INTRODUCTION

Tuberculosis (TB) now ranks as one of the leading causes of death. According to the World Health Organization (WHO) global tuberculosis report in 2015, 9.6 million people are estimated to have fallen ill with TB in 2014. Globally, an estimated 3.3% of new TB cases and 20% of previously treated cases have multidrug-resistant TB (MDR-TB). Extensively drug-resistant TB (XDR-TB) had been reported by 105 countries by 2015. An estimated 9.7% of people with MDR-TB have XDR-TB. The number of reported cases of tuberculosis in Egypt according to World Health Statistics was 7, 467 new cases in 2014 with an incidence rate of 15 per 100,000 population. Fortunately, Egypt is still not on the WHO's list of 22 high-TB-burden countries or the 27 high MDR-TB burden countries. In Egypt, percentage of MDR among new TB cases was 3.4% while that among previously treated TB cases was 15% according to most recent year available data ***(WHO, 2015)***.

MDR-TB cases are difficult to treat and cure rates are low, whereas XDR-TB cases are virtually untreatable, since none of the standard drugs or the reserve drugs are effective ***(Alam et al., 2013).*** There is a global need for new reliable and affordable methods of the detection of drug resistance, as rapid identification of MDR- TB is vital for the prompt initiation of adequate treatment and interruption of further transmission of resistant strains ***(Minion et al., 2010).***

Two drug susceptibility testing (DST) strategies are currently in routine use: phenotypic and genotypic (molecular) methods. Molecular tests have the advantage of a shorter turnaround time, yet require expensive equipment and most also require specialized expertise. Phenotypic methods are in general simple to perform and might be closer to implementation on a routine basis in clinical laboratories, but some methods can take weeks to yield reliable results. Therefore new simple and rapid methods are needed especially for low-income countries with a high incidence of TB and a growing problem of drug resistance ***(Toit et al., 2012).***

Among the phenotypic methods for DST are rapid colorimetric methods such as MTT (3-[4, 5-dimethyl-thiazol-2-yl]-2, 5-diphenyltetrazolium bromide) and resazurin based on the ability of live bacteria to reduce an indicator and produce a change of visual color ***(Yajko et al., 1995; Abate et al., 1998).*** They appear promising techniques and have been described with success ***(Martin et al., 2005).***

Aim of the Work

The aim of this study is to evaluate the colorimetric MTT and resazurin methods as rapid and low cost methods for testing *Mycobacterium tuberculosis* (*M. tuberculosis)* susceptibility to Rifampicin (RMP) and Isoniazid (INH). The performance of these methods will be determined by comparing the results with drug susceptibility test (DST) using the conventional proportion method on Löwenstein-Jensen (LJ) medium.

MYCOBACTERIUM TUBERCULOSIS

History:

Mycobacterium tuberculosis (*M. tuberculosis)* was first discovered on 24 March 1882 by Robert Koch and he received the Nobel Prize in physiology of medicine for this in 1905. It's known as the tubercle bacillus, as well as Koch's bacillus ***(Murray and Nadel, 2010).***

Global Burden of Tuberculosis (TB):

Tuberculosis (TB) now ranks as one of the leading causes of death. According to the World Health Organization (WHO) global tuberculosis report in 2015, 9.6 million people are estimated to have new TB infection in 2014 ***(WHO, 2015)***. The increasing number of Human Immunodeficiency Virus (HIV) infections is a main cause for this comeback ***(Frieden et al., 2003).*** The African Region accounts for about four out of every five HIV-positive TB cases and TB deaths among people who were HIV-positive ***(WHO, 2014a).***

The disease delays socioeconomic development as 75% of people with TB are within the economically productive age group of 15-54 years ***(Dye, 2006).***

Situation in Egypt:

The number of reported cases of tuberculosis in Egypt according to World Health Statistics was 7, 467 new cases in 2014 with an incidence rate of 15 per 100,000 population ***(WHO, 2015).***

