بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

﴿ وَأَنْزَلَ اللهُ عَلَيْكَ الْكِتَابَ
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وَكَانَ فَضْلُ اللهِ عَلَيْكَ عَظِيمًا ﴾

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Study of the High resolution melting curve assay And the ResazurinMicrotiter assay for the rapid identification of multidrug resistant *Mycobacterium tuberculosis*

THESIS

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Presented by

Mona SaadAbdou

M.SC., M.B.BCH-Ain Shams University

Supervised by

Prof. Dr. Amany Mostafa Kamal

Head of Department of Medical Microbiology and Immunology
Faculty of Medicine - Ain Shams University

Dr.Iman Hussein Shehata

Assistant Professor of Medical Microbiology and Immunology
Faculty of Medicine - Ain Shams University

Dr. Mona Hamed El-Shokry

Assistant Professor of Medical Microbiology and Immunology
Faculty of Medicine - Ain Shams University

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رسالة

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(الميكربيولوجيا الطبية والمناعة)

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طبيبة / منى سعد عبده أحمد

ماجستير العلوم الطبية الأساسية (الميكربيولوجيا الطبية والمناعة)
بكالريوس الطب والجراحة

تحت إشراف

الأستاذ الدكتور / أمانى مصطفى كمال

أستاذورئيس قسم الميكربيولوجيا الطبية والمناعة
كلية الطب – جامعة عين شمس

الدكتور /إيمان حسين شحاتة

أستاذ مساعد الميكربيولوجيا الطبية والمناعة
كلية الطب – جامعة عين شمس

الدكتور / منى حامد الشقرى

أستاذ مساعد الميكربيولوجيا الطبية والمناعة
كلية الطب – جامعة عين شمس

كلية الطب
جامعة عين شمس
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INTRODUCTION

Tuberculosis (TB) is an infectious disease of global impact, killing nearly two million people every year. In Egypt, this disease represents a major problem with incidence rate of 21new cases for every 100,000 of population, prevalence of 31 cases for every 100,000 of population and it kills about 10,000 people every year ***(WHO, 2008a)***. The global spread of TB is further complicated by the ubiquitous appearance of drug-resistant and especially multidrug-resistant strainsto most effective (first-line) anti-TB drugs ***(Harries and Dye, 2006)***.

Multidrug-resistant tuberculosis (MDR-TB) is defined as *M. tuberculosis* resistant to at least the two major anti-TB drugs, isoniazid (INH) and rifampicin (RMP). It has become one of the major problems throughout the world since fatality rates for MDR-TB are much higher ***(WHO, 2006a).***Treatment of MDR-TB requires prolonged and expensive chemotherapy using second line drugs of increased toxicity***(Schmid et al., 2008).*** In Egypt, MDR-TB accounted for 2.2% of all cases of TB documented at the World Health Organization between 2005 and 2007***(WHO, 2008b)***.

The resistance of *M. tuberculosis* strains to anti-TB drugs develops due to mutations in drug target genes and MDR-TB strains evolve due to sequential accumulation of these mutations.

Mutations in the *rpoB*gene, encoding the b-subunit of RNA polymerase, occur in 90% to 95% of RMP-resistant strains. In contrast, mutations in the *katG*and *inhA*genes can cause INH resistance, however, mutations in the *katG*gene, encoding catalase-peroxidase, occur more frequently ***(Ahmad and Mokaddas, 2004).***

In Egypt, laboratory diagnosis of TB is usually done by conventional methods including smears, culture and isolation and BACTEC system. Drug susceptibility tests also are usually the conventional ones.

In the last few years, several methods have been proposed for the rapid detection of drug resistance of *M. tuberculosis* compared with conventional drug susceptibility testing (DST) that can take several weeks. Among them, the use of molecular techniques based on PCR amplification of genes involved in resistance mechanisms, followed by the detection of key mutations associated with resistance***(Pietzka et al., 2009)***.

Based on this knowledge, high resolution melting (HRM) curve assay has been developed for accurate identification of mutations in PCR products**.** This PCR-based assay characterizes nucleic acid samples based on their disassociation (melting) behavior. Samples can be discriminated according to their sequence, length, GC content or strand complementarity. HRMcurve assay can be used for simultaneous detection of

resistance of *M. tuberculosis* to RMP and INH***. (Brossier et al., 2006)***

Other techniques that have been proposed for rapid detection of MDR-TB includethe resazurinmicrotiter assay (REMA assay) which is one of the new colorimetric methods. This method uses redox indicator to detect mycobacterium growth. The test is based on the reduction of the colored redox indicator added to the culture medium after *M.tuberculosis* has been exposed in *vitro* to different antibiotics. Resistance is detected by change in color of the indicator, which is directly proportional to the number of viable mycobacterium in the medium ***(Palmino et al., 2004).***

A key element in the control of drug-resistant TB is the early identification of MDR-TB strains to start an effective chemotherapy coupled with surveillance that enable successful intervention to reduce thetransmission and spread of the disease***(Hoek et al., 2008)***.

