufficient GI is observed. Using this system mycobacteria can be detected in half the time compared to conventional culture (50). However the BACTEC 460 procedure is labour intensive and involves the use of radioactive material, which requires special safety and regulatory approvals (51-53).

1.4.2.3 MGIT 960 mycobacteria detection system

It is an automated system for the growth and detection of mycobacteria with a capacity to incubate and continuously monitor 960 mycobacteria growth indicator tubes (MGIT) every 60 minutes for increase in fluorescence (54-56). Growth detection is based on the AFB metabolic O_2 utilisation and subsequent intensification of an O_2 quenched fluorescence dye contained in a tube of modified MGIT. A series of algorithms are used to determine presumptive positivity and alert the operator to the presence and location of the tubes.

1.4.2.4 MB/BacT System

This is a non-radiometric continuous monitoring system with computerised data base management. The system is based on calorimetric detection of CO_2 (54)

1.4.2.5 ESP Culture system II

This is a fully automated continuous monitoring system based on the detection of pressure changes within the headspace above the broth culture medium in a sealed bottle, i.e. either gas production or gas consumption due to microbial growth (57, 58). A special detection algorithm is present in this system for detection of very slowly growing mycobacteria

1.4.2.6 Microscopic observation of broth cultures

This is a rapid and relatively inexpensive method, which compares very well with other established systems in terms of both sensitivity as well as specificity, and also to some extent with speed when compared to solid media. Although this technique may be appropriate for high burden countries, it requires relatively expensive Middlebrook 7H9 broth, oleic acid dextrose catalase (OADC) and anti-microbial supplements and a relatively high technical skill (59).

A comparison between detection time and isolation rates for mycobacterial isolates between the BACTEC 460 method and conventional culture media is shown in table II over leaf.

Table II: Comparison of detection times of the different culture systems

CULTURE MEDIUM	AVERAGE DETECTION TIME IN DAYS	ISOLATION RATE IN %*
BACTEC 460 - 7H12	14	72
LOEWENSTEIN-JENSEN	28	62
MIDDLEBROOK 7H10	21	56
MIDDLEBROOK 7H11	21	52
L.J + 7H10 + 7H11	26	89

Rates calculated using the total number of isolates detected by all four methods as denominator

1.4.2.7 Micro colony detection on solid media

In this method plates poured with thin layer of Middlebrook 7H11 agar medium are incubated and examined microscopically on alternate days for the first 2 days and less frequently there after. In less than 7 days, micro colonies of slow growing mycobacteria such as *M.tuberculosis* can be detected. Though this method is less expensive and

requires about half the time needed for conventional culture, the recovery of mycobacteria is less efficient and is labour intensive.

1.4.2.8 Septi-check AFB method

The Septi-check AFB system consists of a capped bottle containing 30.0mls of Middlebrook 7H9 broth under enhanced (5-8%) CO_2, a paddle with agar media enclosed in a plastic tube, and enrichment broth containing glucose, glycerine, oleic acid, pyridoxal, catalase, albumin, polyoxyethylene 40 stearate, azlocillin, nalidixic acid, trimethoprim, polymyxinB, and amphotericin B. One side of the paddle is covered with non-selective Middlebrook 7H11 agar and the reverse side is divided into two sections: one contains 7H11 agar with para-nitro-a-acetylamino-b-hydrodxypropiophenone (NAP) for differentiation of *M.tuberculosis* from other mycobacteria, the other section contains chocolate agar for detection of contaminants.

This non-radiometric approach has the potential to expedite processing, obviate CO_2 incubation requirements and facilitates early detection of positive cultures since it requires about three weeks of incubation. The unique advantage of this technique is the simultaneous detection of *M.tuberculosis*, non-tuberculous mycobacteria, other respiratory pathogens and even contaminants.

1.5 Identification of mycobacterial species

Mycobacteria isolated from clinical specimens should be rapidly identified to species level whenever possible for a better understanding of the etiology and for proper interpretation of drug surveillance test results (49, 60-63).

Mycobacterial speciation can be carried out by various methods ranging from conventional biochemical tests (60, 62, 64-67) to modern high-tech molecular biological methods. These speciation methods can be briefly classified as:

(i) Phenotypic characterization, which is mainly based on growth rate, pigment production, and temperature preference.

(ii) Biochemical typing tests for identification of mycobacterial species include susceptibility to thiophene-2-carboxylic acid hydrazide, susceptibility to p-nitrobenzoic acid, as well as tests for nitrate reductase, niacin accumulation, catalase and pyrazinamidase (40, 68-69). All these biochemical tests are subjective, tedious, slow and inconsistent. For example, although *M.bovis* is inherently resistant to pyrazinamide and *M.tuberculosis* is considered pyrazinamide-sensitive, some pyrazinamide resistant isolates of *M.tuberculosis* have been found (70).

(iii) Analysis of lipids by gas chromatography, mass spectrum and high pressure liquid chromatography (HPLC).

(iv) Probe based identification, which includes peptide nucleic acid (PNA) and florescence in-situ hybridization assays for identification of mycobacteria species. PNA is a novel DNA mimic in which sugar phosphate backbone of

DNA has been replaced by a polyamide backbone. The uncharged nature and high conformational flexibility of PNA allows PNA probes to hybridise DNA or RNA with excellent affinity and specificity. The sensitivity of *M.tuberculosis* (MTB) probe targeting MTB complex was reported to be 98%. The sensitivity of NTM species, however, was only 57% since these do not target all NTM species (71).

(v) Sherlock mycobacteria identification system (SMIS) that uses computerized software to identify mycobacterial species on the basis of the mycolic acid pattern generated by HPLC (72).

(vi) PCR restriction enzyme analysis that exploits the 65kDa hsp gene and 16s rRNA gene for identification purposes (73, 74).

(vii) DNA chips: this is a technology still under development that appears promising and involves oligonucleotide arrays or DNA chips (molecular biology coupled with computer technology), which have been designed to determine the specific nucleotide sequence diversity of the rpoB and 16s rRNA genes for species identification (75).

It is important to note that each of the above method has its merits and demerits and the choice of the test to be used has to be guided by feasibility in a testing facility.

1.6 Detection and identification of mycobacteria directly from clinical samples

Mycobacterial isolates have traditionally been identified to the species level based on their reactions in a series of phenotypic and biochemical tests. However, the biochemical

reactions of the same species may vary from each other and from time to time, and in many cases no definitive identification is obtained (76). Because biochemical testing is slow, cumbersome, and may yield ambiguous results, laboratories are increasingly using molecular methods for species identification. Both genotypic and phenotypic methods are available and modifications to them are continuously being made. Some of these methods are briefly discussed in the sections below.

1.6.1 Genotypic methods

1.6.1.1 Polymerase chain reaction (PCR)

PCR allows sequences of DNA present in only a few copies of mycobacteria to be amplified in vitro such that the amount of amplified DNA can be visualized and identified. If appropriate sequences specific for *M.tuberculosis* are selected, 10-10,000 organisms can be rapidly identified (77). The PCR method is rapid, with results being available within a day of DNA extraction from the sample. A number of target genes of mycobacterial DNA have been evaluated for diagnosis by PCR and various other genotypic methods. The most common target used in the PCR is the IS6110 (78). This sequence is specific for *M.tuberculosis* complex and is present up to 20 times in the genome, thus offering multiple targets for amplification. PCR detection of IS6110 in sputum (in pulmonary TB) and peripheral blood (in extra pulmonary TB), when compared to culture has a sensitivity, specificity and positive predictability of 83%, 99%, and 94.2% respectively. A variety of PCR methods have been described in the search for a sensitive and reliable screening test for tuberculosis in clinical specimens.

Species-specific and genus-specific PCR methods are being used with various modifications of PCR (79). These include Transcription mediated amplification (TMA) and Nucleic acid amplification (NAA) (80).

1.6.1.2 Transcription Mediated amplification (TMA) and nucleic acid amplification (NAA)

This approach identifies the presence of genetic information unique to *M.tuberculosis* complex directly from pre-processed clinical specimens (81). The NAA technique uses chemical rather than biological amplification to produce nucleic acid, so that within a few hours these tests distinguish between *M.tuberculosis* complex and NTM in an AFB positive specimen. It is currently used only for respiratory specimens; use for non-respiratory specimens is likely in the near future.

A positive direct amplified test in conjunction with an AFB positive smear is highly predictive of TB disease. A negative NAA with an AFB positive smear is probably NTM.

The results of NAA are preliminary and mycobacterial culture is still needed for species identification/ confirmation and for drug susceptibility testing.

The *M.tuberculosis* direct test (MTD) and amplified mycobacterial direct test (AMDT) are highly sensitive (96%) and specific (100%) for *M.tuberculosis* on specimens that are smear positive for AFB (82). However occasional false positives or false negative results have been reported which are either due to the presence of few bacilli or due to

contamination (83, 84). Another disadvantage of the technique is that both viable and dead bacilli can give positive results as the DNA of both can be amplified (85, 86).

1.6.1.3 The Ligase chain reaction:

This method is a variant of PCR, in which a pair of oligonucleotides is made to bind to one of the DNA target strands, so that they are adjacent to each other. A second pair of oligonucleotides is designed to hybridise to the same region on the complementary DNA. The action of DNA polymerase and ligase in the presence of nucleotides results in the gap between adjacent primers being filled with the appropriate nucleotides and ligation of the primers. The LCX *M.tuberculosis* assay kit (ABOTT) is mainly being used for respiratory samples and has a high overall specificity and sensitivity for smear positive and negative specimens (87)

Other modifications of PCR include; strand displacement amplification (SDA), nucleic acid sequence based amplification (NASBA), branched DNA (b-DNA) and line probe assay (LiPA) (49, 88). Despite the numerous advantages of these techniques, the high cost of the technology, limits their routine use in clinical laboratories in low-income countries (89).

1.6.2 Phenotypic methods

1.6.2.1 FAST plaque TB ™

This is an original phage based test which uses the mycobacteriophage to detect the presence of *M.tuberculosis* directly from sputum specimens. It is a rapid manual test,

easy to perform and, in newly diagnosed smear positive TB patients has an overall sensitivity of (70.3-75.2%) and specificity of (98.7-99.0%) compared with sputum smear microscopy whose sensitivity and specificity are (34-61.3%) and (97.3-97.4%) respectively (90, 91). The simplicity of this method makes it a potential tool for detection of mycobacteria and for drug susceptibility testing. However, more data needs to be gathered before this method is recommended for use in routine diagnostic laboratories (92)

1.7 Treatment of HIV-Related Tuberculosis

HIV-seropositive patients with tuberculosis respond well to antituberculosis therapy, as long as the regimen contains INH and a rifamycin (93). There is little or no difference in relapse rate between HIV infected and uninfected patients when rifampicin based short course treatment is used (93). Thus it is essential to adopt strategies of directly observed therapy (DOT) in order to ensure completion of treatment (94) and also help to limit the emergence of drug resistant tuberculosis.

The treatment of HIV-related tuberculosis requires close monitoring because of frequent drug toxicities, possible drug-drug interactions, and other paradoxical reactions.

Thiacetazone based regimens, though cheap, are associated with an unacceptably high incidence of severe toxic reactions in HIV infected persons and they have been universally abandoned (93).

In TB patients infected with HIV, it is important to be aware of the potential problems that can occur when antituberculosis medications and antiretroviral agents are administered concurrently. Gastrointestinal complaints and rash are not uncommon with antituberculosis medications and these can be quite common with certain antiretroviral medications. A flulike illness has been described with rifampin, which could be confused with an abacavir hypersensitivity reaction. Peripheral neuropathy is an adverse effect of INH as well as stavudine and didanosine. Elevated liver function tests can occur with INH, rifampin, pyrazinamide, and most of the NRTIs, NNRTIs, and PIs.

There is considerable evidence that HIV seropositive patients with tuberculosis are at a higher risk of dying than their HIV seronegative counterparts during or after treatment for tuberculosis, with death usually being caused by complications of HIV infection rather than tuberculosis itself (95, 96). This risk is reportedly higher in patients with smear negative and extrapulmonary tuberculosis (97) than among those patients with sputum positive disease, as the former tend to be associated with more profound immunosuppression. This risk of death is also related to the anti-tuberculosis regimen used (98).

