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PULMONARY TUBERCULOSIS AND *PNEUMOCYSTIS JIROVECI* PNEUMONIA IN HIV-INFECTED PATIENTS IN ETHIOPIA

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ABSTRACT

The objective of this study initially was to determine the prevalence of culture-verified pulmonary tuberculosis in TB suspects and investigate the impact of human immunodeficiency virus (HIV) infection on the prevalence, clinical and radiological presentation and diagnosis of tuberculosis. During the study, it soon became clear that the HIV sero-prevalence in tuberculosis (TB) suspects who could not be verified to be culture-positive was too high to deserve an explanation. We, therefore, secondarily set out to look for possible causes of other pulmonary opportunistic infections including pneumocystis pneumonia (PCP), to explain the excess HIV and determine their relative importance in such a setting.

In the first part of this study, among 509 consecutive PTB suspects attending the outpatient department of a university hospital in Addis Ababa, 33% could be culture-verified as having PTB. PTB patients, non-TB patients (culture-negative PTB suspects) and controls were HIV-1 positive in 57.1%, 38.5% and 8.3% of cases respectively. Independent predictors of culture-verified TB were age <25yrs, male gender and the presence of HIV and fever whereas profound weight loss indicated HIV infection. Diagnosis of PTB based on clinical symptoms, direct sputum microscopy for acid fact bacilli (AFB) and chest radiography (CXR) was significantly less sensitive and specific in HIV- positive patients.

A closer look at the relationship between disease pattern and disease burden by chest x-ray, mycobacterial load and HIV infection also demonstrated that: (1) atypical chest x-ray with interstitial infiltrates, pleural effusion, miliary mottling, normal CXR and absent cavitations occurred more frequently in HIV-infected than non-HIV-infected patients (2) Mycobacterial load as assessed by the number of colonies of *Mycobacterium tuberculosis* (MTB) culture was significantly less in HIV-infected patients than non-HIV-infected, (3) the occurrence of high number of culture-verified PTB cases with normal CXR was identified as one of the challenges in the diagnosis of PTB in patients with HIV infection.

An unexpected finding in this study was the high rate of HIV infection in the culture-negative TB suspects (38.5 %), compared to 156 age and sex matched diabetic controls which was 8.5% (P= <0.001). Since few cases of possible PCP were reported from the chest x-rays, we, therefore, looked if selection of PCP had occurred in HIV positive patients. Amongst 119 culture-negative, HIV-positive TB suspects, *P. jiroveci* was detected in 10.9%

by single polymerase chain reaction (PCR) and immunofluorescence (IF) and 30.3% by nested PCR in expectorated sputum sample. This observation prompted us to design a follow up study to find out the relative importance of *P. jiroveci* and other pulmonary opportunistic diseases in patients who are smear-negative and presenting with atypical chest x-rays. We studied 131 consecutive HIV-1 infected patients presenting with atypical chest x-rays and negative sputum smear for AFB. Patients whose CXR's were read typical for PTB or bacterial pneumonia were carefully excluded from the study and given therapeutic trial. Only those who failed to respond to their respective treatment were re-enrolled into the study. Using toluidine blue O (TBO) and IF stain alone, the results of this study showed that the prevalence of PCP was 29.8% and that of bacterial infection 33.6% and tuberculosis 23.7%. Pulmonary Kaposi sarcoma (PKS) and interstitial pneumonitis (NIP) occurred in 4 patients each. Double infection occurred in 18(13.7%) patients. Cryptococcal pneumonitis was conspicuously absent in this study population. We also evaluated the usefulness of a simple diagnostic method, Toluidine Blue O stain for the diagnosis of *P. jiroveci* in expectorated sputum sample and bronchoalveolar lavage (BAL) and compared it to immunofluorescence and PCR. Comparison of these diagnostic tests showed that the sensitivity of TBO in sputum and BAL samples was 71.4 % compared to immunofluorescence. The overall sensitivity for the diagnosis of PJ was 42.7 % by PCR, 29.8% by IF and 20.6% by TBO. PCR was the most sensitive test and detected additional 18 patients than immunofluorescence. Considering cost, simplicity and efficacy, we recommend TBO as the most practical diagnostic test and expectorated sputum (ES) as the most practical biologic specimen for the diagnosis of *P. jiroveci* in resource-constrained, high HIV-settings such as Ethiopia. We also argue that empiric treatment for PCP should not be encouraged in such societies because other competing diagnosis such as bacterial infections and PTB are equally important and the sensitivity of both clinical and radiological diagnosis is very low.

LIST OF PUBLICATIONS

This thesis is based on the following papers which will be referred to by their roman numerals:

I. Bruchefeld J, Aderaye G, Berggren Palme I, Bjorvatn B, Britton S, Feleke Y, Källenius G, Lindquist L. Evaluation of outpatients with suspected pulmonary tuberculosis in a high HIV prevalence settling in Ethiopia: Clinical, Diagnostic and Epidemiological Characteristics. **Scandinavian Journal of Infectious Diseases 2002 (34); 331-337.**

II. G. Aderaye, J. Bruchfeld, G. Assefa, D. Feleke, G. Källenius, M. Baat, L. Lindquist. The relationship between disease pattern and disease burden by chest radiography, *M. Tuberculosis* load and HIV status in patients with pulmonary tuberculosis in Addis Ababa. **Infection. 2004; 32(6) : 333-38.**

III. Aderaye G, Bruchfeld J, Olsson M, Lindquist L. Occurrence of *Pneumocystis carinii* in HIV-positive patients with suspected pulmonary tuberculosis in Ethiopia. **AIDS 2003 ; 17: 435-40.**

IV. Aderaye G, Bruchfeld J, Melaku K, Woldeamanuel Y, Asrat D, Assefa G, Nigussie Y, G-Egziabher H, Worku A, Lebbad M, and Lindquist L. *Pneumocystis jiroveci* pneumonia and other opportunistic pulmonary infections in smear-negative, HIV-infected patients in Ethiopia. **(Manuscript)**

V. Aderaye G, Woldeamnael Y, Asrat D, Lebbad M, Baser E, Worku A, Fernandez V and Lindquist L. Evaluation of toluidine blue O stain for the diagnosis of *P. jiroveci* in an expectorated sputum sample and bronchoalveolar lavage from HIV infected patients in a tertiary care referral center in Ethiopia. **(Manuscript)**

CONTENTS

II. The Current study

LIST OF ABBREVIATIONS

A-a	Alveolar-arterial
AB	Antibiotics
AFB	Acid fast bacilli
AHRI	Armauer Hansen Research Institute
AIDS	Acquired Immunodeficiency Syndrome
ANOVA	Analysis of variance
ART	Anti-retroviral treatment
BAL	Bronchoalveolar lavage
CXR	Chest x-ray
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked Immunosorbent assay
ES	Expectorated sputum
ESR	Erythrocyte sedimentation rate
ER	Emergency room
HAART	Highly active anti-retroviral treatment
HIV	Human immunodeficiency virus
IF	Immunofluorescence
IS	Induced sputum
KS	Kaposi sarcoma
LDH	Lactic dehydrogenase
LJ	Lowenstein Jensen
LSU	Large sub unit
MGIT	Mycobacterial growth indicator tube
MTB	Mycobacterium tuberculosis
NIP	Non-specific interstitial pneumonitis
nPCR	Nested polymerase chain reaction
PCP	Pnemocystis pneumonia
PCR	Polymerase chain reaction
PJ	Pneumocystis jiroveci
PKS	Pulmonary Kaposi sarcoma
PLWHA	People living with HIV|AIDS
PTB	Pulmonary tuberculosis
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RR	Respiratory rate
SMI	Swedish Institute for Infectious Disease Control
sSA	Sub-Saharan Africa
TB	Tuberculosis
TBBX	Transbronchial biopsy
TBO	Toluidine blue O
WBC	White blood cell
WHO	World Health Organization

I. INTRODUCTION

Part I – Tuberculosis

1. Historical Background

Tuberculosis is an ancient disease. It was present in Egypt as early as 3700 BC (1) and probably may have evolved during the Neolithic period (seventh and sixth millennia BC) (2). Tuberculosis was well recognized during the times of Hippocrates, 460-377 BC (3). Tuberculosis being population-density dependant disease, owes its increase to urbanization or at least to the development of aggregate population groups. The study of tuberculosis probably started during the period of renaissance by Girolamo Fracostoro (1483-1553) who recognized the contagiousness of the illness (4). Franciscus Sylvius (1614-1672) deduced from autopsies that tuberculosis was characterized by the formations of nodules which he named tubercles (4). Pasteur, in 1862 suggested that tuberculosis was transmitted via the airborne route (5) but this remained controversial up until in the 1930s, through the elegant studies of William wells at the Harvard School of public Health, conclusively proved airborne transmission via droplet nuclei (6). Indeed, it was Robert Koch who first isolated *M. tuberculosis* in 1882 and convinced the world the communicable nature of the disease. At that time, TB was responsible for one seventh of all deaths. The development of an acid fast stain by Ehrlich in 1885 and the discovery of x-rays by Roentgen in 1895 made it possible early and accurate diagnosis of the disease (7).

2. The organism

The genus mycobacterium belongs to the order of actinomycetales and the family mycobacteriacae (8). There are three species under this genus and these include M. tuberculosis complex, the non-tuberculosis mycobacteria and M. leprae (8). Mycobacterium tuberculosis complex, the human form of mycoabacteria includes *M. tuberculosis, M. bovis, M. Africanum* and the recent additions of *M. microtti and M. Canetti* (9). These are genetically closely related sub-species and repetitive DNA elements such as insertion sequence IS 6110 and direct repeat have been found to be restricted to the M. tuberculosis Complex. The natural reservoir of *M. tuberculosis* and *M. Africanum* is limited to humans while that of *M. microtti* is rodents and *M. bovis* is a wide range of wild and domestic animals. *M. tuberculosis* is a rather fastidious, slow growing, strictly aerobic, lipid-rich, hydrophobic and acid fast bacterial rod which resists decolorization with acid-alcohol. MTB has no outer membrane, rather have a cell wall made of three macromolecules, namely peptidaglycans, arabinogalactan, mycolic acid and lipopolysaccharide/ lipoarabinomana (LAM) which is anchored to the plasma membrane. Mycolic acid is the major component of the cell envelope, >50% by weight and defines the genius mycobacteriae. Glycolipids are attached to it outwardly. The staining characteristic of MTB is due to the mycolic acid which resists decolorization by acid-alcohol.

The complete genome of the mycobacterial strain H37 RV has been sequenced and is known to contain 4,411,529 base pairs, about 4000 genes with a G+C content of 65.6%.

M. tuberculosis can be grown on a simple carbon and inorganic nitrogenous sources. However, it is a slowly growing organism. Artificial media used to support growth include:

(a) Solid media
- Potato and egg-base media (Middle brook 7H 10 and &7H 11)
- Albumin in an agar base (Lowenstein Jensen media)

(b) Liquid media
- Bactec (BBL) system: This contains Middle brook 7H 12 medium containing 14 C palmitic acid with a mixture of antibiotics (PANTA) to suppress other bacterial growth. Bacterial growth is indicated by the detection of 14C released by MTB as it metabolizes the palmitic acid. In smear positive cases, Bactec can detect MTB in 8 days.
- Mycobacterium Growth Indicator Tube (MGIT): The MGIT BBL is based on Middle brook 7 H9 broth containing silicon rubber impregnated with ruthenium pent hydrate that serves as a fluorescence quenching oxygen sensor. As oxygen is consumed by metabolizing bacteria fluorescence of the liquid media is visually detected. The MGIT is less sensitive than the Bactec but is faster to detect MTB.

3. Pathogenesis and immune response

Virtually all new infections with *M. Tuberculosis* are acquired via airborne transmission (10). The sources of infection are persons with tuberculosis who are coughing. Cough produces infectious droplets, 1-5 μ in size, which is inhaled by the next individual and depending upon the concentration of the inoculum and duration of breathing this contaminated air, transmission and acquisition of infection occurs. Whether an inhaled tubercle bacillus establishes an infection in the lung depends on the bacterial virulence and the inherent microbicidal ability of the alveolar macrophages that ingests it. Alveolar macrophages in some individuals might have innate mycobacterial resistance and in these persons the bacilli are presumably destroyed before an infection is established (11). If the bacillus survives the initial defenses, it multiplies within the alveolar macrophage until it bursts and is re-ingested by other macrophages (both alveolar and non-alveolar) attracted by chemokines and cytokines released by the inflammatory process. Subsequent spread from the primary lesion to hilar nodes as well as hematogenous spread throughout the body occurs, with a predilection for the apices of the lung, lymphnodes, kidneys, bone growth plate, vertebrae, meninges etc. During this period a cell-mediated immune response is mounted by the host involving CD4 T cell lymphocytes (Th1 cells) with subsequent granuloma formation. The surrounding tissue necrotizes into solid caseous tubercles and bacillary proliferation is arrested. Once infected, the individual remains so perhaps for the remaining of his life, a situation referred to as latent TB and only few develop clinically apparent disease. However, infected persons can develop progressive pulmonary disease or extra pulmonary TB or disseminated disease if they have a weak immune system as occurs in small children and infants, if they come under physical or emotional stress or if thy become immunocompromised as occurs in HIV infection.

4. Natural History and Interaction of HIV and TB

4.1 Natural history, HIV infection

The natural history of HIV infection is one of progressive decline in immune function, best characterized by loss of CD_4 T lymphocytes.

Data from prospective cohort studies show that after 11 yrs of HIV infection, 54% of patients have AIDS (12). The remaining of patients is also likely to progress to full disease eventually. Over the past 10 yrs, however, the introduction of highly active anti-retroviral treatment (HAART) has allowed us to tame HIV disease into a chronic illness with frequent and multiple drug resistance.

4.2 Natural history, tuberculosis

Exposure to tubercle bacilli usually results from contact with a person who is sputum smear positive. Infection is controlled by cell-medicated immunity but is generally not eliminated (13). The lifetime risk of developing tuberculosis disease from this infection is about 10%, half of the cases occurring within 5 yrs of infection and the other half at a more remote time (14). Early disease is especially likely in children. These data are from studies conducted in Japan and USA. Whether it applies to societies where the prevalence of TB infection is high and the immune status of individuals have been seriously affected by other co-morbid conditions including malnutrition has not been studied and is, therefore, not clear.

4.3. Interaction between HIV and TB

4.3.1 Effect of HIV on PTB

In the normal host, a fragile balance exists between humans and M tuberculosis. The substantial increase in susceptibility for TB and for advanced clinical disease in otherwise normal young children and older individuals exemplifies that only a marginal decrease in host defense can displace this delicate balance. Therefore, it is not surprising that also a marginal impairment of cell mediated immunity in HIV infected patients with apparently normal CD4 counts also can result in a significantly increased TB-associated morbidity and death (15, 16). In contrast, an opportunist like PJ which is well controlled under all circumstances in the normal host causes manifest disease in HIV infection only if a substantial decrease in CD4 counts has occurred. Accordingly, in an environment with a high burden of tuberculosis infection, TB is a prominent pulmonary opportunistic infection in HIV co-infected patients with an importance by far surpassing other opportunistic pathogens (17).

In sub-Saharan Africa, it has been shown that various forms of tuberculosis occur across a broad spectrum of HIV associated immunodeficiency (Fig 1).

Studies in Zaire has shown that one third of the tuberculosis diagnosed occurred at a CD4 count of above 500 cells/mm3, another third at CD4 level between 200 and 500 and the remaining third when the CD4 count dropped below 200 cells/mm3 (18). Conversely, co-infected patients also have an increased risk for progression in their HIV disease compared to non-TB infected HIV patients (19), which may, at least partly, be due to increased apoptotic cell death triggered by the chronic immune activation in TB (20).

