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Transmission dynamics and tuberculosis control among HIV/AIDS patients

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Résumé

Introduction: Les efforts globaux pour contrôler la tuberculose sont présentement restreints par la prévalence croissante du VIH/SIDA. Quoique les éclosions de la tuberculose multi résistante (TB-MDR) soient fréquemment rapportées parmi les populations atteintes du SIDA, le lien entre VIH/SIDA et le développement de résistance n'est pas clair.

Objectifs: Cette recherche visait à : (1) développer une base de connaissances concernant les facteurs associés à des éclosions de la TB-MDR parmi les patients atteints du VIH/SIDA; (2) utiliser ce cadre de connaissances pour accroître des mesures préliminaires pour mieux contrôler la tuberculose pulmonaire chez les patients atteints du VIH/SIDA; et (3) afin d'améliorer l'application des ces mesures, affiner les techniques bactériologiques existantes pour *Mycobacterium tuberculosis*.

Méthodologie: Quatre études ont été réalisées : (1) Une *étude longitudinale* pour identifier les facteurs associés avec une éclosion de la TB-MDR parmi les patients atteints du SIDA qui ont reçu le traitement directement supervisé de courte durée (DOTS) pour la tuberculose pulmonaire au Lima et au Pérou entre 1999 et 2005; (2*)* Une *étude transversale* pour décrire différentes étapes de l'histoire naturelle de la tuberculose, la prévalence et les facteurs associés avec la *mycobactérie* qu'on retrouve dans les selles des patients atteints du SIDA; (3) Un *projet pilote* pour développer des stratégies de dépistage pour la tuberculose pulmonaire parmi les patients hospitalisés atteints du SIDA, en utilisant l'essaie Microscopic Observation Drug Susceptibility (MODS); et (4) Une *étude laboratoire* pour identifier les meilleures concentrations critiques pour détecter les souches MDR de *M. tuberculosis* en utilisant l'essaie MODS.

Résultats : Étude 1 démontre qu'une épidémie de TB-MDR parmi les patients atteints du SIDA qui ont reçu DOTS pour la tuberculose pulmonaire ait été causée par la superinfection du clone de *M. tuberculosis* plutôt que le développement de la résistance secondaire. Bien

que ce clone ait été plus commun parmi la cohorte de patients atteints du SIDA, il n'avait aucune différence de risque pour superinfection entre les patients avec ou sans SIDA. Ces résultats suggèrent qu'un autre facteur, possiblement associé à la diarrhée, peu contribuer à la prévalence élevée de ce clone chez les patients atteints du SIDA. Étude 2 suggère que chez la plupart des patients atteints du SIDA il a été retrouvé une mycobactérie dans leurs selles alors qu'ils étaient en phase terminale au niveau de la tuberculose pulmonaire. Or, les patients atteints du SIDA ayant été hospitalisés pendant les deux dernières années pour une autre condition médicale sont moins à risque de se retrouver avec une mycobactérie dans leurs selles. Étude 3 confirme que la tuberculose pulmonaire a été commune à tous les patients hospitalisés atteints du SIDA, mais diagnostiquée incorrectement en utilisant les critères cliniques présentement recommandés pour la tuberculose. Or, l'essaie MODS a détecté pour la plupart de ces cas. De plus, MODS a été également efficace quand la méthode a été dirigée aux patients soupçonnés d'avoir la tuberculose, à cause de leurs symptômes. Étude 4 démontre les difficultés de détecter les souches de *M. tuberculosis* avec une faible résistance contre ethambutol et streptomycine en utilisant l'essai MODS avec les concentrations de drogue présentement recommandées pour un milieu de culture. Cependant, l'utilité diagnostique de MODS peut être améliorée ; modifier les concentrations critiques et utiliser deux plaques et non une, pour des tests réguliers.

Conclusion: Nos études soulèvent la nécessité d'améliorer le diagnostic et le traitement de la tuberculose parmi les patients atteints du SIDA, en particulier ceux qui vivent dans des régions avec moins de ressources. Par ailleurs, nos résultats font ressortir les effets indirects que les soins de santé ont sur les patients infectés par le VIH et qu'ils peuvent avoir sur le développement de la tuberculose.

Mots clés: La tuberculose pulmonaire ; VIH/SIDA ; la tuberculose multi résistante ; l'essaie Microscopic Observation Drug-Susceptibility

Summary

Background: Global efforts to control tuberculosis are currently being hampered by a continuing rise in the prevalence of HIV/AIDS. Although outbreaks of multidrug resistant tuberculosis (MDR-TB) are commonly reported among AIDS populations, the link between HIV/AIDS and the development of drug-resistance remains unclear.

Objectives: This thesis aimed to: (1) build a knowledge foundation regarding underlying factors associated with outbreaks of MDR-TB among HIV/AIDS patients; (2) use this knowledge framework to develop preliminary health measures for controlling pulmonary tuberculosis among HIV/AIDS patients; and (3) in an effort to better implement these health measures, refine existing culture-based diagnostics for *Mycobacterium tuberculosis*.

Methods: Four studies were conducted: (1) a *longitudinal study* to identify the underlying factors associated with an epidemic of MDR-TB among AIDS patients receiving Directly-Observed Therapy Short-course (DOTS) for pulmonary tuberculosis in Lima, Peru between 1999 and 2005; (2) a *cross-sectional study* to characterize the prevalence and factors associated with gastrointestinal shedding with *mycobacteria* among AIDS patients at different stages in the natural history of tuberculosis; (3) a *pilot study* to develop screening strategies for pulmonary tuberculosis among hospitalized HIV/AIDS patients using the Microscopic Observation Drug Susceptibility (MODS) assay; and (4) a *laboratory-based study* to define the optimal critical concentrations needed for detecting drug resistance in *M. tuberculosis* using MODS.

Results: Study 1 revealed that an epidemic of MDR-TB among AIDS patients receiving DOTS for pulmonary tuberculosis was due to super-infection with a specific clone of *M. tuberculosis* rather than the development of secondary drug-resistance. Although this epidemic clone was more common among patients in the AIDS cohort, risk of super-infection did not differ between AIDS and non-AIDS patients after adjusting for baseline risk

of exposure, suggesting that another factor possibly associated with diarrhea may be contributing to the strain's high prevalence among AIDS patients. Study 2 showed that the majority of AIDS patients in the later stages of pulmonary tuberculosis exhibited gastrointestinal shedding with *mycobacteria*. Stool shedding was rare in the absence of pulmonary tuberculosis. AIDS patients were also less likely to shed *mycobacteria* if they had been hospitalized during the previous two years for another medical condition. Study 3 confirmed that pulmonary tuberculosis was common among hospitalized AIDS patients but frequently misdiagnosed using currently recommended diagnostic algorithms. The MODS assay detected most cases and was equally effective when targeted to patients clinically suspicious for tuberculosis. Study 4 demonstrated that low grade drug resistance in *M. tuberculosis* to ethambutol and streptomycin was difficult to detect with MODS using currently recommended drug-concentration standards in broth. Its diagnostic utility could be improved by modifying drug-concentration standards, and including two versus one critical concentration well for standardized testing.

Conclusion: Our studies underscore the need to improve the diagnosis and treatment of tuberculosis among AIDS patients living in resource-constrained settings, all in an effort to prevent morbidity, mortality and the transmission of drug-resistant strains. They also highlight the indirect effect that general health care among HIV-infected patients can have on the development of tuberculosis.

Keywords: Pulmonary tuberculosis; HIV/AIDS; Multi-drug resistance; Gastrointestinal shedding; Microscopic Observation Drug Susceptibility assay

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List of Abbreviations

AFB	Acid-Fast Bacilli Smear Stain
AIDS	Acquired Immunodeficiency Syndrome
BCG	Bacille Calmette Guérin vaccine
BMI	Body Mass Index
CI	Confidence Interval
DNA	Deoxyribonucleic Acid
DOTS	Directly Observed Treatment, Short-Course
DR-TB	Drug-Resistant Tuberculosis
DS-TB	Drug-Susceptible Tuberculosis
ELISA	Enzyme-linked Immunosorbent Assay
HAART	Highly Active Antiretroviral Therapy
HEPA	High Efficiency Particulate Air
HIV	Human Immunodeficiency Virus
IQR	Interquartile Range
LJ	Löwenstein-Jensen
MABA	Microplate Alamar Blue Assay™
MDR-TB	Multi-Drug Resistant Tuberculosis
MGIT	*Mycobacteria* Growth Indicator Tube
MIC	Minimum Inhibitory Concentration
MIC_{50}	Minimum Inhibitory Concentration for 50% of Samples
MIC_{90}	Minimum Inhibitory Concentration for 90% of Samples
MODS	Microscopic Observation Drug Susceptibility Assay
OADC	Oleic Acid-Albumin-Dextrose-Catalase
OR	Odds Ratio
PCR	Polymerase Chain Reaction
PPV	Positive Predictive Value
PTB	Pulmonary Tuberculosis

RFLP	Restriction Fragment Length Polymorphism
ROC	Receiver Operating Curve
SIR	Standardized Incidence Ratio
TB	Tuberculosis
TEMA	Tetrozolium Microplate Assay
TST	Tuberculin Skin Testing
UNAIDS	Joint United Nations Programme on HIV/AIDS
WHO	World Health Organization
XDR-TB	Extensively drug-resistant tuberculosis
7h9GC	Middlebrook 7h9 Broth with Glycerol and Bacto Casitone

Dedication

In loving memory of:

my grandmother

G.A.P.

whose commitment to helping others (IRC) continues to inspire.

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Preface

The first three studies in this thesis were based on existing larger cohort studies conducted in Lima, Peru between 1999 and 2005 and aimed at:

- Evaluating the validity of different diagnostic methods for *Mycobacterium tuberculosis.*
- Studying the longitudinal effects of HIV and multidrug resistance on time to culture conversion and mortality during Directly Observed Treatment, Short-course for tuberculosis.

In this regard, the first three studies of this thesis represent original attempts to: (1) identify the underlying factors associated with an epidemic of multidrug-resistant tuberculosis (MDR-TB) among AIDS patients in Lima, Peru using molecular data; (2) characterize the prevalence and risk factors for *mycobacteria* stool shedding among AIDS patients at different stages in the natural history of tuberculosis; and (3) evaluate the utility of targeted versus blanket screening of HIV-infected patients with the Microscopic Observation Drug Susceptibility (MODS) assay. The strength of the first two studies lies not only in the topic selected but in the way we analyzed the data, using both cohort and individual-level differences in order to study these phenomena. For the third study in this thesis, our research represented the first to take into consideration the clinical context of diagnosis when evaluating the utility of MODS.

While the datasets used for all three studies were not originally designed to answer any of these research questions, careful examination of the study protocols and data collection sheets revealed that they could be used to answer the research questions proposed for this thesis. Following this decision, datasets were cleaned, missing data was retrieved and all RFLP gels and spoligotype results were re-analyzed It should be noted that new data collection for the first study would not have possible today, given that the epidemic of MDR-TB has now subsided.

The final study in this thesis represents completely original work. During my initial research on the Microscopic Observation Drug Susceptibility assay (prior to the initiation of

the PhD), it became apparent that critical concentrations for this test had never been evaluated for multiple drugs across a range of concentrations. This research gap propelled me to begin designing and conducting experiments in this area. Part of this research (i.e., for two of the drugs) is presented in Chapter 7 of this thesis.

CHAPTER 1.

Introduction

Tuberculosis remains a major cause of morbidity and mortality [1-3]. In 2006, the World Health Organization (WHO) identified 9.2 million new cases and 1.7 million deaths from tuberculosis [1]. Although public health efforts to control tuberculosis since the mid 1990's, have helped to stem the global incidence of this disease, in more recent years, there has been growing concerns regarding a steady rise in the incidence of tuberculosis in regions with a high prevalence of HIV/AIDS [1]. The link between tuberculosis and HIV/AIDS has been particularly evident in Africa, which not only experiences the highest rates of tuberculosis in the world (363 cases per 100 000 in Africa versus 139 cases per 100 000 in the world) but also accounts for 85 percent of the world's cases of AIDS-associated tuberculosis [1,4].

Tuberculosis remains the single most common cause of morbidity and mortality from opportunistic infections among AIDS patients [5]. Of 33 million people who are currently infected with HIV/AIDS and 2 million people who die from AIDS each year, 700 000 cases and 200 000 deaths are attributable to tuberculosis [5 1]. The WHO has also noted that in sub-regions of Africa where the prevalence of HIV infection is high, changes in the incidence of tuberculosis are directly proportional to fluctuations in the prevalence of HIV [1].

Part of this problem is due to HIV-infected patients being not only at greater risk of infection with tuberculosis, but also of progressing from latent to active stages of the disease, once infected with the tubercle bacilli [6]. Of the one-third of the world's population who are currently infected with *Mycobacterium tuberculosis* (the main causative agent of tuberculosis disease) [2], between five to ten percent of tuberculosis-infected individuals will develop active forms of the disease during their lifetime [4,6]. This number increases to 50 percent when patients are co-infected with HIV [4,6].

Further complicating worldwide efforts to control tuberculosis are significant geo-economic differences in disease burden. Aside from affecting the most economically productive age groups of society (75 percent of active tuberculosis cases occur among patients between 15 and 50 years of age [6]), 95 percent of new tuberculosis cases and 98

percent of deaths due to tuberculosis occur in developing countries where health resources to control the disease are often limited [7].

Amidst this health crisis, reports of ongoing outbreaks of multi drug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) among hospitalized AIDS patients and prison populations with a high prevalence of HIV have also led to concerns regarding a potential link between AIDS and the development of drug-resistant tuberculosis. Published studies on hospital outbreaks of MDR-TB frequently cite delays in the diagnosis and treatment of tuberculosis, an increased likelihood of defaulting from treatment among AIDS patients, and poor infection control measures as key contributing factors to these public health events [8,9]. However, epidemiological studies have rarely identified a link between MDR-TB and AIDS during non-outbreak periods of time, making it unclear whether an association actually exists.

In this context, the purpose of this thesis was to:

(1) Build a knowledge foundation regarding underlying factors associated with an outbreak of MDR-TB among HIV/AIDS patients;

(2) Use this knowledge framework to develop preliminary health measures for controlling pulmonary tuberculosis among HIV/AIDS patients; and

(3) Refine existing culture-based diagnostics for *Mycobacterium tuberculosis,* in an effort to better implement these health measures.

CHAPTER 2.

Review of the Literature

2.1. Biomedical Framework for Tuberculosis Control

Most public health efforts to contain tuberculosis center on interrupting person-to-person transmission of *M. tuberculosis*. While the tubercle bacillus was first identified by Robert Koch in 1882, its mode of transmission was not formally established until the 1960's, when it was shown that *M. tuberculosis* could be transmitted through aerosolized droplet nuclei [10]. Since then, much of the scientific literature on transmission dynamics has focused on elucidating the biophysical processes which enable dissemination of tuberculosis to occur [11].

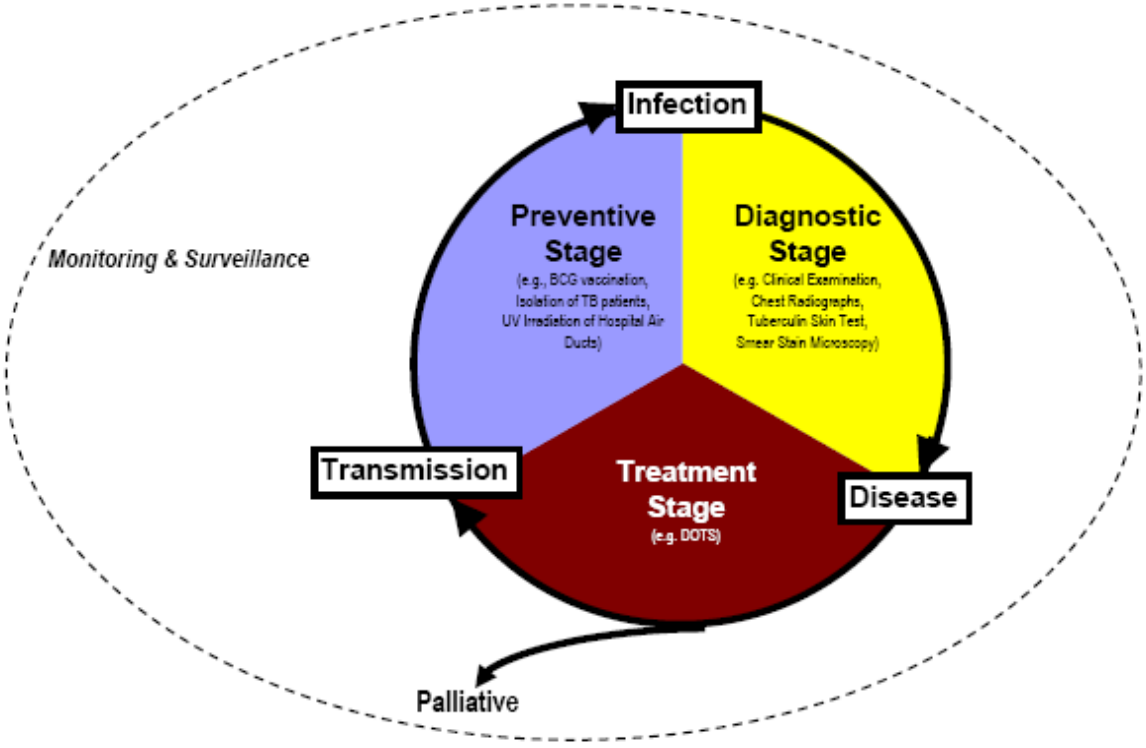
Person-to-person transmission of *M. tuberculosis* takes place when the tubercle bacilli are spread through respiratory droplets from a contagious person to a susceptible host. At the patient-level, the probability of transmission will depend on the manner in which the infector releases *M. tuberculosis* (e.g., talking, coughing or sneezing), and the extent to which an infector is ill at the time of transmission (e.g., cavitation in the lung or positive smear status). Once *M. tuberculosis* is released into the air, the probability that the microorganism will survive depends not only the innate viability of the bacteria to survive but also on environmental stressors which the bacteria is exposed to (e.g., ambient temperature, presence of ultraviolet rays or level of humidity) and size of aerosolized droplets. If the tubercle bacilli are able to survive this transmission process, the probability that a person will be infected will then depend on their level of immunological resistance towards the microorganism (e.g., genetic factors or HIV/AIDS). A person who becomes infected may then enter one of two stages of tuberculosis disease: a dormant asymptomatic phase or an active symptomatic phase. When patients with active pulmonary tuberculosis release *M. tuberculosis* into the environment, the entire tuberculosis transmission cycle is reinitiated.

At the community-level, the risk of transmitting *M. tuberculosis* will depend on the number of cases capable of transmitting the *mycobacterium*, the duration of infectiousness for each case, as well as the number and/or duration of encounters between the source of

infection and susceptible individuals [12]. All of these factors are influenced by how rapidly case detection and treatment of an infected individual occurs.

The classic biomedical model for controlling communicable diseases focuses on interrupting person-to-person transmission through one of three stages of the disease (figure 2.1), namely the diagnostic, treatment, and preventive phases. It is within this disease-centered framework that the Directly Observed Treatment, Short-Course (DOTS) strategy for tuberculosis control was developed [13]. The program is based on five pillars. They include: [1] Political commitment to combating tuberculosis, [2] Case detection using quality-assured bacteriology, [3] Standardized treatment for tuberculosis during at least five months with supervision and patient support, [4] Regular supply of anti-tubercular drugs, and [5] Standardized monitoring and surveillance of treatment results [14].

Figure 2.1. Biomedical framework for tuberculosis control



2.1.1. Diagnostic Stage

The first critical element in transmission control efforts has been the proper diagnosis of tuberculosis [15]. The International Standards for Tuberculosis Care currently recommend that any patient with otherwise unexplained productive cough for more than two to three weeks; or, chest radiographic findings suggestive of tuberculosis, be asked to provide sputum samples for bacteriological confirmation of pulmonary tuberculosis [16]. Appropriate specimens should also be collected from patients with clinical manifestations suggestive of extra pulmonary tuberculosis [16].

Although, numerous laboratory tests have been developed to rapidly and accurately detect *M. tuberculosis* in clinical specimens (please refer to Appendix 1), cost and the need for complex laboratory facilities often limits their use in resource-constrained settings [17]. Instead, most laboratory-based diagnosis are made using the Acid Fast Bacilli (AFB) Smear Stain, which detects 50 to 60 percent of tuberculosis cases in countries with access to high quality microscopy services [18,19]. Patients with culture-positive tuberculosis who test negative using AFB smear stain are commonly referred to as smear-negative culture-positive cases, and remain a key limitation for current tuberculosis control efforts. Immunosuppressed individuals with pulmonary tuberculosis may be at particular risk for smear-negative culture-positive disease since they are less likely to undergo lung cavitation, which increases the bacillary load in sputum. AFB smear stain also cannot differentiate between different *mycobacterium* species or determine whether the tubercle bacilli are still viable.

While Löwenstein-Jensen culture remains a widely available culture-based method of detection in low and middle-income countries, the six to eight week delay needed to process samples substantially limits its clinical practicality [20,21]. Clinical presentation of symptoms, radiological findings and other laboratory-based indicators may also be used to identify smear-negative patients [21-25]. However, co-infection with HIV can make this diagnostic process more complex, particularly if patients are immunosuppressed. For example, while constitutional symptoms such as weight loss and fever, can be useful markers for tuberculosis among HIV-infected patients, a differential diagnosis must also be made between pulmonary tuberculosis and other opportunistic pulmonary diseases such as acute bacterial pneumonia,

Kaposi sarcoma or *Pneumocystis carinii* pneumonia [6]. Chest radiographic patterns may be atypical depending on the extent of patient immunosuppression [6]. Tuberculin skin tests may also be of limited value in these settings since the test cannot differentiate between the infectious and disease forms of tuberculosis or the presence of *M. tuberculosis* versus other environmental *mycobacterium*. False-negative results are common among patients who are immunosuppressed, suffer severe malnutrition or have miliary tuberculosis [26].

Similarly, the diagnosis of patients with MDR-TB [i.e., *M. tuberculosis* that is resistant to the two first-line anti-tubercular drugs isoniazid and rifampicin] and XDR-TB [i.e. MDR-TB that is also resistant to fluoroquinolone and at least one second-line injectable drug, namely amikacin, capreomycin or kanamycin [27]] is an essential component to treating tuberculosis effectively. The only means of identifying a patient with drug-resistant tuberculosis is through either treatment failure or drug-susceptibility testing.

While the proportion method by agar is often considered the reference standard of drug-susceptibility testing, it can take as much as 12 weeks in order to provide results, thus increasing morbidity and mortality among patients [28]. Although molecular techniques such as line probe assays can be used to detect drug-resistance in samples in 1 to 2 days, cost and the need for complex laboratory facilities often limits their utility in the developing world [28]. The diagnostic also has a lower sensitivity for detecting smear-negative samples, and transferability of technology for other pathogens can be difficult since it only detects a limited number of mutations [28,29].

On a similar note, while numerous rapid and reliable drug-susceptibility tests exist for MDR forms of TB, drug-susceptibility testing for XDR-TB cases continues to be poor, hence the use of MDR-TB as a first criteria to define all XDR-TB cases [28]. This brings us to the second dimension of tuberculosis control: treatment.

2.1.2. Treatment Stage

Since the discovery of streptomycin for treating pulmonary tuberculosis in 1948, a second element for controlling tuberculosis has been the use of antibiotics [30]. If active tuberculosis is left untreated in otherwise healthy individuals, approximately 50 percent of patients will die,

25 percent will develop the chronic form of the disease and 25 percent will become self-cured [6]. While the number of anti-tubercular drugs and length of time needed to treat tuberculosis creates significant challenges for treatment compliance (Table 2.1), the DOTS program has significantly improved cure rates for the disease through patient support and daily monitoring of drug intake [31,32]. Within a well-functioning DOTS program, cure rates for drug-susceptible tuberculosis now exceed 85 percent [1].

Table 2.1. Treatment regimens used for Directly Observed Therapy Short-Course of tuberculosis in Peru

DOTS Treatment Scheme Two
(Total of 6 months of treatment – 82 doses)

Phase	Duration	Frequency	Medication & Dose	Total Medication
First	2 months (50 doses)	Daily, except Sunday and holidays	Rifampicin x 300 mg (2 capsules) Isoniazid x 100 mg (3 tablets) Pyrazinamide x 500 mg (3 tablets) Ethambutol x 400 mg (3 tablets)	Rifampicin x 300 mg = 164 capsules Isoniazid x 100 mg = 406 tablets Pyrazinamide x 500 mg = 150 tablets Ethambutol x 400 mg = 150 tablets
Second	4 months (32 doses)	Two doses per week	Rifampicin x 300 mg (2 capsules) Isoniazid x 100 mg (8 tablets)	

DOTS Treatment Scheme Two
*(*Total of 8 months of treatment – 115 doses)

Phase	Duration	Frequency	Medication & Dose	Total Medication
First	2 months (50 doses)	Daily except Sundays & holidays	Rifampicin x 300 mg (2 capsules) Isoniazid x 100 mg (3 tablets) Pyrazinamide x 500 mg (3 tablets) Ethambutol x 400 mg (3 tablets) Streptomycin x 1 g (1 ampoule)	Rifampicin x 300 mg = 230 capsules Isoniazid x 100 mg = 545 tablets Pyrazinamide x 500 mg = 225 tablets Ethambutol x 400 mg = 465 tablets Streptomycin x 1 g = 50 ampoules
	1 month (25 doses)	Daily except Sundays & holidays	Rifampicin x 300 mg (2 capsules) Isoniazid x 100 mg (3 tablets) Pyrazinamide x 500 mg (3 tablets) Ethambutol x 400 mg (3 tablets)	
Second	5 months (40 doses)	Twice per week	Rifampicin x 300 mg (2 capsules) Isoniazid x 100 mg (8 tablets) Ethambutol x 400 mg (6 tablets)	

Source: Mazzetti PS. Norma técnica de salud para el control de la tuberculosis. Ministerio de Salud del Perú. Lima: 2006.

Notwithstanding these advances, there have been growing concerns in recent years regarding the emergence of MDR-TB and XDR-TB, particularly among populations with a high prevalence of HIV [4,33]. It is estimated that five percent of tuberculosis cases in the world are multi-drug resistant, from which seven percent of these cases are XDR forms of the disease [34]. Country rates for MDR-TB range from zero percent in several Western European countries to more than 35 percent in some former Soviet Union countries. Forty-five countries have reported at least one XDR-TB case since 2002 [34].

Rapid bacteriological confirmation of drug-susceptibility status and administration of appropriate drug-therapy remain the most important determinants for treatment success of patients with drug-resistant tuberculosis. Heymann et al. [35] underscore the importance of rapid drug-susceptibility testing by predicting that effective use of testing can reduce time to diagnosis by 38 percent, time to acceptable therapy by 70 percent and mortality by 31 percent. Among HIV positive individuals, drug-susceptibility testing is equally effective, reducing the time to diagnosis by 39 percent, time to acceptable therapy by 59 percent and mortality by 24 percent.

Despite this evidence, it is estimated that only two percent of the world's MDR-TB cases are currently detected and appropriately treated [36]. In many resource-constrained settings, selection of drug-therapy is based on the patient's history of tuberculosis rather than bacteriological confirmation of drug-susceptibility status due to cost and complex laboratory facilities needed in order to bacteriologically confirm drug-resistance. Until recently, the WHO had only advocated testing in resource-constrained settings for patients who failed at least five months of initial treatment or a supervised re-treatment regimen [35]. Selection of a standardized drug-therapy regimen is thus often based on a patient's treatment history for TB. In Peru, for example, Table 2.1 illustrates that DOTS scheme 1 is administered to "never treated" patients; and DOTS scheme 2 to "previously treated" patients who interrupted or failed treatment [37]. Such strategies can not only lead to inappropriate treatment for patients infected with drug-resistant tuberculosis for the first time, but also prolong infectivity and the further development of drug-resistant tuberculosis.

The treatment of tuberculosis among HIV/AIDS patients may be further complicated by their increased risk for gastrointestinal malabsorption of anti-tubercular drugs [38-43]. Lower bioavailability of antibiotics in the bloodstream can increase, in turn, a patient's risk of developing drug-resistance [44]. Although the link between HIV-infection and multi-drug resistance remains unclear, several observational studies and randomized clinical trials have detected a link between HIV status and the development of resistance to rifamycins (the family group of rifampicin) [8,9,45]. Treatment for tuberculosis may also be complicated by HIV/AIDS patients' higher risk of adverse reactions from anti-tubercular drugs.

2.1.3. Preventive Stage

The third element in tuberculosis control efforts has been preventing infection with *M. tuberculosis* through vaccination, administration of prophylaxis, or isolation of infectious patients.

Although immunizations have long been considered a powerful tool in public health efforts to control communicable diseases, the Bacille-Calmette Guerin (BCG) vaccine remains the only means of immunizing against *M. tuberculosis* [46]. Derived from *Mycobacterium bovis*, BCG is most effective at preventing miliary or meningeal forms of tuberculosis in children [46]. In spite of these benefits, the vaccine has shown a wide range of efficacy, with BCG vaccines often being least effective in countries with the highest disease burdens from *M. tuberculosis* [47]. This variability is often attributed to genetic differences in vaccines and decreased immunity caused by co-infection with environmental *mycobacteria* and intestinal helminthes [47,48]. BCG vaccines are also not recommended for HIV-infected individuals [6].

A second method for preventing infection with tuberculosis has been providing prophylactic treatment to high-risk patients such as household contacts of patients with tuberculosis [49]. Isoniazid remains the most common form of prophylaxis, and exhibits bactericidal effects against *M. tuberculosis* [50]. Despite its efficacy, widespread implementation of isoniazid programs are often avoided due to concerns regarding the development of drug-resistant strains of *M. tuberculosis* [51]. Prophylaxis is usually not

provided with the same level of patient support or observation that is found in DOTS programs for tuberculosis. Intermittent use of an antibiotic ultimately decreases the level of protection provided by prophylaxis against tuberculosis infection [52,53]. If during the process, a patient does become develop tuberculosis disease then the prophylaxis would become mono drug-therapy, potentially causing amplification of drug-resistance. The potential for developing drug resistance may be particularly high for HIV/AIDS patients, due to malabsorption of drugs and a higher risk of undetected tuberculosis [38-43,54]. As previously mentioned, HIV/AIDS patients are less likely to exhibit chest cavitation, more likely to present false-negative tuberculin skin test results, and at increased risk of extra pulmonary forms of tuberculosis, depending on their level of immunosuppression.

The most commonly used method for preventing tuberculosis is by minimizing the potential for airborne transmission of tuberculosis [55]. This can be done in a number of ways, including administrative controls to isolate infectious patients; environmental controls such as modifying general ventilation or the use of air cleaning methods such as High Efficiency Particulate Air (HEPA) filters, or ultraviolet irradiation of ducts; and providing respiratory protection through the use of respirators or masks [55]. Screening health care workers for pulmonary tuberculosis through yearly chest x-rays can also be an important element in preventing transmission due to their increased risk of acquiring tuberculosis, and their potential to act as transmission vectors within a health care setting [56,57]. Extra pulmonary and non-infectious forms of childhood tuberculosis are usually not considered of *primary* importance in transmission control efforts due to the low probability of infectious particles being aerosolized [58].

2.2. MODS: A Potential Diagnostic Solution?

The identification of a rapid yet cost-efficient method for detecting tuberculosis and its drug susceptibility status remains a key limitation for current global efforts to control tuberculosis [59]. Although molecular techniques such as line probe assays can be used to detect drug-resistance in samples in under days, cost and the need for complex laboratory facilities often limits their utility in the developing world [28]. In addition to having a lower sensitivity for

smear-negative samples, the molecular methods often detect a limited number of mutations making transferability of technology for other pathogens difficult [28,29]. Cumulative evidence suggests that the Microscopic Observation Drug Susceptibility (MODS) assay may be one potential candidate for meeting these goals [60-66].

The development of MODS was based on three principles, namely: [1] *M. tuberculosis* can be cultured more quickly in liquid rather than solid media; [2] Culture growth of *M. tuberculosis* can be detected more rapidly using a light microscope instead of with the naked eye; and [3] *M. tuberculosis* grows in distinctive strings and tangles within liquid culture, making general species identification based on morphology at the microscopic level possible. Its cost can range between $0.53 to $0.77 US per sample to perform depending on the type of test being conducted (e.g., culture versus drug susceptibility status) and regional differences in labor and laboratory costs [61,67]. Using Middlebrook 7h9 broth, MODS can detect both culture and drug-susceptibility status for *M. tuberculosis* when antibiotics are introduced into broth.

Several studies indicate that culture and drug-susceptibility results for the MODS method are tightly correlated with reference standard methods [60-66]. Table 2.2 shows that concordance results for MODS range from 92 to 98 percent for culture detection; 99 to 100 percent for rifampicin testing; 97 to 100 percent for isoniazid testing; 58 to 95 percent for ethambutol testing; and 51 to 92 percent for streptomycin testing. The median time to culture-positivity for MODS ranges from seven to nine days. Research has also revealed little differences in the diagnostic validity of MODS using direct versus indirect testing [65]; diluted versus undiluted sputum samples [62]; smear negative versus smear positive sputum samples [64]; and sputum samples obtained before and during tuberculosis treatment [64]. More recently, the MODS method was validated for culture-based testing of pleural and cerebral spinal fluid from adults [67,68], as well as gastric aspirate, nasopharyngeal aspirate and stool specimens from children [69].

Despite these promising results, several issues remain for the optimization of MODS. First, because the cording patterns of *M. tuberculosis* in Middlebrook 7h9 broth are similar to

Table 2.2. Clinical evidence regarding the diagnostic validity of the Microscopic Observation Drug Susceptibility (MODS) assay for detecting pulmonary tuberculosis and its drug-susceptibility status.

Author (Reference) Year Origin of Samples	Study Sample	Culture Detection		Drug Susceptibility Testing	
		Reference Standard Test	Diagnostic Results	Reference Standard Test	Diagnostic Results
Caviedes [61] 2000 Peru	172 samples from patients suspected of having tuberculosis based on presence of clinical manifestations and/or smear positive sample	*Middlebrook 7h11*	Sensitivity: 92% Medium TCP: 9 days (Range: 4 - 31 days)	Microplate Alamar Blue Assay (MABA)	**Concordance** Rifampicin: 99% (0.5 µg/mL) 99% (1.0 µg/mL) Isoniazid: 100% (0.1 µg/mL) 100% (0.4 µg/mL)
Park [65] 2000 Peru	53 Isolates from patients at risk of MDR-TB	*n/a*	n/a	Middlebrook 7h10 Agar	**Concordance** Rifampicin: 100% (2.0 µg/mL) Isoniazid: 100% (0.1 µg/mL) Ethambutol: 70% (2.5 µg/mL) 58% (7.5 ug/mL) Streptomycin: 77% (2.0 µg/mL) 51% (6.0 µg/mL)
Moore [64] 2004 Peru	207 Pre-treatment Samples 69 On-treatment Samples Subjects part of two cohorts: (i) Patients receiving DOTS treatment for tuberculosis (ii) Patients with respiratory symptoms	*Lowenstein-Jensen*	Sensitivity: 94% Median TCP: 8 days	*Microplate Alamar Blue Assay (MABA)* & *Tetrozolium Microplate Assay (TEMA)*	**Pre-treatment Sample** **On-Treatment Sample** Rifampicin (1.0 µg/mL): Sensitivity 100% 92.9% Specificity 98.3% 89.7% Isoniazid (0.4 µg/mL): Sensitivity 81.1% 90.3% Specificity 96.9% 98.3% Ethambutol (2.5 µg/mL): Sensitivity 42.3% 64.7% Specificity 91.9% 91.1% Streptomycin (6.0 µg/mL): Sensitivity 44.4% 50.0% Specificity 98.3% 98.0%
Moore [63] 2006 Peru	3760 Sputum & gastric samples obtained from: (i) Patients with suspected tuberculosis (ii) Patients with suspected tuberculosis who were at high risk of	*Lowenstein-Jensen* & *MbBacT*	Sensitivity: 97.8% Median TCP: 7 days (IQR: 6 - 8 days)	*MbBacT* & *Lowenstein-Jensen*	**Concordance** **Kappa** Isoniazid: 97% 0.89 (0.4 µg/mL) Rifampicin: 100% 1.0 (1.0 µg/mL) Ethambutol: 95% 0.71 (2.5 µg/mL) Streptomycin: 92% 0.72

	MDR-TB (iii) Unselected hospitalized HIV-infected patients				(2.0 µg/mL)	
Arias [60] 2007 Brazil & Honduras	180 Respiratory samples (one specimen per patient) obtained from patients with: (i) Suspected tuberculosis treatment failure (ii) Suspected tuberculosis relapse (iii) Treatment default (iv) Close contact with MDR-TB patient	*Lowenstein-Jensen* & *MGIT 960*	**Per-subject Analysis** Sensitivity: 97.5% (95% CI: 96 - 99) Specificity: 94.4% (95% CI: 93 - 95) Median TCP: 7 days (IQR: 5 - 10 days)	*n/a*		n/a
Mello [62] 2007 Brazil & Honduras	1369 Respiratory Samples obtained from 854 subjects who had: (i) New suspected pulmonary tuberculosis (ii) Suspected treatment failure (iii) Suspected relapse (iv) Treatment default	*n/a*	n/a	*Lowenstein-Jensen*		**Undiluted Sample** / **1:10 Diluted Sample**
					Isoniazid (0.1 µg/mL):	
					Sensitivity 96.7%	96.7%
					95%CI (92 – 99)	(92 – 99)
					Specificity 78.4%	83.0%
					95%CI (74 – 81)	(78 – 85)
					Rifampicin (2.0 µg/mL):	
					Sensitivity 96.0%	96.0%
					95%CI (90 – 99)	(90 – 99)
					Specificity 82.9%	84.8%
					95%CI (79 – 85)	(81 – 87)
Ejigue [66] (2008) Ethiopia		*n/a*	n/a	*BACTEC* & *MGIT*	**Multi-drug Resistance (combined):** Sensitivity: 95% Specificity: 100% Accuracy: 98.3% Kappa = 0.981	

TCP= Time to culture-positivity; CI=Confidence Interval; IQR=Interquartile Range

those of *Mycobacterium chelonae* and *M. smegmatis* [61,63], lab readers must be trained to intuitively differentiate each species by their culture rates: both species overgrow rapidly as compared to *M. tuberculosis*. Second, although MODS has been shown to accurately detect rifampicin and isoniazid resistance in *M. tuberculosis*, detection results for ethambutol and streptomycin results have been poor. Possible explanations for these results have included sampling errors and a tendency to overcall drug resistance [65]. The critical concentrations of MODS for both of these drugs have also never been identified. Third, the use of MODS with

other anti-tubercular drugs such as pyrazinamide, ciprofloxacin, capreomycin, kanamycin and amikacin has also not been validated.

An additional concern with using MODS has been the occupational hazards from transmitting *M. tuberculosis* through liquid culture [70]. In hospital laboratories, where the infectious nature of sputum and gastric samples are initially unknown, the risk of occupational transmission may be high. Two studies found that hospital lab personnel were three to nine times more likely to be infected with *M. tuberculosis* as compared to the general population. However, lab-associated cases are very difficult to identify due to the long incubation period for *M. tuberculosis* and the unknown outside sources of infection that hospital personnel may be exposed to, particularly when the prevalence of tuberculosis is high in the general population [71].

As with other liquid culture methods, the risk of lab transmission with MODS is greatest if microbial droplets or aerosols produced during sample decontamination were to be inhaled or if cultures were to splash or spill during the inoculation phase [72,73]. Aerosols can be ubiquitous in nature and difficult to detect [72]. However, the risk of infection during traditional processing of samples for MODS can be minimized by using standard laboratory equipment such as biological safety cabinets, centrifuge safety caps, sealed rotors and plastic screw-lid vials [73]. Additionally, efforts are being made to develop agar versions and liquid cultures for MODS, which do not require decontamination or centrifugation of samples [74]. As for accidental spillage of liquid culture when microplates are being transported from the incubator to the microscope, this problem can be avoided by placing inoculated microplates in polyethylene Ziploc bags immediately following sample inoculation [73,75]. Sealing each microplate well with adhesive tape also avoids having to repeat sputum testing, if an inoculated plate falls during sample processing and the sputum must be re-cultured. More importantly, training and experience in handling *M. tuberculosis* samples can significantly reduce the overall risk of lab-associated infections [73].

2.3. The synergistic effect between HIV/AIDS and tuberculosis

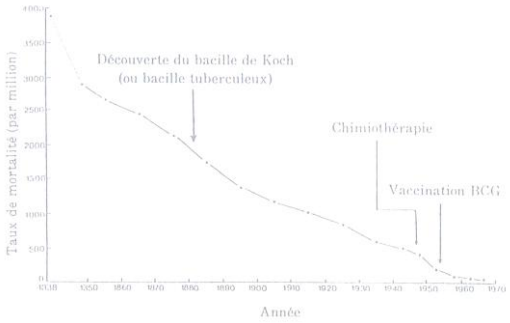
In more recent years, efforts to control tuberculosis have been hampered by the emergence of HIV. The link between HIV/AIDS and tuberculosis is most evident in the epidemiological burden of tuberculosis. While worldwide rates of pulmonary tuberculosis have been dropping since DOTS was instituted in 1995, in settings with a high prevalence of HIV/AIDS the incidence of pulmonary tuberculosis continues to increase.

HIV often targets IFN-γ and $CD4^+$ T lymphocytes needed to protect patients not only from infection with tuberculosis but also its progression from latent to active disease [76,77]. It is estimated that while the lifetime risk of developing active tuberculosis disease from latent infection among otherwise healthy individuals is 10%, this risk increases to 50% for those infected by HIV.