1.8 Drug resistant tuberculosis

Wild strains of *M.tuberculosis* that have never been exposed to anti-TB drugs are almost never clinically resistant (99). Exposure to a single drug as a result of poor adherence to

treatment, inappropriate prescription, and or irregular drug supply suppresses the growth of bacilli susceptible to that drug but permits the multiplication of preexisting drug resistant organisms. This phenomenon is called acquired drug resistance. Subsequent transmission of such resistant bacilli to other persons may lead to disease that is drug resistant from the outset, a phenomenon known as primary resistance.

The emergency of drug-resistant *M.tuberculosis* has been associated with a variety of management, health provider and patient-related factors. In some countries, management factors may include the lack of availability of standardized therapeutic regimen, or poor implementation compounded by frequent or prolonged shortages of drug supply in areas with inadequate resources or political instability. Use of Anti-TB drugs of unproven quality is an additional concern, as is the sale of such medication over the counter and on the black market. Moreover incorrect management of individual cases, difficulties in selecting the appropriate chemotherapeutic regimen with the right dosage, and patients' non-adherence to prescribed treatment contribute to the development of drug resistance. In summary, anti-TB drug resistance results from the absence of a properly organized system to ensure prompt diagnosis and effective treatment within a well-implemented TB control strategy. HIV infection *per se* is not a predisposing factor for the development of multidrug resistance and such an association does not occur in many other parts of the world (100, 101).

1.9 Methods of drug susceptibility testing

Investigation of the magnitude and trend of drug resistance among new and retreatment cases provides useful information for individual treatment and drug resistance surveillance which are essential components of any TB control programme.

Drug susceptibility testing can be done by a variety of techniques that measure the ability of mycobacteria to multiply in the presence of a drug. This can be done either directly using an acid fast positive clinical sample or indirectly using mycobacteria isolated in culture media. Some of the commonly used anti-TB drug susceptibility testing methods are discussed in the subsections below.

1.9.1 Direct Method

Drug susceptibility testing can be performed based on mycobacterial cultivation on solid media, either egg or agar based. In the direct test a set of drug containing and drug free media is inoculated directly with a concentrated sample. The advantage of the direct method over indirect method is that the results are available sooner (within 3 weeks on agar plates), and better represent the patient's original bacterial population.

1.9.2 Indirect method

In the indirect test the pure culture is inoculated in drug containing and drug free slopes either in egg based Löwenstein- Jensen medium or agar based 7H11 medium. These follow either of the three different varieties of susceptibility testing; the absolute concentration method, the proportion method, or the resistance ratio method (46, 47).

These methods could be phenotypic or genotypic (molecular) and are discussed in the subsections below.

1.9.2.1 Phenotypic methods

The absolute concentration method uses a standardized innoculum grown on drug free media and media containing graded concentrations of the drug(s) to be tested. Several concentrations of each drug are tested, and resistance is expressed in terms of the lowest concentration of the drug that inhibits growth; i.e. minimal inhibitory concentration (MIC). This method is greatly affected by the viability of organisms.

In the resistance ratio method the growth of unknown strain of tubercle bacilli is compared with that of standard laboratory strain (H37Rv). Parallel sets of media containing two fold dilutions of the drug are inoculated with standard strains of tubercle bacilli. Resistance is expressed as the ratio of the MIC of the test strain to the MIC of the standard strain in the same set.

This test is also greatly affected by the innoculum size as well as the viability of the strains. In addition, any variation in the susceptibility of the standard strain also affects the resistance ratio of the test strain.

The proportion Method enables a precise estimation of the proportion of mutants resistant to a given drug. Several 10-fold dilutions of innoculum are planted on both control (drug free) and drug containing media; at least one dilution should yield isolated colonies (50-100) colonies. When these numbers are adjusted by multiplying by the dilution of the innoculum used, the total number of viable colonies on the control

medium, and the number of mutant colonies resistant to the drug concentrations tested may be estimated. The proportion of bacilli resistant to a given drug is then determined by expressing the resistant proportion as a percentage of the total population used.

The proportion method is currently the method of choice for estimating drug resistance. This principle is being applied to the following rapid testing methods i.e. BACTEC, MGIT, ESPMyco and MB/BacT, which are already developed for commercial use (47). In all these systems a modified 7H9 broth is used. An indirect susceptibility test has a turn around time of 18-22 days for BACTEC 460, 10-12 days for MGIT, about 24 days for ESP and also for MB/BacT systems (47).

The BACTEC 460 radiometric system uses the proportion method and has been used as one of the standard methods of drug susceptibility testing. The growth of mycobacteria in the drug free controls and in the drug containing bottles is quantified based on the amount of $^{14}CO_2$ produced. The BACTEC 460 machine converts the amount of $^{14}CO_2$ into growth index (GI) values ranging from 0 - 999. The results are interpreted by calculating the difference in GI values from one day to the next when the GI of a 1 in 100 control is greater or equal to 30.

A strain is defined as susceptible if the difference in the GI of the control is greater than that of the drug-containing bottle.

In the MGIT system, growth is measured by detecting fluorescence from silicon embedded compound in the tubes under UV light .The fluorescence is quenched by Oxygen. Growth of Mycobacteria reduces the oxygen and therefore increases the fluorescence.

In the ESP Myco system, growth detection is based on the consumption of Oxygen, which creates a negative pressure in the liquid media. The negative pressure is recorded by a computer system.

In the MB BacT system, growth is detected using a calorimetric sensor. The release of CO_2 by growing mycobacteria changes the colour of the sensor from green to yellow, which is then measured by a computerized system.

The E-test (AB BIODISK) is based on determination of drug susceptibility using strips containing gradients of impregnated antibiotics. There are reports about high rates of false resistance by this method when compared with BACTEC or conventional LJ proportion methods (102).

The Micro well alamar blue assay and micro plate tetrazolium reduction assay, are calorimetric based tests based on oxidation-reduction of the dye Alamar blue or MTT 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide). Drug resistance is detected by the reduction of the dye from blue to pink due to oxidation-reduction metabolism of viable organisms (103, 104).

The Mycolic acid index susceptibility testing is a modification of the original Mycolic acid analysis by HPLC where a coumarin compound is used as a fluorescent derivatizing agent of Mycolic acid instead of p-bromophenacyl bromide. The drug sensitivity is assessed by measuring the total area under Mycolic acid (TAMA) chromatographic peaks of culture of *M.tuberculosis*, and this area has a very good correlation with log CFU per milliliter. Depending on the signal and quantification of this procedure, drug susceptibility pattern can be carried out as a rapid method (105).

The Microscopic observation of broth cultures – drug susceptibility assay is a novel method of microscopic observation of broth cultures with drugs and is used for drug sensitivity testing. It is relatively inexpensive and fairly rapid drug susceptibility testing method with a high sensitivity and specificity and is suited for disease endemic countries (59).

Micro colony detection employs observation of micro-colonies of *M.tuberculosis* with the help of a microscope, on a thin layer of 7H11 agar plate and is used for drug sensitivity testing. It is less expensive than the conventional proportion method, and may be a good low –cost alternative for resource poor countries (106).

The Pha B assay is a new phenotypic culture drug susceptibility testing method named as phage amplified biologicaly (Pha B), and is based on the ability of viable *M.tuberculosis* to support the replication of an infecting mycobacteriophage; non-infecting exogenous phages are inactivated by chemical treatment (107-109). The number of endogenous phages, which is an indication of the original number of viable *M.tuberculosis*, is determined after cycles of infection, replication and release in rapidly growing mycobacteria. In the case of drug resistant *M.tuberculosis*, bacilli will remain viable and will protect the mycobacteriophage. Any mycobacteriophages protected within viable bacilli replicate and ultimately lyse their host.

For rapid detection, the released mycobacteriophages are mixed with rapidly growing *M.smegmatis* host in which they undergo rapid cycle of infection, replication and lysis (107-109). Lysis is easily seen as clear areas or plaques in a lawn culture of *M.smegmatis*. The number of plaques generated from a given sample are directly

proportional to the number of tubercle bacilli that remain viable after drug treatment (i.e. drug resistant). Gingeras et al (75) reported successful application of this assay.
In the luciferase reporter phage assay, viable mycobacteria are infected with reporter phages expressing firefly luciferase gene. Easily detectable signals are seen a few minutes after the infection of *M.tuberculosis* with reporter phages. Light production requires metabolically active *M.tuberculosis* cells, in which reporter phages replicate and luciferase gene is expressed. When drug susceptible *M.tuberculosis* strains are incubated with specific anti-tuberculous drugs, they fail to produce light after infection with luciferase reporter genes. In contrast, drug resistant strains are unaffected by the drugs and produce light at levels equivalent to those documented for untreated controls after infection with reporter phages (110). The other reporter molecule described is the green fluorescent protein (GPF) of the jellyfish Aequorea victoria. This reporter system does not require cofactors or substances due to intrinsic fluorescence nature of the GPF (111). These tests have generally good sensitivity and reproducibility but are yet to be used in routine clinical laboratories.

1.9.2.2 Genotypic (molecular) methods

Molecular methods that rely on the detection of mutation(s) in the region of the gene(s) associated with drug action could also be useful for a rapid diagnosis of drug resistance. Virtually all (96%) rifampicin resistant isolates have point mutations in an 81-bp region of *rpoB* gene, and these mutations are absent in susceptible isolates, making it an ideal target for development of molecular drug susceptibility testing methods (112).

In contrast to rifampicin, the genetic basis of resistance to other TB drugs is more complex. Isoniazid (INH) is a synthetic, bactericidal agent that is used only for treatment of TB because basically all other bacteria are inherently resistant to INH. Alterations in a relatively large number of genes have been associated with INH resistance, but mutations in two genes, *katG* and *inhA*, are found in 75–85% of INH-resistant *M. tuberculosis* isolates. Approximately 65–75% of streptomycin-resistant *M. tuberculosis* isolates have mutations in the 16S rRNA gene or the *rpsL* gene, which codes for the ribosomal protein S12. More than 70% of the pyrazinamide-resistant *M. tuberculosis* isolates have mutations in the *pncA* gene, which encodes for pyrazinamidase, an enzyme that converts pyrazinamide to its active form. Mutations in the *embB* gene are associated with ethambutol resistance in about 70% of resistant isolates (112).

The accuracy of the genotypic methods varies for the different drugs. This is because of failure to understand the complete mechanism of action of most anti-TB drugs and the presence of multiple genes associated with resistance to key drugs (113-116). Mutation(s) in these genes associated with resistance to an anti-TB drug could ideally be detected by sequencing of specifically amplified products. However sequence based detection is difficult and expensive for wide clinical use, therefore simple post-amplification methods of detection of mutations have been developed allowing a better clinical applicability. These include single strand confirmation polymorphism (SSCP) analysis (117-118), heteroduplex formation (119), Dideoxy finger printing (ddF) (120), RFLP analysis of amplicon digested with restriction enzymes (121, 122), a reverse

hybdrisation-based line probe assay (LiPA) (123), RNA/RNA mismatch assay (124) and a molecular beacons-based assay (125).

2.0 STATEMENT OF THE PROBLEM

Tanzania is among the 22 countries with the highest TB burden in the world despite having a well-functioning TB control programme based on WHO-DOTS strategy for the last 25 years (16). Between 1977, when the TB control programme was launched and 1982, the notification rate of smear positive TB increased from 20/100,000 to 38/100,000, mainly because of increasing national coverage (126).

The notification rate continued to increase and reached about 70/100,000 in the period 1993-1997. By the year 2000, the notification rate had increased to 81/100,000, which was only 52% of the WHO-estimated incidence of 153/100,000 for smear positive TB cases (127).

There is enough evidence that that the HIV pandemic in the country has contributed significantly to the increase in TB notification in Tanzania (128).

Studies conducted in Tanzania by the national TB and Leprosy Programme (NTLP) in collaboration with the WHO from 1991 to 1998 in two phases; phase I (1991-1993) and phase II (1994-1998), showed TB and HIV co-infection of 32% and 44%, respectively. In between the two surveys, the notification rates of new smear positive TB increased from 54 to 74/100,000 and it is estimated that approximately 60% of this increase was directly attributable to HIV. It is logical to expect the magnitude of TB in Tanzania to continue to rise, in parallel with the increase in the magnitude of HIV infection (128).

