The Challenges and Opportunities in Tuberculosis Control,
from Texas to Afghanistan

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FOREWORD

Tuberculosis is one of the most ancient human pathogens. Very early in the history of humans, tuberculosis spread throughout the world and currently infects one third of the world’s population\(^1\). Tuberculosis, both as a pathogen and a disease state, is fascinating in its own right. Considering the global public health significance of tuberculosis, which infects one third of the world’s population and kills over 1.5 million people per year, tuberculosis remains a threat and demands further research. For these reasons, my undergraduate research has largely centered on tuberculosis. Chapter 1 gives a broad overview of tuberculosis, applicable but not specific to my research. Each subsequent chapter focuses on a research project, with relevant additional background on tuberculosis. Chapter 2 is based on an internship at the Texas Department of State Health Services analyzing reasons for delayed completion of tuberculosis therapy for patients whose therapy initiated in Texas in 2006. Chapter 3 examines the potential for molecular methods of tuberculosis in low-resource setting, building off of my experience using mutations in the rpoB gene as a surrogate marker of rifampin resistance in Afghanistan. Chapter 4 briefly expands upon further work done in the lab of Dr. Andrew Ellington at the University of Texas at Austin under the supervision of Xi Chen, developing non-enzymatic methods of nucleic acid detection to indicate the presence of tuberculosis and other pathogens.
I. Introduction to Tuberculosis

**Historical Context**

Tuberculosis is an ancient pathogen. Documents by Hippocrates and Galen describe symptoms of tuberculosis\(^2\). As well, archaeological data show bodies with signs of tuberculosis. With industrialization and increased population density during the eighteenth and nineteenth centuries, tuberculosis created a significant health burden, known as consumption and the white plague. During this period of time, tuberculosis caused one in seven deaths in Europe\(^3\). Efforts to cure and contain the disease, such as bleeding, were based on a limited and often mistaken understanding of the etiology of tuberculosis, and usually did more harm than good. The failure to control tuberculosis partially stemmed from the fact that though the infectiousness of tuberculosis was posited as early as 1790, much of Northern Europe continued to discount the possibility of tuberculosis infectiousness and no precautions against transmission were taken. Because tuberculosis often affected multiple members of the same family, tuberculosis was sometimes considered hereditary.

The body of evidence for a microbial basis for tuberculosis continued to grow over the nineteenth century, culminating in 1882 with the isolation of *Mycobacterium tuberculosis* by Robert Koch. Koch was also first to discover and promote the tuberculin skin test as an accurate diagnostic for asymptomatic tuberculosis, a test that continues to be used to this day\(^2\). Dr. Herman Biggs pioneered a public health approach to tuberculosis, in view of its contagious nature. As early as 1889, he convinced New York City to require all health providers to report cases of tuberculosis to the city health
officials. To this day, tuberculosis is monitored, managed, and fought by many level of public health agencies, from a city health department all the way to the WHO\textsuperscript{2,53}.

Mortality and prevalence due to tuberculosis dropped dramatically in the time between Koch and the nineteen fifties, when the first real cure appeared- streptomycin. For a time, the only challenge presented by tuberculosis treatment was getting the medicine to all those who needed it. However, strains resistant to antibiotics quickly rose in the bacteria population. The rise of HIV, with its deadly synergy with tuberculosis, slowed and sometimes reversed the steady decline in tuberculosis incidence observed for the past 150 years. Today, tuberculosis remains a global pandemic, with the added danger of MDR, multiple drug resistant, and XDR- extremely drug resistant strains. The public health approach which drove the substantial reduction in tuberculosis incidence prior to the introduction of antibiotics comprises an integral role in the continuing efforts to combat tuberculosis around the world.

**Mycobacterium Tuberculosis: Infection and Pathogenesis**

*Mycobacterium tuberculosis* is the most common etiological agent of tuberculosis in humans, although humans can also be infected with *Mycobacterium bovis* and *Mycobacterium avium*. Like all bacteria, mycobacteria have a layer of peptidoglycan surrounding the plasma membrane. Mycobacteria have a thick lipid cell wall containing large amounts of mycolic acid. Other prominent molecules are lipoarabinomannan(LAM), a lipopolysaccharide, arabinogalactans, and glycolipids. Technically, mycobacteria are gram positive, but the cell wall excludes the Gram stain, leaving the bacteria only weakly stained. To address these challenges of microscopy, mycobacteria are identified in samples with an acid-fast or Ziehl-Neelsen stain\textsuperscript{3}. The cell
wall is instrumental in bacterial immune evasion within unactivated macrophages and excludes many antibacterial compounds. The cell wall is also thought to be the reason why most mycobacteria can only replicate very slowly, as production of the thick lipid layer is very energy intensive. Embedded within the cell wall proteins, such as porins, proteins that help molecules get through the thick lipid layer. Unlike the lipids, these proteins are antigenic, recognized by T-cells, and the major component of the Purified Protein Derivative, PPD, that forms the TST⁴.

MTB are intracellular pathogens that escape destruction by macrophages and can replicate within them⁵. Naive macrophages are able to phagocytose the bacteria, but the bacteria prevent the fusion of the phagosome with the lysosome. Once phagocytosed, lipids from the mycobacterial cell wall, namely LAM, diffuse into the phagosome and other cellular membranes, fundamentally altering their properties and behaviors. Specifically, phagosomes embedded with LAM do not fuse with the lysosome. Inside the macrophage, the bacteria are temporarily protected from antibodies and other parts of the immune system³. Studies with mice infected with M. avium demonstrate that if tuberculosis spreads to the thymus, new T-cells do not respond to the presence of M. avium antigens. When the immature T-cells differentiate within the thymus, any T-cells that respond to “self” antigens are destroyed. Apparently, infected dendritic cells in the thymus present tuberculosis antigens as “self” antigens. When the T-cells produced by infected thymi encounter mycobacterial antigens, they do not release the INF-γ that activates macrophages and dendrites, as do T-cells from healthy thymi⁶.

The majority of people infected with tuberculosis never become ill because the immune system, even if unable to eliminate the pathogen entirely, can normally contain
the infection. CD4\(^+\) T cells (helper T cells) specific for MTB antigens release cytokines, such as interferon (IFN)-\(\gamma\), that activate macrophages. Activated macrophages have increased expression of NOS2, nitric oxide synthase 2, a protein that produces nitric oxide, a reactive compound essential in macrophage destruction of MTB\(^7\). While CD4\(^+\) cells are key to controlling the initial infection, CD8\(^+\) cells are necessary to prevent the bacteria from reactivating. CD8\(^+\) T cells kill infected macrophages and appear to also kill mycobacteria directly with granulysin and other compounds\(^8\). Studies in mice demonstrate that mice without an effect CD8\(^+\) (killer T cells) response have a delayed control of the bacterial infection (40 as opposed to 20 days), and that the bacterial population grows to a higher level before reaching a plateau than in WT mice. On the other hand, mice lacking CD4\(^+\) response are unable to control the infection at all, and quickly die off\(^9\). Immunosuppressive drugs that target tumor necrosis factor (TNF), result in the targeted destruction of the subset of CD8 cells, CD8\(^+\)CCR7\(^-\)CD45RA\(^+\) effector memory T cells (T\(_{EMRA}\) cells), that suppress reactivation of tuberculosis\(^10\). Patients with inflammatory diseases such as rheumatoid arthritis who are treated with immunosuppressive drugs are at a greatly increased risk of tuberculosis reactivation.

A specific immune response is key to controlling a tuberculosis infection; an improper immune response can be ineffective, as seen with naïve macrophages, or even pathogenic. For example, in patients with pulmonary tuberculosis, the majority of the damage to lung tissue stems from the strong inflammatory response rather than directly from the bacteria\(^11\).

*Mycobacterium tuberculosis* most commonly initially infects the lungs and forms a pulmonary infection, although the bacteria can infect wounds in other parts of the body.
Infection occurs when a person inhales small liquid nuclei laden with bacteria from a coughing individuals or some other form of aerosolization. Large droplets get trapped in the upper respiratory tract and are expelled by cilia, thus only small droplet can cause disease\(^4\). From the initial site of infection, the bacteria can spread to most tissues in the body, including the brain, liver and bone\(^3\). If the bacteria enter the body through the lungs, the initial immune response can often contain the infection in a granuloma while the specific immune response builds up. If the granuloma is small enough, less than 3 mm in diameter, the cellular immune system can penetrate the granuloma, killing infected macrophages, with the released bacteria able to be successfully destroyed by activated macrophages. If the granuloma is too large, it merely contains the bacteria, out of the reach of competent immune cells. In this low oxygen environment, the bacteria enter a semi-dormant state, able to reactivate and spread at a later time\(^4\). This state is referred to as a latent tuberculosis infection (LTBI). Patients with LTBI are diagnosed by a positive skin test but negative chest X-ray. Latent tuberculosis is treated with isoniazid for nine months (CDC 2003). Approximately 10% of LTBI cases later progress to active disease\(^12\). Active disease occurs when the bacteria escape from the granuloma and start replicating, causing the classic symptoms of night sweats, weight loss, and a persistent cough.

Based on these symptoms, a doctor who suspects tuberculosis will confirm the diagnosis with a tuberculin skin test (TST). This test, originally described by Koch, demonstrates whether or not the immune system recognizes tuberculosis antigens from an infection, either recent or past, with tuberculosis\(^2\). One modern advancement in diagnosis of LTBI comes in the form of the QuantiFERON-TB Gold In-Tube test (QFT). Since
QFT looks for the response of effector T-cells to tuberculosis antigens rather than for the delayed type hypersensitivity reaction, it has several advantages over the traditional TST. First, QuantiFERON can differentiate between vaccinated and infected individuals. Additionally, individuals successfully treated for tuberculosis will have a positive TST, but can be shown to be negative by the QFT. Finally, Quantiferon Gold is more suited for mass screenings or contact investigation, able to be administered and interpreted with only one patient contact, unlike the TST which requires a follow-up visit for interpretation.

