

Chapter 7

DRUG SUSCEPTIBILITY TESTING OF MYCOBACTERIA

7.1 Introduction

Drug resistance occurs spontaneously in *M. tuberculosis* at a different rate for each drug. For example, mutations resulting in resistance to rifampicin occur at a rate of 10^{-10} per cell division and 10^{-7} to 10^{-9} for isoniazid. Overall, in drug-free environments this creates an estimated prevalence of resistant organisms of 1 in 10^8 and 1 in 10^6 , respectively. As lung cavities frequently contain 10^7 bacilli, resistant bacilli emerge naturally without antimicrobial pressure and mycobacterial drugs then select the resistant population. By the 1950s, it was established that combination chemotherapy could prevent the emergence of clinical resistance, which occurred when patients were treated with a single drug. Today, monodrug therapy occurs only when inappropriate chemoprophylaxis or treatment is given, where there is poor adherence to therapy, an irregular drug supply or drug malabsorption.

The lack of uniformity and reproducibility in the methods used for antitubercular drugs was first noted in the late 1950s and 1960s. For isoniazid at least eight different criteria were in operation to define resistance. Significant differences were noted in the media used, the inoculum size, minimum concentrations of drug tested and the criteria used to establish resistance. Symposia organized by the WHO

and the International Union Against Tuberculosis produced agreed definitions for drug resistance and three categories of acceptable methods were defined:

1. Absolute concentration (effectively minimum inhibitory concentration).
2. The resistance ratio method.
3. The proportion method.

Detailed descriptions of these methods have been published for first line drugs and more recently for second line drugs. These documents form the bedrock of drug susceptibility testing for *M. tuberculosis* internationally.

International comparative studies in 1985 indicated that there were no significant differences between the absolute concentration and proportion methods for isoniazid, rifampicin and *para*-aminosalicylic acid, but they were not always in agreement for streptomycin, ethambutol, prothionamide (ethionamide), thiacetazone. The WHO Global Network of SRLs was established to act as a quality control network and to maintain a high level of proficiency in the diagnosis of drug resistant tuberculosis. In practice, all three principal methods perform adequately, providing that the technical protocols published are followed exactly.

The absolute concentration method

The drug is incorporated into solid agar or Lowenstein-Jensen medium as two-fold dilutions or used as a broth-dilution method. Solid media methods are more easily standardized. Resistance is defined as the lowest concentration of the drug that inhibits growth (less than 20 colonies). Drug concentrations, and particularly inoculum size, must be carefully standardized with reference to wild type cultures. Variations in inoculum size are the major source of error in this method. This conventional or agar proportion method of antimycobacterial susceptibility testing

requires growing *M. tuberculosis* on medium with and without therapeutic agents for two or three weeks of incubation before results are obtained and reported.

The resistance ratio method

This is a refinement of the absolute concentration method that controls variations in the minimal inhibitory concentration (MIC) of a given isolate when tested on different batches of drug-resistance media. It is defined as the MIC of the test isolate divided by the MIC of a standard susceptible strain such as H37Rv or by recently isolated susceptible wild-type strains. If the ratio is two or less, or eight or more, the isolate is considered to be fully sensitive or highly resistant, respectively. Intermediate or low level resistance is difficult to measure accurately. Inoculum size needs to be standardized but the critical concentration does not need to be determined because of the direct comparison with susceptible isolates.

Proportion method

In this method, the strain is classified as susceptible below a critical proportion of resistant bacteria and as resistant above it. The proportion varies with different drugs, e.g. 1% for isoniazid and rifampicin. This correlates with an effective clinical outcome. In practical terms, the proportion of drug resistant mutants comes from the ratio of the number of colonies growing in a drug-containing medium and on a drug-free medium.