Isoniazid can be used both as a preventive therapy to lower a patient's risk of infection with *M. tuberculosis*, as well as treatment for latent tuberculosis. However, it is rarely used as part of a widespread public health initiative to control for tuberculosis due to long-term difficulties in drug delivery and concerns regarding the development of isoniazid-resistant forms of tuberculosis [78]. The use of prophylaxis can be complicated by difficulties in diagnosing HIV-infected individuals with tuberculosis, who depending on the extent of immunosuppression may be more likely to present with smear-negative disease, atypical chest radiographs, false negative tuberculin skin test results and present with extrapulmonary rather than pulmonary forms of disease [6,26]. Similarly, testing of HIV-positive patients for latent tuberculosis is often more difficult since infection status is based on immune responses for *M. tuberculosis* (which are frequently suppressed by HIV) rather than direct identification of the pathogen [79].

The therapeutic management of tuberculosis can also be problematic when used in conjunction with Highly Active Antiretroviral Therapy (HAART) for HIV-infection. Aside from HIV-infected patients being at increased risk of side effects from anti-tubercular drugs, drug interactions between protease inhibitors and non-nucleoside reverse transcriptase inhibitors (standard drugs used to treat HIV) with rifampicin (the standard first line drug for tuberculosis), can significantly lower plasma concentrations of antiretroviral drugs [80].

Figure 2.2. Annual mortality rates from pulmonary tuberculosis in England, from 1838 to 1970.



* Record collection of mortality rates began in 1838.

Source: McKeown T. Les déterminants de l'état de santé des populations depuis trois siècles: le comportement, l'environnement et la médicine. In: Bozzini, L, Renauld M, Gaucher, D, Llambas-Wolff. Médecine et société. Les années 80. Editions Coopératives Albert Saint-Martin: Montréal. 1981.

Rifampicin can be replaced by rifabutin when treating HIV-infected patients. However, complications with treatment often cause physicians to postpone the use of HAART among HIV-infected patients until after the initial phase of tuberculosis treatment is completed [6].

This synergy between HIV/AIDS and tuberculosis has led to renewed calls by the medical community to strengthen the public health infrastructure in many resource-constrained settings, namely by improving health care systems, diagnostic laboratories, human resources and public health services for the disease [4,5]. It has also been suggested that current public health programs for tuberculosis and HIV be better integrated, on the basis that the "whole may be greater than the parts" when it comes to dealing with HIV-associated tuberculosis [81].

Yet, while current failings in the biomedical approach towards tuberculosis control may be due to the limited resources in many developing countries, historical demographics for tuberculosis suggest that other explanations may exist. It has been noted that reductions in mortality from pulmonary tuberculosis were achieved in England (figure 2.2) several decades before the discovery of the tubercle bacilli, the advent of anti-tubercular agents or the discovery of BCG vaccines (i.e., the root elements of a biomedical framework for tuberculosis control) [82]. Such an observation raises complex questions, including whether

declining trends during the middle 1800's were a reflection of natural selection or improvements in social conditions [82,83].

Tuberculosis has long been recognized as a disease of extreme poverty [84]. However, if an increase in the prevalence of pulmonary tuberculosis among HIV-infected individuals is due to socio-economic differences, then why are incidence rates for tuberculosis only increasing among HIV-infected patients and not among other poverty-stricken groups? More importantly, why have other poverty-related diseases such as malaria, not been impacted by AIDS to the same extent as pulmonary tuberculosis?

2.4. HIV/AIDS: A cause of multi-drug resistant tuberculosis?

Reports of MDR-TB and XDR-TB outbreaks among hospitalized AIDS patients and prison populations with a high prevalence of HIV have also raised concerns regarding a potential link between AIDS and multi-drug resistant forms of tuberculosis [85-93]. While the effect of HIV/AIDS on the incidence of tuberculosis has been well described [1], the role of this factor in relation to multidrug resistance is less certain [10]. The low prevalence of multi-drug resistance and HIV in many tuberculosis settings, and the lack of drug-susceptibility data in joint population-based surveys of HIV and tuberculosis has made studying the association between HIV/AIDS and MDR-TB more difficult [34].

The development of drug-resistance during anti-tubercular treatment may be particularly problematic for AIDS patients due to their increased risk of gastrointestinal malabsorption [9,38-43,54]. This can lead to a lower bioavailability of anti-tubercular drugs in the bloodstream thus increasing a patient's risk for developing drug-resistance [9,38-43,54]. Many of these conditions may be further complicated by the introduction of the Beijing strain into a population of AIDS patients. In addition to this strain being more virulent, it is also more commonly associated with amplifications in drug-resistance.

Although observational studies and randomized clinical trials have confirmed these findings by identifying a link between AIDS and the development of resistance to rifamycins (the family group of rifampicin) [9,10,4], several papers have noted that outbreaks of MDR and XDR-TB usually occurred among AIDS patients who were previously uninfected with

tuberculosis and were living in industrialized countries where the prevalence of tuberculosis was low to begin with [8,11,17,8]. In other words, patients had probably not developed drug-resistance from past treatment of tuberculosis.

Ultimately, this has led to alternative theories for outbreaks of MDR and XDR-TB. They include diagnostic or treatment delays for tuberculosis; a higher risk of defaulting from DOTS due to adverse drug reactions; and poor infection control measures such as placing AIDS patients in the same ward regardless of whether they were infected with pulmonary tuberculosis or not [6,10,94]. However, inferring causal links from outbreak studies can be difficult since they are frequently evaluated using a case-only rather than a case-comparison or case-control approach. It is also interesting to note that during non-outbreak periods of time, few epidemiological studies have identified an association between HIV/AIDS and drug-resistant tuberculosis [95].

2.5. Summary

Tuberculosis remains a major cause of morbidity and mortality [1-3]. Although biomedical strategies such as DOTS have helped to decrease the global incidence of tuberculosis, efforts to control tuberculosis are currently being hampered by a continuing rise in the prevalence of HIV/AIDS (a major risk factor for tuberculosis). At the same time, outbreaks of MDR-TB among AIDS populations have led to concerns regarding a potential link between HIV/AIDS and the development of drug-resistance. Although this problem is frequently attributed to pathophysiological characteristics of HIV/AIDS and deficiencies in biomedical efforts to control tuberculosis, it is currently unclear whether an association between MDR-TB and HIV/AIDS actually exists.

CHAPTER 3.

Overview and Objectives

It is against this backdrop that the first part of this thesis aimed to develop a knowledge foundation regarding the underlying causes that lead to outbreaks of MDR-TB among HIV/AIDS patients.

To do this, we made use of several datasets collected as part of a grand study of tuberculosis between 1995 and 2005 in Lima, Peru. During this period, AIDS patients were experiencing a widespread outbreak of MDR-TB. The Ministry of Health was also on the verge of implementing a comprehensive national MDR-TB program which included drug-susceptibility testing. Before the MDR-TB program was fully instituted in 2005, drug-susceptibility testing took on average five months (almost equivalent to one full round of DOTS treatment for newly diagnosed cases). At the time, patients with and without AIDS in Lima, Peru were also being referred to separate hospitals for medical care, thus providing an opportunity to identify and compare two contemporary groups of patients exposed to pulmonary tuberculosis during an ongoing outbreak of MDR-TB in Lima, Peru, based on cohort prevalence of AIDS.

In the second part of this thesis, the knowledge framework regarding MDR-TB outbreaks was used to develop public health strategies for preventing tuberculosis among HIV/AIDS patients. This included using the Microscopic Observation Drug Susceptibility (MODS) assay to screen hospitalized AIDS patients for tuberculosis. At the technology development level, we also refined the MODS assay in order to detect drug-susceptibility status in *M. tuberculosis*.

The specific objectives of this thesis include:

<u>Knowledge Generation:</u>

Study #1

1. To investigate the role of AIDS in an epidemic of MDR-TB among patients receiving DOTS treatment for pulmonary tuberculosis between 1999 and 2005 in Lima, Peru.

1.1. To describe the prevalence of MDR-TB among AIDS and non-AIDS patients.

1.2. To estimate the proportion of patients who acquired primary versus secondary multidrug resistance during DOTS.

1.3. To describe the strains and clusters of *M. tuberculosis* among AIDS and non-AIDS patients receiving DOTS treatment for pulmonary tuberculosis.

1.4. To identify risk factors for infection with the epidemic clone of MDR-TB.

Study #2

2. To characterize the prevalence and factors associated with stool shedding with *mycobacteria* among AIDS patients in Lima, Peru.

    2.1. To calculate the frequency and pattern of shedding viable *mycobacteria* in the sputum and stool of patients at two different stages in the natural history of pulmonary tuberculosis.

    2.2. To identify factors associated with shedding *mycobacteria* in the stool of AIDS patients in the later stages of tuberculosis disease.

Knowledge Integration/Technology Transfer:

Study #3

3. To begin developing screening strategies for tuberculosis among hospitalized HIV/AIDS patients using the Microscopic Observation Drug Susceptibility assay.

    3.1. To evaluate how screening hospitalized patients using the standard diagnostic criteria of sputum microscopy, symptom review and chest radiography, compares with an alternative approach of using MODS to screen **all** patients.

    3.2. To determine whether MODS screening should be targeted to pre-selected sub-groups of patients

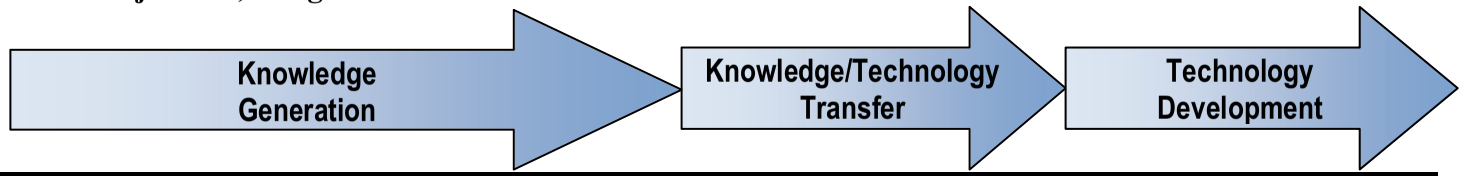
    3.3. To identify the clinical impact of targeted versus blanket screening using MODS.

Technology Development:

Study #4

4. To define the optimal critical concentration needed for detecting drug resistance in *M. tuberculosis* using the Microscopic Observation Drug Susceptibility assay.
    4.1. To identify and validate the critical concentration needed to detect ethambutol and streptomycin resistance in *M. tuberculosis* using MODS.

Table 3.1. Overview of objectives, design and methods for studies included in the thesis



	Study #1 (Chapter 4)	Study #2 (Chapter 5)	Study #3 (Chapter 6)	Study #4 (Chapter 7)
Objective	Identify the role of AIDS during an ongoing epidemic of MDR-TB	Characterize the prevalence and factors associated with shedding viable *mycobacteria* in stool	Examine the clinical utility of MODS for screening hospitalized HIV-infected patients for PTB.	Define the optimal critical concentration in MODS needed to detect drug-resistance in *M. tuberculosis*.
Study Populations	• Patients receiving DOTS for PTB • Divided into 2 cohorts: 1. Patients with AIDS 2. Patients without AIDS	• AIDS patients with tuberculosis • Divided into 2 groups: 1. Patients in early stages of PTB disease (wet vs. dry cough) 2. Patients in later stages of TB disease (EPTB vs. PTB)	• Hospitalized HIV-infected patients	• Stored sputum samples • Selected based on frequency distribution of drug-resistance
Study Design	Longitudinal Study	Cross-sectional Study	Pilot Study	Laboratory-based Study
Study Measures	**Cohort-level analysis: (AIDS vs. non-AIDS cohort)** • Relative risk of presenting MDR-TB at start of DOTS (Incidence Rate Ratio) & during DOTS (Odds Ratio)	• Culture yield by type of media in: - Sputum - Stool • Time to culture positivity • Prevalence of shedding in patients -- stratified measures by:	• Evaluated four screening strategies: 1. Sputum smear microscopy 2. Current diagnostic criteria (Clinical suspicion for TB, Sputum Smear Microscopy, Chest X-	• For each critical concentration: - Sensitivity - Specificity - Youden's Index - Test Efficiency - Amount of growth observed

- Patients' Strain Conversion Status during DOTS
- Comparison of strain distribution within each cohort (χ^2 Test for proportion or Fisher's Exact Test)
- Risk of infectivity per strain (Standardized Incidence Ratio)

Individual-level analysis: (Patients who presented with MDR-TB at start of DOTS regardless of whether they were part of the AIDS or non-AIDS cohort)

- Factors associated with cluster clone infection (Odds Ratio; Positive & Negative Likelihood Ratio)
  - Grade of Smear Status
  - Early vs. Late stage of PTB
  - Wet vs. Dry Cough (only using early stage cohort)
  - EPTB vs. PTB (only using late stage cohort)
  - Cohorts compared using χ^2 Test or Fisher's Exact Test
- Risk factors for shedding among patients in the late stage of tuberculosis (Odds ratio)
- Clinical predictors of patients with tuberculosis who were shedding *mycobacteria* in stool during the later stages of disease (Positive & Negative Likelihood Ratio and Odds Ratio)

ray)

3. Universal MODS screening
4. Targeted MODS screening

- Evaluated:
  - Number & Proportion of patients with PTB identified by each screening strategy
  - Sensitivity & specificity per screening factor

- Values summarized using:
  - ROC curve
- MIC_{50} & MIC_{90}

CHAPTER 4.

Role of AIDS in an epidemic of multi-drug resistance among patients receiving Directly Observed Therapy Short-Course for pulmonary tuberculosis

Knowledge Generation

For this first study, we identified the underlying factors associated with an outbreak of MDR-TB among AIDS patients receiving DOTS treatment for pulmonary tuberculosis in Lima, Peru.

4.1. Study Summary

Background: We examined the role of AIDS in an epidemic of multi-drug resistant tuberculosis (MDR-TB) among patients receiving Directly Observed Therapy Short-Course (DOTS) for pulmonary tuberculosis in Lima, Peru between 1999 and 2005. **Methods**: We identified two patient populations receiving DOTS based on group prevalence of AIDS [n=205 in *AIDS* cohort; n=386 in *non-AIDS* cohort]. Before starting treatment, all patients had physical exams and were interviewed. Sputum samples were collected at enrolment, at week 1, month 1, month 2, and month 4 of tuberculosis treatment and once at the end of treatment. We obtained DNA fingerprints for MDR-TB samples using spoligotype and IS6110 Restriction Fragment Length Polymorphism. **Results**: Patients in the AIDS cohort were at increased risk of presenting MDR-TB at the start of treatment [Crude Odds Ratio (OR): 2.89, 95% CI: 1.4 to 5.8], and at increased risk of acquiring multi-drug resistance during treatment [Crude Rate Ratio: 2.07, 90% CI: 1.0 to 4.3], relative to those in the non-AIDS cohort. 92% (11/12) of AIDS cohort patients who developed multi-drug resistance during treatment had acquired primary drug resistance, from which 55% (6/11) were superinfected by one particular strain of *Mycobacterium tuberculosis*. Although this strain was more common among AIDS cohort patients (χ^2=5.00, p-value=0.02), we found no cohort difference in the risk of superinfection with this strain, after adjusting for patients' baseline risk of exposure to circulating strains [Standardized Incidence Ratio (SIR) for AIDS cohort:

1.63, 95% CI: 0.5 to 3.8 and for non-AIDS cohorts 1.75, 95% CI: 0.4 to 5.1]. **Conclusion**: An epidemic of MDR-TB among AIDS patients receiving DOTS for pulmonary tuberculosis was due to probable super-infection with one specific strain of *M. tuberculosis* rather than the development of secondary drug-resistance. While this epidemic strain was more common among patients in the AIDS cohort, risk of super-infection did not differ between patients in the AIDS and non-AIDS cohort, suggesting that another factor possibly associated with diarrhea may be contributing to the strain's high prevalence among AIDS patients.

4.2. Background

In recent years, while the global incidence of tuberculosis disease has declined, the incidence of tuberculosis has increased in regions where HIV/AIDS is common [1]. This in conjunction with outbreaks of multi-drug resistant (MDR-TB) and extremely drug-resistant (XDR-TB) tuberculosis among hospitalized AIDS patients and prison populations with a high prevalence of HIV, have caused some to suggest that we are now entering the worst epidemic of tuberculosis since the advent of the antibiotic era [85-93,96,97]

Anti-tubercular treatment may be particularly problematic for AIDS patients, due to an increased risk for gastrointestinal malabsorption of anti-tubercular drugs [38-43,54]. Lower bioavailability of antibiotics in the bloodstream can increase a patient's risk of developing drug-resistance [9]. AIDS patients are also more likely to default from DOTS programs, due in large part to adverse drug reactions experienced during treatment [6]. Several observational studies and randomized clinical trials have confirmed these findings by identifying a link between AIDS and the development of resistance to rifamycins (the family group of rifampicin) [8,9,45]. The introduction of the Beijing strain into populations with a high prevalence of AIDS may also hamper effective management of tuberculosis due to the strain's increased virulence and greater susceptibility to amplification of drug-resistance, relative to other strains of *Mycobacterium tuberculosis* [10,98].

Despite this evidence, several papers note that reported outbreaks of MDR-TB and XDR-TB usually occurred among AIDS patients who were previously uninfected with tuberculosis and living in industrialized countries where the prevalence of tuberculosis was

low [85-93,96]. Many of these outbreaks were attributed to diagnostic and treatment delays, as well as poor infection control measures such as placing AIDS patients in the same ward regardless of whether they were infected with pulmonary tuberculosis or not [10,94]. However, inferring causal links from these studies may be difficult since these outbreaks were frequently evaluated using a case-only rather than a case-comparison approach.

In light of these limitations, we examined the underlying factors associated with an epidemic of multi-drug resistance in Lima, Peru, by comparing an AIDS and non-AIDS population receiving Directly Observed Treatment Short-Course (DOTS) for pulmonary tuberculosis.

4.3. Methods

4.3.1. Study Setting:

Data collection occurred between May 1999 and April 2005 in Lima, Peru. During this period, AIDS and non-AIDS patients who were living in the same community were often referred to separate hospitals for medical care, thus enabling us to compare two concurrent patient populations exposed to MDR-TB based on group prevalence of AIDS. During our study, the Peruvian Ministry of Health was in the process of implementing a comprehensive national MDR-TB program which included drug-susceptibility testing for MDR-TB. The program was fully instituted in 2005 [99]. Before then, testing for MDR-TB took on average five months (i.e., almost equivalent to the duration of one round of DOTS treatment) [100].

4.3.2. Study Populations:

We identified two patient cohorts about to commence DOTS treatment for pulmonary tuberculosis. The first group (*AIDS cohort*) consisted of AIDS patients attending the Infectious Disease Clinic of the Hospital Dos de Mayo, the main referral hospital for persons with AIDS in Lima, Peru. The second group (*non-AIDS cohort*) consisted of patients referred to the Pulmonary Clinic of the Hospital Maria Auxiliadora. Patients were excluded from our study if they experienced an adverse reaction to anti-tubercular drugs. Our final analysis

included patients who attended at least 90% of treatment days in the DOTS program and had bacteriological confirmation of *Mycobacterium tuberculosis* in sputum (please refer to Appendix 2 and 3). All subjects provided written informed consent. Institutional review boards at Johns Hopkins Bloomberg School of Public Health and A.B. PRISMA approved the original study protocols.

4.3.3. Data Collection:

Figure 4.1 illustrates that before the start of DOTS treatment (day 0), a complete physical examination was conducted. This included measuring weight in kilograms, height in meters, and body mass index (BMI) in kilograms/meters^2, and collecting blood samples. A questionnaire was completed regarding socio-demographic information and medical history. Two consecutive sputum samples were obtained at enrolment (day 0), as well as during follow-up visits at week 1, month 1, month 2, month 4 of treatment, and once at the end of treatment. A small proportion of patients also provided sputum samples at week 2 of treatment. A study physician conducted the study interview and physical exams (please refer to Appendix 4). All laboratory specimens were sent for testing to the research laboratory at the Universidad Peruana Cayetano Heredia in Lima, Peru.

4.3.4. Laboratory Methods and Definitions:

Sputum samples were tested for smear status using Auramine Smear Microscopy; culture status using Lowenstein-Jensen or Middlebrook 7h9 Broth (Difco, Detroit, Mich.); and drug-susceptibility status using the Microplate Alamar Blue Assay (MABA) [101]. Samples were categorized as drug-resistant if the Minimum Inhibitory Concentration cut-off for MABA was greater than 0.25 μg/mL for isoniazid, and greater than 0.25 μg/mL for rifampicin [102]. If samples were resistant to both isoniazid and rifampicin, they were classified as multi-drug resistant (MDR). Patients had drug-susceptible tuberculosis at study entry if all sputum samples they produced were susceptible to isoniazid and rifampicin before and during the first two weeks of anti-tubercular treatment. Patients who developed drug-resistance after at least one month of treatment were classified as having acquired drug-resistance [103].

DNA fingerprinting by spoligotype was performed on the first sputum sample collected at study entry and on the first sample collected from patients at the time that they acquired drug-resistance [104]. We identified strain genotypes by comparing octal code numbers for each isolate with those recorded in the SpolDB4 database [105]. Strain conversion status for patients who acquired drug-resistant tuberculosis was identified by comparing the strain genotype for isolates collected from a patient at baseline and at the time that they acquired drug-resistance. We identified clones within each strain genotype by conducting IS6110 restriction fragment length polymorphism (RFLP) on isolates from all MDR-TB patients in the AIDS cohort and MDR-TB patients in the non-AIDS cohort who were infected with Lam9 or T1 strains of *M. tuberculosis*. IS6110 RFLP was conducted using previously described methods [106]. We used primers INS-1 and INS-2 to amplify probes to a length of 245 base pairs. Isolates with matching IS6110 RFLP band patterns were ascertained manually and with PRO-SCORE (DNA Proscan, Nashville, TN).

To check for evidence of laboratory cross-contamination in patients who acquired multi-drug resistance but only produced one multi-drug resistant sputum sample during follow-up, we compared DNA fingerprints for the sputum sample in question with fingerprints for all other multi-drug resistant samples processed on the same day. We identified two patients who acquired MDR-TB and whose IS6110 RFLP fingerprint matched another MDR-TB sample processed on the same day. The sputum sample results from both these patient were excluded from our final analysis.

We measured CD4 lymphocyte counts of patients within the AIDS cohort using the Manual CD4 Cell Count Kit (Coulter). Patients in the non-AIDS cohort were tested for HIV-1 and HIV-2 antibodies using enzyme-linked immunosorbent assay (ELISA).

4.3.5. Data Analysis:

Our analysis had six main objectives. The first was to identify whether patients in the AIDS cohort were at greater risk of: 1. presenting MDR-TB at the start of DOTS treatment for tuberculosis; and, 2. acquiring multidrug resistance during DOTS, if infected with drug-susceptible tuberculosis at the start of treatment. For each study cohort, we estimated the

proportion of patients with drug-resistant pulmonary tuberculosis at the start of DOTS treatment (cohorts compared using the odds ratio), and the incidence of acquiring drug-resistant pulmonary tuberculosis per 1000 person-months of treatment (cohorts compared using the incidence rate ratio).

Our second objective was to: 1. identify for each study cohort the proportion of drug-susceptible patients being treated for tuberculosis with DOTS who developed primary versus secondary multidrug resistance during treatment; and, 2. determine whether patients in the AIDS cohort were more likely to acquire secondary multidrug resistance during treatment as compared to patients in the non-AIDS cohort. We identified strain conversion status for each patient who acquired drug-resistant tuberculosis by comparing the strain genotype for isolates collected from a patient at baseline and at the time that they acquired multi-drug resistance. We defined primary drug-resistance as infection with a drug-resistant strain; and, secondary drug-resistance as the development of drug-resistance within a previously susceptible strain of *M. tuberculosis* (Figure 4.6). Using strain conversion status, we classified patients as having acquired primary drug-resistance if their strain of MDR-TB was different than the drug-susceptible (DS) TB strain at baseline. Patients were classified as having acquired secondary multidrug resistance if the strains for both DS-TB at baseline and acquired MDR-TB were the same. We compared the proportion of patients who acquired secondary multidrug resistance from each study cohort using the Fisher's Exact test.

Our third objective was to determine whether the outbreak of multi-drug resistance among the AIDS cohort was due to a particular strain of *M. tuberculosis*. For each study cohort, we calculated the frequency distribution of strain genotypes among patients with MDR-TB at baseline and patients who acquired primary multidrug resistance during DOTS treatment. We then compared the strain distributions from each study cohort using the Chi-square test. We classified any strain that occurred more frequently in the AIDS cohort as an epidemic strain of MDR-TB.

Our fourth objective was to determine whether the risk of superinfection with the epidemic strain differed between patients in the AIDS and non-AIDS cohort, after adjusting for the patients' baseline risk of exposure to all circulating strains. This was done by calculating the

standardized incidence ratio for acquiring an MDR-TB strain during tuberculosis treatment. For each study cohort, we estimated a strain's standardized incidence ratios by dividing the observed number of cases by the expected number of cases. We determined the expected number by assuming that the distribution of strains among patients who acquired MDR-TB strains would be the same as those with MDR-TB at the start of treatment. Confidence intervals were determined using a Poisson distribution.

Our fifth objective was to characterize whether AIDS patients infected with the epidemic strain of *M. tuberculosis* shared a common clone. Using RFLP data, we identified the number of clones per strain type found in isolates from patients in the AIDS cohort, as well as the number of clusters per clone type found to be circulating within the AIDS cohort. We also identified whether cluster clones isolated from patients in the AIDS cohort were circulating within the non-AIDS cohort, by reviewing RFLP data from patients in the non-AIDS cohort who shared the same MDR-TB strain as cluster strains found in the AIDS cohort.

Our final objective was to identify which factors may have contributed to the epidemic clone of MDR-TB becoming predominant among AIDS patients. Using data from MDR-TB patients in both the AIDS and non-AIDS cohorts, we estimated for each factor the univariate odds ratio using an exact logistic regression model and the likelihood ratio for a positive and negative test using standard formula [107]. We assessed the measure of association for the following covariates: sputum smear stain status [positive or negative], HIV-infection status [positive or negative]; sex [male or female]; previous infection with TB [yes or no]; known contact with a TB case during the previous two years [yes or no]; known hospitalization during the previous two years [yes or no]; cough [yes or no]; productive cough [yes or no]; fever [yes or no]; shortness of breath [yes or no]; hemoptysis [yes or no]; recent weight loss [yes or no]; loss of appetite [yes or no]; night sweats [yes or no]; fatigue [yes or no]; past prophylaxis for TB [yes or no]; previously hospitalized [yes or no]; underweight [yes or no]; and presence of diarrhea [yes or no]. We classified any patient with a BMI less than 18.5 kg/m^2 as being underweight [108].

We calculated confidence intervals for all measures using a two-sided alpha of 0.05. We conducted all statistical analysis using STATA v.7 (College Station, TX).

4.3.6. Role of the Funding Source:

The funding source had no role in study design, data collection, data analysis, and data interpretation, writing of the manuscript or the decision to publish.

4.4. Results

From 1999 through 2005, we recruited 730 patients referred to the Peruvian National Tuberculosis Control Program for treatment of pulmonary tuberculosis. 712 patients provided sputum samples before the start of DOTS treatment (figure 4.2). 601 patients were culture-positive for *M. tuberculosis* and thus deemed eligible to participate in our study. 10 or 2% of these patients were missing drug-susceptibility results and thus excluded from our analysis.

Patients in the AIDS cohort resided in more geographically dispersed locations, relative to those in the non-AIDS cohort (figure 4.3). Patients in the AIDS cohort (table 4.1) were also more likely to be men, have been previously diagnosed with tuberculosis, and been hospitalized during the previous two years. All patients in the AIDS cohort were infected with HIV, while less than 5% of patients in the non-AIDS cohort patients tested positive for HIV. HIV-infected patients in the non-AIDS cohort were excluded from the final analysis. Most patients in the AIDS cohort were in advanced stages of HIV and thus extremely immunosuppressed. Of the 158 AIDS-cohort patients that we were able to test for CD4 counts at the start of treatment, 112 or 71% had counts less than 100 cells/μL, 22 or 14% had counts between 100 and 200 cells/μL, and 24 or 15% had counts greater than 200 cells/μL.

Among the 591 patients who entered our study with culture-positive tuberculosis, 100 or 17% were multi-drug resistant, 27 or 5% were mono-resistant to rifampicin, and 42 or 7% were mono-resistant to isoniazid. Mono-resistance to isoniazid was significantly more common in the non-AIDS cohort, while multi-drug resistant TB was significantly more common in the AIDS cohort (table 4.2(a)). The predominant genotypes of MDR-TB in both

study cohorts at the start of DOTS treatment (figure 4.4a) were T1 (36/97 or 37%) and Lam9 (26/97 or 27%). T1 was equally present in both the AIDS and non-AIDS cohorts (χ^2=0.94, p-value=0.33). Lam9 was more common in the AIDS cohort (χ^2=4.02, p-value=0.04).

Of 355 patients who began DOTS treatment with drug-susceptible TB and had follow-up data, 25 or 7% acquired multi-drug resistance, 3 or 1% acquired rifampicin mono-resistance, and 10 or 3% acquired isoniazid mono-resistance. Patients in the AIDS cohort acquired multi-drug resistance at a higher rate than patients in the non-AIDS cohort (table 4.2(b)). We found no group difference in the rate of acquiring mono-resistance to isoniazid or rifampicin.

Our analysis of strain conversion status revealed that 92% (11/12) of AIDS cohort patients and 92% (12/13) of non-AIDS cohort patients who acquired multi-drug resistance during DOTS treatment had developed primary multidrug resistance. 0% (0/12) of AIDS cohort patients and 8% (1/13) of non-AIDS cohort patients had developed secondary multidrug resistance. Conversion status was undetermined for 8% (1/12) of patients in the AIDS cohort due to insufficient quantity of sample. We found no cohort difference in the proportion of patients who developed secondary versus primary multidrug resistance (Fisher's Exact test=1.00).

Among those who acquired primary multi-drug resistant tuberculosis during DOTS treatment, we identified Lam9 in 55% (6/11) of AIDS cohort patients and 17% (2/12) of non-AIDS cohort patients. Although Lam9 was more common among AIDS cohort patients who acquired MDR-TB during treatment (χ^2=5.00, p-value=0.02), we found no difference in the risk of superinfection with Lam9 between AIDS cohort patients [Standardized Incidence Ratio (SIR): 1.63 (95% CI: 0.5 to 3.8] and non-AIDS cohort patients [SIR: 1.75 (95% CI: 0.4 to 5.1)] after adjusting for the baseline risk of exposure to circulating MDR-TB strains.

To evaluate whether patients in the AIDS cohort were being infected by the same Lam9 clone, we typed isolates collected from all MDR-TB patients using IS6110 RFLP band patterns. We identified 4 clusters and 25 unique clones. The largest clusters came from: Lam9 which was divided into one cluster of 29 isolates, and 4 unique clones; and, T1 which was divided into one cluster of 23 isolates, and 7 unique clones. All patients who acquired a

different strain of MDR-TB were infected with T1 or Lam9 "cluster" clones (figure 4.5). The remaining clustered isolates in our analysis accounted for 4 cases of MDR-TB, but did not include an acquired MDR-TB case. Using a sub-analysis of 12 MDR-TB isolates (i.e., 6 from baseline cases and 6 from acquired cases), we found that Lam9 and T1 "cluster" clones were also circulating within the non-AIDS cohort. We observed that 100% (4/4) of Lam9 isolates and 63% (5/8) of T1 isolates shared identical band patterns as "clustered" clones detected in the AIDS population.

In order to identify factors that may have contributed to the epidemic clone of Lam9 becoming predominant among HIV-infected patients, we conducted a univariate analysis among all patients with MDR-TB at the start of DOTS treatment. Table 4.3 shows that patients infected with the epidemic clone of MDR-TB were more likely to experience diarrhea as compared to patients infected with other clones of MDR-TB. The most useful factors for ruling "in" infection with the Lam9 epidemic clone among all patients with MDR-TB included being underweight, shortness of breath, weight loss and having had prophylaxis for tuberculosis.

4.5. Discussion

Our prospective study showed that AIDS patients with drug-susceptible tuberculosis between 1999 and 2005 were at greater risk of acquiring multi-drug resistance during DOTS treatment than non-AIDS patients, and that the majority of these patients were infected with a different multidrug resistant strain during treatment. Our findings support previous scientific evidence that DOTS can effectively prevent the development of drug-resistance among pan susceptible strains of *M. tuberculosis* [109]. However, these results also suggest that malabsorption of anti-tubercular drugs may be an unlikely cause of MDR-TB outbreaks in settings with a high prevalence of AIDS [38-43,54].

Using DNA fingerprinting, we determined that the majority of AIDS patients who acquired multi-drug resistance were infected by just one strain (i.e., Lam9). While Lam9 was found to be circulating within both the AIDS and non-AIDS cohorts, it was more common among patients in the AIDS cohort both at the start and during DOTS treatment. Taken

together, these observations would tend to suggest a synergistic effect between immunosuppression and infection with particular clones of MDR-TB. However, further analysis revealed that when the baseline prevalence of MDR-TB strains was adjusted for, AIDS and non-AIDS patients shared the same risk of superinfection with Lam9.

Identifying why AIDS patients had such a high baseline prevalence of Lam9, was a major issue in our study. Our regression models of patients with MDR-TB at the start of DOTS treatment, revealed an unexpected association between the risk of infection with the epidemic clone of Lam9 and diarrhea. Although the mechanisms underlying this association are purely speculative, an increased susceptibility for acquiring MDR-TB following infection with other diarrhea associated diseases might explain our results. The gastrointestinal tract has long been recognized as the most common site for opportunistic infections among HIV-infected patients [45] [110], and humoral immune responses against diarrhea-associated parasites are thought to favor infection with *M. tuberculosis* and HIV [111] [112] [113]. We were unable to test this hypothesis in our study since we only evaluated patients for the presence of an underlying medical conditions (that included diarrhea), rather than detailed information specifically concerning diarrhea. However, future epidemiological studies on tuberculosis and diarrhea might be designed to examine the role that infection with different diarrhea-associated parasites has on the risk of infection and the development of active tuberculosis.

We also found that administration of isoniazid prophylaxis was a useful factor for "ruling in" infection with the Lam9 epidemic clone of MDR-TB. It is feasible to assume that mono-isoniazid therapy of latent tuberculosis or undetected active tuberculosis could have partially contributed to the outbreak of MDR-TB. Between 52% and 63% of HIV-positive individuals with active tuberculosis have the extra pulmonary form, and approximately 30% of HIV-infected patients have latent tuberculosis [3] [114]. Although we found no difference in risk factors for infection with tuberculosis, namely past history of tuberculosis, risk of known contact with TB, hospitalization during the previous two years, or cavitation (a marker for past tuberculosis infection) between patients with Lam9 versus other MDR-TB strains at baseline, our study may not have had sufficient statistical power to detect these associations. Still, our results highlight the need to rule out all forms of tuberculosis (not just pulmonary

tuberculosis) and provide the same level of patient support found in DOTS programs, when administering prophylaxis to HIV-infected patients.

In terms of strain distribution, we noted that patients in the AIDS cohort who resided in more geographically dispersed regions, had more strains in common than patients in the non-AIDS cohort. Although transmission of tuberculosis rarely occurs over large geographical distances, this is often due to the fact that individuals tend to socialize within rather than outside their own community [115]. Several studies have noted that TB clusters may be more closely linked to social networks rather than geographical location per se [116,117]. At the time that data collection for this study took place, HIV infection was extremely uncommon in the general population of Peru and largely restricted to marginalized populations such as injection drug users, men who have sex with men and commercial sex workers [118]. Consequently, the paradoxical differences in strain distribution between both study cohorts may have been a reflection of socialization practices which led to infection with *M. tuberculosis* at the time of HIV infection.

Several methodological issues should be considered when reviewing our results. First, while our study identified one particular strain of *M. tuberculosis* (i.e., Lam 9) that was strongly associated with the MDR-TB outbreak in the AIDS cohort, we do not believe that this represented a strain effect. Nosocomial outbreaks in Argentina and New York City among HIV-infected populations during the early 1990's had been caused by M and W strains, respectively [89] [119], suggesting that a co-factor other than strain type, may be responsible for MDR-TB outbreaks.

Second, although our study had a large sample size relative to other previously published studies on MDR-TB and AIDS [109,120,121], the statistical power for our regression analysis remained low. We estimate that in order to detect a 50% increase in risk among MDR-TB patients infected with Lam9 using an alpha level of 0.05 and a beta level of 0.80, we would have needed 279 Lam9 MDR-TB cases and a total of 1116 MDR-TB patients, using a ratio of three controls per case. Enrolling this number of subjects would probably not be feasible given that MDR-TB is relatively rare in Peru [i.e. In 2006, 96 patients or 5.3% of all new tuberculosis cases were identified as MDR-TB [34]] and the molecular dynamics of *M.*

tuberculosis could change over a span of one or two decades particularly if the tuberculosis cases in our study represented recent rather than reactivated forms of the disease [122]. To remedy this problem, we included in our study analysis both the 90 and 95 percent confidence interval, when a cell was less than 5 patients. Still, our study may have failed to detect certain true associations.

Third, our study was unable to differentiate between acquired MDR-TB cases due to super-infection versus undetected polyclonal infection at the start of DOTS. It has been hypothesized that for patients infected by multiple strains of *M. tuberculosis*, MDR-TB may be allowed to grow uninhibited when first-line treatment suppresses non-MDR-TB strains [123,124]. These MDR-TB strains would only be detected later on during DOTS (figure 4.6a), thus giving the appearance of a super-infection with MDR-TB. A sub-analysis of 25 culture-positive samples obtained from our study participants at the start of DOTS revealed that 16% shared more than one spoligotype at testing: a number that is within previously reported ranges of 10 to 19% [123-126]. If patients were infected with a new strain of MDR-TB during DOTS, it is also difficult to determine where transmission occurred at the DOTS clinic or within the general community since most study participants were outpatients. Still, if transmission did occur at the clinic, one option would be to treat MDR-TB patients separately from patients with drug-susceptible TB [e.g., in different rooms of a clinic or, if this is not possible, at different times of day (morning vs. afternoon)]

Fourth, all study participants were unaware of their drug-susceptibility status and strain of infection, at the time that patient interviews were conducted, thus minimizing the possibility of selection or recall bias. Additionally, selective recall of diarrheal status was minimized by the fact that our questionnaire asked patients whether they had one of six underlying medical conditions (i.e., diabetes, diarrhea, cardiopathy, renal disease, cancer, liver disease and/or other), rather than diarrhea alone.

In conclusion, most AIDS patients with drug-susceptible pulmonary tuberculosis who acquired multidrug resistance during DOTS treatment in Lima, Peru had been infected with a different MDR-TB strain rather than developed secondary drug resistance. While this strain was more common among patients in the AIDS cohort, the risk of super-infection did not

differ between AIDS and non-AIDS patients, suggesting that another factor (possibly associated with diarrhea) may be contributing to the strain's high prevalence among AIDS patients.