The specific mechanism responsible for an increased risk for TB in HIV infection is not fully understood. It is well known that immune reconstitution during long-term HAART decreases the risk for TB and improves survival in both high and low TB prevalence settings (21, 22, 23, 24). This immune reconstitution can be marked and cause a paradoxical immune response to mycobacterial antigens (25), commonly known as the

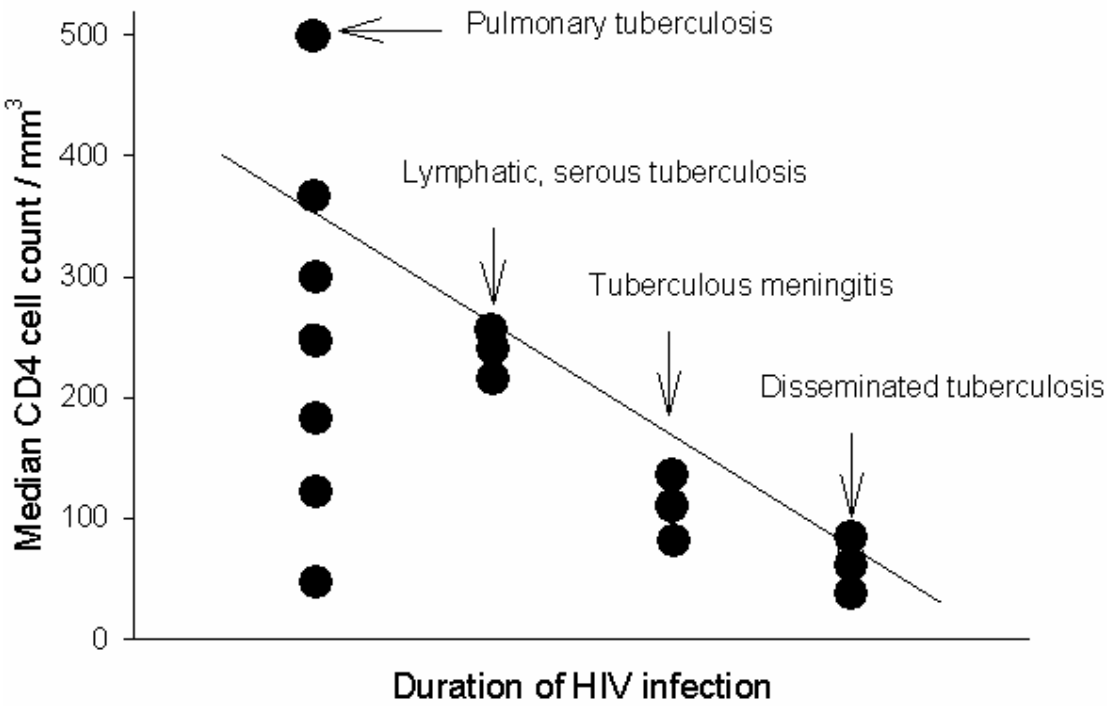


Figure 1: Schematic correlation between extent of HIV-induced immunodeficiency, as expressed by CD4 cell counts, and clinical manifestations of tuberculosis (modifyed after De Cock KM et al. J Am Med Association 1992;268:1581-7)

systemic immune reconstitution syndrome. However, despite an obvious normalization of CD4 counts a several-fold increase in the risk for TB persists also after effective anti-retroviral treatment (21, 26, 27, and 28). An impaired interferon-gamma secreting capacity of *M. tuberculosis* antigen-specific stimulated CD4 cells are seen during the progression of the HIV-1 disease, a defect not fully restored by HAART (29). This finding may indicate a partly irreversible loss of functional TB specific T-cell repertoire during the progress of the HIV infection. It has also been reported that the reduced expression of apoptotic receptors seen in lymphoid tissue in HIV-1 infection neither is fully restored by HAART despite recovery of CD4 counts and inhibited viral replication (30). This may indicate that a more general, not only for TB specific, mechanisms can contribute to the increased risk for TB in the HIV-infected individual. Hence HIV-positive individuals still have increased susceptibility to TB infection and disease. The capacity of TB-specific CD4 cells to secrete INF gamma is significantly impaired despite the recovery of CD4 T-cells. As a result, potent ART may be unable to restore the central memory population of CD4 cells (31, 32).

Three mechanisms that appear relevant for the development of HIV-associated tuberculosis and these include:

a) Reactivation

The work of Selwyn (33) has demonstrated a 24 fold increased risk of developing TB in HIV-positive patients who had a previously documented

positive tuberculin skin test compared to HIV-positive patients with a negative skin test. Approximately 50% of adults in sub-Saharan Africa are skin test positive at the age limit which is highest for HIV infection too. This, so called "Dual Curse", is the driving mechanism for the development of excess TB in HIV infected patients.

b) Re-infection

Conventional thinking obliges us to understand that immuno-competent patients once infected with *M. Tuberculosis* are resistant to subsequent infection. However, RFLP studies have shown that re-infection can occur and particularly so in HIV-infected patients (13, 34). This raises the possibility that apparent cases of relapses after therapy for PTB may in fact represent re-infection.

c) Progression from recent infection

Nosocomial out breaks of tuberculosis (35, 36) including multi-drug resistant disease (37) have been observed in patients with HIV infection who are exposed to *M. Tuberculosis* and are at risk of infection and rapid progression to disease. This has occurred in health care settings and attack rates as high as 30-40% with disease occurring in as little as one month after infection has been reported.

4.3.2. Effect of PTB on HIV infection

PTB results in immune activation affecting T cells and macrophages, witnessed by increased serum levels of different cytokines (38). Increased levels of beta-2-microglobulin have also been demonstrated early in HIV-associated tuberculosis which is a marker of progression to AIDS. Although firm evidence is limited, PTB has been suggested to lead to immune activation, increased viral expression and more rapid progression to AIDS.

5. Epidemiology of TB and the impact of HIV

5.1 Global situation

The frequency of HIV-associated PTB depends on the prevalence of tuberculosis and HIV infection in the society and the overlap between these two populations (39). Because of the high risk of developing TB in HIV-infected persons by either endogenous reactivation or de-novo infection, there has occurred a tremendous increase in case load in high HIV, and TB endemic areas and particularly in sub-Saharan Africa where reported case rates have increased by over 50% (40). By the year 2000, the estimated number of new TB cases has increased from 7.5 million in 1990 to 8.7 million and was mostly due to an increase in the incidence of TB in African countries heavily affected by the HIV epidemics (41). The overall TB-HIV co-infection rate in sub-Saharan Africa was estimated 32% in 1997 (42).

In 2003, the estimated number of new TB cases was 8.8 million with 1.7 million deaths. 27% of the new cases and 31.5 % of the deaths arose in Africa compared to 11% of the world's population. HIV prevalence in tuberculosis patients is less than 1% in the Western Pacific compared to 38% in Africa (43). In countries with the highest HIV prevalence, particularly Southern Africa, more than 75% of cases of tuberculosis are HIV-associated (44). At the end of 2005, an average of 40.3 million adults and children are estimated to be living with HIV/AIDS. Of these, 25.8 million (64%) are in sub-Saharan Africa. Worldwide, about 11.5 million people are co-infected with M. tuberculosis, 70% of whom are in sub-Saharan Africa. HIV infection is highly prevalent among newly diagnosed TB patients in

sub-Saharan Africa. Prevalence rates ranging between 50 and 72% have been described in several countries (45, 46, and 47). Also relatively higher rates have been described in smear-negative and extra-pulmonary TB cases (48, 49). This observation probably reflects the strong association between these conditions, but may also be attributed to the misdiagnosis of other HIV-related diseases as TB (50, 51)

5.2. Ethiopian situation

Ethiopia ranks 8th amongst the 22 global TB high-burden countries with an estimated incidence rate of 154 and 353 per 100,000 populations for new smear-positive pulmonary and all forms of TB respectively. The estimated prevalence of TB and mortality is 533 and 79 per 100,000 populations respectively and one of the highest in the world. The national prevalence of HIV is estimated to be 4.4 % in 2005. The prevalence for the urban and rural population during the same period was 12.6% and 2.8% respectively. The total number of people living with HIV/AIDS is estimated to be 1.7 million and the number of new AIDS cases 143,129 in 2005. Around 50 % of the TB patients in Addis Ababa are HIV-positive. The average prevalence of HIV in adult TB patients (15-49 yrs) is estimated to be 21% by WHO (31).

6. Clinical manifestations of TB and the impact of HIV infection

The clinical presentation of PTB in patients infected with HIV is dependent upon the degree of immunosuppression. Patients with mild to moderate immunosupperssion present like the pre-HIV post-primary form of PTB while those with severe immunodeficiency present like primary PTB. The so called constitutional symptoms such as chronic cough, afternoon fever, night sweats, loss of appetite and weight loss can occur irrespective of the presence or absence of PTB, due to advanced HIV disease or as symptoms of other pulmonary opportunistic infections. Haemoptysis, once considered the hall mark of PTB in developing countries, is not as frequently complained by the patients particularly with advanced HIV infection. Symptoms more specifically associated with HIV infection such as prolonged diarrhea, presence of herpes zoster scar, generalized lymphadenopathy and oral candidiasis are more valuable markers of a dual infection, as they are uncommon in HIV-negative, TB patients (52, 53). PTB can occur concomitantly with other opportunistic infections such as PCP and pneumonia making diagnosis even more difficult. The diagnosis of tuberculosis, which is highly dependant on a positive sputum smear microscopy for AFB in developing countries, has also suffered a set back with an even lower sensitivity compared to the pre-HIV era. The response to treatment of PTB patients co-infected with HIV is believed to be the same as the non-HIV infected but the mortality is quite high (30%) compared to 10% in the non-HIV infected.

7. Diagnosis of TB - sputum smear microscopy and the impact of HIV

Sputum smear microscopy using the Ziehl Neelsen method is known to have high specificity but low sensitivity. During the pre-HIV era, up to 65-75% of culture-proven cases of tuberculosis became smear-positive with repeated smear examinations (54). This relative sensitivity has been reduced due to HIV, particularly in far-advanced immunosuppession. Rates as low as 38% have been reported with HIV (55). The identification of sputum smear-positive patients is crucial for the control of tuberculosis, as these are the most contagious patients, particularly to their close contacts (56, 57). It is estimated that one undiagnosed smear positive patient infects between 10-20 contacts over a one year period if untreated (58). While, the reduced smear positivity in HIV-co-infection appears to favor reduced transmission of the

disease, in actual fact, the total number of smear positive patients produced as a result of the increase in the total TB burden favors increased transmission and particularly in the HIV-infected population. An additional effect of the tremendous increase in TB burden is that it over-stretches the already meager diagnostic facilities resulting in under-reading of slides (false- negative smears). Such under-reading has been noted to occur in up to 21-29% of patients registered as smear-negative PTB who were confirmed to be smear- positive when re-examined in reference laboratories (59,60). The worst scenario is when sputum smear may not be done at all, as suggested from studies in Botswana, where 48% of patients reported with PTB had no smear examination performed (61, 62). Factors which improve the sensitivity of sputum smear examination include homogenization of sputum with sodium hypochlorite (household bleach) followed by concentration either by centrifugation or keeping it to sediment by itself over 12hrs. This method was first described in 1942 (63) but fell out of favor probably due to the introduction of sensitive methods such as fluorescence microscopy and culture until it was re-investigated and introduced by Miorner and co-workers in Ethiopia (64). The relative increase in smear positivity has differed between studies and ranges from a non-significant increase by 2% up to 125% (64, 65). In developing countries, where culture facilities are remote, the relative importance of sputum concentration method cannot be under estimated.

8. Chest X-ray in TB and the impact of HIV infection

The chest x-ray has been used to assist in the diagnosis of PTB particularly in smear-negative patients both in adults and children in the past. Typical features of upper lobe involvement with or without cavitations suggested adult post-primary form of TB while the presence of hilar/mediastinal adenopathy, lower lung involvement and miliary distribution suggested either childhood PTB or tuberculosis occurring in the back ground of severe malnutrition or immunodeficiency. Other findings including pleural effusion have occurred both in primary and port-primary TB. Pre-HIV, studies from Kenya and Tanzania have shown that 66% of the smear -positive patients had cavitary disease (66). However, interpretation of CXRs of individuals suspected to have PTB is difficult and both experience and studies have shown that no radiographic pattern is absolutely typical for tuberculosis (67). Post-HIV and particularly in advanced HIV disease, lack of cavitation, frequent involvement of the middle and lower lobes, interstitial and miliary patterns, hilar and mediastinal adenopathy and pleural effusion which may be bilateral is frequently encountered.(68,69). Incorrect reading of a CXR has been observed in about one third of patients with under-reading and between 21 - 29% of over- reading (70).

9. Differential Diagnosis and issues in smear-negative TB

9.1. Magnitude of the Problem

The HIV epidemic has been associated with significant increase in the incidence of smear-negative pulmonary TB in HIV-positive than HIV-negative patients (71,72). The diagnostic criteria for smear-negative pulmonary tuberculosis includes at least three negative sputum specimen for AFB, radiographic abnormalities consistent with active tuberculosis (TB), no response to a course of broad spectrum antibiotics and decision by a clinician to treat with a full course of anti-tuberculosis chemotherapy (73). A positive acid fast bacilli (AFB) smear requires about 5000-10,000 AFB present per ml of sputum and the AFB are released intermittently from the lungs. This gives AFB smear a sensitivity of only 50-60% (74). Smear-negative PTB could also be a result of poor quality smear microscopy (e.g. inadequate

sputum collection, storage and staining, human error etc.) and misdiagnosis of other HIV-related pulmonary conditions (66). The proportion of smear-negative pulmonary TB patients ranges from 24% to 61% (58, 75) with a weighted average of 28% for most developing countries. This shows that smear positive PTB is the predominant type of disease among HIV-infected patients. These percentages however, vary according to the host immune status. In advanced immunosuppression, it is likely to be high where as in mild to moderate immunosuppression it is likely to be comparable to HIV sero-negative patients. Despite, compared to the pre-HIV era, the proportion of smear negative TB patients is still high. None the less, HIV-positive smear-negative PTB patients had inferior outcome to treatment including death than HIV-positive smear-positive patients (76, 77). This is likely to be due to the fact that smear-negative patients tend to be in advanced state of HIV disease. Moreover, autopsy studies of HIV-positive patients have identified TB as a cause of death in 14% to 54% of deaths and in most cases TB was not diagnosed before death. (78, 79). In several of these studies, over-diagnosis of TB in patients with pneumocystis pneumonia, pulmonary cryptococcosis, pulmonary nocardiosis etc has been made in post-mortem examination without any clue to the diagnosis before death. Besides, it has been shown that in patients who were diagnosed to have smear-negative PTB and were to be started on treatment for TB, 9% and 38% were found to be cases of PCP in Malawi and Uganda respectively (80,81). Clearly, the explanation for the increase in the diagnosis of smear-negative TB is far beyond poor quality smear microscopy and has a lot to do with the non-specificity of the diagnostic criteria and other respiratory opportunistic infections interfering in the diagnosis either separately or along PTB.

9.2. Diagnostic algorithm and delay in diagnosis

In order to improve the diagnosis of smear-negative TB while minimizing over-treatment of those without the disease, sub-Saharan Africa countries use the WHO diagnostic criteria (73) and an adapted diagnostic algorithm in their national guidelines. A recent review of the functioning of this algorithm has demonstrated that there is a major delay in the diagnosis of smear-negative PTB requiring 11 to 34 days to establish the diagnosis (82,83). Although all countries recommend examination of sputum smear before putting a patient on antibiotic trial, the number of sputum examined ranged from two to six samples. If the cough persists, most countries recommend a second set of smear examination after the antibiotic trial followed by CXR. Several studies have shown longer health service delay in the diagnosis of smear-negative than smear-positive TB (83). Such a delay in diagnosis may compromise the chance of survival and could be costly to the individual patient.

9.3. Diagnostic antibiotic trial

Bacterial pneumonias, pneumocystis pneumonia, cryptococcosis and pulmonary nocardiosis are believed to be common among PLWHA and may be misdiagnosed as smear-negative TB. Antibiotic trial, hence, is a useful tool to exclude other chest infections and improve the specificity of the diagnostic protocol. Although the current WHO treatment guidelines for TB continue to recommend a single antibiotic trial (73), in a more recent guide for clinicians working on TB/HIV, two courses of antibiotics were suggested particularly for HIV-infected TB patients (84). The danger behind using trial of antibiotics to improve the performance of the guideline is that PTB patients can lose their respiratory symptoms transiently after a course of antibiotics, possibly due to the mycobacteirostatic action of some drugs

but more likely due to a superimposed double infection. The use of broad spectrum antibiotics such as fluoroquinolines may have detrimental effect in the overall TB control activities through generating drug resistance and fueling delay in the diagnosis. The latest recommendation of WHO guidelines for the diagnostic algorithm of smear-negative TB has excluded antibiotic trial as a screening tool for the diagnosis of TB (publication in press).

