CHAPTER II

LITERATURE REVIEWS

Tuberculosis

In 1882, Robert Koch was the first to establish the causal relationship between the tubercle bacillus and the disease tuberculosis (TB). The organism was initially named *Bacterium tuberculosis* by Zoppf in 1883, an epithet later changed to *Mycobacterium tuberculosis* by Lehmann and Neumann in 1886, presumably because it resembled a fungus in its slow growth and colony morphology (32-35). One-third of the world's population has been in contact with *M. tuberculosis*, but not all develop the disease. Within 2 years, 5-10% will have active TB; these individuals have usually been exposed to a high inoculum, for instance by repeated contact with an active TB patient. The remaining 90-95% will not develop TB unless they are subjected to a severe stress factor, such as immunodeficiency, human immunodeficiency virus (HIV) or malnutrition (36). Patients with HIV have a 10 to 15% risk per year for progression to infection (4).

Tuberculosis incidence and mortality

Tuberculosis remains a major global public health problem in many countries. TB morbidity and mortality are presented for the decade 1990-99 by World Health Organization (WHO). An estimated 88 million new cases of TB, of which 8 million will be attributable to HIV infection, will occur in the world during the decade; 30 million people are predicted to die of TB in the same period, including 2.9 million attributable to HIV infection. The number of new TB cases occurring each year is predicted to increase from 7.5 million in 1990 to 8.8 million in 1995 and 10.2 million in the year 2000. In 1990, 2.5 million persons were estimated to have died of TB; at

the same level of availability of treatment, it is predicted that 3.0 million TB deaths will occur in 1995 and 3.5 million in 2000 (4).

Chemotherapy for tuberculosis

In 1943, Schatz, Bugie and Waksman discovered streptomycin (S). Unfortunately, the use of S alone was impaired by the emergence of S-resistant mutants of *M. tuberculosis* in the sputum of patients with cavitary pulmonary TB, which results in treatment failure. In 1946 and 1951, paraamino-salicylic acid (P) and isoniazid (H) respectively, became available as drugs active against *M. tuberculosis*. Used in combination the three drugs prevented the selection of drug-resistant organisms in the great majority of cases because each drug was active against the mutants resistant to other drugs. For chemotherapy to be a success, it must not only prevent drug resistance, but also kill all, or almost all, of the drug-susceptible organism, to prevent their subsequent multiplication and a resultant relapse after causation of treatment. Because a small number of drug-susceptible organisms survived despite chemotherapy with the three-drug combination, 18-24 months of drug administration were required to permit the host defense to eliminate persisting organisms (37). Ethambutol (E) was introduced by Lederle Laboratories in 1961, and in 1966 was recommended in a leading article in tubercle for practical use (38). Rifampin, introduced in 1968 (13,39), was demonstrated effective not only against actively multiplying organisms, but also against the remaining or persisting organisms responsible for relapses after treatment completion. In combination with S, H and R effected the cure of patients in a treatment period of 9 months. The addition of pyrazinamide (Z) was shown to have considerable anti-*M. tuberculosis* activity in 1952, but it became an important component of short-course chemotherapy only in the mid-1980s. Pyrazinamide, active against organisms located in acid environment, to the S H R combination further increased the effectiveness of chemotherapy, and resulted in 6 months course of chemotherapy, with the 4 drugs combination S H R Z, that is becoming routine practice in a majority of countries (37). In Thailand, treatment regimens for TB patients is revised in 1996 to be in line with WHO. TB is

treated with an initial intensive 2-month regime comprising multiple antibiotics R H Z and E or S to ensure that mutants resistant to a single drug do not emerge. The next 4 months, only R and H are administered to eliminate any persisting tubercle bacilli (Table 1) (9).