The difficulty is that the diagnosis of TB in HIV-infected persons is more difficult due to a number of reasons such as; more non-TB respiratory disease, more smear-negative and extra-pulmonary TB and X-rays are even less specific. This is likely to result in lower detection and cure rates leading to increased spread of TB (129). Thus, the diagnosis of TB in HIV infected individuals need to be re-evaluated to avoid misdiagnosis and failure to give appropriate treatment and monitor progress accurately. The emphasis of the national tuberculosis and leprosy programme in Tanzania, as in many other resource poor countries, has been to detect the infectious cases - those whose sputum contains enough bacilli to be detected by microscopy. This approach is likely to miss a large number of smear negative TB cases, a frequent phenomenon in HIV-associated tuberculosis (130). The use of cultures in the NTLP programme is limited to cases that have failed to respond to first line anti-TB chemotherapy and who require drug susceptibility results for re-treatment.

Comparison of smear and culture results was used to provide a measure of missed TB cases among HIV infected (131) and also provide an important internal quality control measure (132). Although drug resistance to TB drugs has been found to be low, this has not been evaluated for HIV infected individuals per se, who comprise a distinct and growing sub-population of TB patients (133). In addition, due to limited culture studies done in the National TB Reference Laboratory of the NTLP programme there is very limited information regarding the prevailing mycobacterial species. Of special interest was the isolation of non-tuberculous mycobacteria (NTM) which some studies seem to indicate occurrence at high frequency among the HIV-infected patients (134). Finally,

there is a need for information on the drug susceptibility patterns of mycobacteria isolated from HIV-infected individuals, which is the current global concern in the treatment of tuberculosis and is also an epidemiological indicator to assess the success of the TB control programme (135).

3.0 RATIONALE

Even among HIV-infected patients, TB can be cured. More than 90% of surviving HIV-infected TB patients can be cured of TB, reducing transmission, morbidity and mortality and at the same time increase the length and quality of life (136). The laboratory has a crucial role in diagnosis of TB cases, monitoring of treatment and conducting surveillance of drug resistance (49,137-139). This study has provided useful information, which can be used by the National TB Reference laboratory in fulfilling its expected roles in national TB control and possibly beyond Tanzania. In temperate countries NTM are frequent causes of tuberculosis in HIV infected individuals and it will be useful to know the current situation in Tanzania.

4.0 OBJECTIVES OF THE STUDY

4.1 Broad Objective

To determine, among ambulant HIV infected individuals in Dar es Salaam, the prevalence of tuberculosis, aetiological mycobacterial species and their susceptibility to first-line anti-TB drugs.

4.2 Specific objectives

4.2.1 To determine the prevalence of tuberculosis in ambulant HIV positive patients Dar es Salaam by microscopy and culture.

4.2.2 To determine sensitivity, specificity, positive and negative predictive values of smear microscopy in ambulant HIV –infected patients by correlating smear and culture results.

4.2.3 To speciate mycobacteria isolated from ambulant HIV patients by growth characteristics and biochemical tests.

4.2.4 To determine the drug susceptibility pattern of mycobacteria isolated from ambulant HIV seropositive patients to the first line anti-TB drugs by the proportion method.

4.2.5 To study the correlation between occurrence of tuberculosis with CD4 counts among ambulant HIV patients in Dar es Salaam.

5.0 HYPOTHESES

5.1 Tuberculosis occurs in about one-third of ambulant HIV-infected patients in

Dar es Salaam.

5.2 The majority of TB cases are not due to MTB.

5.3 Multi-drug resistant (MDR) TB occurs in about five percent of HIV infected patients with TB.

5.4 There is no correlation between CD4 counts and occurrence of TB.

6.0 MATERIALS AND METHODS

6.1 Study design and Study population

This was a laboratory-based study conducted from January to July 2004 at the National tuberculosis reference laboratory in Dar es Salaam. The study included 5064 sputum specimens that were collected from 1688 (473 males and 1215 females) ambulant HIV sero-positive patients who were taking part in the *Mycobacterium vaccae* vaccine study and brought to the National Tuberculosis Reference Laboratory at Muhimbili National Hospital for sputum laboratory investigations.

6.2 Study site

This study was conducted in Dar es Salaam which is the business capital of Tanzania with a population of about 4 million and has the highest number of Tuberculosis cases in Tanzania (126). The central tuberculosis reference laboratory, where this study was conducted, is the apex of TB microbiology in Tanzania and does perform sputum culture and drug susceptibility testing for the whole country.

6.3 Sample size

Sample size was calculated from the following formula

$n = Z^2P(1-P)/d^2$

Where:

n = is the sample size

z = standard normal deviate corresponding to a two sided specified significance level.

P = (expected) prevalence of tuberculosis

d = margin of error or boundary of error on p

Setting a significance level of 0.05, gives Z = 1.96

Putting P = 25 % (0.25) and d = 5% (0.05) yielded a minimum sample size of 288 patients

6.4 TB case definition

A patient with a verified case of pulmonary TB was defined as one with relevant clinical symptoms and 2 out of 3 smears positive for AFB's or one positive smear and one positive culture (140) or a negative smear but with a positive culture.

6.5 Sputum collection

This took place in open air or in a well-ventilated room. Whenever possible, three specimens (spot-morning-spot) were collected from each patient in a plastic, wide

mouth, and leak proof container with a screw top lid. Specimens were examined for quality and adequacy and their appearance was reported as purulent, muco-purulent, or blood stained and processed within 2 days of collection.

6.6 Sputum smear microscopy

In a Class II biosafety cabinet, a portion of sputum that appeared abnormal, e.g. thick, blood stained or containing pus was picked using a sterile, disposable 1ul loop and spread in the middle of the slide 2 x 1 cm and allowed to dry and then fixed by passing over a flame 3 times.

Smears were stained by auramine (see Appendix II) for screening and positive samples were confirmed by over staining with ZN stain without removing the auramine stain. ZN (see appendix III) staining was performed according to standard procedures. (141-142)

6.7 Reporting of Microscopy results

Results are reported as recommended by the International Union Against Tuberculosis and Lung Disease.

If any definite bacilli were seen the smear was be reported as Acid fast Bacilli and an indication of the number of bacilli present was given as shown in table III.

Table III: Reporting of microscopy results

Finding	Result	Grading

No AFB in 100 microscopic fields	Negative	0
1-9 AFB in 100 microscopic fields	Positive	Report exact number
10-99 AFB in 100 Microscopic fields	Positive	1+
1-10 AFB per field	Positive	2+
>10 AFB per field	Positive	3+

6.8 Sputum cultures

Sputum samples were decontaminated using the modified Petroff's method (143) (See appendix III). Each sample was then cultured in both pyruvate and glycerol containing Lowenstein Jensen media incubated at 37^0C for up to 10 weeks. Plates were examined weekly for growth. When colonies appeared, they were identified according to criteria based on the speed of growth, macroscopic features (e.g. roughness and pigment production) and by their response to biochemical tests; a thermolabile catalase activity (positive at 22^0C, destroyed by heat at 68^0C), a nitrate reductase activity, accumulation of niacin (niacin test), growth in LJ media containing 500ug/ml of 4(p)-nitro benzoic acid (PNB) and sensitivity to thiophen-2-carboxylic acid hydrazine (TCH).

Table IV Preliminary identification of *M. tuberculosis* complex

	Growth in	Pigment	Growth on	Niacin	Nitrate	TCH	Growth at

	3-6 d		PNB				room temp
Rapid NTM	+	+or-	+	-or+	+or-	X	+
M. tub	-	-	-	+	+	+	-
classical							
M. africanum	-	-	-	+	+or-	-	-
M. bovis	-	-	-(rare+)	+	-	-	-
Slow NTM	-	-or+	+	-or+	-or+	X	+or-

Rapid growers include *M. fortiutum*, *M. chelonae*, and tens of other possible species. At least *M. chelonae* may be positive for niacin. *M. fortuitum* is always positive for nitrate and may also be positive for niacin. Niacin positive isolates may be found among *M. avium*, *M. kansasii*, and *M. simiae*.

The preliminary diagnostic criteria for *M. tuberculosis* are:

1. Typical ZN result: stains deeply red and forms clumps and occasional cords.
2. Growth after 7 days.
3. Nonpigmented typical colonies.
4. Niacin positive.
5. Does not grow on PNB or at room temperature
6. Growth in the TCH tube.

6.9 **Recording of culture results**

The number of colonies present in the culture tubes was counted and the results were expressed as the number of colonies per tube, except if their number was so high that they were confluent. The recording was done as follows:

Table V: Recording of culture results

Number of colonies	Reading code
0	Negative
1-10	+
10-100	++
> 100	+++

6.10 Drug susceptibility testing by proportion method

Drug susceptibility testing was performed using the proportion method. In this method, the number of colonies growing on drug-free medium was compared with the number on drug-containing medium. If the proportion of organisms growing on the drug containing medium was ≤ 1%, the organism was considered to be susceptible, while growth >1% was associated with resistance. The steps involved in this method are outlined below.

6.10.1 Preparation of innoculum

1. Add 0.5ml of sterile 0.01% polysorbate Tween 80 solution/0.85%Nacl into a bijou bottle containing 5-8glass beads

2. Using a 22 SWG Nickel chrome wire loop, 3mm external diameter or a plastic inoculating wire loop, scrape growth culture from a wide area of LJ slant and transfer into the bijou bottle.
3. Homogenize by vortex for 30 seconds to 1 minute and allow larger particles to settle.
4. With a sterile Pasteur pipette, withdraw supernatant suspension and transfer into a new sterile test tube with a thin wall of 16x 125 mm.
5. Add some more of the diluting fluid into the test tube so as to adjust density to that of McFarland No1.

6.10.2 Dilution of the innoculum

1. Adjust Ependorff pipette to 500ul volume
2. From the adjusted suspension in step I above take 0.5mls and transfer to a 10 ml plastic tube containing 4.5ml of diluent (this is 1/10 or 10^{-1} dilution) and mix well.
3. From the above dilution transfer 0.5ml into another 10ml plastic test tube containing the same amount of diluent, mix well (this is 1/100 or 10^{-2} dilution).
4. Transfer 0.5ml from the previous test tube into the last 10ml plastic test tube containing the same amount of diluent, mix and transfer and discard 0.5ml (this is 1/1000 or 10^{-3}).

6.10.3 Inoculation of the drug-containing medium

1. Arrange the LJ slopes in the media rack in the following sequence: two drug free LJ slopes, followed by thiophene-2-carboxylic acid hydrazide (TCH) containing LJ slopes, followed by para-nitro benzoic acid (PNB) containing LJ slopes then 0.2ug/ml and 1ug/ml of isoniazid LJ slopes, 40ug/ml rifampicin LJ slopes, 5ug/ml streptomycin LJ slopes and 2 ug/ml ethambutol LJ slopes.
2. Label all tubes on the sides.
3. Using a plastic loop inoculate a loopful of 10^{-3} suspension into the first drug free LJ slope
4. Inoculate 10^{-1} bacterial suspension to the remaining slopes.
5. Make a smear from the 10^{-3} bacterial suspension for ZN stain
6. Incubate slopes at 37°C for 3- 4 weeks.
7. Record the characteristics of colonies from the control medium (DF slopes) and from both dilutions and then calculate the percentage of resistance

6.11 CD 4 enumeration

1. Vortex reagent pair upside down for 5 seconds (set vortex at mid-range speed).
2. Vortex reagent pairs upright for 5 seconds.
3. Open tubes with the coring station.
4. Mix patient blood by inverting the tube 5 times.
5. Pipette 50 ul of whole blood into each of the 2 reagent tubes. Change tips between tubes.

6. Cap the tubes and vortex upright for 5 seconds.
7. Incubate for 60-120 minutes at room temperature. Place reagent pairs in the work station and close the cover to protect the reagents from light.
8. Uncap tubes and pipette 50 ul of fixative solution into each reagent tube. Change tips between tubes.
9. Recap the reagent tubes with new caps and vortex upright for 5 seconds.
10. Run the tubes on the FACScount within 48 hours of preparation. Store samples at room temperature in the workstation until they are run on the instrument. Vortex upright for 5 seconds before running

6.12. Quality assurance

Quality assurance ensures that the information generated from the laboratory is accurate, credible and reproducible. This was achieved by assessing the quality of specimens, monitoring the performance of decontamination, digestion and culture procedures, reagents, media and equipment, reviewing culture results and documenting the validity of culture methods.

