CHAPTER III

LITERUTURE REVIEW

MYCOBACTERIA

The genus *Mycobacterium* is classified in the class Actinomycetes, Order Actinomycetales, and Family Mycobacteriaceae. The mycobacteria are acid-fast, alcohol-fast, aerobic or microaerophilic, non-sporeforming, nonmotile bacilli, slightly curved or straight bacilli, 0.2 to 0.6 by 1.0 to 10 μm in size, sometimes with braching filamentous or mycelium-like growth may occur, but it easily fragments into rods or coccoid elements.

Mycobacteria produce cell walls unusual structure with a total thickness of about 20 nm. The peptidoglycan contains *N*-glycolylmuramic acid instead of the usual *N*-acetylmuramic acid (30). Sixty percents of the weight of the mycobacterial cell wall is occupied by lipids that consist mainly of unusually long-chain fatty acids containing 60-90 carbons, the mycolic acids (Figure 1) (31, 32).
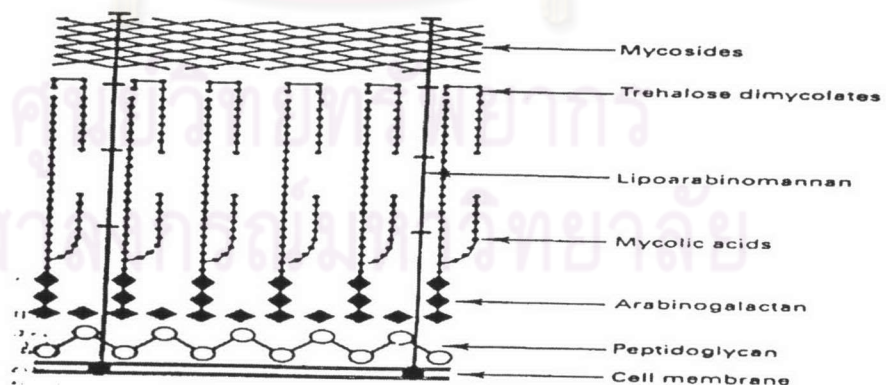

Figure 1. Diagrammatic section of the mycobacetrial cell wall (32)

Mycobacteria are not readily stained by the Gram method but are considered gram positive. Special staining procedures are used to promote the uptake of dye, and once stained, mycobacteria are not easily decolorized even with acid-alcohol. This resistance to decolorization by acid-alcohol is termed acid fastness. Acid fastness may be partly or completely at some stage of growth by some proportion of the cells of some species. Cells of rapidly growing mycobacteria may be less than 10% acid fast.

With the advent of better biochemical and molecular techniques for culture and identification, close to 100 mycobacterial species have now been described. Mycobacteria are devisible into the rapid growers, slow growers and those not yet cultivated *in vitro* as show in Table 1 (33-35).

Mycobacteria are infectious agents of serious human disease, such as tuberculosis and leprosy which caused by *Mycobacterium tuberculosis* complex and *Mycobacterium leprae,* respectively. Those species are true pathogen in human, whereas *Mycobacterium* Other Than Tubercle (MOTT) or Nontuberculous mycobacteria (NTM) are opportunistic pathogens of humans, which usually cause infections in immunocompromised hosts and chronic underlying diseases particularly those with human immunodeficiency virus infection and AIDS (32).

The *Mycobacterium* causing disease in human being can be divided into two major groups: the obligate pathogens and enviromental saprophytes that occasionally cause opportunistic disease. The principal NTM species are listed in Table 2.

The most prevalent NTM infection which is a major clinical problem in immunocompromised patients, particularly in AIDS patients, is *Mycobacterium avium - intracellulare* complex (MAC). In non AIDS patients, MAC mainly causes pulmonary infection in adults with chronic respiratory disease and cervical lymphadenitis in children, whereas disseminated disease are highly frequent in AIDS patients (1-5).

Table 2. The species of mycobacteria (33-35)

Slowly growing

M. tuberculosis	M. bovis	M. africanum	M. shimoidei
M. microti	M. kansasii	M. marinum	M. cookii
M. celatum	M. simiae	M. asiaticum	M. gordonae
M. interjectum	M. scrofulaceum	M. szulgai	M. paratuberculosis
M. canettii	M. intracellulare	M. lepreamurium	M. avium
M. malmoense	M. haemophilum	M. farcinogenes	M. hiberniae
M. triviale	M. terrae	M. nonchromogenicum	M. conspicuum
M. ulcerans	M. gastri	M. xenopi	M. branderi
M. genavense	M. intermedium	M. heckeshornonse	M. heidelbergense
M. lentiflavum	M. triplex	M. tusciae	M. doricum
M. kubicae	M. palustre	M. lacus	M. shottsii
M. botniense			

Rapid growing

M. chelonae	M. fortuitum	M. phlei	M. peregrinum
M. smegmatis	M. aurum	M. gadium	M. immunogenum
M. neoaurum	M. flavescense	M. gilvum	M. mageritense
M. komossense	M. senegalense	M. parafortuitum	M. alvei
M. thermoresistible	M. confluentis	M. mucogenicum	M. septicum
M. goodii	M. wolinskyi	M. brumae	M. chitae
M. diernhoferi	M. agri	M. vaccae	M. duvalii
M. archense	M. chubuense	M. austroafricanum	M. gilvum
M. rhodesiae	M. tokaiense	M. shinshuense	M. komossense
M. porcium	M. fallax	M. pulveris	M. madagascariense
M. sphagni	M. methylovorum	M. moriokaense	M. obuense
M. poriferae	M. shanghaiense	M. yannanense	M. hossiacum
M. novocastrense	M. elephantis	M. holsaticum	M. chlorophenolicum
M. frederiksbergense	M. hodleri	M. murale	M. vanbaalenii

Non-cultivable

M. leprae

Table 2. Classification of NTM recovery from human based on principal site of involvement (36)

Pulmonary disease	Lymphadenitis
M. avium complex	*M. avium* complex
M. kansasii	*M. scrofulaceum*
M. abscessus	*M. fortuitum*
M. xenopi	*M. abscessus*
M. szulgai	*M. kansasii*
M. malmoense	
M. simiae	
Disseminated disease	**Cutaneous disease**
M. avium complex	*M. marinum*
M. kansasii	*M. chelonae*
M. abscessus	*M. fortuitum*
M. fortuitum	*M. abscessus*
M. chelonae	*M. ulcerans*
M. xenopi	*M. avium* complex
M. genavense	*M. kansasii*
	M. nonchromogenicum
	M. smegmatis

GENERAL CHARACTERISTIC OF *MYCOBACTERIUM AVIUM* COMPLEX

The members of MAC which cause disease in humans are classified in the same class *Actinomycetes*, order *Actinomycetales*, family *Mycobacteriaceae* and genus *Mycobacterium*. The MAC are Runyon group III slowly growing nonphotochromogenic mycobacteria, obligatory aerobic, rod shape, with round extremities, about 1-2 μm long and 0.2-0.5 μm in thick, non-motile, without capsule and non-sporeforming. MAC cell wall is related to gram-positive bacteria, which have no such outer membrane. MAC can not be stained with gram stain reagents because their cell walls have a high lipid content, which prevent most dyes from entering the cell. Mycolic acid is the major lipid component of the cell wall, which formed lipid-dye complex with carbol-fuchin or auramine-O, when there were stained with Ziehl-Neelsen (ZN) and fluorescent staining procedure, respectively. The acid-fastness bacilli provided the basis for making a presumptive identification of these organisms.

