CHAPTER III

LITERATURE REVIEW

Mycobacterium tuberculosis (MTB) and the Mechanism of Resistance to Fluoroquinolones (FQs)

FQs as antimycobacterial agents were first described in 1984 and have primarily been used as therapeutic alternatives in MDRTB cases (40). DNA gyrase (Gyr) is the primary target for FQ action (41). Gyr introduces negative supercoils in closed circular DNA molecules (42-43). Quinolone sensitivity is determined by the GyrA protein, which contains the clevage/religation activity (43).

FQs, synthetic derivatives of nalidixic acid, act by inhibiting DNA supercoiling and relaxation activity of Gyr (44). Gyr catalyzes the cutting of DNA, denaturation of the overhang, and strand separation as shown in Fig 1.

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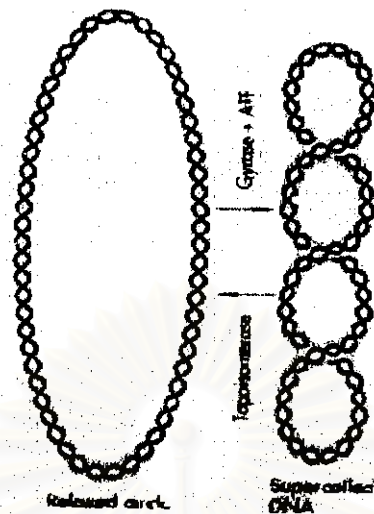

Fig 1. Gyrase action (45)

The exact mechanism of inhibition of Gyr activity with respect to quinolones remains unknown. Consequently, the quinolones may inhibit religation, thereby imposing an effective transcriptional block (46), culminating in cellular death. However, questions about the specific interaction of quinolones and the Gyr/DNA complex remain unsolved (12).

Cloning and expression of the MTB *gyr*A and *gyr*B genes allowed mapping of mutations that confer resistance to FQs (47) as shown in Table 1 along with those of other drugs.

Drug	**Gene**	**Product**	**Reported frequency in resistant strains (%)**
Rifampicin	*rpo*B	B-subunit of RNA pol	>95
Isoniazid	*kat*G	Catalase-peroxidase	60-70
	*Oxy*R-*ahp*C	Alky hydro-reductase	~20
INH-Ethionamide	*inh*A	Enoyl-ACP reductase	<10
Streptomycin	*rps*L	Ribosomal prot. S12	60
	rrs	16S rRNA	<10
Fluoroquinolone	***gyr*A**	**DNA gyrase**	**>90**
Pyrazinamide	*pnc*A	Amidase	70-100
Ethambutol	*emb*CAB	EmbCAB	69

Table 1. Gene loci involved in conferring drug-resistance in MTB (49)

Mutations were found to be clustered in a small region in GyrA, approximately 40 residues amino-terminal that is close to the active site tyrosine, Tyr 122 (*E. coli* numbering) (48). Other single amino substitutions, for residues 88 to 94, were also identified in ciprofloxacin-resistant MTB isolates, as shown in Fig 2 (12,49). Because polymorphism encountered at codon 95 (Ser 95> Thr 95) occurred in both resistant and susceptible isolates, it may not be involved in acquiring the FQ-resistant phenotype.

88	89	90	91	92	93	94	95
GGC	GAC	GCG	TCG	ATC	TAC	GAC	AGC
TGC		**GTG**	**CCG**			**AAC**	ACC
						GGC	
						GCC	
						TAC	
						CAC	

Fig 2. Single-amino acid substitutions responsible for conferring resistance to fluoroquinolones in *gyr*A gene (12,49)

Codon 88, 90, 91,and 94 (boldface type), were found to be associated with ciprofloxacin resistance. Codon 95 may display a serine or a threonine in ciprofloxacin-susceptible strains.

It is necessary for FQ susceptibility testing in the patients to prevent the selection and grow up of FQ-resistant strains (50).

The Studies about Mutation in *gyr*A and *gyr*B in *M. tuberculosis*

DNA gyrase is the target of a number of antibacterial agents. The best known of these are the quinolone and coumarin groups of drugs, which have been shown to inhibit the DNA supercoiling reaction *in vitro* (41,43,51).