For patients with pulmonary tuberculosis, a sputum sample is placed on a slide and examined microscopically for acid fast bacilli. Culture and biochemical assays give definitive proof of a mycobacterium as well as identifying the species, such as *M. tuberculosis* or *M. bovis*. Smear microscopy is also used to monitor treatment and is often used as a proxy measure of infectiousness in a patient. Sputum conversion from positive to negative signals either a significant decrease in bacillary load or cavity closure, both of which indicate a reduction in infectiousness. In many ways, sputum smear microscopy meets the criteria for a perfect diagnostic. Smear microscopy is fast, with results available within an hour, cheap and has low infrastructure requirements, able to be performed in settings with inconsistent electricity or water. Nevertheless, smear microscopy does have limitations. First, smear microscopy is unspecific, unable to differentiate between *Mycobacterium tuberculosis*, *Mycobacterium bovis*, and nonpathogenic Acid-Fast bacilli. Additionally, children are rarely able to produce sputum, forcing clinicians to forgo microscopy and depend entirely on patient

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1 Some technicians with long experience with acid-fast microscopy perceive identifiable nuance between AFB based on bacillus size, clumping of bacteria, and relative width, but these distinctions can not be easily taught and standardized.
presentation. Smear microscopy can only reliably detect bacteria in sputum at a level of 5,000/mL, a relatively high limit of detection.

Finally, smear microscopy cannot differentiate between live and dead bacteria in the lungs. Individuals receiving therapy often become culture negative before they become smear negative. Given the limits of smear microscopy as the standard point-of-care diagnostic, as well as the challenges of phenotypic susceptibility testing, there is a future in alternative point-of-care diagnostics.

The infectiousness of a tuberculosis patient depends on several factors, including proximity and time spent with contacts, number of contacts, bacterial load, and frequency of coughing. Due to the difficulty of measuring these variables after transmission has occurred, no good estimates of patient infectivity have been obtained from epidemiology evidence, such as the number of possible contacts who became infected. Escombe et al. attempted to remove those variable by pumping the air from a ward of HIV/TB coinfected patients over guinea pigs and testing the guinea pigs. The guinea pigs were later tested for tuberculosis, and the DNA fingerprint matched to the strains carried by patients in the ward. For patients not yet on therapy, patients produced on average 6.7 infectious quanta/hr. Infectiousness varied greatly by patient, however, with one MDR patient producing 226 infectious quanta/hr. The only significant determinant of infectiousness discovered were MDR and smear-positive. Other factors may be involved, but the small sample size, 43 patients, limited the power of the study16. The results of this study contradict the controversial observation that MDR strains are less infectious than susceptible strains. Infectiousness sharply declines once a patient begins appropriate therapy.
Treatment

Streptomycin and isoniazid gave the first real hope for curing tuberculosis\(^2\). Soon, however, strains resistant to streptomycin began to arise. Since then, identifying and treating resistant strains has played a major role in combating tuberculosis world wide. Because the bacteria can reproduce inside host cells and remain dormant in a latent state, any drug treatment must last a long time to ensure that all of the bacteria have been eliminated. Standard treatment for active pulmonary tuberculosis can be completed in as few as six months. The first two months include pyrazinamide, ethambutol, rifampin, and isoniazid. These four front-line drugs are used because they are more effective and have fewer side effects than the second-line medications, which are only used if the first-line drugs are ineffective. Using these four drugs reduces the probability of developing resistance, giving a window during which to identify the susceptibility pattern of the isolate. If the isolate is pan-susceptible, rifampin and isoniazid are continued for the remaining four months. Complications, such as INH resistance or a reaction to medication, can extend the treatment longer. The only accepted reasons for extending therapy past 365 days are meningial involvement, disseminated tuberculosis in children under the age of 15, and rifampin resistance. These guidelines include suggested lengths of treatment, as well as the targeted number of doses. Each suggested regimen is rated regarding whether it is based on expert opinion, one trial, or multiple trials\(^{17}\).

*Drug Resistance and Susceptibility Testing*

Culture is more sensitive than direct microscopy for tuberculosis diagnosis and also allows phenotypic susceptibility testing. Drug susceptibility testing is extremely important. Susceptibility tests for rifampin and isoniazid are prioritized as the most
clinically relevant, since they are front line drugs. In fact, MDR (multiple-drug resistant) tuberculosis is defined as resistance to isoniazid and rifampin, with or without resistance to other drugs. If resistance to rifampin and isoniazid is detected, second line drugs must be administered, with the potential for greater toxicity. XDR (Extremely Drug Resistant) is MDR plus resistance to a quinolone and at least one of the following drugs: kanamycin, capreomycin, or amikacin. As culture-based sensitivity testing is quite slow, a clinician may have to adjust the prescription for a patient with a failing treatment regimen without knowing of the exact sensitivities of the isolate. If a person has a high load of bacteria resistant to two antibiotics, random mutations may allow a very small subpopulation to gain resistance to a third antibiotic. If a clinician adds that third antibiotic to the regimen, all of the bacteria resistant to only two antibiotics will die off, and the triply resistant strain will grow unchecked until the individual now carries a large bacillary load of bacteria resistant to three antibiotics. The process by which a population of bacteria resistant to a certain number of antibiotics gains resistant to further antibiotics is termed amplification of resistance. The simplest rule of thumb to avoid the amplification of resistance is to never add a single drug to a failing regimen. The odds are extremely low that a single bacterium could develop resistance to two additional antibiotics through random mutation.

Patterns of drug resistance overlaid on drug intolerance can be complicated, so designing a successful drug regimen often requires an expert consult. Getting a patient with suspected resistance on the proper drug regimen quickly reduces the potential for the amplification of resistance to other antibiotics and reduces the time spent in isolation. Isolation is extremely expensive, especially if drawn out up to a month. Quickly
diagnosing and treating MDR also prevents the development of XDR through improper drug regimens\textsuperscript{52}.

For isoniazid and rifampin, unlike ethambutol, the extent of resistance can vary, but there is a sharp distinction between susceptible and resistant strains. The bacteria do not gradually develop resistance to these antibiotics; rather, any individual bacterium is either resistant or susceptible. The susceptibility of an isolate changes as the proportion of susceptible to resistant bacteria changes. By definition, an isolate is resistant to rifampin or isoniazid if 1\% of the bacterial population is resistant. The preferred method of phenotypic susceptibility method is making identical plates on Löwenstein-Jensen medium, both with and without antibiotics. The proportion of colonies on the antibiotic plate compared to the normal plate gives a measure of the resistance of the population. \textit{Mycobacterium tuberculosis} grows very slowly, however, making culture very slow, expensive, and dangerous to laboratory workers. Culturing mycobacteria requires biosafety cabinets and special facilities with controlled air flow (12).

Isoniazid (INH) has been used to treat tuberculosis since the 1950’s, but its mechanism of action is still not completely understood. In general, INH blocks the synthesis of mycolic acids, which form the lipid coat of the bacilli. INH resistance occurs naturally in \(10^{-7}\) bacteria of \textit{M. tuberculosis}. Other (sub)species of mycobacteria, such as \textit{M. bovis}, are innately resistant to INH. Multiple resistance mechanisms exist against INH. The mechanism first discovered involves mutations in \textit{katG}, which codes for a catalase-peroxidase. Resistance can also stem from mutations in \textit{inhA}, which codes for a protein in the mycolic acid synthesis pathway. \textit{InhA} mutations, but not \textit{katG} mutations, are associated with cross-resistance to ethambutol, another commonly used antibiotic.
Other INH resistance mechanisms remain to be discovered, as almost 20% of strains resistant to INH do not have a mutation in either gene (13).

Rifampin was discovered as a broad-spectrum antibiotic in 1968. However, rifampin’s effectiveness against tuberculosis and fears of resistance arising from widespread use caused the health community to dedicate rifampin as a tuberculosis drug. Since then, rifampin has been a cornerstone of tuberculosis treatment\textsuperscript{20}. Rifampin spreads easily through tissues, reaching the bacteria and diffusing through the cell wall. Rifampin binds the beta subunit of the RNA polymerase, exercising bactericidal activity as it blocks elongation of the transcript. Once elongation has initiated, rifampin no longer binds and does not inhibit transcription. The subunit, coded by the \textit{rpoB} gene, is highly conserved across bacterial species, especially in the 12 residues that interact directly with rifampin.

Other antibiotics used to supplement these core drugs are ethambutol, pyrazinamide, fluoroquinolones, and aminoglycosides. Ethambutol interferes with the metabolism of mycobacteria. Pyrazinamide is an analogue of nicotinamide that lowers the pH of the bacterial cytoplasm. Fluoroquinolones act against DNA gyrase and are used when resistance to a first line drug has been identified\textsuperscript{21}. Aminoglycosides, which include Amikacin and Kanamycin, bind tightly to ribosomes and disrupt protein synthesis\textsuperscript{4}. Bacteria are able to develop resistance to any single one of these antibiotic through random mutations, so proper drug management focuses on using a combination of antibiotics such that any individual bacterium would be unlikely to simultaneously develop resistance to all at the same time. Continuing research for novel antibiotics is underway, but clinicians and health policy officials recognize that the appropriate
framework for the use of antibiotics is necessary to prevent the widespread development of strains resistant to any and all antibiotics.