Aim of the work:

The aim of this study is to evaluate the genotypic method HRM curve assay and the phenotypic method REMA assay for the rapid identification of MDR *M. tuberculosis* strains to start an effective treatment of TB.

TUBERCULOSIS

Historical Aspects:

TB has been present in the human population since antiquity. It was documented in ancient Egypt, India, and China as early as 5000 years ago, respectively**.** Fragments of the spinal column from Egyptian mummies from 2400 B.C. show definite signs of tubercular decay ***(Morse et al., 1964)***.

TB has been known with different names, since ancient times. The ancient disease called phthisis had references of symptoms, similar to that of TB. Later, it was known as 'consumption' because a person used to just 'waste away' after contracting the disease. Around 460 BC, Hippocrates identified phthisis as the most widespread disease of the times ***(Brown, 1941***). The TB epidemic in Europe, later known as the "Great White Plague", started at the beginning of the 17th century ***(Dubos and Dubos, 1952).***

Sylvius, a Dutch physician and scientist, was first to identify characteristic changes in lungs due to TB, in the 17th century. He recorded the progress of the disease in his book Opera Medica. He also noticed that abscesses and cavities were

caused due to 'consumption'. In 1720, an English physician Benjamin Marten was first to hypothesize that tuberculosis could be caused by micro-organisms. However, the exact cause of TB was still unknown. The concept of sanatorium cure, where the patients were isolated and taken care of, was introduced for TB patients. The peak of the TB epidemic reached Europe, at the end of the 18th and early 19th centuries ***(Doetsch, 1978).***

Afterwards, a major reversal occurred in industrialized countries and death rates began to fall; three explanations have been advanced;

1. Improved socioeconomic conditions that led to better living standards.

2. Application of primitive public health measures.

3. The dawning realization that TB was probably an infectious disease and the beginning sequestration of (contagious) consumptives in hospitals and sanatoriums ***(Shimao, 1956).***

On 24 March 1882, Robert Koch revealed that the disease was caused by Tubercle bacilli ***(Koch, 1882)***. In 1895 Wilhelm Roentgen, discovered the X-ray, which allowed physicians to diagnose and track the progression of the disease, and although an effective medical treatment would not come for another

fifty years, the incidence and mortality of TB began to decline. However, the steady fall in the incidence of TB was confounded by a steep rise during and after the two world wars ***(Doetsch, 1978)***

French scientists Calmette and Guerin had discovered the BCG vaccine by the year 1919. In the year 1944, streptomycin, the first antibiotic drug to cure TB was discovered by Selman Abraham Waksman, making a significant contribution to medicine ***(Bloom and Murray, 1992)***. Then there was a rapid succession of discoveries of anti-tuberculous drugs after streptomycin including isoniazid, pyrazinamide, rifampicin, ethambutol. These drugs are the anti-tuberculosis drugs used today. The general improvement in public health with TB program activities reinforced by successful chemotherapy, resulted in reduction of infection and death rates. The disease became greatly controlled but never disappeared ***(Petrini and Hoffner, 1999).***

Since the mid-1980s, this decreasing trend slowed down and even reversed in some countries, such as the USA. The resurgence of the disease was attributed to the epidemic of human immunodeficiency virus (HIV) infection, diminished public health efforts to control TB, rising poverty, homelessness, overcrowded conditions, and immigration from countries with a high prevalence of TB ***(Lienhard, 2001).***

In poorly developed countries, TB remains a significant threat to public health, as incidences remain high, even after the introduction of vaccination and drug treatment ***(Shah et al., 2011).***

Global Burden of TB:

More than a decade after it was declared by the WHO as a global health emergency in 1993, TB remains one of the world's leading infectious causes of death among adults. After years of steady decline in incidence rates, TB is making a dramatic comeback and is on the rise since 1980s ***(WHO, 2009).*** The increasing number of Human Immunodeficiency Virus (HIV) infections is a major reason for this comeback ***(Frieden et al., 2003).*** A person is newly infected with *M. tuberculosis* every second. Left untreated, a person with active TB will infect an average of 10 to 15 other people every year. Worldwide, one person out of three is infected with *M. tuberculosis* – two billion people in total ***(Dye et al., 2005).***

World Health Organization (WHO) fact sheet released in ***(2010a)*** estimated that there were 9.4 million new cases of TB in 2009 including 1.1 million among people with HIV. Most of these cases were in Asia (55%) and Africa (31%), with small proportions of cases in the Eastern Mediterranean Region (6%), the European Region (5%) and the Region of the Americas (3%).