9.4. The chest X-ray in smear-negative PTB

The reliability of chest x-ray (CXR) in the diagnosis of pulmonary TB is mainly complicated by its non-specificity and interpretation problems and this has been alluded to in the previous chapter. Despite this decreased specificity, the pattern of CXR finding in HIV-infected patients has been well characterized and is only atypical in relation to what is known as typical in the pre-HIV era, hence can be used to suggest a diagnosis of PTB. Therefore, the use of CXR to speed up the diagnosis of smear-negative TB is to be encouraged. However, there is a need to define its best timing along the course of the diagnostic algorithm. A major pitfall of the CXR is that several diseases including PCP and bacterial infection resemble that of PTB such that there is both an over-diagnosis PTB and under- diagnosis of the other infections. The other problem is that many primary care physicians lack knowledge of how to read a chest x-ray; therefore, there is a need to train physicians and other paramedical how to read a chest x-ray.

9.5. Sputum culture

Culture is the gold standard for the diagnosis of tuberculosis. Using a solid or liquid culture media requires 10-100 viable bacilli per ml of sputum (62). Culture is currently recommended selectively mainly for surveillance of drug sensitivity and to confirm treatment failure and relapse cases and in PTB patients with repeated negative smear results. However, mycobacteria are slowly growing organisms, therefore, require 6-8 weeks and need relatively more sophisticated equipments. Sputum culture of HIV-infected individuals requires more incubation time than non-HIV-infected patients (85). Conventional culture media using either egg or agar is 5-10 times more expensive per sample than smear microscopy. These factors have hampered the routine use of culture in resource-constrained countries.

9.6. Conclusion

Clearly there is a need to improve the diagnosis of not only smear-negative PTB but also other non-PTB conditions which resemble it. Improving AFB smear microscopy by methods such a sputum concentration with sodium hypochlorite (NaOCl) is one approach while improving sputum culture for MTB in terms of reduced cost and rapid delivery of results is another one. The recently introduced mycobacterial growth indicator tube (MGIT) is hoped to deliver culture results within 8-16 days (in some cases less than 5 days) compared to the conventional Lowenstein Jensen (LJ) median which takes between 4-8 weeks (86). However, MGIT requires the same infrastructure and technical expertise as the conventional method and is costly to install which limits its wider use in developing countries. Besides high rate of contamination (4-15%) had been a concern for its wider use. For the now, we can only depend on our diagnostic algorithm which has to consider the other differential diagnosis of smear-negative TB including PCP, bacterial infection etc depending upon the relative prevalence of these diseases in that specific society. Another alternative is to develop a simple and cheap diagnostic capability for these diseases. This is a task we should continue to pursue for.

10. Strategies for the prevention of TB in HIV infection

Despite well-implemented TB control programs using directly-observed, rifampicine based short-course chemotherapy and active case detection using an annual miniature chest radiography screening programs, a rising HIV prevalence has resulted in an increasing TB incidence (87). One of the strategies adopted to reduce TB incidence is isoniazid preventive treatment (IPT), isoniazid given at 300 mg/day for 6 to 12 months. A meta-analysis of clinical trials in the past have shown that IPT reduces TB incidence by 42% overall and 60% among individuals who have positive tuberculin skin test (88). Apart from efficacy studies, a recent IPT effectiveness study has demonstrated that it can reduce TB incidence by 38% overall and by 46% among individuals with no previous history of TB treatment. (89). Hence, there is growing evidence that not only primary IPT but also secondary IPT may be important in reducing the TB incidence in high TB prevalent settings (90).

Another strategy to reduce TB incidence is rolling out anti-retroviral treatment (ART) in the community. ART has been shown to reduce TB incidence at the individual level (21, 22). However, there are concerns that it can paradoxically increase TB incidence at the community level if the net effect is to increase survival among HIV-infected individuals without completely restoring immunocompetence (91).

In general, the available strategies do not seem to be adequate to reduce the TB incidence in high-prevalent settings. Additional and more radical approaches are required to further reduce the TB incidence.

Part II- Pneumocystis pneumonia (PCP)

1. History of PCP

Pneumocystis jiroveci (PJ) was first identified by Carlos Chagas in 1909 from the lungs of guinea pigs (92) and subsequently by Antonio Carinii in infected rat lungs (93). It was initially thought to be a trypanosome until Delanoes in 1912 recognized that this was a new species with a unique tropism to the lung, hence the name *Pneumocystis* carinii (94). It was not until 1952 that the organism was associated to human disease by Vanek and Jirovec (95) when outbreaks of interstitial plasma cell pneumonia occurred in malnourished young children in orphanages in Iran. Since then, it has been increasingly noted in patients using immunosuppressive drugs for malignancy and connective tissue diseases, congenital immunodeficiency syndromes, and transplant patients. The AIDS epidemic, however, marked the beginning of the impact of the disease in a substantial number of patients and clusters of PCP cases in homosexual men and intravenous drug users were one of the first indications of the HIV epidemic (96). *Pneumocystis jiroveci* was initially considered as a protozoan on the basis of morphologic features of the small trophic form, the large cyst form and the development of up to eight progeny within the cyst. In 1988, however, an analysis of the small rRNA subunit from PJ established a phylogenetic linkage to the fungal kingdom within the ascomycetous fungi. Additional DNA data showed that pneumocystis organisms in different mammals were distinctly different, hence, in 1999 a new binomial nomenclature classified the organism as *Pneumocystis jiroveci* Frankel 1999 (in place of *Pneumocystis carinii*) in honor of the Czech parasitologist Otto Jirovec who is credited for describing the microbe in humans (97).

2. The biology of the organism

Pneumocystis organisms have been identified in virtually every mammalian species. In humans, serologic surveys have shown nearly universal sero-positivity of pneumocystis by two years of age. Molecular methods have shown that pneumocystis organism are host specific. *P. jiroveci* DNA has never been found in lung samples from any other mammals including non-human primates (98, 99) The reason for this strange host specificity is unclear. Perhaps pneumocystis organisms might be obligate parasites that have evolved to survive in a particular host species (100).

The major obstacle in studying pneumocystis is the inability to achieve sustained propagation of the organism outside the host lung. Both cell-free and tissue culture systems including attempts to simulate the intra-alveolar environment has failed to grow the organism in vitro (101). Pneumocystis has a unique tropism for the lung, where it exists primarily as an alveolar pathogen without invading the host. Rarely pneumocystis can disseminate in the setting of severe underlying immunosuppression or overwhelming infections. The life cycle of pneumocystis is complex. Microscopically, one can identify the small trophic (1-4 min in diameter) forms more abundantly than the larger cysts (8 min in diameter) cysts during infection. The trophic forms are haploid whereas the cyst contains two, four or eight nuclei. As of recent years, using molecular techniques, major advances has been made in the understanding of the biology of the organism including the identification of key molecules involved in the various activities of the parasite.

3. Pathogenesis and immune response

Severe pneumocystis pneumonia is characterized by neutrophilic lung inflammation that may result in diffuse alveolar damage, impaired gas exchange and respiratory failure. Respiratory impairment and death are closely correlated with the degree of lung inflammation than parasite burden in pneumonia (102). The most important subsets of lymphocytes responsible for host defense against pneumocystis are CD_4 + cells, and the risk of infection increases with a CD_4 count of less than 200/mm^3 (103,104). Alveolar macrophages, neutralizes and several soluble mediators also facilitate the clearance of infections.

4. Transmission, reactivation, re-infection and colonization

The transmission of pneumocystis is not fully understood nor has its environmental niche been identified. An asymptomatic infection is believed to occur in early childhood, since about 80% of children less than 4 yrs of age have been reported as serologically positive (105). A presumed reactivation of a latent infection may then occur late in life if the patient becomes immunosuppressed. The reactivation theory has been the most popular theory for decades in the past.

There is now evidence that person to person transmission is the most likely mode of acquiring new infection (re-infection), although acquisition from environmental sources may also occur (106). In humans, the occurrence of clusters of PCP cases and the absence of detectable *P. jiroveci* in bronchoalveolar lavage (107) and autopsy lung specimen (108,109) in asymptomatic HIV-infected patients further supports the re-infection theory. *P. jiroveci* DNA has also been detected in samples of airborne fungal spores (110) and in sample of pond water (111) though their number in the environment seems to be very few. Despite the results of studies that favor air-borne transmission, respiratory isolation of patients with pneumocystis pneumonia is not currently recommended.

P. jiroveci has been isolated from respiratory specimen of HIV-infected persons who do not have clinical disease and this has been defined as colonization or sub-clinical carriage. Pneumocystis DNA has been detected by PCR alone and the organism is not seen on routine histochemical staining. The clinical significance of pneumocystis in respiratory specimen and the viability of the organism detected only by PCR are unknown. However colonization may be important for several reasons. (a) It may increase the risk of progression to clinical PCP. (b) Carriers of the organism may transfer the infection to others and (c) latent infection may lead to inflammation that is detrimental to the lung. Rates of colonization in HIV-infected patients could be as high as 69% (112). Recent evidences suggest that non-HIV-infected persons may also be colonized, thus increasing the potential number of persons affected (113)

5. Epidemiology of PCP in adults in Africa

In contrast to the situation in many developed countries, PCP has been thought to be rare in African adults. Most early studies reported a prevalence rate of 0-22% with a median prevalence of 5% in 11 studies compared to a median prevalence of 21% (average 0-39%) in 12 studies conducted in the latter part of the epidemic (Table I).

Table 1: Prevalence of PCP and other respiratory conditions in HIV positive adults in studies from sub-Saharan Africa

Country, year of publication (ref), N= sample size	***Inclusion criteria*** [a b]	***Exclusion criteria***	***Material for PJ***	***Detection method PJ***	***PCP n (%)***	***Other diagnosis n (%)***
Congo, 1991 (114) N=45	AIDS patients,- pulmonary manifestations	AFB +	BAL	SM	5 (11)	Not sought
Zimbabwe, 1989 (115) N=37	-In patients, resp. symptoms + CXR infiltrates	AFB+	BAL, BB, TBB lung tissue	GM, SM TL	8 (22)	TB 12(32) Bacteria 16(43) Fungi 2(5) KS 7(19)
Zambia, 1989 (116) N=27	Inpatients, resp. symptoms & CXR+	AFB+	IS	TL IF	0	TB 11/22 (50)
Rwanda, 1994 (117) N=111	In and out-patients, referred with PDUE, CXR+/-	AFB+	BAL, TBB	GB, SM	5 (5)	TB 25(23) Crypto14(13) KS 10(9) NIPB 42(38)
Zambia, 1992 (118) N=44	Resp. symptoms, no response to AB	AFB+	BAL	TL	4 (9)	Not sought
Zimbabwe, 1995 (119) N=64	In patiens, CXR with diffuse pneumonia, no response to pcG	AFB+	BAL	SM, TL, DQ	21 (33)	TB 24(39) KS 6(9) Unknown 21(33)
Malawi, 2001 (120) N=352	Outpatietns, registered for TB treatment HIV+n=278 (89%)	AFB+	BAL (173 patietns)	IF, n PCR	17 (9)	TB 137 (39) KS 10(3) Non -TB chest Infection 48(14) Unknown=128 (37)
South Africa, 1991 (121) N=67	Retrospective chart review	AFB+, PC+ in sputum	BAL	HE, SM	9(27)	TB 9(13) KS 4(6) NIPB 13 (19) Cryptococci 1(2)

Uganda, 1989 (122) N=40	Inpatients, CXR infiltrates	-	BAL	GM, SM	0	TB 6(15) (AFB+) Cryptococci 2(5)
Tanzania, 1993 (123) N=83	Resp. symptoms +/- CXR findings	-	IS	GM, TL DQ	3 (4)	TB 32 (39) (AFB+)
Cote d'Ivoire, 1992 (124) N=78	In-hospital deaths. HIV+ n=53 HIV- n=25	-	Lung tissue	SM HE	5 (9)	TB HIV+21(40) HIV-1(4) PN HIV+18(34) HIV-7(28) KS HIV+3(6)
Senegal, 1993 (125), N=27	+/- resp. symptoms, +/- CXR infiltrates.	-	IS	TL	6 (22)	Not sought
Burundi, 1993 (126) N= 302	Inpatients, resp. symptoms HIV+ve=222 HIV –ve= 80	Chronic lung dis	BAL	GM, TL, IF	11 (5)	TB HIV+ve=49% HIV–ve=49% PN HIV+=36% HIV- = 41% KS HIV+ = 1%
Cot d'Ivoire, 1993 (127) N=247	In hospital deaths	-	Tissue sample	SM HE	7(3)	TB 94 (38) Bacteremia 40(16) CMV 45 (18) Cerebral toxo 37 (15) PN 94 (30)
Cote d'Ivoire, 1998 (128) N=200	Inpatients, referral centre for respiratory disease HIV+ n=150 (75) HIV-n=50	AB treatment	BAL (5 HIV+)	IF	0	TB HIV+91(61) HIV-32(64) PN HIV+23(15) HIV-1(2) Gram-neg. Septicemia HIV+14(9) HIV-0(0) PDUA HIV+15(10) HIV-4(8)
Tanzania, 1996 (129) N=237	Inpatients, acute resp. disease and CXR findings HIV+n=127 (54) HIV-n=110	Previous TB, chronic lung dis. immunosuppr.treat	BAL (32 HIV+)	GM, TL	1(1)	TB: HIV+95(75) HIV-87(79) PN: HIV+18(14) HIV-17(16) KS: HIV+2(2)
Botswana, 1997/98 (130) N=104 HIV+ve	Adult death inpatients	-	Lung tissue	Growth SM	11%	TB=40% PN = 23% KS = 11%
Senegal, 1999 (131) N=29 HIV +ve	Smear -ve pneumopathy	AFB+ve	BAL	TBO GM	2/29 (7)	Not sought
Tunisia, 1994/97 (132); N=27 HIV+	Respiratory symptoms	-	BAL	MS GM	9/27 (33)	Not sought
Algeria, 1998 (133) N=14	Respiratory troubles	-	BAL Expectorated sputum	?	3/14 (21)	Not sought

S. Africa, 2001 (134); N=39	AFB +ve, pneumonia Inpatients	-	Sputum+ others	?	-	39 pts had both TB & PCP
Uganda, 1999/2000 (135) N=83	Smear negative Lower resp. tract infection HIV+ve	AFB +ve	BAL	IF	32/83 (39)	TB = 20(24%) KS = 9(11%) PN=7(8%),No Dx in 24 (30%)
Kenya, 1999/2000 (136) N=51 HIV +ve	-Sub-acute onset of cough +SOB -Smear -ve -Bilateral CXR infiltrates	AFB+ve	BAL	TBO IF	19/51 (37)	Not given
Ethiopia, 1996/97 (137) N=119 HIV +ve	Resp. sympt. AFB-ve M.TB culture-ve	AFB+ve TB culture +ve	Expectorated sputum	IF S-PCR n-PCR	11% 8% 30%	Not sought

Abbreviations: PDUE**=** pulmonary disease of undetermined etiology; LIP= lymphocytic interstitial pneumonia; NIPB= non-specific interstitial pneumonia or bronchiolitis; KS= Kaposi sarcoma**;** CMV= cytomegalovirus; PN= pneumonia; AFB= acid fast bacilli. **Material:** IS= induced sputum; BAL= bronchoalveolar lavage; NPA= nasopharyngeal aspirate; BB= bronchial brushing; TBB= trans-bronchial biopsy. **Method:** SM= silver methenamine; TL= toluidine blue; GM= giemsa; HE= hematoxylin-eosin; IF= immunofluorescence; PCR= polymerase chain reaction; DQ= diff-quick

[a] = in or outpatients not-specified; [b] =inclusion of HIV-ve patients specified, if not, HIV +ve patients only

PCP might not have been commonly reported in Africa for several reasons. First, there is limited resource for the diagnosis including bronchoscopy, sputum induction and experienced laboratory personnel to prepare and interpret diagnostic specimen. Second, HIV infected African adults may die of other causes such as bacterial pneumonia and tuberculosis while their CD_4 count is still high and may not survive until their CD_4 is low enough for PCP to occur. Third, environmental factors such as seasonal variation may affect the occurrence of PCP. Fourth, regional strains in Africa may be less virulent or the population may be resistant, as HIV infected African Americans have been shown to have lower rates of PCP compared to white Americans (138). However high rates of anti pneumocystis antibodies among African children suggest that exposure to the organism is common (139).

If has also been observed that the prevalence of PCP differed in the different regions of Africa. One obvious reason for this could be a difference in the study design of the reported cases. As of recent, however, this geographical variation appears to be narrowing. Countries which have never reported the occurrence of PCP in the past are now reporting high rates of PCP (136,137). A possible explanation for such a difference could be that *P.jiroveci* might be in the process of penetrating the African continent and these differences simply represent the temporal sequence of this spread (140). The incidence of PCP in Africa may be growing as the AIDS epidemic progresses. A recent review concluded that cases of PCP seem to have increased over time (141). Unless and until preventive treatment with co-trimoxazole and use of antiretroviral treatment (ART) is widely practiced, the incidence is likely to increase in the foreseeable future.