Conventional methods of susceptibility testing require growing *M. tuberculosis* on medium containing therapeutic agents for 2 to 3 weeks of incubation before results are obtained. The most frequently used susceptibility testing method is the BECTEC TB-460 system, however, 4 to 12 days of incubation is still required before results are available. This method involves the measurement of $^{14}\text{CO}_2$

produced by mycobacteria growing in broth containing ^{14}C -labeled palmitic acid with or without antimycobacterial agents. Some studies reported susceptibility testing of *M. tuberculosis* could be performed rapidly by using flow cytometry. Flow cytometry has increasingly been used to perform susceptibility testing for bacteria and yeast. The main advantages are accuracy, reproducibility, sensitivity, objectivity and most importantly, speed. Results of susceptibility tests are available within 24 hours after *M. tuberculosis* organisms are incubated in the presence and absence of antimycobacterial agents. This study developed antituberculosis drugs as dry powder inhalers with the biocompatibility and biodegradable carriers. In addition, to study the susceptibility testing of dry powder inhalers using *M. bovis* because of more safety than *M. tuberculosis* (Drobniewski, 2002 ; Kirk *et al.*, 1998a ; Kirk *et al.*, 1998b ; Norden *et al.*, 1995).

7.2 Materials

BCG vaccine (Aventis Pasteur Ltd., Toronto, Ontario, Canada)

Fluorescein diacetate (Sigma chemical company, St. Louis, MO, USA)

Middlebrook 7H9 broth (Difco™, Detroit, MI, USA)

OACD enrichment (Difco™, Detroit, MI, USA)

96-Well microtitre plate (Biosigma S.r.l., Venice, Italy)

7.3 Equipment

Flow cytometer (FACSCalibur, Becton-Dickinson, Mountain View, California, USA)

Laminar flow, biohazard hood (Model Canadian Cabinets DND-DP, USA)

Microplate reader (Molecular Devices, Sunnyvale, CA, USA)

7.4 Methods

7.4.1 Drug susceptibility testing of *M. tuberculosis* by the broth microdilution method

M. tuberculosis (H37Ra) was used for this study. Culture suspensions were prepared by growing the inoculum in Middlebrook 7H9 broth containing 0.5% glycerol and 10% oleic acid, albumin, dextrose and catalase (OADC) enrichment for 21 days at 37°C, without shaking. To prepare the suspension for inoculation, the cultures were vortexed, left for 30 seconds to allow the settling of heavy particles then the suspension was diluted to reach a turbidity that matched the optical density of a Mcfarland 1.0 standard.

The broth microdilution method was performed in 96-well microtitre plates with U-shaped wells. The plates were arranged to give 12 rows by eight lanes and these were filled with 0.1 ml amounts of Middlebrook 7H9 broth, supplemented with 0.1 ml OADC enrichment. All of selected formulations [RIF-1 (A), RIF-2 (C), RIF-3 (A), INH-1 (A), INH-2 (C), INH-3 (A), INH-1 (sd), INH-3 (sd) and RIF-1 (50)] were weighed and diluted in 10% DMSO to obtain concentrations 60 µg/ml and two fold dilutions for each formulation were prepared. The standard antibiotics gradient was initial concentration of 60 µg/ml for rifampicin and isoniazid. The standard antibiotic concentrations were prepared as similar to sample drugs.

The 0.1 ml volumes of all tested formulations and standards were dispensed into plates. Plates were stored at -20°C until use. Each well was inoculated with 5 µl of 1.0 McFarland standard bacterial suspension. A well without antimycobacterial agents was also inoculated with 10⁻² dilution of 1.0 McFarland standard as a growth control. The plates were sealed, placed in plastic bags and incubated at 37°C for 21 days in a moisturized incubator.

MIC was defined as a lowest drug concentration that exhibited no growth by microplate reader, and the strains were considered susceptible to each drug, if their MICs were below or equal to the critical concentration (Coban *et al.*, 2004 ; Leite *et al.*, 2000).

7.4.2 Drug susceptibility testing of *M. bovis* by using flow cytometry

The flow cytometric susceptibility was performed as followed. Isolates of *M. bovis* are grown in 5.0 ml of Middlebrook 7H9 broth at 37°C in the presence of 5% CO₂ until the turbidity of the suspension is equivalent to McFarland 1.0 standard. Some cultures of *M. bovis* are diluted by the addition of Middlebrook 7H9 broth. Once sufficient *M. bovis* organisms is obtained, serial dilutions (volume 0.5 ml, concentration 30-0.03 µg/ml) of the selected drugs [RIF-1 (A), RIF-2 (C), RIF-3 (A), INH-1 (A), INH-2 (C), INH-3 (A), INH-1 (sd), INH-3 (sd) and RIF-1 (50)] and standard rifampicin and isoniazid were inoculated with 0.5 ml of 1 × 10⁶ *M. bovis* organisms. Drug-free suspensions of *M. bovis* organisms were also included as controls. The suspensions were then incubated for 24 hours at 37°C in the presence of 5% CO₂.