Coumarins are inhibitors of the ATP hydrolysis and DNA supercoiling reactions catalysed by DNA gyrase. Their target is the B subunit of gyrase (GyrB), encoded by the *gyr*B gene. The exact mode and site of action of the drugs is unknown (46). In 1992, Asuncion

Contreras and Anthony Maxwell identified four mutations conferring coumarin resistance to *E. coli* : Arg136 to Cys, His or Ser and Gly164 to Val (52), and descussed the significance of Arg136 and Gly164 in relation to the notion that coumarin drugs act as competitive inhibitors of the ATPase reaction.

In 1994, Takiff and his colleages (12) investigated the frequency and mechanisms of fluoroquinolone resistance in *M. tuberculosis*, that were cloned and sequenced the wild-type *gyr*A and *gyr*B genes. On the basis of the sequence information, they performed DNA amplification for sequencing and single-strand conformation polymorphism analysis (SSCP) to examine the presumed quinolone resistance region of *gyr*A and *gyr*B from reference strain (n=4) and clinical isolates (n=55). Mutations in codons of *gyr*A analogous to those described in other fluoroquinolone-resistant bacteria were identified in all isolates (n=14) for which the ciprofloxacin MIC was >2 μg/ml, a level which appears to be useful in the evaluation of clinical isolates and which has been proposed as a cutoff for clinical resistance (53) : Ala90 to Val (n=3), Ser91 to Pro (n=1), Asp 94 to His (n=1), Asp 94 to Asn (n=1), Asp 94 to Gly (n=5), Asp 94 to Tyr (n=2), Asp 94 to Ala (n=1).

In 1994, Cambau and his partners (21) studied a strain of *M. tuberculosis* resistant to ofloxacin, was selected in a patient with a long history of multidrug-resistant tuberculosis eventually treated by ofloxacin combined with other second-line drugs. A mutation in the *gyr*A gene was hypothesized to be the mechanism of acquired resistance

to ofloxacin in this strain. Chromosomal DNA of strains MTB1, isolated before treatment and susceptible to ofloxacin (MIC, 1μg/ml), and MTB2, isolated during treatment and resistant to ofloxacin (MIC, 32μg/ml), was amplified by polymerase chain reaction (PCR) using two oligonucleotide primers highly homologous to DNA sequences flanking the quinolone resistance-determining region (QRDR) in *gyr*A of mycobacteria. Comparison of the nucleotide sequences of the PCR product (by DNA sequencing) revealed a point mutation in MTB2 leading to the substitution of histidine for aspartic acid (Asp87 to His) at a position corresponding to residues involved in quinolone resistance in *E. coli.*

In 1995, Alangaden et. al (29) tried to examine the mechanism of resistance to fluoroquinolones in *M. tuberculosis*, they selected spontaneous fluoroquinolone-resistant mutants from a susceptible strain, H37Rv, and studied the susceptibilities of these mutants and two fluoroquinolone-resistant clinical isolates (A-382, A-564) to various fluoroquinolones and to isoniazid and rifampin. Furthermore, since mutations within the quinolone resistance-determining region of the structural gene encoding the A subunit of DNA gyrase are the most common mechanism of acquired resistance, they amplified this region by PCR and compared the nucleotide sequences of the fluoroquinolone-resistant strains with that of the susceptible strain. Fluoroquinolone-resistant mutants of H37Rv appeared at frequencies of $2x10^{-6}$ to $1x10^{-8}$. For three mutants selected on ciprofloxacin, ofloxacin, and sparfloxacin, respectively, and the two clinical isolates, MICs of ciprofloxacin and

ofloxacin were as high as 16 μg/ml, and those of sparfloxacin were 4 to 8 μg/ml. They displayed cross-resistance to all fluoroquinolones tested but not to isoniazid or rifampin. After sequencing, all of the fluoroquinolone-resistant strains had mutations in the quinolone resistance-determining region which led to substitution of the Asp residue at position 87 (Asp87) by Asn or Ala or the substitution of the Ala83 by Val in the A subunit of DNA gyrase.