Table 1. Treatment regimens

Category	Regimen	Indication
1	2HRZE(S)/4HR	New smear positive
		Severe smear negative
		Severe extrapulmonary
2	2HRZES/1HRZE/5HRE	Relapse and failure
3	2HRZ/4HR	New smear negative
4	Second line drugs	Chronic cases

Mechanism of drug resistance

In the tubercle bacilli, drug resistance appears to be chromosomal in origin, caused by specific mutations that occur independent genes (Table 2) (40). Natural resistance is a species-specific resistance which can be used as a taxonomic marker useful in species identification. For example, *M. bovis* is naturally resistant to pyrazinamide, and drug susceptibility is one test used to differentiate *M. bovis* from *M. tuberculosis* (7,41). Meanwhile, wild type resistant mutants occur as a result of genetic mutations that precede contact with the drug. These spontaneous mutants are found in wild *M. tuberculosis* strains with frequencies which vary for different drugs (15). The frequency of drug-resistance mutations for H and S is one in 1 million (10^6) organisms. The rate for rifampin is 10^{-8} (12), and for ethambutol 10^{-4}. Consequently, the probability of a mutation is directly proportional to the bacterial load. A bacillary load of 10^9 will contain several mutants resistant to any one antituberculous drug.

Resistance to the various anti-TB drugs occurs independently; therefore, the chance of an isolate having wild-type resistance to two drugs is the product of the individual probabilities (40). Drug-resistant mutants exist at different frequencies for given drugs and have a selective advantage in the setting of monotherapy as they are able to survive therapy while the drug susceptible bacilli are killed. When two drugs are included in the treatment, mutants resistant to one drug are killed by the other drug (15).

Table 2. Gene targets for anti-tuberculosis drugs and mutations associated with drugs resistance

Drug	Probable targets	mutation in gene conferring resistance	Function of gene
Rifampin	RNA synthesis	*rpoB*	DNA-dependent RNA polymerase (β subunit)
Isoniazid	Mycolic acid biosynthesis	*katG*	Catalase/peroxidase
		inhA/mabA	Fatty acid biosynthesis
		ahpC	Alkyl hydroperoxide C reductase
		oxyR	oxidative stress regulator
Pyrazinamide	pyrazinamide (Pzase)+?	*pncA*	Pzase nicotinamidase ; loss of Pzase gives resistance

Table 2. (cont.) Gene targets for anti-tuberculosis drugs and mutations associated with drugs resistance

Drug	Probable targets	mutation in gene conferring resistance	Function of gene
Streptomycin	Protein synthesis	*rrs*	16S rRNA
		rpsL	Ribosomal protein S12
Ethambutol	Cell wall synthesis	*embA, B, C*	Lipoarabinomannan and arabinogalactan synthesis
Ethionamide	Cell wall synthesis	*inhA* + ?	Cross-resistance associated with *inhA* mutations
Ciprofloxacin	DNA synthesis	*gyrA, gyrB, nor*	DNA gyrase subunit A and B (principally)

Rifampin resistance

Rifampin, or rifampicin, is 3,4-(methylpiperazinyliminomethylidene)-rifamycin SV (12,13,39). It was introduced in 1968 as a broad-spectrum antibiotic which, along with isoniazid, is central to current tuberculosis therapy. Rifampin is rapidly bactericidal against *M. tuberculosis* and its use has greatly shortened the duration of chemotherapy necessary for the successful treatment of drug-susceptible tuberculosis. High-level resistance to rifampin occurs at a rate of 10^{-8} in *M. tuberculosis* in vitro and is thought to be a one-step mutational event (11-13). Rifampin is well established in other bacteria that rifampin inhibits RNA synthesis by it interaction with the β subunit of the RNA polymerase molecule (42-43). Point mutations with the *rpoB* gene, encoding the β subunit, confer resistance to rifampin (43). The *rpoB*