After incubation, 0.2 ml of each assay suspensions were placed in a sterile screw-cap micro-tube containing 0.2 ml of fluorescein diacetate prepared at 500 ng/ml in phosphate buffered saline at pH 7.4. Samples were then incubated at 37°C for 30 minutes before being analyzed using flow cytometer and CellQuest™ software for data acquisition and analysis. Initially, 7H9 medium alone, 7H9 medium containing unstained viable *M. bovis* cells, 7H9 medium containing viable *M. bovis* cells stained with FDA and 7H9 medium containing viable *M. bovis* cells incubated with selected drugs for 24 hours and then stained with FDA were detected and differentiated from 7H9 particles by using FDA fluorescence. Live gating was performed on profiles of *M. bovis* during data acquisition to exclude all 7H9 particles. Data were acquired for 50 seconds to obtained approximately 50,000 counts from gate events. Samples were analyzed by histogram profiles of FDA fluorescence by using CellQuest™ software. Gates establishing for viable and non-viable mycobacteria on the basis of their incorporation of FDA (Kirk *et al.*, 1998a ; Kirk *et al.*, 1998b ; Norden *et al.*, 1995).

7.5 Results and discussion

7.5.1 Drugs susceptibility testing of *M. tuberculosis* by broth microdilution method

Table 7.1 shows the MICs of selected formulations compared with standard rifampicin and isoniazid alone by broth microdilution method. The results show that the MICs of isoniazid formulations were 1.7 or 3.4 times lower than standard isoniazid alone. The MICs of rifampicin formulations are equally to standard

rifampicin alone except the formulation sprayed into antisolvent [RIF-1 (50)] is higher than standard rifampicin (about 30 times). These results may cause the stability of rifampicin in this formulation according to the stability after storage period. Other reports of the MICs against *M. tuberculosis* of isoniazid were 0.05-0.2 µg/ml by broth microdilution or agar dilution method (Kirk *et al.*, 1998a ; Mohamad *et al.*, 2004 ; Norden *et al.*, 1995). The MICs of rifampicin against *M. tuberculosis* were 0.031-0.250 µg/ml (Kirk *et al.*, 1998a ; Norden *et al.*, 1995) and MICs of rifampicin against *M. bovis* were 0.063-0.125 µg/ml depended on the source of microorganism (Rastogi *et al.*, 2000).

Table 7.1 MICs of standard rifampicin, standard isoniazid and the selected dry Powder formulations obtained from physical mixing, spray drying and spraying into antisolvent method

Formulation code	MIC (µg/ml)
standard rifampicin	0.0047
RIF-1 (A)	0.0037
RIF-2 (C)	0.0073
RIF-3 (A)	0.0018
RIF-1 (50)	0.1571
standard isoniazid	0.1
INH-1 (A)	0.0293
INH-2 (C)	0.0586
INH-3 (A)	0.0293
INH-1 (sd)	0.0586
INH-3 (sd)	0.0586

Rifampicin is a semisynthetic antibiotic derived from rifamycin. The antibiotic affects mainly gram positive bacteria. Gram negative bacteria are much less sensitive to rifampicin. Gram negative bacteria possess an outer membrane that is probably too great a barrier for the transport of rifampicin in to the cell. Rifampicin's bactericidal effect is caused by the inhibition of RNA synthesis in bacteria. The antibiotic interacts with RNA polymerase, thus inhibiting the synthesis for long RNA strands. Rifampicin's binding site lies on the β -subunit of the enzymes. Rifampicin does not interact with eukaryotic RNA polymerases. For the results of rifampicin stems from its high affinity binding to, and inhibition of, the bacterial DNA-dependent RNA polymerase (RNAP) therefore the protein synthesis of bacteria was inhibited (Campbell *et al.*, 2001 ; White *et al.*, 1971). Isoniazid is exact mechanism of action is unknown but it is thought to prevent the bacteria from making components called mycolic acids which are needed to form cell walls. It also seems to combine with an enzyme which interferes with the cell metabolism of the bacteria. As a result of the disruption in it's metabolism and without a cell wall the bacteria die (Bardou *et al.*, 1998 ; McClatchy, 1971).