In 1996, Chen Xu et. al (54) were interested in fluoroquinolones because fluoroquinolones are potent antibacterial agents being used clinically against multidrug-resistant tuberculosis. Treatment failure is thought to arise from acquisition of fluoroquinolone resistance by *M. tuberculosis*. So they collected 13 resistant clinical isolates of *M. tuberculosis* and examined for ciprofloxacin sensitivity relative to controls. For ciprofloxacin-resistant isolates, they founded the specific alleles were associated with distinct levels of drug susceptibility for 11 isolates that contained nucleotide changes expected to alter the amino acid sequence of the A subunit of DNA gyrase. Five different *gyr*A (ciprofloxacin resistance) alleles were presented; Ala90 toVal (n=2), Asp94 to His (n=1), Asp94 to Asn (n=3), Asp94 to Tyr (n=3), Asp94 to Gly (n=2). The rest two isolates lacked a mutation in the quinolone-resistance region of *gyr*A.

Rapid Automated Susceptibility Testing Method (BACTEC)

There are three so-called conventional methods employing the critical concentrations to determine whether a *Mycobacterium tuberculosis* strain is susceptible or resistant: (a) the proportion method, (b) the resistance ratio (RR) method, and (c) the absolute concentration method. These methods were described in publications by the WHO panel (55-56). When conventional methods are employed, it takes a long process, at least 7 to 8 weeks in 7H10 agar plates and up to 3 months when Lowenstein-Jensen is the medium of choice (57).

The need for rapid methods in mycobacteriology was emphasized in the first WHO report (56). Attempts to develop a rapid method, particularly by using radiometric techniques, were made in the late 1960s and early 1970s (58-62). Developing a technique for automated detection of metabolism of bacteria by measuring of $^{14}CO_2$ liberated during the decarboxylation of ^{14}C labelled substrates present in the medium.

This technique has been applied successfully to blood culturing, detection of antibiotic effect on bacterial growth, *Neisseria* sp. differentiation by substrate metabolism, and serum assay of aminoglycoside antibiotics. Cummings and co-workers carried out preliminary work that showed the same principle could be applied to detect growth of *Mycobacterium tuberculosis* (62). A major advancement occured in 1977 when a radiometric system using 7H12

broth containing ^{14}C-labelled palmitic acid as a source of carbon was introduced (63).

A cooperative study by five institutions show that the overall mean time required for the radiometric technology (BACTEC), including isolation and indirect drug susceptibility test, was 18 days vs. 38.5 days by conventional methods. Several published studies have reported that results obtained by the BACTEC method compared well with the conventional proportion method (employing 7H10/7H11 media) or the resistance ratio method (employing LJ egg medium). The accuracy and reproducibility of the BACTEC method has also been evaluated, with excellent results. It's the significant time saving in the primary isolation of mycobacteria from clinical specimens using the new radiometric medium (64-70).

For the principles, the BACTEC TB medium (12B) is an enriched Middlebrook 7H9 broth base. Mycobacteria utilize a ^{14}C labelled substrate (fatty acid) present in the medium and release $^{14}CO_2$ into the atmosphere above the medium. When the 7H12 medium vials with growth are tested on the BACTEC 460 instrument, the $^{14}CO_2$ is aspirated from the vial and its radioactivity is determined quantitatively in terms of numbers on a scale from 0 to 999. These numbers are designated as the Growth Index (GI). The daily increase in the GI output is directly proportional to the rate and amount of growth in the medium. If an inhibitory agent is introduced into the medium, inhibition of metabolism is indicated by reduced production of $^{14}CO_2$ when compared to a control

having no inhibitory agent. This basic principle is applied for drug susceptibility testing.

To determine the 1% proportion of resistance, the bacterial inoculum used in the control vial is one-hundred fold less than that used for the drug-containing vials. The drug and control vials are tested daily after inoculation. The rate of increase in the GI, or the amount of change over that of the previous day, called delta (Δ) GI, is compared for the control vial and the vials containing drugs. If the daily GI increase in the drug vial is equal to or greater than that in the control vial, the test organisms are considered resistant to the drug. For a susceptible population, the daily GI increase for the control would be higher than that of the drug vial. For example, if 1% of the mycobacterial polulation is resistant to isoniazid (INH), then 99% of the organisms would be inhibited by INH and only 1% will grow in the drug vial. The growth rate in the drug vial would be similar to the growth rate in the control vial in which the original bacterial inoculum was only 1/100 of that in the drug vial. Thus, instead of counting colonies in the control and the drug medium, the GI values are utilized to determine susceptibility results (98).