For smear microscopy, every batch of slides that was stained, the reference strain H37RV and a known negative sample were included in a blind manner by a senior technical staff. The known negative and positive slides were read before the study slides could be read.

For media quality control, the medium pH was tested using pH strips both prior to inspissation and after inspissation. The condensed liquid at the bottom of the culture tube was used for testing after inspissation. The pH was adjusted as necessary to 6.6-6.8 using HCl or NaOH. Inspissation increases pH by 0.1 – 0.2. For quality control of the media every fresh batch was left at room temperature for up to 48 hours and then inoculated. Ten uninoculated LJ slopes were incubated together with the inoculated slopes to further check for contamination. Performance of each batch of media was carried out using a light suspension of MTB (H37RV) and the ability to support growth followed.

Quality measures in cultures included calculating the percentage of contaminated cultures, comparison of smear and culture results and determining the proportion of non tuberculous mycobacteria (NTM) among the mycobacterial isolates

Susceptibility testing was performed on the standard H37RV strain with each new batch of LJ medium and for each drug (144).

In addition, , the laboratory participated in the UK National External Quality Assurance Scheme for TB smear microscopy and culture, and has cumulative scores of 100% and 98% for microscopy and culture, respectively.

6.13 Ethical consideration

Ethical clearance for carrying out this research was obtained from the Muhimbili University College of Health Sciences ethics committee. There were no major ethical issues since the examined specimens were obtained from known HIV positive patients submitted to the central TB reference laboratory and there was no direct contact with patients. Patients found to have tuberculosis were treated according to the existing national guidelines.

7.0 RESULTS

During the study period January to June 2004 a total of 1688 known HIV sero-positive patients, 473 (28%) males and 1215 (72%) females, aged between 18 and 70 years (mean 44 years) submitted 5064 sputum samples for smear microscopy and culture. Of these 34 (2%) were AFB positive and1654 (98 %) were AFB negative. As for the culture results, 81(4.8%) were culture positive, 1607 (95.2%) were culture negative, while 43 (2.4%) were contaminated. Compared with culture results, smear microscopy had sensitivity, specificity, positive and negative predictive value of 34.6%, 99.6%, 82.4%, 96.8%, respectively. The difference in the recovery of mycobacteria by smears and culture was highly significant ($p > 0.001$). Out of these 1688 HIV sero-positive patients, a total of 87 (5.2%) patients were shown to have mycobacterial disease based on a positive sputum smear and/ or positive bacterial culture.

Table VII shows growth on pyruvate and glycerol containing LJ media. Of the 132 culture positives, 31(23.5%) grew on glycerol alone, 41 (31%) grew on Pyruvate alone and 60 (45.5%) grew on both. Table VIII shows the correlation between CD4 counts and tuberculosis. Apparently there was no correlation between the level of CD 4 counts and the occurrence of tuberculosis among the study subjects.

Fifty-two isolates were available for biochemical speciation and drug susceptibility testing. Using our classification scheme, combining growth characteristics and biochemical tests, 28 (53.8%) of the isolates were classified as *Mycobacterium tuberculosis* complex, 9 as non-tuberculous mycobacteria (NTM), 10 were *M.fortuitum* and 5 were untypable using conventional methods. Table X summarizes the drug

susceptibility test results. Overall, majority (59.6%) of isolates were fully sensitive to all the four drugs tested. Resistance to any drug was found among 21 (40.4%) of the isolates and resistance to all the four drugs was found among 14 (26.9%) of the isolates. Table XI shows the drug susceptibility pattern of the different groups of isolates. Majority (89.3%) of the MTB isolates were fully sensitive, while mono resistance to INH and SM was 3.6% for each of the two drugs.

Of the NTM, 2 (22%) of the isolates were fully sensitive to all the tested drugs, while mono resistance to RMP was 11.1% and resistance to INH, RMP and Ethambutol and INH, RMP, and SM was 11.1% for each. Of the *M.fortuitum* 1 (10%) of the isolates were fully sensitive while the majority (90%) were resistant to all the four drugs tested. Of the untypable isolates 60% were fully sensitive. Mono resistance to SM was 20% while resistance to all drugs was 20%. Multidrug resistance (MDR) was 3.6 %, 66.7%, 90%, and 20% for MTB, NTM, *M.fortuitum* and untypable isolates respectively.

Table VI: Correlation between culture and smear results

	Culture		
Smear	Positive	Negative	Total
Positive	28	6	34
Negative	53	1601	1654
Total	81	1607	1688

Sensitivity =34.6% (24.5-46.0)

Specificity = 99.6% (99.1-99.8)

PPV = 82.4% (64.8-92.6)

NPV = 96.8% (95.8-97.6)

X^2 = 456.8 $p < 0.001$

Table VII: Growth of mycobacteria on LJ media supplemented with either pyruvate or glycerol

Media	Number	Percentage
Growth on glycerol alone	31	23.5
Growth on Pyruvate alone	41	31.0
Growth on Both	60	45.5
Total	132	100

Table VIII: Correlation of CD4 counts with tuberculosis

CD4 counts	No. of patients with TB	No. of patients without TB	Total
<200	9	122	131
201-500	31	201	232
>500	13	133	146
Total	53	456	509

$X^2 = 4.342$ df = 3 p >0.05

Table IX: Speciation of mycobacterial isolates

Strain	Number	Percentage (%)
TBC	28	53.8
NTM	9	17.3
M.fortuitum	10	19.2
Untypable	5	9.7
Total	52	100

Table X Drug susceptibility pattern of all the isolates

Tested Strains	Number	Percentage (%)
`Total no of isolates	52	100
Fully Sensitive	31	59.6
Any drug	21	40.4
Resist. RMP	1	1.9
Resist. SM	2	3.8
Resist INH & SM	1	1.9
Resist. INH & RMP	1	1.9
Resist. INH, RMP & EMB	1	1.9
Resist. INH, RMP & SM	1	1.9
Resist all drugs	14	26.9

Table XI: Drug susceptibility pattern of the different categories of isolates

	TBC	NTM	*M.fortuitum*	Untypable
Total no of isolates	28	9	10	5
Fully sensitive	25	2	1	3
Resist. To any drug	3	7	9	2
Resist RMP	0	1	0	0
Resist SM	1	0	0	1
Resist. INH & RMP	1	0	0	0
Resist INH & SM	1	0	0	0
Resist. INH, RMP & EM	0	1	0	0
Resist. INH, RMP & SM	0	1	0	0
Resist all 4 drugs	0	4	9	1

Table XII: summary **of internal quality control measures**

Reagents	Proper labelling, checking expiry dates, fresh preparation of reagents where possible
Smears	Known positive and negative samples included in a blind manner and read first.
Media	pH regulation using pH strips leave uninoculated media at room temperature for 48 hours Incubate 10 uninoculated media together with inoculated media Inoculate a light suspension of H37Rv
Culture	Proportion of NTM Percentage of contamination Proportion of smear positive culture negative samples
Speciation	H37Rv
DST	H37Rv

Table XIII: Summary results of UK national External Quality control Assessment results

Total No of specimens received	Tests performed	Cumulative score
16	AFB Microscopy	64/68 (94%)
16	Mycobacteria culture	64/70 (91.4%)

Table IV: Smear positive culture negative results

No	Smear grade	Clinical notes
1	1+	Clinical notes were not available
2	1+	Clinical notes were not available
3	X1+	Diagnosed with abdominal tuberculosis (sputum smear and culture negative) and embarked on antiTB treatment. These were samples after 2 months of treatment.
4	3+	3rd sputum positive sample that made TB diagnosis; other 2 samples had growth on culture.
5	X6AFBs	Diagnosed with TB (sputum and culture positive) in October 2003 and embarked on antiTB treatment. These were samples after 2 months on treatment
6	1+	The only sputum sample that was positive; the other 2 were AFB negative

X= these patients had received antituberculous therapy before submitting the sputum samples

8.0 DISCUSSION

TB remains the leading cause of death among HIV infected patients (145). Thus, special efforts must be made to thoroughly screen HIV patients for TB, so that prompt treatment could be instituted (146). In addition, adequate monitoring of TB patients, which may involve serial examination of sputum and for some cases, drug susceptibility testing is essential (49,137-139).

The objectives of this study were to study the prevalence of tuberculosis, the etiological mycobacterial species and their drug susceptibility patterns and to correlate CD4 levels with tuberculosis among ambulant HIV patients attending a TB vaccine trial clinic in Dar es Salaam.

To achieve the above objectives, a number of quality assurance measures were undertaken and are summarized in Table XIII. Briefly these included; fresh preparation of reagents and their labelling, pH regulation of the media, incubation of un-inoculated LJ slopes, examination of sputum for adequacy and quality, staining and reading of known positive and negative slides, inclusion of a standard H37Rv strain, calculation of the proportion of NTM and participation in external quality assurance programmes.

The results of this study indicate that the prevalence of tuberculosis among ambulatory HIV infected patients was 5.2%, a magnitude which is much lower than the 40-50% national estimate for Tanzania among HIV infected patients in different stages of HIV infection (16). The prevalence of TB found in the present survey is similar to that reported in Haiti (5.8%) (147) and South Africa (3%) (148) among ambulatory HIV patients. The fact the that majority (83%) of our patients had high CD4 counts ($\geq$ 200)

may explain, at least in part, the lack of association between CD4+ counts and occurrence of TB seen in this study. This observation lends support to the findings of a cohort study of HIV-infected Ethiopians that showed that low CD4 counts (<200 cells) and high viral load (median 4.97 log copies/ml, range 3.70–5.58), preceeded the diagnosis of TB (149).

Of the 5.8% of the pulmonary tuberculosis patients, 2% were picked by smear, 4.8% were identified by culture and 1.7% were both smear and culture positive. The higher sensitivity of culture is due to the lower detection limits (5-10 bacilli/ml) (150) as compared to the 5000-10000 bacilli /ml of smears (150).

In this study, the smears were screened first by auramine staining and those that were positive were counter-stained by the Ziehl-Neelsen staining technique. The reason is that the fluorescent staining method offers the advantage of screening the smear under low power where a wider field can be viewed and a large number of smears can be screened in a short time compared to the Ziehl-Neelsen technique, which was used for confirmation (151). The sensitivity of sputum smear microscopy in this study was 34.6% and is comparable to the 33 % found in Kenya (152). This low sensitivity indicates the limited usefulness of direct sputum microscopy, especially in HIV patients, a phenomenon that has been reported in other studies (153). Smear negative pulmonary TB has several implications that include; infectious risk to others (154) lowered notification rates (152, 155, 156), severe immunosuppression and poor prognosis (154, 157, 158,) and poses a challenge to the concept of isoniazid (INH) prophylaxis (159-160). In order to increase the diagnostic value of sputum microscopy in the light of the

growing HIV pandemic, concentration of decontaminated sputum specimens, which has been shown to increase sensitivity of microscopy for up to 30%, has been recommended (38, 39 161-165). In diagnostic facilities where the intended end point is smear microscopy, decontamination can be done by using 5% sodium hypochlorite (44, 166). However, if the specimen is to be cultured, decontamination and concentration should be done either using either 4% NaOH or NALC –2% NaOH for 30 minutes.

On comparison of smears and cultures there were a number of smear positive but culture negative samples (Table XIV). Of these, cases 3 and 5 could be explained by the fact that these patients were on anti-TB treatment at the time of submitting their specimens. It is known that *M.tuberculosis* looses viability after a few weeks of treatment but could still be seen in sputum smears for up to 2 months following initiation of treatment (167). Cases 1, 2, and 6 for which only one smear was positive and all cultures were negative, are most likely to be false positive cases (168-174). False-positive smears may result from; contamination the water or solutions used in staining, transfer of acid-fast organisms from positive to negative smears during the staining process or via microscope immersion oil or objective lenses, improperly filtered and irregular replacement of reagents, staining of artifacts present in the smear, insufficient de-staining of non-acid-fast organisms and from retention of acid-fast stain by non-mycobacterial organisms (175-178). Auramine staining procedures may produce a higher false-positive rate than carbol fuchsin methods, presumably because auramine may stain inanimate objects. Studies conducted in laboratories in Africa showed that about 1% of false positive smears could be attributed to transfer of organisms and

another 1% to various administrative errors. Careful quality control of microscopy procedures and review of positive smears can therefore markedly decrease the incidence of false-positive smears.