was characterized and mutations conferring the resistant trait were identified (14,20,21,24). Most mutations were determined to be restricted to an 81-bps core region and are dominated by single nucleotide changes, resulting in single amino acid substitutions, although inframe deletions and insertions also occur at lower frequencies. Changes in the codons Ser-531 and His-526 have been documented in more than 70% of the rifampin-resistant isolates. A very small number of mutations in rifampin-resistant isolates do not map in this 81-bp core region; it is speculated that additional mechanisms, including rifampin permeability and mutations in alternate subunits of RNA polymerase, may also be involved in conferring the resistance phenotype. The consistency of mutations in the *rpoB* locus and the rifampin-resistant phenotype (>95%) has marked clinical implications. Because it may act as a surrogate marker for MDR-TB, rifampin resistance has prompted development of various diagnostic tests to improve the susceptibility of mutation detection (44). Although automate sequencing has been unambiguously applied to characterize mutations associated with rifampin resistance, a number of other techniques such as PCR-SSCP (20,24,28,45-47), dideoxy fingerprinting (6), PCR heteroduplex analysis (14,25-26), and line probe hybridization (15,27,29,48) have been successfully applied to detecting these mutations. In Thailand, Vattanaviboon et al. (23) determined the sequence alteration in the central region of *rpoB* gene in three different phenotypes of rifampin resistant *M. tuberculosis* isolated from TB patients. The results showed different mutations within codon 511-533 in all these drug resistant isolates.

Epidemiology of drug resistance

Primary resistance is defined as the presence of resistance to one or more antituberculosis drug resistance in a tuberculosis patient who has never received prior treatment. Acquired resistance is defined as resistance to one or more antituberculosis drugs, which occurs during the course of treatment, usually as a result of nonadherence to the recommended regimen or incorrect prescribing.

The World Health Organization-International Union against Tuberculosis and lung Disease Working Group on Anti-Tuberculosis Drug Resistance Surveillance, report data on drug resistance in 35 countries between 1994 and 1997. Among patients with no prior treatment, a median of 9.9 percent of *M. tuberculosis* strains were resistance to at least one drug (range, 2 to 41 percent); resistance to isoniazid (7.3 percent) or streptomycin (6.5 percent) was more common than resistant to rifampin (1.8 percent) or ethambutol (1.0 percent). The prevalence of primary multidrug resistance was 1.4 percent (range, 0 to 14.4 percent). Among patients with histories of treatment for one month or less, the prevalence of resistance to any at the four drugs was 36.0 percent (range, 5.3 to 100 percent), and the prevalence of multidrug resistance was 13 percent (range, 0 to 54 percent). The overall prevalences were 12.6 percent for single-drug resistance (range, 2.3 to 42.4 percent) and 2.2 percent for multidrug resistance (range, 0 to 22.1 percent). Particularly high prevalences of multidrug resistance were found in the former Soviet Union, Asia, The Dominican Republic, and Argentina. Resistance to antituberculosis drugs was found in all 35 countries and regions surveyed, suggesting that it is a global problem. Most countries affected by the HIV pandemic and increases in TB also have poorly functioning TB-control programs and cannot afford the antituberculosis-drug regimens that are most effective at preventing multidrug-resistant disease as well as treating it. Thus, the stage is being set for a substantial increase in the incidence of drug-resistant TB in many countries. If this is allowed to occur, the developing countries will not be the only ones affected. International travel, migration between countries, and trade with emerging economies continue to increase, and the proportion of TB cases in developed countries that originate in developing countries has been increasing as well (49).

Tuberculosis has recently become a very significant reemerging disease in Thailand again after considerable decline to some extents during the previous decade. TB was fifth-ranked among over all mortality rates reported in 1995 (9). The annual risk of infection in 1997 is estimated to be 1.40% while approximately 100,000 new TB patients of which 50,000 cases are smear positive develop each year. Among

these only 40% of total new TB patients and 50-60% of new smear positive patients are diagnosed for treatment. The most recent TB mortality in 1994 was 5.8 per 100,000 population or 3,473 patients (9). Drug Resistance Surveillance project has been conducted in Thailand, covering 46 provinces, since 1996. The preliminary report revealed overall figure of MDR-TB of 3.15% and monoresistance to rifampin = 6.1%, isoniazid=5.7%, ethambutal=1.02%, and streptomycin=7.3% in 1998 (10).