Generally, sugars have antibacterial activity at high concentrations. Trehalose, mannose and lactose can be improved the antibacterial activity of rifampicin and isoniazid. These sugars may be dehydrate of mycobacteria and may be enhanced permeability of cell membrane. In the case of mannose, it acts as an antibacterial agent. Bacteria have lectins on their surfaces that bind to host cells, resulting in infection. However, mannose completes with the bacterial, occupying sites that would normally bind to host cell mannose receptors, thus preventing

attachment and thwarting possible infection. Mannose also has antiviral, antiparasitic, and antifungal properties.

7.5.2 Drug susceptibility testing of *M. bovis* by flow cytometry

The method was based on the ability of viable *M. bovis* organisms to hydrolyze FDA to free fluorescein with detection of fluorescent mycobacteria by flow cytometric analysis. It is known that FDA is nonpolar, non fluorescent molecule capable of diffusing across the cell wall and cell membranes of mycobacteria and other bacteria by active transport and passive diffusion (Norden *et al.*, 1995). Once in the cytoplasm, FDA is rapidly (5 minutes) hydrolyzed by esterases to fluorescein. Metabolically inactive and non-viable bacteria have decreased quantities of active esterases that result in these organisms demonstrating less fluorescein.

Few particles were detected in the 7H9 medium alone [Figure 7.1 (A)]. Subsequently, suspensions of 7H9 medium containing unstained viable *M. bovis* cells [Figure 7.1 (B)], 7H9 medium containing viable *M. bovis* cells stained with FDA [Figure 7.1 (C)] and 7H9 medium containing viable *M. bovis* cells incubated with isoniazid 3.0 µg/ml of isoniazid for 24 hours (are shown for example) and then stained with FDA [Figure 7.1 (D)] were used to obtain histogram profiles of the fluorescence of events in the suspensions. The 7H9 medium containing viable *M. bovis* cells incubated with other sample drugs for 24 hours and then stained with FDA were obtained histogram profile similarly with Figure 7.1 (D). Viable *M. bovis* cells were detected in the 7H9 medium although the cells were unstained. *M. bovis* organisms incubated with FDA, only viable *M. bovis* organisms were incubated with FDA, only viable *M. bovis* organisms demonstrated a fluorescent intensity, with a

mean channel fluorescence of 1538 [Figure 7.1 (C)]. Unstained *M.bovis* or non-viable *M. bovis* organisms did not hydrolyze FDA and had a mean channel fluorescence of approximately 2.68 [Figure 7.1 (B)]. When these experiments were repeated, similar results were obtained. The mean channel fluorescence correlated with viability or non-viability of mycobacteria organism (Norden *et al.*, 1995). In addition, the mean channel fluorescence was determined after 48 hours of incubation, similar results were obtained as incubated for 24 hours (Kirk *et al.*, 1998a).

Kirk *et al.* (1998a) suggested that multiplication occurred in some of assay suspensions but multiplication was not necessary to discern differences between drug-free suspensions of mycobacteria and those treated with antimycobacterial agents.