**Tuberculosis/HIV coinfection**

*Impact of tuberculosis on HIV patients*

Tuberculosis is one of the major opportunistic infections of HIV patients. Tuberculosis kills 30% of people with HIV. HIV patients who are infected with tuberculosis experience increased viral loads in both their blood and other organs. This phenomenon most likely occurs because immune activation stimulates viral replication. In response to an infection of tuberculosis, the immune system releases cytokines, such as tumor necrosis factor (TNF)-α, which stimulate viral replication. Another contributing mechanism appears to be that immune activation in response to tuberculosis increases the proportion of CD4+ cells that express CCR5 and CXCR4, two receptors that HIV uses to gain entry into the cell. With more available host cells, the virus is able to replicate faster. While all HIV patients are susceptible to tuberculosis, their risk of infection depends on their probability of coming into contact with someone with tuberculosis.

*Impact of HIV on TB patients*

Tuberculosis diagnosis and treatment is complicated by coinfection with HIV, particularly advanced stages of HIV. Because the traditional PPD skin test for tuberculosis measures an immune reaction to tuberculosis antigens, rather than testing directly for the presence of tuberculosis, individuals with severely depressed immune systems may have a false negative test. Furthermore, the classic radiographic signs of tuberculosis on a chest x-ray stem from the accumulation of macrophages and T-cells around bacteria, with the subsequent tissue damage and cavitation due to inflammation.
Tuberculosis bacteria replicating unchallenged in the body of an immune compromised host do not cause cavitary lung tissue damage that is visible to a clinician. In fact, most tuberculosis patients with CD4 count under 200 cells/mm³ have normal X-rays. Even sputum smears cannot reliably diagnose HIV+ patients with active pulmonary tuberculosis. In immunocompromised individuals, TB disease progresses much more quickly than in individuals with a robust immune system. Rather than being confined to the lungs, TB is able to disseminate throughout the body, presenting atypical extrapulmonary forms of the disease. Given these complementary mechanisms, HIV+ individuals infected with tuberculosis have a 20 times higher annual probability of progressing to active disease than noninfected individuals, and that ratio reaches 170 for individuals with AIDS. The increased susceptibility to tuberculosis can be observed as early as seroconversion and the susceptibility continues to increase and the CD4+ count falls. Initiating Anti-RetroViral Therapy (ARV), with the subsequent resurgence of CD4+ cells, somewhat mitigates the susceptibility to tuberculosis, but even HIV patients on ARVs are at an increased risk of tuberculosis than individuals without tuberculosis.

Treatment of coinfected patients is also complicated. First of all, both HAART (Highly Active Anti-Retroviral Therapy) HIV treatment and tuberculosis medication involves taking multiple medications simultaneously to decrease the probability of the pathogen developing resistance. Patients taking up to eight different medications have increased chance for drug interactions and negative side effects, e. For example, rifampin activates CYP450 enzymes in the liver that speed the metabolism of many other drugs. Rifampin also blocks protease inhibitors, thus most TB/HIV drug regimens substitute rifabutin for rifampin. Additionally, both HIV and TB drugs can
result in hepatotoxicity, or liver damage. Initiating HAART therapy increases the
probability of the so-called Paradoxical Reaction (PR), wherein a patient undergoing
effective anti-tuberculosis therapy experiences a worsening of symptoms. ART-induced
PR is most likely due to the resurgence of the immune system, with subsequent
inflammation and tissue damage that is symptomatic of tuberculosis. In the long-term, the
PR is eventually reversed and treatment proceeds successfully. For a person in a fragile
state of health, however, the PR may be severe enough to cause death. Based on the
severity of both diseases, a clinician might decide to delay initiation of treatment for one
of the diseases.

From a public health perspective, the increased susceptibility of HIV patients can
cause a surge in tuberculosis incidence in regions with otherwise effective tuberculosis
programs and low baseline levels of resistant strains. In Botswana, despite consistent
DOTS implementation, TB prevalence increase by 120% over a seven year period,
almost entirely due to the rise of HIV.

**Tuberculosis Control in Emergency Situations**

Control of tuberculosis is further complicated by large upheavals due to either
conflict or natural disasters. These upheavals present special challenges in both
transmission prevention as well as continuity of therapy. The steady decline in
tuberculosis incidence and mortality suffered a check during World War I. Health
professionals recognized the problem and quickly instituted new interventions to address
the rise in tuberculosis cases. After hurricane Katrina, one fifth of individuals being
treated for tuberculosis could not be located after the hurricane. Treatment interruptions
present a worse prognosis for individuals. Incomplete therapy also poses a risk for the development of resistance to antibiotics, with broader public health implications.

**Pediatric tuberculosis**

Tuberculosis behaves differently in children than adults. Children infected with tuberculosis develop disease in a matter of weeks or months, much faster than adults. Children are also more prone to develop extrapulmonary tuberculosis, including many deadly and debilitating forms of tuberculosis. Some tuberculosis control programs tend to overlook children, however, both because they are difficult to diagnose, and because children are not considered infectious\(^{29}\). Prompt recognition and treatment of pediatric tuberculosis not only protects the child, but also helps doctors and public health officials locate the infectious contacts of children before adults infected at the same time become sick.

Diagnosis of pediatric tuberculosis is difficult, often relying on clinical symptoms, a positive skin test, and a history of contact with an contagious adult. Children with pulmonary tuberculosis have difficulty producing sputum, and since the bacteria are often contained in sealed lesions, only 5% of pediatric cases are smear positive. Furthermore, even under the best sample collection methods, only 40% of children with tuberculosis are culture positive. To overcome these diagnostic challenges, current US guidelines state that all children who live in the household of newly diagnosed adult cases of tuberculosis should be placed on precautionary regimens of isoniazid\(^{31}\).

Another measure aimed mainly at pediatric tuberculosis is the use of the BCG(Bacille Camile- Guillot) vaccine. The BCG vaccine is a live attenuated form of *M. bovis*. Much debate surrounds the safety and efficacy of the vaccine. The BCG vaccine
appears less effective in regions where there is a high likelihood of exposure to environmental mycobacteria, which is mainly in the tropical climates. While studies conflict on the measure of protection, most meta-analyses indicate that the BCG is 50% effective at preventing tuberculosis disease. While 50% effectiveness is rather small, the real power behind the BCG vaccine, and the main justification for its near global use today, lies in the over 80% efficacy at preventing life-threatening forms of tuberculosis in children.32

The BCG vaccine is not routinely used in the United States. Individuals vaccinated with the BCG will react to the PPD skin test, giving a false positive result for tuberculosis infection. Given the low incidence of tuberculosis in the United States, the ability to diagnose and treat subclinical cases of tuberculosis is considered more effective overall than potentially preventing disseminated tuberculosis in children.33 BCG vaccination is also not recommended for individuals know to have HIV, due to an increased risk for an adverse reaction34. Finally, though the risk of adverse reactions to the vaccine are normally quite small, the rise of pediatric HIV has created a population of immunosuppressed children who can become sick from the attenuated bacteria.

Development of New Vaccines

New vaccines are being sought, but these efforts face several challenges. First, Mycobacterium tuberculosis is a human pathogen, and finding an appropriate animal model poses a challenge. Mice, while cheap to infect and maintain, mount an immune response quite different from the human response. Guinea pigs are very susceptible to tuberculosis and die quickly, not demonstrating the spectrum of disease observed in humans. Rabbits appear to be a more appropriate immunological model than mice or
guineau pigs, but research involving rabbits, as with guineau pigs, lacks the wide range of immunological tools available for mice. Vaccines that show promise in one of the above models can then be tested with macaques, which pose a greater expense and biohazard to laboratory personnel. Another challenge in vaccine development is that the early immune response to tuberculosis infection, which is often successful in destroying or controlling the bacteria, is only starting to be understood. Finally, triggering an appropriate immune response is especially important with tuberculosis, as many of the pathologies of tuberculosis are due to an improper immune response.

**Molecular tools for tuberculosis control**

Molecular biology has introduced new tools in the fight against tuberculosis. DNA fingerprinting allows strain typing of tuberculosis, giving useful epidemiological information. For example, DNA fingerprinting allows epidemiologists to determine whether cases occurring in the same region are the result of coincidence or whether they are clustered. Fingerprinting can also answer the question of whether an individual with a second episode of tuberculosis is suffering a relapse of the previously treated strain, or whether the individual has been reinfected by a new strain. Finally, fingerprinting can help identify false positive cultures and track down nosocomial transmission.

There are several strategies for DNA fingerprinting of tuberculosis. In general, strain typing analyzes differences in non-coding DNA, which is not under selective pressure and is thus free to undergo steady change. In general, two isolates will match only if representing a recent transmission. IS6110 restriction fragment length polymorphisms (RFLP) fingerprinting was the first commonly accepted molecular method to assign epidemiological linkages. IS6110 is a movable element in the TB
genome that exists in multiple copies. The multiple copies allow for frequent recombination, altering the DNA length between instances of the fragment. Because RFLP fingerprinting relies on an intact genome, PCR amplification cannot be used. Rather, an isolate must be grown in culture to the point that lysing the cells and treating the DNA with restriction enzymes specific for the IS6110 sequence will result in fragment in high enough quantities that they can be visualized when separated out on a gel. A newer method of strain typing, spoligotyping, is a PCR based system, and thus can analyze diagnostic specimens directly. Additionally, spoligotyping can differentiate between strains with less than five IS6110 elements\textsuperscript{38}.

\textit{Genotypic tests for Rifampin Resistance}

Given the time delay and biohazard posed by culturing tuberculosis, molecular methods began to be sought for susceptibility testing, especially for rifampin. Over 90\% of rifampin resistant strains are also resistant to isoniazid\textsuperscript{39}. The \textit{rpoB} gene codes for the beta subunit of the RNA polymerase, the target of rifampin. Mutations in the \textit{rpoB} gene compose the major resistance mechanism for rifampin. Specific mutations cause specific levels of resistance, as well as having various cross-resistances to other antibiotics.\textsuperscript{40} Identifying the exact mutation could suggest which alternative antibiotics will or will not be effective\textsuperscript{41}.