Treatment of Tuberculosis

Proper TB treatment requires accurate and early diagnosis, screening for drug resistance and HIV, the administration of effective regimens under supervision, and the support to patients for compliance throughout the course of treatment ***(WHO, 2012).***

Before effective drugs were available, 50% of patients with active pulmonary tuberculosis died within 2 years, and only 25% were cured ***(Kasperbauer and Daley, 2008).***

Directly observed treatment (DOTs) strategy uses the four- drug treatment regimen of first-line drugs (isoniazid, rifampicin, pyrazinamide, and ethambutol). Treatment requires a minimum of 6 months in two phases: 2 months of all four drugs in the intensive phase and 4 months of isoniazid and rifampicin in the continuation stage ***(WHO, 2012).***

I- First-line Agents

A) Isoniazid:

Isoniazid (INH) is the most widely used first-line anti-tuberculous drug. It is a cornerstone of regimens used to treat both TB disease and latent infection. INH is activated by the catalase-peroxidase enzyme encoded by the katG gene ***(CDC, 2014).***

Mechanism of action:

The primary target of INH is the mycolic acid synthesis pathway inhibition. A lack of mycolic acid synthesis results in loss of cellular integrity and the bacteria die ***(Johnson et al., 2006; Shi et al., 2007).***

Mechanism of resistance:

Resistance to INH involves many mutations in different genes. It is mainly (approximately 70–80%) due to mutations in the *katG* and *inhA* regulatory region ***(CDC, 2014).***

B) Rifampicin:

Mechanism of action:

Rifampicin (RMP) acts by inhibition of the mycobacterial transcription by inhibiting DNA-dependent RNA polymerase of *M. tuberculosis* and is able to hinder RNA synthesis even when the organism has minimal metabolic activity ***(CDC, 2014).***

Mechanism of resistance:

More than 96% of RMP-resistant isolates contain a mutation in the central region of the rpoB gene that encodes the β-subunit of the bacterial DNA-dependent RNA polymerase ***(Ahmad and Mokaddas, 2009; CDC, 2014).***

C) Pyrazinamide:

Mechanism of action:

Pyrazinamide (PZA) is a bactericidal drug that is active only against *M. tuberculosis*. PZA acts as a structural analogue of nicotinamide ***(Heifets, 2002; Zhang and Mitchison, 2003).***

Mechanism of resistance:

PZA susceptibility testing is not done routinely in many countries due to technical difficulties. Thus the extent of PZA resistance globally is unknown. In most cases, resistance to PZA is associated with mutations in pncA. PZA-resistant strains have shown a wide range of alterations in the 630 bp of the open reading frame or the 82 bp of the promoter region ***(Sekiguchi et al., 2007).*** Some PZA-resistant strains do not present any alterations in the coding region or the promoter of the pncA gene. For these strains, it has been postulated that PZA resistance could be due to mutations in an unknown pncA regulatory gene ***(Cheng et al., 2000).***

D) Ethambutol:

Ethambutol (EMB) has a broad spectrum of activity. It is included in initial treatment regimens until drug susceptibility results are received and resistance to the other first-line drugs has been excluded, at which time it can be discontinued ***(Rivers and Mancera, 2008).***

Mechanism of action:

It acts by affecting the cell wall synthesis. It interacts with three homologous membrane associated arabinosyl transferases enzymes as its target. It has been suggested that it plays a rule in increasing the susceptibility of *M. tuberculosis* to other drugs ***(Da Silva and Aínsa, 2007).***

Mechanism of resistance

The molecular basis of resistance to EMB is not fully defined ***(Ahmad and Mokaddas, 2009).*** Mutations in the *embCAB* operon were identified in up to 65% of EMB resistant clinical isolates of *M.tuberculosis.* Mutations at codon 306 of *embB* occur most frequently but mutations at amino acids residues *Asp 328, Gly 406* and *Glu 497* are also found to be associated with EMB resistance ***(Garg et al., 2006).***

Additional mutations in the *embC- embA* region have been found in strains that also had resistance associated amino

acid substitutions in *embA or embB,* and these may play a role in resistance ***(Ramaswamy et al., 2000).***