MAC does not grows on ordinary culture media but only on enriched media, which containing an egg-potato base or a serum (albumin) base. MAC is a slow grower growth rate, the generation time of mycobacteria in the best conditions of culture being 17-18 hr, therefore 2 to 6 weeks are required for development of visible colonies on solid media (37). On subculture, colonies of MAC on an egg media at 14 to 21 days are about 1 mm in diameter, smooth, and white and domed, but growth may be effuse. Some strains of MAC can produce a feeble yellow pigment (38).

Taxonomically, the MAC comprises *M. avium*, *M. intracellulare*, *M. paratuberculosis*, *M. lepraemurium*, and the "wood pigeon" bacillus. Recently, it has been proposed that *M. avium*, *M. paratuberculosis*, and the wood pigeon bacillus be placed in one species with three supspecies (*M. avium* subsp *avium*, *M. avium* subsp *paratuberculosis*, and *M. avium* subsp *silvaticum* [wood pigeon bacillus]) (39); it has been suggested that *M. lepraemurium* be reduced to a supspecies of *M. avium*, as well (40). Only the *M. avium* complex organisms that cause human infections are discussed and include *M. avium*, *M. intracellulare*, and *M. paratuberculosis*. Unfortunately, the nomenclature is somewhat confusing. Although *M. avium* and *M. intracellulare* are clearly different organisms, these

organisms so closely resemble each other that the distinction cannot be made by routine laboratory determinations or on clinical grounds. Futhermore, because the isolation of *M. paratuberculosis* in a routine laboratory setting is exceedingly rare, the term *M. avium* complex is most commonly used to report the isolation of *M. avium-M.intracellular*. In general, *M. avium* complex (MAC) is used and includes only *M. avium* and *M. intracellulare* (41).

MAC strains vary in pathogenicity, which may Types 4 and 8 are the most common organisms to infect patients with AIDS (42, 43). Markers of the pathogenicity of the organism include the presence of plasmid, antibiotic susceptibility patterns, restriction fragment length polymorphism patterns, and multilocus enzyme electrophoresis pattern. Studies suggest that transparent colonies are more virulent by virtue of being more drug resistant, isolated more frequently from blood of patients with AIDS, and appearing more virulent in macrophage and animal models (44-46).

EPIDEMIOLOGY

Mycobacterium avium complex (MAC) is the most frequent isolates among the nontuberculous mycobacteria found in specimen taken from patients. It is ubiquitous in the environment, can be recovered from fresh water, sea water and dairy product as well as from a wide variety of animals including chicken, pig, dog, cat and insect (1, 47, 48). The source of MAC infection in human remains uncertain. Water and aerosols have been implicated as possible environmental sources of MAC (49, 50). Infection caused by this organism is not transmissible from person to person (6).

Prior to the emergence of the AIDS epidemic, MAC infection most often manisfested in human as localized pulmonary disease which in occurred in 2,000 to 3,000 patient annually in the United states (51, 52). Most noticeable among other localized manifestations was cervical lympadenopathy in children. (51, 53). The epidemic of disseminated MAC infection is concurrent with the AIDS epidemic. MAC infection was recognized in AIDS

patients in 1982 and after that the number of cases increased dramatically (1). The prevalence of disseminated MAC infection in HIV infected patients varies widely in the medical literature reviews. Havlik et al. reported the incidences in cases were from 5.7% in 1985-1988 to 23.3% in 1989-1990. It is well known that 25-50% of patients with AIDS were infected with MAC in the United states (7, 54, 55). Martin et al. (56) reported the incidence, trends and clinical significance of acid-fast bacilli (AFB) isolates at Bridgeport hospital from 1995-1999. One hundred twenty-two isolates of nine different type of mycobacteria and nocardia were from 117 patients. About of patients 30% of patients were HIV-positive, 34% were HIV-negative and the HIV status of 36% was unknown. The predominant isolates were MAC (60%) and *M. tuberculosis* (MTB) (21.3%). Pulmonary sources accounted for 74% of the isolates. The incidence of MTB remained stable as 2.9-4.1 cases/100,000 total patient discharges/year. In contrast, the incidence of MAC soared from a baseline rate of 1.6/100,000 total patient discharges/year in 1995/96 to 19.5/100,000 total patient discharges/year in 1999. The increase was consistent across pulmonary and non-pulmonary sources, HIV status and across disease likelihood. Seventy- three percent of MAC isolates were associated with definite or probable disease.

According to the International MAC Study Group, the incidence of disseminated MAC infections in AIDS patients of developed countries and developing countries were 10.5% to 21.6% and 2.4% - 2.6% respectively (9). In Thailand, Suwanagool et al. reported the prevalence of disseminated MAC infection 24 per cent of advanced AIDS patients from Siriraj hospital (11). In 1999, Chuchottawon et al. studied on AIDS patients who had fever with unknown etiology more than 2 week from 4 referral hospitals and founded that 17.4% of patients were infected with MAC (12). The results of these studies demonstrated that MAC infection, indeed, exist among Thai AIDS patients. The prevalence of MAC infection in Thailand was rather high and comparable to that in the western countries.

The predominant isolates from AIDS patients were *M. avium* (97%), although *M. intracellulare* (3%) also causes infection. However, 40% of pulmonary infection in immunocompetent patients was estimated to be from *M. intracellulare* (13, 42, 55).

MAC infection was associated CD4 lymphocyte counts values, exposure to environment source of the organisms, mycobacterial immunity of individual persons (due to prior infection with *M. tuberculosis*, enviromantal mycobacteria or BCG vaccination) and chemotherapy of other microorganisms (57). There were no differences in the frequency of disseminated MAC infection according to age, gender, race, geographic region or the route of HIV transmission (9).

CLINICAL MANIFESTATION

Localized disease caused by MAC includes cervical lymphadenitis, pneumonitis, hepatic dysfunction, skin lesions, endophthalmitis, and abscesses. Patients with localized disease have somewhat higher CD4 T-lymphocyte counts than those with disseminated disease. It is logical to assume that patients with localized disease and perhaps those who are colonized are at high risk for disseminated disease, although this has not been clearly established (57).

Disseminated MAC disease may appear as fever, weight loss, night sweats, diarrhea, abdominal pain, anemia, or an elevated serum concentration of alkaline phosphatase (1, 8, 54, 58). Patients often have intraabdominal lymphadenopathy and hepatosplenomegaly. These symptoms, signs, and laboratory abnormalities are nonspecific, however, and may be caused by a variety of infectious and neoplastic processes. Although MAC may be a marker for severe immunosuppression in patients whose fever or inanition is caused by another process, case-control studies have demonstrated that MAC can cause systemic symptoms, especially in patients with bacteremia. Therapeutic trials correlating reduction in bacteremia with improvement in symptoms also support MAC as a cause of morbidity.

The principal predictor of an increased risk of MAC disease is the peripheral-blood CD4 T-lymphocyte count (1, 7, 8, 54, 59). In several published series, the median count in adult patients with disseminated disease ranged from 10 to 50 cells per cubic millimeter, with very few patients having counts higher than 100 cells per cubic millimeter (1, 7, 8, 59, 60).