6. Epidemiology of PCP in children in Africa

In contrast to adults, HIV-infected children in Africa have higher rates of PCP(Table II)

Table II: Prevalence of PCP in children in sub-Saharan African countries

Country, year of publication (ref), N=sample size	***Inclusion criteria*** [a b]	***Exclusion criteria***	***Material for PJ***	***Detection method PJ***	***PCP; n (%)***	***Other diagnosis; n (%)***
S. Africa, 1996 (142) N=172	Pneumonia, died in ICU; HIV+36 HIV-36		Lung tissue	SM. IF	16/31 (52)	TB HIV+,1(3)
Cote d'Ivoire, 1996 (143) N=155	Consecutive child deaths, in-and out patients HIV+ n=78 HIV- n=77	-	Tissue	Not given	11(14) <15 months 31%	TB HIV+ 1(1) HIV-0 PN HIV+33(42) HIV-34 (44) LIP HIV+1(1) NIPB HIV+14 (18) HIV-14 (18)
Zimbabwe, 1997(144) N=184	Child deaths, out patients HIV+122(66) HIV-62	-	Lung tissue	HE SM.	19(22)	TB HIV+6(5) HIV-2(3) CMV HIV+9(7) LIP HIV+11(9)
Malawi, 2000 (145) N=150	Inpatient, severe PN, HIV +ve=93 HIV –ve=57	-	NPA	IF	16(17)	PN HIV +ve=12(13)
Malawi, 1997 (146) N=60	Inpatients, acute lower resp. tract infect. <23 months old	PN Associated with measles or pertussis	NPA	IF	5(8)	Not sought
S. Africa, 2000 (147) N=151	Inpatients with pneumonia	CF, other immunodefi ciency	IS BAL NPA	SM IF	15(10)	Not sought
Zimbabwe, 2001 (148) N=24	Child deaths, inpatients with pneumonia		Lung tissue	SM. PCR	16(67)	TB 0 CMV 2(10)
S. Africa, 2002 (149) N=105	Inpatients, severe pneumonia<2 years	Bronchiolitis	IS, NPA Lung tissue	IF	51 (48)	CMV 8/18(44) TB 3/18(17)
S. Africa, 1998/99 (150) N=93	Post- mortem Clinical lung diseases	-	Lung tissue Liver tissue	HE Grocott IF	21/93 (23)	TB = 4 (4.3%) CMV =30 (32.3%) LIP = 10.8% NIP = 11 (11.8%)
Botswana, 1997/98 (151) N=32	Post mortem all causes of resp. dis. and non resp dis.	-	Lung tissue	?	10/35 (28)	TB = 4/35 = 11% BP = 31/35=88% LIP= 2= 5.7%
South Africa, 2002 (152), N=185	episodes of pneumonia	-	?	?	101/231 (44)	Not looked for
Uganda, 2004 (153) N=43	Severe pneumonia	-	IS	IF	18/43 (42)	Not looked for
Zambia, 2002 (154) N=180	Autopsy Respiratory illness	-	Lung tissue	?	58/180 (32)	Pneumonia 74/180 = 41% TB=?

Abbreviations: PDUE= pulmonary disease of undetermined etiology; LIP= lymphocytic interstitial pneumonia; NIPB= non-specific interstitial pneumonia or bronchiolitis; KS= Kaposi sarcoma**;** CMV= cytomegalovirus; PN= pneumonia; AFB= acid fast bacilli. **Material:** IS= induced sputum; BAL= bronchoalveolar lavage; NPA= nasopharyngeal aspirate; BB= bronchial brushing; TBB= trans-bronchial biopsy. **Method:** SM= silver methenamine; TL= toluidine blue; GM= giemsa; HE= hematoxylin-eosin; IF= immunofluorescence; PCR= polymerase chain reaction; DQ= diff-quick

[a] = in or outpatients not-specified; [b] = inclusion of HIV-ve patients specified, if not, HIV +ve patients only.

Seven autopsy series described rates of PCP from 14% to 67% (142-146, 148, 150-151, 149) and most of the cases occurred in infants than older children (143-144, 154). Because autopsy studies examine terminal diseases, their assessment of disease prevalence might be biased. Prevalence studies among children in clinics or hospital settings will estimate disease frequency more accurately. In six such studies the prevalence of PCP ranged from 8% to 48%. Once again, most of the cases occurred in children under 2yrs of age.

The validity of these studies, both in adults and children, vary considerably. There were only limited number of prospective studies that included consecutive patients (115, 117-120, 123,126,129,135,136). Most of the studies were descriptive in type and differed very much in their inclusion criteria. In most, the numbers of patients included were too few to make any reasonable conclusion. If, in addition, only studies with a sample size of at least 100 HIV-positive patients are considered, the PCP prevalence is in the range of 0-17.9% (117,120,127,128,129,130, 137).

7. Clinical presentation of PCP

HIV-infected patients present with gradual onset dyspnoea, non-productive cough, fever and weight loss. On physical examination, they are tachypneic, febrile and cyanotic. Auscultation of the chest is often times unremarkable although inspiratory crackles may be heard on deep inspiration. Other AIDS-associated illnesses such as oral thrush are commonly observed.

8. Laboratory tests

8.1 Lactic Dehydrogenase (LDH)

The most useful test appears to be serum level of lactic dehydrogenase (LDH) which is elevated during infection (155). However LDH levels could be elevated in other similar conditions such as pneumonias and lymphomas, rendering the test relatively non-specific (156,157). Perhaps serial changes of LDH may be useful in the follow up of patients. Also the degree of elevation appears to be a useful marker of prognosis (158,159). However, in a multivariate regression analysis, LDH was not found to be an independent risk factor for mortality (160).

8.2. CD4 count

Determination of CD_4 count is important as the risk of *P.jiroveci* infection rises sharply as the CD_4 count falls below 200 cell/mm^3 (161,162).

8.3 Arterial blood gas

Both resting and exercise oximetry will be important to determine the severity of illness. One needs to calculate the alveolar-arterial (A-a) gradient after exercise to detect an early disease. An A-a gradient of over 35mmHg has been associated with increased mortality. In such a situation, the use of corticosteroids has been shown to improve survival (163).

8.4 Chest X-ray

The classic CXR pattern of PCP is a bilateral fluffy infiltrate with a ground glass appearance and resembling pulmonary edema (164). An unusual finding is pneumothorax. *P. jiroveci* pneumonia may be associated with a normal chest x-ray in up to 25% (165, 166). High resolution CT (HRCT) has 100 % sensitivity but reduced specificity.

8.5. Gallium 67 scanning

This test is highly sensitive but non-specific. It is used to screen patients suspected to have PCP but with a normal chest x-ray.

9. Diagnosis of PCP

The diagnosis of PCP is based on staining for the organism from respiratory samples including expectorated or induced sputum, bronchial washings, bronco-alveolar lavage, trans-bronchial biopsy or open lung biopsy. The relative sensitivity of the various respiratory samples for the diagnosis of PJ when stained by the silver methenamine technique is shown in table III. Silver methenamine stain is considered to be highly specific but less sensitive compared to immunofluorescence stain (167, 168).

Table III. Diagnostic yields using silver stain for each specimen

SOURCE OF SPECIMEN	DIAGNOSTIC YIELD Median (range)
Sputum	50% (15-94%)
Bronchial Washings	65% (60-70%)
Bronchoalveolar lavage (a single area lavaged)	90% (60-100%)
Bronchoalveolar lavage (two areas lavaged)	95% (85-100%)
Transbronchial biopsy	97% (89-100%)

9.1. Diagnostic specimen

9.1.1. Induced sputum (IS)

Induction of sputum has been popularized particularly in high endemic areas where bronchoscopy might not be available for the diagnosis of all cases. The yield has been quite variable from good (169) to bad (170) but the median yield has been < 50% in the majority. It is particularly less sensitive than BAL, for the detection of organisms other than *P.jiroveci* and MTB, therefore, offers very little advantage over empiric treatment under such circumstances.

9.1.2. Bronchoalveolar lavage (BAL) and bronchial washing

BAL is superior to bronchial washings in the diagnosis of *P. jiroveci.* Bronchial washings is the pooled sample from the airways collected during the entire bronchoscopy while BAL is a specific task of wedging a bronchoscope in a distal airway and instilling aliquots of saline and immediately retrieving the fluid by either a low suction or a hand held syringe. Done properly, BAL has an excellent sensitivity (95-100%). Two lobe lavage in the same lung, usually the upper and middle lobes, have been shown to have higher yield than single lobe lavage (171). In patients with severe hypoxemia, however, the procedure has to be done fast and a single lobe lavage might suffice.

9.1.3. Trans-bronchial biopsy (TBBX)

This technique allows sampling of a lung tissue. It is particularly useful in detecting organisms other then *P.jiroveci* such as MTB and fungi. In PJ

infection, TBBX is complimentary to lavage. Many series document that both techniques are over 90% sensitive (172, 173) Complications of this procedure include pneumothorax and haemoptysis. Pneumothorax occurs in up to 22% (174) and approximately half of the patients who develop pneumothorax will require chest tube drainage. The technique appears to be more useful in non-HIV related PCP, particularly transplant patients, where the pathogen burden is less than in AIDS patients. Because of the comparable yield to BAL and the associated complications, TBBX is rarely employed for the diagnosis of PJ currently.

9.1.4. Open lung biopsy

This procedure has been abandoned for the diagnosis of PCP in favor of induced sputum and BAL. Although few cases missed by bronchoscopy could be diagnosed by lung biopsy, however, the risks of the procedure do not warrant its routine use. (175)

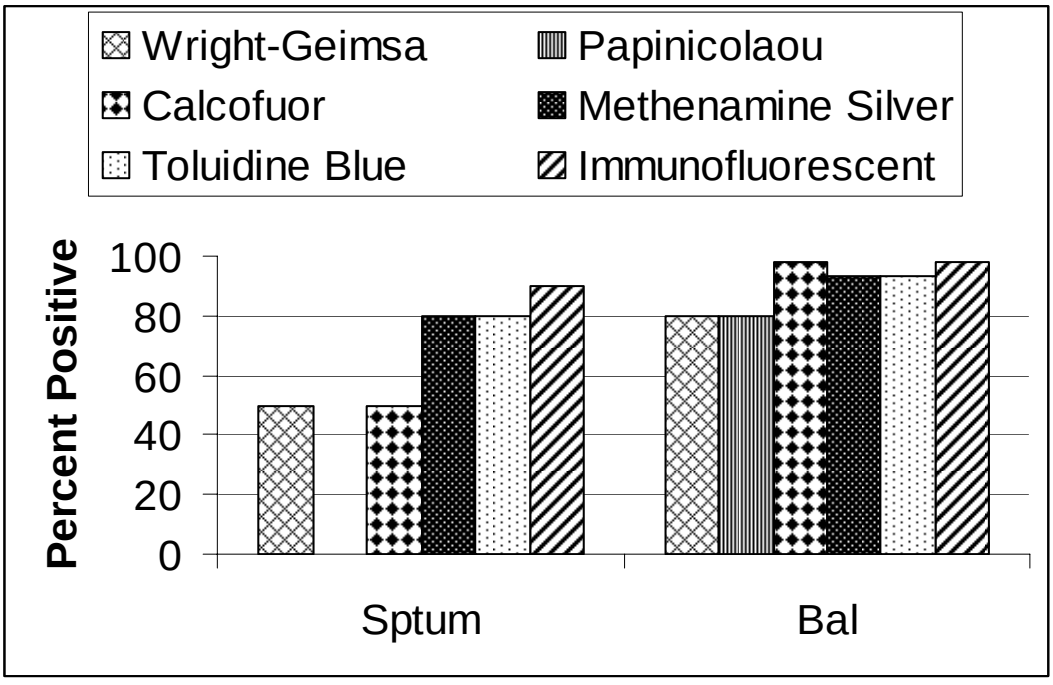
9.1.5. Expectorated sputum

There has been recent reports that expectorated sputum could be a useful specimen for the diagnosis of PJ (176,177). This is particularly true with the use of PCR as a diagnostic method. In countries where induction of sputum and bronchoscopy are expensive and not practical, expectorated sputum could be an alternative respiratory specimen to BAL.

9.2. Stains for identification

Several types of stains exist for the identification of PJ. These are broadly classified into conventional stains and immunofluorescence stain. The conventional ones either stain for the cyst wall or trophozites while immnofluorescence stain both cysts and trophic forms. The relative sensitivity of the various stains is shown in figure 2.

Figure 2: Comparison of conventional stains for the diagnosis of PCP



9.2.1 Traditional conventional stains

These are divided into those which stain the trophic forms and those which stain the cyst forms. The most commonly used stains for the trophic forms are Wright-Giemsa, Diff quick and Papanicolaou. The Wright-Giemsa stain and its modification (Diff Quick) do not require special fixation, therefore, the slides can be read quickly, allowing a rapid diagnosis of PCP. Unfortunately, the over all sensitivity is lower than the cyst staining techniques (178,179). The most popular cyst staining techniques are methenamine silver (Grocott, Gomori) and Toluidine Blue O (TBO) stains. Over all, stains for cyst wall are more sensitive and accurate in the diagnosis of PJ although other micro-organisms and particularly fungi might have positive stain making the diagnosis slightly difficult. PJ cysts are similar to the size of the red cell, tends to form clumps and reside within the cell. Fungi on the other hand tend to clump in alveolar macrophages.

The most commonly employed cell wall stain is the silver stain which has been considered as the "gold standard" stain for PJ since it was more sensitive and specific than the other stains (180). Unfortunately, the procedure is tedious to perform, and requires several hours to do the staining. Toluidine blue O (TBO) stain, on the other hand, is simpler to perform and less time consuming than the silver stain and has been used instead or in addition to the silver stain with comparable sensitivity and specificity (181, 182). A modified TBO has recently been shown to improve the background material hence allowing distinction between fungi and PJ (183).

Non-specific fluorescent staining for the cyst wall such as calcofluor can be used to identify PJ but the major limitation is that it can also stain the wall of fungi. (184). It´s major advantage is for the rapid screening of sputum samples for a few positive staining cells.

9.2.2 Immunofluorescence staining

Despite the availability of antibodies for PJ for many years, the use of antibody-based fluorescent stain started quite recently. The development of antibody based fluorescent stain is directed into two general categories, the direct fluorescent antibody technique which relies on a monoclonal antibody and the indirect fluorescent antibody technique which relies on the use of polyclonal antibody and have a wider range of stains (185). Both techniques have comparable sensitivity, although the direct monoclonal technique appears to be more specific than the indirect polyclonal technique. Immunofluorescence staining in general is more sensitive than the conventional stains because it stains both the cyst and trophic forms. Currently, it is considered as the gold standard for the diagnosis of PJ although both the initial and running cost of the procedure makes it prohibitive for routine use in developing countries.

9.3. Polymerase Chain Reaction (PCR)

Following the advent of the gene library of PJ, it has been possible to use genetic-based technology for the diagnosis of the organism. The study of genetic markers of PJ has demonstrated distinct species specificity of the organism. It has also allowed us to note that repeated infections of PJ may be due to new infection rather than reactivation of pre-existing organisms (186). PCR in the diagnosis of PJ has been

applied to both sputum and BAL. As conventional stains have low yield in induced sputum, PCR has been shown to be more sensitive and cost-effective (187). Overall, PCR appears to be more sensitive than fluorescent technique and particularly so in sputum samples (table IV) (188,189).

Table IV. Comparison of polymerase chain reaction to immunofluorescence for diagnosis of P. jiroveci

Sensitivity				**Specificity**				
Sputum FA	**Sputum PCR**	**BAL FA**	**BAL PCR**	**Sputum FA**	**Sputum PCR**	**BAL FA**	**BAL PCR**	**Ref.**
		82%	100%			85%	85%	190
53%	100%	100%	95%	100%	93%	93%	93%	191
43%	86%	100%	100%	N.A.	N.A.	N.A.	N.A.	188
50%	74%			N.A.	N.A.			192
		60%	66%			97%	100%	193
67%	100%			100%	100%			194

PCR however has also been shown to be positive in HIV infected patients without clinical evidence for infection. This has been termed as sub-clinical carriage or infection. Since PCR detects DNA particles only, whether such positivity arises from a viable or parts of a non-viable micro-organisms is also not known. Despite, some of these sub-clinical infections have been shown to progress into clinical disease (195). Recently, PCR has been shown to be positive for PJ in respiratory samples of HIV-non infected persons also. Due to these considerations, the over all role of PCR in the diagnosis PCP remains unclear.