4.6. Figures and Tables

Figure 4.1. Timeline for data collection and analysis

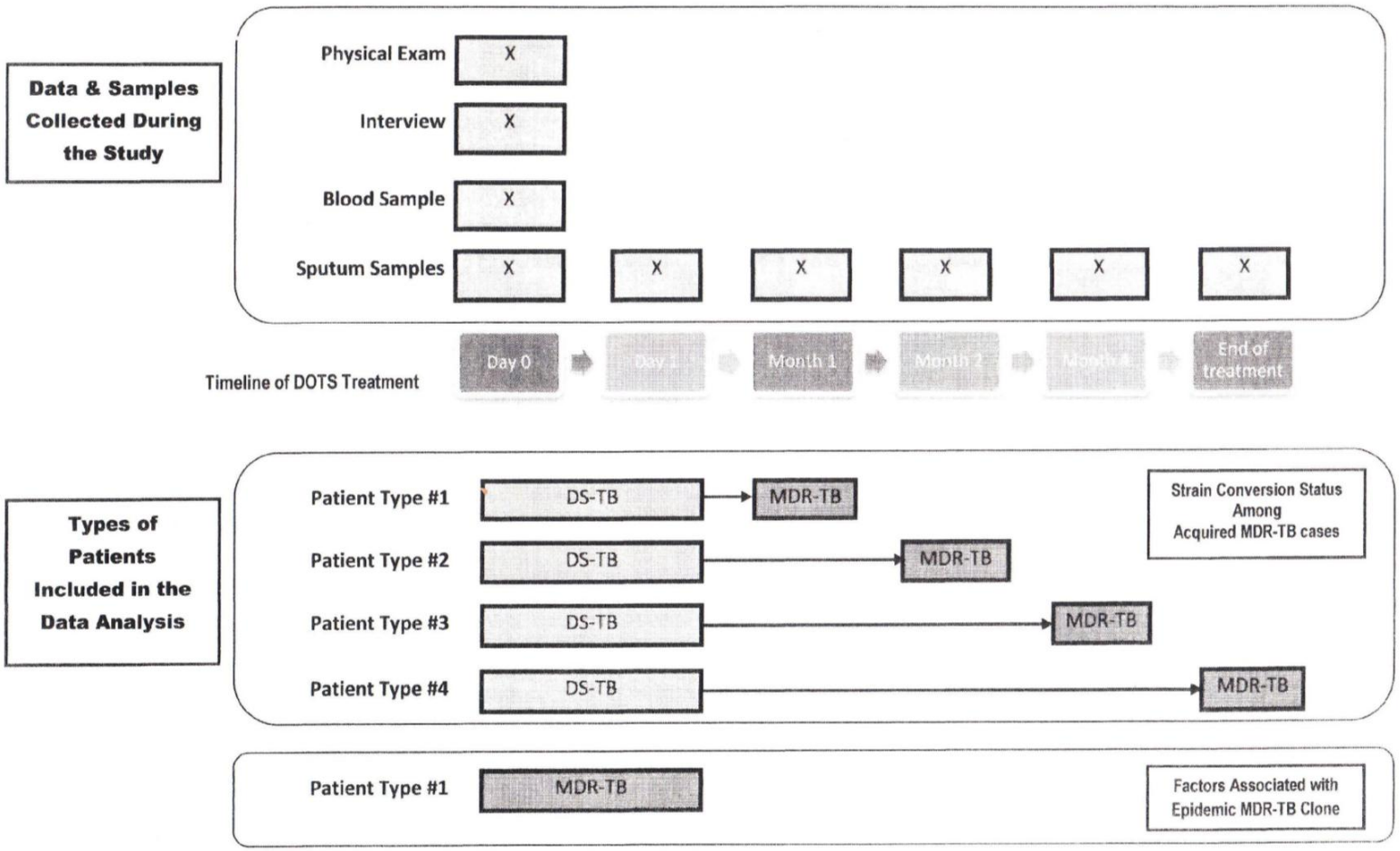


Figure 2. Study Flow Chart

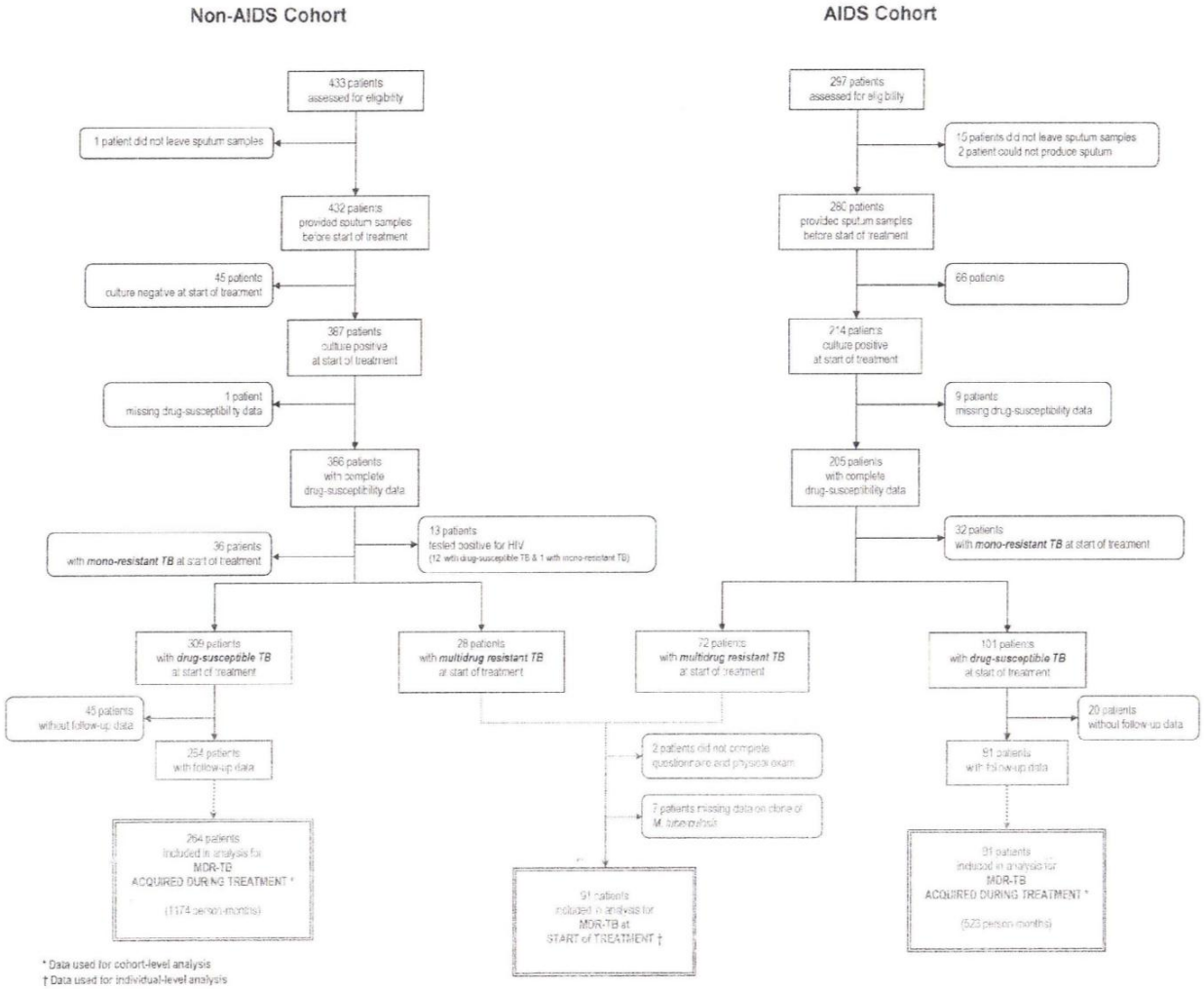


FIGURE 4.3. Map showing patients' place of residence in Metropolitan Lima, by study cohort

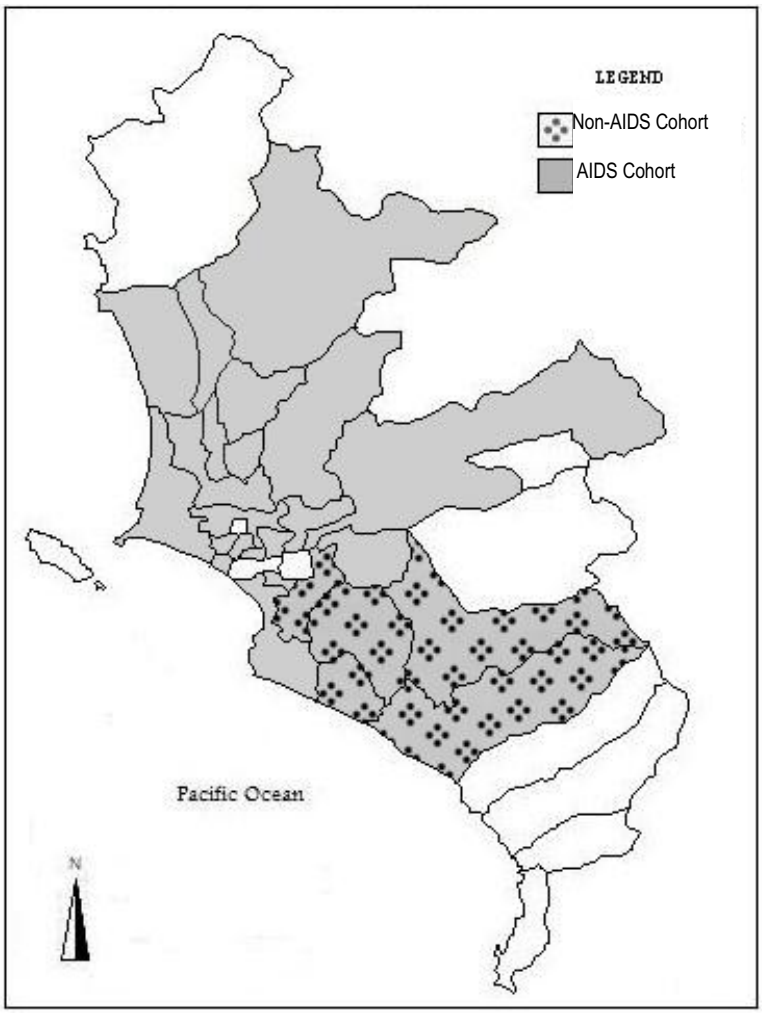
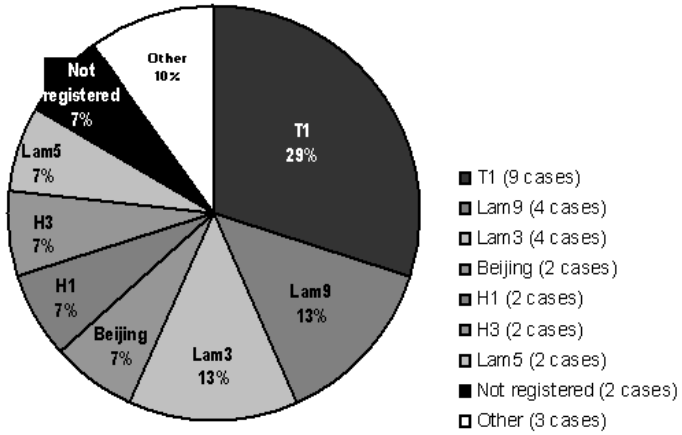


FIGURE 4.4. Frequency distribution of genotypes in sputum isolates from patients with multi-drug resistant *Mycobacterium tuberculosis*

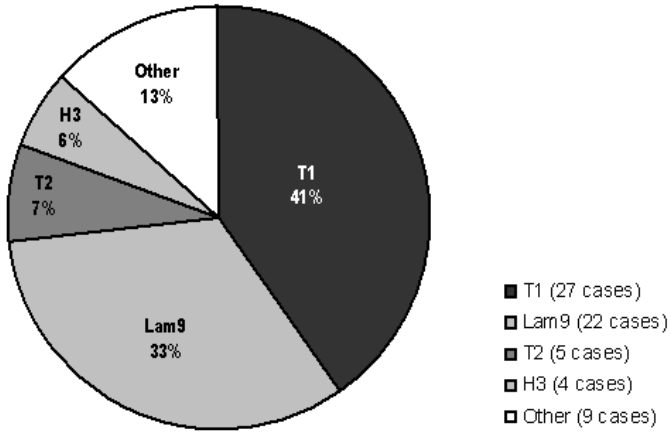
(a) Multi-drug resistant tuberculosis at the start of DOTS treatment

Non-AIDS Cohort (n=28)*



* Two samples were polyclonal.

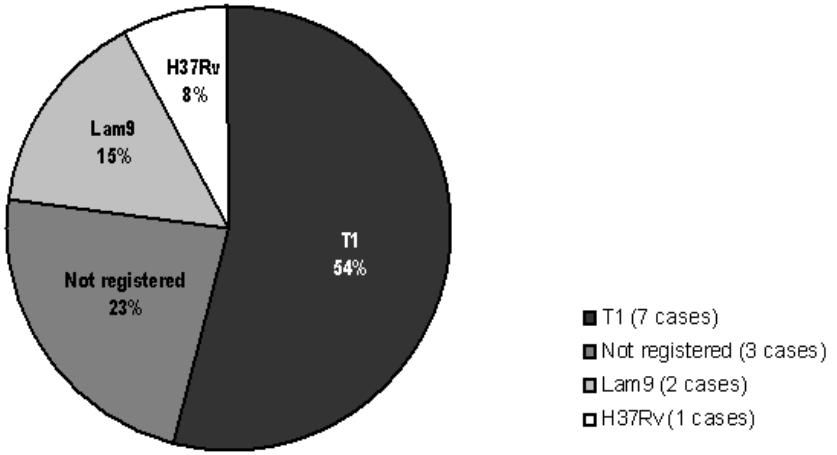
AIDS Cohort (n=72) †



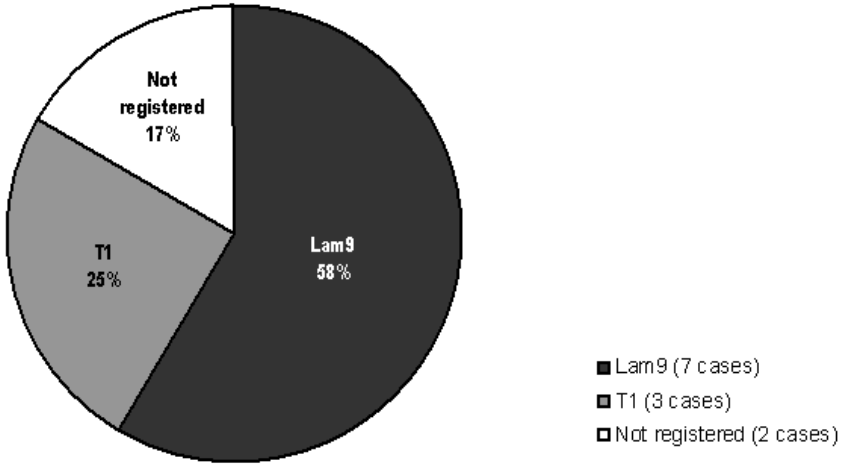
†Five samples had insufficient volume to test

(b) Multi-drug resistant tuberculosis acquired during DOTS treatment ‡

Non-AIDS Cohort (n=13)

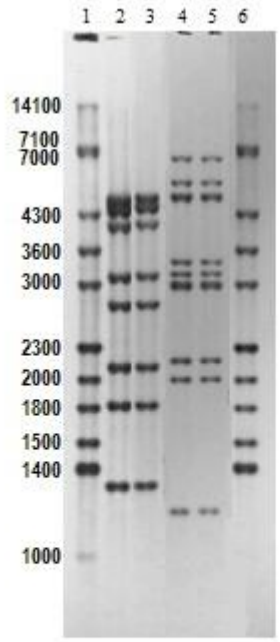


AIDS Cohort (n=12)



‡ Includes both primary and secondary MDR-TB.

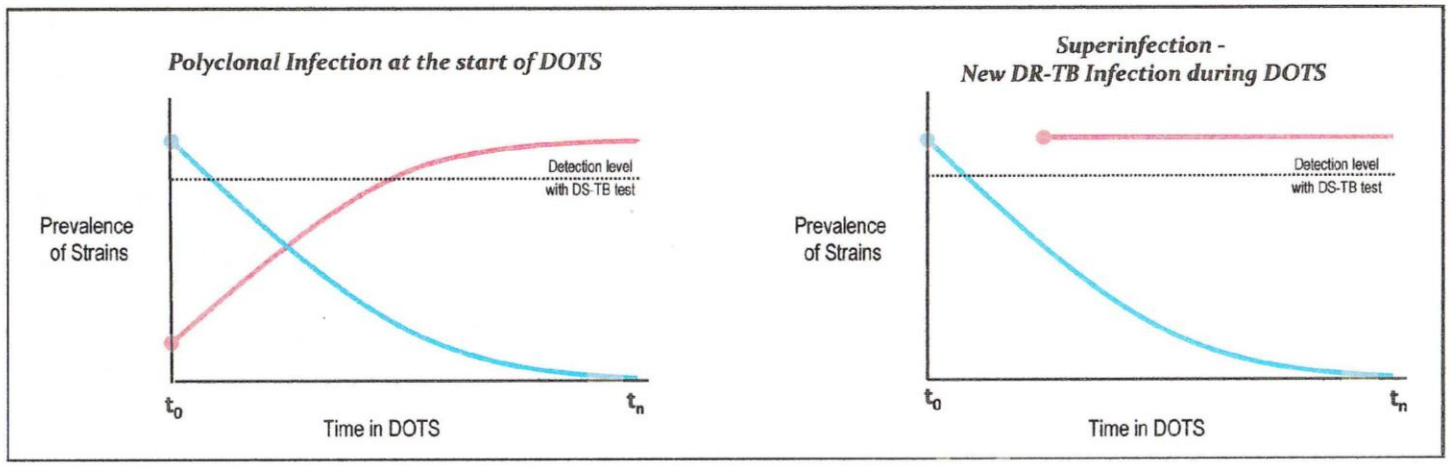
FIGURE 4.5. IS6110 RFLP hybridization pattern for Lam 9 and T1 cluster clones of multi-drug resistant *Mycobacterium tuberculosis* identified in Lima, Peru between 1999 and 2005.



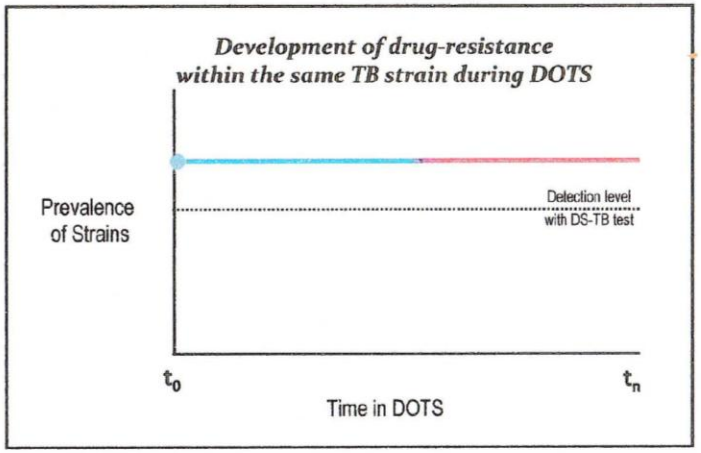
Lanes 1 and 6 indicate the M. tuberculosis 14323 reference strain used as a positive control. Numbers on the left indicate the molecular weight in base pairs (bp). Lanes 2 and 3 indicate the Lam9 cluster clone identified in patients. Lanes 4 and 5 indicate the T1 cluster clone identified in patients.

FIGURE 4.6. Illustration of different types of drug-resistance acquired during DOTS

(a) Primary Drug-Resistance (i.e., infection with a DR-TB strain)



(b) Secondary Drug-Resistance (i.e., development of drug-resistance within a DS-TB strain)



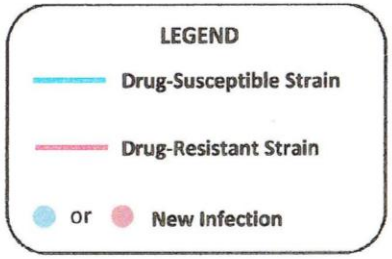


TABLE 4.1. Baseline characteristics of 591 culture-positive patients with complete drug-susceptibility data at the start of DOTS treatment in Lima, Peru between 1999 and 2005.

Characteristic	Non-AIDS Cohort (n=386)	AIDS Cohort (n=205)
Age in years, median (IQR)	25 (20 – 33)	31 (26 – 37)
Male, n (%)	222 (58)	147 (72)
HIV-positive, n (%)	13 (3)	205 (100)
Previously diagnosed with tuberculosis, n (%)	60 (15)	98 (48)
Hospitalized during the previous two years, n (%)	51 (13)	88 (43)

TABLE 4.2.

(a) Proportion of patients with drug-resistant tuberculosis at the start of DOTS treatment for tuberculosis*

Type of Drug Resistance	Number of Patients		Crude Odds Ratio†		
	Non-AIDS Cohort n=64 (DR_{type}/DR_{other})	AIDS Cohort n=104 (DR_{type}/DR_{other})	Point Estimate	90% Confidence Interval	95% Confidence Interval
Isoniazid mono-resistance	26/ 38	16/88	**0.27**	**0.1 – 0.5**	**0.1 – 0.6**
Rifampicin mono-resistance	10/54	16/88	0.98	0.4 – 2.3	0.4 – 2.6
Multi-drug resistance	28/36	72/32	**2.89**	**1.6 – 5.2**	**1.4 – 5.8**

* The odds of presenting each type of drug resistance was calculated by dividing the number of patients who presented with a specific type of drug-resistance (DR_{type}) by the number of patients infected who presented all other types of drug-resistance (DR_{other}).
† The non-AIDS cohort was used as the reference category when estimating the odds ratio.

(b) Incidence rate of acquiring drug-resistance during DOTS treatment for tuberculosis

Type of Drug Resistance	Non-AIDS Cohort (Total: 1174 person-months of observation)		AIDS Cohort (Total: 523 person-months of observation)		Crude Incidence Rate Ratio*		
	Number of patients who acquired DR-TB n=23 (%)	Incidence per 1000 person-months (95% Confidence Interval)	Number of patients who acquired DR-TB n=15 (%)	Incidence per 1000 person-months (95% Confidence Interval)	Point Estimate	90% Confidence Interval	95% Confidence Interval
Isoniazid mono-resistance	9 (39)	7.67 (3 – 14)	1 (7)	1.91 (0 –11)	0.25	0 – 1.5	0 – 1.8
Rifampicin mono-resistance	1 (4)	0.85 (0 – 5)	2 (13)	3.82 (1 – 14)	4.49	0.4 – 130	0.2 – 264
Multi-drug resistance	13 (57)	11.1 (6 – 19)	12 (80)	22.9 (12 – 40)	**2.07**	**1.0 – 4.3**	0.9 – 4.9

* The non-AIDS cohort was used as the reference category when estimating the incidence rate ratio.

TABLE 4.3. Factors associated with infection with the epidemic clone among 91 patients with MDR-TB at the start of DOTS treatment in Lima, Peru between 1999 and 2005.

Clinical Characteristic	Characteristic Present		Characteristic Absent		Likelihood Ratio		Univariate Odds Ratio (95% Confidence Interval)	P Value
	No. of patients infected with epidemic clone	No. of patients infected with other clones	No. of patients infected with epidemic clone	No. of patients infected with other clones	Positive Test (95% Confidence Interval)	Negative Test (95% Confidence Interval)		
Sputum smear staín positive	20	52	3	16	1.14 (0.9 – 1.4)	1.80 (0.6 – 5.6)	2.05 (0.5 – 12)	0.28
HIV-infected	21	44	2	24	**1.41 (1.1 – 1.8)**	4.06 (1.0 – 16)	**5.73 (1.2 – 54)**	**0.01**
Female	3	22	20	46	0.40 (0.1 -1.2)	0.79 (0.6 -1.0)	0.31 (0.1 – 1.2)	0.07
Underweight	10	16	13	52	**1.85 (1.0 – 3.5)**	1.35 (0.9 – 2.0)	2.50 (0.8 – 7.5)	0.07
Clinical Features								
Cough	21	57	2	11	1.09 (0.9 – 1.3)	1.86 (0.4 – 7.8)	2.03 (0.4 – 20)	0.38
Productive Cough	21	58	2	9	1.06 (0.9 – 1.2)	1.55 (0.4 – 6.6)	1.63 (0.3 – 17)	0.55
Fever	16	48	7	19	0.97 (0.7 – 1.3)	0.93 (0.4 – 1.9)	0.90 (0.3 – 3.0)	0.85
Shortness of breath	18	40	5	27	**1.31 (1.0 – 1.8)**	1.85 (0.8 – 4.2)	2.43 (0.8 – 9.3)	0.11
Hemoptysis	5	23	18	44	0.63 (0.3 – 1.5)	0.84 (0.6 – 1.1)	0.53 (0.1 – 1.8)	0.26
Weight Loss	21	53	2	13	**1.14 (1.0 – 1.4)**	2.27 (0.6 – 9.3)	2.58 (0.5 – 25)	0.23
Loss of Appetite	15	42	8	25	1.04 (0.7 – 1.5)	1.07 (0.6 – 2.0)	1.12 (0.4 – 3.5)	0.83
Night Sweats	10	37	13	30	0.79 (0.5 – 1.3)	0.79 (0.5 – 1.2)	0.62 (0.2 – 1.8)	0.33
Fatigue	19	50	4	17	1.11 (0.9 – 1.4)	1.46 (0.6 – 3.9)	1.62 (0.4 – 7.4)	0.44
Diarrhea	9	10	14	58	**2.66 (1.2 – 5.7)**	**1.40 (1.0- 2.0)**	**3.73 (1.1 -12)**	**0.01**
Risk factors for TB								
Previously infected with TB	3	7	20	61	1.27 (0.4 – 4.5)	1.03 (0.9 – 1.2)	1.31 (0.2 – 6.4)	0.72
Contact with TB	10	33	13	35	0.90 (0.5 – 1.5)	0.91 (0.6 – 1.4)	0.82 (0.3 -2.1)	0.68
TB prophylaxis	13	24	10	44	**1.60 (1.0 – 2.6)**	1.49 (0.9 – 2.4)	2.38 (0.8 – 7.0)	0.07
Previously hospitalized	12	46	11	22	0.77 (0.5 – 1.2)	0.68 (0.4 – 1.2)	0.52 (0.2 – 1.5)	0.18

*CI: Confidence Interval

TABLE 4.4. Association between grade of smear status and infection with the Lam9 epidemic clone among 91 patients with MDR-TB at the start of DOTS treatment in Lima, Peru between 1999 and 2005.

Grade of Smear Status	No. of patients infected with epidemic clone		Likelihood Ratio		Univariate Odds Ratio (95% Confidence Interval)	P Value
	Positive	Negative	Positive Test (95% Confidence Interval)	Negative Test (95% Confidence Interval)		
0	3	16	1.0	1.0	1.0	-
1+	8	28	1.41 (0.4 – 4.7)	1.08 (0.8 – 1.4)	1.52 (0.4 – 6.6)	0.57
2++	4	8	2.11 (0.6 – 7.8)	1.26 (0.8 – 2.0)	2.67 (0.5 – 15)	0.26
3+++	8	17	2.03 (0.6 – 6.6)	1.24 (0.9 – 1.7)	2.51 (0.6 – 11)	0.23

CHAPTER 5.

Prevalence and factors associated with shedding of *mycobacteria* in the stool of AIDS patients at different stages in the natural history of tuberculosis

Knowledge Generation

Based on our observation in study #1, that gastrointestinal-related factors were associated with AIDS patients being superinfected with particular clones of MDR-TB during DOTS, for our second study we characterized the prevalence and factors associated with gastrointestinal colonization with tuberculosis among HIV/AIDS patients.

5.1. Study Summary

Background: We characterized the prevalence and factors associated with shedding viable *mycobacteria* in stool, among AIDS patients with tuberculosis. **Methods**: We identified two groups of AIDS patients at different stages in the natural history of tuberculosis [n=30 in the *Early-stage cohort*; n=69 in the *Late-stage cohort*]. Sputum and stool samples were collected from all patients at study entry. Patients in the *late-stage cohort* were also interviewed, had physical exams, and were followed-up during Directly Observed Therapy Short-course for tuberculosis. **Results:** Culture positive *mycobacteria* were isolated from the stool of 45% (13/29) of pulmonary tuberculosis patients in the *early-stage cohort* and 77% (35/46) of pulmonary tuberculosis patients in the *late-stage cohort*. In both cohorts, shedding was more common among those with smear-positive disease (χ^2=2.04, p=0.04), and rarely occurred in the absence of pulmonary tuberculosis, even when patients were diagnosed with extra-pulmonary tuberculosis (χ^2=24.1, p<0.001). Hospitalization during the previous two years was a protective factor for shedding, even after adjusting for patients' history of tuberculosis (Odds Ratio: 0.14, 95% Confidence Interval: 0 to 0.7). **Conclusion**: Shedding of viable *mycobacteria* in stool was common among AIDS patients with pulmonary tuberculosis, particularly among those in the later stages of the disease. Previous hospitalization was a

protective factor against colonization suggesting that treatment for another opportunistic infection may provide indirect health benefits for HIV-infected individuals who develop tuberculosis later on.

5.2. Background:

Tuberculosis is the leading causes of morbidity and mortality among HIV-infected individuals. However, the gastrointestinal tract remains the most common site of opportunistic infections among HIV-infected individuals [45] [110,127]. The elevated risk of infection at this site is often attributed to the gastrointestinal tract being a major site of HIV replication. This eventually causes a depletion of CD4 cells within the lamina propia of the stomach and gastrointestinal tract making the person vulnerable to opportunistic infection [110,127,128]. HAART has helped to decrease the risk of gastrointestinal complications among these patients [110]. Still, gastrointestinal disease continues to be a problem in developing countries where access to HAART may be limited.

Despite a long standing recognition that low CD4 counts can lead to infection and the development of active tuberculosis, few studies have examined the prevalence of gastrointestinal colonization with *mycobacteria* among AIDS patients. *Mycobacterium tuberculosis* has long been recognized as an obligate aerobe that is only transmissible through air. However, the bacteria have also been known to survive the low pH high anoxic conditions found in the stomach [13,129-131]. Several case studies have also reported isolating *M. tuberculosis* in the stool of children and adults with intestinal tuberculosis [132-135]. Gastrointestinal tuberculosis may be an important issue among AIDS patients, since they are more likely to experience extra pulmonary tuberculosis and are at greater risk of developing disseminated forms of the disease during the later stages of active tuberculosis as compared to HIV-negative patients [6].

In this study, we characterized the prevalence of viable *mycobacteria* in the stool of AIDS patients at different stages in the natural history of pulmonary tuberculosis; and identified risk factors for shedding *mycobacteria* among AIDS patients in the later stages of tuberculosis disease.

5.3. Methods:

5.3.1. Study Setting:

Data for this study was collected at the Infectious Disease Clinic of the Hospital Dos de Mayo between March 2002 and January 2004. During this time period, the hospital was the main referral center for HIV-infected individuals living in the greater metropolitan region of Lima, Peru. In 2003, the HIV-seropositivity level in Peru was estimated at 3.4 per 100,000 persons [136], from which 50% of HIV-infected patients developed active tuberculosis during at least one point in their illness [94].

5.3.2. Study Populations:

We identified two groups of AIDS patients at different stages in the natural history of tuberculosis. The first group (*Early-stage cohort*) consisted of AIDS patients who were exhibiting respiratory symptoms at the time of study entry and diagnosed with pulmonary tuberculosis during our study. These patients were identified by screening consecutively enrolled HIV-infected patients who reported coughing during at least one week. The second group (*Late-stage cohort*) consisted of AIDS patients who were already diagnosed with tuberculosis (pulmonary or extra pulmonary form) at the time of study entry; and, were about to commence Directly Observed Therapy Short-Course (DOTS) as part of the Peruvian National Tuberculosis Control Program. None of the study participants in either cohort were receiving tuberculosis treatment at the time of study enrolment. Patients in the *early-stage cohort* were also eligible to participate in the *late-stage cohort*, if they were recommended for tuberculosis treatment. All study participants provided written informed consent. Institutional Review Boards at the Johns Hopkins Bloomberg School of Public Health, Hospital Dos de Mayo and A.B. PRISMA approved the original study protocols.

5.3.3. Data Collection:

For the *early-stage cohort*, patients with a productive or wet cough were asked to provide two sputum, one stool and one urine sample. Sputum samples were collected on consecutive days with all samples being collected within a one-week period. Patients with a non-productive or dry cough were asked to provide one sputum and one stool sample on the same day. Sputum was induced using a hypertonic saline solution. For the *late-stage cohort*, we asked patients to provide one sputum and one stool sample at the time of study enrollment (day 0) as well as day 1, month 1, month 2, month 4, month 6 of DOTS treatment, and once at the end of treatment. They were also measured for weight in kilograms and height in meters; observed for the presence of scars from a Bacille-Calmette Guerin (BCG) vaccine; had blood samples collected for CD4 counts; and interviewed for socio-demographic characteristics, clinical manifestations and history of exposure to tuberculosis (please refer to Appendix 4). Information on diarrheal status was not available for this study.

5.3.4. Laboratory Methods and Definitions:

Sputum samples were tested for smear status using Auramine Smear Microscopy; culture status using Lowenstein-Jensen and Middlebrook 7h9 Broth (Difco, Detroit, Mich.); and drug-susceptibility status using the Microalamar Blue Assay (MABA) [101]. S*tool samples* were tested for smear status using Auramine Smear Microscopy; and were then cultured using Lowenstein-Jensen, Middlebrook 7h9 Broth (Difco, Detroit, Mich.) and Middlebrook 7h10 Agar (Difco, Detroit, Mich.). Drug-susceptibility status in stool samples was assessed using MABA, only when the matching sputum sample from a patient was culture-negative. We categorized samples as drug-resistant if the MABA Minimum Inhibitory Concentration (MIC) was greater than 0.25 µg/mL for rifampicin; 0.25 µg/mL for isoniazid; 5.0 µg/mL for ethambutol; and, 2.0 µg/mL for streptomycin [102]. We classified a sample as drug-resistant if it was resistant to at least one drug; *multi-drug resistant* if it was resistant to both rifampicin and isoniazid; and *culture-positive* if tuberculosis was grown on at least one type of growth media. Patients had *pulmonary tuberculosis* if they produced at least one sputum sample that was culture-positive for tuberculosis.

For patients in the *late-stage cohort*, we conducted spoligotype DNA fingerprinting on the first culture-positive sputum sample collected from each patient. In order to determine whether the strain of tuberculosis found in sputum was the same as that found in stool, we identified and compared spoligotypes octal code numbers for seven pairs of sputum and stool samples. We identified strain genotypes for sputum samples by comparing each isolate's spoligotype octal code number with those recorded in the SpolDB4 database [105]. CD4 lymphocyte counts were obtained from blood samples using the Manual CD4 Cell Count Kit (Coulter).

5.3.5. Data Analysis:

Using data from both the *early* and *late-stage cohorts*, we presented categorical variables as numbers with proportions; and, continuous variables as medians with inter-quartile ranges. We estimated the *culture yield* by dividing the number of samples from which we isolated culture-positive tuberculosis by the total number of samples tested; and *time to culture-positivity* in days by subtracting the date on which growth in a particular culture-media was first observed from the date that the sample was inoculated. We tested for differences in paired means using T-test; differences in proportions using the McNemar's χ^2 test or, when a cell had less than 5 samples or patients, the Fisher's Exact test; and the presence of trends using the χ^2 test for trend. We summarized the extent of concordance and discordance between different types of diagnostic media per sample and different isolates per patient using Venn diagrams. We drew proportional Venn diagrams using Euclidian geometry, with the area of the circle representing the size of the population and the area of intersecting circles representing the degree of overlap between two or more populations.

Using data from the *late-stage cohort*, we estimated the univariate odds ratio using exact logistic regression and the positive and negative likelihood ratio using standard formula for each clinical manifestation reported by patients [107]. We asked patients regarding the presence (or absence) of the following clinical manifestations: cough, productive cough, fever, shortness of breath, hemoptysis, weight loss, loss of appetite, night sweats, and fatigue. We also identified risk factors for shedding *mycobacteria* in stool using an exact logistic

regression model. Our multivariate model included the following covariates: age in years, sex [male or female], Body Mass Index in kilograms per meters^2, CD4 counts in cells per μL, receiving antiretroviral treatment [yes or no], history of prophylaxis for tuberculosis [yes or no]; presence of BCG scar [yes or no/not not known], previous diagnosis with tuberculosis [yes or no/unknown], known contact with a tuberculosis patient [yes or no/unknown], previously worked or lived in a prison/shelter [yes or no], and hospitalized during the previous two years [yes or no]. All statistical analysis were conducted using STATA (College Station, TX).

5.3.6. Role of the Funding Source:

The funding source had no role in study design, data collection, data analysis and data interpretation, writing of the manuscript or the decision to publish.

5.4. Results:

From 2002 to 2004, we screened 473 consecutive respiratory symptomatic patients for pulmonary tuberculosis (*Early-stage cohort*), and enrolled 106 patients referred to the Peruvian National Tuberculosis Control Program for the treatment of tuberculosis (*Late-stage cohort*). Figure 5.1 illustrates the flow of study participants throughout our study. 403 or 85% of patients in the *early-stage cohort* and 72 or 68% of patients in the *late-stage cohort* provided both a sputum and stool sample for testing. 12 patients in the *early-stage cohort* and 3 patients in the *late-stage cohort* provided either contaminated or insufficient volumes of samples to be tested, and were thus excluded from our analysis.

5.4.1. Growth characteristics of sputum, stool and urine samples

We isolated culture-positive tuberculosis in 20% (115/589) of sputum samples, 8% (46/551) of stool samples, and less than 1% (1/124) of urine samples. Of these, 52% (48/115) of sputum isolates, 14% (20/46) of stool isolates, and 0% (0/1) of urine isolates were smear-positive for acid-fast bacillus. Sputum samples were more likely to be smear-positive than stool samples from the same patient (χ^2=12.6, p<0.001). The culture yield from sputum

samples was the same regardless of the type of media used (χ^2=0.50, p=0.73). For stool samples, Middlebrook 7h10 agar yielded the same quantity of culture-positive isolates as Lowenstein-Jensen (χ^2=3.27, p=0.12) but fewer culture-positive isolates than Middlebrook 7h9 broth (χ^2=10.89, p=0.001). We found no difference in the culture yield of stool samples using Lowenstein-Jensen versus Middlebrook 7h9 broth (χ^2=3.77, p=0.09). We also found no difference in time to culture-positivity for sputum and stool samples grown on Lowenstein-Jensen (t-test: 1.15, p=0.25). However, stool samples took longer to culture than sputum samples, when grown on Middlebrook 7h9 broth (t-test: 2.61, p<0.001).

5.4.2. Patients characteristics for shedding mycobacteria in stool

Of the total 460 patients who provided complete sputum and stool samples, 75 (16%) had culture-positive sputum, and 49 (11%) had culture-positive stool (Table 5.1). 64% (48/75) of patients with culture-positive sputum also had culture-positive stool. Figure 5.3 shows that shedding rarely occurred in the absence of pulmonary tuberculosis, even when patients in the *late-stage cohort* were diagnosed with extra-pulmonary tuberculosis. Of the 75 patients with pulmonary tuberculosis, we identified *mycobacteria* in the stool of 72% (21/29) of patients with high-grade (3+) smear-positive disease; 100% (5/5) with medium-grade (2+) smear-positive disease; 62% (10/16) with low-grade (1+) smear-positive disease; and, 48% (12/25) with smear-negative (0-) disease. While patients with smear-positive pulmonary tuberculosis were more likely to shed *mycobacteria* in stool than those with smear-negative disease (χ^2=2.04, p=0.04), we found no trend in stool shedding by grade of smear status (χ^2=6.50, p=0.09). A sub-analysis of paired sputum and stool samples from seven patients in the *late-stage cohort* showed that each pair of samples had matching spoligotype octal code numbers.

We found no difference in shedding (Fisher's Exact Test=0.45, p=0.25) between pulmonary tuberculosis patients in the *early stage cohort* with productive (33% or 4/12) and non-productive cough (53% or 9/17). However, stool shedding was more common among pulmonary tuberculosis patients in the *late-stage cohort* (χ^2=7.54, p=0.006), with 45% (13/29) of pulmonary tuberculosis patients in the *early-stage cohort* and 77% (35/46) of pulmonary tuberculosis patients in the *late-stage cohort* had culture-positive stool. Patients in

the *late-stage cohort* were also more likely to have smear-positive pulmonary tuberculosis as compared to those in the *early-stage cohort* (χ^2=19.65, p<0.001).

The predominant strains of pulmonary tuberculosis among patients in the *late-stage cohort* with stool shedding were T1 (31%), H3 (17%) and T2 (11%). However, each strain was equally present among pulmonary tuberculosis patients who were not shedding [χ^2 for T1: 2.17 (p=0.14), H3: 0.55 (p=0.46), and T2: 0.05 (p=0.83)]. Table 5.2 shows that patients in the *late-stage cohort* with gastrointestinal colonization were less likely to have been hospitalized during the previous two years, after adjusting for a patient's history of tuberculosis. Table 5.3 shows that the most useful factors for identifying patients who may have been shedding included cough and loss of appetite. Of the 33 patients in the *late-stage cohort* who were followed-up during DOTS and had culture-positive stool, 28 (85%) stopped shedding by the first month of treatment. The remaining 5 (15%) patients who did continue to shed were all infected with rifampicin-resistant tuberculosis, and thus may not have been responding to DOTS.

5.5. Discussion

Our cross-sectional study revealed that shedding of viable *mycobacteria* in stool was common among AIDS patients with pulmonary tuberculosis, particularly amongst those in the later stages of the disease. Although stool shedding was more prevalent in pulmonary tuberculosis patients with smear-positive disease, it rarely occurred in the absence of pulmonary tuberculosis even when patients were diagnosed with extra pulmonary disease. Using spoligotype data on a subset of paired sputum and stool samples, we identified that the strain of *M. tuberculosis* isolated from a patient's sputum was identical to the strain isolated in their stool.

Taken together, these results support the hypothesis that when *M. tuberculosis* is swallowed, the bacteria may remain viable in the gastrointestinal tract and eventually be shed in stool. However, it is unclear whether our study results represented a unique phenomenon to AIDS patients in Lima, Peru or immunosuppressed patients as a whole. At the time that

data was collected, the AIDS population were undergoing an outbreak of MDR-TB with one particular clone of *M. tuberculosis;* a clone that was associated with diarrhea.

If shedding is a common event, the high prevalence of viable tuberculosis in patients' stool raises the question as to whether non-airborne transmission of *M. tuberculosis* may be possible in areas with a high prevalence of AIDS. It has long been established that transmission of other *mycobacteria* species may occur through water supply systems, contaminated food and wastewater [137-141]. If the same is true for *M. tuberculosis*, then currently recommended health strategies that only target airborne modes of tuberculosis transmission (e.g. irradiation of air ducts with ultraviolet light) could be less effective in high AIDS-burden settings. These findings also highlight the need to diagnose and treat patients with pulmonary tuberculosis as quickly as possible, regardless of whether they have productive cough or not.

We did find that hospitalization during the previous two years was a protective factor against shedding of *mycobacteria* in stool, even after adjusting for patients' previous history of tuberculosis. Although the reasons for this link are unclear, hospitalization and previous residence in a prison or shelter may have been acting in our study as markers for access to health care. In particular, several studies have suggested that when humoral immune responses against other diarrhea-associated parasites are induced, individuals may be at increased risk of infection with *M. tuberculosis* [111 112 113]. Rapid detection and administration of anti-parasitic treatment could prevent this process from occurring. However, additional research will be needed in order to clarify these issues since study participants were not evaluated for diarrhea in this study.

Our results showed that shedding was more common among patients in the later stages of pulmonary tuberculosis. One explanation for these results is that smear-positive disease was more common among patients in the later stages of pulmonary tuberculosis. Conversely, our regression analysis of risk factors for shedding also revealed that cough and loss of appetite were useful predictors for identifying patients who may be shedding. Although our study screened AIDS patients for respiratory symptoms during the previous 15

days, loss of appetite was not included in our screening criteria. The prevalence of shedding among patients in this cohort may have thus been underestimated.