Gene sequencing is the gold standard in mutation detection. Sequencing identifies the exact mutations and allows silent mutations to be differentiated from amino acid substitutions. However, while sequencing is very accurate on a pure culture, it has trouble showing mutants that form a small percent of the bacterial population, thus misidentifying clinically resistant strains that can be diagnosed with other molecular
methods. Many in house assays have been developed to quickly identify rifampin and isoniazid resistance in research laboratories. The DNA must be extracted from the sample. Some methods require the DNA to be extracted from a pure culture, but testable DNA can be extracted from sputum. In fact, DNA extracted from Ziehl-Neelsen stained slides can diagnose rifampin resistance by looking at substitutions in codons 516, 526, and 531 with 84% sensitivity.

Although in house assays abound, clinical applications require a reliable, standardized assay. The main commercial method developed is the reverse line probe assay. The sample DNA is extracted. The \( rpoB \) gene is amplified with biotinylated primers. The PCR product is incubated with a membrane strip, allowing it to hybridize with immobilized sequences representing either wild-type or mutant sequences for various codons. After washing, the hybridized sequences are visualized with streptavidin-coupled enzymatic colorization. The streptavidin irreversibly binds the biotinylated PCR product. The entire procedure can be performed in two days. It detects mutations in mixed cultures. As with any PCR test, cross-contamination poses a threat of false positives, and must be rigorously controlled. INNO-LiPA Rif TB, a reverse line probe assay from Invitrogen, detects rifampin resistance with 100% specificity and 98% sensitivity. A study performed in Rwanda using individuals with no background in molecular biology to run and interpret these tests demonstrates the field potential for molecular diagnostics. Like other countries, Rwanda has received funding for treating MDR, but MDR cannot be treated until routine susceptibility testing is performed. While the commercial tests are currently prohibitively expensive for low-resource countries, the

\[2\] Despite the greater resources and training available in industrialized nations, FDA approval lags behind the rate of advances in technology, creating a gap between the development of new diagnostic technologies and their routine implementation (Personal communication, Dr. Kenneth Jost).
Foundation for Innovative Diagnostics (FIND) is subsidizing another line probe assay, the Genotype MTB-DR plus from Hain Lifesciences for use in low-resource countries\textsuperscript{46}.

Humans have battled tuberculosis for thousands of years. Several key advances in that battle include the isolation of \textit{Mycobacterium tuberculosis} in 1882 and the introduction of antibiotics in the middle of the twentieth century. Arguably, some of the biggest advances have occurred in the past twenty years, as advances in technology have given unprecedented insight into the virulence and genetics of tuberculosis. In addition to furthering the scientific understanding of tuberculosis, advances in technology are creating new tools for tuberculosis vaccination, diagnosis, and treatment. Alongside an increased understand of details such as environmentally dependent expression of transcription factors has come an increase understanding that effective control of tuberculosis must also address the barriers to diagnosis and consistent treatment that individuals with tuberculosis sometimes face.
II. Barriers to Timely Completion of Treatment of \textit{Mycobacterium tuberculosis} in Texas

**ABSTRACT**

Tuberculosis remains a serious problem across the United States (US) as well as in Texas. In 2006, 1585 new cases of tuberculosis (TB), comprising over 10\% of cases in the entire US, were reported to the Texas Department of State Health Services (DSHS). The CDC Division of Tuberculosis Elimination has codified performance evaluation criteria to aid measures to control TB. In order to ensure the best quality care to patients and best utilize the resources of the state of Texas, DSHS decided in 2006 to focus on evaluating and improving the performance of Texas on a single measure, Timely Completion of Therapy (COT). Timely COT is the proportion of cases eligible to complete therapy in less than a year that successfully completed therapy in less than a year.

The purpose of the study was to look more closely at the 132 TB cases first reported in 2006 indicated as having completed therapy in greater than a year. Of the 116 cases evaluated, 21(18\%) had had erroneous information reported to DSHS and did not qualify as delayed completion of therapy. Of the remaining 95 cases, 41 were delayed due to medical factors, 25 were delayed due to noncompliance, and 18 were delayed due to a lack of coordination among care providers. Three additional cases had no determinable reason for delayed completion of therapy. While the mean therapy duration was 469.9 days, 42 cases (44\%) finished therapy within one year and one month. Risk factors associated with delayed completion of therapy were homelessness, resistance to
isoniazid, HIV, extrapulmonary involvement, diagnosis in a correctional facility, and alcohol abuse.

INTRODUCTION

Barriers to Timely Completion of Therapy

Treatment guidelines are supposed to be communicated to physicians who can rely and follow the guidelines. Nonetheless, some doctors continue to prescribe longer than necessary treatment regimens, increasing the cost and inconvenience of treatment. Doctors tend to prescribe longer regimens for patients with extrapulmonary tuberculosis or HIV, despite studies demonstrating that these patients can be successfully treated in less than one year\textsuperscript{47, 48}.

Interruptions in therapy allow the concentration of antibiotics in the blood to drop, giving the bacteria a chance to develop resistance. Dangerous side-effects of the therapy are a significant cause of interruptions in therapy. For mild side effects, the importance of consistent tuberculosis treatment outweighs patient discomfort. Other side effects are more severe and even life-threatening, requiring treatment to be suspended until the responsible drug can be identified. One relatively common severe side effect is hepatotoxicity. One meta-analysis combined the data from 19 studies that evaluate the risk of hepatotoxicity due to INH or when combined with RIF. Alone, INH carries a 1.6% chance of hepatotoxicity, but combined with RIF carries a 2.7% risk of hepatotoxicity. Hepatotoxicity is more of a risk for patients with liver damage due to hepatitis or alcohol. Depending on the severity of the reaction, hepatotoxicity forces the physician to hold tuberculosis therapy until the drug responsible can be identified and removed from the
regimen. To quickly identify hepatotoxicity, physicians monitor patient LFT (liver function test). With such long regimens, patient compliance can be quite low. Patients either forget to take medication, or decide not to take medication. To combat the problem of noncompliance, the WHO recommends, and most countries implement, DOT, Directly Observed Therapy, where the patient is observed taking each dose of medication. DOT has been proven to increase completion and has had a positive impact on treatment in Texas. In the 25 cities and counties with over 100 new cases of tuberculosis in at least one year between 1990 and 1994, the jurisdictions with more cases tend to have lower 12-month completion rates. For example, Houston, TX had the fifth largest number of cases per year, and ranked near the bottom of 12-month completion rates, with only 50% completion rate before the large influx of federal funding in 1993 that supported the expansion of DOT programs. Dallas-Dallas County, El Paso, Ft. Worth-Tarrant County, and Austin-Travis County on the other hand, average over 90% completion rates. Over the study period, Dallas rates of DOT increased ten-fold from 4.9% to 49.4%, but the 12-month completion rate remained constant at 96%. Most often, treatment is given free of charge. For patients with complicating life circumstances, such as homelessness, additional incentives and enablers to encourage treatment, such as food stamps and assisted housing, may be made available. For completely intractable situations, patients may be treated as in-patients at the Texas Center for Infectious Disease (TCID) and Heartland Medical Center, both located in San Antonio, as well as the UT Tyler tuberculosis hospital. Given the enormous cost of in-patient stays of over six months, this final treatment option is a last resort.
To help simplify the efforts to control tuberculosis, the CDC Division of Tuberculosis Elimination published 10 fundamental recommendations with corresponding performance indicators. Once reliable data on those indicators are available, each state, city and provider is encouraged to set performance goals based on the indicators. The CDC recommends focusing on evaluating improving one indicator at a time. In 2006, Texas chose “Timely Completion of Therapy,” and set a goal of 83% by the year 2010. The nationally set goal for 2015 states: “To account for gaps in therapy, which may occur because of drug intolerance or nonadherence, the performance indicator is for 90% of patients with TB caused by a drug-susceptible isolate to complete a course within 12 months of initiation of treatment.”

The purpose of this study was to examine the barriers to timely completion of tuberculosis therapy in Texas. Identifying the major barriers to timely completion of therapy can help suggest more effective strategies to overcome these barriers and meet national and state performance goals.

**METHODS**

*Study Population*

This study looks at confirmed cases of tuberculosis reported in 2006. The CDC reports 13,767 confirmed new cases in the entire United States. Over ten percent, or 1,585, of those cases were identified in Texas. One hundred and thirty two of those cases eligible to complete therapy within 365 days did not complete therapy during that time, but had successfully completed therapy by the time of this study. Exclusion criteria include death, *M. bovis* resistant to pyrazinamide, resistance to rifampin, meningeal involvement, and disseminated tuberculosis in children under the age of fifteen. All of these criteria greatly
complicate therapy, and a patient with one of these risk factors is expected to require more than a year of therapy.

Data Sources

Cases meeting the criteria for this study were first identified through TIMS, the database for tuberculosis cases in Texas. Additional patient information, mainly the DOT records and the progress reports, were requested from the county health department that reported the case. TIMS contains both social information (such as housing status, substance abuse pattern, date of entry into the United States) as well as medical information (HIV status, primary site of tuberculosis, start and completion of therapy dates). Additional information, such as interruptions in therapy, drug intolerance, and other sites of disease can be gleaned from the DOT records and the progress reports. Selected information concerning the cases was gathered from these sources into a new database for analysis.

Data Analysis

Initially, the start and completion of therapy dates listed in TIMS were confirmed based on the information in the DOT records. If the revised therapy duration was less than 366 days, the case was removed from the study pool. Additionally, if factors such as meningial involvement that disqualify a patient from completing therapy were discovered in the progress notes, the case was removed from the study pool. Based on the progress notes and DOT records, an underlying reason for delayed completion of therapy was assigned to each patient. These reasons fit into four major categories: lack of coordination between health providers, medical complications, compliance issues, and provider decision. County health departments take primary responsibility for following patients
with tuberculosis, but when patients are hospitalized, incarcerated, deported, or move, other health entities take partial or complete control over patient therapy. Medical complications may consist of medicine intolerance, extrapulmonary spread, resistance to isoniazid, or poor response to therapy. Patient noncompliance occurred both with and without DOT. Finally, provider decision included any reason not mentioned above that made the provider continue therapy past 365 days. The most common barriers to timely treatment completion were identified.