DNA sequencing (Dideoxy sequencing)

The dideoxy or enzymatic method as originally developed by F. Sanger (71) utilizes *E. coli* DNA polymerase I to synthesize a complementary copy of a single-stranded DNA template. After primer is

annealed to DNA template, the deoxynucleotide added to the growing chain is selected by base-pair matching to the template DNA. Chain growth involves the formation of a phosphodiester bridge between the 3′-hydroxyl group at the growing end of the primer and the 5′- phosphate group of the incoming deoxynucleotide. Overall chain growth is in the 5′→ 3′ direction.

The Sanger sequencing method capitalizes on the ability of *E. coli* DNA polymerase I to use 2′,3′- dideoxynucleotides as substrates. When a dideoxynucleotide is incorporated at the 3′ end of the growing primer chain, chain elongation is terminated selectively at G,A,T or C because the primer chain now lacks a 3′- hydroxyl group as shown in Fig 3a.

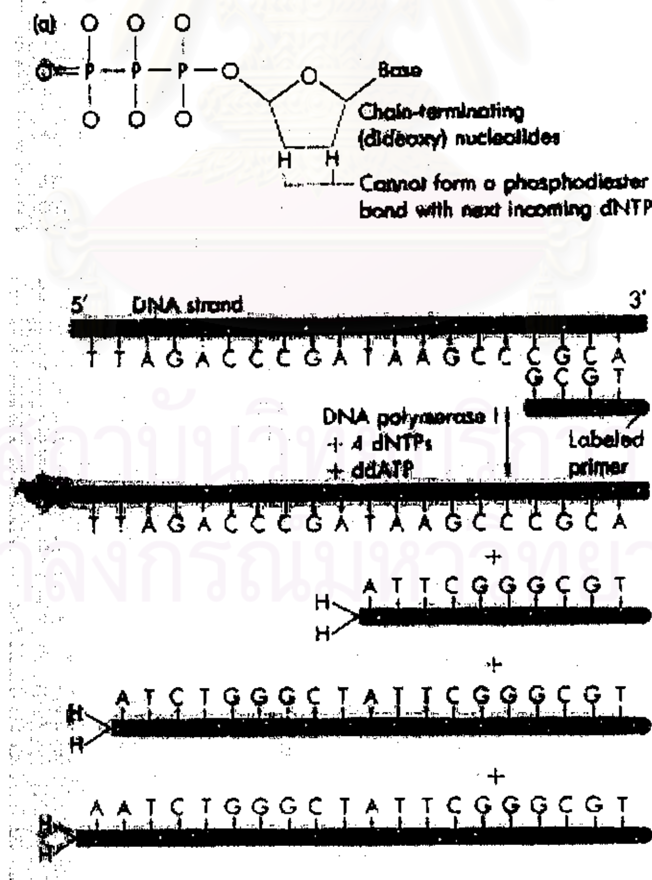

Fig 3a. Dideoxynucleotide and Sanger sequencing principle (72)

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In practice, the annealed template-primer is devided into four aliquots, and polymerase is used to synthesize a radiolabeled oligodeoxynucleotide complementary to the template. Each of the four chain elongation reactions contains all four deoxynucleotides, one or more of which is radiolabeled, and one of the four 2′,3′-dideoxynucleotides. When the elongation reactions are terminated, and the radiolabeled primer-extended single-stranded molecules are separated from the template DNA by heating and then electrophoresed on a sequencing gel. The primer-extended oligodeoxynucleotides are electrophoresed on adjacent lanes, and the DNA sequence can be read directly from the autoradiograph of the gel, as shown in Fig 3b.