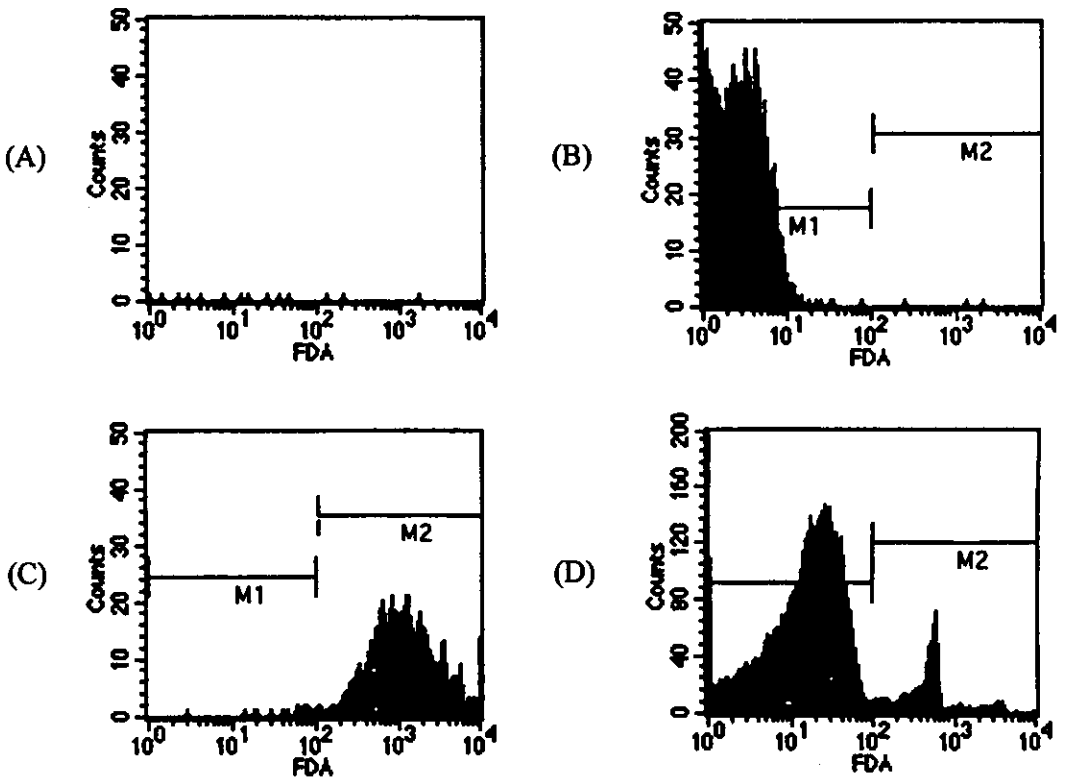


Figure 7.1 Histogram profiles of the intensity of fluorescence of the number of events (non *M. bovis* particles or *M. bovis* cells) in 7H9 medium alone (A), 7H9 medium containing unstained viable *M. bovis* cells (B), 7H9 medium containing viable *M. bovis* cells stained with fluorescein diacetate (C), and 7H9 medium containing viable *M. bovis* cells incubated with 3.0 $\mu\text{g/ml}$ of isoniazid for 24 hours and then stained with fluorescein diacetate

Figure 7.2 (A and B) shows the viable cells of *M. bovis* when incubated with rifampicin and isoniazid dry powder formulations and standard isoniazid. All concentrations of isoniazid formulations killed *M. bovis* organisms more than 99.9% of the bacterial population. The results show that these formulations killed *M. bovis* cells equally to standard isoniazid alone ($P > 0.05$) at all

concentrations. At low concentrations of rifampicin (0.03-1.5 $\mu\text{g/ml}$) viable cells of *M. bovis* organism of all formulations are lower than standard rifampicin ($P < 0.05$) because the dry powder formulations were kill mycobacteria more than rifampicin alone. At higher concentrations (3-30 $\mu\text{g/ml}$) the viable cells of *M. bovis* incubated with these formulations are not different of the viable cells ($P < 0.05$).

Generally, oral dose of rifampicin and isoniazid are not equal. Dose of rifampicin is 10 mg/kg daily (maximum dose is 600 mg daily) but dose of isoniazid is 5 mg/kg daily (maximum dose is 300 mg daily). Isoniazid is more potent than rifampicin (Raynolds, 1993). In this study, the concentrations of both drugs are equal, therefore the viable cell of *M. bovis* when incubated with isoniazid were lower than incubated with rifampicin.

According to this study, isoniazid dry powder formulations have better therapeutic effects than isonizid alone. Therefore, isoniazid dry powder inhalers may destroy *M. tuberculosis* in the lungs of TB patients to a satisfied level. The antibacterial activity of these formulations is similar with broth microdilution method.