Using the data available through TIMS, the odds ratio of delayed completion of therapy based on risk factors was determined. The risk factors examined in this analysis were HIV status, housing status, substance abuse, age, site of disease, diabetes status, country of origin, and previous history of incarceration. Data analysis was completed using Microsoft Excel and Access.

**RESULTS**

A review of TIMS revealed 132 cases eligible to complete therapy in less than a year who were treated for greater than a year. One hundred and sixteen of the 132 cases were included in the study based on DOT logs and progress notes, however, the DOT logs and progress notes were not received for 16 cases. Twenty one additional cases were removed from the study following a review of the more accurate information available in the charts. Figure 2 examines the 132 cases originally included in the study.

A careful look at the data revealed that record errors inflated the number of cases that were reported as delayed completion of therapy. As seen in Figure 3, minor errors in start and stop dates changed the reported length of therapy for 15 cases, but did not result in their removal from the study population. Major errors resulted in 21 cases being
removed from the study population either because the case had completed therapy in less than 365 days, or because the case fit the study exclusion criteria. The specific errors are shown in the bar on the right. For twelve cases, the closure date was listed as the stop therapy date. Four cases had the prescription date listed as the start date, three met exclusion criteria, and 2 had the wrong year recorded. The rest of the results below analyze the final 95 cases included in the study.

Figure 4 shows how many days the 95 cases took to complete therapy. Sixteen cases completed therapy within one year and one week. Almost half of the cases finished within one year and one month. If those cases had had attention focused on completing therapy within one year, along with successful treatment, the number of cases of delayed COT in 2006 would have been closer to 50 rather than 95. The mean of 469.9 days is skewed by small number of cases that took very long time to complete therapy.

Based on the DOT logs and progress notes, each case was assigned a reason that resulted in delayed completion of therapy. Only the single most important contributing factor was counted. For example, a patient that missed a few doses over the holidays was not listed as noncompliant if the real reason why they took longer than a year to complete therapy was that they were resistant to INH.

The individual reasons for delayed COT were grouped into the convenient categories seen in Figure 5. Fifty-two percent of the cases of delayed completion of therapy were rooted in some medical issue. Patient noncompliance, 26%, and lack of coordination between health providers, 19%, accounted for most of the rest of the cases. Three percent of cases had no apparent reason for delayed completion of therapy and were listed as delayed completion of therapy due to provider decision.
Figure 6 breaks down the medical reasons that contributed to delayed completion of therapy. Forty-nine cases in total had medical issues that led to delayed completion of therapy, but some patients were counted in more than one category. For instance, a case of tuberculosis of the spine that was resistant to isoniazid was counted as both Bone/Spine and INH-R. Fourteen cases with bone and joint TB and 6 with disseminated disease completed their therapy in more than one year as did 33 cases who experienced complications from the therapy. The first two medical reasons, involved with site of disease, appear at time of diagnosis. The last three become an issue during treatment and can extend therapy for a patient who was initially diagnosed for less than one year. The most commonly encountered complication of intolerance was hepatotoxicity. According to the guidelines, these medical issues, including bone and joint tuberculosis should not push therapy past one year. Nevertheless, physicians are hesitant to treat these patients for less than one year.

Figure 7 shows the 25 patients for whom noncompliance was the reason their therapy was delayed. Noncompliance of patients occurs when the patient moves without informing the health department, misses DOT appointments, refuses medication, or otherwise obstructs treatment. Nonadherence to medication continues to challenge successful completion of therapy, even after the widespread adoption of directly observed therapy. It was not possible to verify that only 3 patients were no-compliant with self medication because the missed doses were self-reported.

Sometimes, a lapse in TB therapy occurs due to a lack of coordination between care providers, as illustrated in Figure 8. One difficulty encountered in providing consistent care occurs when a patient is incarcerated or released from jail, moves to the
jurisdiction of another health department, or is admitted to the hospital. These cases fall into one of two categories: 1) the patient did not receive therapy from new provider once they moved, or 2) the patient received therapy in hospital or another county, but when they moved, the new health department decided to restart treatment. Both of these issues stem from a lack of communication and trust between care providers.

A large part of the story of delayed COT is interruptions in therapy. As seen in Figure 9, interruptions varied in length from less than 30 days to more than 300 days and occurred for various reasons. Some of the major reasons are noncompliance, reaction to the medication, complications due to another medical condition, and incarceration. Depending on when during therapy the interruption occurred as well as how long the interruption lasted, the physician will decide to either add the missed doses to the end of treatment, or to restart therapy from the beginning. As you can see in the bar on the left of the graph, 34 cases had minimal interruptions in therapy but still took longer than a year to complete therapy. Generally, those were cases of extrapulmonary tuberculosis who were initially prescribed for twelve months of therapy followed by a smooth course of therapy.

**Risk Factors**

By comparing the characteristics of those who had delayed COT with those who completed therapy in less than one year, we can identify risk factors related to delayed COT. Risk factors are characteristics that are present at diagnosis that are statistically associated with delayed COT. Knowing significant risk factors can help a care provider be aware of which cases are in danger of delayed COT. Risk factors can include
demographic characteristics, behavioral and lifestyle factors, concurrent medical conditions, laboratory results, and the presentation of the disease.

There was not a statistically significant difference for eight of the risk factors when comparing those who completed therapy on time and those with delayed completion of therapy. Four demographic characteristics, age, gender, ethnicity, and immigrant status were not predictive of delayed COT. Disease factors that did not influence timely completion of therapy included smear positivity and concurrent diabetes mellitus. Lifestyle factors that were not significant include previous history of TB and residence in a long-term care facility such as a nursing home.

Figure 10 presents the risk factors that were found to be statistically significant, including the strength of the association between the risk factor and delayed COT. They include both lifestyle factors, such as homelessness, diagnosis in jail, alcohol abuse, and unemployment, as well as medical factors such as resistance to isoniazid, HIV, and extrapulmonary involvement. These risk factors are not completely independent- for instance, a large proportion of the homeless individuals are also unemployed.

**DISCUSSION AND FUTURE DIRECTIONS**

What main conclusions can be drawn from these results, specifically related to augmenting the proportion of cases who finish therapy in less than a year? With a 2006 performance of 81.4% of patients achieving timely completion of therapy, Texas is close but still short of the Texas 2010 goal, and still more than 10% away from the national goal for 2015 for this indicator. This study examined the reasons why 116 patients took longer than the recommended year to complete therapy for tuberculosis. This study dug deeper than the information reported to the DSHS, going back to the original DOT logs.
and progress notes. This approach revealed that a large percent of the records had misreported data. Misreporting the case closure date as the stop therapy date made the biggest difference when therapy was completed outside of the supervision of the reporting entity, demonstrating the importance of coordination between health providers. Simply tightening up reporting practices will not only improve the accuracy of the records in general, but will let Texas get proper credit for all of the cases that are treated on time.

An analysis of the length of therapy for the 95 cases of delayed COT reveals that while some of the cases have extremely drawn out therapy due to complicating factors, 17 percent finished within a year and one week. The analysis further suggests that a focused effort could bring almost half of the cases under a year of therapy, allowing Texas to reach the national goal of 90% completion by 2015. This improvement might be accomplished by the health department communicating the value of timely COT to the providers.

An examination of risk factors revealed that homelessness is the strongest predictor of delayed completion of therapy. Physicians and care providers are already aware of the challenges presented in providing continuous care to a person without a permanent address. Two other risk factors were statistically associated with delayed completion of therapy because physicians use those conditions, such as HIV and extrapulmonary involvement, to prescribe for 12 months or longer.

These insights suggest several concrete measures that can be taken to improve Texas' performance on the performance evaluation criterion. Unlike doctors, who only see patients one at a time, health departments, both at the county and the state level, can
see a bigger picture and approach tuberculosis strategically. There will always be cases with complications both medically and socially that will be treated in over one year. Many other cases, however, could be treated within a year with a small amount of focused effort.

Future studies at DSHS semester will look at cases who completed therapy at least once, but then suffered a relapse of tuberculosis. Some of the questions asked will be whether the second disease is a reactivation of the old strain of tuberculosis or was the result of a new exposure to tuberculosis. Additionally, the risk factors associated with relapse will be identified, in part to determine whether some of the same factors that cause delayed COT also increase the probability of a relapse. For example, if patients with extrapulmonary tuberculosis are predisposed to suffering a relapse, then perhaps doctors are justified in treating them for longer periods of time.

According to the recommendations, patients with TB in the bone or joints, or patients with HIV can be successfully treated within one year. Nevertheless, physicians are hesitant to treat these patients for less than one year. A survey of physicians in Texas could show whether the doctors are aware of the current recommendations. Further studies demonstrating the effectiveness of nine months of treatment might be more convincing to physicians hesitant to prescribe shorter therapy for patients with extrapulmonary involvement or HIV.
III. A Retrospective Analysis of Rifampin-Resistant tuberculosis in Afghanistan

ABSTRACT

The countries with the highest burden of tuberculosis are often those with the least resources available to combat it, challenging global efforts to stop tuberculosis. Afghanistan, for example, faces a high burden of tuberculosis in a low-resource country. Little data exists concerning the rate of drug resistance, a key factor in both planning and implementing public health interventions. This study used resistance conferring mutations in the \textit{rpoB} gene as a surrogate marker of rifampin resistance. With an initial sample of 511 sputum slides representing approximately 5\% of cases of tuberculosis diagnosed in the first quarter of 2008, full sequences of the region of interest were obtained for 136 samples. The results of this study give the first survey data of rifampin resistance across Afghanistan, as well as suggesting further implications concerning the potential benefit of molecular diagnostics in low-resource settings.