Laboratory diagnosis of drug resistance

Measuring the drug susceptibility of isolates has three main purposes. Firstly, it confirms the efficacy of an empirical treatment regimen in a given individual, or permits the tailoring of treatment in the case of drug resistance. Secondly, it enables medical and public health services to plan appropriate empirical regimens to address problems of emerging drug resistance. Thirdly, it is a useful crude indicator of the efficacy of a treatment programme. For example, poor case handing and compliance lead to increasing levels of acquired drug resistance (40).

Phenotypic methods

Culture-based phenotypic method, in which the survival of a test strain in comparison with a drug-susceptible control is assayed on a solid or liquid medium. Four methods have been described. These include the resistance ratio method, the absolute concentration method, the proportion method and the radiometric or BACTEC method. Testing may be direct or indirect, i.e. performed on the original specimen or on a subculture, respectively.

1. The resistance-ratio (RR) method

A description of this method in the first WHO report (50) was given by D. A. Mitchison, with more details offered in the second WHO report (16). The method is described for use in Lowenstein-Jensen (L-J) medium without potato

starch. The RR is defined as a ratio of the minimal inhibitory concentration (MIC) for a tested strain to the MIC of the same drug for H37Rv strain. The description of the RR method includes details of preparation of inoculum that ensures that the suspension contains mostly viable bacteria. Reading after 4 weeks of incubation defines "growth" on any slope as the presence of 20 or more colonies. Though not specified in the above quoted reports, MIC was probably defined as the lowest drug concentration in the presence of which the number of colonies was less than 20 to 0. The range required for the test strain is determine a resistance ratio of 2 or less for susceptible strains and a resistance ratio of 8 or more for resistant strains (38).

2. The absolute concentration method

The description of this method for the first WHO (50) report was prepared by G. Meissner. For this susceptibility test, bacterial suspensions are adjusted by the optical standard to have the turbidity equivalent to 1 mg/ml of wet weight, and diluted 1:50, which gives about $2x10^5$ to 10^6 bacteria per ml. The actual inoculum per tube made with a loop should contain $5x10^3$ to 10^4 bacteria. The drug-containing cultures and two drug-free controls are incubated at $37^{O}C$ for 5 to 6 weeks if the growth is insufficient at the 4 weeks reading. The inhibition of growth is reported if the number of colonies is less than 20 with confluent growth in the drug-free controls. It is assumed that the growth of at least 1% of the bacterial population is clinically significant to consider the isolate resistant, and appearance of 20 or more colonies on the drug-containing L-J slopes is indicative of resistance if the growth on the drug-free medium shows that the inoculum was sufficient for such judgment (38).

3. The proportion method

This method was described in the first WHO report by G. Canetti and J. Grosset (50) and in the second by G. Canetti and N. Rist in collaboration with J. Crosset(16). This origin proportion method was develop for L-J medium without potato starch. The inocular are prepared from a well-dispersed bacterial suspension

adjusted to the optical density of a standard suspension containing 1mg/ml wet weight of tubercle bacilli; 10^{-3} and 10^{-5} dilutions are inoculated in two sets of drug-containing and drug-free tubes, 0.1 ml per each slop. After 28 days of incubation, the colonies on the drug-containing and drug-free slopes are counted to calculate the ratio between them, which indicates the proportion of resistant bacteria (38). For R, 1% is the criterion for resistance using this technique. That is to say that if 1 out of every 100 bacilli being tested is able to grow above a certain concentration of drug in the growing medium the strain is said to be resistant. For R, this concentration is defined as 40 μg/ml (7).

In the United States, the proportion method is performed most frequently using Middlebrook agar medium. The advantage of performing test in 7H10 agar plates is that the final results can be reported within 3 weeks instead of the 4 to 6 weeks or more required by the original method in L-J medium. For R, this concentration is defined as 1.0 μg/ml. This critical concentration is different from that used in L-J medium in particular because of the deterioration of drugs by inspissation L-J medium, and possibly by the difference in protein content (38). The critical proportion of bacteria in the population designating the strain "resistant" is 1% rather than 10%.

