DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* IN HUMAN SPUTUM SAMPLES USING *mpt40* GENES AND PREDICTING ANTIMICROBIAL RESISTANCE USING RAPID SINGLE-STEP DNA EXTRACTION, POLYMERASE CHAIN REACTION AND POLYACRYLAMIDE GEL ELECTROPHORESIS

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DEDICATION

I would like to dedicate this thesis to the United States Army. It was the Army that made this study possible.

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ABSTRACT

Boiling, boiling coupled with sonication and the use of a capture resin, Genereleaser(TM) (GR), were compared as a means of extracting DNA for polymerase chain reaction (PCR) amplification of *Mycobacterium tuberculosis* directly from human sputum samples. The primers TB*mpt*1 [5'-CAACGCGGCCGCGGTGG-3'] and Tb*mpt*-2 [5'-GCGGTGCCGTGGGGGG-3'] specific to the insertion sequence *mpt*40 which is specific to *M. tuberculosis* were used in the PCR amplification. Out of 77 human sputum samples that were analyzed, 11 were positive for *M. tuberculosis* as per PCR amplification compared to nine by BACTEC 460, six by growth on Middlebrook 7H12 and four by Acid Fast Staining (AFB). The results indicate the *mpt40* gene was specific for *M. tuberculosis* and shows promise for use in a clinical laboratory setting for diagnosing *M. tuberculosis* . The 11 samples that were positive for *M. tuberculosis* by PCR were screened for mutations in the genes *rpoB* (which encodes for RNA polymerase) and *katG* (which encodes for catalase peroxidase). Mutations in these genes lead to resistance to Rifampin and Isoniazid (INH), respectively. There were no amplification of *kat*G in clinical samples, however, *rpo*B was amplified in all 11 samples. Both genes were amplified from an experimental model consisting of sputum inoculated with the wild type (ATCC 27294) strain of *M. tuberculosis*. A 3-12% gradient polyacrylamide gel electrophoresis (PAGE) of the *rpo*B gene amplicon detected larger size fragments in two of the 11 human sputum *M. tuberculosis* positive samples. These two specimens showed positive hybridization against a 411 bp *rpo*B gene probe derived from a Rifampin resistant *M. tuberculosis* strain (ATCC 35838).

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INTRODUCTION

1.1 PURPOSE OF STUDY

Since 1981 there has been a steady increase in the outbreak of tuberculosis (TB) world-wide, in particular multidrug resistant strains of *Mycobacterium tuberculosis*. With this increase in the outbreak of TB, it has become necessary to be able to diagnose TB in the shortest time possible. Thus the purpose of this study was to investigate the specificity and sensitivity of the polymerase chain reaction (PCR) in the detection of multidrug resistant *M. tuberculosis* (MDRT) in human sputum samples.

1.2 TUBERCULOSIS IN THE UNITED STATES

Mycobacteria are slender curved or slender rod-shaped organisms 0.2-0.4 to 10 μm in size. They have linear double stranded DNA, are non-motile and do not form spores. Their cell wall has a high lipid content; thus, mycobacteria resist staining with commonly used basic aniline dyes at room temperature. They take-up dye with increased staining time and temperature and resist decolorization with up to 3% hydrochloric acid and 95% ethanol. These characteristics referred to as acid fastness, are basic to distinguishing different species of mycobacteria, in particular *M. tuberculosis*. Organisms that are closely related to *M. tuberculosis* with respect to their acid fastness and their attack on the respiratory system, are *Norcardia asteriodes* and some *Streptomyces specie (spp)*. which are aerobic actinomycetes (Eisenstadt et al., 1995).

Epidemiologically, *M. tuberculosis* and most aerobic *Actinomycetes* are air borne and are contracted through inhalation although structurally they are different (Eisenstadt et al., 1995). *Nocardia* species are usually seen with a partially acid fast appearance. A variety of bronchial fungal infections such as *Histoplasmosis* and *Coccidiomycosis* cause similar damage to human lungs on X-ray film analysis, but the major difference is that the fungal cell walls are made of chitin while different species of mycobacteria have a waxy lipid cell wall.