E) Streptomycin:

Streptomycin (SM) was the first antibiotic shown to be active against *M. tuberculosis* ***(Weitzman et al., 1950).*** However, significant levels of resistance occurred when SM was used as monotherapy, and some side effects, SM usage declined greatly in the 1960s ***(Bloom and Murray, 1992).*** Recently, the emergence of strains of *M. tuberculosis* displaying resistance to some or all of the major anti-tuberculosis drugs (INH, RMP, EMB, PZA and FQs) has led to renewed interest in SM and its family, Kanamycin (KAN) and Amikacin (AMK) ***(Shi et al., 2007).***

Mechanism of action:

SM has been shown to interact directly with the 30S ribosomal subunit, thus preventing protein biosynthesis**.** It is a very active bactericidal drug ***(Rivers and Mancera, 2008).***

Mechanism of resistance:

Mutations in *rpsL* and *rrs* genes lead to SM resistance ***(Shi et al., 2007).*** These mutations make the strain resistant only to SM not to the other aminoglycoside agents, such as KAN and AMK ***(Petrini and Hoffner, 1999).***

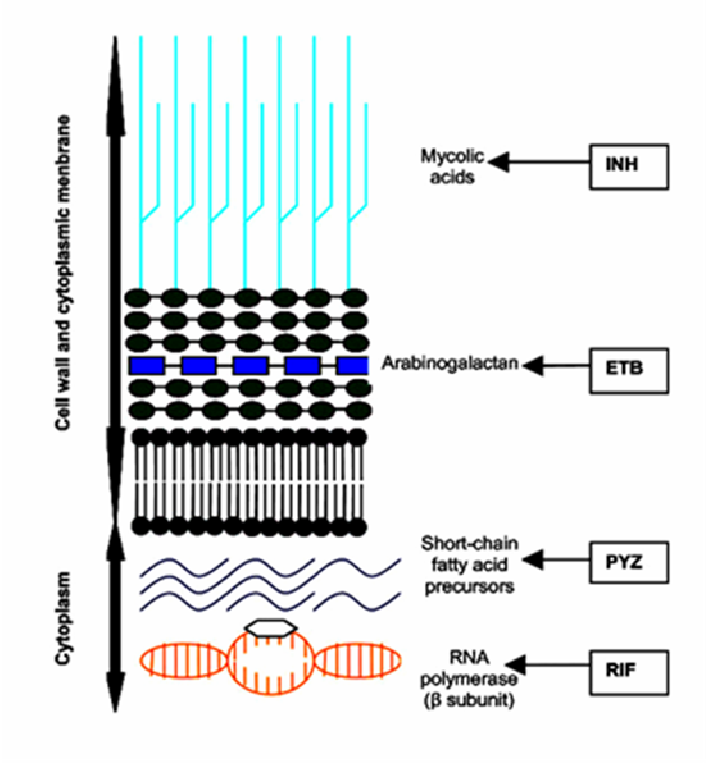

Figure (1): Sites of action of the principal anti-tuberculous drugs ***(du Toit et al., 2006).***

II- Second-Line drugs (SLDs)

Drugs in this group are used when there is resistance to first-line anti-tuberculosis drugs in suspected or confirmed cases, or when severe adverse effects to other anti-tuberculosis drugs develop ***(Seaworth and Longfield, 2006).***

Second-line agents are less effective, more toxic, or both, than first-line drugs ***(Rivers and Mancera, 2008).***

The second line drugs include:

A. Injectable drugs:

The second-line injectable drugs include a cyclic-peptide antibiotic, capreomycin (CAP), and two aminoglycoside antibiotics, kanamycin (KAN) and amikacin (AMK). The three drugs affect protein translation, share a molecular target, and bind at similar locations. As a result, cross-resistance has frequently been detected ***(CDC, 2014).***

B. Non injectable drugs:

1-Thioamides:

Mechanism of action:

Thioamides (ETH) is a structural analogue of INH that is also acts by inhibiting mycolic acid biosynthesis ***(Johnson et al., 2006).***