An additional factor could be the low incubation temperature (33^0C) that was frequently encountered due to improper calibration of the incubators and frequent power cuts, which affects growth of mesophilic mycobacteria (42).

In this study each sputum specimen was grown on both pyruvate and glycerol containing LJ medium. The former is known to favour the growth of *M.bovis* (42), which was not isolated from our patient population. Though most isolates (45.5%) in this study grew on both pyruvate and glycerol LJ slopes a significant proportion (54.5%) grew on one medium alone. The reason for the latter observation seems to be associated with the low gravitational force of 2000g used in this study for the concentration of sputum that led to insufficient dispersion and therefore unequal distribution of mycobacteria in the sample.

The culture contamination rate of 2.4% found in this study was within the acceptable range of 2-5% recommended by the World Health Organization (179). However, it is important to note that the modified Petroff's method used for decontamination has been found to be toxic, both for contaminants and for tubercle bacilli and may kill up to 60% of tubercle bacilli in clinical specimens and therefore, strict adherence to the indicated timing is required (42,). In order to avoid over exposure to sodium hydroxide, there is a need to process a small number of specimens, not more ten at a time (42). Other milder decontamination methods such as the N-acetyl-L- cysteine-sodium hydroxide (NALC-

NaOH) method (66) as well as the use of 2% NaOH) and cytocentrifugation, Zephiran-trisodium phosphate (Z-TSP) method (66) and oxalic acid method (66) should be considered that are able to support growth of mycobacteria while maintaining a low contamination rate (66).

In this study, speciation done by a combination of growth characteristics and biochemical tests indicate that majority (53.8%) of the isolates were *M tuberculosis*. However, the high proportion of NTM found in this study warrants further investigations. Although studies have indicated the etiological role of NTM in TB causation in HIV infected patients (180), this could also result from laboratory contamination of cultures with, for example, water containing saprophytic mycobacteria (175-177). In this study it was not possible to ascertain whether the isolated NTM were isolated from the patients or were mere contaminants since this would require genetic fingerprinting (168). The clues for cross-contamination normally include acid-fast smear negativity, growth only in broth or solid medium, and growth in solid medium with 5 or fewer colonies (181). Laboratory cross contamination can be minimised by instituting measures such as reducing the number of specimens processed at time, having one specimen container uncapped at a time in the safety cabinet, using aliquots of buffer and other reagents and not larger multi-use volumes and waiting after the specimen centrifugation step to allow time for aerosol settling in the test tube (182).

The fact that 5 (9.6%) of the isolates were untypable could be due to the speciation scheme used in this study. Mycobacterial identification based on their reactions in a series of phenotypic and biochemical tests, has been shown to vary from each other and

from time to time, and in many cases no definitive identification is obtained (76). Because biochemical testing is slow, cumbersome, and may yield ambiguous results, laboratories are increasingly using simple rapid molecular methods like AccuProbe and Line immunoprobe assay (LiPA) mycobacteria for species identification (183-188). These simple rapid speciation methods are quite sensitive, involve a few steps and are potentially cost-effective even in resource deprived settings (188).

In this study there were significant species differences with regard to anti-TB drugs susceptibility. A high proportion (89.3%) of the *M tuberculosis* isolates were fully susceptible to the first line drugs. The low level of MDR (3.6%) among the isolates from these HIV positive patients is similar to that of HIV negative TB patients in Tanzania (126). This finding does indicate the effectives of the national tuberculosis control programme in controlling the issue of emergence and spread of drug resistance through strict enforcement of the DOTS programme. Furthermore, the low magnitude of MDR in these patients indicates that HIV infection per se may not be a risk factor for multi-drug resistant tuberculosis, which is in keeping with previous observations (189). The high magnitude of resistance to first line anti-TB drugs among the NTM, including *M.fortitum* is not surprising because these mycobacteria do not respond to the first line drugs used for TB treatment (190). The drugs normally used for these species include amikacin, capreomycin, ciprofloxacin, clarithromycin, cycloserine, ethionamide and rifabutin (190). The significant difference in the drug susceptibility patterns of MTB, NTM and *M.fortitum* indicates that speciation is an important adjunct to TB treatment (60-62) and further emphasises the need to evaluate the etiological role of the NTM

species in our setting. A diagnosis of tuberculosis (TB) is rarely disputed if *Mycobacterium tuberculosis* is isolated from a clinical specimen; however, specimen contamination may occur (191-193). Identification of TB strain patterns through molecular typing or DNA fingerprinting is a recent advancement in TB laboratory techniques that could be used to determine the frequency of clustering among *M. tuberculosis* strains and possibility of laboratory cross-contamination (191). This could also be used to investigate the etiological role of the NTM isolates and therefore avoid erroneous diagnoses and unnecessary therapy and public health investigations. Of particular priority are specimens whose smears are negative and only one culture is positive. An inconsistent clinical course and a DNA fingerprint that matches those of other culture-positive specimens processed concurrently, coupled with the lack of an epidemiological connection would suggest cross-contamination.

9.0 CONCLUSION

In this population of ambulant HIV patients, majority of whom with CD 4 counts above 200 cells/ml, the prevalence of tuberculosis was 5.2% and was not correlated with CD4 counts. Majority (53.8%) of the isolates were *M. tuberculosis* and the others were NTM (17.3%), *M. fortitum* (19.2%) and a few were untypable. Majority (89.3%) of the *M. tuberculosis* isolates were fully sensitive to the first line drugs and only one isolate (3.6%) was multi drug resistant. NTM isolates including *M. fortitum* isolates were highly resistant to the first line drugs and had high levels of MDR (78.9%). The high isolation rate of NTM is worthy investigating further.

10.0 RECOMMENDATIONS

Based on the experience gained during this study the following recommendations are suggested:

1. To ensure and improve the quality of results obtained in this laboratory, the transition time between specimen collection and processing needs to be documented, media pH needs to be properly adjusted, the quality of water used in the preparation of stains should be checked, carbol fuchsin should be filtered regularly, and incubator temperatures should be regularly monitored. As a matter of internal quality control, it is important that smear results are compared with corresponding culture results. Furthermore, participation in external quality assuarance programmes for smear microscopy, cultures, speciation and drug susceptibility testing needs to be sustained, and improvement should be made on the basis of the scores obtained.

2. To avoid erroneous diagnoses and unnecessary therapy and public health investigations, specimens from patients with tuberculosis whose smears are negative and only one culture is positive should be investigated for cross-contamination. Laboratory cross contamination can be minimised by instituting measures such as; i) reducing the number of specimens processed at time, ii) having one specimen container uncapped at a time in the safety cabinet, iii) using aliquots of buffer and other reagents and not larger multi-use volumes iv) waiting after the specimen centrifugation step to allow time for aerosol settling in the test tube.

3 There is a need of substituting the rather cumbersome, slow and often-inconclusive biochemical scheme of speciation, with simple and rapid methods like gene probe and

LiPA mycobacteria. The introduction of fingerprinting technology will provide conclusive identification of isolates and evaluate the etiological role of the numerous NTM isolated in this study.

4 To improve drug susceptibility testing, there is a need to; i) ensure standardization of innoculum preparation, ii) the quality control strains, need to be replaced regularly to avoid loss of phenotypic characteristics with successive subcultures, iii) continue participating in external quality assuarance programme and strive to attain the required scores, and iv) improve turn around time of DST results by exploring rapid and simple methods such as phage replication assays that give results within 48hours of culture results.

11.0 REFERENCES

1. AIDS Control and Prevention (AIDSCAP) Project of Family Health Internal, The Francois-Xavier Bagnoud Center for Public Health and Human Rights Of the Harvard School of Public Health, UNAIDS. The Status and Trends of the Global HIV/AIDS Pandemic. Final Report July 5-6, 1996.
2. Raviglione MC, Narain JP, Kochi A. HIV-associated tuberculosis in developing countries: clinical features, diagnosis, and treatment. *Bull WHO* 1992;70:515-526.
3. Raviglione MD, Snider DE, Kochi A. Global epidemiology of tuberculosis: morbidity and mortality of a worldwide epidemic. *JAMA* 1995;273:220-226. Small PM. Tuberculosis research. Balancing the portfolio. *JAMA* 1996;276:1512-1513.
5. Dye C, Scheele S, Dolin P, Pathania V, Raviglione MC. Global burden of tuberculosis. Estimated incidence, prevalence, and mortality by country. *JAMA* 1999;282:677-686.
6. World Health Organisation. *TB; a global emergency*. Geneva: WHO, 1994
7. Coker R, Miller R. HIV associated tuberculosis. *BMJ* 1997;314:1847-1850.
8. World Health Organisation. *The world health report 1998*. Geneva: WHO, 1998.
9. Ustianowski A, Mwaba P, Zumla A. Tuberculosis and HIV; perspectives from sub-Saharan Africa. In: Porter JDH, Grange JM, eds. *Tuberculosis; an interdisciplinary perspective*. London: Imperial College Press, 1999.

10. Blinkhoff P, Bukanga E, Syamalevwe B, *et al. Under the mupundu tree.* Strategies for Hope Series No 14. London: Actionaid, 1999.

11. Havlir DV, Barnes PE. Tuberculosis in patients with human immunodeficiency virus infection. *N Engl J Med* 1999; 340:367-373.

12. Dolin PJ, Raviglione MC, Kochi A. Global tuberculosis incidence and mortality during 1990-2000. *Bull World Health Organ* 1994;72:213-220.

13. Chretien J. Tuberculosis and HIV. The cursed duet. *Bull Int Union Tuberc Lung Dis* 1990;65:25-28.

14. Alland D, Kalkut GE, Moss AR, *et al.* Transmission of tuberculosis in New York City; an analysis by DNA fingerprinting and conventional epidemiologic methods. *N Engl J Med* 1994;330:1710-1716.

15. Leitch AG, Rubilar M, Watt B, *et al.* Why disease due to *Mycobacterium tuberculosis* is less common than expected in HIV-positive patients in Edinburgh. *Respir Med* 1995;89:495-497.

16. United Republic of Tanzania. Ministry of Health. Manual of the National Tuberculosis and Leprosy programme in Tanzania, 2003.

17. Lucas S, Nelson AM. Pathogenesis of tuberculosis in human immunodeficiency virus-infected people. In: Bloom BR, ed. *Tuberculosis: pathogenesis, protection and control.* Washington: *ASM Press* 1994;503-513.

18. Jones BE, Young SMM, Antoniskis D, Davidson PT, Kramer F, Barnes PF. Relationship of the manifestations of tuberculosis to CD4 cell counts in patients

with human immunodeficiency virus infection. *Am Rev Respir Dis* 1993;148:1292-127.

19. Chintu C, Zumla A. Pediatric tuberculosis and the HIV epidemic. In: Zumla A, Johnson M, Miller RF, eds. *AIDS and respiratory medicine*. London: Chapman and Hall 1997;153-163.
20. Huebner RE, Castro KG. The changing face of tuberculosis. *Ann Rev Med* 1995;46:47-55.
21. Whalen C, Horsburgh CR, Hom D, *et al*. Accelerated course of human immunodeficiency virus infection after tuberculosis. *Am J Resp Crit Care Med* 1995; 151:129-134.
22. Harries AD. Tuberculosis in HIV-infected persons with special emphasis on sub-Saharan Africa. *J Infect* 1998;37:205-209.
23. WHO 1996. TB/HIV. A clinical manual .WHO/TB/96.200
24. Haries AD. Tuberculosis in Africa: Clinical presentation and management. *Pharmacol Ther*1997;73:1-50.
25. Teklu B. Symptoms of pulmonary tuberculosis in consecutive smear-positive cases treated in Ethiopia. *Tuberc. Lung Dis* 1993; 74: 126-128.
26. Ghidey Y, Habte D. Tuberculosis in childhood: an analysis of 412 cases. *Ethiop Med J* 1983; 21:161-167.
27. Miglori GB,Borghesi,Rossanogo P, *et al*. Proposal f an improved score method for the diagnosis of pulmonary tuberculosis in childhood in developing countries. *Tuber Lung Dis* 1992;73:145-149.