Tuberculosis has become a revitalized threat in the United States (Centers For Disease Control and Prevention, 1993). It is a problem not only of developing countries but developed countries as well. Previously, industrialized countries experienced a decline in TB but since 1981, this trend has been reversed with increases of 18.4% in the United States (Telenti et al., 1994). The increase in tuberculosis frequency in the U.S. can be correlated with the AIDS epidemic, the influx of infected immigrants, outbreaks of TB in congregative facilities and numbers of homeless people (Altamirano et al., 1994). To complicate the problem, outbreaks of MDRT have also been on the increase with the mortality rate exceeding 80% (Stoeckle et al., 1993; Centers For Disease Control and Prevention, 1993; Altimirano et al., 1994). Since 1984, however, the incidence has increased each year. Due to the increase in the number of immunocompromised patients, the number of patients hospitalized with active TB has also been on the increase (Centers For Disease Control, 1993; Heym et al., 1994). This combination is responsible for numerous nosocomial outbreaks of TB. Hospital associated cases of TB often go undiagnosed

for extended periods, as these patients often develop extrapulmonary TB, with or without the symptoms associated with active TB. These isolates are resistant to as few as two, and as many as seven, antimycobacterial agents (Pearson et al., 1992; Centers for Disease Control, 1993). While the major risk group for these strains is immunocompromised patients, hospital workers have also been infected (Telenti et al., 1993). New York, Texas, Florida and California were and still are the states within the continental U.S with the highest outbreaks of MDRT (Altimirano et al., 1994).

1.3 ANTITUBERCULOUS AGENTS

According to Van Scoy et al. (1992), some of the drugs currently used in short-course chemotherapy of TB include Isoniazid (INH) (isonicotinic acid hydrazide), Rifampin, Streptomycin and Ethambutol. INH is a chemotherapeutic agent that is a white, water-soluble, crystalline substance. It is bactericidal, and is absorbed well when administered orally or parenterally. It acts primarily by inhibiting the synthesis of mycolic acid, an important component of the cell walls (Heym et al., 1994). Peak blood concentrations occur 1 to 2 hours after oral administration and most strains of *M. tuberculosis* are susceptible to this drug (minimum inhibitory concentration MIC min < 0.02 ug / ml) (Zang et al., 1994;Van Scoy et al., 1992). To determine the susceptibility radiometrically, an indirect drug susceptibility test is performed using the BACTEC 460. In this process, a growth index (G.I.) replaces the MIC, and the reduction in labeled carbon dioxide as compared to a drug free control determines resistance or susceptibility. If the change in the growth index is equal to or greater

than the change of the growth index in the control (drug free organism) then the organism is deemed resistant (Heym et al., 1994). Adverse effects of INH include hepatitis, particularly in people 35 years or older (Van Scoy et al., 1992).

Rifampin is a potent antituberculous drug that also has antibiotic activity against other bacteria, and *in vitro* activity against some viruses (Van Scoy et al., 1992). It is derived from the bacterium *Streptomyces mediterranei* and seems to be as potent an antituberculous agent as INH. Rifampin is a large, fat soluble molecule that acts by inhibiting synthesis of RNA. More specifically, it inhibits bacterial DNA-dependent RNA polymerase. Rifampin has well absorbed when taken orally; 600 mg doses produce peak serum concentrations of about 7 μg / ml in approximately 2-4 hours after administration. Rifampin is the broadest spectrum of the antituberculous agents. Disk and agar susceptibilities are rarely performed since most species of mycobacteria are slow growing (Heym et al., 1994). Most microbiology laboratories use the radiometric susceptibility method to determine the susceptibility of *M. tuberculosis* to Rifampin (Van Scoy et al., 1992). Rifampin has numerous side effects ranging from nausea and vomiting to the discoloration of virtually all body fluids. Another antituberculous agent is Streptomycin. It belongs to the aminoglycoside group of antibiotics and its side effects include renal toxicity and ototoxicity manifested by vomiting and vertigo (Van Scoy et al., 1992). Hearing loss may also occur. Ethambutol on the other hand, is a synthetic antituberculous drug that is orally administered. It is one of the very few antituberculous drugs that have been administered to pregnant women. Ethambutol is dextro isomer of 2,2'-

(ethylenediimino) di-1-butanol dihydrochloride. The drug is effective against actively growing cells with a peak serum concentration of 2 to 5 μg / ml occurring 2-4 hours after administration. Side effects include ocular toxicity, decreased visual activity, loss of green color perception, central scotomas and peripheral visual field defect (Van Scoy et al., 1992).