10. Prophylaxis and treatment of PCP

10.1. Prophylaxis for PCP

Primary prophylaxis against PCP in patients infected with HIV is indicated when the CD_4 count falls below 200 cells/mm^3 or when there is a history or physical finding of oropharyngeal candidiasis or when a patient has WHO clinical stage III or IV disease (including tuberculosis) (196,145). More recent evidence supports the use of co-trimoxazole prophylaxis among people with higher CD4 count and those with less advanced HIV disease (WHO clinical stages 1 & 2) and studies from sub-Saharan Africa have shown a reduction in the number of deaths and hospital admissions among patients with varying CD4 counts with and without TB (196, 197). The recommended medication is trimethoprim-sulfamethoxazole. This is the first drug of choice and in a sub-Saharan African setting; co-trimoxazole prophylaxis has been shown to reduce all cause mortality and morbidity, including diarrhea and malaria in HIV-infected patients (198). Since the diagnosis of PCP is rarely made in sub-Saharan Africa, both prevention and treatment of PCP is probably a missed opportunity. Co-trimoxazole is also associated with several side-effects which are believed to be less frequent in African AIDS patients (199). Under those circumstances, alternative drugs to co-trimoxazole include pentamidine, dapsone, dapsone plus pyrimethamine, plus leucovarin, primaquin plus clindamycin, atovaquone plus pentamidine, most of which are not readily

available in sub-Saharan Africa.. In well-resourced countries, secondary prophylaxis may safely be discontinued when the patient's immune status is restored to a CD_4 level of above 200 using highly active antiretroviral therapy (HAART). In resource-limited settings, however, the data is limited and there is no randomized clinical trial that has assessed the safety and timing of the discontinuation of co-trimoxazole prophylaxis following immune recovery in response to HAART. Primary prophylaxis is the most cost-effective intervention that has brought a significant decline in the incidence of PCP.

10.2. Treatment of PCP

No prospective controlled treatment trial to assess the response of PCP to therapy has been performed in sub-Saharan Africa. Nevertheless death is reported in association with the use of co-trimoxazole. Among adult patients, the mortality rate ranges from 10 to 27%, a range comparable to that reported from the US (118). In children, however, mortality is much higher than in industrialized countries ranging from 10 to 80% (146). This might be due to late initiation of treatment or rapid progression of HIV due to infrequent use of HAART. Besides, some of the causes of death may have been due to an AIDS-associated complication other than PCP. Medications used to treat PCP are essentially the drugs used for prophylaxis. Co-trimoxazole is the drug of choice. Additional corticosteroids is given for the patient with severe hypoxemia (Pa $0_2 \leq$ 70mm Hg). Such patients should receive prednisolone 40mg twice a day for the first five days followed by 40mg daily for the next 5days and 20mg daily then after until the completion of treatment (200).

II. THE CURRENT STUDY

1. Aims of the study

1.1. Overall aims:

- To determine the prevalence of culture-verified PTB and HIV amongst TB suspects.
- To demonstrate the interaction between TB and HIV in terms of the clinicalpresentation, diagnosis and chest x-ray pattern.
- Based on the observation that unexpected and high rates of both HIV and PCP occurred in patients who were suspected but could not be verified to have PTB, to investigate for the various opportunistic pulmonary disorders that are differential diagnosis of PTB.
- To evaluate a simple and inexpensive diagnostic test for PCP that could be applied in resource poor settings such as Ethiopia.

1.2. Specific objectives

- To investigate the prevalence of HIV infection amongst TB patients and characterize the clinical and epidemiological features of HIV-associated TB and evaluate the usefulness of standard diagnostic methods (Paper I).
- To investigate the relationship between disease pattern and disease burden by chest radiography with M. Tuberculosis load and HIV status.(Paper II)
- To investigate the occurrence of pneumocystis pneumonia in HIV- positive patients with suspected pulmonary tuberculosis. (Paper III)
- To establish the prevalence and relative importance of PCP in relation to other opportunistic respiratory diseases in Ethiopian patients infected with HIV and smear negative for AFB presenting with atypical chest x-ray. (Paper IV)
- To evaluate the usefulness of a simple diagnostic test, toluidine blue O (TBO) stain for the diagnosis of PCP in expectorated sputum sample in patients infected with HIV. (Paper V)

2. Patients and Methods

2.1. Setting

This study consists of two prospective and consecutive patient cohorts, cohort I and II, both included at Black Lion University hospital in Addis Ababa, Ethiopia, during the period of March 1996 to February 1997 (Cohort I, paper I-III) and March 2004- July 2005 (Cohort II, Paper IV-V). All patients were either referred or self-referred. In cohort I patients came to either the regular or emergency outpatient department while patients in cohort II included admitted patients too.

2.2. Patients and controls - Cohort I (Paper I-III)

512 consecutive patients aged ≥ 15 years and attending the medical OPD and ER were initially assessed by medical residents and enrolled into the study if (1) the clinical suspicion of PTB was strong enough to warrant a sputum smear examination for AFB and (2) consented for the study. In principle, Ethiopian guidelines defining a PTB suspect as a patient with cough lasting more than 3 weeks and presence of other constitutional symptoms such as fever, weight loss were followed. (201). Patients unable to produce sputum were not included in the study.

Exclusion criteria were treatment for TB within the last 3 months or initiation of TB treatment before sampling was performed. Physicians were specifically instructed not to include patients with extra-pulmonary TB including isolated pleural effusion. Two research nurses registered the patient's demographic data, previous TB treatment and current clinical symptoms.

The treating physician re-evaluated the patients along the sputum smear and CXR findings and classified them into 3 categories (ii) smear positive PTB, (ii) smear negative PTB or (iii) respiratory tract infection.

Of the 512 patients, 3 patients were excluded, 2 because their age was less than 15 and one because he can not produce adequate sputum. Hence 509 patients remained for further analysis of whom 488 (96%) provided 3 consecutive sputum samples, 7 (1.4%) provided 2 samples and 14 (3%) one sample.

One hundred and fifty six age and sex matched, asymptomatic consecutive outpatient diabetics coming for their regular checkups were used to control for HIV prevalence in the non-TB group. All controls were screened for active PTB using a CXR and sputum smear for AFB whenever indicated.

2.3. Patients- Cohort II (Paper IV and V)

131 consecutive smear negative, HIV-positive patients with respiratory complaints of unlimited duration of illness and whose CXR's were judged by radiologists as atypical localized or diffuse pneumonias and patients with normal CXRs but were too sick with RR > 26/min and/or oxygen saturation less than 90% were enrolled into the study. Those patients whose CXR's was read as typical for lobar/segmental pneumonia and PTB were initially excluded from the study and given therapeutic trial and later on included if they failed to respond to their treatment. Sputum smear-negative patients with atypical CXR, were then counseled and asked to undergo HIV testing and if tested positive to consent for the study and undergo bronchosocpy. Those who consented for the study and had adequate expectoration were asked to provide sputum sample after brushing their teeth and rinsing their

mouth under tap water for PJ staining. Patients whose sputum tested positive for PJ by immunofluorecence (IF) were considered as definite cases of PCP and were spared of bronchoscopy. Those who sputum were negative by both IF and TBO or positive by TBO alone (false positive) and all other patients who could not produce sputum underwent bronchoscopy and bronchoalveolar lavage (BAL). The BAL material was then investigated for (1) PJ using TBO, IF, and PCR (2) culture for common respiratory bacterial pathogens (3) culture for common fungi (4) acid fast stain and (5) culture for MTB. Bronchoscopy was not done in patients being treated for PCP if the duration of treatment exceeded 7 days. All clinical, laboratory and radiographic data were recorded in a data collection sheet. Physicians were asked to put their initial diagnosis based on their clinical evaluation and /or chest x-ray finding.

2.4. Case definitions

Pulmonary tuberculosis: A verified case of PTB was a patient with suggestive clinical symptoms whose sputum or BAL culture turned positive for *M. tuberculosis*.

Pneumocystis pneumonia: A diagnosis of PCP was made whenever *Pnemocystis jiroveci* was isolated from sputum or BAL by IF.

Bacterial infection: Was diagnosed whenever there was excessive amount of purulent secretion coming out of the tracheo-bronchial tree during bronchoscopy and the BAL specimen was negative for MTB, PCP or fungal pathogen.

Pulmonary Kaposi sarcoma (PKS): Was considered likely whenever there was typical macular, violaceous lesions observed over the tracheo-bronchial tree during bronchoscopy.

Non-specific interstitial pneumonitis (NIP): Was considered likely whenever there was bilateral diffuse interstitial infiltrate with indolent course that failed to clear with treatment for bacterial infection and/or PCP and BAL is negative for TB, PCP or fungi.

Clinical PCP: Was considered whenever a patient with clinical and CXR findings typical for PCP responded to high dose co-trimoxazole in spite of a negative study for PCP.

Double Infections: Was considered whenever any two of the above diagnosis occurred together.

2.5. Chest X-ray

2.5.1. Cohort I (Paper I and II)

All CXRs were initially read by a chest physician and later by two radiologists who were blinded of the clinical and laboratory data. Radiographic patterns that suggested TB were apical infiltrates, cavities with surrounding nodular shadows miliary pattern, localized small nodular shadows, collapse, unilateral hilar adenopathy, pulmonary nodule with calcification and pleural effusion. PCP was defined as diffuse, bilateral and symmetric fine granular and reticular shadows. Cystic lesions and

pneumothorax also suggested PCP. Apart from the pattern of involvement, CXRs were also categorized according to extent of disease as normal, minimal, moderately or far-advanced using the diagnostic standard of the classification of the American Thoracic Society (202). The extent of disease was also assessed by a total zonal score representing the number or intra-thoracic regions (out of six) involved by any lesion. Chest x-rays were also categorized by site of involvement as upper, middle and lower lung zones.

2.5.2. Cohort II (Paper IV)

All chest x-rays were independently read by two radiologists (one of whom also participated in part I of the study.) who were unaware of the bacteriological and clinical diagnosis. Chest x-rays were read as highly likely (typical), likely or unlikely for PTB, pneumonia, PCP, KS or NIP based on an already prepared diagnostic format. Accordingly, typical pulmonary TB was defined as upper lobe infiltrates with or without cavitations, intrathoracic-lymphadenopathy, miliary mottling and pleural effusion. Typical PCP was defined as fine, bilateral, perihilar granular infiltrates with bat wing configuration. Typical bacterial pneumonia was defined as lobar/segmental or sub-segmental consolidation with or without pleural effusion. Kaposi sarcoma was highly likely when bilateral coarse reticulo-nodular infiltrates that follow septal lines with peri-bronchovascular dominance were seen. Non-specific interstitial pneumonitis was considered when diffuse bilateral interstitial infiltrates with indolent course and failure to treatment for bacteria infection and PCP.

2.6. Laboratory procedures

2.6.1. Direct smear microscopy (Paper I, II and IV)

Direct microscopic examination for acid fast bacilli (AFB) after staining according to Ziehl Neelsen was done in three consecutive sputum samples (spot, morning, morning) at Black lion Hospital TB laboratory in both part I and part II of the study. However the technicians in the first study were different from the technicians in the 2nd study. Sputum samples were kept at 4^{0}C and later sent to the TB laboratory of the Armauer Hansen Research Institute (AHRI) for further examination including culture for MTB. BAL sampled retrieved in part II of the study also underwent acid fast stain for AFB.

2.6.2. Sputum AFB after concentration with household bleach (Paper I and II)

In both studies, sputum was examined by the concentration technique using household bleach. At the Armauer Hansen Research Institute (AHRI), the three sputum samples of individual patients were pooled and transferred to a 15ml screw-capped tube and mixed with an equal volume of ordinary bleaching agent (NaOCl, 5%). The tube was incubated at room temperature for 15min and shaken by hand at regular intervals. After addition of 8ml of distilled water, the tube was centrifuged at 3000 rpm for 15minutes. The supernatant of each tube was carefully discarded, the sediment was mixed with the remaining fluid and direct smear were prepared by applying a drop of the re-suspended sediment with a sterile pipette to a slide. The slides were dried in air, heat fixed and stained by the Ziehl Neelsen technique (203).

2.6.3. *Mycobacterial culture (Paper I-IV)*

Mycobacterial culture on conventional Lowenstein (LJ) egg medium and LJ egg medium containing 0.6% sodium pyruvate was performed in all pooled sputum sample of the cohort I patients and in pooled pre-bronchoscopy sputum and BAL samples in cohort II of the patients at the Armauer Hansen Research Institute (AHRI), in Addis Ababa, Ethiopia. Before culture, the samples were digested and decontaminated from non-mycobacterial micro-organisms by the sodium lauryl sulphate method (204). The tubes were incubated at 37^0C in 5% CO_2 for one week, there after at 37^0C in air for another 7 weeks and were checked once a week for mycobacterial growth. Growth of mycobacterium was confirmed by detection of typical colony morphology and by microscopy for AFB in both studies, and by additional thiophene 2 carboxylic acid hydroxide (TCH) and the pyrazinamide test in part II of the study. In part I of the study, cultures confirmed to be positive by ZN stain were transported at room temperature on LJ medium to the Swedish Institute for Infectious Disease Control (SMI), Stockholm, Sweden, for further characterization. After sub-culturing, cultures with typical macro and microscopic features were tested with nucleic acid probe specific for the *M. tuberculosis* complex and *M. avium* complex (MAC) using Accuprobe Systems (Gen-probe, San Diego, CA, USA). Species identification was not done in the 2nd part of the study.

2.6.4. *Bacterial and Fungal Culture (Paper IV)*

In cohort II patients, BAL specimen, apart for identification of PJ and MTB, were inoculated directly on blood, chocolate, mannitol salt and Macon key agars (Oxoid Ltd, Basingstoke Hampshire, England) which are recommended for the isolation of the most common respiratory pathogens. BAL fluid was also cultured on Saborauds dextrose agar supplemented with chloroamphenicol (without cyclohexamide) for the isolation of the most common fungi.

2.6.5. *Toluidine blue O stain for P.jiroveci (Paper V*)

In cohort II patients, staining for PJ was done on sputum and BAL samples using toluidine blue O according to the method described by Gosey et al (205). After centrifugation and several washing steps, two drops of the pellets was added on a slide, one drop with 25ml and another one with 50ml. The material was spread out to a diameter of about 10mm and let dry completely. Six staining jars were prepared, one with fixing solution, one toluidine blue O stain, three with isopropanol and one with xylene. Each slide was treated separately. The slides were then mounted under cover slip with DPX while the preparation is still damp and allowed to dry. The slide was observed under light microscope with objective 40x and 100x for confirmation. A positive result was taken if at least one cluster with characteristic cyst was detected and a cluster was defined as equal to or ≥ 2 cysts. If positive, the number of clusters were counted and graded.

2.6.6. Immunofluorescence assay for P. jiroveci (Paper III and V)

In both cohort I (ES) and cohort II (ES & BAL) of the study, the IF method was based on a mouse monoclonal antibody to *P. jiroveci* and fluorescin isothiocyanate conjugated sheep anti-mouse immunoglobulin (206). In part I of the study, IF was done at the Swedish Institute for Infectious Diseases

(SMI), Sweden, by two technicians in a blinded fashion on PCR positive samples and comparable number of PCR-negative samples serving as controls. IF was considered as the gold standard for the diagnosis of PCP. In the event of discordant or doubtful results (14 samples), a new sample from the sputum pellet was re-stained and read again. A result was reported positive when there was at least one cyst and/or 5 trophozites, counted per well containing 10-25 ml of sample material. In the second part of the study, the same method was applied but IF was done in all expectorated sputa samples (85 samples) and all BAL specimen (120 samples). 11 patients who had their sputum IF positive for *P.jiroveci* were spared of bronchoscopy, therefore, had no BAL IF done. The staining and reading was done by a microbiologist at Tikur Anbessa hospital. For external quality control, an extra slide from every fifth sample was prepared, dried and then stored at $+4^0$C until it was sent to (SMI), Sweden. These slides were subsequently stained at SMI and the results recorded. A positive result was taken if at least one cluster with characteristics cysts was detected and a cluster was defined as ≥ 2 cysts.