4. Radiometric (BACTEC) method

The isolation of *M. tuberculosis* from the patient's specimen followed by an indirect drug susceptibility test is a long process when conventional method are employed; it takes at least 7 to 8 weeks in 7H10 agar plates and up to 3 months when L-J is the medium of choice. Only if the direct test is successful can the time be reduced to a 3-or4-week period. The need for rapid methods in mycobacteriology was emphasized in the first WHO report. Attempts to develop a rapid method, particularly by using radiometric techniques, were made in the late 1960s and early 1970s (38). A major advancement occurred in 1977 when a radiometric system using 7H12 broth containing ^{14}C-labeled palmitic acid as a source of carbon was introduced

(51). The BACTEC procedure for drug susceptibility testing of *M. tuberculosis* is based on the modified proportion method (16). The critical proportion for resistance is taken as 1% for all antituberculosis drugs. The number of organisms added to the control medium (without antimicrobial agent) is 100-fold less than that added to medium containing antimicrobial agent. The inoculated vials are incubated and subsequently read daily, and the results are interpreted by evaluating the rate of increase in the growth index (GI) of the control compared with the rate of increase in the GI in the antimycobacterial agent is less than that in the control, the isolate is considered susceptible; if it is greater than increase in the control, the isolate is considered resistance at the 1% level. Several published studies have reported that the radiometric method correlated at a 90-98% level with the standard proportion indirect test method for isoniazid, rifampin, streptomycin, and ethambutol (52,53). Results were generally available in 4-5 days for the radiometric method and the routine plate method required 21 days. A study which compared recovery rates and drug susceptibility results for the radiometric method and conventional methodology has been carried out (18). For this investigation, 463 acid-fast smear positive specimens were inoculated onto solid media and into 7H12. Average detection times for *M. tuberculosis* were 8.3 days for the radiometric method and 19.4 days for the conventional method. Drug susceptibility results were in 95% agreement and reporting times were 4.2-6.9 days (radiometric) and 13.7-21 days (conventional). An average of 18 days was required for complete recovery and susceptibility testing using the BACTEC method and was 38.5 days of the conventional proportion method using 7H10 or 7H11 agar. The radiometric method has also been favourably compared with the proportion method using L-J medium and the absolute concentration and resistance ratio drug susceptibility testing methods (17) for isoniazid, streptomycin, and rifampin (54).

The BACTEC radiometric antimicrobial susceptibility test is a rapid method based on the principal used for primary isolation of mycobacteria. Mycobacteria are inoculated into vials of BACTEC 12 B (Middlebrook 7H12) medium with and without antimycobacterial agents and incubate the vials at 37^{O}C in

the dark. The bottles are read once each day on the BACTEC 460. In the absence of antimycobacterial inhibition the mycobacteria grow and utilize ^{14}C-labeled palmitic acid as a growth substrate present in the medium. The numbers generated are expressed in terms of GIs, which represent the quantity of $^{14}CO_2$ produced. The GIs are directly proportional to the amount of growth of mycobacteria in the medium. Susceptibility testing of *M. tuberculosis* can be performed by using either a direct or an indirect procedure. In the direct test, a smear-positive specimen is inoculated directly into the antimicrobial agent-containing medium and a control. In the indirect test, a pure culture of organisms grown on primary isolation medium is tested. Generally, results of the direct test are available 3 to 4 weeks earlier than results of the indirect test (55).

Genotypic methods

Genotypic assays combine three main steps: (a) sample preparation to release DNA from mycobacterial cells; (b) amplification of the relevant part of the gene, usually by the polymerase chain reaction (PCR); and (c) detection of the mutation in comparison with the equivalent region of a wild-type strain. Methods which have been used for genotypic susceptibility testing (40).

1. DNA sequencing

DNA sequencing remains the gold standard, as all mutations will be detected. Manual DNA sequencing requires a high level of technical competency and employs a radioactive material, which must be safely disposed of, making the technique unsuitable for busy clinical laboratories receiving hundreds of specimens. Automated sequencers that use fluorescent-based chemistry can provide accurate sequence data within 24-48 hr, but are costly, relatively expensive to maintain, and required a high degree of operator expertise for consistently reliable data. In the case of rifampin, the presence of mutations appears to be fully predictive of resistance; however, this is not necessarily the case with other genes associated with drug

resistance, as silent mutations that do not lead to any significant change in phenotype also occur (14,20,21,24).