Additional factors correlating with the development of disseminated MAC disease include the time since the diagnosis of AIDS, the presence of substantial anemia (hemoglobin level, <8 g per deciliter), previous opportunistic infection, and any interruption in zidovudine therapy (7, 8).

No prospective study has precisely defined the effect of untreated disseminated MAC disease on prognosis, but several cohort and case-control studies suggest that this entity is associated with reduced survival (8, 61). One study suggested that the median survival among patients with untreated disseminated MAC disease was 4 months, as compared with 11 months among patients without disseminated MAC (61). Another study calculated the median survival in these two groups as 3.5 and 9.1 months, respectively (59). A prospective cohort study of more than 1000 patients with advanced HIV disease showed that patients in whom MAC disease developed had a significantly increased risk of death, even when other predictors of mortality were controlled for (8). Although such studies clearly indicate an association between disseminated MAC disease and reduced survival, they do not conclusively attribute the mortality to MAC.

TREATMENT OF MAC INFECTION

There are 2 principles in the treatment of MAC infection : never use a single drug and always include an extended-spectrum macrolide in the regimen. This applies to both HIV infected and non-HIV infected patients. For most MAC organisms, clarithromycin is the most active drug and azithromycin is an alternative. Ethambutol and rifabutin are added to enhance bactericidal activity and to prevent emergence of resistant organism.

Therapy of disseminated MAC disease

The goal of therapy for disseminated MAC disease is to reduce the amount of mycobacteria in patients or to eradicate it from patients altogether and thereby improve the quality or duration of their survival. A previous analysis of survival of patients with disseminated MAC showed or revealed decreased survival compared with AIDS without

disseminated MAC (61). In monotherapy studies, clarithromycin reduced MAC bacteremia by 2.5 logs, compared to 1.5 logs for azithromycin, 0.4 logs for ethambutol, and no change for rifampin or clofazimine (62). Clarithromycin or azithromycin have been associated with increased survival among AIDS patients with disseminated MAC (63). But azithromycin is less active than clarithromycin (64). Rifabutin, a rifamycin derivative, has demonstrated activity against MAC, but adverse drug reaction (esp. uveitis) and drug interactions (rifabutin reduced the blood level of zidovudine and clarithromycin) limit rifabutin use in disseminated MAC (65, 66).

Clarithromycin or azithromycin monotherapy are not recommended for treatment of disseminated MAC because of the rapid development of clarithromycin and azithromycin resistance in MAC (18, 64). Clarithromycin resistance developed in 21% of the patients within 12 weeks; the rate later climbed to 46% (17). Presently, it is recommended that therapy for disseminated MAC include clarithromycin 500 mg bid and at least one other agent (67). Ethambutol (15 mg/kg/day), with or without clofazimine (100 mg/kg/day), is the agent most often combined with clarithromycin (68). But adding both ethambutol and clofazimine to clarithromycin for treatment MAC is still controversial (68, 69). Azithromycin can be used as an alternative for patients intolerant to clarithromycin (70). For the patient with far advanced disseminated MAC, amikacin 7.5 mg/kg should be added (69). For the disseminated MAC patient who has been on prophylactic clarithromycin or azithromycin, rifabutin 300 mg/day or rifampicin 600 mg/day plus ethambutol 15 mg/kg/day plus ciprofloxacin 750 mg bid is suggested. If there is recurrence of disseminated MAC during therapy, antibiotic susceptibility test should be performed and the addition of amikacin should be considered prior to susceptibility test results (70).

PROPHYLAXIS OF MAC

Most evidences suggest that disseminated MAC infection is the result of recent acquisition from environmental sources such as water, food and soil, the recognition habitats of these organisms. It is not possible to avoid being exposed. Prevention of disseminated MAC is an important goal in the management of HIV infection and low CD4-T lymphocyte counts, since disseminated MAC disease occurs so frequently and appears to increase morbidity.

The Public Health Service Task Force on Prophylaxis and Therapy for *Mycoabacterium avium* complex recommended prophylaxis with rifabutin (300 mg/day) for patients with AIDS with CD4 T-lymphocyte below 100 cells per cubic millimeter (71). Rifabutin has been a significant advance in prophylaxis MAC infection. Its efficacy is only modest, and there are a number of drawbacks, including the numerous potential drug interaction, high cost, problem with compliance associated with daily therapy and the potential of *M. tuberculosis* to produce cross-resistance to rifampin.

On the basis of new macrolide are antimicrobial agents with demonstrating *in vitro* and *in vivo* activity against MAC infection. Pierce et al. recommended clarithromycin at 500 mg / twice daily for prophylaxis against MAC in AIDS patients with all CD4 count status. Clarithromycin prophylaxis is safe and can reduce the risk of disseminated MAC and prolong survival in these patients (72). In the study of the National Institute of Allergy and Infectious Diseases, AIDS Clinical Trials Group and Community Program on Clinical Research on AIDS (ACTG 196/CPCRA 009), clarithromycin was found to be more effective than rifabutin, but the combination of clarithromycin plus rifabutin was not better than clarithromycin alone. Azithromycin (1200 mg once per weekly) was also found to be another prophylaxis regimen for MAC in AIDS patients (73).Current guidelines suggest that all patients with CD4 cell count of less than 50 cells per cubic millimeter receive MAC prophylaxis. Azithromycin and clarithromycin are considered preferred prophylactic agent (74).

CLARITHROMYCIN

Clarithromycin is a semi-synthetic macrolide antibiotic, structurally similar to erythromycin (a 14-membered macrolide) and have modification that improve their acid stability and increase their potency, half life, achievable concentration in tissue, and bioavailability without causing toxicity. Chemically, it is 6-0-methylerythromycin (Figure 2). The molecular formula is $C_{38}H_{69}O_{13}$, and the molecular weight is 747.96. Clarithromycin is a white to off-white crystalline powder. It is soluble in acetone, slightly soluble in methanol, ethanol and acetonitrite, and practically insoluble in water. This macrolide is bacteriostatic agents and inhibit the growth of microorganism by binding to the 50S subunit of the prokaryotic ribosome, blocking protein synthesis at the peptidyltransferase step.

Clarithromycin is important agent in the treatment of all form of MAC disease, infections caused by rapidly growing mycobacteria, and leprosy. In addition, clarithromycin is effective and approved prophylactic agents for preventing disseminated MAC (72). Indeed, they are viewed as potential cornerstone in the treatment of nontuberculous mycobacterial infection (75).
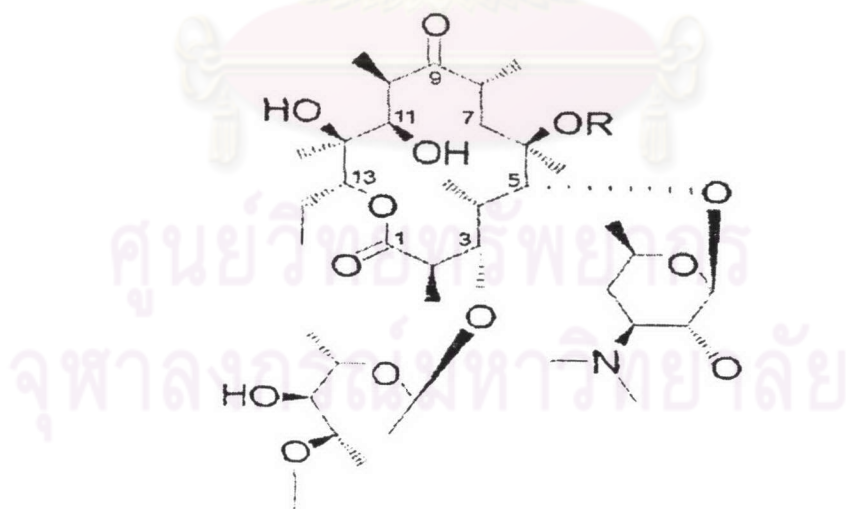