2.6.7. PCR assay for P.jiroveci (Paper III and V)

In the first cohort of patients, both single and nested PCR was done in (i) 119 HIV-positive TB-negative patients, (ii) 96 HIV positive, TB-positive patients to detect co-infection (iii) 72 HIV-negative, TB-positive patients and (iv) 97 HIV-negative, TB-negative patients serving as controls. The frozen sputum samples were thawed and treated with dithiotreitol and ethanol (205) and the pellets were the origin for the DNA preparations and IF assays. The DNA was extracted with commercial kits (wizard DNA clean up kit, Promega, Falkenberg, Sweden) (207). The PCR assay was performed in two steps (single and nested PCR) and based on amplification of a portion of the large subunit (LSU) of the mitochondrial ribosomal RNA gene of P. jiroveci. The nested PCR was performed with the outer primers PAZ 102-H and PAZ-102-E, the inner primers pAZ 102-X/RI and PAZ 102-Y/RI and a protocol based on that described by Wakefield (208) with some modification. The single PCR was carried out with 35 cycles and the nested PCR with an initial denaturation step at 940C for 10 minutes and 15 cycles instead of 30 in the second cycling stage. Two percent (vol/vol) of the first PCR was used as template in the nested PCR and both the reaction mixes contained 0.0125 U Ampli, Taq, /Gold (PE Corporation, Stockholm, Sweden) per ml instead of Taq polymerase. The PCR reaction mixes were kept at -40C until use. All DNA preparation was controlled for content and inhibition by a PCR based on amplification of a portion of the human B-globin gene (209).

In the 2nd cohort of patients, PCR was done both in expectorated sputum and BAL samples and the methods employed were the same except nested PCR was not done. The prevalence of PJ was then compared in the three methods, TBO, IF and PCR. Besides, we hope to study the locus for dihydropteorate synthase gene, the gene which confers resistance to co-trimoxazole, in the near future and correlate it to the clinical material.

2.6.8. HIV serology (Paper I-V)

In the first cohort of patients, serum samples were screened for the presence of HIV antibodies by using ELISA both for HIV-1 and HIV-2 (Enzygnost

anti HIV-1 /HIV-2, Behring werke AG, Marbug, Germany). Positive samples were re-analyzed by the same ELISA and if still positive, the results were confirmed by an HIV-1 ELISA (Wellcozyme anti HIV-1 UK 57, Murex diagnostics Ltd, Dartford, UK). IF the sample was positive by all three ELISA, the result was considered positive. If the test result was inconsistent by the different ELISA, the Western Blot test (Version 2.2, Diagnostic biotechnology, Genelab Diagnostics SA Geneva, Switzerland) was done at SMI.

In the 2nd cohort of patients, initial screening was done using the Determine rapid spot test. Positive results were confirmed by micro-ELISA system (Vironostika Uniform II Ag/Ab). Unigold was the tie-breaker. As there were no discordant results, Uni-Gold was not employed. Besides, CD4 count was determined at the time of enrollment.

2.6.9. Other Laboratory tests

In the 2nd cohort of patients, complete blood count was determined in all patients. Oxygen saturation was also recorded at the time of enrollment to the study.

2.7. Ethical consideration

In both studies, patients were included in the study after they verbally provided informed consent. However, in part I, HIV testing was done anonymously with coded serum samples that were unlinked to the patient's identity. IF HIV status was required on clinical grounds, testing was performed with the patient's approval and outside the protocol of the study. In part II, both HIV testing and bronchoscopy were done following written informed consent. Both parts of the study were approved by the National Ethical Committee in Ethiopia and the ethical committee of Karolinska Institute in Stockholm, Sweden.

2.8. Data management and analysis

In cohort I, JMP software (version 3.2, SAS Institute Inc., Cary, NC, USA), Statistical software (version 5.1, Stat soft In., Tulsa, OK, USA) and Epi-Info 2000 software (version 1.0.5; Centers for Disease control and Prevention, Atlanta, Ga) were used for statistical analysis. The McNemar test was used to assess differences in proportions in paired observations and the X^2 test to assess differences in proportions in unpaired observations. In the analysis of factors predictive of PTB and the outcome of diagnostic methods calculations were made based on mycobacterial culture outcome as the reference method (paper I-IV). Odds ratios for paired observations were calculated using the SAS software (version 7.1, SAS Institute Inc. Cary, NC, USA). Patients were stratified by age groups in the analysis of TB prevalence and by age groups and sex in the analysis of HIV prevalence (paper I). Variables from the univariate analysis were tested in a multivariate model for the analysis of factors associated with PTB or clustering, but only significant variables were included in the final regression model (Paper I, III). P-values of 0.05 or less were regarded as significant.

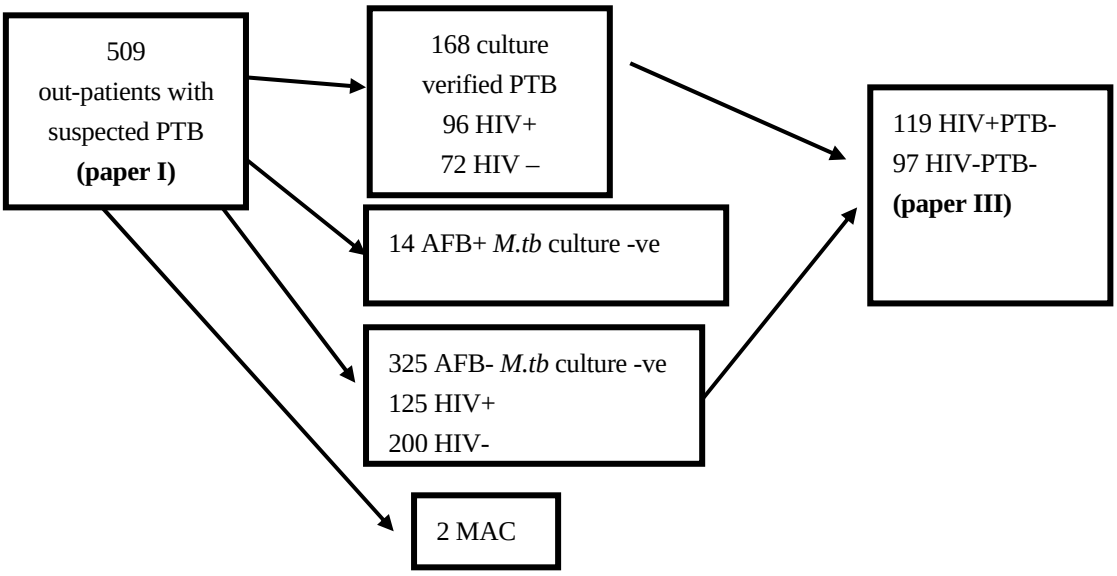
In cohort II, after pre-coding of the variables, the raw data was entered into computers using EPI-Info version 6.04D statistical package and exported to SPSS for windows version 11 statistical package for further analysis. Frequencies, proportions and summary statistics were used to describe the study subjects in relation to relevant variables. ANOVA was used to compare the mean values of

continuous variables among the different disease patterns. Chi-Square statistic and Fisher's Exact test were used to assess significant associations among categorical variables as appropriate. Multiple logistic regressions were employed to assess the independent predictors of the outcome variables. For comparing the results of different test procedures, sensitivity, specificity and predictive values were calculated. For assessing level of agreement between two raters, Kappa statistics was used.

3. Results: Cohort I (Paper I-III)

3.1. Outline of study population (Paper I –III)

Figure 3: The distribution of the study population in cohort I (Paper I-III)



Of the 509 patients suspected of tuberculosis, only 1/3 (168 patients) could be proved to have culture-verified PTB and are referred as PTB group in what follows. 14 patients whose sputum smear was positive for AFB were negative on sputum culture, therefore, were excluded from the study on the ground that these may be false positive cases. In the remaining 325 sputum AFB-ve / culture-ve patients and are referred as non-TB group in what follows, 125 (38.5%) tested positive for HIV. The difference in HIV prevalence between the non TB group (38.5%) and the 156 age and sex matched diabetic controls (8.5%) was statistically highly significant ($P<0.001$). This high rate of sero-positivity in the non-TB patients was the reason that prompted us to look for possible cases of PCP amongst this group of patients (Paper III).

3.2. Clinical and epidemiological characteristics (Paper I)

The overall HIV sero-prevalence among culture-verified PTB patients, non-TB patients and controls was 57.1%, 38.5% and 8.5% respectively. The peak HIV-sero-prevalence in the TB group was between 35-44 years of age and appears to have been distributed equally among the sexes. The corresponding age for the non-TB patients was between 25-34 yrs. The median age for the HIV positive and HIV-negative patients was 31 (range 17-55) and 25 (range 15-62) respectively.

Independent predictors of culture-verified PTB were age < 25yrs, male sex, HIV-positive sero-status, fever and profound weight loss. In a multivariate regression model, however, age <25yrs, male sex, HIV positive sero-status and fever remained discriminators of culture-verified PTB. When HIV status was removed as a variable, profound weight loss was also identified as an independent clinical predictor. The clinical symptoms in PTB and non-TB patients with and without HIV is shown in Table V.

Table V. Clinical symptoms in pulmonary TB and non-TB patients in relation to HIV status

	PTB patients		Non-TB patients	
	HIV- positive n = 96 (%)	HIV- negative n = 72 (%)	HIV- positive n= 125 (%)	HIV -negative n = 200 (%)
Cough > 3 weeks	93 (97)	69(96)	118(94)	186(93)
Hemoptysis[a]	21 (22)	17(24)	35/124(89)	61(31)
Fever[b]	91 (95)	66(93)	110/124(89)	166(83)
Night sweat[c]	88/95(93)	66(92)	111(89)	172(86)
Pronounced weight loss[d] (≥ 10kg)	38/87 (44)	13/65(20)	39/121(32)	32/190(17)

a,b,c,d As symptoms were not registered for all patients, available patients for analysis are specified.

3.3. Diagnostic outcome (Paper I)

The outcome of diagnostic tests for PTB in culture-verified PTB patients and non-TB patients is shown in Table VI below.

Table VI. Outcomes of diagnostic tests for PTB in culture-verified PTB patients and non-TB patients. Values given in parentheses are percentages.

	PTB patients		P value	Non-TB patients		P value
Diagnostic test	HIV positive n = 96	HIV negative n = 72		HIV positive n = 125	HIV negative n = 200	
Positive direct smear for AFB	37/96 (38.5)	54/72 (75)	<0.001	--	--	-
TB diagnosis by CXR[a]	72/90 (80.0)	63/70 (90.0)	0.078	46/121(38.0)	60/195(30.8)	0.19
Physician's TB diagnosis[b]	77/94(81.9)	66/71(93.0)	0.033	52/120 (43.3)	61/195(31.3)	0.031

[a,b]As CXR and physician's diagnosis were not registered for all patients, available patients for analysis are specified.

The overall sensitivity and specificity of sputum microscopy for AFB was 54.2% (91/168) and 96.8% (330/341). Sputum smear microscopy was positive in 38.5% of the 96 HIV-positive patients compared to 75% (P<0.001) in 72 HIV-negative patients. A diagnosis of TB by CXR was made in 84.3% (135/160) of the PTB group compared to 33.5% (106/316) in the non-TB group. 8 patients in the non-TB group were diagnosed to have PCP by chest radiography, 7 of whom were HIV

positive. Physicians correctly categorized 86.7% (143/165) of the culture-verified PTB cases but they also wrongly categorized 36% of the non-TB patients as PTB giving an overall specificity of 64.1%.

3.4. Chest X-ray findings (Paper II)

A total of 163 (97%) of the 168 culture-verified TB patients had CXRs available for analysis. Fifteen (9.4%) patients of whom ten were HIV positive had normal CXRs. These ten patients were initially diagnosed to have respiratory tract infection by the treating physician. HIV-positive patients were less likely to have cavitary disease and more likely to have miliary, interstitial and normal pattern. The majority of patients (66%) presented with moderate or far-advanced disease. HIV-positive patients tended to have either a normal CXR or minimal involvement (P=0.059). The mean number of lung zones involved by disease was slightly less in HIV-positive patients compared to HIV-negative patients (P=0.08) Middle and lower lung zone involvement was significantly more common in HIV- positive patients (P< 0.05).

3.5. M. Tuberculosis colony count and bacillus load (Paper II)

In general, HIV-positive patients had an increased proportion of negative sputum smear for AFB than HIV-negative patients. Of the 96 HIV-positive patients with culture-verified PTB, 46 (48%) were smear-negative for AFB by the concentration method compared to only 14 of the 72 (19.4%, P<0.001) of HIV-negative patients. The results of MTB colony count and AFB smear grades for HIV-positive and HIV-negative patients are shown in Table VII.

TableVII: M. tuberculosis load by culture and bacillary load by concentrated sputum smears in HIV-negative patients with culture verified pulmonary tuberculosis.

	HIV positive		**HIV negative**			
	Culture +ve	smear +ve	Culture +ve	Smear +ve	P-value Culture +ve	P-value Smear +ve
M. tuberculosis colony count and bacilli load[a]	(No.=96) No.(%)	(No.=50) No. (%)	(No.=72) No.(%)	(No.=59) No.(%)		
1+(50-100colonies)	20(20.8%)	14(28%)	11(15%)	20(34.5%)	P=NS	NS
2 + (100-200colonies)	32(33.3%)	14(28%)	10(14%)	12(20.6%)	P=0.003	NS
3 + (200-500colonies)	28(29%)	14(28%)	26(36%)	13(22.4%)	NS	NS
4 + (>500colonies, confluent)	16(16.6%)	8(16%)	25(34.7%)	13(22.3%)	P=0.007	NS
Combined 1 + and 2+	52 (54%)	28(56%)	21(29%)	32(55%)	P=0.002	NS
Combined 3 + and 4 +	44 (45.8%)	22(44%)	52(71%)	26(45%)	P=0.001	NS

Bacillus load determined by concentration technique and not by direct sputum smear.

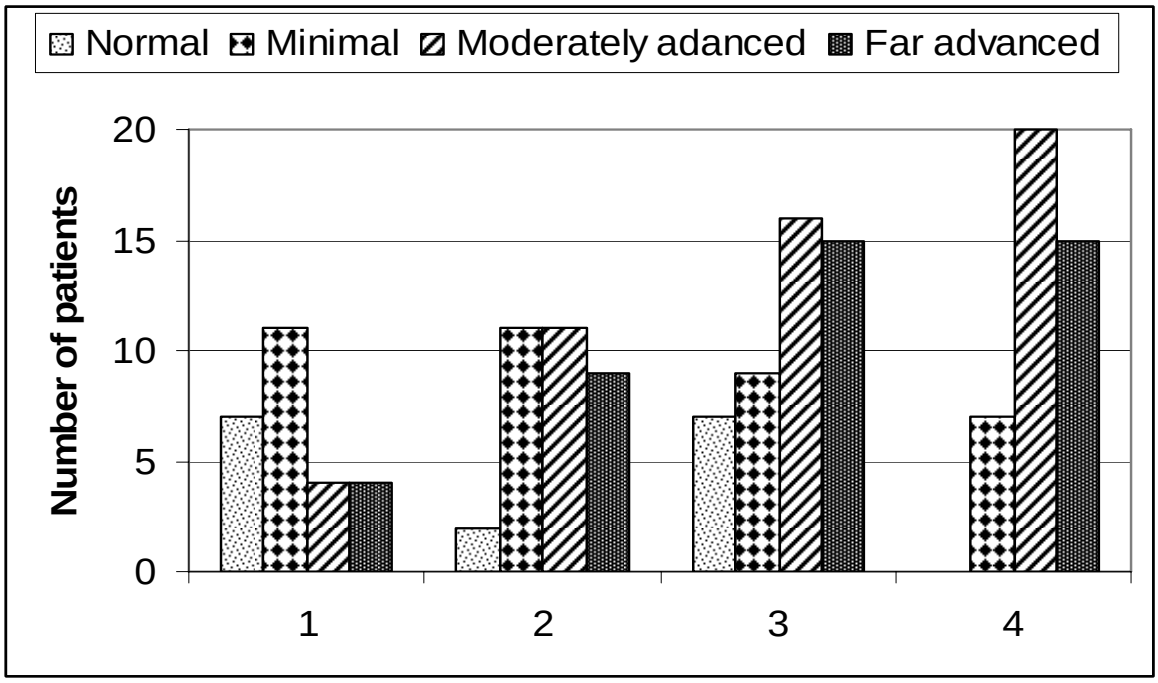
HIV-positive patients had lower colony count than HIV-negative patients (54% versus 29%, P=0.002) whereas the AFB smear grade did not differ between the two groups when sputum concentration technique was employed. However, in direct

sputum smears, HIV-positive patients had significantly lower AFB smear grades than HIV-negative patients (180).