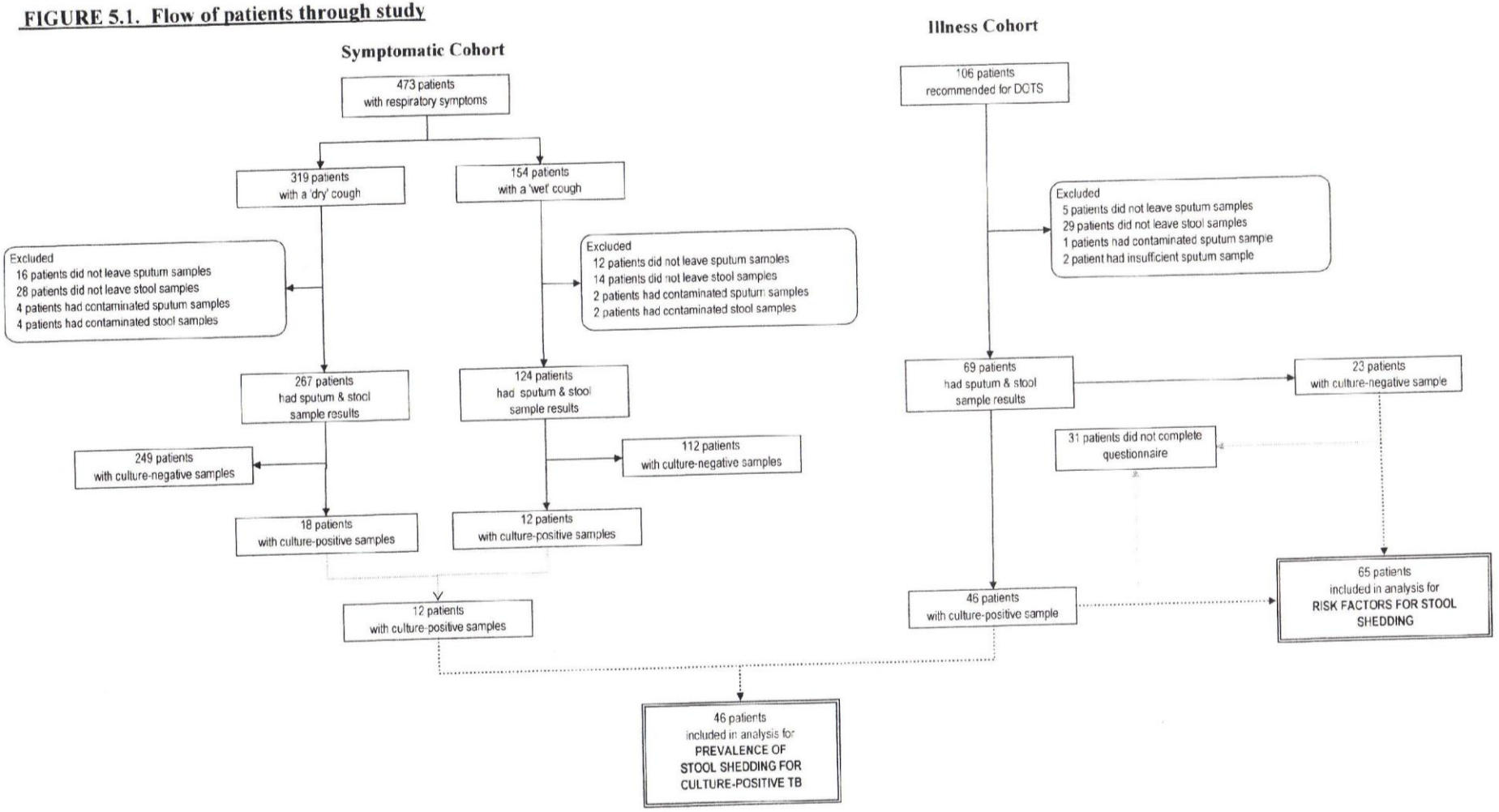
The identification of *mycobacteria* in stool may have important implications for developing tuberculosis diagnostics, particularly among individuals who exhibit non-productive cough. Invasive procedures are often needed to extract sputum from patients with a dry cough. Such procedures can include nasal-gastric aspiration or inducing sputum with hypertonic solutions [142]. Examination of stool rather than sputum could limit the use of such invasive procedures, facilitating the diagnostic process in turn.

Several additional study limitations should be considered. First, our results should not be generalized beyond persons with AIDS. As previously mentioned, the gastrointestinal tract represents a major site of HIV replication and CD4 depletion [110,127,128]. Consequently, the pathophysiology of gastrointestinal colonization with *mycobacteria* may differ between immunosuppressed and otherwise healthy patients. Second, our study may have failed to detect certain factors associated with shedding due to the small sample size. Using an alpha of 0.05 and a beta of 0.80, we estimate that the lowest odds ratio that our model could detect was ≤ 0.60 or ≥ 4.6. Finally, we refer to culture-positive samples in stool as *mycobacteria* rather than *M. tuberculosis* since our study genetically verified the presence of *M. tuberculosis* only in a subset of stool samples. All culture-positive sputum samples were tested using spoligotype.

In conclusion, shedding of *mycobacteria* in stool was common among AIDS patients, but rarely occurred in the absence of pulmonary tuberculosis. Cough and loss of appetite were useful predictors for identifying patients who might be shedding. Previous hospitalization was a protective factor against colonization, suggesting that treatment for another disease may provide indirect health benefits for HIV-infected patients who develop tuberculosis later on.

5.6. Figures & Tables

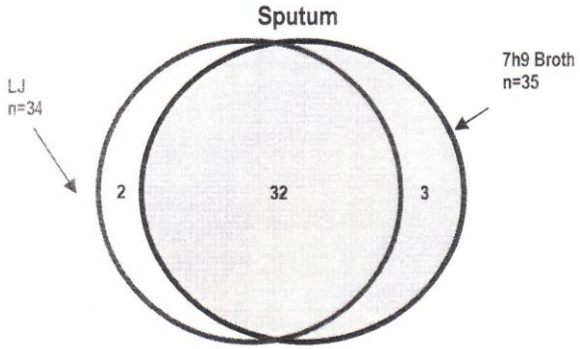
FIGURE 5.1. Flow of patients through study



* 11 Patients with culture-positive sputum

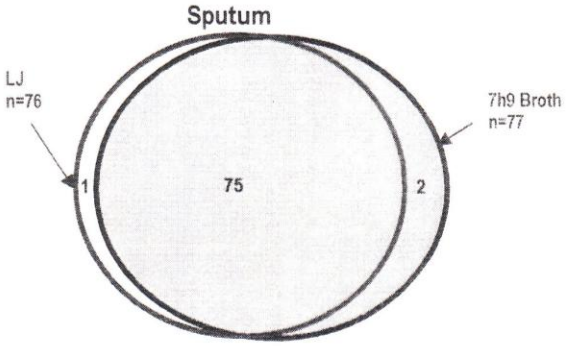
FIGURE 5.2. Proportional Venn diagram showing the type of culture medium used to identify *Mycobacterium tuberculosis* in sputum and stool isolates. Samples with contaminated or missing results in at least one growth media were excluded from Venn diagram analysis.

(a) "Respiratory Symptomatic" Cohort

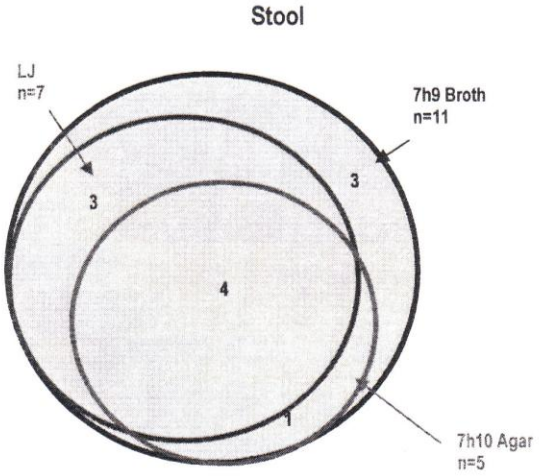


	Number of Culture-Positive Isolates Detected (%)	Median Time to Culture Positivity (IQR)
Middiebrook 7h9 Broth	35 (95)	10 (8 – 12)
Lowenstein-Jensen	34 (92)	30 (22 – 36)
Total	37 (100)	-

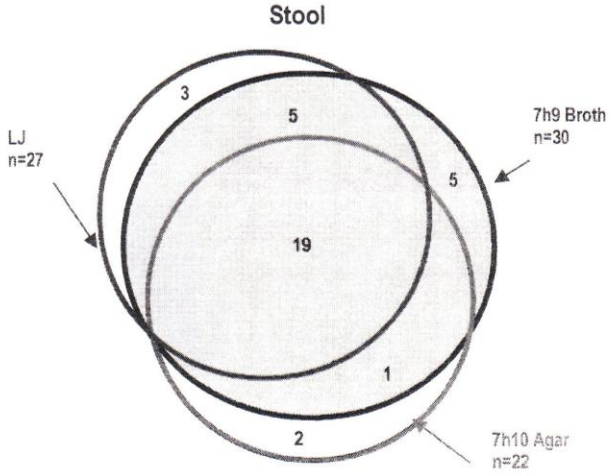
(b) "DOTS Treatment" Cohort



	Number of Culture-Positive Isolates Detected (%)	Median Time to Culture Positivity (IQR)
Middlebrook 7h9 Broth	77 (99)	7 (6 – 9)
Lowenstein-Jensen	76 (97)	21 (18 – 33)
Total	78 (100)	-



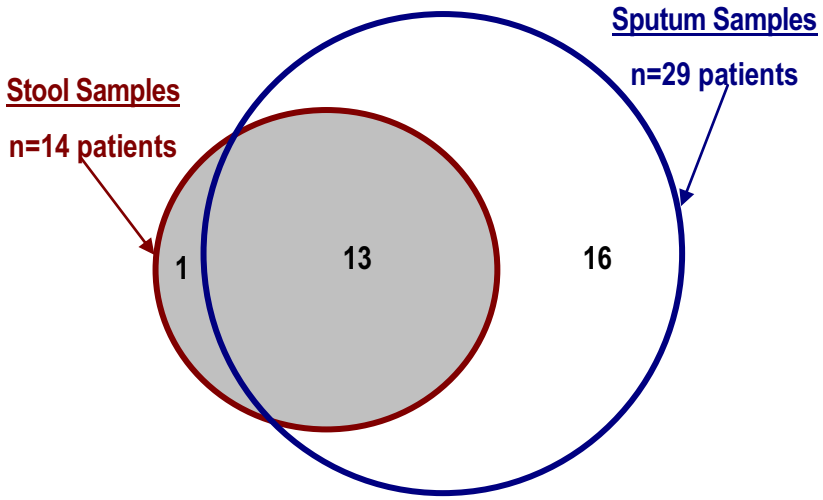
	Number of Culture-Positive Isolates Detected (%)	Median Time to Culture Positivity (IQR)
Middlebrook 7h9 Broth	11 (100)	12 (7 – 14)
Lowenstein-Jensen	7 (64)	22 (19 – 33)
Middlebrook 7h10 Agar	5 (45)	16 (15 – 21)
Total	11 (100)	-



	Number of Culture-Positive Isolates Detected (%)	Median Time to Culture Positivity (IQR)
Middlebrook 7h9 Broth	30 (86)	10 (8 – 13)
Lowenstein-Jensen	27 (77)	29 (24 – 34)
Middlebrook 7h10 Agar	22 (63)	17 (13 – 21)
Total	35 (100)	

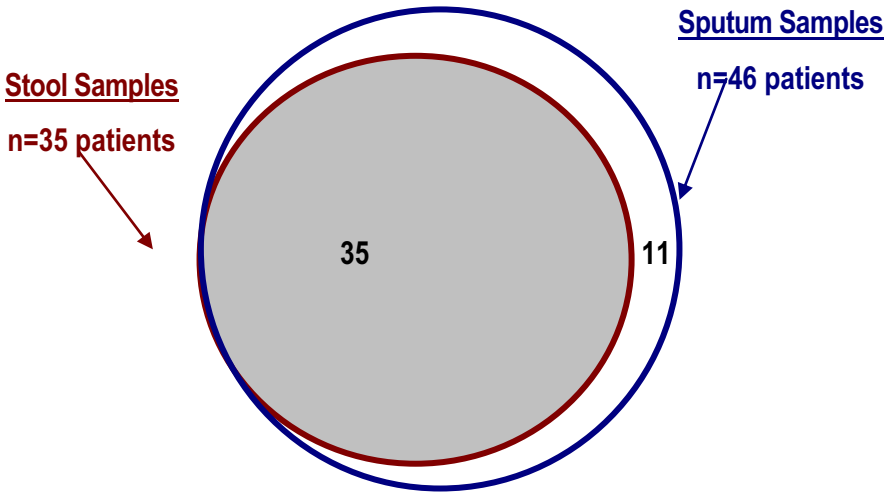
FIGURE 5.3. Proportional Venn diagram showing the type of isolate used to identify patients with culture-positive tuberculosis

(a) "Early-Stage" Cohort (Total number of Patients= 391)



Pr {Shedding in stool | Pulmonary tuberculosis}:	45% (13/29)
Pr {Shedding in stool | No pulmonary tuberculosis}:	0.3% (1/362)
Pr {Pulmonary tuberculosis | Shedding in stool}:	93% (13/14)

(b) "Late-stage" Cohort (Total number of Patients= 69)



Pr { Shedding in stool | Pulmonary tuberculosis}:	77% (35/46)
Pr {Shedding in stool | No pulmonary tuberculosis}	0% (0/23)
Pr {Pulmonary tuberculosis | Shedding in stool}:	100% (35/35)

TABLE 5.1. Number and proportion of AIDS patients with culture-positive tuberculosis in sputum, stool and urine isolates.

Type of Isolate	Early-stage Cohort			Late-stage Cohort n (%)	Overall n (%)
	"Wet Cough" n (%)	"Dry Cough" n (%)	Overall n (%)		
Sputum	12 (9.7)	17 (6.4)	29 (7.4)	46 (67)	75 (16)
Stool	4 (3.2)	10 (3.7)	14 (3.6)	35 (51)	49 (11)
Urine	1 (0.8)	-	-	-	-
Sputum or Stool	12 (9.7)	18 (6.7)	30 (7.7)	46 (67)	76 (17)
Total Number of AIDS Patients	*124 (100)*	*267 (100)*	*391 (100)*	*69 (100)*	*460 (100)*

TABLE 5.2. Diagnostic validity of clinical manifestations for identifying DOTS patients who were shedding tuberculosis in stool

Clinical manifestation	No. of patients without clinical manifestation		No. of patients with clinical manifestation		Likelihood Ratio		Univariate Odds Ratio (95% Confidence Interval)
	Not Shedding	Shedding	Not Shedding	Shedding	Positive Test (95% Confidence Interval)	Negative Test (95% Confidence Interval)	
Cough	15	6	16	28	**2.23 (1.1 – 4.5)**	**1.96 (1.2 – 3.2)**	**4.38 (1.3 – 16)**
Productive Cough	15	8	16	26	1.78 (0.9 – 3.3)	**1.71 (1.0 – 2.8)**	3.05 (0.9 – 10)
Fever	19	13	12	21	1.57 (0.9 – 2.6)	1.63 (0.9 – 2.8)	2.56 (0.8 – 7.9)
Shortness of breath	14	10	17	24	1.41 (0.8 – 2.4)	1.41 (0.9 – 2.3)	1.98 (0.6 – 6.3)
Hemoptysis	27	30	4	4	0.95 (0.4 – 2.0)	0.95 (0.4 – 2.0)	0.90 (0.2 – 5.4)
Weight loss	6	3	25	31	1.67 (0.6 – 4.3)	1.49 (0.9 – 2.6)	2.48 (0.5 – 17)
Loss of appetite	16	8	15	26	**1.90 (1.0 – 3.5)**	**1.82 (1.1 – 3.0)**	**3.47 (1.1 – 12)**
Night sweats	19	14	12	20	1.47 (0.9 – 2.4)	1.54 (0.9 – 2.6)	2.26 (0.8 – 6.9)
Fatigue	13	9	18	24	1.40 (0.8 – 2.5)	1.38 (0.8 – 2.3)	1.93 (0.6 – 6.3)

TABLE 5.3. Risk factors for shedding tuberculosis in stool among 65 patients in the later stage of tuberculosis in Lima, Peru between 1999 and 2005

Characteristic	No. of Patients Not Shedding TB (n=31)	No. of Patients Shedding TB (n=34)	Unadjusted Odds Ratio (95% CI)	Adjusted Odds Ratio (95% CI)
Age in years	29 (25 – 38)	31 (27 – 33)	0.98 (0.9 – 1.1)	1.06 (0.9 – 1.2)
Gender				
Female	8	5	Ref	Ref
Male	23	29	2.01 (0.6 – 7.0)	5.82 (0.9 – 36)
Body Mass Index in kilograms per meters²	19.2 (18 – 21)	18.6 (17 – 20)	**0.79 (0.6 – 1.0)**	0.89 (0.6 – 1.2)
CD4 Count in cells/μL	<30* (<30 – 68)	<30 (<30 – 40)	0.99 (0.9 – 1.0)	0.99 (0.9 – 1.0)
Receiving anti-retroviral treatment				
No	22	29	Ref	Ref
Yes	9	5	0.42 (0.1 – 1.4)	0.50 (0.1 – 2.7)
Past prophylaxis for tuberculosis				
No	13	13	Ref	Ref
Yes	18	21	1.17 (0.4 – 3.2)	1.16 (0.2 – 6.6)
Presence of BCG Scar				
No/Not known	6	9	Ref	Ref
Yes	25	25	0.66 (0.2 – 2.2)	0.91 (0.2 – 4.3)
Previously diagnosed with tuberculosis				
No/ Not known	21	24	Ref	Ref
Yes	10	10	0.88 (0.3 - 2.5)	0.94 (0.2 – 5.5)
Known contact with tuberculosis case				
No/Not known	22	22	Ref	Ref
Yes	9	12	1.33 (0.5 – 3.8)	2.25 (0.5 – 11)
Previously in prison/shelter				
No	22	33	Ref	Ref
Yes	9	1	**0.07 (0 – 0.6)**	0.15 (0 – 2.4)
Hospitalized during the previous two years	17	27	Ref	Ref
No	14	7	**0.32 (0.1 – 0.9)**	**0.14 (0 – 0.7)**
Yes				

* CD4 counts could not be measured below 30 cells/μL. We thus substituted all values equal to "<30" by 29 in our model.

CHAPTER 6.

Screening HIV-infected patients for pulmonary tuberculosis using the Microscopic Observation Drug Susceptibility (MODS) assay: A pilot study

Knowledge Integration/Technology Transfer

Our previous two studies illustrated the importance of screening HIV/AIDS for pulmonary tuberculosis regardless of whether they had productive or non-productive cough. In light of this data and the potential for nosocomial transmission of pulmonary tuberculosis among AIDS patients, in the following study we evaluated the utility of MODS for culture-based screening of hospitalized HIV-infected patients.

6.1. Study Summary

Background: To examine the clinical utility when using the Microscopic-Observation Drug-Susceptibility (MODS) assay to screen hospitalized HIV-infected patients for pulmonary tuberculosis. **Methods**: Consecutive newly hospitalized HIV-positive patients not previously diagnosed with tuberculosis (n=150) at two public hospitals in Lima, Peru, were recruited and prospectively screened for pulmonary tuberculosis using clinical assessment, chest radiography and sputum smear microscopy. Sputum and/or gastric samples were cultured for *Mycobacterium tuberculosis* using MODS, Lowenstein-Jensen and automated MbBacT. **Results**: We found that 19% (28/150) of patients had pulmonary tuberculosis. Screening all admissions with sputum microscopy would have detected 39% (95%CI: 32-47) of patients with tuberculosis. Standard diagnostic criteria identified 32% (95%CI: 16-52) of patients with tuberculosis. Screening all patients using MODS correctly identified 96% (95%CI: 82-100) of patients with tuberculosis, and provided concurrent drug-susceptibility results. Alternatively, targeting MODS testing to the 63% (95/150) of patients who were sputum smear-positive or smear-negative with clinically suspicious symptoms for tuberculosis, detected 93% (95%CI: 77-100) of patients with pulmonary tuberculosis, with 93% (14/15) of these cases being identified within two weeks of admission. **Conclusion**: Sputum smear-

negative tuberculosis was common in newly hospitalized HIV-positive patients. Targeted culture-based screening with universal drug-susceptibility testing using MODS accurately and rapidly detected these cases.

6.2. Background

Globally, tuberculosis is the most common opportunistic infection affecting HIV-infected individuals, and can be particularly difficult to control for in hospitals caring for AIDS patients in resource-limited settings. Although nosocomial transmission to health-care workers and other susceptible patients can be prevented by early case detection and administration of effective drug therapy [87,143,144], diagnosing HIV-infected patients with pulmonary tuberculosis is an important challenge. HIV modifies the typical clinical presentation of tuberculosis, decreasing the diagnostic sensitivity of chest radiographs and sputum microscopy [22,23].

In developing countries, where 95% of tuberculosis cases occur, diagnosis is further complicated by the limited availability of sensitive culture-based laboratory diagnostics [7]. Barriers to using these tests include complexity, high capital and unit costs, and prolonged test duration [19,145]. These diagnostic difficulties increase morbidity and mortality, and permit continuing tuberculosis transmission in a hospital setting [35]. Modeling dynamics of tuberculosis transmission also suggest that improving diagnostic capability for tuberculosis among adult populations with a high HIV prevalence may reduce tuberculosis prevalence and mortality in the general community by approximately 20% [145].

The World Health Organization has identified the development of rapid, sensitive, and affordable diagnostic tools for tuberculosis detection as a research priority [146,147]. In recent years, the non-proprietary Microscopic-Observation Drug-Susceptibility (MODS) technique has been shown to detect *Mycobacterium tuberculosis* and its drug-susceptibility status in sputum samples more rapidly and efficiently than current standards of diagnosis yet at one-tenth the cost ($2) of gold-standard rapid assays [60,61,63,64,69].

In this paper, we examine how well the laboratory performance of MODS for detecting tuberculosis in sputum samples translates into clinical usefulness when screening

hospitalized HIV-infected patients for active pulmonary tuberculosis disease. Firstly, we evaluate how screening hospitalized patients using the standard diagnostic criteria of sputum microscopy, symptom review and chest radiography, compares with an alternative approach of using MODS to screen all patients. Second, considering the need for immediate diagnosis and the limited resources that exist in most high HIV-TB burden settings, we analyze whether MODS screening could be targeted to pre-selected sub-groups of patients. Finally, in order to determine the wider generalizability of these results we model the effect that tuberculosis prevalence would have on the diagnostic efficiency of targeting screening using MODS.

6.3. Study Population & Methods

The study was conducted at two large public hospitals (Hospital Arzobispo Loayza and Hospital Hipolito Unanue) in Lima, Peru, between March 2003 and June 2004 as part of an operational evaluation of MODS [63]. Regardless of clinical diagnostic suspicions, all adult (≥18 years) admissions to hospital who were known to be HIV-infected were consecutively recruited. Patients unwilling or unable to give consent or already diagnosed with tuberculosis were excluded. Patients who provided written informed consent underwent chest radiography, venesection for CD4 count, and an interview recording demographic characteristics, medical history, and tuberculosis-associated risk factors (please refer to Appendix 5).

6.3.1. Sample Testing:

Two sputum samples were collected on consecutive days. Morning (pre-breakfast) gastric washings were obtained from patients who could not produce sputum. All samples were processed in the research laboratory at Universidad Peruana Cayetano Heredia as described [148,149]. Briefly, samples were decontaminated by the NaOH n-acetyl cysteine method and then tested for acid fast bacilli using auramine sputum smear microscopy, and cultured in parallel using Löwenstein-Jensen slants, automated MbBacT broth culture (Biomériux), and MODS technique. Culture-positive samples were tested for drug-susceptibility to isoniazid, and

rifampicin using MbBacT, Löwenstein-Jensen proportion method [63], MODS and the Micro-Alamar Blue Assay (MABA), with discrepant results for drug-susceptibility tests being interpreted as previously described [102]. Laboratory technicians were blinded to the results of other test methods. Results were reported to the attending physician for use in patient management.

6.3.2. Definitions:

A patient was classified as having pulmonary tuberculosis if *M. tuberculosis* was isolated from sputum in at least one culture media. An infectious disease specialist blinded to clinical and study data but aware that all patients were HIV-infected graded chest radiographs on a five-point scale. A radiograph was considered to be *"consistent"* with tuberculosis if it was graded as possible or likely tuberculosis (e.g. presence of pleural lesions, cavitation, infiltrates or hilar enlargement), and *"not consistent"* with tuberculosis if it was normal, minimally abnormal but not consistent with pulmonary tuberculosis in an HIV-positive person (e.g. hyperinflation), or clearly abnormal but with a definite diagnosis other than tuberculosis (e.g., known hydatid cyst) [150].

Patients were considered *at increased risk for tuberculosis* if they had been previously treated for tuberculosis, been in contact with a known tuberculosis case, worked in a health care facility or worked/resided in a penitentiary. Patients were considered *at risk for drug-resistant tuberculosis* if they had been previously treated for tuberculosis or been in known contact with an MDR-TB case. Patients were categorized as having *constitutional symptoms of tuberculosis* if they reported fever, night sweats or weight loss at the time of study entry; *respiratory symptoms of tuberculosis* if they experienced coughing; and, *prolonged cough* if they experienced coughing for the last 15 days or more. Patients with cough and at least one constitutional symptom of tuberculosis (i.e., fever, night sweats or weight loss) were categorized as having symptoms *clinically suspicious* of pulmonary tuberculosis. *Standard diagnostic criteria* refer to the use in these hospitals of clinically suspicious symptoms, sputum microscopy, and chest radiography for diagnosing pulmonary tuberculosis (figure 6.1).

6.3.3. Statistical analysis:

The number and proportion of HIV-infected patients who were identified with pulmonary tuberculosis were calculated for each screening strategy. Sensitivity and specificity with 95% confidence intervals (95%CI) were estimated for each screening factor [151]. For both screening strategies that used MODS, we estimated the effect of tuberculosis prevalence on the proportion of patients who would need MODS assays. The footnote to Figure 6.3 defines the sensitivity and specificity parameters used in the model. Analysis was performed using STATA v7 (Stata Corp, College Station, TX, USA). All tests were two-sided and p-value<0.05 was considered to be the threshold for statistical significance.

6.3.4. *Ethics review:*

Institutional review boards at AB PRISMA (Peru), Universidad Peruana Cayetano Heredia (Peru), Hospital Arzobispo Loayza (Peru), Hospital Hipolito Unanue (Peru), Imperial College London (UK) and Johns Hopkins Bloomberg School of Public Health (USA) approved the study.

6.4. Results

6.4.1. Patient recruitment and characteristics:

Between March 2003 and June 2004, 184 patients who met the entry criteria were identified at the two hospitals. Twenty-five of these individuals either died before study procedures could be initiated or were unwilling or unable to provide written informed consent. The remaining 159 eligible patients contributed a total of 296 samples: 267 were sputum and 29 were obtained by gastric lavage. Subjects were excluded from analysis if: no sputum or gastric samples were collected for testing (n=2); if all sputum cultures were repeatedly contaminated by bacterial/fungal overgrowth in all cultures (n=1); or if chest radiographic information was missing (n=6, all were culture-negative by MODS, MbBacT and Löwenstein-Jensen). The study analysis was based upon the remaining 150 patients who provided 284 samples.

Table 6.1 shows the demographic, radiographic, clinical and risk factor data for this population. On admission, 67% (101/150) of patients reported cough, 75% (112/150) weight loss, 54% (81/150) fever, and 39% (59/150) night sweats. Ninety-three percent (139/150) of patients provided two samples on two separate days, the remainder provided only one sample. Ninety-four percent (141/150) of patients had a CD4 count ≤200 cells/μL, 89% (125/150) of patients had a CD4 count <100 cells/μL, and 69% (104/150) of patients had a CD4 count <50 cells/μL. Twenty-eight patients with culture-positive pulmonary tuberculosis were identified using Löwenstein-Jensen, MbBacT or MODS (18%). Seventy-nine percent (22/28) of tuberculosis patients had concordant culture-positive results by all three methods, 11% (3/28) were culture-positive by MODS and either MbBacT or Löwenstein-Jensen, 3.6% (1/28) were culture-positive only by MbBacT, and 7.1% (2/28) were culture-positive only by MODS.

6.4.2. Screening using Standard Diagnostic Criteria:

Figure 6.1(a) shows that screening all patients with sputum smear microscopy only identified 39% (95%CI: 32-47) of culture-positive patients. Alternatively, the policy of using smear microscopy and chest x-rays among patients with symptoms suspicious for tuberculosis, failed to identify 68% (95%CI: 48-84) of patients with tuberculosis, and falsely identified 2% (95%CI: 0-6) of patients as having tuberculosis (figure 6.1(b)). Table 6.1 shows that clinical suspicion (defined as any cough and at least one constitutional symptom) was the best predictor of tuberculosis. However, in general all clinical characteristics had poor reliability for identifying patients with pulmonary tuberculosis.

6.4.3. Culture-based Screening using MODS:

Figure 6.1(c) shows that by using MODS to screen all participants for pulmonary tuberculosis 96% (95%CI 82-100) of patients with pulmonary tuberculosis were identified. This includes 95% (95%CI: 74-100) of the patients with pulmonary tuberculosis who were misdiagnosed by standard diagnostic criteria. None of the hospitalized patients would have completed this screening process on the same day. MODS culture of the second sputum

sample from each smear-negative patient only contributed one additional tuberculosis diagnosis compared with the first MODS culture from each patient. Median days to detect patients with tuberculosis was 6 days (Interquartile Range (IQR): 6-8) for MODS, 15 days (IQR: 12-17) for MbBacT, and 28 days (IQR: 23-24) for Löwenstein-Jensen.

Figures 6.1(d) and illustrates that targeting MODS testing to the 63% (95/150) of patients who were sputum smear-positive or smear-negative with clinically suspicious symptoms for tuberculosis, detected 93% (95%CI: 77-100) of patients with pulmonary tuberculosis and 100% (95%CI: 97-100) who were culture-negative. Although this type of targeted screening missed one patient with tuberculosis, 37% (55/150) fewer patients had to be tested with MODS, enabling them to complete screening on the same day.

We evaluated the generalizability of these results to other settings by modeling the effect of tuberculosis prevalence on the number of MODS tests done, using each screening strategy. Figure 6.3 shows that targeted MODS screening would require significantly fewer MODS assays than universal MODS screening provided that the TB prevalence was lower than 70%.

6.4.4. Drug Susceptibility Testing

Fifty-four percent (15/28) of pulmonary tuberculosis patients had drug-resistant tuberculosis: 18% (5/28) resistant to isoniazid; 3.6% (1/28) to rifampicin; 36% (10/28) to streptomycin; and 0% (0/28) to ethambutol. 3.6% (1/28) of patients had MDRTB. Targeting drug-susceptibility testing to the 25% (38/150) of patients with recognized risk factors for MDR-TB failed to identify 80% (4/5) of patients with isoniazid and/or rifampicin resistant tuberculosis and 93% (14/15) of all patients with drug resistance.

6.5. Discussion

In this group of unselected HIV-positive people admitted to hospital, undiagnosed pulmonary tuberculosis was common and usually missed by screening strategies based on sputum microscopy, symptoms and chest radiographs. In contrast, sputum culture with MODS was more reliable and provided rapid concurrent drug-susceptibility testing. MODS was most

efficient when restricted to patients who reported symptoms clinically suspicious for tuberculosis at the time of admission. Mathematical modeling suggested that this increased efficiency from targeting MODS would be greatest in settings with lower tuberculosis prevalence.

Previous studies have shown that duplicate sputum microscopy, chest radiography and symptomatology fail to detect a large proportion of HIV-associated pulmonary tuberculosis cases [152]. In particular, patients who have CD4 counts <200 cells/μL or more advanced stages of HIV-infection, have a greater risk of sputum microscopy-negative tuberculosis [153] and atypical chest radiographs [6,154]. This is important for patient morbidity and because smear-negative tuberculosis contributes to tuberculosis transmission [18]. The data reported here characterized this problem and demonstrated that a single sputum culture with MODS was a more reliable screening strategy for hospitalized HIV-infected patients.

Our study also supports the use of targeted culture-based screening in combination with sputum smear microscopy and clinical symptoms in HIV-positive patients. The microbiological performance of the MODS technique has been well-defined in comparison with traditional egg-based culture and automated tests [148,149,155]. This study expands on these findings by documenting that most HIV-positive patients with pulmonary tuberculosis can be identified when MODS is used with sputum smear microscopy, and that screening with MODS was almost as effective when targeted only towards sputum microscopy-negative patients with cough and constitutional symptoms at the time of hospital admission.

We also found that screening HIV-positive hospital admissions for tuberculosis without culture misclassified the majority of patients with pulmonary tuberculosis as being free from disease. These results reveal that in addition to facilitating early case detection and administration of appropriate treatment of tuberculosis disease, rapid, sensitive tuberculosis diagnostics also have potential importance in 'ruling-out' tuberculosis disease so that HIV-positive patients may safely receive isoniazid preventive therapy.

Although the sample size for our current study was small, drug resistance was common and without rapid drug-susceptibility testing, most patients with drug-resistant tuberculosis in our study would have received inappropriate empiric tuberculosis treatment.

Moreover, because most drug resistance was not associated with recognized risk factors for drug-resistant tuberculosis, targeted screening for drug-susceptibility status would have been of limited benefit. In settings with a high prevalence of drug-resistant tuberculosis, universal MODS testing of both sputum smear positive and negative patients with clinical suspicion for tuberculosis may be the most appropriate screening strategy needed in order to provide early appropriate drug therapy.

One limitation to the current study is that detection of extra-pulmonary tuberculosis using MODS was not investigated. While extra-pulmonary tuberculosis is associated with a very low risk of transmission, it still causes morbidity and mortality [156,157]. A study in 2002 by Corey et al. [158], characterizing the natural history of untreated HIV-infection among patients in Lima, Peru found that 40% of AIDS patients had tuberculosis. Although our study identified 19% of AIDS patients with TB, this is likely due to the fact that we were only looking for pulmonary TB rather than extrapulmonary TB (a more common form of TB among AIDS patients).

An additional limitation is that given the number of smear-negative culture-positive patients is small, our screening results may require further validation in a second, independent dataset or study before clinical recommendations can be confidently made.

In conclusion, this research demonstrates that active pulmonary tuberculosis was common among hospitalized HIV-infected patients and was usually missed by screening based on smear microscopy, chest radiography and clinical symptoms. Screening sputum microscopy-negative patients with MODS identified the great majority of pulmonary tuberculosis allowing early potentially curative therapy. Targeted MODS screening to only those patients with symptoms consistent with tuberculosis had similar sensitivity and greater efficiency. Screening HIV-positive hospital patients with MODS has the potential to reduce TB-related morbidity, mortality and nosocomial tuberculosis transmission.

6.6. Figures & Tables

Table 6.1. Utility of clinical symptoms when screening for pulmonary tuberculosis among all hospitalized HIV-infected patients and those who are sputum smear microscopy-negative

Figure 6.1. Diagnostic algorithms for screening pulmonary tuberculosis among hospitalized HIV-infected patients using MODS

Figure 6.2. Sensitivity, specificity and proportion of patients who completed screening process on same day, for each screening strategy used to detect pulmonary tuberculosis

Figure 6.3. Percentage of HIV-positive patients screened with MODS using each strategy, by hospital prevalence of pulmonary tuberculosis

Table 6.1. Utility of clinical symptoms when screening for pulmonary tuberculosis among all hospitalized HIV-infected patients and those who are sputum smear microscopy-negative

Clinical Symptom	Number of patients with clinical symptom			Diagnostic value in all patients		Diagnostic value in smear-negative patients	
	Culture Negative (n=122)	Culture Positive (n=28)	Culture-positive Smear Negative (n=17)	Sensitivity (95% CI)	Specificity (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)
Smear-microscopy positive	0	11	-	39 (22 – 59)	100 (97 – 100)	-	-
Chest radiograph consistent with tuberculosis	119	6	1	21 (8 – 41)	98 (93 – 100)	6 (0 – 29)	98 (93 – 100)
Cough	77	24	16	86 (80 – 91)	37 (29 – 45)	94 (90 – 98)	37 (29 – 45)
Prolonged Cough	38	28	7	43 (35 – 51)	69 (61 – 76)	41 (33 – 49)	69 (61 – 77)
Productive Cough	81	21	11	75 (68 – 82)	34 (26 – 41)	65 (57 – 73)	34 (26 – 42)
Fever	60	21	12	75 (68 – 82)	51 (43 – 59)	71 (63 – 78)	51 (42 – 59)
Night Sweats	44	15	10	54 (46 – 62)	64 (56 – 72)	59 (51 – 67)	64 (56 – 72)
Weight Loss	89	23	15	82 (76 – 88)	27 (20 – 34)	88 (83 – 94)	27 (20 – 34)
Clinical Suspicion of pulmonary tuberculosis	68	24	16	86 (80 – 91)	44 (36 – 52)	94 (90 – 98)	44 (36 – 52)

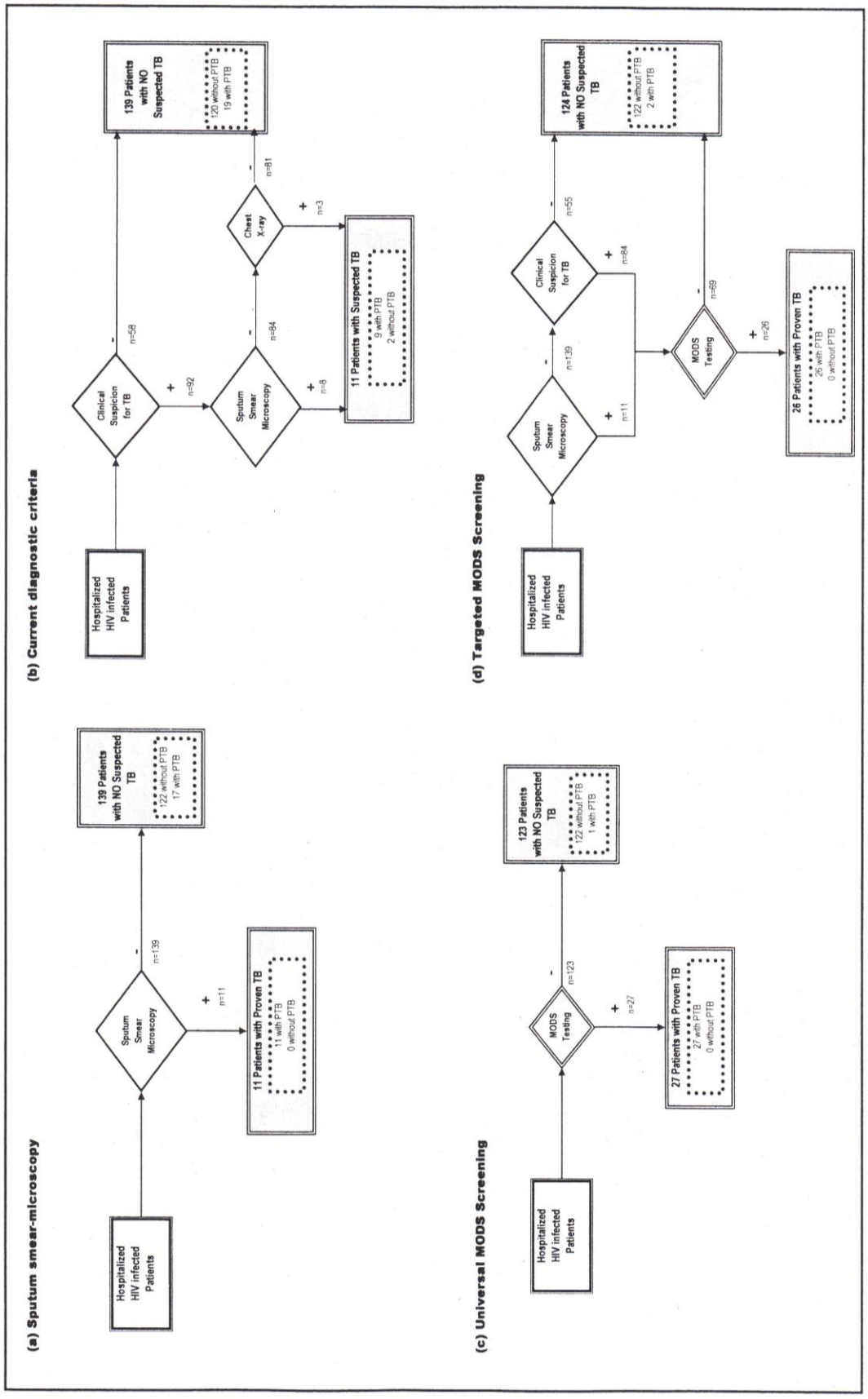


Figure 6.1. Diagnostic algorithms for screening pulmonary tuberculosis among hospitalized HIV-infected patients using MODS

Figure 6.2. Sensitivity, specificity and proportion of patients who completed screening process on same day, for each screening strategy used to detect pulmonary tuberculosis

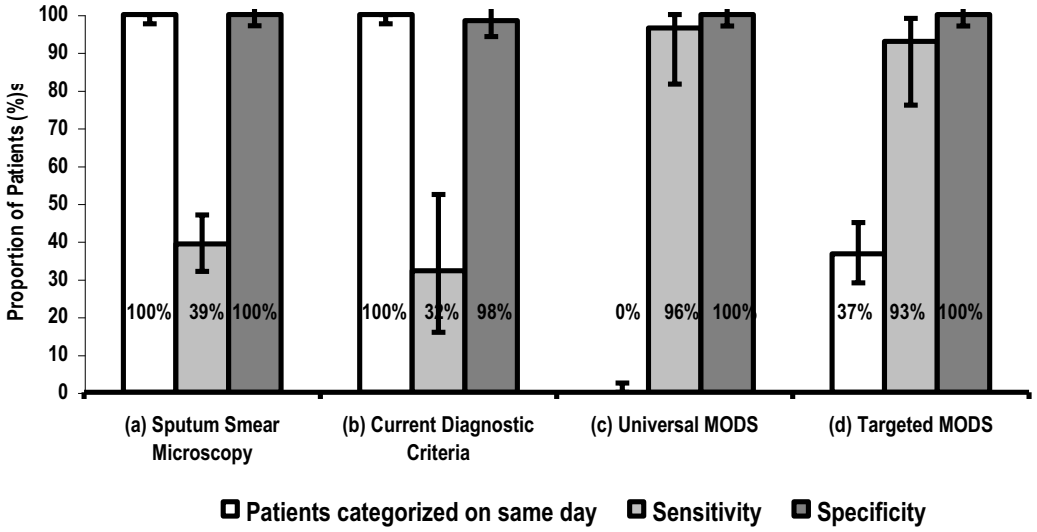
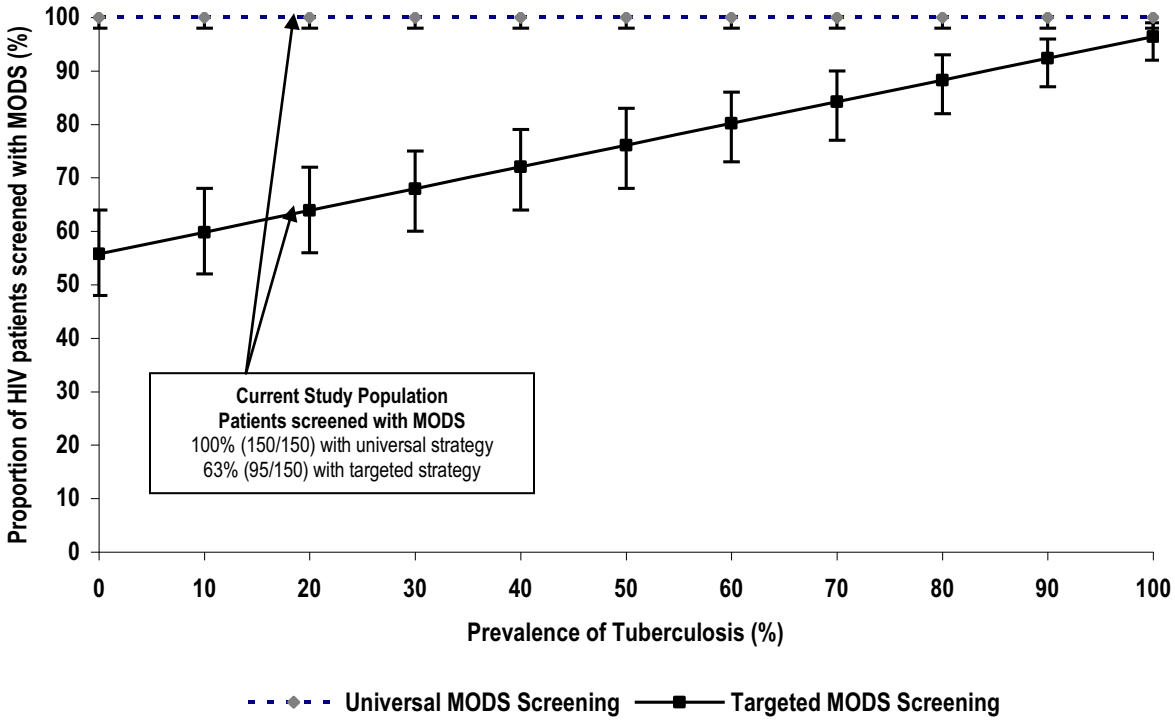


Figure 6.3. Percentage of HIV-positive patients screened with MODS using each strategy, by hospital prevalence of pulmonary tuberculosis*



* ***Universal MODS screening*** assumed that all patients would be tested using MODS.
Targeted MODS screening assumed that sputum microscopy had a sensitivity of 39% (95%CI: 22 to 59) and a specificity of 100% (95%CI: 98 to 100), and cough among sputum microscopy-negative patients had a sensitivity of 94% (95%CI: 90 to 98) and a specificity of 44% (95%CI: 36 to 52).