2. Heteroduplex formation analysis (HDF)

In this method, amplified DNA from test organisms and reference drug-susceptible strains is mixed, denatured and cooled to produce double-stranded hybrid DNAs through complementary base pairing. In regions where mutations occur, e.g., in a drug-resistant test strain, there will be a mismatch of the two strands and the resulting heteroduplex will run with a different mobility to the homoduplex (where the test strain has no mutations) on a nondenaturing electrophoresis gel system. This method has been applied to the detection of rifampin resistance (14,25-26). However, HDF require long electrophoresis steps under highly controlled conditions and also require expertise to perform and interpret in order to ensure reproducibility can be very similar to wild type patterns (56).

3. PCR single-strand conformation polymorphism (PCR-SSCP) analysis

Single-stranded DNA folds into a complex tertiary structure whose shape is determined by the DNA sequence. If any two single strands of DNA differ by one or more bases, i.e., a mutation is present, they will fold into structures with different mobility on a nondenaturing polyacrylamide gel. This is termed a single-strand conformation polymorphism (SSCP). In conjunction with PCR used to amplify the region of the gene of interest, SSCP analysis has been used to detect rifampin (20,24,28,45-47). Normally the method uses a radioactive detection system, but silver staining has been described recently as an alternative and susceptible method. Automation of the process permits a higher throughput of specimens, but increases the cost as an automated sequencer is required to detect migration of the bands (24). However, SSCP may not offer the level of mutation discrimination needed for reliable differentiation of rifampin-resistant strains from susceptible strains with functionally silent sequence changes. The sensitive of SSCP varies with the sequence content of

the DNA strands, position of the mutation within the fragment, temperature, and salt conditions (57-59). Additionally, the sensitivity of SSCP is influenced by the size of the DNA fragment, a feature that may not be modifiable for some assays (6).

4. Dideoxy fingerprinting (ddF)

Dideoxy fingerprinting (ddF), a hybrid between dideoxy sequencing and SSCP that can detect the presence of single base and other sequence changes in PCR-amplified segments. As implemented herein, ddF involves a Sanger sequencing reaction with one dideoxynucleotide followed by nondenaturing gel electrophoresis. Dideoxy fingerprinting provides information about the location of the sequence change, and the efficiency of detection is in dependent of the length of the amplified product. In ddF, procedure, a ladder of bands is generated by performing one of the four standard dideoxy sequencing reactions and resolving the products by electrophoresis on a nondenaturing polyacrylamide gel (59). Felmlee et al. (6) described the use of ddF as a postamplification screening method to identify rifampin-resistant genotype. The results of this analysis were concordant with DNA sequence analysis and conventional clinical laboratory method. ddF analysis does have advantage over DNA cycle sequencing in a clinical setting. Because of the fingerprinting patterns defined for each mutation cannot rule out the possibility of two different mutations generating the same fingerprint.

5. Solid-phase hybridisation analysis

Mutations can be detected by solid-phase hybridisation analysis. For example, in order to detect any mutations which may encode rifampin resistance, a series of overlapping probes that are complementary to the 81-bp regions of the *rpoB* gene are immobilised on to a solid membrane support. In addition, probes complementary to the most common rifampin-resistance mutations can be included. The *rpoB* gene fragment is amplified from a test strain by PCR and then reverse hybridised to the membrane containing the probes (15,27,29,48). Such a system, the

Line Probe Assay (Innogenetics, Ghent, Belgium) has been used with some success (15,27,29,48). This assay includes probes for four mutations, including the two commonest (His 526→Tyr and Ser 531→Leu), but the ability to identify less frequent mutations correctly is unknown. It is technically much simpler than SSCP and HDF, remains relatively expensive, and quickly provides results. However, because insertions, deletions, and mutations in external regions cannot be detected with the Kit, negative results with the line probe assay kit do not rule out rifampin resistance (15).