3.6. Relationship between disease burden by CXR and MTB bacillus load (Paper II)

More patients with normal /minimum disease CXR had lower mycobacterial colony count compared to patients with moderate or advanced disease (Figure 4). The number of colony count in the combined normal/ minimal CXR group was significantly less than in the combined moderate/ far-advanced group (P=0.01).

Figure 4. Disease burden by chest x-ray correlated to colony count graded 1+ to 4+ by culture in patients with culture-verified pulmonary tuberculosis (When the number of patients with normal chest x-ray and minimal disease were combined and compared to the number of patients with moderate and far advanced disease, the relationship between bacillus load and extent of disease was significant, p<0.01.



3.7. Detection of P.jiroveci by IF and PCR (Paper III)

Amongst the 119 patients who were TB-negative but HIV-positive, *P.jiroveci* was found in 10.9% by IF, 8.4% by single PCR and 30.3% by nested PCR (Table VIII). In the PTB-positive/ HIV-positive group, 3.1% were positive for PJ by IF and 13.5% by nested PCR, suggesting a co-infection. All IF and single PCR positive samples were positive by nested PCR too.

When we consider all HIV-positive patients with negative sputum smears for AFB, *P.jiroveci* (by nested PCR) and *M. Tuberculosis* were equally common with a prevalence of 27.1% (45/166) and 28.3% (47/166) respectively.

Table VIII. Detection of P. jiroveci in expectorated sputum samples by immunofluorescence (IF), single and nested PCR in HIV positive (HIV+) and negative (HIV-) patients without (PTB-) and with (PTB+) culture verified pulmonary pulmonary tuberculoses (PTB).

Patient category	IF Positive[a]	Single PCR Positive[a]	Nested PCR Positive[b]	IF-negative controls[b]
HIV+PTB- (n = 119)	13 (10.9%)	10(8.4%)	36(30.3%)	38
HIV+PTB+ (Total n=96)	3 (3.1%)	3 (3.1%)	13(13.5%)	9
AFB+ (n = 49)	0	0	4	
AFB- (n = 47)	3	3	9	
HIV-PTB+ (Total n = 72)	0	0	3(4.2%)	8
AFB+ (N=60)	0	0	3	
AFB- (N=12)	0	0	0	
HIV-PTB- (N=97)	0	0	3(3.1%)	7

[a]*Patients Positive in IF and single PCR are also positive in nested PCR.* [b]*The nested PCR-positive samples were analyzed by IV IF with a comparable number of nested PCR-negative controls by staff unaware of the clinical data and PCR results. AFB, Microscopy for acid fast bacilli in sputum with Ziehl-Neelsen stained conventional direct smears and after digestion with sodium hypochlorite and concentration.*

3.8. Chest X-ray and physician's diagnosis in relation to IF and PCR

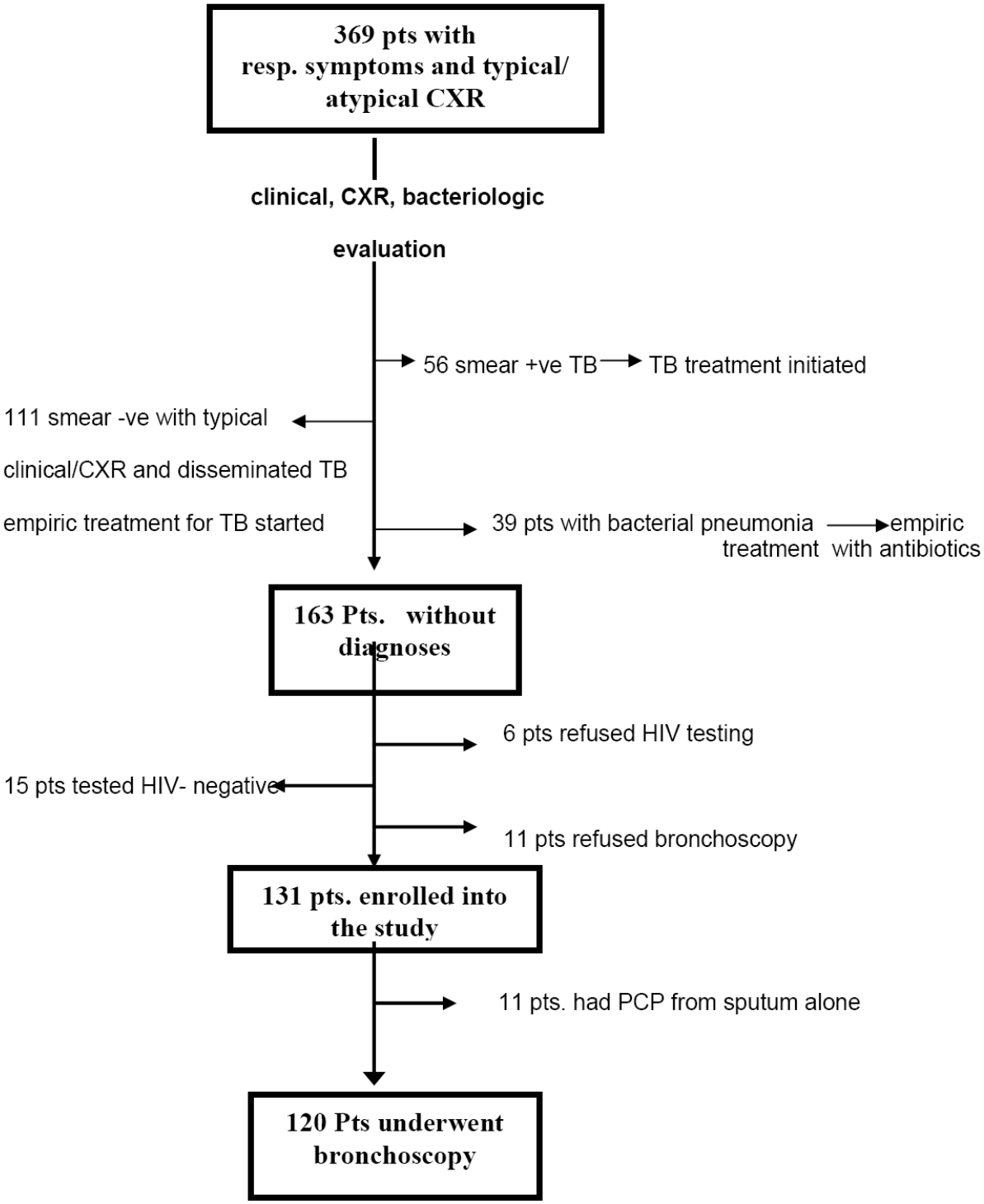
In the HIV-positive PTB-negative patients, 8 chest x-rays were read as suggestive of PCP of whom 6 (75%) were positive for *P.jiroveci by* IF and/or PCR. In this group of patients , more than one third of the IF-positive and nested PCR positive patients were wrongly read as having PTB by CXR as well as by physicians, depicting the difficulty in differentiating PCP from TB by CXR and clinical means. Only one out of 13 IF-positive patients was suspected of having PCP by physicians.

In the PTB-positive, HIV-positive patients, there was neither a clinical nor radiological suspicion of PCP in the 13 patients with nested PCR positive samples of which 3 were IF positive. These three patients were smear-negative for AFB and their CXRs were interpreted as suggestive of PTB, hence were started on anti TB medication alone.

4. Results: Cohort II (Paper IV-V)

Between March 2004 and July 2005, 369 patients were screened for entry into the study (Figure 5). A total of 131 patients were enrolled into the study of whom 120 required bronchoscopy. 91 (69.5%) were seen as outpatients and 40 (30.5%) as inpatients. 58% were female and the median age was 35 yrs (range 15-65). The majority were either married (52%) divorced (13) or widowed (9.2%). Only 25% were single.

Figure 5. Screening protocol for patients entering the study



4.1. Final diagnosis (Paper IV)

A total of 148 diagnosis were made in 131 patients, of which 17(13%) were due to double infection. (Table IX). The 3 most common diagnosis made were bacterial infections (33.6%), pneumocystis pneumonia (29.7%) by immunofluorescence and pulmonary tuberculosis (23.7%) in that order. Diagnosis could not be confirmed in 24 patients, of whom 17 were suspected to have PCP on clinical and radiological grounds. Kaposi sarcoma and non-specific interstitial pneumonitis were rare (4

patients each) and one patient had pulmonary strongloides. The most common form of fungi isolated were candida and aspergillus species, both of whom were commensals in the respiratory tract. 68% of patients who had candida isolated from BAL specimen also had oropharyngeal candidiasis suggesting a contamination from the respiratory tract. In more than 90% of patients in whom candida and aspergillus were isolated from BAL specimen, there were other definite diagnoses to explain the underlying pathology.

Table IX. Disease rates among the 131 HIV positive AFB negative patients with interstitial pneumonia included in Paper IV

Disease	***Rate (%)***	***Remarks***
A) Definitive diagnosis		
Bacterial infection	44 (33.6%)	12 had double infections
PCP	39 (29.7 %)	8 had double infection
TB	31 (23.7%)	27 culture +ve for MTB, 4 culture-ve disseminated TB, 10 had double infection
Kaposi sarcoma	4 (3.1%)	
NIP	4 (3.1%)	
Others	2(1.5%)	Bronchiectasis = 1, strongloidiasis = 1 (both had double infection)
B) Undiagnosed		
Clinical PCP	17(12.98 %)	2 double infections
Diagnosis unknown	7 (5.3 %)	
* Total	148	17 patients had double infection

****** Because of presence of double infections percentages do not add up to 100%*

4.2. Clinical features and predictors of diagnosis (Paper IV)

The relative prevalence of the clinical and radiographic findings, blood count, CD_4 count and oxygen saturation for each of the major diagnosis is shown (Table X). PCP patients had less chest pain, severe dyspnoea, lower O_2 saturation, lower ESR, lower CD_4 count and high sensitivity for radiographic diagnosis significantly more than patients with bacterial infection and PTB. Both respiratory rate and presence of cyanosis did not discriminate PCP from the other infections. The presence of other opportunistic infections such as oral candidiasis and Herpes zoster were equally distributed between the three major diagnoses. The presence of purulent sputum was significantly associated with bacterial infections while haemoptysis was associated with PTB. In a multivariate regression model, low CD_4 count and typical CXR finding for PCP and yellowish sputum for bacterial infections were the only independent clinical predictors.

Table X. Clinical, radiographic, laboratory and O_2 saturation features of PCP, TB and bacterial infection

	PCP (41pts) mean %	***TB (27pts) Mean %***	***Bacterial Infection (44) Mean %***	***P- Value***
Symptoms				
Duration of illness (wks)	7.2	6.07	13.23	* P = 0.025
Cough (present)	87.8%	88.9%	95.5%	P = 0.419
Non productive /scanty sputum	51.2%	55.5%	31.8%	P = 0.249
Sputum type:	58.5%	44.4%	38.6%	P = 0.175
whitish	7.5%	25%	50%	* P = 0.001
Yellowish	2.4%	27.3%	11.4%	* P = 0.013
haemoptysis	66%	70.4%	68.2%	P = 0.92
Night sweats	70.7%	44.4%	54.5%	* P = 0.083
Absent chest pain	36.6%	11.1%	18.2%	* P = 0.0308
Severe SOB				
Signs				
Mean temperature	35.9	37.58	35.87	P = 0.36
Resp. rate	33.9	30.41	30.68	P = 0.146
Weight loss > 10kg	15.4%	18.5%	9.1%	P = 0.5
Cyanosis	25%	11%	16.7%	P = 0.362
Normal auscultatory finding	67.5%	63%	45.2%	P = 0.102
Oral thrush	61%	70.4%	59.1%	P = 0.615
Radiography				
CXR sensitivity	90%	42.3%	18.6%	P < 0.001
CXR positive predictive value	45.6%	40.7%	32%	P = 0.482
Laboratory data				
CD_4 count	59.49	88.35%	119.68	* P = 0.005
WBC count (total)	7473.75	5169.23	7076.74	* P = 0.007
ESR	70.85	89.83	92.63	* P = 0.023
Oxygen saturation	81.34	86.56	87.93	* P = 0.015

4.3. Chest X-ray findings (Paper IV)

All the 131 patients had their CXRs taken and read before enrollment into the study. Four (3.1%) of these CXRs were read as normal and these 4 patients were included into the study on the basis of decreased O_2 saturation (<90 %) and /or rapid respiratory rate (RR >26/min). In the remaining 127 patients, the sensitivity of the CXR for the diagnosis of the three major diseases is shown in (Table XI). The sensitivity of the CXR for the diagnosis of PCP was 90% but the CXR also wrongly classified 50% of the other diagnosis into PCP. The sensitivity of the CXR for the diagnosis of TB and bacterial infections was dismally low, 42.3% and 18.6% respectively. A diagnosis of NIP was made in 4 patients. The basis for the diagnosis was mostly radiological in a patient with minimal symptoms and abnormal CXR with bilateral interstitial infiltrates and failure to respond to treatment for

pneumonia and PCP. The CXRs of these 4 patients were initially read as suggestive of PCP in 2 and PTB in another 2. In 2 patients with suspected NIP, the CXR finding disappeared after initiation with anti-retroviral treatment. Pulmonary Kaposi sarcoma was suspected in 2 out of the 4 patients by the radiologists. All the 4 patents with KS also had cutaneous Kaposi.

Table XI. Initial Physician Diagnosis and CXR findings

	PCP = 39	**TB = 26**	**Bacterial Infections = 44**
Initial physician Dx			
Sensitivity	24/39 = 61.5 %	7/26 = 26.9%	7/43 = 16.2%
Specificity	49/90 = 54.4%	77/103 = 74.7%	75/86 = 87%
CXR findings			
Sensitivity	36/39 = 92.3 %	11/26 = 42.3 %	8/44 = 18 %
Specificity	48/90= 53.3 %	87/105 = 82.9 %	69/87 = 79.3 %

Discordant results between the two radiologists were observed in 25 (19.5%) cases. The most common discordance was between PTB and PCP in 15 (60%) patients. However, the level of agreements between the two radiologists was 79.3% (kappa coefficient = 0.792 +/- SE of kappa = 0.095) and was rated as very good.

4.4. Initial physician diagnosis (Paper IV)

Physicians were asked to put a diagnosis based on their clinical assessment and/or radiographic findings. The sensitivity and specificity of initial physician diagnosis against final diagnosis is shown in (Table 9). For all the three diseases and particularly for PTB and pneumonia, the sensitivity of physician's diagnosis was low. In general, a diagnosis of PCP was made in 65 (49.6%) of the 131 patients and a diagnosis of bacterial infection was the most difficult to make by physicians. The majority of bacterial infections were diagnosed as either PCP (46.5%) or PTB (30.2%).

4.5. Comparison of TBO to IF in sputum and BAL samples (Paper V)

Overall, 85 (65%) of the 131 patients could produce sputum for TBO and IF stain. In 8 patients, the sputum was considered inadequate; therefore, only 77 sputa were analyzed for PJ infection. Bronchoalveolar lavage was done in 120 patients. In the remaining 11 patients, diagnosis was already made from sputum examination; therefore, there was no need for bronchoscopy. 39 (30%) patients were already started on treatment for PCP with high dose co-trimoxazole for a median period of 5 days (range 1-7 days). Considering IF as the gold standard for the diagnosis of PCP, the sensitivity**,** specificity positive and negative predictive values of TBO against IF is shown in (Table XII).

Table XII. Comparison of TBO against IF in Sputum and BAL Specimen

	Sensitivity	***Specificity***	**Positive predictive value**	**Negative predictive value**
Sputum	10/14 (71.4 %)	63/63 (100 %)	10/10 (100%)	63/67 (94 %)
BAL	17/25 (68 %)	92/92 (100 %)	17/17 (100 %)	92/100 (92 %)

A total of 14 sputum samples tested positive for PJ by immunofluorescence of which 10 (71.4%) were positive by TBO too. Seventeen of the 25 (68%) IF positive BAL samples were positive by TBO too. Hence, the sensitivity of TBO was comparable in sputum and BAL specimen. In sputum samples, the mean number of PJ clusters by TBO staining was 8.55 (50=6.98) compared to 12.3 (SD=10.79) by IF staining, hence an increase by 33.8%. In BAL samples, the mean number of clusters by TBO staining was 16.68 (SD = 11.39) compared to 21.2 (SD= 13.88) by IF staining, an increase by 20%. This increase, however, was not statistically significant. The difference in the mean number of cluster was significant when comparison was made between sputum TBO and BAL TBO (8.55 Vs 16.68 cluster, p=0.059) and sputum IF and BAL IF stain (12.3 Vs 21.2 clusters, p= 0.041), suggesting that a difference in the type of specimen is more important than the staining technique with regard to parasite density. 21 of the 39 (53.8%) PCP patients have been on co-trimoxazole treatment for a median period of 4 days (range 1-7 days) prior to bronchoscopy. However, there was no relationship between the number of clusters and the duration of treatment with co-trimoxazole (corrélation coefficient (r) = 0.089; P=0.618). The number of clusters of PJ tended to increase, although not significant, as the CD_4 count decreased (correlation coefficient (r) = -0.122, P=0.500).