CHAPTER 7.

Critical Concentrations for detecting drug-susceptibility status of *Mycobacterium tuberculosis* using the Microscopic Observation Drug Susceptibility Assay: A laboratory-based study

Technology Development

As previously mentioned, MODS can be used to detect both culture and drug-susceptibility status of tuberculosis. While its diagnostic efficiency for identifying rifampicin and isoniazid resistance is superior, for other first line drugs its validity remains sub-optimal. In this final study, we aimed to improve the diagnostic validity of MODS for phenotypic detection of ethambutol and streptomycin drug resistance, particularly in its low-grade resistant form (also known as "intermediate" drug susceptibility status).

7.1. Study Summary

Background: The MODS assay is a rapid method for diagnosis and drug-susceptibility testing of *Mycobacterium. tuberculosis*. The validity of MODS for ethambutol and streptomycin drug-susceptibility testing has been poor relative to other first-line drugs such as rifampicin and isoniazid. This study aimed to improve its validity by defining the optimal critical concentration needed for detecting drug resistance. **Methods**: We tested 48 pre-frozen sputum samples using the MODS method with concurrent serial 1:2 dilutions of ethambutol and streptomycin solutions in standard 7H9 broth. Drug concentrations ranged from 20 to 0.3625 μg/mL for ethambutol, and 8.0 to 0.125 μg/mL for streptomycin. Plates were incubated at 37ºC. *M. tuberculosis* growth was observed by light microscopy. Reference standard drug-susceptibility testing was conducted using the Tetrazolium Microplate Assay. **Results**: We found that the optimal critical concentrations for defining drug resistance in MODS were 5.0 μg/mL for ethambutol (Sensitivity: 80%, Specificity: 88%) and 1.0 μg/mL for streptomycin (Sensitivity: 87%, Specificity: 50%). The Minimum Inhibitory Concentration for 50% of isolates (MIC_{50}) using MODS was 1.68 μg/mL for

ethambutol and 2.16 μg/mL for streptomycin. The Minimum Inhibitory Concentration for 90% of isolates using MODS (MIC_{90}) was 8.25 μg/mL for ethambutol and 8.85 μg/mL for streptomycin. **Conclusion**: The accuracy of ethambutol and streptomycin drug-susceptibility testing in MODS was improved by changing currently used drug concentrations.

7.2. Background

Mycobacterium tuberculosis remains one of the leading causes of infectious diseases worldwide [1]. Bacteriological confirmation and administration of appropriate drug-therapy remain the most important determinants for treatment success.

Most standardized treatment regimens include ethambutol and/or streptomycin during the initial months of drug therapy [159]. Although the mechanisms of drug action for streptomycin remain unclear, ethambutol appears to inhibit growth by blocking the synthesis of arainogalactan on the cellular wall of *M. tuberculosis* [160]. About 60 percent of ethambutol resistant isolates share a common alteration in the embB306 codon [161]. However, genetic mutations in embB306 do not appear to confer the same classical properties of drug-resistance found with other drugs [160]. *M. tuberculosis* isolates with the embB306 mutation have an increased ability to be transmitted, are more likely to share cross-resistance to other drugs such as rifampicin and isoniazid, and usually have a Minimum Inhibitory Concentration greater than 16 μg/mL for ethambutol [160]. Differentiating between phenotypic and genotypic resistance to streptomycin and ethambutol may thus be necessary when testing patients for drug-susceptibility status [162].

Culture-based testing is often limited by the length of time needed to obtain drug-susceptibility results [24]. Cumulative evidence suggests that the Microscopic Observation Drug Susceptibility (MODS) assay may be one potential candidate for meeting these goals. Based on the principles that *M. tuberculosis* can be cultured more quickly in liquid rather than solid media; and that culture growth of *M. tuberculosis* can be detected more rapidly using a light microscope instead of with the naked eye, several studies have shown a high correlation and a shorter turn-around time for detecting isoniazid and rifampicin resistance using MODS than reference-standard culture-based techniques [60,61,63,64,163]. Despite these

promising findings, the diagnostic validity of MODS for ethambutol and streptomycin has been limited, with results often being attributed to sampling errors and a tendency to overcall drug resistance [163].

This study aimed to improve the diagnostic validity of MODS for detecting ethambutol and streptomycin resistance by defining for the first time the optimal critical concentrations needed to phenotypically differentiate between drug resistant and drug-susceptible samples of *M. tuberculosis*.

7.3. **Materials and Methods** (please refer to Appendix 6)

7.3.1. Preparation of Mycobacterium tuberculosis isolates:

Sputum samples were tested for smear status using Ziehl-Neelson Smear Stain, then decontaminated using the NaOH-NALC method [164] and cultured in Middlebrook 7H9 broth (Difco, Detroit, Mich.). Reference standard drug-susceptibility status was conducted using previously described methods for the Tetrazolium Microplate Assay (TEMA) [102]. Drug susceptibility status for an isolate was characterized with reference standard MIC's using two different approaches. For the first method of categorization, isolates were classified dichotomously (i.e., drug-susceptible or drug-resistant). A sample was drug resistant if the TEMA Minimum Inhibitory Concentration (MIC) was greater than 5.0 μg/mL for ethambutol, and 2.0 μg/mL for streptomycin [102]. For the second categorization approach, isolates were classified into three levels (i.e., susceptible, intermediate, resistant) using the TEMA MIC breakpoints listed in Table 7.1. Unused portions of the samples were then stored at –20^{o}C. Culture-positive samples were selected for our study from this lot, and retested using TEMA.

7.3.2. Preparation of 7h9 broth:

2.95 g of Middlebrook 7h9 broth base (Difco, Detroit, Mich.) was dissolved in a solution of 1.35 mL glycerol, 0.625 g Bacto casitone and 450 mL deionized water. 7h9 broth was autoclaved for 15 minutes at 121 to 124ºC, and then cooled to room temperature. One

SELECTATAB (Mast Group Ltd., Bootle, UK) and 50mL of OADC were added to the broth. To verify sterility, the broth was then incubated at 37ºC for 48 hours. Broth was stored at 6 to 8ºC until day of use.

7.3.3. Preparation of antibiotic solutions:

Ethambutol and streptomycin were obtained from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Two *antibiotic stock solutions* were prepared by dissolving 5.00 mg of ethambutol in 2.5 mL of sterile distilled water; and 5.00 mg of streptomycin in 6.2 mL of sterile distilled water. Solutions were filtered through an aqueous solvent filter (pore size: 0.2 µm; Fisher Scientific, Pittsburgh, PA), and then stored at -20ºC. On the day of use, *antibiotic starting solutions* were made by diluting 15 µL of defrosted stock solution in 1485 µL of 7h9GC-OADC broth. The final drug concentrations were 20 µg/mL for ethambutol, and 8 µg/mL for streptomycin.

7.3.4. Inoculation of microplates:

240 µL of antibiotic stock solution were pipetted into the first row of a 96-well microplate. 120 µL of 7h9 broth was then pipetted into each remaining well. Figure 7.1 illustrates how antibiotic starting solutions were diluted using six concurrent serial 1:2 dilutions of ethambutol and streptomycin solutions. Final drug concentrations ranged from 20 to 0.3625 µg/mL for ethambutol, and 8.0 to 0.125 µg/mL for streptomycin. The last row of microwells was used as drug-free control wells. Stored sputum samples were defrosted on ice, and re-cultured by adding 20 µL of sample to each broth-containing microwell. In order to avoid drying, inoculated wells were sealed using scotch-tape. Microplates were then placed in ziplock bags and incubated at 37ºC.

7.3.5. Identification of colonies using the MODS method.

Starting on the fifth day of incubation, control wells were examined for *M. tuberculosis* growth using an inverted light microscope at 40x magnification, every three to four days. On the first day that at least ten colonies were observed in the control well or day 21 (whichever

came first), the numbers of colonies in each drug-containing well were counted (i.e., First Colony Count). Microplates were then re-incubated until day 40, when the numbers of colonies in each well were counted again (i.e., Second Colony Count) (please refer to Appendix 9). Minimum Inhibitory Concentration was defined as the lowest drug-concentration that inhibited visible tuberculosis growth.

7.3.6. Determination of critical concentrations.

Sensitivity, specificity, Youden's Index and test efficiency measures were calculated for each drug concentration using data from the first colony count using previously described formula [151,165]. Differences in proportions were compared using McNemar's χ^2 test. Receiver Operating Curves (ROC) were constructed by plotting 1-specificity on the x-axis and sensitivity on the y-axis. Area under the ROC curve was then estimated using the trapezoidal method. Drug concentrations that inhibited 50 percent (MIC_{50}) and 90 percent (MIC_{90}) of samples were calculated for each drug. Optimal drug-concentration breakpoints for detecting drug-resistance in *M. tuberculosis* (i.e., drug-susceptible versus drug-resistant) were selected based on the maximum Youden's Index and test efficiency measures. In order to maximize the test efficiency of MODS for detecting samples with intermediate levels of drug-resistance, we selected a second drug-concentration that had the highest sensitivity across a range of drug concentrations and a specificity greater than 0. Amount of growth observed in a critical concentration well was calculated by dividing the number of colonies observed in the drug-containing well by the number of colonies observed in the control well.

7.4. Results and Discussion

Forty-eight clinical isolates of *M. tuberculosis* were used to evaluate susceptibility testing towards antimicrobials using MODS. At the beginning of the experiment, we noted that the prevalence of ethambutol resistance was relatively rare in Peru, occurring in about two percent of *M. tuberculosis* samples collected in 2006 [34]. In order to ensure that isolates with a wide range of ethambutol and streptomycin MIC's were tested, previously processed sputum samples rather than newly collected sputum samples were used. Thirty-two of the samples

(67%) were resistant to streptomycin and 24 (50%) were resistant to ethambutol. Table 7.1 shows the grades of drug resistance for these isolates.

Of the 96 "control" wells that were inoculated (48 control wells per drug), 2 (2%) wells were dry and 4 (4%) wells were contaminated. This supports previous studies on MODS which showed contamination rates between 0 and 2%. [61,163] Although 17 of the 48 clinical isolates (35%) used failed to grow in both ethambutol and streptomycin control wells, culture rates did not differ between samples concentrated 1:1 and 1:100 (χ^2: 0.82, p=0.32); by grade of smear status (z=1.41, p=0.157); or by grade of drug-resistance for ethambutol (z=1.41, p=0.16) or streptomycin (z=0.71, p=0.48). To validate the use of previously processed samples, isolates were re-tested using TEMA. Classification of drug-susceptibility status remained the same using original and re-cultured TEMA MIC's for all but two samples per drug. These discordant results may be explained by the fact that the MIC's for these isolates occurred just at the breakpoint used to classify samples as drug-resistant or not.

Table 7.3 displays the diagnostic validity of MODS using different critical concentrations. The MODS MIC_{50} was 1.68 μg/mL for ethambutol and 2.16 μg/mL for streptomycin, while the MODS MIC_{90} was 8.25 μg/mL for ethambutol and 8.85 μg/mL for streptomycin. Area under the ROC curve was 0.68 [95% CI: 0.21 to 0.96] for streptomycin and 0.87 [95% CI: 0.23 to 1.0] for ethambutol.

The currently recommended antimicrobial concentrations for testing drug-susceptibility status with MODS are 2.50 μg/mL for ethambutol and 2.00 μg/mL for streptomycin [63,64,163]. Table 7.3 shows that in our experiment, the optimal drug concentration for detecting ethambutol and streptomycin drug-resistance using MODS was 5.0 μg/mL and 1.0 μg/mL, respectively. For ethambutol, a change to these new critical concentrations from the currently recommended standards resulted in a 13% [95% CI: 2 to 38] increase in specificity while the sensitivity remained unchanged. For streptomycin, the change resulted in a 27% [95% CI: 8 to 55] increase in sensitivity and a 12% [95% CI: 0 to 53] decline in specificity. Although the critical concentrations for both ethambutol and streptomycin increased by two-fold when the incubation period was extended from 21 days (i.e., first

colony count) to 40 days (i.e., second colony count), cut-offs remained the same regardless of whether the original or re-cultured TEMA MIC's were used as reference standards.

The median number of colonies observed in critical concentration wells that were inoculated with isolates resistant to streptomycin and ethambutol resistant were 7 colonies [IQR: 2 – 20] and 8 colonies [IQR: 2 – 10], respectively. Figure 7.2 illustrates the number of colonies observed in wells containing these new critical concentrations by grade of resistance for the isolate being tested. For culture-positive control wells, the median number of colonies observed were 18 colonies [IQR: 10 – 30] at the first count, and 500 colonies [IQR: 200 – 800] at the second count.

Figure 7.3 shows that the test efficiency of MODS using these new critical concentrations was lowest for *M. tuberculosis* isolates with intermediate levels of drug resistance. Identifying these patients early on is vitally important since amplification of drug-resistance in *M. tuberculosis* is more common among partially resistant strains rather than fully susceptible strains [109]. Additionally, misdiagnosing drug-resistant tuberculosis may have a greater impact on the clinical effectiveness of tuberculosis treatment than misdiagnosing fully susceptible tuberculosis as drug resistant.

Considering these observations, we evaluated whether the use of two critical concentration wells rather than one would increase the test efficiency of MODS for detecting *M. tuberculosis* with intermediate levels of drug-resistance. Figure 7.3 shows that for ethambutol while the test efficiency for detecting drug-susceptible samples dropped by 33% [95% CI: 10 to 65], it increased by 33% [95% CI: 1 to 91] for isolates with intermediate levels of drug resistance, and 25% [95% CI: 1 to 81] for isolates with high grade drug resistance. For streptomycin, there appeared to be little benefit for including two critical concentration wells rather than one, since all isolates were classified as resistant. However, further validation with larger sample sizes may be needed.

One criticism of MODS has been the biohazard risk in using liquid culture. Our study minimized this danger by using standard laboratory equipment such as biological safety cabinets, centrifuge safety caps, sealed rotors, plastic screw-lid vials and inserting plates into ziplock bags. Sealing each microplate well with adhesive tape also avoided having to

reculture samples if an inoculated plate fell during sample processing. More importantly, all lab workers involved in this experiment were trained and experienced in handling *M. tuberculosis* samples (i.e., significant factors when reducing the overall risk of lab-associated infections) [166].

It should be noted that using currently recommended drug concentrations, the sensitivity of MODS in our study was superior than previously published studies which showed a sensitivity of 42 to 65% for ethambutol resistance and a sensitivity of 44 to 50% for streptomycin resistance [64,163]. Similarly, the median time to culture-positivity for isolates in control wells were 14 days [Interquartile range (IQR): 13 to 21 days] or twice as long as previously reported studies on MODS [60,61,63,64]. Both of these observations raise the question as to whether freezing samples may inhibit the growth of fast growing strains of *M. tuberculosis*. streptomycin and ethambutol are used as sterilizing agents against semi-dormant strains of *M. tuberculosis* during the early stages of anti-tubercular treatment regimens, thus critical concentrations selected for this type of *mycobacteria* would be valid [167]. However, future studies evaluating the diagnostic validity of MODS and treatment outcome at the second month of treatment (i.e., proxy of semi-dormant strains) may be needed to evaluate MODS for detecting rifampicin resistance.

Similarly, we do not believe that the use of stored samples will influence the validity of our study findings. Logistical difficulties and a lack of diagnostic facilities in rural settings can often delay testing of sputum samples [168,169]. While refrigeration can be used to maintain the longevity of samples, about 50% of samples die during the first 8 weeks of storage after which the viability of samples stabilizes [170,171]. As illustrated in our study, there is little evidence to show that storage of samples may influence levels of drug-resistance. However, the samples we tested were likely more virulent since the strains most apt to survive storage are those with the greatest fitness [136].

In conclusion, this study showed that the accuracy of ethambutol and streptomycin drug-susceptibility testing in MODS was improved by changing currently used drug concentrations. Additionally, detection of isolates with intermediate levels of ethambutol resistance could be improved by including two critical concentration wells rather than one.

7.5. Tables and Figures

TABLE 7.1. Growth characteristics of *Mycobacterium tuberculosis* isolates used to validate the Microscopic Observation Drug Susceptibility assay

Level of Drug Resistance	Range of MABA Minimum Inhibitory Concentration	Number of Samples Tested	Number of Samples Grown in Control Well*	% of Samples Grown in Control Well* (95% CI)
Ethambutol				
Susceptible	Less than 4.0	18	12	67 (41 – 87)
Intermediate	4.0 to 8.0	11	4	36 (11 – 69)
Resistant	Greater than 8.0	18	5	28 (10 – 55)
Streptomycin				
Susceptible	Less than 2.0	3	1	33 (1 – 91)
Intermediate	2.0 to 4.0	27	15	56 (35 – 75)
Resistant	Greater than 4.0	17	7	41 (18 – 67)

CI: Confidence Interval

* Control wells did not contain antibiotics.

Table 7.2. Dataset regarding drug-susceptibility results from patients with pulmonary tuberculosis who provided more than one sputum sample in study*

Patient	Sample Number	Sputum Microscopy	MABA Ethambutol	MABA Streptomycin
1	1	2	S	I
	2	3	S	I
2	1	3	I	R
	2	3	I	I
3	1	2	R	S
	2	2	R	I
4	1	2	R	I
	2	2	R	I
5	1	2	R	I
	2	3	R	I
	3	3	R	I
	4	2	R	I
6	1	2	R	I
	2	3	R	I
	3	3	R	R
	4	3	R	R
7	1	3	S	I
	2	2	I	I
8	1	3	S	I
	2	3	S	I
	3	3	S	I
	4	3	S	R
	5	3	S	R
	6	3	S	R
	7	3	I	R
9	1	3	I	R
	2	1	R	R
	3	1	R	R
10	1	2	I	I
	2	2	I	R
	3	3	R	I
	4	1	R	I

Number of Discordant Results per Patient		
Susceptible & Resistant	0	0
Susceptible & Intermediate	2	1
Intermediate & Resistant	2	4
Total	*4*	*5*

*Isolates from a patient was graded with the following levels of drug resistance: S=Susceptible; I=Intermediate; R=Resistant

TABLE 7.3. Diagnostic validity of the Microscopic Observation Drug Susceptibility assay for detecting *Mycobacterium tuberculosis* resistant to streptomycin and ethambutol, by level of drug concentration added to broth.

	Type of Antimicrobial Agent													
	Ethambutol (n=21)							Streptomycin (n=23)						
Drug Concentration (µg/mL)	**0.3625**	**0.625**	**1.25†**	**2.50**	**5.00*†**	**10.0**	**20.0**	**0.125**	**0.25**	**0.50†**	**1.00*†**	**2.00**	**4.00**	**8.00**
Sensitivity	1.00	1.00	1.00	0.80	**0.80**	0.20	0.00	0.92	0.87	0.87	**0.87**	0.60	0.33	0.27
Specificity	0	0	0.31	0.75	**0.88**	1.00	1.00	0	0	0.125	**0.50**	0.62	0.88	1.00
Youden's Index	0	0	+0.31	+0.55	**+0.68**	+0.20	0	-0.08	-0.13	-0.01	**+0.37**	+0.22	+0.21	+0.27
Test Efficiency	0.20	0.20	0.45	0.76	**0.86**	0.81	0.80	0.57	0.56	0.61	**0.74**	0.61	0.52	0.52

* Critical concentration using one well for drug-susceptibility testing.
† Critical concentrations using two wells for drug-susceptibility testing.

FIGURE 7.1. Layout of 96-well microplate used for the Microscopic Observation Drug Susceptibility assay. Each plate was used to test 6 samples (columns 1, 3, 5, 7, 9 and 11) per drug. 7h9 broth was pipetted into all wells within these columns. Antibiotic starting solutions were then placed in row b and double-diluted in the direction of the arrow (→). Each grey circle (●) represents a control well with 7h9 broth that was free of antibiotic.

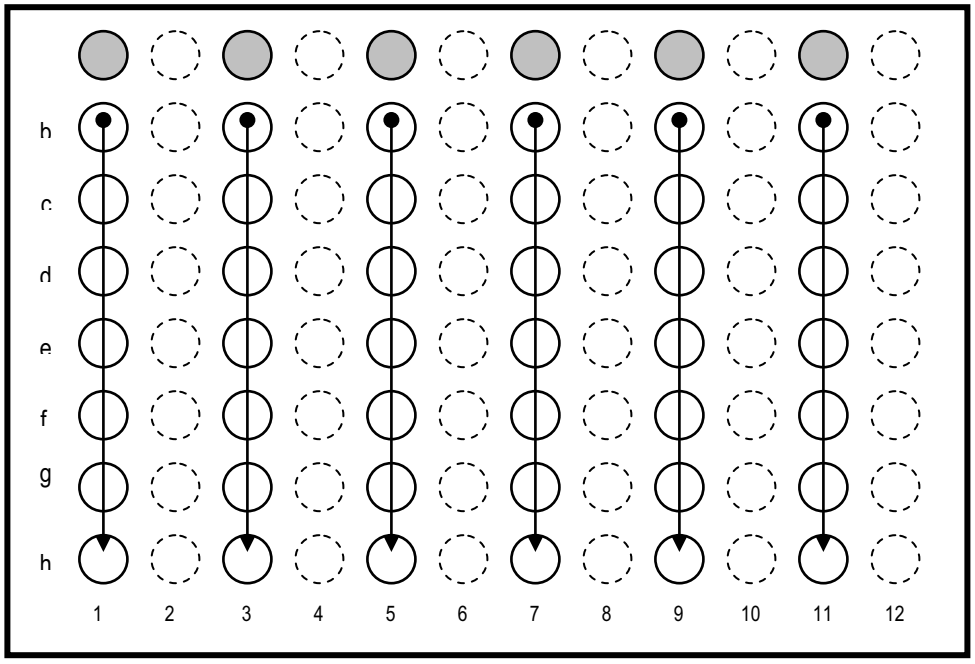
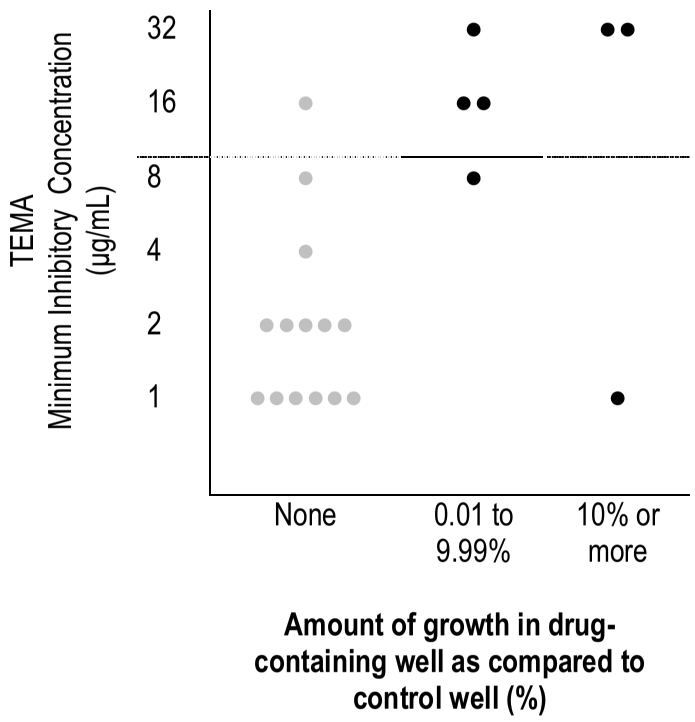


FIGURE 7.2. Amount of growth observed using the Microscopic Observation Drug Susceptibility assay at the optimal drug concentration, by grade of drug resistance for the sample being tested. Each grey circle (●) represents one isolate that did not grow in MODS at the optimal drug concentration. Each black circle (●) represents one isolate that did grow in MODS at the optimal drug concentration. Samples below the dotted line are those that were classified as drug-susceptible using MABA Minimum Inhibitory Concentration.

(a) Ethambutol at a drug concentration of 5.0 µg/mL



(b) Streptomycin at a drug concentration of 1.0 µg/mL

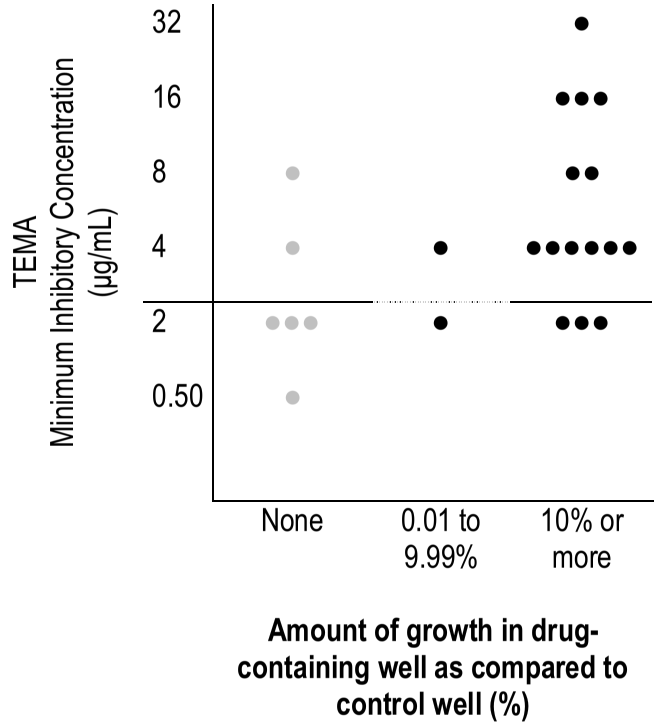
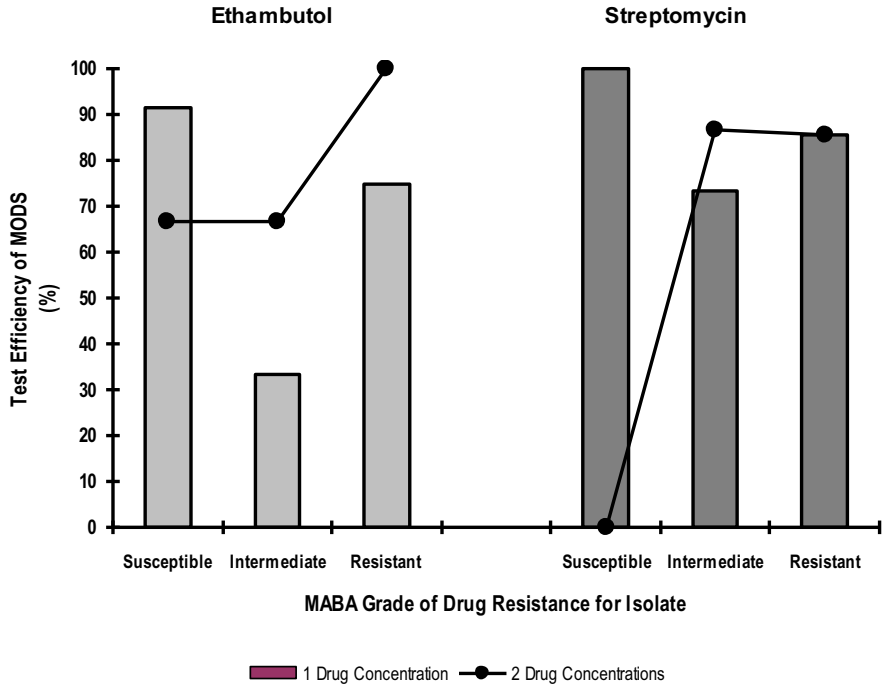


FIGURE 7.3. Test efficiency of the Microscopic Observation Drug Susceptibility Assay for detecting ethambutol and streptomycin resistance in *Mycobacterium tuberculosis* using one versus two critical concentration wells. Drug resistance was determined for ethambutol using one drug concentration well (5.00 µg/mL) or two drug concentration wells (5.00 µg/mL or 1.25 µg/mL); and for streptomycin using one drug concentration well (1.00 µg/mL) or two drug concentration wells (1.00 µg/mL or 0.50 µg/mL). An isolate was classified as drug-resistant if *M. tuberculosis* grew in at least one drug-containing well.



CHAPTER 8.

General Discussion

8.1. Overview of Results

The research undertaken in this thesis represented an effort to: [1] build a knowledge foundation regarding the underlying factors associated with outbreaks of MDR-TB among HIV/AIDS patients; [2] use this knowledge framework to develop health measures for controlling pulmonary tuberculosis among HIV/AIDS patients; and [3] in an effort to better implement these health measures, refine existing culture-based diagnostics for *M. tuberculosis*.

To increase understanding of the role of AIDS on the worldwide epidemic of MDR-TB, we conducted a longitudinal study within AIDS and non-AIDS populations who were receiving DOTS treatment for pulmonary tuberculosis in Lima, Peru, and who were undergoing an ongoing epidemic of MDR-TB. Our study revealed that while DOTS for drug-susceptible tuberculosis was protective against amplification of drug-resistance in pan susceptible strains of *M. tuberculosis*, it did not confer protection against infection with a different MDR-TB strain. Our molecular epidemiological analysis also revealed that while one particular strain of MDR-TB occurred more frequently among patients in the AIDS cohort (i.e., Lam9), the risk of superinfection with this epidemic strain of MDR-TB was the same for both groups of patients after adjusting for baseline prevalence of circulating strains. Considering this evidence, our study then focused on answering the question: Why did AIDS patients in Lima, Peru have such a high incidence of MDR-TB to begin with?

An analysis of patients from both the AIDS and non-AIDS cohorts revealed that those infected with the epidemic clone of MDR-TB were more likely to present with diarrhea as compared to patients infected with other MDR-TB clones. One explanation for these results is that infection with another enteric pathogen may have increased patients' susceptibility to superinfection with an MDR-TB strain during DOTS. Humoral immune responses against diarrhea-associated parasites are thought to favor infection with *M. tuberculosis,* making HIV-infected patients particularly vulnerable to disease. Similarly, the gastrointestinal tract

has long been recognized as a major site of opportunistic infections among HIV-infected patients, due in part to the tract being a major site of HIV replication, causing in turn the lamina propia of the stomach to undergo extensive depletion of CD4 cells [110,127].

In order to understand the role of gastrointestinal-related factors and infection with *M. tuberculosis*, the next study in this thesis involved characterizing the prevalence and factors associated with shedding *mycobacteria* in the stool of AIDS patients [45 110 110,128]. Our study revealed that among AIDS patients in the early stages of pulmonary tuberculosis, about 45% shed viable *mycobacteria* in stool. In contrast, 77% of AIDS patients who were in the later stages of pulmonary tuberculosis shed *mycobacteria*. The higher rate of shedding among late stage pulmonary tuberculosis patients may have been due in part to smear positive disease being more common in this group. However, AIDS patients are also more likely to experience disseminated disease in the later stages of pulmonary tuberculosis [6]. We also noted that hospitalization during the previous two years was a protective factor against shedding, after adjusting for history of tuberculosis. These results suggest that gastrointestinal colonization with *M. tuberculosis* is less common among AIDS patients who had previously received medical care for another opportunistic infection.

The next stage of this thesis aimed to develop screening strategies for pulmonary tuberculosis among AIDS patients. Our study findings revealed that pulmonary tuberculosis was common among hospitalized AIDS patients but frequently misdiagnosed using currently recommended diagnostic algorithms. Using blanket culture-based screening with the Microscopic Observation Drug Susceptibility Assay, we were able to identify almost all cases of pulmonary tuberculosis. Moreover, MODS was equally effective when targeted to patients clinically suspicious for tuberculosis. Although we used MODS to detect both the presence and drug susceptibility status of tuberculosis among these patients, for other first line drugs such as ethambutol and streptomycin the results have been suboptimal.

In our final study, we refined the ability of MODS for detecting resistance to ethambutol and streptomycin by evaluating its diagnostic validity across a range of drug-concentrations. We found that by modifying currently used drug concentrations we could increase the test efficiency for ethambutol and streptomycin by 10% and 13% respectively.

Despite these promising results, the test efficiency of MODS was lowest for strains with low grade drug resistance. This form of drug resistance may pose the greatest challenge for clinical management of tuberculosis since patients with low grade resistance are more likely to develop high grade resistance as compared to patients with completely susceptible strains of *M. tuberculosis* [109]. Our study revealed that the diagnostic efficiency of MODS for detecting low grade drug resistance could be improved by using two drug-concentration wells instead of one.

8.2. General Implications

These results have several implications, both at the clinical and public health level. First, our studies highlight the need for careful administration of tuberculosis prophylaxis. This includes accurately ruling out all forms of tuberculosis (not just pulmonary tuberculosis) before administering mono-drug prophylaxis, and providing patient support and observation at the same level as that found in DOTS programs. For HIV-infected patients, detection of tuberculosis is particularly important due to their increased risk of presenting with extra-pulmonary tuberculosis and their tendency to exhibit atypical symptoms depending on the extent of immunosuppression [6]. Considering the high risk of drug complications among HIV infected patients and the need to adequately prevent infection with tuberculosis during prophylaxis, patient support may also be important for increasing patient compliance.

Second, our results showed that while DOTS can adequately protect against the development of drug-resistant tuberculosis, it does not confer protection against superinfection with drug-resistant strains. This may be particularly problematic in hospital settings where HIV-infected patients may be placed in wards regardless of whether they are infected with TB or not [10,94]. There is thus a need to identify and isolate patients with drug-resistant tuberculosis early on.

Third, our study illustrated how AIDS patients with pulmonary tuberculosis had more strains in common than non-AIDS patients, despite residing in more geographically dispersed regions. We attributed this paradoxical difference in strain distribution to socialization practices. At the time that data collection for this study took place, HIV infection was

extremely low in the general population of Peru and largely restricted to marginalized populations such as injection drug users, homosexual men and those involved in prostitution [118]. The socialization practices that initially led to HIV infection may have thus been the same practices as those that led to TB infection. In light of these findings, contact investigations should be based not only on household contacts but also on social contacts.

Fourth, our study demonstrated how current standards of diagnosis in resource-constrained settings (i.e., sputum smear stain microscopy, chest radiographs or clinical manifestations) may be of limited clinical utility among HIV-infected individuals. These results highlight the importance of culture-based diagnosis and drug-susceptibility testing of tuberculosis. Similarly, identification of tuberculosis with low grade drug resistance is essential since these strains may pose the greatest challenge for clinical management of tuberculosis. Amplification of drug-resistance is more likely to occur among samples with low-grade resistance rather than fully susceptible strains. Until now, the main focus for selecting diagnostic cutoffs for drug-susceptibility tests has been to differentiate between isolates with extremely high or extremely low MIC's. For the MODS assay, this problem may be minimized by using two drug-concentration wells instead of one.

Finally, our study revealed that hospitalization during the previous two years was a protective factor against shedding *mycobacteria* in stool, even after adjusting for previous history of tuberculosis. Although the reasons for this link are unclear, hospitalization and previous residence in a prison or shelter may be acting as markers for access to health care in this deprived population for which health care is not free of charge. Our results thus highlight the indirect effect that general health care among HIV-infected patients can have on the development of tuberculosis. They also support recent suggestions to better integrate public health programs for tuberculosis and HIV in order to control HIV-associated tuberculosis [81].

8.3. Comments on Study Design and Limitations

8.3.1. Sample Size

The greatest limitation of this thesis was sample size. On several occasions, we used an exact logistic regression model or Fisher's exact test in order to adjust in our analysis for cells with less than five patients.

For our first study (i.e., evaluating the role of AIDS in an epidemic of MDR-TB), we estimate that in order to detect a 50% increase in risk among MDR-TB patients infected with Lam9, we would have needed to enroll 279 Lam9 MDR-TB cases and a total of 1116 MDR-TB patients using an alpha level of 0.05, a beta level of 0.80 and using a ratio of three controls per case.

Recruiting this number of subjects would probably not have been very feasible, considering the rarity of MDR-TB in Peru. Although in 2006, the Peruvian Ministry of Health identified 96 patients or 5.3% of all new tuberculosis cases as having MDR-TB in all of Peru [34], this number has declined steadily since the full introduction of the national MDR-TB program in 2005. Our study identified 100 MDR-TB patients over a 6 year span, from which 23 were infected with the Lam9 strain of MDR-TB. As compared to other previously published studies on MDR-TB and AIDS, this number represented a relatively large sample size [109,120,121]. A further complicating factor when selecting accrual periods for molecular epidemiological studies is that the molecular dynamics of *M. tuberculosis* may change over the span of one or two decades, particularly if tuberculosis cases represent recent rather than reactivated forms of disease.

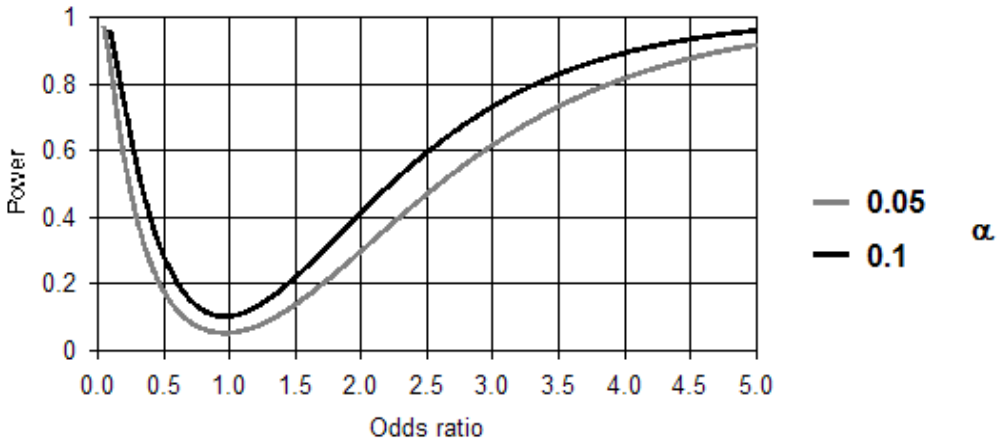
Sample size also remained a key issue in our second study (i.e., stool shedding of *mycobacteria)*. We estimate that the lowest odds ratio that our models could detect was ≤ 0.60 or ≥ 4.6 using an alpha of 0.05 and a beta of 0.80. As previously mentioned, the literature regarding stool shedding among tuberculosis patients is limited. Aside from the fact that we were using a secondary dataset, anticipating sufficient sample sizes would have been very difficult to predict, given the information available to us at the time, regarding stool

shedding. It is also interesting to note that in order to identify 30 patients with early stage tuberculosis, 473 respiratory symptomatic patients needed to be screened.