Figure 2. Chemical structures of macrolide antibitotics. Erythromycin, a 14-membered macrolide (R = H) and its semi-synthetic derivative clarithromycin (R = CH₃)

MECHANISMS OF MACROLIDE RESISTANCE

Various macrolide resistance mechanisms, intrinsic and acquired, encountered in different bacteria, belong to one of the following categories:

1. Modification of the target

1.1 Methylation of rRNA

This mechanism of resistance, which was the first described in terms of specific modification of the target of macrolides (76) is, today, the most prevalent in pathogenic bacteria (77). It is mediated by the acquisition of an *erm* gene, encoding a methyltransferase which methylate the N(6) position of adenine 2058 (A-2058, *E. coli* numbering) in 23S rRNA gene (78). More than 30 *erm* genes from a variety of sources have been described but they all show large similarities suggesting that they all derive from common ancestor (78, 79). Resistance is more probably due to the steric hindrance created by the methyl(s) into the macrolide binding site, which prevents the correct positioning of the amino-sugar, thanks to a modification of the binding site conformation (80). The expression of the methylase is either constitutive or inducible. Because macrolides inhibit protein synthesis, this mechanism implies that the efficiency of induction is critically dependent on the macrolide concentrarion. If the concentration is low, too few ribosomes will be occupied to allow for sufficient erm synthesis. Conversely, if the concentration is too high, the antibiotic will rather inhibit the translation of the *erm* mRNA (81).

1.2 Mutation of 23S rRNA

Among the mutations detected in the binding site of macrolides, substitution of adenine 2058 with a guanine (G) in peptidyl tranferase loop of 23S rRNA gene is the most common one in bacterial pathogens (80, 82). It usually defines a macrolides and lincosamides phenotype of resistance, with high MICs for erythromycin, azithromycin, the 16-membered macrolides and lincosamides, a slightly reduced susceptibility to clarithromycin, but no streptogramins and ketolides (83). This mechanism is so far mainly

found in *Helicobacter pylori*, *Mycoplasma* and *Mycobacterium* spp. probably because these bacteria possess only one or two copies of the rRNA operons (82).

1.3 Mutation of r-proteins

Mutations in the protein L4 and L22 have also been recently associated with the appearance of resistance to macrolides in clinical strains of streptococci (84, 85). Mutations in the L4 protein are located in a conserved motif which interacts with the rRNA (84) and perturb the binding of the macrolide to its target (160); confer a macrolides resistance phenotype, with MIC remaining low (83). Mutations in L22 protein are localized on a β-sheet making part of the exit tunnel (83). They cause a wider opening of the tunnel, allowing the nascent peptide for slipping by the macrolide (86). These mutation confer a low level of resistance to macrolides (83).

2. Small peptides

This mechanism has been described fortuitously in *E. coli* while searching for small rRNA fragments able to bind antibiotics. It was found thereafter that deletion of some of these small fragments render cells resistant to erythromycin but that translation of these fragments was sufficient to confer resistance, indicating that it was the encoded peptide rather than the RNA which was the resistance determinant (87, 88). These peptides act in *cis*, so that they confer resistance only to the ribosome on which they were translated and their sequence defines the macrolides to which they confer resistance. Thus, E-peptides conferring resistant to erythromycin are characterized by the consensus sequence M-(L)-L/I-(F)-V, while K-peptides conferring high resistance to ketolides, have the consensus sequence M-K/R-(F/L/V)-X-X (87, 89). The mechanism of resistance proposed here is that of a "bottle brush", where the ribosome produces a short peptide that binds to the macrolide, kicks it out of its binding site on the ribosome, and thereby, makes the ribosome available for protein synthesis (90, 91).

3. Antibiotic inactivation

Unlike target modification, this mechanism confers resistance to structurally-related antibiotics only. At the present time, phosphorylases and esterases conferring resistance to 14-, 15- and 16-membered macrolides have been mainly reported in Enterobacteriaceae. Enterobacteria are intrinsically resistant to high levels of erythromycin and two types of erythromycin-inactivating esterases have been identified in *E. coli* strains. These esterases are encoded by two genes, *ere*(A) and *ere*(B), the first of which has been shown recently to be organized as an integron gene cassette the mobility of which has been demonstrated (92). The clinical significance of this resistance remains, however, minor, since these bacteria are not the primary target of macrolides. Yet, a few strains of *S. aureus* producing phosphotransferases have already been reported (93), which suggests that this mechanism may become more significant in the future.

4. Efflux

Expression of efflux pumps is now recognized as a general mechanism developed by cells to protect themselves against the invasion by diffusible, foreign substances. In this respect, constitutively expressed pumps able to transport macrolides are probably responsible for the poor susceptibility of several gram-negative to macrolides (94, 95). Moreover, these pumps have a wide spectrum of substrates, and are therefore often involved in multiresistance phenotypes (95). In gram-positive bacteria, the expression of efflux pumps conferring resistance to macrolides is induced by the exposure of the bacteria to the antibiotic. Two main classes of pumps have been described so far. In contrast with what is described in gram-negative, these efflux pumps have a narrow spectrum. Thus, the *Msr*(A) pump of *Staphylococci* species and which is inducible by 14- and 15-macrolides, confers resistance to these macrolides (96). This pump belongs to the superfamily of ABC transporters (ATP-binding cassette) (95) which require ATP hydrolysis as energy source. The *mef*(A) and *mef*(E) efflux systems of streptococci, described in several species of streptococci including *S. pneumoniae* and *S. pyogenes* as well as in enterococci, are inducible and confer resistance only to 14- and 15-membered macrolides (94, 97, 98). They belong to the MFS

(major facilitator superfamily) of transporters (95) driven by protion-gradient motive force. The *mef*(A) gene is located on a conjugative transposon, and can therefore easily spread between bacteria or even between streptococci species (99, 100).

CLARITHROMYCIN RESISTANCE IN MAC

Ribosomal mutation are a rare cause of antibiotic resistance among bacterial species, in part related to the finding that most gram-positive and gram-negative bacterial contain multiple copies of the 23S rRNA gene in chromosome. Hence, a mutation involving only one of these genes would have lesser impact (probably not a clinical one) than mutations involving other genes that are present as single copies. Previous studies in MAC have shown that they contain only a single chromosomal copy of the 23S rRNA gene (21, 101) and hence would be highly susceptible to mutation involving the gene.