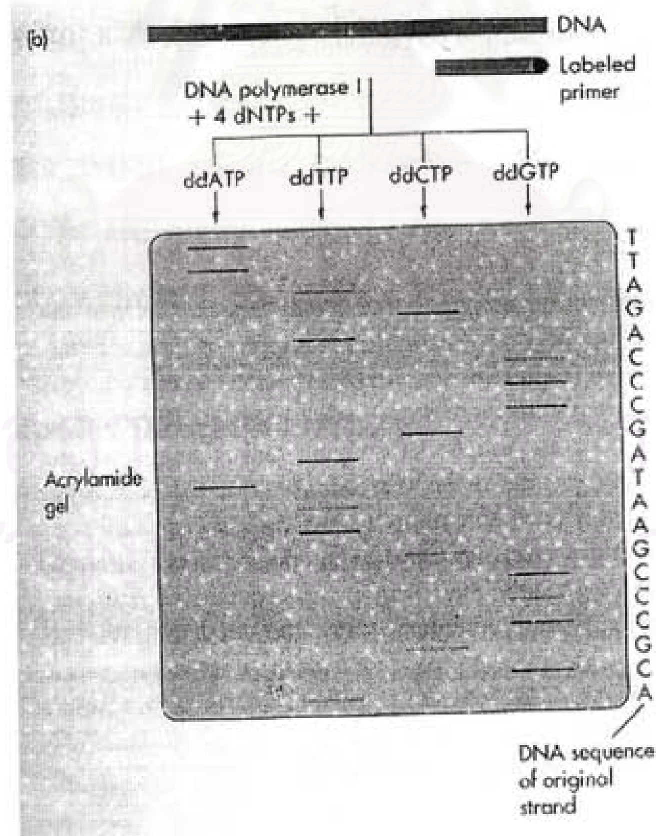

Fig 3b. Sanger sequencing method (72)

Rapid Genetic Methods for Detecting Antibacterial Resistance Genes

Resistance to antimicrobial agents in bacteria can be mediated by several mechanisms, including (a) changes in the permeability of the cell envelope that limit the amount of drug that has access to cellular targets, (b) changes in or elimination of the site of drug action, (c) provision of alternate enzymatic pathways around those blocked by antibacterial therapy, and (d) destruction or inactivation of the antimicrobial agent (32,73-74). While some of these changes are due to random mutations in the bacterial genome, most resistance to antimicrobial agents in bacteria is mediated by acquired genes whose presence in a cell is usually synonymous with a resistance phenotype. Genetic tests aimed at the detection of resistance genes in bacterial isolates by using DNA probes or the PCR are based on the supposition that gene carriage equals resistance. There are many methods for detection mutation and now were used in many clinical laboratories.

Heteroduplex formation (HDF)

Different strains of the same "species" of microorganism often display distinctive properties. In a growing number of cases, linkage of phenotypic traits with genetic markers is allowing complicated biological assays to be replaced by genetic typing. The detection of such genetic variation has been revolutionized by the polymerase chain reaction (PCR) which allows fragments of even the most complex genomes to be isolated in an essentially pure form in a matter of hours. Differences between gene segments can then be determined by direct

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sequencing of the PCR product. To further expedite, and thereby to extend genetic screening assays to greater numbers of samples, multiple non-sequencing methods have been developed that are simplier, typically require less complex apparatus, and are of lower cost than DNA sequencing. For example, heteroduplex analysis has been used in the field of medical genetics(75-81) and for the detection of genetic polymorphisms in human populations (82-83).

Heteroduplexes are formed by simply denaturing and reannealing (usually by heating and cooling) partially complementary DNA strands. Sequence variation can then be detected by noticing a reduced electrophoretic mobility of DNA heteroduplexes following electrophoresis through a polyacrylamide gel. The structural distortious of the DNA double helix caused by mismatched nucleotides (resulting from base substitution mutations) and unpaired nucleotides or "gaps" (resulting from insertions or deletions in aligned regions) reduce the mobility of the DNA through the pores of the gel.

In 1993, Delwart et al. (84) studied the genetic diversity of human immunodeficiency virus (HIV) by the DNA heteroduplex mobility assay, simple and rapid methods for the detection and estimation of genetic divergence between HIV strains on the basis of the observation that DNA heteroduplexes formed between related sequences have a reduced mobility in polyacrylamide gels proportional to their degree of divergence. Reliable phylogenetic subtypes were assigned for HIV-1 strains from around the world. Relationships

between viruses in a given geographic region correlated with the length of time HIV-1 had been detected in the population and the number of strains initiating widespread infection. They discussed, heteroduplex mobility analysis provides a tool to expedite epidemiological investigations by assisting in the classification of HIV and is readily applicable to the screening and characterization of other infectious agents and cellular genes.