28. Castila J, Gutierrez A,. Guerra L, *et al.* Pulmonary and extra pulmonary tuberculosis at AIDS in Spain: epidemiological differences and implications for control. *AIDS* 1997;11:1583-1588.

29. Whalen C, Horsburgh CR, Hom D, Lahart C, Simberkoff M, Ellner J. Site of disease and opportunistic infection predict survival in HIV- associated tuberculosis. *AIDS* 1997;11:455-460.

30. Daley CL. The typically "atypical" radiographic presentation of tuberculosis in advanced HIV disease. *Tuber Lung Dis* 1995;76:475-476.

31. Tshibwabwa-Tumba E, Mwinga A, Pobee JOM, *et al.* Radiological features of pulmonary tuberculosis in 963 HIV-infected adults at three central African hospitals. *Clin Radiol* 1997;52:837-841.

32. Saks AM, Posner R. Tuberculosis in HIV positive patients in South Africa: a comparative radiological study with HIV negative patients. *Clin Radiol* 1992;46:387-390.

33. Greenberg SD, Frager D, Suster B, *et al.* Active pulmonary tuberculosis in patients with AIDS: spectrum of radiographic findings (including a normal appearance). *Radiol* 1994;193:115-119.

34. Long R, Maycher B, Scalcini, M *et al.* The chest roentgenogram in pulmonary tuberculosis patients seropositive for immunodeficiency virus type-1. *Chest* 1991;99:123-127.

35. Nelson SM, Deike MA, Cartwright CP. Value of examining of multiple sputum specimens in the diagnosis of pulmonary tuberculosis. *J Clin Microbiol* 1998; 36:467-469.

36. Warren JR, Bhattacharya M, De Almeida KN, Trakas K, Peterson LR. A minimum of 5.0mls improves the sensitivity of acid fast smears for *Mycobacterium tuberculosis*. *Am J Respir Crit Care Med* 2000;16:1559-62.

37. Falkinham JO, III. Epidemiology of infection by non-tuberculous mycobacteria. *Clin Microbiol Rev* 1996; 9:177-215.

38. Bruchfeld J, Aderaye G, Palme IB, Bjorvatn B, Kallenius G, Lindquist L. Sputum concentration improves diagnosis of tuberculosis in a setting with a high prevalence of HIV. *Trans R Soc Trop Med Hyg* 2000;94:677-80.

39. Apers L, Mutsvangwa J, Magwenzi J, *et al*. A comparison of direct microscopy, the concentration method and the Growth Indicator Tube for examination of sputum for acid-fast bacilli. *Int J Tuberc Lung Dis* 2003; 7:376-381

40. Heifets LB, Good R. Current laboratory methods for the diagnosis of tuberculosis. In Tuberculosis: pathogenesis, protection and control. Bloom BR, *ASM press* Washington DC 1994.

41. Paramasivan CN, Sputum smear examination, Tuberculosis research centre. Indian council of Medical Research Chennai.

42. Collins C H; Grange John M; Yates M D. Organization and practice in tuberculosis bacteriology. London; Boston: Butterworths, 1985

43. Tomani K. Tuberculosis case finding and chemotherapy. Questions and answers, Geneva: *World Health Organisation*; 1979

44. Gebre-Selassie S. Evaluation of the concentration sputum smear technique for the laboratory diagnosis of pulmonary tuberculosis. *Trop Doct* 2003; 33:160-162.

45. Heifets LB, Cangelosi GA. Drug susceptibility testing of *Mycobacterium tuberculosis*: a neglected problem at the turn of the century. *Int J Tuberc Lung Dis* 1999;3:564-581.

46. Canetti G, Froman S, Grosset J, *et al.* Mycobacterial laboratory methods for testing susceptibility and drug resistance. *Bull World Health Organ* 1963;29:565-570.

47. Caneetti G,Fox W,Khomenko A, et al. Advances in techniques of testing mycobacterial drug sensitivity, and the use of sensitivity tests in tuberculosis programmes. *Bull World Health Organ* 1969;41:21-43.

48. Heifets L.B. Drug susceptibility in the management of chemotherapy of tubeerculosis. In Heifets L B (ed) Drug susceptibility in the chemotherapy of mycobacterial infections. 1991;89-120 *CRC press*, Inc. USA.

49. Tenovar FC, Crawford JT, Heubner RE, Geiter LJ, Horshburgh CR Jr, Good RC. The resurgency of tuberculosis: Is your laboratory ready? *J Clin Microbiol* 1993; 31:767-770.

50. Siddiqi SH, Libonati JP, Middlebrook G. Evaluation of rapid radiometric method for drug susceptibility testing of *Mycobacterium tuberculosis*. *J Clin Microbiol* 1981;13:908-912.

51. Chew WK, Lasaitis RM, Schio FA, Gilbert GL. Clinical evaluation of the Mycobacteria Growth Indicator Tube (MGIT) compared with radiometric (Bactec) and solid media for isolation of mycobacterium species. *J Med Microbiol* 1998;47:821-827.

52. Tartoli E, Cichero P, Piersimoni C, Simonetti MT, Gesu G, Nista D. Use of Bactec MGIT 960 for recovery of mycobacteria from clinical specimens. Multicentre study. *J Clin Microbiol* 1999;37:3578-3582.

53. Somoskeovi A, Keodmeon C, Lantos A, et al. Comparison of recoveries of *Mycobacterium tuberculosis* using the automated BACTEC MGIT 960 system, the BACTEC 460 system and Lowen-stein Jensen medium. *J Clin Microbiol* 2000;37:25-30.

54. Yan JJ, Huang AH, Tsai SH, Ko WC, Jin YT, Wu JJ. Comparison of the MB/BacT and BACTEC MGIT 960 system for recovery of mycobacteria from clinical specimens. *Diagn Microbiol Infect Dis* 2000; 37:25-30.

55. Pfyffer GE, Welscher HM, Kissling P, *et al*. Comparison of Mycobacteria Growth Indicator Tube (MGIT) with radiometric and solid culture for acid fast bacilli. *J Clin Microbiol* 1997; 35:364-8

56. Katila ML, Katila P, Erkinjuntii-Pekkman R. Accelerated detection and identifaiction of mycobacteria with MGIT 960 and COBAS AMPLICLOR systems. *J Clin Microbiol* 2000; 38:960-4.

57. Riuz P, Zerolo FJ, Casal MJ. Comparison of susceptibility testing of *Mycobacterium tuberculosis* using ESP culture system II with that using the BACTEC method. *J Clin Microbiol* 2000; 38:4663-4.

58. Woods GL, Fish G, Plaunt M, Murphy T. Clinical evaluation of the Difco ESP culture system II for growth and detection of mycobacteria. *J Clin Microbiol* 1997;35.121-124.

59. Caviedes L, Lee TS, Gilman RH, *et al*. Rapid, efficient detection and drug susceptibility testing of *Mycobacterium tuberculosis* in sputum by microscopic observation of broth cultures. The tuberculosis working group in Peru. *J Clin Microbiol* 2000;. 38:2395-2397.

60. Huebner RE, Good RC, Tokars JI. Current practices in mycobacteriology: results of a survey of state public health laboratories. *J Clin Microbiol* 1993;31:771-775.

61. Metchock G, Nolte FS, Wallace R J. Mycobacterium, 1999. p. 399-437. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), Manual of clinical microbiology, 7th ed. American Society for Microbiology, Washington, D.C.

62. Salfinger M, Pfyffer GE. The new diagnostic mycobacteriology laboratory. *Eur J Clin Microbiol Infect Dis* 1994;13:961-979.

63. Springer BL, Stockman K, Teschner GD, Roberts E, *et al*. A two-laboratory collaborative study on identification of mycobacteria: molecular versus phenotypic methods. *J Clin Microbiol* 1996;34:296-303.

64. Cernoch PL, Enns RK, Saubolle MA, Wallace RJ. 1994. Cumitech 16A Laboratory diagnosis of the mycobacterioses. Coordinating ed., A. S. Weissfield. American Society for Microbiology, Washington, D.C.

65. Herold, CD. Fitzgerald RL, Herold DA. Current techniques in mycobacterial detection and speciation. *Crit Rev Clin Lab Sci* 1996;33:83-138.

66. Kent PT, Kubica GP. 1985. Public health mycobacteriology. A guide for the level III laboratory. U.S. Department of Health and Human Services, Centers for Disease Control, Atlanta, Ga.

67. Kiehn TE. 1993. The diagnostic mycobacteriology laboratory of the 1990s, p. S447-S454.

68. Wayne LG, Mycobacterial speciation. In Kubica GP and Wayne LG. The Mycobacteria, a source book 1st ed. Marcel Dekker inc. New York 1984.

69. Taylor TB, Paterson C, Hale Y, Safranek WW. Routine use of PCR restriction fragment length polymorphism analysis for identification of mycobacteria growing in liquid media. *J Clin Microbiol* 1997; 35:79-85.

70. Zumarranga M, Bigi F, Alito A, Romano MI, Cataldi A. A 12.7 kb fragment of the *Mycobacterium tuberculosis* genome is not present in Mycobacterium bovis. *Microbiol* 1999; 145:893-897.

71. Hongmanee P, Stender H, Rasmussen OF. Evaluation of a fluorescent in situ hybridization assay for differentiation between tuberculous and non-tuberculous mycobacterium species in smears of Lowenstein Jensen and mycobacteria growth indicator tube cultures using peptide nucleic acid probes. *J Clin Microbiol* 2001;39:1032

72. Kellogg JA, Bankert DA, Withers G, Sweimler W, Keihn TE, Pfyffer GE. Application of the Sherlock mycobacteria identification system using high performance liquid chromatography in a clinical laboratory. *J Clin Microbiol* 2001;39: 964-970.

73. Devalois A, Goh KS, Rastogi N. Rapid identification of mycobacteria to species level by by PCR restriction fragment length polymorphism analysis of the hsp65 gene and and preposition of an alogarithm to differentiate 34 mycobacterial species. *J Clin Microbiol* 1997;35:2969-2973.

74. Karabachew M, Enger, Sandaa RA, Lemma E, Bjorvatn B. Amplified ribosomal DNA restriction anlysis in the differentiation of related species of mycobacteria. *J Microbiol Methods* 2003;55:83-90

75. Gingeras TR, Ghandour G, Wang E, *et al*. Simultaneous genotyping and species identification using hybridisation pattern recognition pattern analysis of generic mycobacterium DNA arrays. *Genome Res* 1998;8: 435-438.

76. Soini H, Musser JM. Molecular diagnosis of mycobacteria. *Clin Chem* 2001;47:809-814.

77. Negi SS, Khan SFB, Gupta S, Pasha ST, Khare S, Lal S, Comparison of the conventional diagnostic modalities, BACTEC culture and Polymerase chain reaction test for diagnosis of tuberculosis. *Ind J Med Microbiol* 2005;23:29-33

78. van Emden JD, Cave MD, Crawford JT, Dale JW, Eisenach KD, Gicquel B, *et al* Strain identification of *Mycobacteria tuberculosis* by DNA finger printing: Recommendations for a standardised methodology. *J Clin Microbiol* 1993;31 :406-9

79. CDC. Diagnosis of tuberculosis by nucleic acid amplification methods applied to clinical specimens. *MMWR* 1993;42:35-38.

80. Shaw RJ, Taylor GM. Polymerase chain reaction: Applications for diagnosis, drug sensitivity and strain identification of *M.tuberculosis* complex. In: Clinical Tuberculosis , (2nd Edition) ED , P.D.O Davies, Chapman and Hall , London, 1998;p.47

81. Nightingale SL. From the Food and Drug Administration: new tuberculosis test approved. *JAMA* 1996;275:585.

82. Gene probe Gen-Probe. Amplified *Mycobacterium tuberculosis* direct test for in-vitro diagnostic use:San Diego, California: Gen-Probe, July 23, 1996.

83. Bennedsen J, Ostergaard Thomsen V, Pfyffer GE, *et al.* Utility of PCR in diagnosing pulmonary tuberculosis. *J Clin Microbiol* 1996;34:1407-1411.