In an effort to increase statistical power using secondary datasets, we analyzed data for this thesis using the 90 and 95 percent confidence intervals. Figure 8.1 illustrates how, in our first study, by decreasing the alpha by 0.05, statistical power could be increased by 10 to 15% depending on the strength of the association. Still, our analysis may have failed to detect certain true associations, and thus should only be used to rule in rather than rule out potential factors.

Aside from statistical power issues, sample size also influenced the type of samples we used in our final study (i.e., defining optimal critical concentrations for MODS). For this experiment, we needed to test samples with a wide range of MIC's using samples from a population with a relatively low prevalence of ethambutol resistance. In 2006, only 36 patients or 2% of all tuberculosis cases in Peru were resistant to ethambutol [34]. In an effort to avoid long accrual periods and maximize the range of MIC's tested, we used stored rather than fresh samples in our study.

Figure 8.1. Curves illustrating the statistical power in study #1 for identifying factors associated with infection with the epidemic clone of MDR-TB, by strength of the association of the factor.



*Our study included a total sample size of 91 subjects with a ratio of 3 controls per case.

8.3.2. External Validity

An essential element to consider when reviewing our results is the issue of generalizability. The first half of this thesis revealed that epidemic clones of MDR-TB were associated with diarrhea and that shedding of *mycobacteria* in stool was common among AIDS patients, particularly among those reporting loss of appetite and lower BMI's. Although these findings suggest greater gastrointestinal involvement for HIV-associated tuberculosis than previously thought, our results should not be generalized beyond AIDS patients. As previously mentioned, the gastrointestinal tract remains the most common site of opportunistic infections among HIV-infected individuals [45] [110]. This is partially attributable to the gastrointestinal tract being a major site of HIV replication resulting, in turn, in a depletion of CD4 cells within the lamina propia of the stomach [110,128]. In this context, the pathophysiology of tuberculosis may differ between HIV-positive and HIV-negative individuals.

Aside from gastrointestinal differences, we also found in our first study that an epidemic of MDR-TB among AIDS patients was due to one particular strain of *M. tuberculosis* (i.e., Lam9). It is difficult to determine whether this outbreak represented a strain effect that could occur under similar conditions in other settings. However, our molecular analysis revealed that while multiple clones of Lam9 strains of MDR-TB were circulating among AIDS patients, only one was associated with an epidemic of MDR-TB. Similarly, other epidemics in Argentina and the United States were caused by W and M strains, and not Lam9 [89] [119].

External validity also remained an issue in our third study. We identified different tuberculosis screening strategies using MODS among hospitalized AIDS patients in a low HIV burden setting. In Peru, HIV-seropositivity rates are estimated at less than 1% [136]. It is unclear whether these results would be generalizable to high HIV-burden settings such as Africa where the prevalence of HIV infection can reach as high as 30%.

8.3.3. Internal Validity

Several issues were raised in this thesis in regards to internal validity. Our study showed that shedding was more common among patients in the later stages of pulmonary tuberculosis. One explanation for these results is that smear-positive disease was more common among patients in the later stages of pulmonary tuberculosis. Our regression analysis of factors associated with shedding also revealed that cough and loss of appetite were useful predictors for identifying patients who may be shedding. Although our study screened AIDS patients for respiratory symptoms during the previous 15 days, loss of appetite was not included in our screening criteria. The prevalence of shedding among patients in this cohort may have thus been underestimated.

Recall bias can often occur when an individual reports information only after learning of their diagnosis. It can be particularly problematic when cases recall to a greater extent than controls, information normally not considered important. While this thesis relieved heavily on interview-derived information, we do not believe that recall bias was an issue since all of the study participants were unaware of their drug-susceptibility status, strain/clone of infection, and stool shedding status at the time of patient interview. It should also be noted that while diarrhea was included as one exposure factor in our first study, our original questionnaire actually asked study participants whether they had one of seven underlying medical conditions (i.e., diabetes, diarrhea, cardiopathy, renal disease, cancer, liver disease and/or other). It was only during the analysis, that it became apparent that several patients had diarrhea, while rather than diarrhea alone, thus further reducing the possibility of selection or recall bias.

8.4. Future Research Directions

The findings from the first half of this thesis suggest that future research must be undertaken in order to better clarify the association between HIV-associated tuberculosis and gastrointestinal related factors such as diarrhea. The link between these two factors was somewhat surprising and was only discovered when patients were asked regarding seven different underlying medical conditions. Although the mechanisms underlying this

association are purely speculative, an increased susceptibility for acquiring MDR-TB following infection with other diarrhea associated diseases might partially explain our results. As previously mentioned, humoral immune responses against diarrhea-associated parasites are thought to favor infection with *M. tuberculosis* and HIV [111] [112] [113]. In light of these observations, future epidemiological studies on tuberculosis and diarrhea might be designed to examine the role that infection with different diarrhea-associated parasites has on the risk of infection and the development of active tuberculosis. This area of research may be particularly relevant for HIV-infected children in the developing world, where the incidence of childhood diarrhea may be high.

Conversely, the identification of *mycobacteria* in stool may have important implications for developing tuberculosis diagnostics. For individuals who exhibit non-productive cough, invasive procedures such as nasal-gastric aspiration or hypertonic solutions may be needed to induce or extract sputum [142]. Examination of stool rather than sputum could limit the use of such invasive procedures, thus minimizing patient discomfort while facilitating diagnosis.

Finally, our study illustrated through paradoxical differences in strain distribution, how transmission of tuberculosis may be more closely linked to social interactions rather than geographical location, per se. One of the questions raised from this finding is how the social structures that exist for a society or a group of individuals enable transmission to occur. This body of research could be based on different aspects of social network theory [172], which focuses on elucidating the characteristic patterns of social relationships or links between different persons in a social network (or community), rather than the individuals themselves [173]. For transmission dynamics, elucidating these links could be achieved by combining data from social and molecular networks of disease transmission.

For example, by identifying the degree of centrality or position of a person within a social network, one may be able to identify whether certain individuals are more “capable” of spreading tuberculosis as compared to others [173]. This analysis would enable public health practitioners to distinguish between a person who is capable of spreading an airborne communicable disease to an entire community (e.g. a school teacher), as compared to one

who, given their small social network, is only capable of transmitting a disease to their close family or friends. Using this information, public health specialists may then be able to better target tuberculosis interventions such as treatment or case-contact investigations, in order to avoid future disease transmission.

Concomitantly, social network theory could be used to identify thresholds for the transmission of certain microorganisms. Also known as "tipping points" [173], social network analysis may be used to analyze how easily *M. tuberculosis* is spread throughout a network. Social network theory may also help in elucidating the density of connections within a social group, through the use of network segmentation. This information could be particularly useful when evaluating and comparing the biological fitness of resistant versus non-resistant bacteria spread during a social interaction.

Unlike other models, the advantage of social network theory lies in its ability to contextualize social interaction dynamics. Although research in this area has largely been dedicated to describing the structure of social organizations, several conceptual elements of this theory may provide significant insight into how tuberculosis is transmitted.

CHAPTER 9.

Overall Conclusions and Recommendations

The following conclusions and recommendations were derived on the basis of the study findings and discussion of the results:

Study #1:

Most AIDS patients with drug-susceptible pulmonary tuberculosis who acquired MDR-TB during DOTS were infected by a different strain of *M. tuberculosis* rather than by amplification of drug-resistance (i.e., secondary drug-resistance). While this strain was more common among AIDS patients, its risk of infectivity did not differ between AIDS and non-AIDS patients, suggesting that another factor may have contributed to the strain's high prevalence among AIDS patients. Diarrhea was associated with risk of infection with this clone. Previous prophylaxis was also a useful factor for "ruling in" infection with this epidemic clone. Finally, our study provided further evidence regarding the role of social networks on TB transmission, particularly among socially marginalized populations.

Recommendations:

- Public health strategies to control for TB and HIV should be better integrated. This includes providing HAART and taking a more holistic approach to patient care.

- Patients with DR-TB need to be identified and isolated quickly in order to avoid nosocomial transmission of DR-TB in hospitals and/or DOTS clinics. For patients, receiving DOTS this can include isolating them in a separate ward or treating them at different times of day (e.g., patients with DS-TB in the morning, those with MDR-TB in the afternoon).

- Contact investigations for tuberculosis should not only include household contacts but also social contacts, especially when controlling the disease among socially marginalized populations.

- In settings with a high burden of MDR-TB, treatment for latent tuberculosis should include two drugs rather than one (e.g., isoniazid + rifampicin or isoniazid + pyrazinamide). Before receiving prophylaxis, patients should be properly screened for all forms of tuberculosis (i.e., pulmonary and extra-pulmonary tuberculosis). Finally, prophylaxis and treatment for latent tuberculosis should be administered with the same level of patient support as DOTS.

Study #2:

Shedding of *mycobacteria* in stool was common among AIDS patients in the later stages of pulmonary tuberculosis, but rarely occurred in the absence of pulmonary tuberculosis. Although, type of cough (productive or not) did not influence the risk of shedding, patients were more likely to shed *mycobacteria* in stool if they had smear-positive disease. Cough and loss of appetite were useful predictors for identifying patients who might be shedding. Past hospitalization was also a protective factor against colonization.

Recommendations:

- Strategies aimed at preventing airborne transmission of tuberculosis (e.g., ultraviolet lights) may not be sufficient for preventing nosocomial transmission of the disease. Efforts should be made to ensure that patients with pulmonary TB are quickly diagnosed and properly treated.

- Although current guidelines for recommend that patients with otherwise unexplained productive cough lasting 2-3 weeks be screened for TB [17], consideration should be given in extending these guidelines to include those with unproductive forms of cough.

Study #3:

Active pulmonary tuberculosis was common among hospitalized AIDS patients but was usually missed by screening based on smear microscopy, chest radiography and clinical symptoms. Blanket screening with MODS identified the majority of these patients. However, targeted screening based on symptoms had similar sensitivity and greater efficiency.

Recommendations:

- MODS (or at the very least some sort of culture or molecular-based method of detection) should be used in addition to standard criteria used for TB diagnosis, when screening hospitalized HIV-infected patients in developing countries.

Study #4:

Our study showed that the validity of MODS for ethambutol and streptomycin drug-susceptibility testing could be improved by modifying currently used drug concentrations to 5.0 μg/mL for ethambutol and 1.0 μg/mL for streptomycin. Similarly, detection of isolates with intermediate levels of drug-resistance could be improved by using two critical concentration wells rather than one.

Recommendations:

- Identification of patients with low-grade drug-resistance should be considered since they are more likely than patients with drug-susceptible tuberculosis to develop high grade drug resistance due to genetic amplification.
- Critical concentrations used in MODS to identify resistance of *M. tuberculosis* to ethambutol and streptomycin should be modified.
- Two critical concentration wells rather than one should be used in order to detect low-grade drug-resistance in *M. tuberculosis*.

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APPENDICES

Appendix 1

Brief summary of diagnostic tests

Staining & Microscopic Detection

Mechanism of Detection:
Microscopic observation of mycobacteria bacilli.

Advantages:
-Easy to use
-Rapid response
-Low cost
-Can determine infectiousness of patients

Limitations:
- Very low sensitivity
-Positive result indicative of mycobacterial disease--not TB
-Experience needed to evaluate slides
-Reagents deteriorate if used infrequently
-Requires positive control to ensure staining done well.

Factors influencing sensitivity of staining:
-staining technique -centrifugation speed
-reader experience
-prevalence of TB in pop'n

2 types of staining:

1. Classic Fuschin (Carbol Fuchsin, Ziehl-Neelsen)	*2. Fluorescent* (Auramine O, Auramine-Rhodamine)

Fluorescent staining more easily detected than classic fuschin-stained bacilli

Culture

Mechanism of Detection:
Solid & Liquid Middlebrook 7h9 Media-- Phenotypic Obsevation
Commercial systems-- Growth detected by increases in metabolic products (radioactive CO2), decreases in O2, or increase in turbidity

Advantages:
-culture more sensitive than microscopy—detect as few as 10 bacteria/mL of material
-growth in egg-based faster than agar & growth in liquid faster than solid—liquid (1-3 wks), solid(3-8 wks)

Limitations:
-need to digest & decontaminate most samples
-tb grows very slowly & usually outgrown by other organisms.
Cell doubling time: 15-24 hours while other bacteria-20 minutes
Colonies on solid media—2-3 weeks but for visible growth—8 weeks,

Indicator for whether decontamination completed well:
>5%--digestion-contamination insufficient
<2%--procedure too strong & likely kills too many mycobacteria.
Most digestion-decontamination procedures kill 50-90%.

2 forms of media :

1. Liquid		**2. Solid**	
Egg-based (Lowenstein-Jensen)	*Enhanced nutrition media (Middlebrook 7h10 & 7h11)*	Middlebrook 7h9	Commercial systems (Modified 7h9)
Advantages: -Less expensive -Longer shelf life than agar -Morphology of mycobacterial colonies more typical Limitations: -more laborious to prepare than agar	Advantages`: -easier to prepare - Compared to egg-based: more rapid growth detection, DR bacteria growth more stable -some TB bacilli prefer it over other media -Colony appearance more distinct than on egg-based -Better standardized than egg-based (used in research	Limitations: -requires CO2 enriched environment, (thus need for Petri dishes.) -exposure to lighr/heat causes formaldehyde production which could inhibit mycobacteria growth	BACTEC 460, MGIT, ESP, BACT/ALERT, MB quicker than solid media

Species Identification

1. Genetic probes

Advantages:
-highly specific but not sensitive
-cannot detect drug-resistance

2. High performance liquid chromatography

Mechanism of Detection
Identify species by amount of mycolic acid

Advantages:
-Test performed in a couple of hours

Limitations:
-Organism must be in pure culture in order to be tested
-High initial cost for obtaining equipment
-Difficult to distinguish *M.tuberculosis* & *M.bovis*

3. Gas Chromatography

Mechanism of Detection
Identify species by amount of mycolic acid
patterns of shorter chained fatty acids produced

4. Restriction Fragment Length Polymorphisms

Mechanism of Detection
Used to determine strain relatedness.
genotyping of cultures org. useful for epi links or lab cross-contamination
Clinical use validity needs to be confirmed

-Replacing old style phage typing in order to identify sources of outbreaks

Other methods (under development/ not in widespread use): Immunoassays for mycobacterial antigen/antibody, Phage typing

Appendix 2

Copy of patient treatment cards for Directly Observed Therapy Short-Course (DOTS) of Tuberculosis completed by the Peruvian Ministry of Health

NORMA TECNICA DE SALUD PARA EL CONTROL DE LA TUBERCULOSIS

ESTRATEGIA SANITARIA NACIONAL DE
PREVENCION Y CONTROL DE LA TUBERCULOSIS

TARJETA DE CONTROL DE ASISTENCIA Y ADMINISTRACIÓN DE MEDICAMENTOS CON ESQUEMA UNO

REGION DE SALUD _____ H C ó F F [ ] N° DE CASO [ ]
RED DE SALUD _____
ESTABLECIMIENTO: _____
APELLIDOS Y NOMBRES _____
OCUPACION _____ EDAD [ ] SEXO [F] [M]
DIRECCION: _____ Telefono: _____
BCG [SI] [NO] PESO: [ ] Kg Talla [ ] mts. DNI: [ ]
BK INICIAL FECHA: _____ RESULTADO: [ ] N° Reg. Lab. [ ]
CULTIVO INICIAL FECHA: _____ RESULTADO: [ ] N° Reg. Lab. [ ]
FECHA: _____ RESULTADO Anatomo patológico: [ ]
TUBERCULOSIS: PULMONAR [ ] EXTRAPULMONAR [ ] Localizacion _____
FECHA INICIO TRATAMIENTO: [ ]

PRIMERA FASE

MEDICAM. / DOSIS		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
FECHA	DIA																									
	MES																									
CONTROL DE PESO																										
Resultado BK de Control																										
N° REG. LAB.																										
Resultado Cultivo																										
N° REG. LAB.																										
RAFA y/o Hospitalizac.																										

MEDICAM. / DOSIS		26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
FECHA	DIA																									
	MES																									
CONTROL DE PESO																										
Resultado BK de Control																										
N° REG. LAB.																										
Resultado Cultivo																										
N° REG. LAB.																										
RAFA y/o Hospitalizac.																										

SEGUNDA FASE

MEDICAM. / DOSIS		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
FECHA	DIA																									
	MES																									
CONTROL DE PESO																										
Resultado BK de Control																										
N° REG. LAB.																										
Resultado Cultivo																										
N° REG. LAB.																										
RAFA y/o Hospitalizac.																										

MEDICAM. / DOSIS		26	27	28	29	30	31	32																		
FECHA	DIA																									
	MES																									
CONTROL DE PESO																										
Resultado BK de Control																										
N° REG. LAB.																										
Resultado Cultivo																										
N° REG. LAB.																										
RAFA y/o Hospitalizac.																										

FECHA DE EGRESO: _____ Condicion egreso: Curado [ ] Abandono [ ] Transf s/conf [ ] Fracaso [ ] Fallecido [ ]

CONTROL DE CONTACTOS

N° de Orden	Apellidos Y Nombres	EDAD		TIPO DE CONTACTO		Relación caso	BCG		Primer Control		Segundo Control		Tercer Control	
		M	F	DOMIC	EXTRA DOMIC	Parentesco	1	2	Fecha	Resultado	Fecha	Resultado	Fecha	Resultado
1°														
2°														
3°														
4°														
5°														
6°														
7°														
8°														
9°														
10°														
11°														
12°														
13°														

Control de administracion de quimioprofilaxis a contactos menores de 15 años

N° Orden	Edad		Peso	Dosis	Administracion de H 5 mg/Kg/día Fecha de Inicio.....																					
	M	F			1er mes				2do mes				3er mes				4to mes				5to mes				6to mes			
					1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24

N° de control	Consulta médica (fechas)	Entrevista de Enfermería (fechas)	Entrevista de Servicio Social (fechas)	Visitas Domiciliarias		Charlas Educativas (fechas)
				Fechas	Motivos	

RAFA: SI ☐ NO ☐ Fármaco (s) Causante (s) _____

Hepática ☐ Cutanea ☐ Digestiva ☐ Renal ☐ Otras ☐ Cual? _____

Suspension del medicamento SI ☐ NO ☐ Cambio del Esquema SI ☐ NO ☐

OBSERVACIONES _____

Firma y sello del responsable del ingreso del paciente

Firma y sello del responsable del Egreso del

NORMA TECNICA DE SALUD PARA EL CONTROL DE LA TUBERCULOSIS

ESTRATEGIA SANITARIA NACIONAL DE
PREVENCION Y CONTROL DE LA TUBERCULOSIS

TARJETA DE CONTROL DE ASISTENCIA Y ADMINISTRACIÓN DE MEDICAMENTOS EN PACIENTES CON ESQUEMA DE TRATAMIENTO DOS

REGION DE SALUD _____ H.C.ó F.F [ ] N° DE CASO: [ ]
RED DE SALUD _____
ESTABLECIMIENTO: _____
APELLIDOS Y NOMBRES _____
OCUPACION _____ EDAD [ ] SEXO: [F] [M]
DIRECCION: _____ Telefono: _____
BCG [SI] [NO] PESO [ ] Kg Talla [ ] mts DNI [ ]
BK INICIAL FECHA: _____ RESULTADO: [ ] N° Reg. Lab. [ ]
CULTIVO INICIAL FECHA: _____ RESULTADO: [ ] N° Reg. Lab [ ]
FECHA: _____ RESULTADO Anatomo Patológico: [ ]
TUBERCULOSIS PULMONAR [ ] EXTRAPULMONAR [ ] Localizacion _____
CONDICION DE INGRESO RECAIDA [ ] ABANDONO RECUP [ ]
FECHA INICIO TRATAMIENTO: [ ]

PRIMERA FASE

MEDICAM / DOSIS		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
FECHA	DIA																									
	MES																									
CONTROL DE PESO																										
Resultado BK de Control																										
N° REG. LAB																										
Resultado Cultivo																										
N° REG. LAB																										
RAFA y/o Hospitalizac																										

MEDICAM / DOSIS		26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
FECHA	DIA																									
	MES																									
CONTROL DE PESO																										
Resultado BK de Control																										
N° REG. LAB																										
Resultado Cultivo																										
N° REG. LAB																										
RAFA y/o Hospitalizac																										

MEDICAM / DOSIS		51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75
FECHA	DIA																									
	MES																									
CONTROL DE PESO																										
Resultado BK de Control																										
N° REG. LAB																										
Resultado Cultivo																										
N° REG. LAB																										
RAFA y/o Hospitalizac.																										

SEGUNDA FASE

MEDICAM / DOSIS		76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
FECHA	DIA																									
	MES																									
CONTROL DE PESO																										
Resultado BK de Control																										
N° REG. LAB																										
Resultado Cultivo																										
N° REG. LAB																										
RAFA y/o Hospitalizac																										

FECHA DE EGRESO: _____ Condicion egreso Curado [ ] Abandono [ ] Transf pricnf [ ] Fracaso [ ] Fallecido [ ]

MEDICAM. / DOSIS		101	102	103	104	105	106	107	108	109	110	111	112	113	114	115
FECHA	DIA															
	MES															
CONTROL DE PESO																
Resultado BK de Control																
N° REG. LAB.																
Resultado Cultivo																
N° REG. LAB.																
RAFA y/o Hospitalizac.																

MEDICAM. / DOSIS	
FECHA	DIA
	MES
CONTROL DE PESO	
Resultado BK de Control	
N° REG. LAB.	
Resultado Cultivo	
N° REG. LAB.	
RAFA y/o Hospitalizac.	

P. Mazzetti S.

MEDICAM. / DOSIS	
FECHA	DIA
	MES
CONTROL DE PESO	
Resultado BK de Control	
N° REG. LAB.	
Resultado Cultivo	
N° REG. LAB.	
RAFA y/o Hospitalizac.	

D. Céspedes M.

MEDICAM. / DOSIS	
FECHA	DIA
	MES
CONTROL DE PESO	
Resultado BK de Control	
N° REG. LAB.	
Resultado Cultivo	
N° REG. LAB.	
RAFA y/o Hospitalizac.	

XXX

NORMA TECNICA DE SALUD PARA EL CONTROL DE LA TUBERCULOSIS

CONTROL DE CONTACTOS

N° de Orden	Apellidos Y Nombres	EDAD		TIPO DE CONTACTO		Relación caso	BCG		Primer Control		Segundo Control		Tercer Control	
		M	F	DOMIC	EXTRA DOMIC	Parentesco	1	2	Fecha	Resultado	Fecha	Resultado	Fecha	Resultado
1°														
2°														
3°														
4°														
5°														
6°														
7°														
8°														
9°														
10°														
11°														
12°														
13°														

Control de administracion de quimioprofilaxis a contactos menores de 15 años

N° Orden	Edad		Peso	Dosis	Administracion de H 5 mg/Kg/día Fecha de Inicio.....																							
	M	F			1er mes				2do mes				3er mes				4to mes				5to mes				6to mes			
					1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24

N° de control	Consulta médica (fechas)	Entrevista de Enfermería (fechas)	Entrevista de Servicio Social (fechas)	Visitas Domiciliarias		Charlas Educativas (fechas)
				Fechas	Motivos	

RAFA: SI ☐ NO ☐ Fármaco (s) Causante (s) _____

Hepática ☐ Cutanea ☐ Digestiva ☐ Renal ☐ Otras ☐ Cual? _____

Suspension del medicamento SI ☐ NO ☐ Cambio del Esquema SI ☐ NO ☐

OBSERVACIONES _____

_____

_____

_____

Firma y sello del responsable del ingreso del paciente

Firma y sello del responsable del Egreso del

Appendix 3

Copy of patient registry and follow-up for tuberculosis treatment completed by the Peruvian Ministry of Health

NORMA TECNICA DE SALUD PARA EL CONTROL DE LA TUBERCULOSIS

MINISTERIO DE SALUD
ESTRATEGIA SANITARIA NACIONAL DE PREVENCION Y CONTROL DE TUBERCULOSIS

LIBRO DE REGISTRO Y SEGUIMIENTO DE PACIENTES CON TUBERCULOSIS

DIRECCION DE SALUD: ____________________ RED DE SALUD: ____________________
ESTABLECIMIENTO DE SALUD: ____________________ RESPONSABLE ____________________

Nº de Orden	APELLIDOS Y NOMBRES	FECHA DE INGRESO Dia/Mes/Año	H.C. F.F	EDAD		TB PULMONAR	TB EXTRAPULMONAR	CONDICION INGRESO			TRANSFE RENCIAS RECIBIDAS	BK INICIAL	B.K. CONTROL								ESQ.TRAT AMIENTO (en siglas)	CONDICION DE EGRESO					FACTOR DE RIESGO	OBSERVACIONES
				M	F			NUEVO	RECAIDA 1,2,3...	ABANDONO RECUPERADO 1,2,3....			1	2	3	4	5	6	7	8		CURADO	FRACASO	ABANDONO	FALLECIDOS	TRANSF. SIN CONFIRMAR		

Appendix 4 (a)

Copy of data collection sheets for patients screened for tuberculosis at Hospital Maria Auxiliadora and Hospital Dos de Mayo

Clinical Data

FICHA DE DATOS INICIALES

Codigo: MDR99HDM_____ **Iniciales: _____**
Fecha de entrevista:_____ **Fecha inicio tratamiento:_____**
Responsable de la entrevista:_____

FILIACION:
1) Sexo: 1.Masc 2.Fem 2) Edad:_____
3) Lugar de nacimiento: Dep:_____ Prov:_____ Ciudad:_____
4) Direccion (Indicar referencias):_____
_____

5)Estado Civil:
1.Soltero
2. Casado
3. Conviviente
4. Separ/Divor
5.Viudo

6) Grado de Instrucción:
1. Ninguno
2. Primaria incompleta
3.Primaria Completa
4.Secundaria Incompleta
5. Secundaria completa
6. Superior incompleto
7. Superior completo

7) Ocupacion:
1. Empleado
2. Obrero
3. Sin trabajo
4. Ama de casa
5. Independiente
6. Otro:_____

8)# de personas que viven en la misma casa con el paciente: Niños_____ / Adultos_____
9) Habitaciones en la casa:_____ 9) Habitaciones para dormir:_____

ANTECEDENTES:
11) Fecha de dx. HIV: _____/_____/_____
12) Cicatriz BCG: 1. Si 2. No
13) Localizacion: 1. Brazo Derecho #_____ 2. Brazo Izquierdo #_____ 3. Ninguna
14) PPD Previo: 1. Si 2. No 3. No sabe/no recuerda
15) Fecha:_____ 1. No recuerda/no sabe 2. Ninguna
16) Resultado: 1. Positivo 2. Negativo 3. No recuerda/no sabe
17) Tuberculosis previa: 1. Si 2. No 3. No sabe/no recuerda # veces_____
18) Fecha:_____/_____/_____ 1. No sabe/ no recuerda 2. Ninguno

19) Localizacion:
1.Pulmonar
2. Extrapulmonar:_____
3. No sabe/ No recuerda
4. Ninguno

20) Diagnostico:
1. Esputo
2. Radiografia
3. Biopsia
4. No sabe/no recuerda
5. Otro:_____

21)Tratamiento: 1.Si 2.No 3. No recuerda
22) Cumplimiento: 1. Completo 2. Incompleto 3. No recuerda/no sabe 4. ninguno

23) Drogas:
1. INH (blancas chiquitas)
2. RFP (capsulas rojas)
3.PZA (blanca grande)
4.ETB(amarilla-crema grande)
5. STM (inyecciones)
6.No recuerda/ No sabe
7. Otro:_____

24) Duracion:
1. Seis meses
2. Un año
3. Otro:_____
4. No recuerda/ no sabe
5.Ninguno

25) Resultado: 1.Curado 2. Abandono 3. Fracaso 4. No sabe/no recuerda 5. Ninguno
26) Contacto TBC en los ultimos dos años: 1. Si 2. No 3. No sabe
27) El contacto vive (vivia) en casa con el paciente 1.Si 2.No 3.Ninguno
28) Fecha de diagnostico:_____/_____/_____
29) Localizacion: 1.Pulmonar 2.Extrapulmonar 3. No recuerda 4.Ninguno
30)Tratamiento: 1. Si 2.No 3.No recuerda
31) Cumplimiento 1. Completo 2.Incompleto 3.Ninguno
32) Duracion: 1.Seis meses 2.Un año 3.Otro:_____ 4. Ninguno
33) Resultado: 1. Curado 2.No curado 3. Actualmente en tratamiento 4.Ninguno
34) Descarte TBC: pt fue estudiado para TBC 1.Si 2.No 3. No sabe/No recuerda

33) Como:
1.PPD Fecha:___/___/___ Positivo ( )____/____mm Negativo ( ) No recuerda ( )
2. Esputo ( ) Fecha:___/___/___ Positivo ( ) Negativo ( ) No recuerda ( )
3.Radiografia ( ) Fecha:___/___/___ Positivo ( ) Negativo ( ) No recuerda ( )
4.Otro:_____ Fecha:___/___/___ Positivo ( ) Negativo ( ) No recuerda ( )
5. No recuerda/no sabe

34) Profilaxis TBC: El paciente recibio profilaxis: 1.Si 2. No 3. No recuerda/no sabe
35) Cumplimiento 1. Completa 2.Incompleta 3. No sabe/No recuerda 4. Ninguno
36) Cual 1. INH 2. Otro:_____ 3.No sabe/No recuerda 4. Ninguno
37) Por cuanto tiempo: 1.Seis meses 2.Un año 3. Actualmente en profilaxis
4.Otro:_____ 5. No sabe/ no recuerda 6. Ninguno
38) Alergico a alguna medicina: 1.Si 2. No 3. No recuerda/No sabe
39)Cual:_____

40) Otras enfermedades: Otra Infeccion diferente a TBC actualmente: 1.Diabetes 2.Diarrea 3.Cardiopatias 4.Enfermedad Renal 5.Cancer 6.Enfermedad hepatica 7.Otra:_____ 8. Ninguna 9. No sabe/no recuerda

41) Hospitalizaciones previas en los ultimos dos años: 1.Si 2.No 3.No recuerda
42) Diagnostico:_____

ENFERMEDAD ACTUAL:
43)Sintomas:

SINTOMA	NO	SI	DURACION
1.Tos			
2.Espectoracion			
3.Fiebre			
4.Falta de Aire			
5.Tos con sangre			
6.Perdida de peso			
7.Decaimiento			
8.Sudorac. nocturna			
9. Falta de apetito			
10. Otro:			

44) Examen Clinico:
Peso: _____ kg Talla: _____mt IMC: _____
PPD 1.Si 2.No Fecha: _____/_____/_____ Horiz:_____ Vertc:_____
Frecuencia Respiratoria: _____ Tirajes: Si ( ) No ( ) Cianosis: Si ( ) No ( )
Cicatriz BCG: Si_____ No_____

HALLAZGO	1.LSD	2.LMD	3.LID	4.LSI	5.LII	6.No realizado
45) Matidez						
46)Sibilantes						
47)Roncos						
48)Crepitos						
49)Frote pleural						
50)Soplo tubarico						
51)Otro						

DATOS DE LABORATORIO (*)

1) Esputo: ( ) Ziehl-Nielsen ( ) Auramina ( ) PCR ( ) Cultivo ( ) Sensibilidad
2) Sangre: ( ) HIV-ELISA ( ) Albumina / pre-albumina ( ) Gamma-IFN
( ) TGF-beta ( ) Vitamina A ( ) Vitamina D

(*) Registrar las muestras tomadas en esta visita y la prueba(s) que se realiza(n) en cada una.

FICHA DE DATOS INICIALES

Codigo: MDR99MA_____ **Iniciales: _____**
Fecha de entrevista:_____ **Fecha inicio tratamiento:_____**
Responsable de la entrevista:_____

FILIACION:

1) Sexo: [1].Masculino [2].Femenino
2) Lugar de nacimiento: Dep:_____ Prov:_____ Ciudad:_____
3) Direccion (indicar referencias):_____

4)Estado Civil:	5) Grado de Instrucción:	6) Ocupacion:
1. Soltero 2. Casado 3. Conviviente 4. Separ/Divor 5. Viudo	1. Ninguno 2. Primaria incompleta 3. Primaria Completa 4. Secundaria Incompleta 5. Secundaria completa 6. Superior incompleto 7. Superior completo	1. Empleado 2. Obrero 3. Sin trabajo 4. Ama de casa 5. Independiente 6. Otro:_____

7)# de personas que viven en la misma casa con el paciente: Niños_____ / Adultos_____
8) Habitaciones en la casa:_____ 9) Habitaciones para dormir:_____

ANTECEDENTES:

10) Cicatriz BCG: [1].Si [2].No

11) Localizacion:	12) PPD Previo:	13) Fecha:	14) Resultado:
1. Brazo Derecho #_____ 2. Brazo Izquierdo #_____ 3. Ninguna	1.Si 2.No 3.No sabe/no rcrda	___/___/___ 1. No rcrda/no sabe 2. Ninguna	1.Positivo 2.Negativo 3.No rcrda/no sabe

15) Tuberculosis previa:	16) Fecha:	17) Localizacion:	18) Diagnostico:
1. Si 2. No 3. No sabe/no rcrda # veces_____	___/___/___ 1. No sabe/ no rcrda 2. Ninguno	1. Pulmonar 2. Extrapulmonar: _____ 3. No sabe/ No rcrda 4. Ninguno	1. Esputo 2. Radiografia 3. Biopsia 4. No sabe/no rcda 5. Otro:_____

19)Tratamiento: [1].Si [2].No [3].No recuerda

20) Cumplimiento	21) Drogas:	22) Duracion:	23)Resultado
1. Completo 2. Incompleto 3. No rcrda/no sabe 4. ninguno	1. INH (blancas chiquitas) 2. RFP (capsulas rojas) 3. PZA (blanca grande) 4. ETB(amarilla-crema grande) 5. STM (inyecciones) 6. No recuerda/ No sabe 7. Otro:_____	1. Seis meses 2. Un año 3. Otro:_____ 4. No rcda/no sabe 5.Ninguno	1. curado 2. Abandono 3. Fracaso 4. No sabe /Rcda 5. ninguna

24) Contacto TBC en los ultimos dos años: [1].Si [2].No [3].No sabe
25) El contacto vive (vivia) en casa con el paciente [1].Si [2].No [3].Ninguno
26) Fecha de diagnostico:____/____/____

27) Localizacion:	28)Tratamiento:	29) Cumplimiento	30) Duracion:	31) Resultado:
1.Pulmonar 2.Extrapulmonar 3. No recuerda 4.Ninguno	1.Si 2.No 3.No recuerda	1.Completo 2.Incompleto 3.Ninguno	1.Seis meses 2.Un año 3.Otro:_____ 4. Ninguno	1.Curado 2.No curado 3.Actualmente Tto 4.Ninguno

32) Descarte TBC: pt fue estudiado para TBC: [1].Si [2].No [3].No sabe/No recuerda

33) Como:	Fecha	Positivo	Negativo	No recuerda
1. PPD	/ /	/___mm		
2. Esputo	/ /			
3. Radiografia	/ /			
4.Otro	/ /			
5. No recuerda/no sabe				

34) Profilaxis TBC: El paciente recibio profilaxis: [1].Si [2]. No [3]. No recuerda/no sabe

35) Cumplimiento	36) Cual :	37) Por cuanto tiempo:
1.Completa 2.Incompleta 3.No sabe/No rcrda 4.Ninguno	1.INH 2.Otro:_____ 3.No sabe/No recuerda 4.Ninguno	1.Seis meses 2.Un año 3. Actualmente en profilaxis 4.Otro:_____ 5. No sabe/ no recuerda 6. Ninguno

38) Alergico a alguna medicina: [1].Si [2]. No [3]. No recuerda/No sabe
39) Cual:_____

40) Otras enfermedades: Otra Infeccion diferente a TBC actualmente:

1.Diabetes
2.Diarrea
3.Cardiopatias
4.Enfermedad Renal
5.Cancer
6.Enfermedad hepatica
7.Otra:_____
8. Ninguna
9. No sabe/no recuerda

41) Hospitalizaciones previas en los ultimos dos años: [1].Si [2].No [3].No recuerda
42) Diagnostico:_____

ENFERMEDAD ACTUAL:

43)SINTOMAS	NO	SI	DURACION
1.Tos			
2.Espectoracion			
3.Fiebre			
4.Falta de Aire			
5.Tos con sangre			
6.Perdida de peso			
7.Decaimiento			
8.Sudorac. nocturna			
9. Falta de apetito			
10. Otro:			

44) Examen Clinico:
Peso: _____ kg Talla: _____mt IMC: _____
Bioimpedancia: Resistencia :_____ Reactancia:_____
Frecuencia Respiratoria: _____ Tirajes: Si ( ) No ( ) Cianosis: Si ( ) No ( )
Cicatriz BCG: Si_____ No_____

HALLAZGO	1.LSD	2.LMD	3.LID	4.LSI	5.LII	6.No realizado
45) Matidez						
46)Sibilantes						
47)Roncos						
48)Crepitos						
49)Frote pleural						
50)Soplo tubarico						
51)Otro						

DATOS DE LABORATORIO (*)

Esputo	Sangre	Heces
( ) Ziehl-Nielsen ( ) Auramina ( ) PCR ( ) Cultivo ( ) Sensibilidad	( ) HIV-ELISA ( ) Albumina/pre-albumina ( ) Gamma-IFN ( ) TGF-beta ( ) Vitamina A ( ) Vitamina D	( ) PCR

(*) Registrar las muestras tomadas en esta visita y la prueba(s) que se realiza(n) en cada una.