In 1994, Bachmann et. al (85) assisted in the preparation for the testing of vaccines against human immunodeficiency virus (HIV), evaluated the genotypic variation of HIV-1 in cohorts from Brazil, Rwanda, Thailand, and Uganda. In all cases in which viral envelope gene fragments could be amplified by polymerase chain reaction, subtypes could be assigned using a heteroduplex mobility assay (HMA) by comparison with HIV-1 strains representing six HIV-1 envelope subtypes. All subtype classifications matched those found by envelope gene sequencing. Phylogenetic relationships were further clarified by heteroduplex formation between samples within each subtype. They found the analysis by HMA is simple for screening of HIV-1 genotypes and it should be generally useful when samples containing at least one variable genetic locus need to be rapidly classified by genotype and/or analyzed for epidemiological clustering.

Hybridization Protection Assay (HPA)

The hybridization protection assay, which was originally developed to identify mycobacteria, has recently been used as a rapid drug susceptibility test for *M. tuberculosis* (86). HPA is a simple and fast *M. tuberculosis* drug susceptibility test using a commercially available and easy identification HPA kit using DNA probe hybridization with rRNA of mycobacteria. Miyamoto et. al (87) demonstrated an excellent relationship between viable bacterial counts and relative light units (RLU). The RLU values of susceptible strains were significantly different form the resistant strains within 72 h of incubation indicating that the test was fast and accurate in identifying *M. tuberculosis* strains resistant to antitubercular drugs. The results also suggested that the drug susceptibility test using HPA might also be useful to detect resistance to antimicrobial agents other than antimycobacterials, even in the ordinary routine laboratory.

Species-Specific Assays for Procursor rRNA

rRNA precursor (pre-rRNA) molecules are intermediates in rRNA synthesis generated by RNase III cleavage of primary transcripts of bacterial *rrn* operons. Leader and tail sequences are removed from pre-rRNA during the secondary steps in rRNA processing, to yield the mature rRNA (88-89). Pre-rRNAs are typically more abundant than even strongly expressed mRNA species (89), making them easier to detect. However, as biosynthetic intermediates, they are less persistent in growth-inhibited cells than are mature rRNAs (88), potentially making

them more-sensitive indicators of bacterial physiological response to antibiotic exposure.

From pre-rRNA molecules characteristics, carry terminal stems which are removed during rRNA synthesis to form the mature rRNA subunits. Their abundance in bacterial cells can be markedly affected by antibiotics which directly or indirectly inhibit RNA synthesis. Gerard and his group (90) evaluated the feasibility of rapidly detecting antibiotic resistant *M. tuberculosis* strains by measuring the effects of brief in vitro antibiotic exposure on mycobacterial pre-rRNA. By hybridizing, extracted *M. tuberculosis* nucleic acid with radiolabeled acid probe specific for pre-16S rRNA stem sequences, they detected clear responses to rifampin and ciprofloxacin within 24 and 48 h, respectively, of exposure of cultured cells to these drugs. Detectable pre-rRNA was depleted in susceptible cells but remained abundant in resistant cells. They suggested that this general approach may prove useful for rapidly testing the susceptibility of slowly growing *Mycobacterium* species to the rifampin and fluoroquinolone drugs and, with possible modifications, to other drugs as well.

Single strand conformation polymorphism (SSCP)

The polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis can easily and rapidly detect mutation in gene, for example detection of *rpo*B gene mutation (91-94), *gyr*A gene mutation (12) and *rps*L gene (95) in Mycobacteria. Mutation screening by PCR-SSCP which involves radiolabeling of PCR fragments

during amplification and analysis of the electrophoretic mobility of the single strands in nondenaturing polyacrylamide gels (96). PCR-SSCP is useful for very small amount of samples (97) and is considered as one of useful routine genetic analysis. However that PCR-SSCP has been analyzed using radioisotope (RI) such as $[^{32}P]$ labeled compounds. So that it was difficult to use this method routinely at clinical laboratories.

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