84. Bradley SP, Reed SL, Catanzaro A. Clinical efficacy of the amplified *Mycobacterium tuberculosis* direct test for the diagnosis of pulmonary tuberculosis. *Am J Respir Crit Care Med* 1996;153: 1606-1610.

85. Hellyer TJ, Fletcher TW, Bates JH, *et al.* Strand displacement amplification and the polymerase chain reaction for monitoring response to treatment in patients with pulmonary tuberculosis. *J Infect Dis* 1996;173:934-941.

86. Moore DF, Curry JI, Knott CA, Jonas V. Amplification of rRNA for assessment of treatment response of pulmonary tuberculosis patients during antimicrobial therapy. *J Clin Microbiol* 1996;34:1745-1749.

87. Lindbråthen A, Gaustad P, Hovig B, Tønjum T. Direct detection of *Mycobacterium tuberculosis* complex in clinical samples from patients in Norway by ligase chain reaction. *J Clin Microbiol* 1997; 35:3248–3253.

88. Chemal K, Portaels F. Molecular diagnosis of non-tuberculosis mycobacteria. *Curr Opin Infect Dis* 2003; 16:77-83.

89. Kambashi B, Mbulo G, McNerney R, *et al.* Utility of nucleic acid amplification techniques for the diagnosis of pulmonary tuberculosis in sub-Saharan Africa. *Int J Tuberc Lung Dis.* 200;5:364-369.

90. Muzaffar R, Batool S, Aziz F, Naqvi A, and Rizvi R. Evaluation of FAST Plaque TB assay for direct detection of *M.tuberculosis* in sputum specimens. *Int J Tuberc Lung Dis* 2002;6: 635-640.

91. Albert H, Heydenrych A, Brookers R, *et al.* Performance of rapid phage based test FAST plaque TB TM to diagnose pulmonary tuberculosis from sputum specimens in South Africa. *Int J Tuberc Lung Dis* 2002;529-537.

92. Mshanga IM, Matee MIN, Nyambo TB, McNerney R. Evaluation of Dot-Blot and Phage replication techniques for detection of drug resistant *Mycobacterium tuberculosis Tanz Med J* (submitted)

93. World Health Organization. TB/ HIV. A Clinical manual WHO/HTM/TB2004.329.

94. Anonymous. Treatment regimens in HIV-infected tuberculosis patients. An official statement of the International Union Against Tuberculosis and Lung Disease. *Int J Tuberc Lung Dis* 1998;2:175-178.

95. Ackah AN, Coulibaly D, Digbeu H, *et al.* Response to treatment, mortality, and CD4 lymphocyte counts in HIV-infected persons with tuberculosis in Abidjan, Cote d'Ivoire. *Lancet* 1995;345:607-610.

96. Kenyon TA, Mwasekaga MJ, Huebner R, *et al.* Low levels of drug resistance amidst rapidly increasing tuberculosis and human immunodeficiency virus co-epidemics in Botswana. *Int J Tuberc Lung Dis* 1999;3:4-11.

97. Perriens JH, Colebunders RL, Karahunga C, *et al.* Increased mortality and tuberculosis treatment failure rate among human immunodeficiency virus (HIV) seropositive compared with HIV seronegative patients with pulmonary tuberculosis treated with "standard" chemotherapy in Kinshasa, Zaire. *Am Rev Respir Dis* 1991;144:750-755.

98. van den Broek J, Mfinanga S, Moshiro C, *et al.* Impact of human immunodeficiency virus on the outcome of treatment and survival of

tuberculosis patients in Mwanza, Tanzania. *Int J Tuberc Lung Dis* 1998;2:547-552.

99. Kochi A, Vareldzis B, Styblo K. Multidrug-resistant tuberculosis and its control. *Res Microbiol* 1993;144:104-110.

100. Anti-tuberculosis drug resistance in the world: the WHO/IUATLD Global project on antituberculosis drug resistance surveillance. Geneva, World Health Organisation, 1997 (Document WHO/TB/97.229).

101. Anti-tuberculosis drug resistance in the world: the WHO/IUATLD Global project on antituberculosis drug resistance surveillance. Report 2: Prevalence and trends. Geneva, World Health Organisation, 2000 (document WHO/CDS/TB/2000.278).

102. Hausdorfer J, Sompeck E, Allerberger F, Dierich MP, Rusch-Gerders S. E-test for susceptibility testing of *Mycobacterium tuberculosis Int J Tuberc Lung Dis* 1998;2: 751.

103. Franzbalau SG, Witzing RS, McLaughin JC, *et al*. Rapid, Low technology MIC determination with clinical Mycobacterium isolates by using microplate alamar blue assay. *J Clin Microbiol* 1998;36: 362-366.

104. Mshana RN, Tadesse G, Abate G, Miorner H. Use of 3 (4,5-dimetheylthiozol-2-yl)-2,5 diphenyl tetrazolium bromide for rapid detection of rifampicin resistant M.tuberculosis. *J Clin Microbiol* 1998;36:1214-1219.

105. Vlader-Salvado JM, Garza-Gonzalez E, de los Angeles M, *et al*. Mycolic acid index susceptibility method for *Mycobacterium tuberculosis*. *J Clin Microbiol* 2001;39:2642-2645.

106. Meija GI, Castrillon L, Trujillo H and Robeldo JA. Micro colony detection in 7H11 thin layer culture is an alternative for rapid diagnosis of *Mycobacterium tuberculosis* infection. *Int J Tuberc Lung Dis* 1993: 138.

107. Hu ZY. Phage amplified biologically assay and its application in rapid detection of Mycobacterium tuberculosis. *Zhonghua Jie He He Hu Xi Za Zhi* 2004;27:850-852.

108. Han XQ, Ma Y, Gao WW, *et al*. Analysis and evaluation of phage amplified biologically assay, DNA sequencing analysis and single-strand conformational polymorphism for the drug susceptibility testing of Mycobacterium tuberculosis. *Zhonghua Jie He He Hu Xi Za Zhi* 2004;27:815-819.

109. Hu ZY, Jin AJ, Chen HP, *et al*. Application of rapid detection for rifampin resistance in clinical isolates of *Mycobacterium tuberculosis* by phage amplified biologically assay. *Zhonghua Jie He He Hu Xi Za Zhi* 2004;27:811-814

110. Riska PF, Su Y, Bardorov S, *et al*. Rapid film based determination of antibiotic susceptibilities of *Mycobacterium tuberculosis* strains using a luciferase reporter phage and Bronx Box. *J Clin Microbiol* 1999;37: 1144.

111. Collin LA, Torrero M N, Franzblau SG. Green Flourescent protein reporter microplate assay for high throughput screening of compounds against *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 42: 334.

112. Ramaswamy S, Musser JM. Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. *Tuber Lung Dis* 1998;79:3-29.

113. Banerjee A, Dubnau E, Quemard *A et al.* inhA a gene encoding a target for INH and Ethionamide in *Mycobacterium tuberculosis. Science* 1994; 263:227-230.

114. Mdluli K, Slaydeen R.A, Zhu Y, et al. Inhibition of a *Mycobacterium tuberculosis* Ketoacyl ACP synthase by isoniazid. *Science* 280:1607-1613.

115. Sharman D R., Mdluli K., Hickey M J., *et al.* Compensatory ahpC gene expression in isoniazid resistance *Mycobacterium tuberculosis*. *Science* 1998 272:1641-1643.

116. ZhangY., Heym B., Alleen B., Young D., Cole S., The catalse peroxidase gene and isoniazid resistance in *Mycobacterium tuberculosis*. *Nature* 1992;358:591-593

117. Telenti A, Imboden P, Marchesi F, et al. Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. *Lancet* 1993;341:647-50.

118. Telenti A, Imboden P, Marchesi F, Schidheini T, Bodmer T. Direct, automated detection of rifampicin-resistant *Mycobacterium tuberculosis* by polymerase chain reaction and single-strand conformation polymorphism analysis. *Antimicrob Agents Chemother* 1993;37:2054-2058.

119. Williams DL, Waguespack C, Eisenach K, et al. Characterization of rifampicin-resistance in pathogenic mycobacteria. *Antimicrob Agents Chemother* 1994;38:2380-2386.

120. Felmlee TA, Lui Q, Whelen AC, Williams D. Sommer SS, Persing DH. Genotypic detection of *Mycobacterium tuberculosis* rifampicin resistance: comparison of single strand conformation polymorphism and Dideoxy fingerprinting. *J Clin Microbiol* 1995;33:1617-1623.

121. Cockerill F R ,Uhl J R, Temesgen Z, *et al*. Rapid identification of point mutations of the *Mycobacterium tuberculosis* catalse peroxidase (KatG) gene associated with Isoniazid resistance . *J Infect Dis* 1995;171:240-245.

122. Dobner P, Rush-Gerdes S, Bretzel G, *et al.* Usefulness of *Mycobacterium tuberculosis* genomic mutations in the genes KatG and inhA for the prediction of Isoniazid resistance. *Int J Tuberc Lung Dis* 1997;1:365-369.

123. Beenhouwer H.De., Lhiang Z., Jannes G., *et al*. Rapid detection of rifampicin resistance in sputum and biopsy specimens from tuberculosis patients by PCR and line probe assay. *Tuber Lung Dis* 1995;76:425-430.

124. Nash K.A., Gaytan A., Inderlied C.B Detection of rifampicin resistance in *Mycobacterium tuberculosis* by use of a rapid, simple, and specific RNA/RNA mismatch assay *J Infect Dis* 1997;176:533-536.

125. Piatek A.S, Tyagi S, Pol. A.C, *et al* Molecular beacon sequence analysis for detecting drug resistance in *Mycobacterium tuberculosis*. *Nat.Biotechnol*.1998;16:359-363..

126. United Republic of Tanzania. Ministry of Health. Annual report of the National TB/Leprosy Programme Tanzania 2001.

127. WHO Report 2002 Global Tuberculosis Control: Surveillance, Planning, Financing. WHO/CDS/TB/2002.295.

128. Egwaga SM. The impact of HIV on transmission of tuberculosis in Tanzania. *Tuberculosis* 2003; 83: 66-67

129. Kelly PM. Local problems, local solutions: improving tuberculosis control at the district level in Malawi. *Bull World Health Organ* 2001;79:111-7.

130. Liberato IR, de Albuquerque Mde F, Campelo AR, de Melo HR. Characteristics of pulmonary tuberculosis in HIV seropositive and seronegative patients in a Northeastern region of Brazil.*Rev Soc Bras Med Trop* 2004;37:46-50.

131. Yassin MA, Takele L, Gebresenbet S, Girma E, Lera M, Lendebo HIV and tuberculosis coinfection in the southern region of Ethiopia: a prospective epidemiological study. *Scand J Infect Dis* 2004; 36:670-673.

132. Kivihya-Ndugga LE, van Cleeff MR, Githui WA, *et al*. A comprehensive comparison of Ziehl-Neelsen and fluorescence microscopy for the diagnosis of tuberculosis in a resource-poor urban setting. *Int J Tuberc Lung Dis* 2003;7:1163-71

133. Murcia-Aranguren MI, Gomez-Marin JE, Alvarado FS *et al*. Frequency of tuberculous and non-tuberculous mycobacteria in HIV infected patients from Bogota, Colombia. *BMC Infect Dis* 2001;1:21.

134. Yang ZH, Mtoni I, Chonde M *et al*. DNA fingerprinting and phenotyping of *Mycobacterium tuberculosis* isolates from human immunodeficiency virus

(HIV)-seropositive and HIV-seronegative patients in Tanzania. *J Clin Microbiol* 1995;33:1064-1069.

135. Paramasivan CN, Venkataraman P. Drug resistance in tuberculosis in India. *Indian J Med Res* 2004;120:377-86.

136. Ya Diul M, Maher D, Harries A. Tuberculosis case fatality rates in high HIV prevalence populations in Sub-saharan Africa. *AIDS* 2001;15:143-152.

137. Salfinger M, Morris AJ. The role of the microbiology laboratory in diagnosing mycobacterial diseases. *Am J Clin Pathol* 1994;101:6–13.

138. National Committee for Clinical Laboratory Standards. Susceptibility Testing of mycobacteria, and Other aerobic actinomycetales*: Tentative standard.* 2nd ed. NCCLS document M24-T2. Wayne, Pennsylvania:NCCLS, 2000.