Mechanism of resistance:

It has been suggested that low-level INH resistance is correlated with resistance to ETH. Mutations in *inhA* regulatory region that are associated with INH resistance cause cross-resistance to ETH ***(Johnson et al., 2006).***

2-Fluoroquinolones:

Fluoroquinolones (FQ) are an important class of drugs used to treat tuberculosis resistant to first-line drugs, but have

the potential to become part of future first-line regimens ***(CDC, 2014).***

Mechanism of action:

The quinolones act by inactivating the DNA gyrase, a type-II DNA topoisomerase composed of two A and two B subunits encoded by *gyrA* and *gyrB*, respectively and DNA topoisomerase IV ***(Drlica and Malik, 2003).***

Mechanism of resistance:

Resistance to FQ is due to mutations in gyrA gene ***(CDC, 2014).*** Of the FQs, there are drugs with different degrees of activity against *M. tuberculosis.* Ciproflaxin (CIP) and ofloxacin (OFL) are the two FQs used in MDR-TB treatment ***(Mitnick et al., 2007).***

The new-generation FQs (moxifloxacin, sparfloxacin and gatifloxacin) have excellent bactericidal activity against *M. tuberculosis* with lower MICs than levofloxacin (Lev), CIP and OFL ***(Rustomjee et al., 2008).***

3-Para-aminosalysilic acid:

Para-amino salicylic acid (PAS) is active only against *M. tuberculosis*. It is bacteriostatic and can be given orally daily. The use of PAS has largely decreased since the introduction of RMP and EMB, however, due to its low cost; it is still in use in resource limited countries ***(Da Silva and Anisa, 2007)***.

Mechanism of resistance:

The mechanism of resistance of PAS is unknown. Interference with folic acid biosynthesis and inhibition of iron uptake have been proposed as two possible mechanisms of resistance for PAS ***(Morlock et al., 2003).***

4-Cycloserine:

Mechanism of action:

Cycloserine acts by inhibiting the synthesis of peptidoglycan by blocking the action of D-alanine recemase and D-alanine synthase ***(Peteroy et al., 2000).***

Mechanism of resistance:

The mutation in D-alanine recemase enzyme, encoded by *alrA*, results in resistance to D-cyclosporine ***(Chacon et al., 2002).***

III- Third- Line Agents

These agents are less effective against *M. tuberculosis* than second-line agents, and are mostly given only as adjunctive therapy to persons with extensively drug-resistant (XDR) tuberculosis. These drugs include amoxicillin-clavulanate, clarithromycin, clofazamine, and linezolid ***(Hauck et al., 2009).***

DRUG-RESISTANT TUBERCULOSIS

Emergence of drug-resistant Tuberculosis (DR-TB):

After the use 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, by the mid-1990s, multi drug-resistant TB (MDR-TB) appeared in most countries. The worst was yet to come in 2006, extensively drug-resistant TB (XDR-TB) emerged ***(WHO, 2008a).***

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 susceptible strains in their capacity to grow in the presence of higher concentration of a drug ***(Sharma and Mohan, 2006).***

Different categories of drug resistance have been established:

- **Mono-resistance:** resistance to one first-line anti-tuberculous drug [either rifampicin (RMP) or isoniazid or streptomycin or ethambutol.
- **Poly-resistance:** resistance to more than one first-line anti-tuberculous drug, other than both isoniazid and rifampicin.
- **Multidrug-resistance (MDR):** resistance to at least INH and RMP.
- **Extensive drug-resistance (XDR):** resistance to any fluoroquinolone, and at least one of three injectable second-line drug (capreomycin, kanamycin and amikacin), in addition to MDR.
- **Rifampicin Resistance (RR):** resistance to rifampicin detected using phenotypic or genotypic methods, with or without resistance to other anti-TB drugs. It includes any resistance to rifampicin, in the form of mono-resistance, poly-resistance, MDR or XDR.
- **Pan-resistant strains:** resistant to all first- and second-line drugs with a proven activity against *M. tuberculosis*, have been reported ***(WHO, 2014a).***