In MAC, clarithromycin resistance results from mutation of the peptidyl transferase loop of 23S rRNA gene (Figure 3) and usually occurs during single-drug therapy (21, 24). This type of mutation was first reported in 1994 in clinical isolates of *M. intracellulare* (24). After this report, many other clarithromycin resistance strains of MAC (clinical isolates or *in vitro* selected mutants) were characterized and show to harbor mutation in 23S rRNA gene either A-2058 or A-2059, all three possible base substitution have been observed at position 2058, whereas a substitution at position 2059 is more restrictive (Table 3) (21-25). Cross-resistance between clarithromycin and azithromycin was confirmed with laboratory mutants and clinical isolates (20, 24).

A-2058 is thought to be involved in macrolide binding for several reasons. The same position in rRNA is the site of methylation by *erm* gene (102), the most common mechanism of macrolide resistance among gram-positive bacteria, which acts by blocking ribosomal binding. In addition, erythromycin binding affinity to the 23S ribosome has been shown to be reduced with mutations involving *E. coli* A-2058 (103), presumably related to conformational changes in the peptidyltransferase loop (21, 103). The effects of base pair

changes at A-2059 on ribosomal structure or macrolide binding are not as well defined as with A-2058. Resistance to erythromycin in *Mycoplasma pneumoniae* (104) and of lincosamides in chloroplast (105) has also been reported following point mutations at locations homologous to A-2059. Thus, this position appears to play an important role in macrolide binding in addition to the previously recognized A-2058.

Figure 3. Peptidyl transferase loop of 23S rRNA according to the model Noller. The nucleotide positions are based on *E. coli* numbering. The base substitutions observed in clarithromycin-resistance MAC isolates are indicated.

Table 3. Mutation in the 23S rRNA gene detected association with macrolide resistance in MAC [modified from (82)]

E.coli 23S rRNA position[a]	Organism	Nucleotide(s)		Phenotype	Reference(s)
		Wild type	Mutant		
2058	M. avium	A	C,G,T	Clr[R]	(21, 22, 25)
	M. avium	A	T	Clr[R]	(22)
	M. avium	A	C,G	Clr[R]	(23)
	M. intracellulare	A	C,G,T	Clr[R]	(24)
	M. intracellulare	A	G	Clr[R]	(22)
	MAC	A	G,T	Clr[R]	(22)
2059	M. avium	A	C	Clr[R], AZM[R]	(24)
	M. avium	A	C	Clr[R]	(22, 25)
	M. intracellulare	A	C	Clr[R], AZM[R]	(24)
	MAC	A	G	Clr[R]	(22)

[a] Nucleotide positions are numbered according to the corresponding positions in *E. coli* 23S rRNA .
Clr[R]: clarithromycin resistance; AZM[R]: azithromycin resistance

METHOD FOR DETECTION OF CLARITHROMYCIN RESISTANCE IN MAC

Phenotypic method

1. NCCLS recommended methods

The susceptibility testing should be performed using a broth-based method, either macrodilution or microdilution. In the United states, there is consensus that both radiometric broth macrodilution and broth microdilution methods are accurate and reliable.

Radiometric broth macrodilution (BACTEC 460 TB method)

The radiometric method is now a widely used methods, especially in the developed countries. The BACTEC system may be used to determined the MIC of drug for MAC by inoculating in bottled contain a range of drug concentration. Mycobacteria easily grow in the BACTEC 12B medium containing [^{14}C] palmitic acid. This compound is metabolized, resulting in the production of $^{14}CO_2$; therefore, growth or inhibition of growth in BACTEC 12B medium is measured as changes in the growth-dependent production of CO_2. The instrument quantitatively detects the amount of $^{14}CO_2$ released, expressed in terms of a growth index (GI), and then automatically replaces the headspace with 5 to 10% unlabeled CO_2 in air, thereby maintaining the recommended CO_2 atmosphere. The rate and amount of $^{14}CO_2$ produced are directly proportional to the rate and amount of growth. This method is not without problem; in particular; a mixture of mycobacterial species or the presence of contaminant, both of which may falsely indicate drug resistance; is not early detectable. The BACTEC system required expensive equipment and hazardous waste from radioisotope.

Broth microdilution method

The broth microdilution method is an adaptation of the macrodilution broth method using small volumes for susceptibility testing . Its utilizes microtiter plastic plates containing 80, 96, or more wells depending on the number and concentration of antimicrobials included in the test. Middlebrook 7H9 or Mueller-Hinton broth supplemented with OADC or ADC can be used for determining the susceptibility of MAC (106). The advantage of the system is,

it utilizes small volumes of reagents and allows large numbers of bacteria to be tested relatively quickly and inexpensively. The disadvatage of this method is laborious and time-consuming for routine use.

2. Conventional method, agar dilution method

In Europe, an agar dilution method with Mueller-Hinton agar supplemented with OADC is used for clarithromycin susceptibility in MAC. The principle of this method is comparable to the microdilution broth method. This method utilizes agar instead broth. The disadvatage of this method is laborious and time consuming (about 3-weeks) for detection of clarithromycin resistance in MAC.

3. New clarithromycin susceptibility method

Epsilometer test (E test)

The E-Test is a novel method for direct quantification of antimicrobial susceptibility using an agar diffusion method. The E-test comprises a plastic strip 50 x 5 mm, of which on one side is a predefined continuous exponential gradient of stabilized antimicrobial along the length of the strip and on the other an exponential MIC reading scale (mg/l). When the strip is applied to the surface of an agar plate, inoculated with the test organism, which are rapidly transferred to the agar medium from the carrier strip. After the adequate incubation an elliptical zone of inhibition is produced and the 'MIC' is read where the zone of inhibition intercepts the strip. The advantage of this method is speed of preparation, allows sensitivity testing of single isolates, including almost all fastidious species. Therefore, this method increasingly is used, if susceptibility is required for critically ill patients. (e.g. septicemia, endocarditis, meningitis, anaerobic infections). In 1996, Lebrun et al. (27), who compared the E test with the agar dilution method to assess the in vitro activites of clarithromycin against MAC, found that 70% and 100% of the results correlated within ± 1 and ± 2 $\log_2$ dilution step, respectively and no major errors resulting in misclassification in susceptibility or resistance categories were detected for the E-test MIC method. In 2002, Thiermann et al. (23), who assess the phenotypic reproducibility, found that

E test 56/59 E test replicates were within ±1 log$_2$ dilution step and 3/59 were ±2 E test log$_2$ dilution step. Both studied suggested that the E test is easy to perform, produces result quickly and is accurate method for determining susceptibility of MAC strains to clarithromycin.

Disk Diffusion method

Diffusion of an antimicrobial agent from a disc in contact with the moist agar surface previously inoculated with a bacterial suspension (standardised inoculum density) gives rise to zones of inhibition. Although direct calculation of the inhibitory concentration is not done in practice, the MIC can actually be calculated with reasonably accuracy if the characteristics of antimicrobial diffusion and bacteria growth are known. The diffusion test still is the most widely used method in routine clinical laboratories worldwide. The diameter of the zone of inhibition is measured (mm) and used to interpret the susceptibility category ('Susceptible', 'Intermediate' or 'Resistant'). These categories are derived by a statistical evaluation (regression analysis), comparing the MICs and the diameter of the zone of inhibition. In 1998, Jaboe et al. (107) evaluated an agar disk diffusion method of determining the susceptibility of MAC to clarithromycin by comparing its results with those of broth microdilution. Isolates were inoculated onto the surface of Middlebrook 7H11 agar, followed by the application of a 15-microgram clarithromycin disk. Zone sizes were read after 5-7 days of incubation. Defining susceptibility as a zone size of >10 mm, disk diffusion test results agreed with the results by the broth microdilution method for 50 of 51 (98%) isolates tested by both methods. This studied suggested that agar disk diffusion is promising method for the determining of clarithromycin susceptibility testing for MAC.