4.6. ***Comparison of TBO to PCR in sputum and BAL samples***

Toluidine blue O stain detected 27 (48.2%) of the 56 PCR-positive sputum and BAL samples. In sputum samples alone, TBO detected 10 (34.5%) of the 29 PCR-positive cases compared to 10 (71.4%) of the 14 samples positive by immunofluorescence. In BAL samples, TBO detected 17 (41.5%) of the 41 PCR-positive cases (Table XIII).

4.7. Diagnosis of bacterial infection (Paper IV)

A total of 272 bacteria were isolated from 116 BAL samples. The median number of bacteria isolated was 2 (range 1-5). There were 24 different types of bacteria isolated. The most common type of bacteria isolated *were Stenotrophomonas maltophilia* in 71 (61.2 %) followed by *coagulase–negative staphylococci* in (27.6%), *non-group A beta-hemolytic streptococci* in (23.3%), *coagulase-positive staphylococci* in (19.8%), *Streptococcus pyogenes in* (19%), *Klebsiella pneumoniae* in (15.5%), *Streptococcus pneumoniae* in (14.7%) in that order. The median number of bacteria isolated was comparable in the three major diseases, 2.5, 2.2 and 2.3 for PCP, TB and bacterial infections respectively. In all the three disease, the commonest bacterium isolated was *S. maltophilia*. The pattern of bacterial isolates did not defer between the three diseases.

Table XIII: Prevalence of P. jiroveci according to different diagnostic methods and specimen

	Prevalence of P.jirovecii		***P-Value**
Diagnostic methods	*Sputum (N= 77)* *No (%)*	*# BAL (N=120)* *No (%)*	
TBO	10/78 (12.8%)	17/117 (14.5 %)	P= 0.73
IF	14/78 (17.9%)	25/117 (21.4 %)	P= 0.56
PCR	29/76 (38.2%)	42/120 (35%)	P= 0.65
**P-value; PCR vs TBO	0.00035	0.00026	
P-value; PCR vs IF	0.005	0.02	
P-value; If vs TBO	0.375	0.17	

the denominator for TBO and IF in BAL samples is 117 because 3 bronchoscopies were done inadvertently when the sputum was already positive.
* Comparison of sputum versus BAL by the same diagnostic techniques. ** Comparison of the various diagnostic techniques in sputum and BAL.

4.8 Diagnosis of Tuberculosis (Paper IV)

A total of the 26 (21.6%) BAL specimen grew *M. tuberculosis.* Additional one specimen grew MTB from sputum culture, hence making the total MTB positive culture 27. Thirteen (15.3%) of the 85 sputa samples negative for AFB by direct microscopy grew mycobacterium tuberculosis on LJ medium. All, except one, samples positive in sputum were also positive in BAL fluid. Nine (34.6%) out of the 26 culture–positive BAL samples were positive for AFB by direct microscopy.

5. Discussion

In both cohort I and cohort II , we have tried to minimize selection bias by recruiting consecutive patients. Most previous studies from sub-Saharan Africa have focused on the etiology of respiratory diseases in smear-negative, HIV-infected patients and have included mainly inpatients or autopsy material. In the 2nd cohort of patients, we have excluded those with typical CXR findings of bacterial pneumonias and pulmonary tuberculosis so that the study addresses the diagnostic challenges in evaluating patients who present with atypical chest-x-ray and sputum smear negative for AFB.

5.1. HIV prevalence and Clinical characteristics

The finding of an HIV sero-prevalence of 57.1% in culture-verified TB cases clearly demonstrates the rapid rise in the sero-prevalence of HIV in the population in general and PTB patients in particular. In 1988, the HIV prevalence in 106 TB patents in Addis Ababa was 6.6% (210). In the subsequent years, prevalence of 22% was reported amongst 90 patients with pleural tuberculosis (211) and 45.3% amongst smear-positive PTB patients recruited from all health centers in Addis Ababa (212). The relatively higher prevalence in our study while reflecting the rising HIV sero-prevalence in our population can also be due to inclusion of only culture-verified TB cases. In studies which have used smear positivity as criteria for the diagnosis of PTB, a lower HIV rate has been detected due to the well-known

association between smear-negative PTB and HIV. In our own study, the HIV prevalence in the direct smear-positive PTB patients was 40.7%. Independent clinical predictors of PTB were fever and HIV sero-status, while marked weight loss was a marker for HIV infection.

In cohort II, however, we did not find any discriminator, including fever, for PTB compared to bacterial infections and PCP. Hemoptysis occurred significantly more in patients with PTB but did not stand out as a predictor of PTB in a multivariate regression analysis. The limited discriminatory value of clinical symptoms demonstrate the non-specific nature of symptoms traditionally associated with PTB, (namely chronic cough, hemoptysis, night sweats, weight loss etc) in a setting with a high prevalence of HIV. In contrast, TB prediction models developed in low-HIV prevalence settings have found classical TB symptoms useful (213). In Malawi, a prediction model based on the presence or absence of specified symptoms predicted smear-negative PTB with 85% sensitivity but only 67% specificity (214). In a recent study in an outpatient setting in Addis Ababa, a clinical scoring system based on symptoms and CXR was developed as a potentially valuable tool for the diagnosis of PTB (215). No HIV data were given. In this study, however, like our study, the rate of false positivity was high. Unfortunately, most of these prediction models have not been evaluated for their performance. In cohort I, where patients were included on the basis of suspected PTB, the sensitivity of physicians diagnosis of TB based on symptoms, CXR and smear in HIV-positive patients was high (86.7%) even though significantly lower in the TB-HIV co-infected group. This is because of the reduced diagnostic impact of AFB microscopy and CXR in HIV infected patients. However, in cohort II, in patients with atypical pneumonia, the sensitivity of physician diagnosis of PTB was only 26.8%. This is probably due to the fact that all patients were smear-negative and patients with typical chest x-ray for PTB have been excluded from the study, hence making clinical diagnosis difficult. Once again, this depicts the difficulty in diagnosing PTB in smear-negative HIV–infected patients. The implication of this is that there will be a delay in initialing anti-TB treatment and this will inevitably be followed by increased morbidity and mortality.

We have also demonstrated a significantly higher rate of smear negativity in HIV-positive patients (38.5%) compared to HIV–negative patients (75%). Comparable results have been shown from studies in several sSA countries (216,217). Other studies have shown reduced smear positivity but the majority of HIV-positive patients, were still smear-positive (218).

5.2. Differential diagnosis of smear-negative TB

One of the most remarkable and unexpected finding in cohort I of this study was the high HIV sero-prevalence (38.5%) in patients with symptoms suggestive of PTB but that were negative by smear-microscopy and culture (Paper I). The sensitivity of mycobacterial sputum culture for the diagnosis of PTB in HIV–positive patients is in the range of 85-100% (219). It is, therefore, unlikely, that we may have missed culture-negative TB cases. The HIV prevalence still remained high after excluding possible undiagnosed TB patients in this group, namely culture-negative patients with cavitary disease or patients with isolated pleural effusion (39.3% and 37.2%). The possible explanation for this phenomenon is the presence of other opportunistic infections mimicking tuberculosis. Based on this finding and some the CXRs which suggested PCP, we retrospectively studied for

the occurrence of *P. jiroveci* in this group of patients. The results of this study showed that 1/3 of the patients were positive for *P.jiroveci* by nested PCR while 10% and 13% were positive by single PCR and IF respectively. Indeed, the clinical significance of nested PCR can not be determined with certainty as it may detect asymptomatic carriage and sub-clinical colonization (220,221). However, the recovery of PJ by n-PCR from expectorated sputum, which has a lower diagnostic yield than induced sputum or BAL, by itself, attaches importance to the finding. The sensitivity for diagnosing PJ in induced sputum (IS) by IF in various studies ranges from 43-67% (222). Using single and/or nested PCR, an increased analytical sensitivity can be obtained (220,223). Besides, in HIV-infected patients with progressively diminishing immune function, nested PCR positive samples may indicate clinical PCP or a risk for a later manifest PCP (224, 225) Hence, a finding of nested PCR positivity of 30% is significant from epidemiological point of view.

In the HIV-positive, non-TB patients, more than 1/3 of the CXRs were interpreted as PTB in the IF-positive and nested PCR-positive patients. These patients will be interpreted as having smear-negative TB and run the risk of drug side effects and increased mortality. Such an overlap and atypical presentation on CXR between PTB, PCP and bacterial pneumonia have been reported in the past too (226, 227). Unfortunately, only one HIV-positive non-TB patient was clinically diagnosed to have PCP confirming the lack of awareness among physicians of the existence of PCP in Ethiopia. This high rate of PCR-positive PCP has prompted as to systematically examine the prevalence of PCP and other pulmonary opportunistic diseases in HIV-positive patients who present with atypical CXR and sputum smear negative for AFB.

In cohort II, we clearly demonstrated that PCP is a frequent and important differential diagnosis to tuberculosis and bacterial infections. The prevalence of PCP was comparable to recent reports from Uganda and Kenya which were 38.6% and 37.2% respectively (136,138).The sensitivity of physician's diagnosis was 58.5%, much better than the finding in the previous cohort, suggesting an improvement in the awareness of physicians for the diagnosis of PCP. Since all the patients were smear negative for AFB, inevitably most of the patients in the remaining 42.5% will be started on anti TB and run the risk of drug side-effects that will further complicate the management of the patients. Conversely the majority of patients with pulmonary tuberculosis (57.7%) were initially diagnosed to have PCP, therefore creating a delay in diagnosis. Such a delay has been associated with excess mortality in HIV-infected persons with smear-negative tuberculosis (2287). PCP has been considered a rare disease in sub-Saharan Africa including Ethiopia at the wake of the HIV epidemic. In our previous study, we demonstrated a prevalence of 11% by IF in smear and culture negative TB suspects. As of recent, however, there is an increasing report of *P.jiroveci* infection, particularly in the pediatric age group. It will be difficult to explain why such a rise occurred but certainly lack of awareness and technical difficulties in inducing sputum, doing bronchoscopy and staining for PJ has been a major limitation for the diagnosis of the disease in the past. What was observed as a major variation in the occurrence of PCP in the various sub-regions of Africa is also closing the gap. Whether this lessened geographic variation and uniform reporting is due to increased awareness and improved diagnostic capacity or the organism is emerging as a new and virulent pathogen in Sub-Saharan Africa is not clear. It has been argued that one of the reasons, amongst many, for the observed geographic variation could be that

P.jiroveci might be in the process of penetrating the African continent and these differences simply represent the temporal sequence of this spread.

Bacterial infections were the number one cause of opportunistic chest infections amongst smear-negative patients with atypical chest x-ray. Culture from bronchoalveolar lavage was not useful to make an etiologic diagnosis. Sputum culture will be even worse. The mean duration of illness was 3 months and comparable to that of tuberculosis and PCP such that it will be difficult to distinguish one from the other. Most of the HIV-associated pneumonias described in the past have been acute onset in nature and there have not been reports of chronic bacterial infections. In that respect, we have demonstrated bacterial infections to be one of the competing diagnoses in HIV-associated chronic chest infections. In a multivariate regression analysis, the presence of purulent sputum predicted bacterial infections; therefore, it is reasonable to consider the diagnosis in patients who expectorate purulent sputum.

Tuberculosis remains to be an important differential diagnosis even when the smear is negative and the CXR atypical. Sputum concentration using household bleach on expectorated sputum could diagnose 38.5% of the sputum culture-positive cases. This is encouraging news particularly for countries such as Ethiopia where there is very little bronchoscopoy service. However, sputum concentration diagnosed only 18.5 % of all the TB cases, including BAL culture, therefore, there is a need to improve the diagnosis of PTB in the absence of culture. One possibility is to evaluate the yield of induced sputum by both direct AFS and concentration technique. Recent techniques in sputum culture using liquid media, which usually have a faster recovery of MTB, need to be encouraged in developing countries provided their cost is not prohibitive.

5.3. Diagnosis of PCP

TBO detected 70% of what could be diagnosed by IF and 35% of what could be diagnosed by PCR. Using IF as the gold standard for the diagnosis of PCP, the sensitivity of TBO for the diagnosis of PCP is comparable to that of sputum AFB for PTB. In a way, both sputum AFB by the Ziehl Neelsen (ZN) stain and sputum for PCP by TBO have identical yield. However, ZN stain is much simpler to perform in expectorated sputum sample than TBO, which requires more skill and experience to distinguish between PJ cysts and fungal spores.

Clinical algorithm using a combination of symptoms and CXR didn't seem to be useful for the diagnosis of PCP. None of the symptoms were clear and independent predictors of PCP. Overall, the sensitivity of the CXR for the diagnosis of PCP was good but had a very low specificity. 12 of the 26(46.2%)of patients with PTB and 24 of 43(55.8%) of patients with bacterial infection were misdiagnosed as cases of PCP. The same holds true using physician's diagnosis. This means a lot of patients who had PTB and bacterial infections would have, unnecessarily, been put on PCP treatment. On the other hand, empiric approach will create a delay in diagnosis of PTB from expectorated sputum using the concentration technique, BAL AFB or culture.

Of the staining techniques, TBO is probably is simpler and less time consuming than Grocott stain which has a comparable sensitivity. Giemsa would have been even more rapid than TBO but since it stains trophozites, it is less sensitive and specific.

Diagnostic test for PCP using BAL or induced sputum is rarely done in sub-Saharan Africa. Besides, widespread use of immunofluorescence or PCR is just not affordable. Under such circumstances, expectorated sputum sample and TBO stain are the most practical biologic specimen and diagnostic technique. Our study has demonstrated that a significant number of patients can be diagnosed by this simple and practical technique. Even if it is not the most ideal test, it can still be useful. We also recognize that TBO staining requires skill and experience, both of which can be achieved through time and dedication.

6. Conclusions

- We found a high prevalence of HIV infection not only in patients with culture-verified PTB but also in patients with clinical symptoms suggestive of PTB where the TB diagnosis could not be confirmed In HIV-positive patients, the sensitivity of sputum smear for the diagnosis of PTB was significantly lower compared to HIV-negative patients.
- Despite the fact that culture–verified PTB is more often under diagnosed in HIV-positive patients by sputum smear, other HIV-related pulmonary infections are also wrongly interpreted as smear-negative TB. The high prevalence of HIV in the culture-negative TB suspects justifies the routine use of HIV screening as part of the diagnostic work-up in this group of patients. It also calls for an increased awareness and improved diagnostic tools and algorithms for the diagnosis of HIV-related pulmonary infections including PTB.
- Atypical presentation of chest x-ray was found to be common in HIV-associated PTB and the disease burden assessed by chest radiography was proportional to the bacillus load. The high prevalence of normal appearing chest x-ray particularly in HIV-infected patients is worrying and has an implication for the diagnosis of smear-negative TB in high HIV-prevalent settings where the chest x-ray is an integral part of the diagnostic work up.
- Pneumocystis pneumonia is highly prevalent in HIV-positive patients presenting with atypical chest x-ray and negative sputum smear for AFB in Ethiopia. Important differential diagnoses under such circumstances include bacterial infections and PTB. The sensitivity of physician's and chest x-ray diagnosis for bacterial infections and tuberculosis was dismally low and many were misdiagnosed as cases of PCP. This calls for an improved and inexpensive

method of making a distinction between PCP and other opportunistic infections in low income countries.

- Toluidine blue O stain detected 70% of the IF positive PCP cases in both expectorated sputum and BAL samples. The performance of TBO in expectorated sputum is very encouraging as bronchoscopy and IF microscopy including PCR is neither available nor affordable in countries such as Ethiopia. We, therefore, recommend the application of this method for a wider clinical use in resource-constrained settings.

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IV. REFERENCES

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