Appendix 4 (b)

Copy of data collection sheets for patients screened for tuberculosis at Hospital Maria Auxiliadora and Hospital Dos de Mayo

Socioeconomic Data

FICHA DE DATOS SOCIO ECONOMICOS

FECHA ☐☐☐ CODIGO FAMILIA ☐☐☐ DIRECCION ☐ RESPONSABLE DE ENTREVISTA ☐ HISTORIA CLINICA ☐

FAMILIARES

	NOMBRES Y APELLIDOS	01 SEXO 1 M 2 F	02 FECHA NACIMIENTO DD/MM/AA	03 EDAD	04 LUGAR DE NACIMIENTO	05 LUGAR DE PROCEDENCIA	06 ESTADO CIVIL 1 MENOR 2 SOLTERO 3 CASADO 4 DIVORCIO 5 CONVIVE 6 VIUDO	07 EDUCACION 1 NINGUNA 2 PRIM INCOM 3 PRIM COMPL 4 SEC INCOMP 5 SEC COMPLE 6 SUP INCOM 7 SUP COMPLE 8 TECNICO 9 OTRO	08 OCUPACION 1 ESTUDIANTE 2 TRAB HOGAR 3 VEND AMBUL 4 OBRERO 5 EMPLEADO 6 TRAB INDEP 7 PROF. INDEP 8 MILITAR 9 DESEMPL 10 AMA DE CASA 11 OTRO	09 MONTO RECIBIDO MENSUAL (NUEVOS SOLES)	10 RELACION CON JEFE DE FAMILIA 1 JEFE 2 ESPOSA 3 HIJO 4 TIO 5 SOBRINO 6 PADRES 7 FAM POLIT 8 HIJASTRO 9 OTRO	11 RAZA 1 BLANCA 2 NEGRA 3 ORIENTAL 4 MESTIZA 5 INDIGENA	12 TIEMPO DE RESIDENCIA EN EL DOMICILIO ACTUAL	13 ALCOHOLISMO	14 DROGADICCION
1															
2															
3															
4															
5															
6															
7															
8															
9															
10															

SOCIOECONOMICOS

1 NUMERO TOTAL DE PERSONAS QUE VIVEN EN LA VIVIENDA ☐

				TOTAL
2	DORMITORIO	SI	NO	
3	BAÑO	SI	NO	
4	COCINA	SI	NO	
5	SALA	SI	NO	
6	NUMERO DE VENTANAS			

06 TENENCIA DE LA CASA
1 PROPIA
2 ALQUILADA
3 ALOJADA
4 GUARDIANIA
5 OTRO

07 PAREDES
1 ESTERAS
2 CARTON
3 MADERAS
4 ADOBE
5 LADRILLO CEMENTO
6 OTRO

08 TECHO
1 ESTERAS
2 ETERNIT
3 MADERAS
4 CARTON
5 PLASTICOS
6 CONCRETO
7 OTRO

09 PISO
1 TIERRA
2 CEMENTO
3 MADERA
4 LOSETA
5 OTRO

10 FUENTE DE AGUA
1 CONEX INTRADOM
2 PILON
3 C CISTERNA
4 OTRO

11 DESAGUE
1 SI
2 NO

12 TIPO DE ALUMBRADO
1 RED PUBLICA
2 KEROSENE
3 VELAS
4 OTRO

13 COMBUSTIBLE PARA COCINAR
1 ELECTRICIDAD
2 GAS
3 KEROSENE
4 LENA
5 OTRO

14 NUMERO DE CAMAS ☐

15 NIÑOS POR CAMA ☐

Appendix 4 (c)

Copy of data collection sheets for patients screened for tuberculosis at Hospital Maria Auxiliadora and Hospital Dos de Mayo

Chest radiographic Data

FICHA DE EVALU[illegible]ON RADIOLOGICA

CODIGO:_____ INICIALES: _____
RADIOGRAFIA #:_____ RESPONSABLE: _____
Fecha: ____/____/____ FIRMA: _____

Sindromes Radiológicos	HTD (1/3)			HTI (1/2)		
	Sup	Med	Inf	Sup	Inf	Comentarios
Sindrome Parenquimal						
acinar						
intersticial						
mixto						
miliar						
cavidades						
nodular						
calcificación						
atelectasia						
hiperinflación						
Sindrome Pleural						
derrame pleural						
neumotorax						
Hidroneumotorax						
Sd Mediastinal						
Rx normal						

Comentarios:

FICHA DE EVALUACION RADIOLOGICA

CODIGO: MDR99MA_____ INICIALES:_____

RADIOGRAFIA #1

Fecha: _____/_____/_____ Responsable:_____

1) Tipo de radiografia:
1. AP+LAT
2. Solo AP
3. Solo LAT
4. Solo PA
5. PA + LAT

2) Extensión de la enfermedad:
1. 00 (radiografia normal)
2. 0 (adenopatia mediastinal/hiliar)
3. 1 (un cuadrante afectado)
4. 2 (dos cuadrantes afectados)
5. 3 (tres cuadrantes afectados)
6. 4 (cuatro cuadrantes afectados)

3) Localizacion:
1. I (LSD)
2. II (LMD)
3. III (LID)
4. IV (CSI)
5. V (CII)

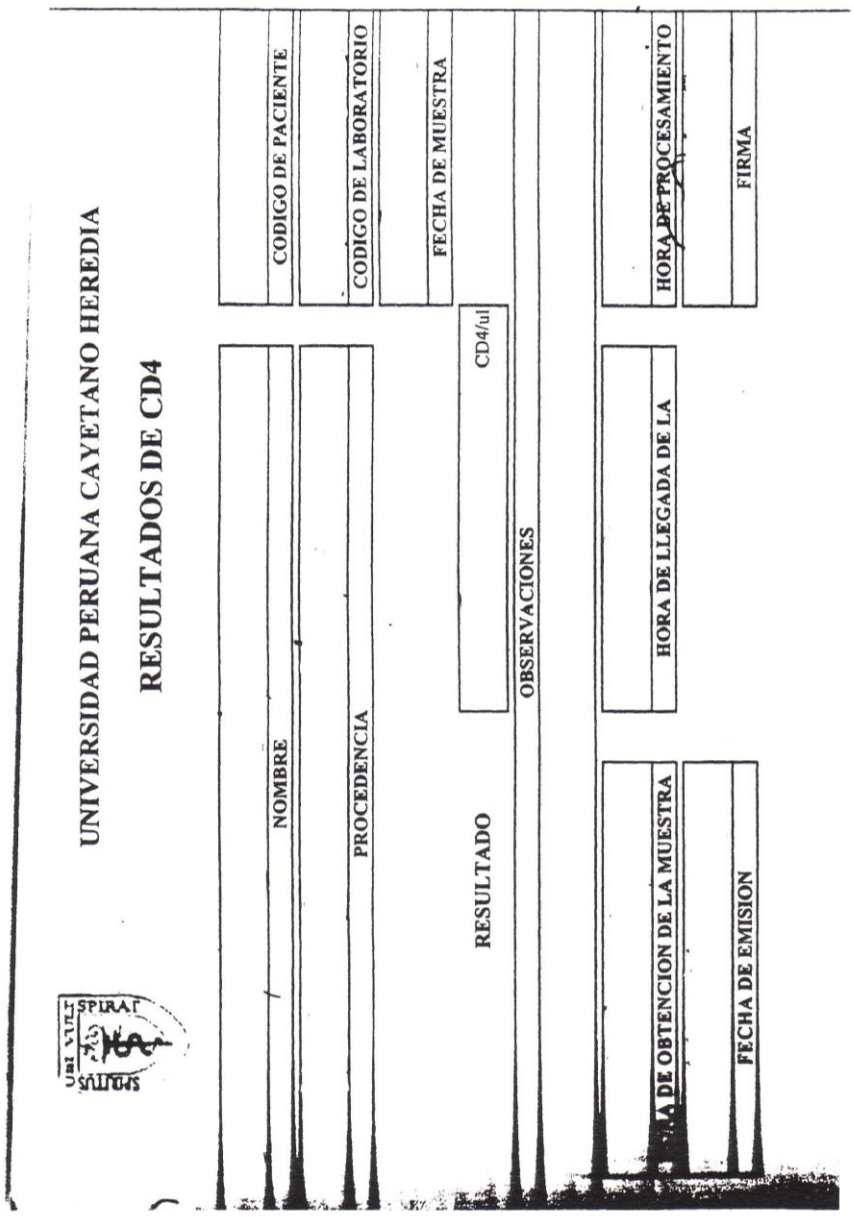
4) Tipo de lesión:
1. a (cavidad/es)
2. b (miliar)
3. c (intersticial)
4. d (alveolar)
5. e (nodular)
6. f (calcificacion)
7. g (atelectasias)
8. h (derrame)
9. i (hiperinflacion)

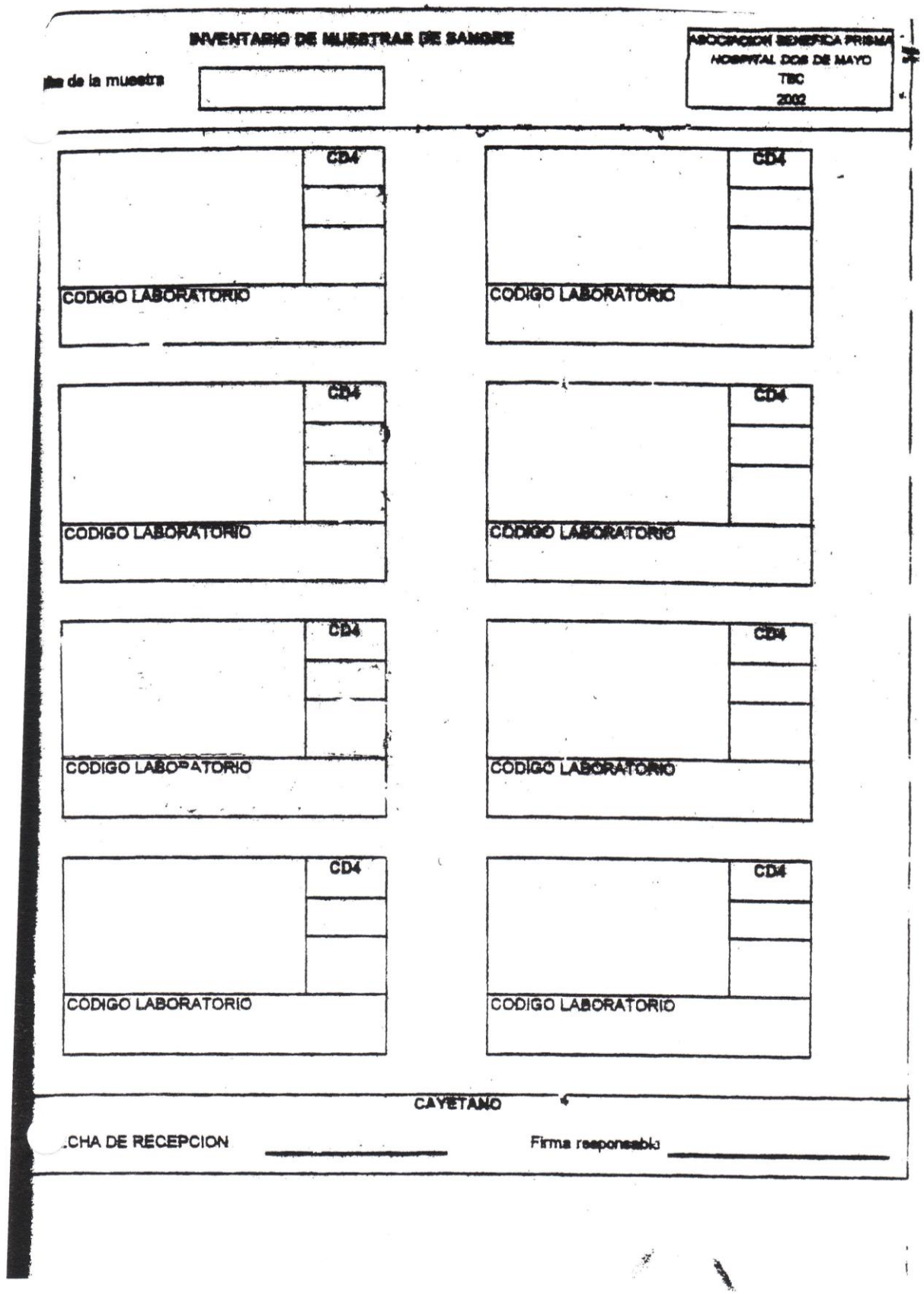
Clasificación según esquema: _____

Appendix 4 (d)

Copy of data collection sheets for patients screened for tuberculosis at Hospital Maria Auxiliadora and Hospital Dos de Mayo

CD4 Count Data


UNIVERSIDAD PERUANA CAYETANO HEREDIA
RESULTADOS DE CD4
NOMBRE
CODIGO DE PACIENTE
PROCEDENCIA
CODIGO DE LABORATORIO
FECHA DE MUESTRA
RESULTADO
CD4/ul
OBSERVACIONES
HORA DE LLEGADA DE LA
HORA DE PROCESAMIENTO
FECHA DE EMISION
FIRMA



INVENTARIO DE MUESTRAS DE SANGRE

ASOCIACION BENEFICA PRISMA
HOSPITAL DOS DE MAYO
TBC
2002

...de la muestra

CD4

CODIGO LABORATORIO

CD4

CODIGO LABORATORIO

CD4

CODIGO LABORATORIO

CD4

CODIGO LABORATORIO

CD4

CODIGO LABORATORIO

CD4

CODIGO LABORATORIO

CD4

CODIGO LABORATORIO

CD4

CODIGO LABORATORIO

CAYETANO

...CHA DE RECEPCION _____ Firma responsable _____

Appendix 4 (e)

Copy of data collection sheets for patients screened for tuberculosis at Hospital Maria Auxiliadora and Hospital Dos de Mayo

Laboratory Data

MDR99DM_____

INICIALES: _____

EP	Fecha	Auramina	PCR	Cultivo L-J	Cultivo 7H9	Cultivo 7H11	MABA				MOBOSA								CD4
							INH	RMP	SM	ETB	INH		RMP		SM		ETB		
											0.4	0.1	1	0.5	8	2	5	2.5	
#1																			
#2																			
#3																			
#4																			
#5																			
#6																			
#7																			
#8																			
#9																			
#10																			
#11																			
#12																			

IDENTIFICACION DEL PACIENTE

APELLIDOS: PATERNO | MATERNO

NOMBRES:

EDAD: SEXO [F] [M]

CODIGO PAC: TBMDR 1

INICIALES:

N° MUESTRA: (1) (2)

COD LAB:

ESTUDIO

- [ ] SR
- [ ] SRTB
- [ ] MDR
- [ ] TBG
- [ ] Vit D
- [ ] Cuerda Encapsulada
- [ ] Esputo Cuantitativo
- [ ] _____

TIPO DE MUESTRAS

- [ ] Esputo normal
- [ ] Esputo Inducido
- [ ] Esputo Cuantitativo
- [ ] Asp. Nasogastrico
- [ ] Asp Faringo Traqueal
- [ ] Cuerda Encapsulada
- [ ] Liquido Pleural
- [ ] CEPA
- [ ] Orina
- [ ] Heces
- [ ] Biopsia
- [ ] Otros

RESPONSABLE

	dd	mm	aa
Fecha Toma de Muestra			
Fecha Envio Muestra			
Fecha de Proceso			

PRUEBAS Y RESULTADOS

			Fecha siembra	Fecha lectura
Auramina		Lowenstein Jensen		
PCR		7H9 Caldo		
BK cuantitativo		7H11 Agar		

CULTIVOS

		Fecha siembra	Fecha lectura
7H10 Agar			
Cultivo Cuantitativo			

Observaciones: _____

SUSCEPTIBILIDAD

MODS : Ensayo de Susceptibilidad Directa por Observacion Microscopica (ug/ml)

Fecha Proceso		Fecha Positivo	
Vol EP (ml)			
INH 0.4		INH 0.1	
RFP 2.0		RFP 1.0	
SM 6.0		SM 2.0	
ETB 5.0		ETB 2.5	

Observaciones: _____

MABA: Ensayo de Susceptibilidad en Microplaca con Alamar Blue (MIC ug/ml)

Fecha Proceso			
INH		ETB	
RMP		CAP	
SM		CIP	

Observaciones: [redacted]

Appendix 5

Copy of data collection sheet for patients screened for pulmonary tuberculosis at the Hospital Arzobispo Loayza and Hospital Hipolito Unanue

Datos de Estudio

Hospital: HAL (1) | DAC (2) | DDM (3) | HHU (4)

Fecha de entrevista: _ _ / _ _ / _ _

Nombre de entrevistadora:

Fecha de hospitalizacion: _ _ / _ _ / _ _

Fecha de alta: _ _ / _ _ / _ _

Codigo estudio

TBHO . _ _ _ _

(Estudio parte . único numero de paciente)

A. Datos del paciente

Nombres (A1)

Apellido Paterno (A2)

Apellido Materno (A3)

Sexo (A4)

Edad (A5)

Fecha de nacimiento: _ _ / _ _ / _ _ (A6)

Direccion (A7)

Referencia (A8)

Telefono (A9)

Nombre codigo (A10): (4 letras y numero único de paciente)

Necesidades basicas?

Habitaciones para dormir	Cuantos?			
Personas que viven en la casa	Cuantos?			
Personas de la casa que trabajan	Cuantos?			
(a) Hacinamiento		Si	No	
(b) Personas que dependen por cada trabajador?	3 o mas?	Si	No	
(c) Material de la casa	No noble?	Si	No	
(d) Servicios higienicos	Sin desagüe?	Si	No	
(e) Hay niños entre 6 y 12 años que no asisten al escuela?		Si	No	

Cada si=1, no=0

Estado socio-economico (0, 1, 2) (A11)

Muestras

TBHO . _ _ _ _ . _ _ _ (Estudio parte . numero de paciente . muestra) | (check) | _ _ / _ _ / _ _ (fecha de muestra)

TBHO . _ _ _ _ . _ _ _ | | _ _ / _ _ / _ _

B. Historia clinica

	Sintomas			Tiempo (dias)
B1	Tos	Si	No	B9
B2	Expectoracion	Si	No	B10
B3	Fiebre	Si	No	B11
B4	Hemoptisis	Si	No	B12
B5	Disminucion de peso	Si	No	B13
B6	Sudoracion nocturno	Si	No	B14
B7	Dolor toracico	Si	No	B15
B8	Falta de aire	Si	No	B16
B17	Otro (especifica)			B18

C. Factores de riesgo de TB/MDR-TB

C1	Terapia con corticoids (actual)	Si	No
C2	Insuficiencia renal (actual)	Si	No
C3	Diabetes mellitus (actual)	Si	No
C4	Cancer (actual)	Si	No
C5	Contacto conocido con caso de TB	Si	No
C6	Infeccion con VIH	Si	No
C7	Hospitalización (los ultimos 12 meses)	Si	No
C8	Fumar — actual — anterior	nunca	
C9	Trabajador de salud	Si	No
C10	Trabajador o ex-interno penales	Si	No
C11	Tratamiento para TB (antes)	Si	No
C12	Contacto conocido con caso de TB-MDR	Si	No
C13	Consumo de drogas (mes anterior)	Si	No
C14	Consumo de alcohol (actual)	Si	No

D. Historia de TB

Antecedente TB ? Si / No D1

Numero de veces de TB D2

BCG ? Si / No D4

Numero de cicatrices vacunas D5

INH profilaxis? No / Si, actual / Si antes D6

Fecha de inicio _ _ / _ _ / _ _ D7

Tiempo de profilaxis (meses) D8

D3 Antecedentes diagnosticos, tratamientos y resultados

Tipo	Fecha de diagnosis	Forma diagnostica			H	R	P	E	S	O	Tiempo (meses)	C	A	F
		BK frotis	cultivo	biopsia										
Pulmonar														
Otro (donde?)														

isoniacida (H), rifampicina (R), pirazinamida (P), ethambutol (E), streptomicina (S), otro (O); curado (C), abandono (A), fracaso (F)

G. Investigaciones

Investigación	Fecha	Muestra ID	Resultados											
			auramina			cultivo LJ			cultivo MB BacT			cultivo MODS		
			pos	neg	desconocido	pos	neg	desconocido	pos	neg	desconocido	pos	neg	desconocido
Esputo 1		TBHO. _ _ _ _ . EP _												
Esputo 2		TBHO. _ _ _ _ . EP _												
Lavado gastrico 1		TBHO. _ _ _ _ .LG _												
Lavado gastrico 2		TBHO. _ _ _ _ .LG _												
Radiographia toracica			Normal / Anormal											
			Si anormal...											
			alto sospecho de TBC activo			TBC activo posible					no sospecho de TBC			
PPD (5iu mantoux)			_ _ _ mm induración (sí tuvo en los ultimós 6 meses, indica resultado y fecha)											
Recuento de CD4			_ _ _ _ cellulos/mm^3											

Notas: Codificación de muestras

- el código de estudio
- y un número único para cada paciente
- y una identificación de muestra (EP por esputo y LG por lavado gastrico)
- seguido por el numero que indica la muestra

Muestras son identificado para:

TBHO
TBHO . **0157**
TBHO . 0157 . **EP**
TBHO . 0157 . EP **1**

Cada paciente tiene 2 muestras para cada hospitalización. Si hubieran subsiguientes hospitalizaciones, el numero de la muestra sería consecutivo con respecto a las muestras anteriores de las anteriores hospitalizaciones. Por ejemplo, muestras de la segunda hospitalización pueden ser EP3 y LG4 (o EP3 y EP 4, o LG3 y LG4 o LG3 y EP 4!). (El nombre codigo es solamente por anonimato en base de datos e.g. JUPS0157)

H. Otras cosas

Numero de hospitalizacion de este paciente en este estudio _____ H1

Causas de ingresso _____ H2

Fecha de diagnosis de VIH _ _ / _ _ / _ _ H3

Appendix 6

Dataset containing drug-susceptibility results from hospitalized AIDS patients with drug-resistant tuberculosis who provided duplicate sputum samples on consecutive days

(Study #3)

Patient	Sample Number	Sputum mcroscopy	Isoniazid					Rifampicin					Streptomycin					Ethambutol					*No. of discordant drug-susceptibility results per sample*
			MODS	MABA on MODS	MbBacT	Proportion Method (LJ)	MABA on LJ	MODS	MABA on MODS	MbBacT	Proportion Method (LJ)	MABA on LJ	MODS	MABA on MODS	MbBacT	Proportion Method (LJ)	MABA on LJ	MODS	MABA on MODS	MbBacT	Proportion Method (LJ)	MABA on LJ	
1	1	1	S	S_1	S	S	S_1	S	S_1	R	S	S_1	S	S_2	S	S	S3	S	S_6	S	S	S_4	***1***
	2	1	S	S_1	S	S	S_1	S	S_1	S	S	S_1	S	S_2	S	S	S_2	S	S_4	S	S	S_4	***0***
2	1	0		S_1	S	S	S_1	.	S_1	R	S	S_1	.	S_2	S	S	S_2	.	S_5	R	S	S_4	***2***
	2	0	S	S_1	S	S	S_1	S	S_1	S	S	S_1	S	S_2	S	S	S_2	S	S_3	S	S	S_4	***0***
3	1	0	S	S_1	S	S	S_1	S	S_1	S	S	S_1	S	S_2	S	R	S_2	S	S_3	S	S	S_3	***1***
	2	0	S	S_1	S	S	.	S	S_1	S	S	.	S	S_2	S	R	.	S	S_3	S	S	.	***1***
4	1	0	S	S_1	S	S	S_1	S	S_2	S	S	S_1	S	S_2	S	R	S_2	S	S_4	S	S	S_3	***1***
	2	1	S	S_1	S	S	S_1	S	S_1	S	S	S_1	S	S_2	S	R	S_2	S	S_3	S	S	S_4	***1***
5	1	1	S	S_1	S	S	S_1	S	S_1	S	S	S_1	S	S_1	S	R	S_1	S	S_2	S	S	S_3	***1***
	2	1	S	S_1	S	S	S_1	S	S_1	S	S	S_1	S	S_1	S	R	S_1	S	S_4	S	S	S_3	***1***
6	1	1	S	S_1	S	S	S_1	S	S_1	S	S	S_1	R		R	R		S	S_3	S	S	S_3	***0***
	2	2	S	S_1	S	S	S_1	S	S_1	S	S	S_1	R		R	R		S	S_2	S	S	S_5	***0***
7	1	1	S	S_1	S	S	S_1	S	S_1	S	S	S_1	R	S_5	R	R	S_3	S	S_3	S	S	S_2	***0***
	2	1	S	S_1	S	S	S_1	S	S_1	S	S	S_1	R	S_4	R	R	S_4	S	S_3	S	S	S_3	***0***
8	1	3	S	S_2	R	R	R_3	S	S_1	S	S	S_1	S	S_2	S	S	S_2	S	S_3	R	S	S_4	***3***
	2	2	S	R_3	R	R	S_2	S	S_1	S	S	S_1	S	S_2	S	R	S_2	S	S_4	R	S	S_4	***3***
9	1	0	R	R_5	R	R	.	S	S_2	S	S	.	S	S_5	R	R	.	S	S_3	S	S	.	***1***
	2	0	R	R_6	R	R	.	S	S_1	S	S	.	S	S_3	S	R	.	S	S_2	S	S	.	***1***
10	1	1	R	R_4	R	R	R_4	S	S_1	S	S	S_1	S	S_4	S	R	S_5	S	S_1	S	S	S_2	***1***
	2	0	R	R_4	R	R	R_5	S	S_1	S	S	S_2	.	S_5	S	R	S_4	.	S_3	S	S	S_4	***1***
11	1	0	R	R_5	R	R	R_5	S	S_1	S	S	S_1	S	S_3	R	R	S_4	S	S_4	R	S	S_4	***2***
	2	0	R	R_6	R	R	R_5	S	S_1	S	S	S_1	S	S_3	R	R	S_3	S	S_4	R	S	S_4	***2***
12	1	3	R	R_7	R	R	R_6	R	R_4	R	R	S_2	R	S_4	R	R	S_5	R	R_6	R	S	S_4	***1***
	2	2	R	R_7	R	R	R_6	S	S_3	S	R	S_3	R	S_5	R	R	S_5	S	S_5	R	S	S_4	***2***
Number of discordant results per patient			***0***	***1***	***0***	***0***	***1***	***1***	***1***	***3***	***0***	***0***	***0***	***0***	***1***	***1***	***0***	***1***	***1***	***1***	***0***	***0***	

*For MODS, MbBacT and Proportion Method: R=Resistant to drug; S=Susceptible to drug
For MABA: the critical concentration (ug/mL) for Isoniazid was 0.125 (S_1), 0.250 (S_2), 0.500 (R_3), 2.00 (R_4), 4.00 (R_5), 8.00 (R_6), 16.00 (R_7); for Rifampicin 0.063 (S_1), 0.125 (S_2), 0.250 (S_3), 2.00 (R_4); for Streptomycin 0.125 (S_1), 0.250 (S_2), 0.500 (S_3), 1.00 (S_4), 2.00 (S_5); for Ethambutol 0.500 (S_1), 1.00 (S_2), 2.00 (S_3), 4.00 (S_4), 8.00 (S_5), 16.00 (R_6)

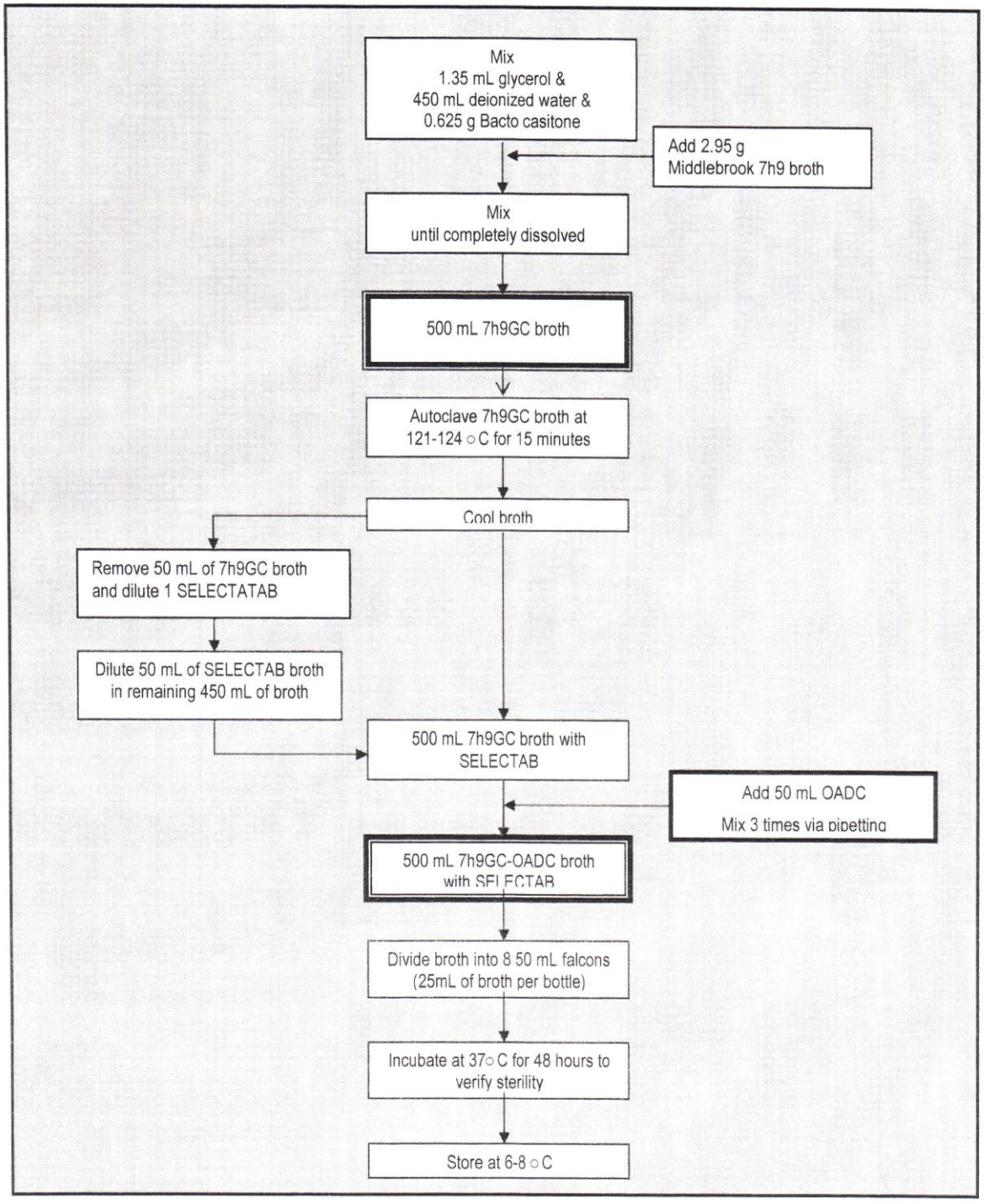
Appendix 7

Laboratory protocol for testing samples with the Microscopic Observation Drug Susceptibility Assay

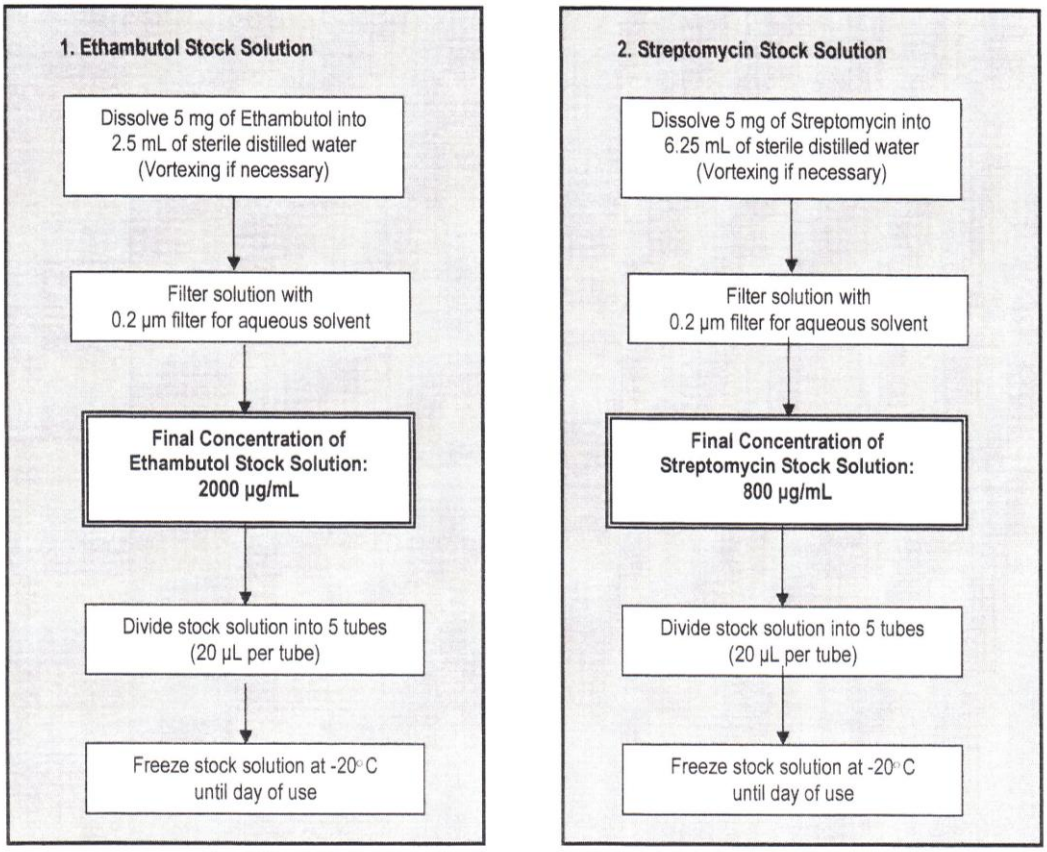
EXPERIMENT #49
CRITICAL CONCENTRATIONS FOR DRUG SUSCEPTIBILITY TESTING USING MODS

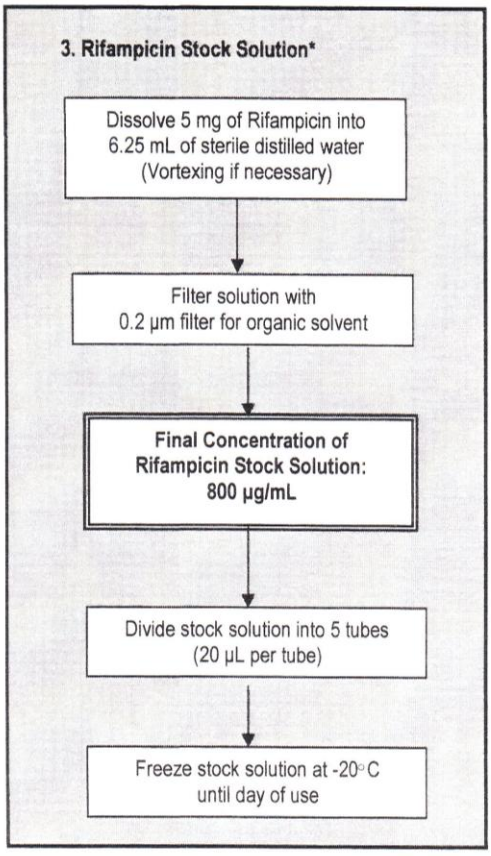
LAB PROTOCOL

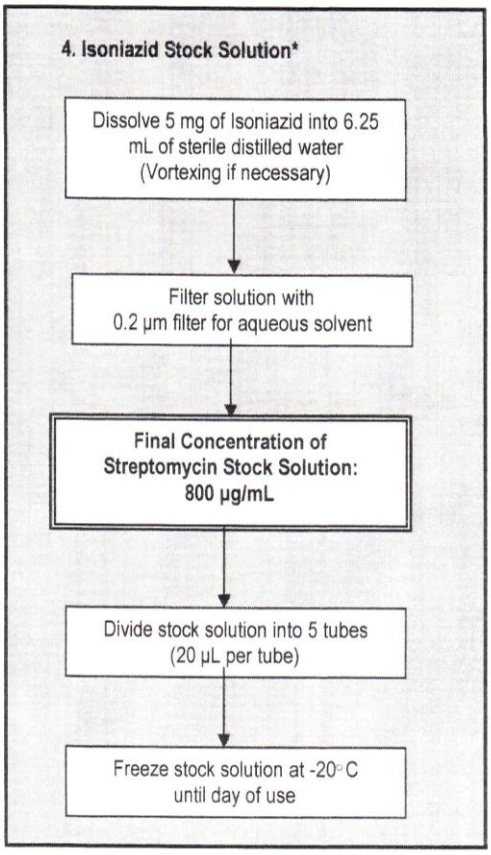
STEP 1. PREPARATION OF 7H9GC-OADC (with SELECTAB) BROTH



STEP 2. PREPARATION OF FROZEN STOCK SOLUTIONS







*For other drugs, replace by:
-5 mg of ciprofloxacin into 6.25 mL of sterile distilled water
-5 mg of capreomycin into 1.25 mL of sterile distilled water

STEP 3. DILUTION OF STOCK SOLUTION & PREPARATION OF MICROPLATES

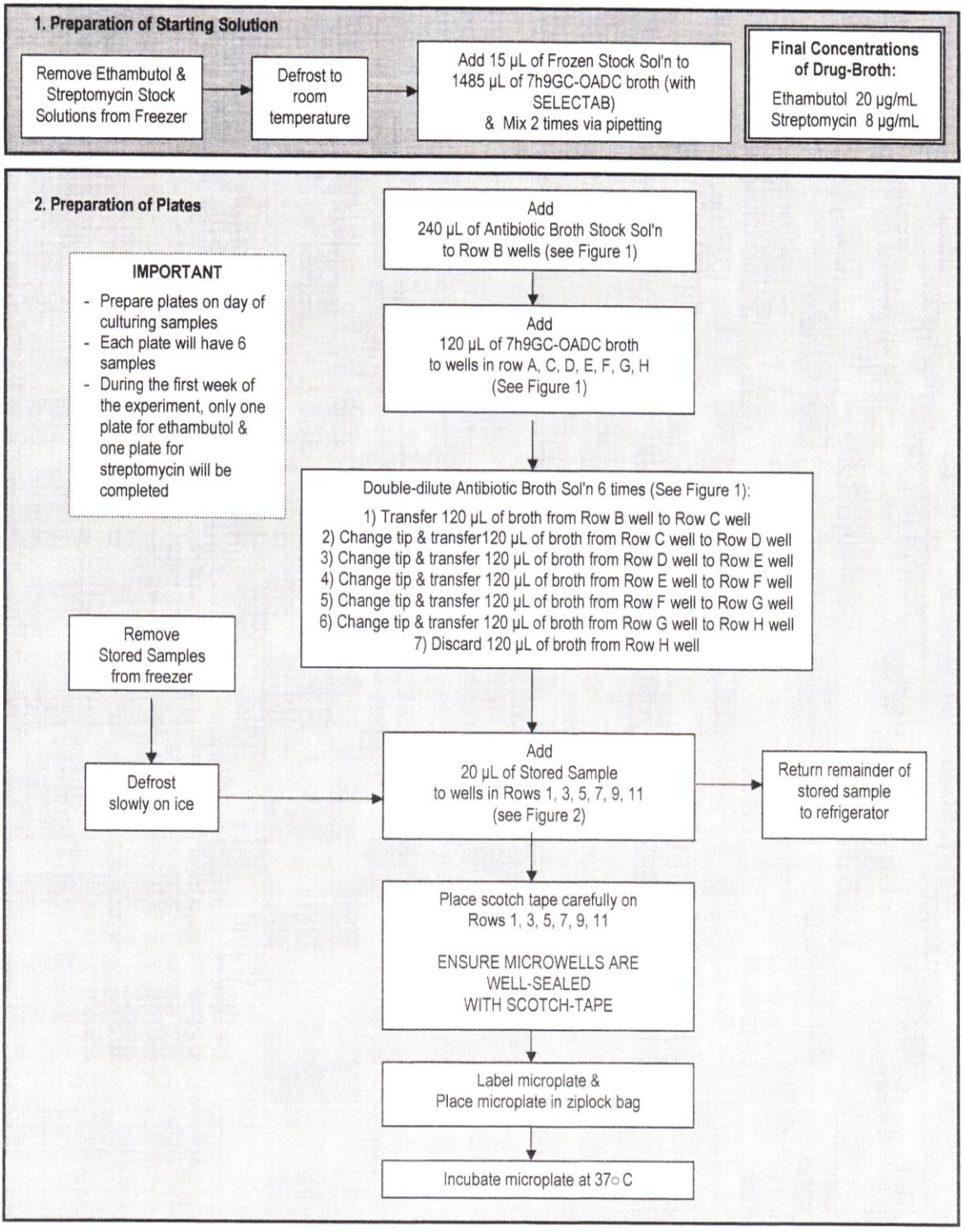
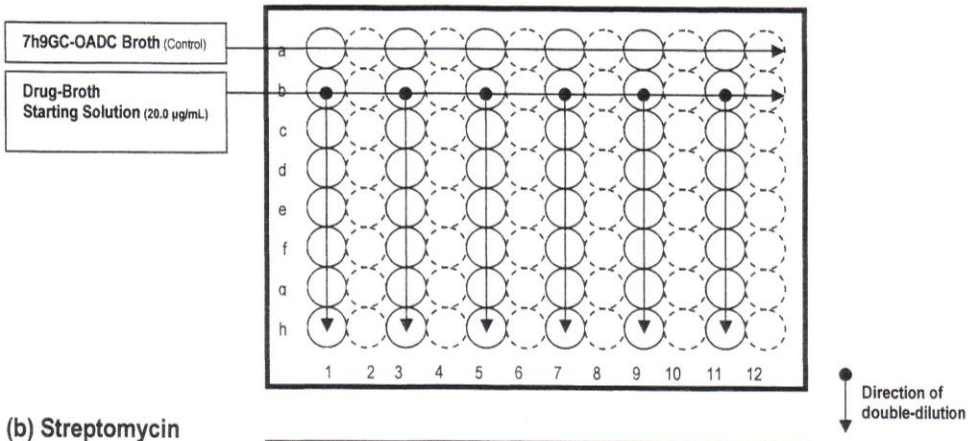


Figure 1. Layout of Microplates for pipetting broth

(a) Ethambutol



(b) Streptomycin

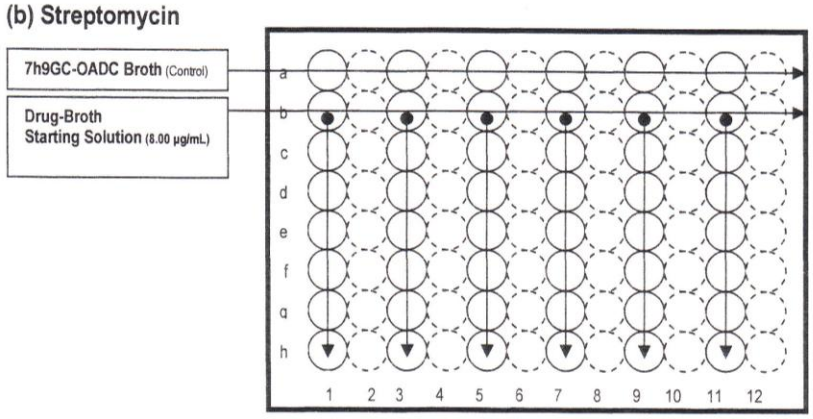
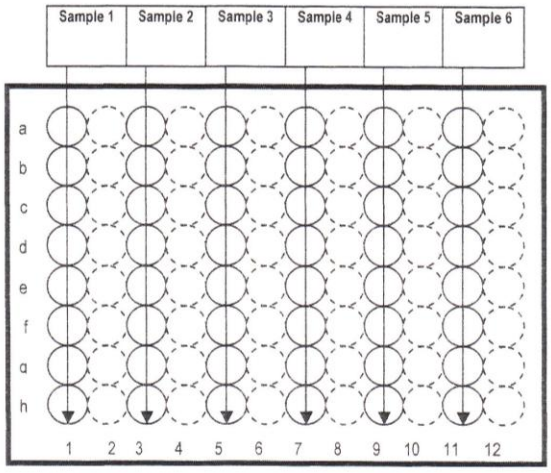