Microplate Alamar blue assay (MABA)

Microplate Alamar blue assay (MABA), a colorimetric drug-susceptibility testing method that uses a redox indicator that changes color from blue to pink to indicate bacterial growth. In 2004, Vanitha and Paramasivan (108) investigated the realibility of the MABA for susceptibility testing of MAC to clarithroycin by comparing the results with those obtained by the BACTEC radiometric method. They found that the overall agreement between MABA and BACTEC was 86%. MABA has 86% sensitivity and 80% specificity. This study

suggested the MABA is a reliable and inexpensive method for drug susceptibility testing of MAC.

Potassium tellurite method

The potassium tellurite method is based on MAC possesses an enzyme that reduces potassium tellurite in less than 3 days and results in formation of black precipitate. In 2001, Afghani and Fujiyama (109) was to determined whether reduction of potassium tellurite by mycobacteria can be used as a means of testing the susceptibility of MAC to clarithromycin. Minimum inhibitory concentrations (MICs) for 104 clinical isolates of MAC were determined by the tellurite method and compared with those tested by a recommended microdilution method. They found that 100% of the results correlated within ± 2 $\log_2$ dilution step and when the MICs were classified into interpretive categories, there was 100% agreement by the two methods. These studied suggested that use of potassium tellurite is a more rapid, reliable and inexpensive method of testing the susceptibility of MAC to clarithromycin.

Mycobacteria growth indicator tube (MGIT)

In 1995, the MGIT was introduced for the growth and detection of mycobacteria from clinical specimens (110, 111). The MGIT medium consist of a modified Middlebrook 7H9 broth conjunction with fluorescence quenching-based oxygen sensor (silicon rubber impregnated with a ruthenium pentahydrate). The fluorescent compound is sensitive to the presence of oxygen dissolved in the medium. The fluorescence of the indicator is quenched in the presence of oxygen, but fluorescence increases as soon as actively respiring microorganisms utilize the dissolved oxygen. Fluorescence is detected using a 365-nm UV transilluminator. Principally, the MGIT system is available as a manual or a fully automated, continuously monitoring system called BACTEC MGIT 960. This method may be used to determined the MIC of drug for MAC by inoculating in bottled contain a range of drug concentration. In 1998, Piersimoni et al. (28), who compared the MGIT system with radiometric BACTEC 460TB method based on the threshold adopted for interpretation of BACTEC system-determined proposed by Heifets. They found that excellent agreement was demonstrated for MAC isolates for clarithromycin and 100% of the

result correlated within ±2 log$_2$ dilution step. This study suggested that this system is reliable, rapid and easy to interpretation.

ESP culture system

The ESP culture system is a nonradioactive system for detection of mycobacteria growth. This system is based on detection of pressure changes (gas production or gas consumption due to microbial growth) within the headspace above the broth culture medium in sealed bottle. The culture medium consists of a Middlebrook 7H9 broth which has been enriched with glycerol and Casitone, and contains a cellulose sponge. Before the medium is inoculated with a test strain, OADC enrichment must be added to a final concentration of 10%. In 2000, Lui et al. (112) validated the non-radiometric, broth-based ESP system for determining MAC susceptibilities. Susceptibilities were determined and comparison made between the agar proportional method and the ESP system for clarithromycin, ethambutol, sparfloxacin and cycloserine. They found that 90% of the MIC generated by the ESP system user identical to or lower than the MICs determined by the agar proportion. This studied suggested that the ESP system is rapid and reliable method for determining MAC susceptibilities.

Flow cytometric susceptibility test method

The flow cytometric susceptibility test method is based on the ability of mycobacteria organisms exposed to fluorescein diacetate (FDA) to rapidly hydrolyze the compound to fluorescein by intrinsic esterases. In nonviable mycobacteria or mycobacteria susceptible to antimycobacterial agents, hydrolysis of FDA is reduced due to the decreased metabolic activity of the organisms. The use of flow cytometry allows rapid measurement (1 min or less per sample) of differences in the amounts of accumulated fluorescein among susceptible organisms and those resistant to, or untreated with, antimicrobial agents. In 2000, Vena et al. (113) investigated the reliability of the flow cytometric susceptibility test method and to compare the results with those achieved by the radiometric method (BACTEC). They found that agreement was 97% between the results of the method. The result of flow cytometric susceptibility test were available 24 h after inoculation of drug-containing medium. This studied suggested that the flow cytometric assay is rapid, safe simple and reproducible.

Genotypic methods

1. Polymerase chain reaction (PCR) - DNA sequencing

DNA sequencing remains the "gold standard" for identifying the products of amplification reactions was generally performed by polymerase chain reaction (PCR).

1.1 The polymerase chain reaction

PCR is a technique for the *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. DNA polymerase carries out the synthesis of a complementary strand of DNA in the 5' to 3' direction using a single-stranded template, but starting from a double-stranded region. This is the primer extension reaction (Figure 4) and is a basis for a variety of the labeling and sequencing techniques. The cycle, which only takes a few minutes, is repeated many times so that after many cycles there may be a million-fold replication of the target DNA (Figure 5) (114).

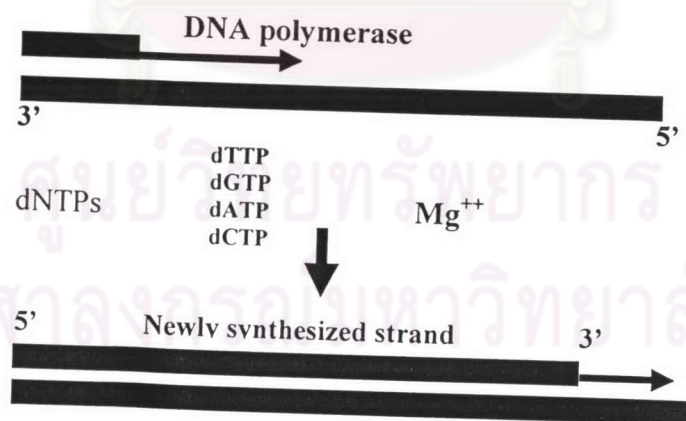**Polymerase chain reaction: basic principle and automation**