INTRODUCTION

Multi-drug resistant tuberculosis (MDR-TB) is defined as tuberculosis that is resistant to isoniazid and rifampin, with or without resistance to other antibiotics. Isoniazid and rifampin are considered front-line antibiotics due to their relative effectiveness and low toxicity compared with other antibiotics. Swift diagnosis of MDR-TB is important both clinically and in term of public health. Once diagnosed, MDR-TB can be properly treated, leading to a better outcome for the patient and preventing further transmission of resistant strains\textsuperscript{55}. Improperly treat MDR-TB can result in the amplification of resistance,
whereby an isolate resistant to a certain subset of antibiotics gains resistance to further antibiotics, eventually leading to extremely drug resistant tuberculosis (XDR)\textsuperscript{56}.

Molecular diagnosis of rifampin-resistance in TB proves to be a general marker for multi-drug resistance for two reasons. First, rifampin monoresistance, isolates that are resistant to rifampin but susceptible to isoniazid, is rare; a global survey found that 95.4\% of rifampin resistant strains were also MDR\textsuperscript{57}. Secondly, 90\% of tuberculosis strains resistant to rifampin contain a mutation in a 308 bp region, which includes an 81bp “hotspot”, near the middle of the *rpoB* gene, making this gene a good predictor of rifampin resistance.\textsuperscript{58} In contrast, only 67\% of isoniazid resistance is detectable by mutations found in *katG*\textsuperscript{59}. However, the proportion of rifampin-resistant isolates that are also MDR, as well as the frequency of specific mutations, varies with geographic area, necessitating caution when interpreting such results.\textsuperscript{60}

Molecular techniques carry the advantage of much faster results than conventional phenotypic susceptibility testing, as well as reduced exposure to infectious materials\textsuperscript{61}. Another advantage over culture-based sensitivity testing is that PCR-analysis can be preformed on non-viable bacteria\textsuperscript{62}. DNA extracted from Zhiel-Neelsen stained slides has been used to detect and type tuberculosis\textsuperscript{63}, as well as to detect rifampin resistance\textsuperscript{64,65}. The slides can be stored and transported at room temperature, reducing logistical challenges.

Afghanistan is twenty-second on the WHO list of high-burden countries, with an estimated burden of 168 cases/100,000 residents/year. Afghanistan is one of the few remaining countries in the world with not survey on the extent of MDR-TB to date, and drug susceptibility tests are not routinely preformed\textsuperscript{66}. Multiple dynamics complicate
efforts to predict the rate of MDR-TB in Afghanistan. For example, Afghanistan neighbors many countries, including former Soviet states, with a high burden of MDR-TB, suggesting that travel between Afghanistan and its neighbors with a high burden may increase the rate in Afghanistan. On the other hand, Afghanistan has a relatively young anti-tuberculosis program, and there is no pressure for untreated tuberculosis to develop resistance, suggesting that Afghanistan might have a low burden on MDR-TB. In the most comprehensive attempt to unravel these parameters, Zignol et al. used a mathematical analysis of factors contributing to MDR-TB in over 70 countries to estimate the rate of MDR-TB in countries that had not been surveyed, suggesting Afghanistan may have a rate of 3.0% of all TB cases, both new and previously treated. However, a study conducted at the National Research Institute of Tuberculosis and Lung Disease, the national TB referral center in Iran, found that 23% of TB isolated from Afghan patients was phenotypically MDR. The rate of tuberculosis is higher for Afghans living in Iran than that of native Iranians. The rate of MDR among Afghans in Iran is not necessarily representative of the local population of Afghanistan, in part because Afghans living in Iran as refugees are more likely to have interruptions in therapy that allow resistance to develop. In Afghanistan, the diagnosis and treatment of tuberculosis is shared between many organizations. Diagnosis relies upon clinical examination, along with Zhiel-Neelsen-stained sputum smears. The slides from clinics are sent to regional laboratories for quality control once every quarter. As routine susceptibility testing is not available in Afghanistan, clinicians infer drug-resistance from treatment failure (defined as a positive sputum smear after five months of treatment).
Studies demonstrate the feasibility of molecular analysis of tuberculosis in low-resource countries. Even so, cost remains a major barrier to the routine implementations of molecular techniques for tuberculosis analysis in the developing world. Herein, we provide the first national survey of rifampin resistance within the country of Afghanistan. Furthermore, this study represents the first use of molecular markers to identify rifampin resistant strains in Afghanistan. Resistance conferring mutations were present in twenty-one samples, representing thirteen of the twenty-six provinces for which data was obtained.

**METHODS**

Slides were obtained from the Quality Assurance Program at the National Tuberculosis Program of Afghanistan with IRB approval from both the University of Texas and the Afghan Ministry of Public Health. Each quarter, every laboratory that performs smear microscopy sends a portion of their slides to a regional laboratory for blind cross-checking. All slides are graded by a technician, then blindly cross-checked at a regional lab, and graded a third time if the first two grades disagree. Slides from every province in Afghanistan were included in this study, matched with the clinic of origin. The grades of the slides were not provided for most of the slides, however. Some small clinics with less than five positive slides during the quarter send all of their positive slides for cross-checking. Larger health centers which diagnosed over 150 smear positive cases in that quarter send around 15 slides, with no standard slide selection procedure. Thus, smaller clinics are disproportionately represented in the sample.

DNA material was recovered from the slides using the procedure described by Suresh, et al. The mineral oil was removed with xylenes (Figure 11). 50 ul of distilled
water was used to wash smear scrapings into an eppendorf tube. 10% Chelex was used to precipitate the cell fragments out of solution as well as remove potential amplification inhibitors such as metal ions\textsuperscript{74}. The tubes were boiled fifteen minutes at 97°C, then cooled and centrifuged. In the protocol described by Suresh \textit{et. al.}, the supernatant was used directly in PCR reactions. Initial DNA samples did not amplify, however, indicating the presence of inhibitors. Subsequently, all DNA samples were purified using phenol-chloroform extraction followed by .3M NaOAc ethanol precipitation, desiccated and resuspended in a total of 100 ul.

\textit{Heminested amplification}

Methods for a multi-stage PCR were originally described by Williams, \textit{et. al.} with the following modifications.\textsuperscript{75} The first stage consisted of 25 ul reaction (.2uM dNTP, 2mM MgCl\textsubscript{2}, 1X PCR buffer, .2ul Platinum Taq (Invitrogen) forward (rpoB105) and reverse (rpoB293) primers, and 5 ul of purified slide DNA). Initial denaturation was performed at 94°C for 5 min, followed by 25 cycles of 95°C for 1 min, 62°C for 1 min, and 72°C for one min. Afterwards, 75 ul of the second stage master mix, (.2uM dNTP, 2mM MgCl\textsubscript{2}, 1X PCR buffer, .2ul Platinum Taq (Invitrogen) forward (rpoB105) and reverse (rpoB273)), was added to the reaction and cycled for thirty more cycles with an annealing temperature of 65°C. Amplification was checked by agarose gel for the 193 bp product (Figure 12), and amplified samples were PCR purified using a PCR-Clean Up kit (Biosci), then sequenced at the Core facility of the University of Texas at Austin using the rpoB105 primer.

\textbf{RESULTS}
In order to estimate the prevalence of MDR-TB in Afghanistan, diagnostic sputum slides were obtained from the Quality Assurance Program of the National Tuberculosis Program. Tuberculosis DNA was extracted from the slide material, and the \textit{rpoB} gene mutations determined by sequence were used as surrogate markers of MDR. Full sequences of the region of interest were obtained for 136 of the 511 samples, 20 of which contained resistance conferring mutations (Figure 13). Although samples from almost every province were collected and tested, for some provinces, no sequencing data was obtained, because those provinces had relatively fewer slides in the sample, and none of the DNA from those slides amplified.

Since both the province and clinic of origin was known for each sample, samples with resistance-conferring \textit{rpoB} mutations had a known geographic origin. According to the WHO country profile of Afghanistan, the Southern, North-Eastern and Eastern regions of Afghanistan have the highest case notification rate of tuberculosis, the same regions that also show the highest rates of rifampin-resistance in this study (Figure 14). High levels of undiagnosed MDR can increase the total incidence of tuberculosis in a population, even with otherwise competent management, as observed in the New York epidemic of the early 1990's\textsuperscript{76}.

TCG531TTG is the most common mutation encountered globally among rifampin resistant strains of tuberculosis and was also the mutation most frequently encountered in this study (Figure 15). In these samples, however, 11 of the 16 mutant sequences had the parallel silent mutation TTC514TTT. These samples may represent a dominant strain of rifampin-resistant tuberculosis that has spread among individuals in Afghanistan. Alternatively, these results may represent PCR cross-contamination. Strain typing the
original DNA could differentiate between these two scenarios: if the samples with the
same mutations belong to the same superfamily, they most likely represent the same
resistant strains. On the other hand, if the samples with the same two rpoB mutations
belong to different families, the similarity is due to PCR contamination. Unfortunately,
after testing the rpoB sequence in this study, insufficient tuberculosis DNA remained to
test further.

**DISCUSSION**

In conclusion, PCR analysis of rifampin resistance approaches the accuracy of
conventional methods. As well, once the DNA has been isolated, it is stable, allowing
future analysis, such as sequencing or spoligotyping. Using the Zhiel-Neelsen slides
provides an easy way to store and transport the samples, allowing for a greater sample
size. These results demonstrate the proof of principle that DNA recovered from slide can
be used to test rifampin resistance. As well, this study demonstrates the feasibility of a
national screening program based on molecular analysis of sputum smears, utilizing the
existing infrastructure. Data from a national survey of MDR can help strategically plan
MDR-TB interventions in Afghanistan.