Figure 2. Layout of Microplates for pipetting sample

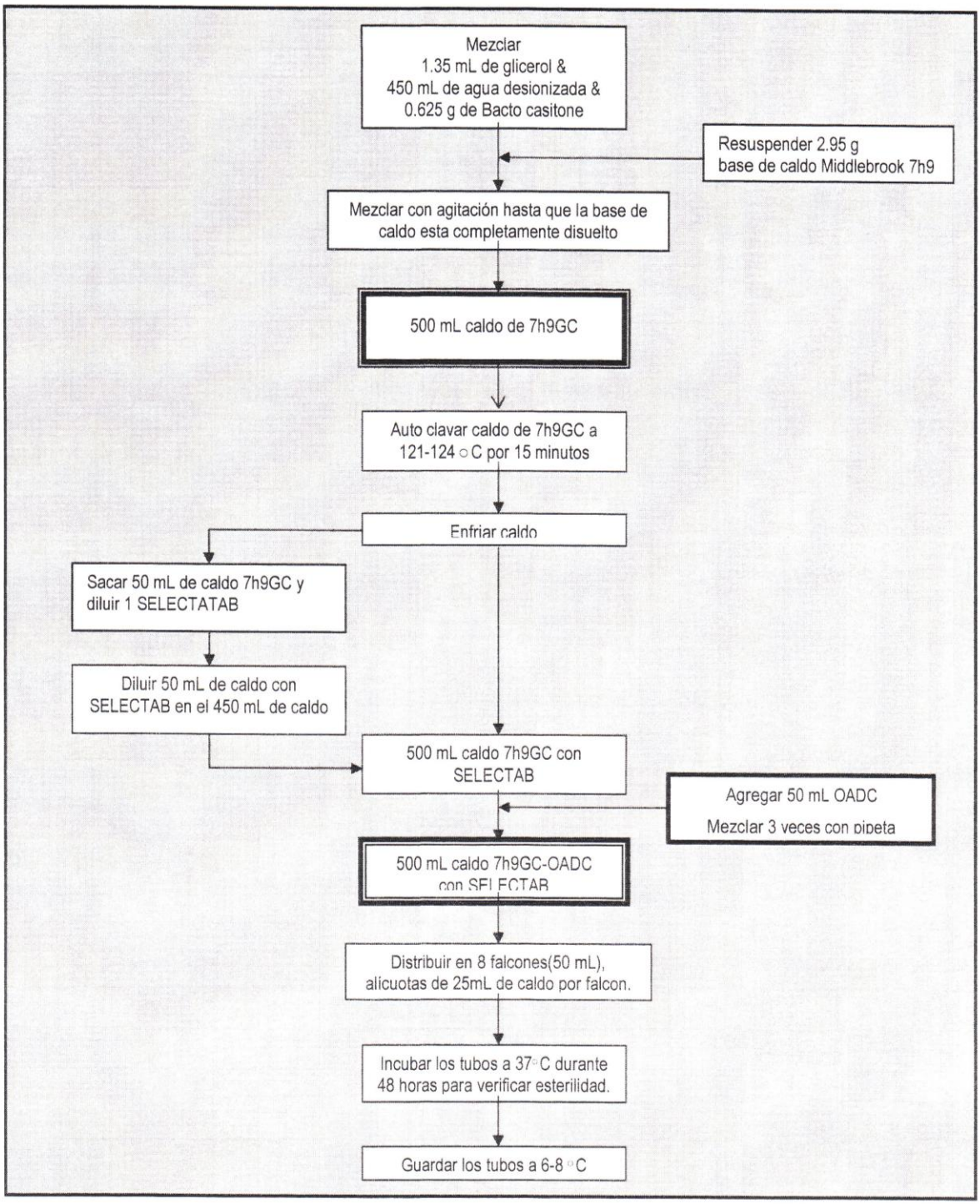


EXPERIMENTO #49

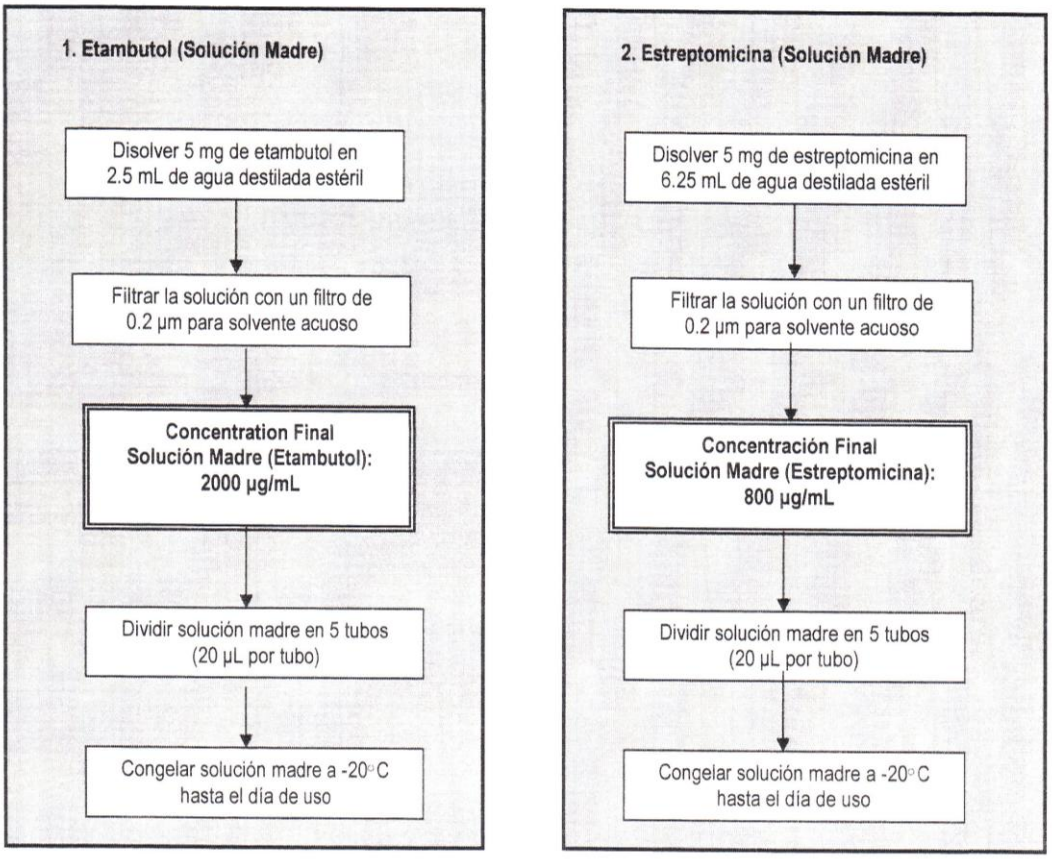
Concentraciones Criticas en MODS para detectar TB resistente a drogas

PROTOCOLO DEL LABORATORIO

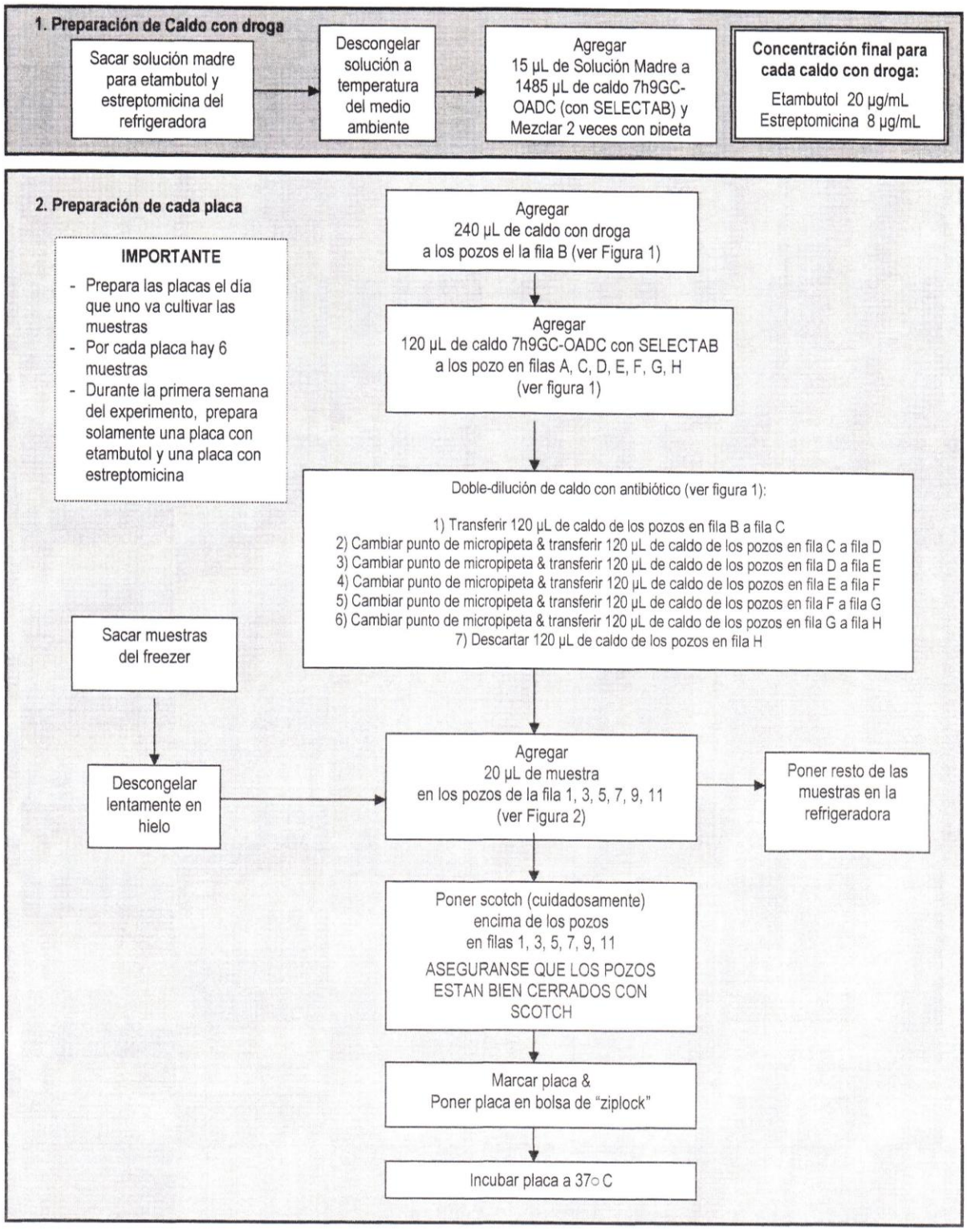
ETAPA 1. PREPARACION DE CALDO 7H9GC-OADC



ETAPA 2. PREPARACION DE SOLUCION MADRE (CONGELADA)



ETAPA 3. DILUCION DE SOLUCION MADRE & PREPARACION DE PLACAS



ETAPA 4. LECTURA DE PLACAS

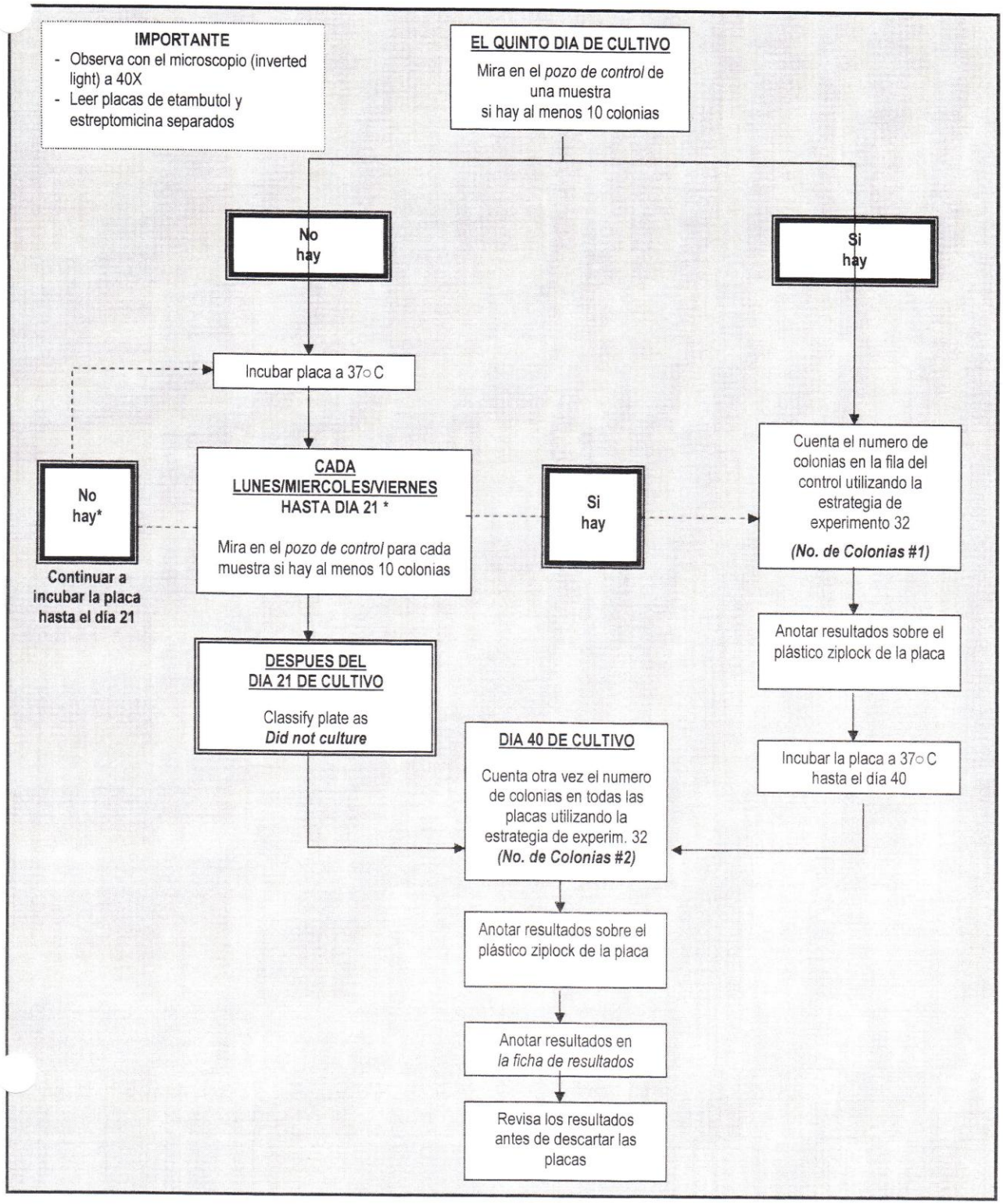
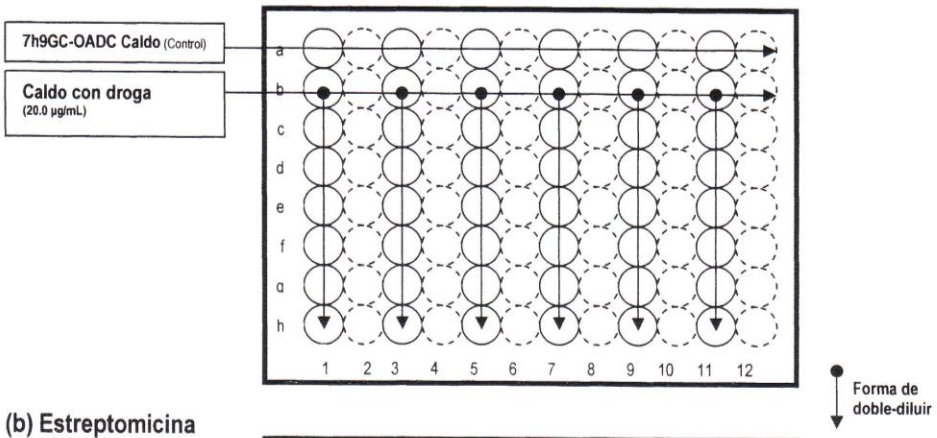


Figure 1. *Esquema de Placas – Forma de distribuir caldo*

(a) Etambutol



(b) Estreptomicina

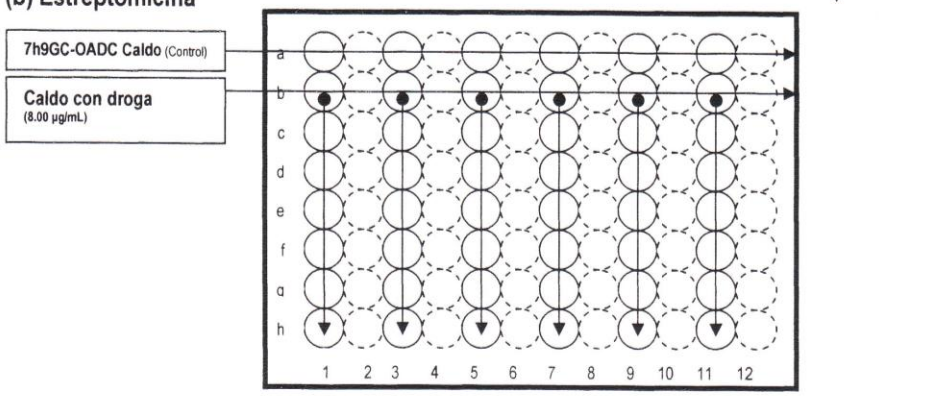
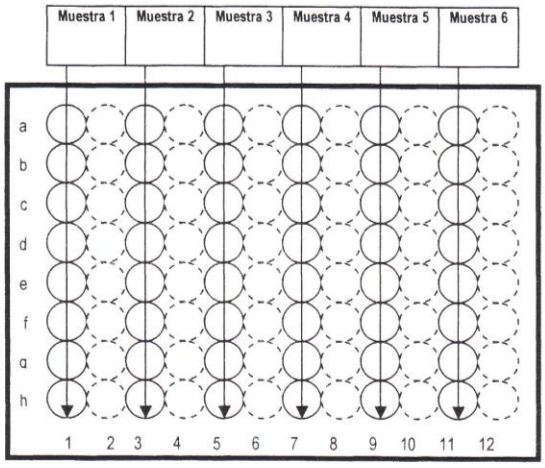


Figure 2. ***Esquema de Placas – Forma de distribuir muestra***



MATERIALES

I. PREPARACION DE CALDO 7H9GC-OADC

- 1.18 g base de caldo Middlebrook 7h9
- 0.25 g Bacto casitone
- 0.62 mL Glicerol
- 20 mL OADC
- 180 mL agua desionizada
- 1000 mL aqua distilada
- 1 SELECTATAB
- 8 falcones (50 mL)
- pipeta

II. PREPARACION DE SOLUCION MADRE (CONGELADA)

- 5 mg etambutol
- 5 mg estreptomicina
- 8.75 mL de agua destilada estéril
- 4 filtros para solvente acuoso (0.2 µm)
- 10 tubos (15 mL)
- 4 jeringas (10 mL)
- 4 agujas (21 x 1 ½)

III. DILUCION DE SOLUCION MADRE & PREPARACION DE PLACAS

Por cada 2 placas:

- 15 µL de Solución Madre para etambutol
- 15 µL de Solución Madre para estreptomicina
- 16 mL de caldo 7h9GC-OADC
- placas (96 posillas)
- 14 eppendorfs
- micropipeta
- puntas para micropipeta
- scotch
- bolsas de ziplock

Appendix 8 (a)

Copy of data collection sheet for tuberculosis samples tested for ethambutol and streptomycin resistance with the:

Microscopic Observation Drug Susceptibility Assay

Placa No.: ____

Fecha de Siembra: ☐☐ - 07 (M D)

Antibiótico en Placa:

Sm ☐ Emb ☐

LECTURA EN POZO DE CONTROL
(crecimiento si hay al menos 10 colonias)
(si= + no=- c=contaminada)

Fecha de Lectura	Fila 1 TBV	Fila 3 TBV	Fila 5 TBV	Fila 7 TBV	Fila 9 TBV	Fila 11 TBV
Lectura 1 ☐☐ - 07 (M D)						
Lectura 2 ☐☐ - 07 (M D)						
Lectura 3 ☐☐ - 07 (M D)						
Lectura 4 ☐☐ - 07 (M D)						
Lectura 5 ☐☐ - 07 (M D)						
Lectura 6 ☐☐ - 07 (M D)						
Lectura 7 ☐☐ - 07 (M D)						
Lectura 8 ☐☐ - 07 (M D)						
Lectura 9 ☐☐ - 07 (M D)						
Lectura 10 ☐☐ - 07 (M D)						

Observaciones: ____

C:\Documents and Settings\Default\My Documents\PhD Thesis\Critical Concentrations for MODS\0707\Ficha de Datos.doc
MG/.

CONTAR NUMERO DE COLONIAS

	Fila 1	Fila 3	Fila 5	Fila 7	Fila 9	Fila 11
Fecha de primer calculo	☐☐ - 07 (M D)	☐☐ - 07 (M D)	☐☐ - 07 (M D)	☐☐ - 07 (M D)	☐☐ - 07 (M D)	☐☐ - 07 (M D)
Fecha de segundo calculo	☐☐ - 07 (M D)	☐☐ - 07 (M D)	☐☐ - 07 (M D)	☐☐ - 07 (M D)	☐☐ - 07 (M D)	☐☐ - 07 (M D)

1. Primer día de crecimiento o día 21 si no hay crecimiento

	Fila 1	Fila 3	Fila 5	Fila 7	Fila 9	Fila 11
Pozo A (Control)						
Pozo B						
Pozo C						
Pozo D						
Pozo E						
Pozo F						
Pozo G						
Pozo H						

2. Día 40 de cultivo

	Fila 1	Fila 3	Fila 5	Fila 7	Fila 9	Fila 11
Pozo A (Control)						
Pozo B						
Pozo C						
Pozo D						
Pozo E						
Pozo F						
Pozo G						
Pozo H						

Appendix 8 (b)

Copy of data collection sheet for tuberculosis samples tested for ethambutol and streptomycin resistance with the:

MicroAlamar™ Blue Assay

Universidad Peruana Cayetano Heredia

PROYECTO DE SALUD EN VENTANILLA

PRISMA

Donación de vitaminas para la búsqueda de protección de contactos sanos de un nuevo caso de TBC
a todos los participantes voluntarios se les ofrecen cultivos de esputo gratuito. y estudios de sensibilidad.
Una colaboración entre: PCT, DISA Callao, Dirección de Salud de Ventanilla, Centros de salud y personal del PCT.
Universidad Peruana Cayetano Heredia, A.B.Prisma.

Prueba de esputo opcional y gratuito. Para todas las muestras Bk positivos (baar,+,++,+++) y todas las muestras de rutinas del PCT con etiquetas de PRISMA. Si tiene preguntas acerca de las pruebas de esputo no dude en comunicarse a los Telfs. : 3820929 o 99517233

CSM/ CSV: _____ Fec.Colec: __/__/200 Fec. De BK : _____200__ C.S.: _____

Nombres: _____ A.Paterno: _____ A.Materno: _____ Cod.Z/ _____

Dirección: _____ Edad: _____

Resultado Bk en Laboratorio Centro de Salud de Ventanilla Un BK positivo es una evidencia fuerte de TBC pulmonar, pero algunos pacientes con TBC pulmonar tienen BK (-)	No tiene	
	BAAR	
	BK (-)	
	BK(+)	
	BK(++)	
	BK(+++)	

Firma de Técnico de Laboratorio: _____

Resultado Cultivo en Universidad Peruana Cayetano Heredia Un resultado positivo es una confirmación de TBC pulmonar. Una proporción de pacientes con TBC MDR tienen curación con tratamiento normal, otros necesitan tratamiento especial.Prueba de sensibilidad directa y rápida (MODS) Journal Clinical Microbiology	Se necesita otra muestra	
	Cultivo negativo (-) No TBC en esta muestra	
	Cultivo Positivo de TBC Sensible (no es MDR)	
	Cultivo Positivo de TBC MDR (multidrogo resistente) Sensibilidad de Segunda Línea después de dos meses	
	Cultivo Positivo de TBC pero Sensibilidad pendiente	

Notas: _____

Cod TBV: _____

Responsable de Procesamiento _____ Jefe del Área de Mycobacterium _____ ó Médico Investigador Principal Fecha: __/__/200__

1.-***Copia en Oficina :*** Ingresado a Base de Datos _____ _____ Fecha: __/__/200__

2.-***PCT de la Posta del Paciente:*** Recibido por: _____ Fecha: __/__/200__

3.-***Centro de Salud de Ventanilla:*** _____ Fecha: __/__/200__

4.-***Dirección de Laboratorio de Salud Pública de la DISA I Callao:*** _____ Fecha: __/__/200__

Fec. Decont: ____/____/200__ CSV / CSM: _____ TBV: _____ Z : _____ - ___
Fec. Siembra: ____/____/200__

Volúmen: _____ Original: + _____ml ssf

Deco por _____min en NaOH : _____%

Si no hay Bk ---> Auramina : ☐

Fecha de primer positivo en '0' / contaminación : ____/____/200___
Fec.de Lectura 3 días despues y conteo : ____/____/200___

	Detección	I	R	I - R
Control				
Muestra 1 : 1				
Muestra 1 : 100				

Morfologia: TBC / probablemente / atipica _____

Resultado Inicial : Sensible INH / RIF / MDR / Pendiente / Negativo / Contaminado / Cont.Negativo

Fecha de chequeo de los 35 días o de lectura negativa: ____/____/200___
El mismo resultado u otro : Sensible / INH RIF / MDR / Pend. / Neg. / Cont. / Cont.Neg.

Coloración : ____/____/200___ Resultado : _____

Resp.Proces.: _____

Resp.Lectura : _____

Fecha de Redeco : ____/____/200___ Fec. Siembra: ____/____/200__
Fecha de primer positivo en '0' / contaminación : ____/____/200___
Fec.de Lectura 3 días despues y conteo : ____/____/200___

	Detección	I	R	I - R
Control				
Muestra 1 : 1				
Muestra 1 : 100				

Morfologia: TBC / probablemente / atipica _____

Resultado Inicial : Sensible / INH / RIF / MDR / Pendiente / Negativo / Contaminado / Cont.Negativo

Fecha de chequeo de los 35 días o de lectura negativa: ____/____/200___
El mismo resultado u otro : Sensible / INH / RIF / MDR / Pend. / Neg. / Cont. / Cont.Neg.

Coloración : ____/____/200___ Resultado : _____

Resp.Proces.: _____

Resp.Lectura : _____

Fecha de Repique: ____/____/200___ MABA : NO SI ____/____/200___ Resultado: _____
Crio : ____/____/200___

CAJA : ☐ UBICACIÓN : ☐

UNIVERSIDAD PERUANA CAYETANO HEREDIA

PROYECTO DE SALUD EN VENTANILLA

PRISMA

Donación de vitaminas para la búsqueda de protección de contactos sanos de un nuevo caso de TBC
a todos los participantes voluntarios se les ofrecen cultivos de esputo gratuito, y estudios de sensibilidad.
Una colaboración entre: PCT, DISA Callao, Dirección de Salud de Ventanilla, Centros de salud y personal del PCT,
Universidad Peruana Cayetano Heredia, A.B.Prisma.

Prueba de esputo opcional y gratuito. Si tiene preguntas acerca de las pruebas de esputo no dude en comunicarse a los Telfs. : 3820929 o 99517233

Cód.Lab.TBV: _____ Cód.Lab.CSV: _____ Fec.de Muestra: ___/___/200__ Centro de Salud: _____

Nombres: _____ A.Paterno: _____ A.Materno: _____ Cod.Z _____

Dirección: _____ Edad: _____

Resultado de Sensibilidad en Universidad Peruana Cayetano Heredia

Prueba de sensibilidad indirecta (MABA) Journal Clinical Microbiology 36:362. Después de un cultivo positivo es necesario ~2 meses para esta prueba

Antibiótico	Concentración critica (µg/ml)	MIC (µg/ml)	
Isoniazida	0.2		Sensible / Resistente
Rifampicina	1.0		Sensible / Resistente
Streptomicina	2.0		Sensible / Resistente
Ethambutol	5.0		Sensible / Resistente
Capreomicina	aproximadamente 10		Sensible / Resistente
Ciprofloxacina	aproximadamente 1		Sensible / Resistente

Notas: _____

_____ _____

Responsable de Procesamiento — Jefe del Área de Mycobacterium ó Médico Investigador Principal — Fecha: ___/ ___/ 200__

1.-***Copia en Oficina :*** Ingresado a Base de Datos : _____ Fecha: _____/ ___/ 200__

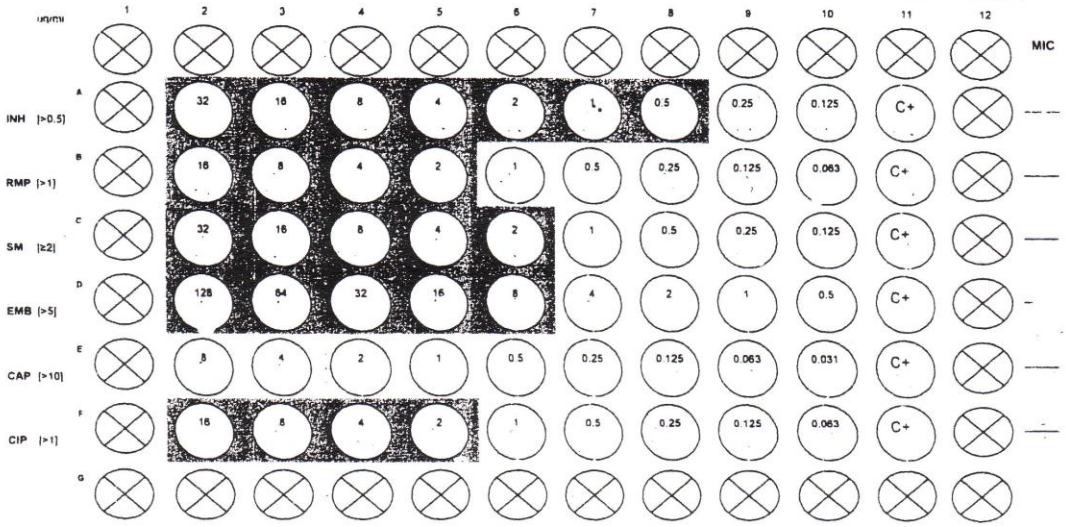
2.-***Centro de Salud de Ventanilla:*** Recibido por _____ Fecha: _____/ ___/ 200__

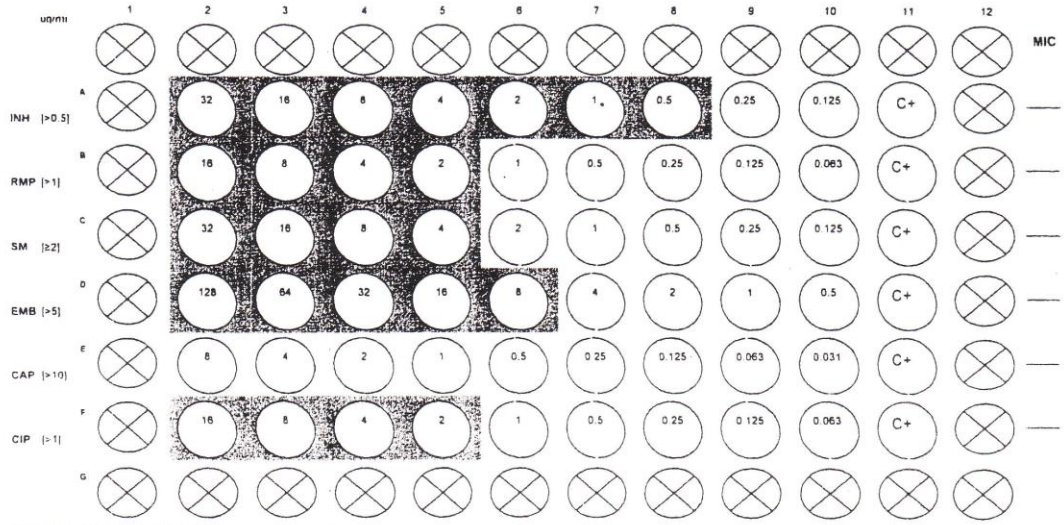
3.-***Dirección de Laboratorio de Salud Pública de la DISA I Callao:*** _____ Fecha: _____/ ___/ 200__

Cod Lab TBV _____ (CSV/CSM _____ Cod Z _____ Fecha Col _____ Fecha Deco _____ Inoculation date _____

Positive at: _____ Day 5 _____ Day7 _____ Day9 _____ Day11 _____ Day13 _____ Day15

Observación _____





M: morado con crecimiento
A: Amarillo sin crecimiento
MIC: Es la mimina concentración inhibitoria. De color amarillo por que existe inhibición.
CC: Concentración critica, SI existe crecimiento de TB en CC entonces la cepa es resistentes
Si hay crecimiento (MORADO) en zonas sombreadas existe resistencia

Appendix 9

Description of study variables included in the thesis

Variables included in Chapter 4 analysis
(Role of AIDS during an ongoing outbreak of MDR-TB)

Variable	Definition	Scale
Outcomes		
Pulmonary tuberculosis	Isolation of culture positive *Mycobacterium tuberculosis* from sputum [Yes, No]	Dichotomous
Drug-Resistant tuberculosis (includes mono-resistance to rifampicin or isonizanid, and multi-drug resistance to both rifampicin and isoniazid)	*Mycobacterium tuberculosis* that is resistant to either rifampicin or isoniazid [Yes, No]	
Strain of *M. tuberculosis*	The lineage of *M. tuberculosis* as characterized by spoligotype	Categorical (multiple strains)
Clone of *M. tuberculosis*	An isolate of *M. tuberculosis* descending from a common precursor strain as characterized by RFLP	
Independent Variables		
Sputum smear status	Presence of acid fast bacilli in sputum [0-, 1+, 2++, 3+++]	Dichotomous (smear positive/negative) or Quartiles
Age	Age in years at the time of diagnosis	Continuous
Sex	Male, Female	Dichotomous
HIV-infection	Tested positive for HIV [Yes, No]	
Underweight	A body mass index less than 18.5 kg/m^2 [Yes, No]	
History of TB	Previous diagnosis of TB [Yes, No/Not known]	
Contact with tuberculosis	Known contact with a tuberculosis patient during the previous two years [Yes, No]	
Past hospitalization	Hospitalized during the previous two years [Yes, No]	

Clinical Manifestations		
Cough	Persistent, urgent or strong cough that is more than just an irritating cough and occurs several times during the day [Yes, No]	Dichotomous
Productive Cough	Urge to cough or clear throat, accompanied by phlegm, saliva, etc. and occurs several times during the day [Yes, No]	Dichotomous
Fever	Body temperature is above 37°C several times in a day [Yes, No]	
Shortness of breath	Sensation of shortness of breath or normal breathing which does not provide enough air [Yes, No]	
Hemoptysis	Same as productive cough but accompanied by blood [Yes, No]	
Weight loss	Evident loss of weight with no explanation [Yes, No]	
Loss of appetite	Evident loss of appetite with no explanation [Yes, No]	
Night Sweats	Recurrent atypical sweating during the night [Yes, No]	
Fatigue	Tiredness or weakness with no explanation [Yes, No]	

Variables included in Chapter 5 analysis
(Shedding of *mycobacteria* in stool of patients with pulmonary tuberculosis)

Variable	Definition	Scale
Outcomes		
Pulmonary tuberculosis	Isolation of culture positive *Mycobacterium tuberculosis* from sputum [Yes, No]	Dichotomous
Shedding in Stool	Isolation of cuture positive *mycobacteria* from stool [Yes, No]	
Shedding in Urine	Isolation of culture positive *mycobacteria* from urine [Yes, No]	
Independent Variables		
Sputum Smear Status	Presence of acid fast bacilli in sputum [0-, 1+, 2++, 3+++]	Dichotomous (smear positive or negative) or Quartiles
Age	Age in years at the time of diagnosis	Continuous
Body Mass Index (BMI)	BMI in kilograms per meters^2	
CD4 Count	Number of CD4 cells per µL	
Sex	Male, Female	Dichotomous
History of TB	Previous diagnosis of TB [Yes, no/Not known]	
History of TB prophylaxis	Patient previously received prophylaxis during the previous year for tuberculosis [Yes, No/Not known]	
Antiretroviral Therapy	Receiving antiretroviral drugs for HIV at the time of diagnosis [Yes, No]	
BCG Vaccination	Presence of a BCG scar on either arm of the patient [Yes, No]	
Contact with tuberculosis	Known contact with a tuberculosis patient during the previous two years [Yes, No]	

Past hospitalization	Hospitalized during the previous two years [Yes, No]	
Worked and/or lived in Prison or shelter	Worked and/or lived in prison or shelter during the past [Yes, No]	

Variables included in Chapter 6 analysis
(Clinical utility of MODS for screening AIDS patients for pulmonary TB)

Variable	Definition	Scale
Outcomes		
Pulmonary tuberculosis	Isolation of culture positive *Mycobacterium tuberculosis* from sputum [Yes, No]	Dichotomous
Sputum smear status	Presence of acid fast bacilli in sputum [0-, 1+, 2++, 3+++]	
Independent Variables		
Cough	Persistent, urgent or strong cough that is more than just an irritating cough and occurs several times during the day [Yes, No]	Dichotomous
Productive Cough	Urge to cough or clear throat, accompanied by phlegm, saliva, etc. and occurs several times during the day for 15 days or more [Yes, No]	
Prolonged Cough	Urge to cough or clear throat, accompanied by phlegm, saliva, etc. which presents itself several times a day [Yes, No]	
Fever	Body temperature is above 37˚C several times in a day [Yes, No]	
Weight loss	Evident loss of weight with no explanation [Yes, No]	
Night Sweats	Recurrent atypical sweating during the night [Yes, No]	
Constitutional symptoms	Reported cough with at least one constitutional symptom (fever, night sweats or weight loss) [Yes, No]	
Radiographic appearance of TB	Chest radiograph consistent with tuberculosis (e.g., presence of pleural lesions, cavitation, infilitrates or hilar enlargement) [Yes, No]	

Appendix 10

Additional study results on

stool shedding of *Mycobacterium tuberculosis*

The gastrointestinal tract continues to be the most common site for opportunistic infections among HIV-infected individuals (1, 2). The elevated risk of infection is often attributed to the gastrointestinal tract being a major site of HIV replication, which causes a depletion in CD4 counts making the patient vulnerable to infection (3, 4). Previous studies have shown that almost half of otherwise healthy patients with smear-positive cavitary pulmonary tuberculosis are infected by gastrointestinal tuberculosis (5). Despite this evidence, there is little research regarding the prevalence of stool shedding with *Mycobacterium tuberculosis* among AIDS patient (6). In this paper, we determined the extent of gastrointestinal involvement in pulmonary tuberculosis by examining the prevalence and risk factors for stool shedding among AIDS patients.

Data was collected between March 2002 and January 2004 at the Infectious Disease Clinic of the Hospital Dos de Mayo. We examined stool shedding among two groups of AIDS patients at different stages in the natural history of tuberculosis. The first group (i.e., *patients in early stages of pulmonary tuberculosis*) was identified by screening 473 consecutively enrolled HIV-infected patients who reported coughing during at least one week. Patients with productive or wet cough (n=154) were asked to provide two sputum and one stool sample. Sputum samples were collected on consecutive days with all samples being collected within a one-week period. Patients with a non-productive or dry cough (n=319) were asked to provide one sputum and one stool sample on the same day. Sputum was induced using a hypertonic saline solution. The second cohort (i.e., *patients in advanced stages of tuberculosis*) consisted of 106 AIDS patients who were already diagnosed with tuberculosis (pulmonary or extra pulmonary forms) at the time of study entry; and, who were about to commence Directly Observed Therapy Short-Course (DOTS) as part of the Peruvian National Tuberculosis Control Program. Patients were interviewed at the start of treatment regarding clinical manifestations and asked to provide one sputum and one stool sample at the time of study enrollment (day 0), day 1 as well as at month 1, month 2, month 4, month 6 of DOTS treatment, and once at the end of treatment. Patients recruited into the *early-stage cohort* were eligible to participate in the *advanced-stage cohort* if they were recommended for tuberculosis treatment at a later time. None of the study participants in either cohort were receiving tuberculosis treatment at the time of study enrolment.

Sputum samples were tested for smear status using Auramine Smear Microscopy; culture status using Lowenstein-Jensen and Middlebrook 7h9 Broth (Difco, Detroit, Mich.); and drug-susceptibility status using the Microplate Alamar Blue Assay (MABA). S*tool samples* were tested for smear status using Auramine Smear Microscopy; and then cultured using Lowenstein-Jensen, Middlebrook 7h9 Broth and Middlebrook 7h10 Agar (Difco, Detroit, Mich.). Stool samples were only tested for drug-susceptibility status using MABA if the matching sputum sample from a patient was culture-negative. All culture-positive sputum and stool samples were tested for the presence of *Mycobacterium tuberculosis* using a hemi-nested PCR assay targeting the IS6110 insertion element. Patients had pulmonary tuberculosis if they produced at least one sputum sample that was culture-positive for *M. tuberculosis*.

We found that from the 458 patients who provided sputum and stool samples at study entry, 4.3% (20/473) of early-stage cohort patients and 41% (43/106) of late stage cohort patients tested positive for pulmonary tuberculosis. Among patients recruited into the early-stage cohort, 31% (48/154) with wet cough and 4.4% (14/319) with dry cough had pulmonary tuberculosis. Stool shedding only occurred in the presence of pulmonary tuberculosis, even when patients were diagnosed with extra-pulmonary tuberculosis. 50% (31/62) pulmonary tuberculosis patients shed culture-positive *M. tuberculosis* in stool. Of the 29 pulmonary tuberculosis patients who provided sputum samples on consecutive days, 19 (65%) shed *M. tuberculosis* in stool on both days. Of the 33 patients with later stage pulmonary tuberculosis who were followed-up during stool shedding, 28 (85%) stopped shedding by the first month of DOTS. The remaining 5 (15%) patients who continued

to shed were infected by rifampicin-resistant tuberculosis, and thus may have been failing to respond to treatment. Stool shedding was more common among pulmonary tuberculosis patients with a dry cough [Adjusted Odds Ratio (aOR): 16.4, 95% Confidence Interval (CI): 1.5 – 177]. Stool shedding was not influenced by stage of disease [aOR: 8.4, 95% CI: 0.8 – 91], but shedding increased by grade of sputum smear status ($\chi 2$ test for trend=8.91, p=0.03). This trend remained in our inferential-based analysis after adjusting for age, sex, stage of disease and type of cough [Adjusted Odds Ratio: 2.48, 95% Confidence Interval: 1.2 to 5.3]. Table 1 also shows that shedding was disproportionately greater for patients with an AFB grade 3+++ sputum smear.

Table 1. Factors associated with pulmonary tuberculosis patients shedding *Mycobacterium tuberculosis* in stool

Characteristic	Number of Pulmonary Tuberculosis Patients		Crude Odds Ratio	Adjusted Odds Ratio (95% Confidence Interval)
	No stool shedding n=31	Shedding in stool n=31		
Age				
Less than 25 years	4	3	-	-
25 to 29 years	6	13	2.89	3.05 (0.3 – 28)
30 to 34 years	12	9	1.0	1.03 (0.1 – 9.2)
35 years or more	9	6	0.89	0.90 (0.1 – 8.6)
Sex				
Male	26	25	-	-
Female	5	6	1.25	1.75 (0.3 – 12)
AFB Grade of Sputum Smear				
0				
1+	10	4	-	-
2++	11	7	1.59	1.41 (0.2 – 10)
3+++	4	3	1.87	2.60 (0.2 – 32)
	6	17	7.08	**8.59 (1.1 – 69)**
Type of Cough				
Wet	25	23	-	**-**
Dry	6	8	1.45	**16.4 (1.5 – 177)**
Stage of disease				
Early	14	6	-	-
Late	17	25	3.43	8.4 (0.8 – 91)

` AFB= Acid Fast Bacilli