Figure 4. Primer extension, DNA polymerase extends a primer by using a complementary strand as a template (114)
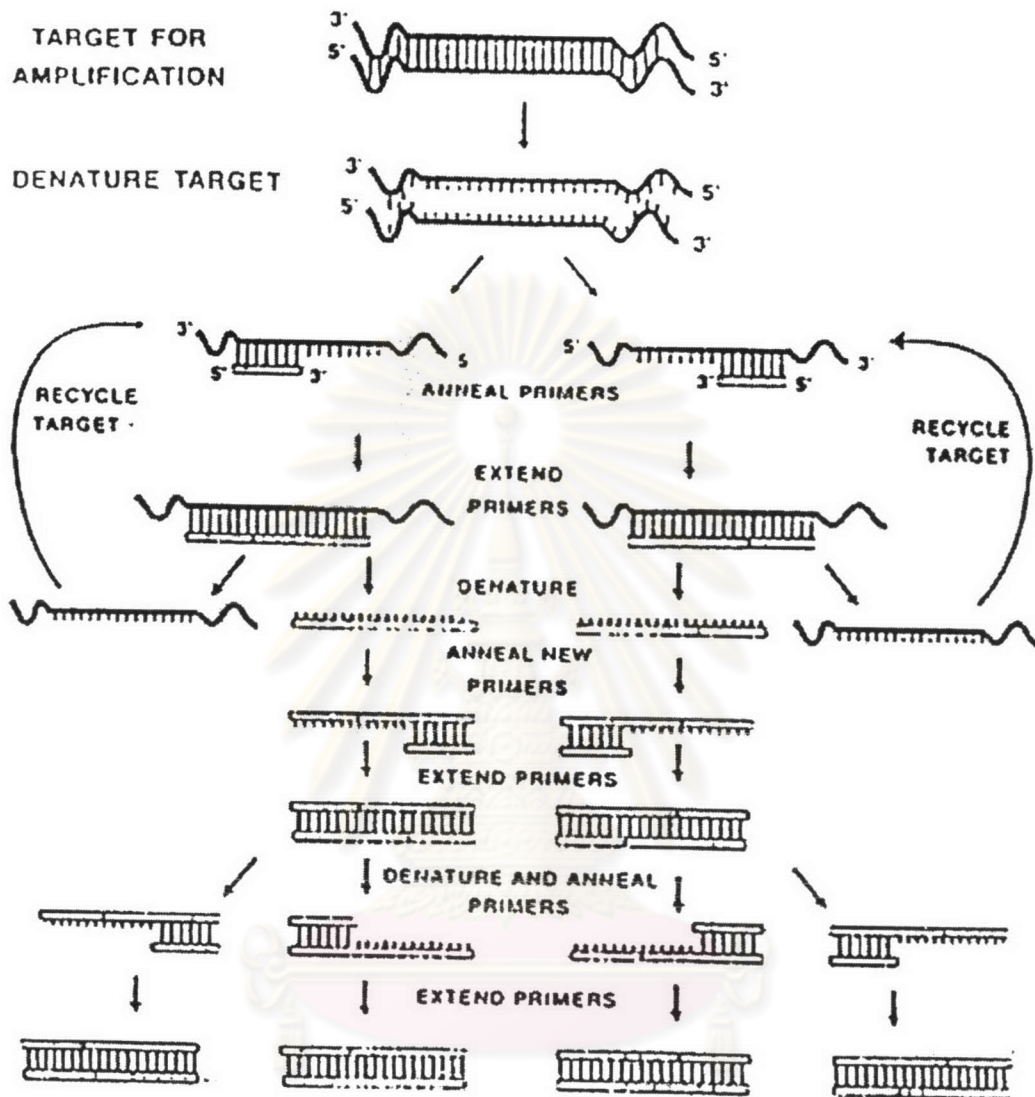
Figure 5. Schematic diagram of PCR (115)

1.2 DNA sequencing (Dideoxy sequencing)

The dideoxy enzymatic method as originally developed by Sanger F. utilizes *E. coli* DNA polymerase I to synthesize a complementary copy of a single-stranded DNA template. After primer extension (Figure 4), DNA polymerase extends a primer by using a complementary strand as a template is annealed to DNA template (115), 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 a substrates. When a dideoxynucleotides 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 (Figure 6) (116).

In an automate sequencer, thermal cycle sequencing is a method of dideoxy sequencing in which a small number of template DNA molecules are repetitively utilized a generate a sequencing ladder. A dideoxy sequencing reaction mixture (template, primer, dNTPs, ddNTPs, and a thermostable DNA polymerase) is subjected to repeated rounds of denaturation, annealing and synthesis steps, similar to PCR using a commercially available thermal cycling machine (117). In practice, automate sequencing that use fluorescent-based chemistry can provide accurate sequence data within 24-48 hr (Finger 7).
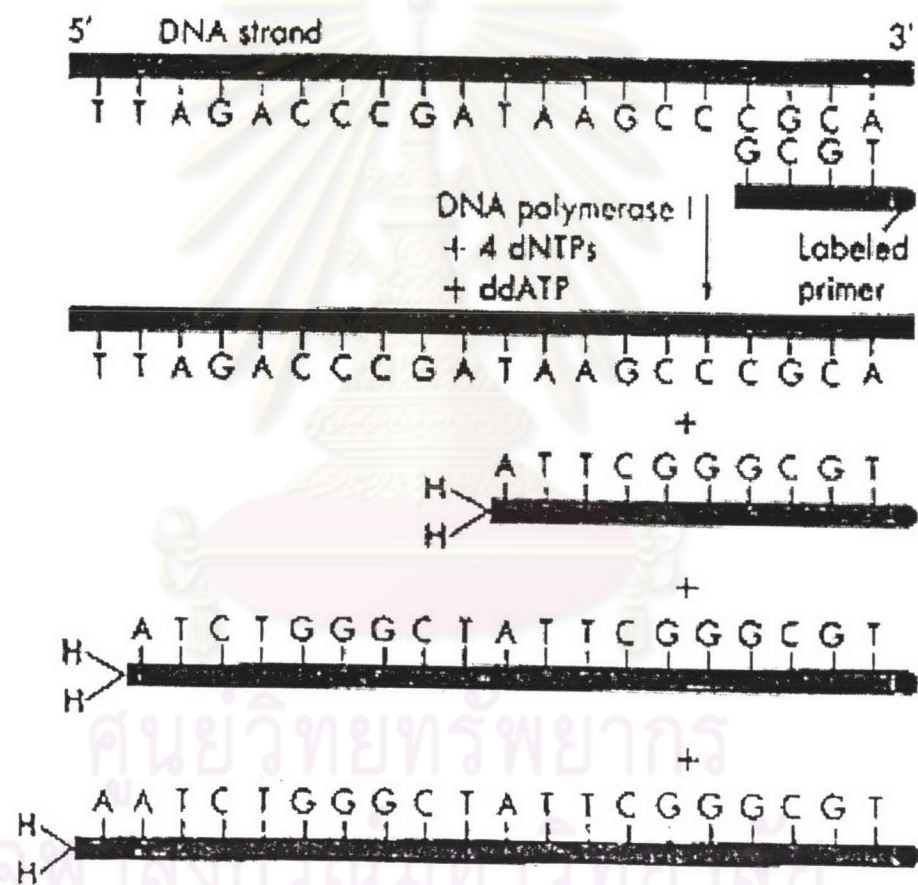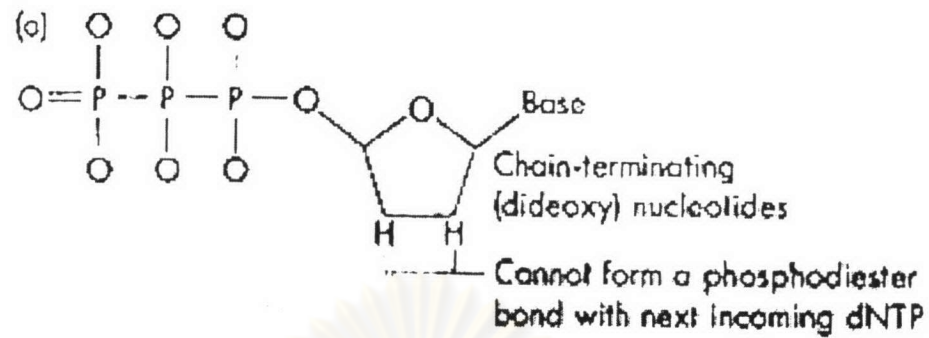
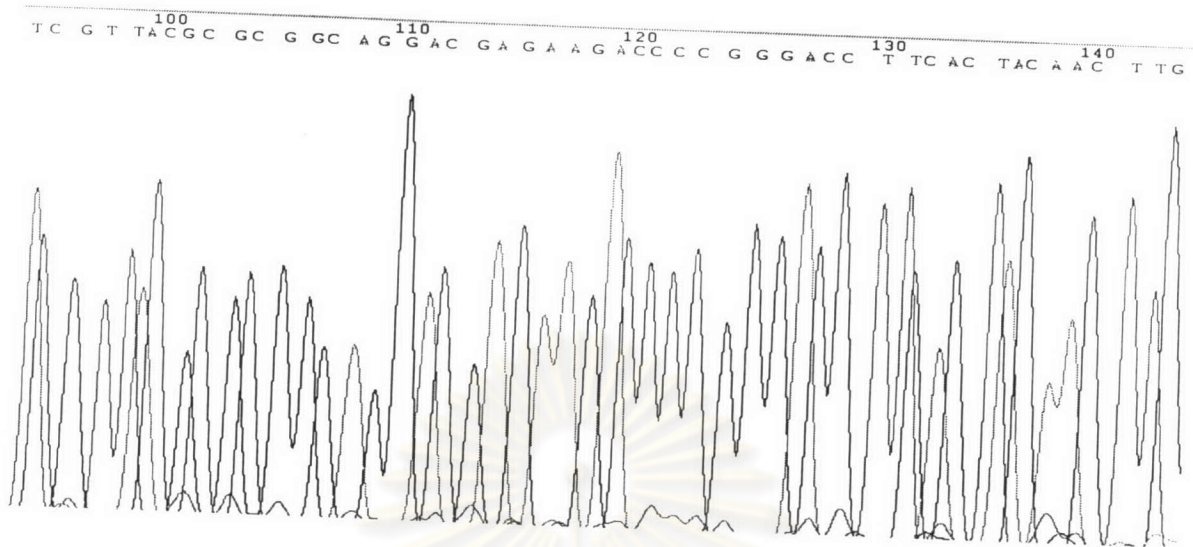**Figure 6. Principle of DNA-sequencing method developed by Sanger. (116)**