Some 22 high-burden countries collectively account for 80% of the global TB burden

Incidence, Prevalence and mortality rates appear to be falling in all six WHO regions. The estimated global incidence rate fell to 137 cases per 100 000 population in 2009, after peaking in 2004 at 142 cases per 100 000. The rate is still falling but too slowly. 1.7 million people died from TB (including 380 000 women) in 2009, including 380 000 people with HIV, equal to 4700 deaths a day ***(WHO, 2010a)***.

TB holds the seventh place in the global ranking of causes of death with 95% of all cases and 99% of deaths occur in developing countries ***(Smith, 2004).*** It is a disease of poverty, and the declining incidence in many relatively wealthy areas is not unexpected, but there are other parts of the globe where health systems are defective, which lead to the sloppy implementation of directly observed treatments (DOTS) programs and exacerbate the TB problem ***(Donald and van Helden, 2009).***

The consequences of TB on society are immense. In most countries, more cases of TB are reported among men than women. Nevertheless, TB is among the three greatest causes of death among women aged 15-44, killing more women than all causes of maternal mortality combined ***(Montoro and Rodriguez, 2007)***.

Although the "direct costs" of diagnosis and treatment are significant for poor families, the greatest economic loss occurs as a result of "indirect" costs, such as loss of employment, travel to health facilities and in particular, lost productivity from illness and premature death ***(WHO, 2005).*** The disease hinders socioeconomic development as 75% of people with TB are within the economically productive age group of 15-54 years ***(Dye, 2006).***

Situation in Egypt

Egypt is not on the WHO list of 22 high-TB-burden countries, yet it contributes to 3% of the total TB cases in WHO's Eastern Mediterranean region. According to WHO reports, TB is the third most important public health problem in Egypt, after hepatitis C and Bilharzia. 66% of TB cases occur among socially and economically productive age groups of 15 to 54 years. In 2008, the estimated number of prevalent TB cases was 20,000, with a rate of 24 per 100,000 population while the estimated number of TB cases was 16,000 with an estimated incidence of 19 per 100,000 population ***(WHO, 2010b).***

DRUG-RESISTANT TB

Emergence of Drug-Resistant TB (DR-TB)

The history of TB had been changed dramatically after the introduction of anti-mycobacterial agents. Drug treatment is fundamental for controlling TB, promoting the cure of the patients and breaking the chain of transmission when the anti-tuberculosis drug regimen is completely and correctly followed ***(CDC, 2003)***.

Anti-tuberculosis drug treatment started in 1944, when streptomycin (SM) and para-aminosalicylic acid **(**PAS) were discovered ***(Weitzman et al., 1950)***

In 1950, the first trial was performed comparing the efficacy of SM and PAS both as monotherapy or combined. The study demonstrated that combined therapy was more effective and resulted in the first multidrug anti-tuberculosis treatment that consisted of a long course of both drugs. In 1952, a third drug, isoniazid (INH), was added to the previous combination, greatly improving the efficacy of treatment, but which still had to be administered for 18-24 months ***(Bloom and Murray, 1992)***.

In 1960, Ethambutol (EMB) substituted PAS, and the treatment course was reduced to 18 months. In the ’70s, with the

introduction of rifampicin (RMP) into the combination, treatment was shortened to just nine months. Finally, in 1980, pyrazinamide (PZA) was introduced into the anti-tuberculosis treatment,which could be reduced further to only six months ***(Petrini and Hoffner, 1999).***

Soon after the introduction of the first anti-mycobacterial drugs, drug resistant bacilli started to emerge, but the launch of both combination therapy and new more effective drugs seemed to be enough to control the disease. In fact, it was thought that TB could be eradicated by the end of 20th century ***(CDC, 2003)***.

However, TB unexpectedly re-emerged in the ’80s, and in the following years there was an important increase in the incidence of poly-, multiple-, and extensively drug resistant strains ***(Pedro and Jose', 2007)***.

Definitions concerning resistance in TB:

- **Susceptible strains** are those that have not been exposed to the main anti-tuberculous drugs and respond to these drugs in a uniform manner.

- **Resistant strains** differ from the sensitive strains in their capacity to grow in the presence of higher concentration of a drug ***(Sharma and Mohan, 2006).***