Compared with a binary resistance test, sequencing offers the advantage of
additional epidemiological data. For example, one particular mutation (TCG531TGG)
was present in two samples. Given the low probability of any specific mutation
occurring, there exists a chance that those two samples are epidemiologically linked. The
fact that these samples were collected in neighboring provinces, and one of the samples
was collected in a district bordering the other province, further suggests that the two
samples represent the same resistant strain. The epidemiological link cannot be
definitively established, however, as information on individual patients was excluded from the study. The two samples might represent unrelated cases, members of the same household, or even the same individual who sought treatment at two separate clinics.

One sequence, from a sample from Takhar Province, aligned more closely with Nocardiosis species than Mycobacteria in a BLAST search. As this environmental actinomycete is not normally associated with human disease, the bacteria probably got on the slide from environmental contaminations.

This study found much lower PCR amplification frequency than that described by Suresh. Contributing factors may include differences in sputum smear preparation and staining, differences in storage conditions, or relative paucity of bacteria present on the slides. Additionally, the freeze-thaw cycles encountered during the transport of the isolated DNA may have damaged the integrity of the DNA. Finally, the primers used in this study may have had a lower efficiency than those used by Suresh.

Sequencing the gene gave an unbiased look for the mutations viewed that are associated with rifampin resistance in Afghanistan. While the numbers of mutant strains encountered in this study are too small for statistical significance, several conclusions can be drawn. First, the mutations found were those that appear most commonly throughout the world. These mutant alleles are targeted in commercial mutation detection kits such as Inno-Lipa-RifTB and MTBDRplus from Hain, which remove the need for a sequencing step.

Currently available molecular tests for drug resistance are still dependent on PCR machines and further equipment, limiting their effectiveness for point of care diagnostics. This study also shows the potential danger of sample contamination, undermining the
reliability of results. Nevertheless, compared to the alternative of conventional phenotypic susceptibility testing, molecular susceptibility testing still carries multiple advantages. First, when transporting sputum specimens with viable bacteria is logistically impossible, the only alternative is for the patients to travel to the nearest reference laboratory to give the sample for susceptibility testing. The personal expense to these patients is inconvenient if not prohibitive. Comparatively, mailing a week’s worth of slides at a time is a minor challenge. Secondly, PCR contamination is detectible with proper controls, and presents a danger only for the integrity of the results. Contamination in culture-based systems, on the other hand, jeopardizes both the integrity of the results, as well as the health of laboratory workers and the general public, as drug-resistant strains are grown in pure culture.

Improvements in technology will continue to make molecular testing cheaper, faster, and less dependent on expensive infrastructure. Molecular testing holds potential for countries at all levels of development, and even as a component of a field test at a sub-national level.
IV. Detection of DNA by Strand Displacement and G-Quadruplex Peroxidase Activity

ABSTRACT

As previously discussed, molecular methods can be cheaper, faster, and safer than conventional susceptibility testing for rifampin-resistant tuberculosis. Molecular methods are also useful for the initial diagnosis and subsequent evaluation of *Mycobacterium tuberculosis*. The goal of this project is to develop a low-cost, point-of-care visual detection mechanism for both TB DNA in general and rifampin-resistant alleles in particular. This research successfully demonstrated the proof of principle that protein independent amplification modules can provide a visual signal to indicate the presence of a specific nucleic acid, with discrimination to the point of a single nucleic acid.

INTRODUCTION

Although molecular methods to detect both MTB complex DNA as well as specific antibiotic resistance marker show great promise, there are still limitations. The vast majority of molecular detection techniques depend on PCR. PCR requires a thermocycler, which represents an initial investment, as well as polymerases, which much be stored in a freezer and maintained in a cold chain. As well, PCR products require some form of subsequent analysis, with further costs and infrastructure requirements.

The diagnostic mechanism has three components: 1) Sequence specific DNA detection 2) Non-enzymatic signal amplification with strand displacement and 3) a G-quadruplex reporter that produces a visual signal (Figure 16).

*Strand Displacement*
Strand displacement is the process by which an invader strand is able to replace one strand in a nucleic acid duplex in a two step process. In the first step, the invader strand binds to a single-stranded portion of the duplex, termed the toe-hold. The second step is branch migration, where the invader strand replaces one of the strands in the duplex, one or two bases at a time. Because the energy of the bond created is equal to the energy of the bond broken, branch migration is an energy neutral and reversible process. Because strand displacement is energy neutral, it can isothermally separate a DNA hybrid that otherwise would be stable to a very high temperature.

Strand displacement can also be used to amplify a signal. Zhang et al. created a catalytic circuit entirely based on strand displacement. In brief, the output strand is originally hybridized to a dock, leaving a toe-hold of several base pairs on the dock. The output strand has an overhanging region that is involved in downstream processes but which is inactive while the output is bound to the dock. A fuel strand, also complementary to a sub length of the dock, is present in excess concentration, but is unable to displace the output at a rate of any significance as the fuel does not match the toe-hold displayed on the dock. The invader strand functions as a catalyst, binding to the toe-hold on the dock and displacing the output, but leaving a corresponding toe-hold on the other end of the dock. As this process is reversible, the output could potential rebind to the dock and displace the catalyst. Given the excess amount of fuel, however, the fuel is more likely to bind the toe-hold than the output, which is then free to activate the reporter. The fuel binds the toe-hold and displaces the catalyst, which is then free to displace another output strand. Through this iterative process one molecule of catalyst can release multiple strands of output.
As the strand to be displaced can be easily labeled, strand displacement can be used as a proxy measure for the presence of an invader stand. The reporter strand can be labeled in a variety of ways that each has its own properties, including price, equipment required to detect the label, and sensitivity of the label. Labels that have been used include radioactivity, fluorescence\textsuperscript{78}, and electronic beads\textsuperscript{79}.

\textit{Visual Detection using G-Quadruplex Peroxidase}

Sequences rich in guanine are able to form both intermolecular and intramolecular quadruplexes, wherein each guanine forms hydrogen bonds with two adjacent strands. Adjacent strands may be either parallel or antiparallel. The G-rich telomeric sequences have been shown to form physiologically significant G-quadruplexes \textit{in vivo}. These G-quadruplexes block telomerase activity and must dissociate in order for the telomeres to be elongated. Compounds that stabilize G-quadruplexes show promise as a means to limit cell proliferation and combat cancer\textsuperscript{80}. Besides role in telomeres, G-quadruplexes have additional interesting properties. In particular, G-quadruplexes can associate with hemin and demonstrate peroxidase activity\textsuperscript{3}. Peroxidases split hydrogen peroxide, producing oxygen radicals which pull electrons away from any neighboring reducing agents. The stability of the G-quadruplexas well as a parallel intramolecular conformation largely determines the strength of its peroxidase activity. Cheng \textit{et al}. examine the kinetics of a variety of natural and designed G-quadruplexes and found that the EAD2 quadruplex, (CTG\textsubscript{3}(AG\textsubscript{3})\textsubscript{3}A) exhibited the highest rate among the sequences measured\textsuperscript{81}.

Peroxidase activity has been harnessed to produce a colorimetric signal by using a reducing agent whose color depends on its oxidation status. ABTS is a commonly used

\textsuperscript{3} Although the peroxidase activity of G-quadruplexes was first observed \textit{in vitro}, Cheng \textit{et. al} suggest that G-quadruplex peroxidases may be responsible for some of the oxidative cellular injury observed in the presence of hemin.
substrate, clear in its reduced form, and deep blue-green when oxidized. The color change can be observed qualitatively or measured quantitatively by reading the absorbance at 414 nm on a spectrophotometer. Weizmann et al. created a DNA detection mechanism using a G-quadruplex reporter, with amplification dependent on two protein enzymes, a polymerase and an endonuclease.

In the circuitry designed by Xi Chen (Figure 16) and tested in this research, part of the quadruplex sequence is bound to a separate dock sequence, preventing quadruplex formation and peroxidase activity. When the dock strand is displaced by the output strand of the amplification process, the quadruplex can fold and peroxidase activity can be observed.

METHODS

Sequences were ordered from IDT and resuspended in ddH2O. Because experiments with fluorescence demonstrated non-specific absorption of DNA at low concentrations onto the surface of the plate wells as well as pipette tips, all DNA at a concentration of less than 10uM was stored in dT dilution buffer (1X TE with 1 uM dT(TTTTTT) to occupy binding sites), and all experiments were performed in dT buffer. Double stranded constructs were formed by mixing the two sequences, heating at 95C for 1 min, then cooling to 25C at .1C/min.

For fluorescence assays, a master mix was prepared with 100nM double stranded fluorescent reporter (fREP) and 11.5uM MgCl₂. After multiple optimization experiments, each condition was prepared in a separate tube with 100 nM Gate (a double-stranded construct of invader with its dock), 300 nM fuel, and varying concentration of the catalyst strands. In order to obtain an accurate time course of the reaction, either the gate or the
catalyst was not added until directly before fluorescent readings. The master mix was simultaneously added to the tubes with a multichannel pipette. 16uL of the total volume of each reaction was then added to a plate and read immediately for the presence of fluorescein. The positive control consisted of 100nM invader. Negative controls consisted of fREP in dT buffer, fREP with 100nM Gate, and fREP with 100nM Gate and 300nM fuel.

Peroxidase activity was assessed by incubating the DNA and equimolar hemin at room temperature for 20 min in a total volume of 20uL before adding 80uL of substrate solution for a final concentration of 2mM hydrogen peroxide and 2mM ABTS. The extent of reaction was determined visually after fifteen minutes of peroxidase activity, or by taking kinetic absorbance readings at 414nM starting immediately after the addition of substrate solution.