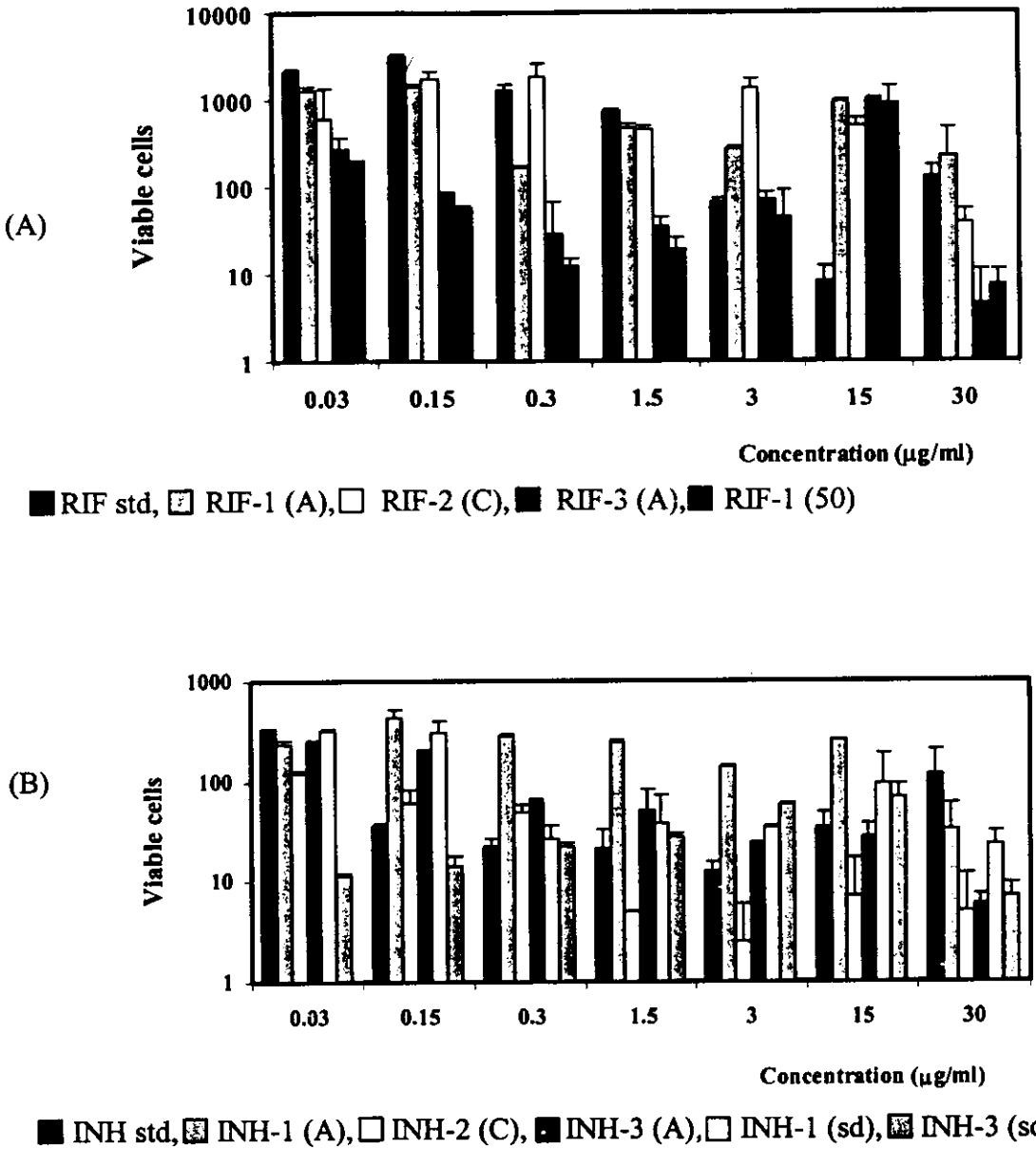


Figure 7.2 Viable *M. bovis* cells after incubated with atandard rifampicin and rifampicin dry powder formulations (A), standard isoniazid and isoniazid dry powder formulations (B), all drugs were incubated for 24 hours and then stained with fluorescein diacetate and detected by flow cytometry