139. Woods GL. Susceptibility testing for mycobacteria. *Clin Infect Dis* 2000; 31:1209–15.

140. CDC. Case definitions for public health surveillance. *MMWR* 1990;39:41-42.

141. Neelsen, F.Ein casuistischer Beitrag zur Lehre von der Tuberkulose. Centrabl. Med. Wissenschaften 1883.28:497-501.

142. Weyer, K.. Laboratory services in tuberculosis control. Part II. Microscopy. WHO technical bulletin World Health Organization, Geneva, Switzerland.1998 98.258.

143. Baker, J. F., and R. E. Silverton.. Introduction to medical laboratory technology, 5th ed. 1978, p. 528-530. Butterworths, London, England.

144. WHO. Guidelines for surveillance of drug resistance in tuberculosis. WHO/TB 2003.320-WHO/CDS/CSR/RMD/2003.3

145. Raviglione MD, Snider DE, Kochi A. Global epidemiology of tuberculosis: morbidity and mortality of a worldwide epidemic. *JAMA* 1995;273:220-226.

146. Mtei L, Matee M, Herfort O *et al*. High rates of clinical and sub-clinical tuberculosis among HIV infected ambulatory subjects in Tanzania. *Clin Inf Dis* (In press).

147. Desormeaux J, Johnson MP, Coberly JS, *et al* Widespread HIV counselling and testing linked to a community-based tuberculosis control program in a high-risk population. Bull Pan Am Health Organ. 1996;30:1-8.

148. Nachega J, Coetzee J, Adendorff T, *et al*. Tuberculosis active case-finding in a mother-to-child HIV transmission prevention programme in Soweto, South Africa AIDS. 2003 13;17:1398-400.

149. Wolday D, Hailu B, Girma M, Hailu E, Sanders E, Fontanet AL Low CD4+ T-cell count and high HIV viral load precede the development of tuberculosis disease in a cohort of HIV-positive Ethiopians. *Ethiop Med J* 2003;41:67-73.

150. David HL. Bacteriology of mycobacterioses. US Department of Health, Education and Welfare, Public Health Service, Communicable Disease Centre, Atlanta, USA, 1996.

151. What is new in the diagnosis of tuberculosis? Part I: Techniques for diagnosis of tuberculosis. *IMCR Bulletin* August, 2003:32, No.8

152. Githui W, Nunn P, Juma E *et al*. Cohort study of HIV-positive and HIV-negative tuberculosis, Nairobi, Kenya: comparison of bacteriological results. *Tuber Lung Dis* 1992;73:203-9.

153. Elliot. A.M, Luo, N, Tembo, G, *et al*. Impact of HIV on tuberculosis in Zambia: A Cross-Sectional Study. *BMJ* 1990; 30:412-415.

154. Behr MA, Warren SA, Salamon H, *et al*. Transmission of *Mycobacterium tuberculosis* from patients smear-negative for acid-fast bacilli. *Lancet* 1999;353:444-449.

155. Hargreaves NJ, Kadzakumanja O, Whitty CJ, Salaniponi FM, Harries AD, Squire SB. 'Smear-negative' pulmonary tuberculosis in a DOTS programme: poor outcomes in an area of high HIV seroprevalence. *Int J Tuberc Lung Dis* 2001;5:847-854.

156. Hawken MP, Muhindi DW, Chakaya JM, Bhatt SM, Ng'ang'a LW, Porter JD. Under-diagnosis of smear-positive pulmonary tuberculosis in Nairobi, Kenya. *Int J Tuberc Lung Dis* 2001;5:360-363.

157. Elliott AM, Namaambo K, Allen BW, *et al*. Negative sputum smear results in HIV-positive patients with pulmonary tuberculosis in Lusaka, Zambia. *Tuber Lung Dis* 1993;74:191-194.

158. Banda H, Kang'ombe C, Harries AD *et al*. Mortality rates and recurrent rates of tuberculosis in patients with smear-negative pulmonary tuberculosis and tuberculous pleural effusion who have completed treatment. *Int J Tuberc Lung Dis* 2000;4:968-974

159. Palmieri F, Girardi E, Pellicelli AM *et al* . Pulmonary tuberculosis in HIV-infected patients presenting with normal chest radiograph and negative sputum smear. *Infect* 2002;30:68-74.

160. Missed opportunities for prevention of tuberculosis among persons with HIV infection--selected locations, United States, 1996-1997. *MMWR* 2000;49:685-687.

161. Bell JC, Rose DN, Sacks HS. Tuberculosis preventive therapy for HIV-infected people in sub-Saharan Africa is cost-effective. *AIDS* 1999;20:1549-1556.

162. Anglaret X, Dabis F, Batungwanayo J, *et al*. Primary chemoprevention of tuberculosis in HIV-infected patients in non-industrialized countries. *Sante* 1997;7:89-94.

163. Saxena S, Mathur M, Talwar VK. Detection of tubercle bacilli in sputum: application of sodium hypochlorite concentration method. *J Com Dis* 2001;33:241-244.

164. Peterson EM, Nakasone A, Planton-Deleon JM, Jang Y, de La Maza LM, Desmond E. Comparison of direct and concentrated acid-fast smears to identify specimen culture positive for mycobacterium spp. *J Clin Miicrobol* 1999;37: 3564-3568.

165. Habeenzu C, Lubasi D, Flemng AF. Improved sensitivity of direct microscopy for detection of acid fast bacilli in sputum in developing countries. *Trans R Soc Trop Med Hyg* 1998; 92:415-6.

166. Ängeby KAK Hoffner SE, Diwan VK. Should the 'bleach microscopy method' be recommended for improved case detection of tuberculosis? Literature review and key person analysis. *Inter J TB Lung Dis* 2004; 8: 806-815.

167. Jaime C Montaya, MD, Edna Santiago MD, Minda Manalo, MD and Edwin Dy MD. Clinical, radiologic, and microbiologic features mycobacterial infection in patients with HIV. *Phil J Mcrobiol infect Dis* 1998; 27: 97-101.

168. Foldy S, Rach J, Tjaden J, et al. Multiple misdiagnosis of tuberculosis resulting from laboratory error –Wisconsin 1996. *MMWR* 1997; 46:797–801.

169. Burman WJ, Reves RR. Review of false-positive cultures for *Mycobacterium tuberculosis* and recommendations for avoiding unnecessary treatment. *Clin Infect Dis* 2000;31:1390–1395.

170. Burman WJ, Stone BL, Reves RR, *et al.* The incidence of false-positive cultures for *Mycobacterium tuberculosis*. *Am J Respir Crit Care Med* 1997;155:321–326.

171. Wurtz R, Demarais P, Trainor W, *et al.* Specimen contamination in mycobacteriology laboratory detected by pseudo-outbreak of drug-resistant tuberculosis: analysis by routine epidemiology and confirmation by molecular technique. *J Clin Microbiol* 1996;34:1017–1019.

172. Bauer J, Thomsen VO, Poulsen S, *et al.* False-positive results from cultures of *Mycobacterium tuberculosis* due to laboratory cross-contamination confirmed by restriction fragment length polymorphism. *J Clin Microbiol* 1997;35:988–91.

173. Ramos M de C, Soini H, Roscanni GC, et al. Extensive cross-contamination of specimens with *Mycobacterium tuberculosis* in a reference laboratory. *J Clin Microbiol* 1999;37:916–919.

174. Shilkret K, Liv Z, Santos F, ***et al.*** Misdiagnoses of tuberculosis resulting from laboratory cross-contamination of *mycobacterium tuberculosis* cultures—New Jersey, 1998 *JAMA*. 2000;283:3191-3193.

175. Peters M, Muller C, Rusch-Gerdes, Seidel C, Gobel U, Pohle HD, Ruf B. Isolation of atypical mycobacteria from tap water in hospitals and homes: Is this a possible source of disseminated MAC infection in AIDS patients. *J Infect* 1995; 31:39-41.

176. Lalande V, Barbut F, Varnerot A *et al*. Pseudooutbreak of *Mycobacterium gordonae* associated with water from refrigerated fountains. *J Hosp Infect* 2001; 48:76-79.

177. Sniadack DH, Ostroff SM, Karlix MA, *et al*. A nosocomial pseudo-outbreak of *Mycobacterium xenopi* due to a contaminated potable water supply: lessons in prevention. *Infect Control Hosp Epidemiol* 1993;14:636-41.

178. Le Dantec C, Duguet JP, Montiel A, Dumoutier N, Dubrou S, Vincent V. Occurrence of mycobacteria in water treatment lines and in water distribution systems. *Appl Environ Microbiol* 2002;68:5318-5325.

179. WHOSEA. Standard operating procedures for laboratory diagnosis of tuberculosis and *M avium* complex disease in HIV positive patients In: Guidelines on Standard Operating Procedures for Laboratory Diagnosis of HIV-

Opportunistic Infections. Ed. Dr Sudarshan Kumari. WHO Regional office for South East Asia. June 2001. SEA-HLM-332

180. Corbett EI, Blumberg L, Churchyard GJ, *et al.* Nontuberculous mycobacteria defining disease in a prospective cohort of South African miners. *Am J Respir Crit Care Med* 1999; 160:15-21.

181. Bhattacharya M, Dietrich S, Mosher L, *et al.* Cross-contamination of specimens with *Mycobacterium tuberculosis*: clinical significance, causes, and prevention. *Am J Clin Pathol* 1998;109:324-330.

182. Breese PE, Burman WJ, Hildred M, *et al.* The effect of changes in laboratory practices on the rate of false-positive cultures for *Mycobacterium tuberculosis*. *Arch Pathol Lab Med* 2001;125:1213–1216.

183. Lebrun L, Gonullu N, Boutros N *et al.* Use of INNO-LIPA assay for rapid identification of mycobacteria *Diagn Microbiol Infect Dis* 2003;46:151-153.

184. Mijs W, De Vreese K, Devos A, *et al.* Evaluation of a commercial line probe assay for identification of mycobacterium species from liquid and solid culture. *Eur J Clin Microbiol Infect Dis* 2002;21:794-802.

185. Tortoli E. Identification of mycobacteria by using INNO LiPA. *J Clin Microbiol* 2002;40:3111-3114.

186. Sarkola A, Makinen J, Marjamaki M, Marttila HJ, Viljanen MK, Soini H. Prospective evaluation of the genotype assay for routine identification of mycobacteria. *Eur J Clin Microbiol Infect Dis* 2004;23:642-645.

187. Richter E, Weizenegger M, Fahr AM, Rusch-Gerdes S. Usefulness of the GenoType MTBC assay for differentiating species of the *Mycobacterium tuberculosis* complex in cultures obtained from clinical specimens. *J Clin Microbiol* 2004;4:4303-4306.

188. Mäkinen JA, Sarkola M, Marjamäki MK, Soini H. Evaluation of GenoType and LiPA MYCOBACTERIA assays for identification of Finnish mycobacterial isolates. *J Clin Microbiol* 2002;40:3478-3481.

189. O'Brien RJ. Drug resistant tuberculosis: aetiology, management and prevention. *Sem Resp Iinfect* 1994; 9:104-112.

190. Wallace RJ Jr, O'Brien R, Glassroth J, Raleigh J, Dutta A. Diagnosis and treatment of disease caused by nontuberculous mycobacteria. *Am Rev Respir Dis* 1990;142:940-53.

191. Braden CR, Templeton GL, Stead WW, Bates JH, Cave MD, Valway SE. Retrospective detection of laboratory cross-contamination of Mycobacterium tuberculosis cultures with use of DNA fingerprint analysis. *Clin Infect Dis* 1997;24:35-40.

192. Jasmer RM, Roemer M, Hamilton J, Bunter J, Braden CR, Shinnick TM, Desmond EP. A prospective, multicenter study of laboratory cross-contamination of *Mycobacterium tuberculosis* cultures. *Emerg Infect Dis* 2002;8:1260-1263.

193. Small PM, McClenny NB, Singh SP, Schoolnik GK, Tompkins LS, Mickelsen PA. Molecular strain typing of Mycobacterium tuberculosis to confirm cross-

contamination in the mycobacteriology laboratory and modification of procedures to minimize occurrence of false-positive cultures. *J Clin Microbiol* 1993;31:1677-82