RESULTS

The goal of this research is to develop an isothermal, colorimetric, protein-independent assay for a specific DNA sequence, aimed towards tuberculosis diagnostics, but adaptable to any target sequence. First, the activity of EAD2 was assessed to determine if 5’ or 3’ extensions disrupted peroxidase activity (Figure 17). Then, the optimal ratio of quencher to fluorophore was determined. If there is too much quencher relative to fluorophore, the invader strand will bind to free quencher strands, reducing sensitivity. If there is little quencher, there will be a high level of fluorescence even in the absence of a signal, contributing to a high background. The same principle applies to the G-quadruplex reporter and its dock. The optimal dock:output ratio and fuel concentrations were also experimentally determined (data not shown). Next, the reporter
can spontaneously recover peroxidase activity when the output strand bound to the dock strand that had been blocking quadruplex folding (Figure 18).

Further experiments led to the achievement of visual amplified detection of a catalyst strand (Figure 19). In the absence of fuel, each catalyst strand can displace a maximum of one output strand, and .25uM of catalyst is not visually observable. In the presence of fuel, however, each catalyst strand can release multiple output strands, which then increase the effective peroxidase concentration, leading to a clear visual signal of .25um catalyst. As G-quadruplex peroxidase activity is sensitive to pH, changing the pH of the reaction buffer lowered the limit of detection of both the G-quadruplex and the catalyst (Figure 20). With amplification, the fluorescence assay can reliably detect 5nM of catalyst in a total volume of 16uL (80 femtmoles) (Figure 21). Furthermore, this sensitivity is maintained while discriminating based on as little as a single base-pair mutation (Figure 22). While even 25 nM of the wild-type catalyst is able to fully activate the fluorescent reporter, 100 nM of the catalyst with a single base-pair mutation does not achieve activation above that of the Gate and fuel alone.

**DISCUSSION AND FUTURE DIRECTIONS**

The goal of this project is to develop a field-ready diagnostic capable of detecting a specific target DNA sequence and producing a visual signal. Herein we present the first experimental proof that a G-quadruplex can reliably report a signal amplified by strand displacement, while discriminating based on a single point mutation. The next step will be to adapt the assay to detect a medically relevant sequence, most likely mutated *rpoB*. Future work will focus on increasing the sensitivity of the assay, and setting up the assay to work with sputum samples spiked with target nucleic acids, sputum spiked with
bacteria, and eventually patient samples. Due to the modular nature of the detection scheme, the initial detection module can be changed without having to redesign the rest of the test. Therefore, this assay should be able to be easily modified to report the presence of any sequence of interest, whether to detect other pathogens, such as HIV, or a particular allelic state.

Running the assay does not require any special equipment and can be performed at room temperature. Both of those characteristics are promising in terms of the development of a field test. Further testing will be required to determine how robust the system is to temperature and other variables, as “room temperature” can vary widely depending on the room. This DNA detection scheme also demonstrates how multiple discoveries and advances in basics sciences, such as G-quadruplex peroxidase activity and catalytic strand displacement, can be combined to create a medically relevant device, in this case a diagnostic assay that is extremely easy to perform and straight-forward to interpret.

V. Conclusion

Compared to many other human pathogens, tuberculosis might appear quite tame and even boring. Tuberculosis produces no known toxins, unlike botulism, which can cause permanent paralysis, or hemorrhagic E. coli, which can rip holes in the digestive tract. Unlike Ebola, with a mortality rate approaching 90%, 90% of people infected with tuberculosis never even get sick. Tuberculosis replicates extremely slowly. To put the growth rate in perspective, if someone started with one bacterium of the nonpathogenic E. coli and one M. tuberculosis bacterium, each in their favored medium, after twenty-four hours, there would be two tuberculosis bacteria and seventeen million E. coli.
Finally, unlike *Staphylococcus* which can instantly gain resistance to many antibiotics through the uptake of a plasmid, tuberculosis, which hardly ever uses horizontal gene transmission, must slowly accumulate resistance to various antibiotics over time. Despite its seemingly unthreatening nature, however, tuberculosis has garnered enormous notoriety and attention.

With the global increase in population, although rates of TB infection have declined, there are more people currently infected with TB than ever before\(^8^4\). The rise of tuberculosis strains resistant to antibiotics in New York caused an upsurge in new cases of tuberculosis in the early 1990’s, after nearly forty years of steady decline. Unless proper measures are taken to prevent and contain anti-biotic resistant tuberculosis, untreatedable strains of tuberculosis will erode the global progress towards the end goal of eradicating tuberculosis. The 2010 WHO report on drug resistant tuberculosis reveals that the rate of MDR in both new and previously treat tuberculosis cases is increasing in many parts of the world, such as Tajikistan, where over 16% of new tuberculosis cases were found to be MDR\(^8^5\). The emergence of TB strains resistant to antibiotics and the growing challenge of TB/HIV coinfection challenge the traditional paradigm of tuberculosis having low mortality and progressing slowly. A chilling outbreak of MDR among AIDS patients in South Africa killed 52 of 53 patients for a mortality rate of 98% and the median time from sputum collection to death was 16 days\(^8^6\).

Along with the new and continued challenges posed by tuberculosis, new signs of promise exist on many fronts. In diagnostics, the QuantiFERON Gold tests have been developed to replace tuberculin skin testing, used since the time of Koch. Molecular methods and other rapid resistance tests, reduce the time for a clinician to know the
susceptibility results for an isolate and be able to treat accordingly. The projects presented in this thesis demonstrate the potential of molecular diagnostics in resource limited settings, as well as demonstrating the importance of addressing social factors when combating tuberculosis. Several new vaccines show promise and are entering clinical trials. Continued advances in the basic understanding of tuberculosis can continue to drive new approaches to prevent and treat tuberculosis infection. New technologies in prevention, diagnostics, and treatment, combined with the technology independent strategies of respiratory isolation of infectious individuals, rest and good nutrition to bolster the immune system, and basic contact investigation to identify clustered cases of the disease offer the promise that tuberculosis can be defeated, once and for all.
VI. Figures
Figure 1. Selection of Study Population

TIMS
1585 cases
Reported, 2006

Eligible to complete within 1 year
Completed therapy
>365 days

TIMS
132 cases
Delayed COT

Requested
DOT logs and
progress notes
from
County HD

DOT logs and progress notes
116 cases
Figure 2. Apparent Cases of Delayed Completion of Therapy for Tuberculosis Reported in Texas, 2006

- Pending Receipt: 16
- Included in study: 95
- Removed due to record error: 21

n=132
Figure 3. Record Errors Discovered Among TB Cases whose DOT Logs and Progress Notes Were Received
Figure 4. Length of Therapy for 95 Cases of Delayed Completion of Therapy
Figure 5. Reasons for Delayed Completion of Therapy

- Coordination Issues: 3%
- Compliance: 19%
- Medical Issues: 52%
- Provider Decision: 26%

n=95
Figure 6. Medical Factors Contributing to Delayed Completion of Therapy
Figure 7. Patient Noncompliance
Figure 8. Coordination Failures Contributing to Delayed Completion of Therapy
Figure 9. Interruptions in Therapy Among Cases
Figure 10. Risk Factors Associated With Delayed Completion of Therapy

- Homelessness
- INH Resistance
- HIV
- Extrapulmonary Involvement
- Diagnosed in Jail
- Alcohol Abuse
- Unemployment

Odds Ratio
Figure 11. Diagnostic Sputum Slide Cleaned with Xylene
Figure 12. Primers and Region Amplified

<table>
<thead>
<tr>
<th>Number</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>rpo105</td>
<td>5'-CGT GGA GGC GAT CAC ACC GCA GCAGTT-3'</td>
</tr>
<tr>
<td>2</td>
<td>rpo293</td>
<td>5' - GACCTCCAGCCCCCGACGCTCACG-3'</td>
</tr>
<tr>
<td>3</td>
<td>rpo273</td>
<td>5'-AGT GCG ACG GGT GCA CGT CGC GGA CCT-3'</td>
</tr>
<tr>
<td>Afghan Cases 1st Q 2008</td>
<td>Slides Received</td>
<td>Slides Chosen</td>
</tr>
<tr>
<td>------------------------</td>
<td>----------------</td>
<td>--------------</td>
</tr>
<tr>
<td>~5000</td>
<td>1120</td>
<td>511</td>
</tr>
</tbody>
</table>
Figure 14. Geographical distribution of mutations. No sequence available for grey provinces.
Figure 15. Mutations Found Among Samples

<table>
<thead>
<tr>
<th>Mutation by codon</th>
<th>Number of samples</th>
</tr>
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<tbody>
<tr>
<td>TCG531 TTG</td>
<td>16</td>
</tr>
<tr>
<td>TCG531 TGG</td>
<td>2</td>
</tr>
<tr>
<td>CAC526 CGC</td>
<td>1</td>
</tr>
<tr>
<td>CAC526 AGC</td>
<td>1</td>
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</tbody>
</table>
Figure 16. DNA Detection via Non-enzymatic Amplification and G-Quadruplex, Adapted from Xi Chen
Figure 17. Conservation of EAD2 Activity With Addition of 5’ or 3’ Extension

Blank
MgCl₂+EDTA
EDTA
MgCl₂
Positive control
Figure 18. Spontaneous Recovery of G-Quadruplex Peroxidase Activity Upon Strand Displacement

Double Stranded (DS)

DS+ Invader

SS EAD.11a
Figure 19. Amplification and Visual Detection of a Sequence of Interest

0.25uM catalyst, fuel
0.25uM catalyst
1uM catalyst
1uM output
Ds reporter
Buffer
1uM gate, 2.5uM fuel
Figure 20. PH Dependence of G-Quadruplex Peroxidase Activity

EAD2

(−)
Figure 21. Limit of Detection of Catalyst Using Fluorescent Reporter
Figure 22. Amplification Discrimination Based on Single Base Pair Mutation
VII. REFERENCES


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