TC GT TACGC GC G GC AG GAC GA GA AG ACC C C G GGACC T TC AC TAC AAC TTG
100   110   120   130   140

Figure 7. Chromatogram of sequencing by automate sequencer

3. PCR-RNA/RNA duplex RNase cleavage assay

Nash and Inderlied have recently used a PCR-RNA/RNA duplex RNase cleavage assay to detect mutations in the 23S rRNA gene of MAC which are associated with macrolide resistance (118). The method is based on the ability of RNase 1 and RNase T1 to cleave mismatches (Figure 8). Duplex RNA is transcribed from PCR product (test strain and wild-type strain) generated with primers containing opposing phage RNA polymerase promoters (T7/T7 or T7/Sp6). Following hybridization and RNase treatment, cleavage products of RNA/RNA duplexes occur due to mismatches. Mismatches are the result of mutation in the test strain compared with the wild-type strain.

This method is commercialized and is available in kit form (Mutation Screener; Ambion, Inc., Austin, Tex.). It is relatively easy to perform but requires a longer time for completion than a PCR alone. Nash and Inderlied note that in their hands the assay can be completed in about 1 working day and that the cost (including reagent, supplies and personnel time) is less than $50 if one or more strains are tested simultaneously. Of course, added to this are expenditures for a thermocycler and electrophoresis equipment.
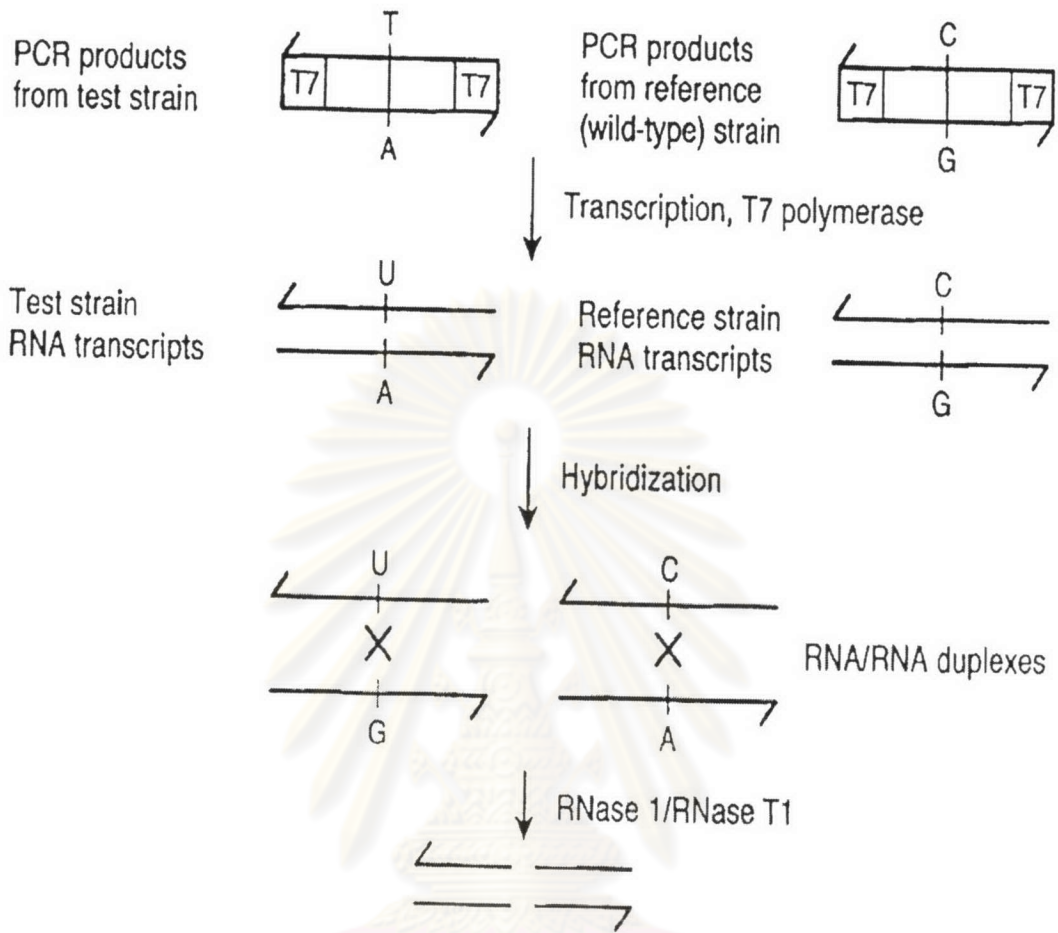
Figure 8. Principle of PCR-nonisotopic RNase cleavage assay (119).