1.4 MOLECULAR BASIS FOR ANTIBIOTIC IN *MYCOBACTERIUM TUBERCULOSIS*

Antibiotic resistance in mycobacteria has been attributed to genetic mutations within the organism (Stoeckle et al., 1993). With respect to *M. tuberculosis*, resistance to INH has been attributed to deletions in the *katG* gene which encodes for catalase-peroxidase (Stoeckle et al., 1993; Altamirano et al., 1994). One mechanism that may explain INH resistance in *M. tuberculosis* is the total deletion of the *katG* gene (Zang et al., 1994). The complete deletion of the *katG* gene alone, however is not the major mechanism for INH resistance (Altamirano et al., 1994). Altimirano et al., 1994, demonstrated that INH-resistant isolates showed positive hybridization with a *katG* probe but had no expression of the catalase peroxidase activity, suggesting the probability of point mutations as the mechanism for INH-resistance. It appears that total deletion of the *katG* gene accounts for 10%-24% of INH-resistant isolates; in the remaining cases, the *katG* gene is present with randomly distributed mutations (Altimirano et al., 1994). A second hypothesis explaining INH resistance to *M. tuberculosis* is that catalase is inactivated by the free hydroxyl radicals produced during metal-catalyzed

autoxidation of INH (Stoeckle et al., 1993). Another suggestion is that catalase oxidizes INH into its analog isonicotinic acid, which then becomes incorporated into a NAD analog that fails to function as a coenzyme (Stoeckle et al., 1993). The existence of *inhA* genes in addition to *katG* genes suggests that more than one mechanism is responsible for INH-resistance. Cloning experiments cited by Altimirano et al. (1994), showed that the absence of *inh*A in mycobacteria conferred resistance too. Shinnick et al. (1995) attributed the loss of resistance to be due to the loss of the catalase gene or from the failure of a key enzyme to bind INH into the cell. Zang et al. (1992) cloned the *katG* from *M. tuberculosis* and demonstrated that this gene restored sensitivity in resistant mutant strains. They transformed INH-resistant strains of *M. tuberculosis* with a plasmid vector carrying the functional catalase-peroxidase *(katG)* gene. Expression of *katG* restored full drug susceptibility in isolates initially resistant to concentrations ranging from 3.2 to > 50 μg / ml. Transformation with the corresponding *katG* gene from *Eschericia coli* resulted in low level expression of catalase and peroxidase activities and conferred partial sensitivity.

Resistance to Rifampin involves alterations of RNA polymerase. The gene that encodes for RNA polymerase subunit beta is *rpoB* (Telenti et al., 1993; Imboden et al., 1993). Most of the mutations are clustered within the region of 23 amino acids, thus substitution of a limited number of highly conserved amino acids encoded by the *rpoB* gene appears to be the molecular mechanism responsible for a "single step" high level resistance to Rifampin although the precise molecular mechanism is

not known (Telenti., 1993), however, the resistance mechanism for the reaction of Rifampin is believed to be changes in the sequence of a small region of the *beta* subunit of RNA polymerase, so that the enzyme no longer binds (Shinnick et al., 1995). Furthermore, Substitution of key amino acids would result in conformational changes and defective binding of the drug (Telenti et al., 1993). Insertions have also been known to occur 2% of the time in-between the CAA ↓ TTC sequences and 1% of other cases in-between the TTC ↓ ATG sequences (Kapor et al., 1994). Nolan et al. (1995) determined that INH resistance occurred 100 times more than Rifampin resistance. This correlates with the fact that more people are on INH therapy. It is therefore possible that increases in the use of INH therapy has lead to the wide spread resistance to INH encountered today. It is important to note that most bacteria contain the *kat*G and *rpo*B, so it is important to digest and decontaminate all sputum samples before the onset of PCR, as traces of either gene from indigenous bacteria during the assay could yield false positives (Whelen et al., 1995). Whelen et al. (1995) demonstrated that a heminested (multiple PCR) protocol not only increased the sensitivity of the PCR assay in the detection of *M. tuberculosis*, but also could be used as a stand alone method for both the direct detection of *M. uberculosis* and the determination of Rifampin susceptibility or resistance. They suggested that since single drug resistance resistance rarely occurs in the United States, *rpo*B may serve as surrogate marker for multi-drug resistant *M. tuberculosis.*