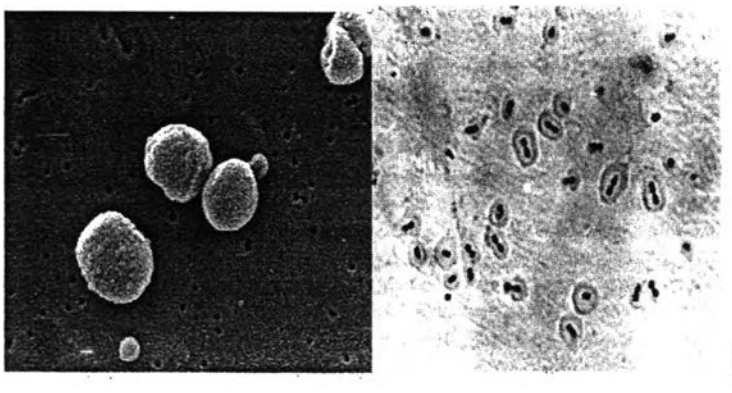
# CHAPTER III

# LITERATURE REVIEW



## 1. BACTERIOLOGY

*Streptococcus pneumoniae* is classified in the Phylum Firmicutes, Class diplococci, Order Lactobacillales, Family Streptococcaceae, Genus Streptococcus, species *S. pneumoniae*. It is gram-positive diplococci, often lancet-shaped. The cells are 0.5 to 1.2 μm in diameter. (54). Colonies of *S. pneumoniae* growing on blood agar are surrounded by a greenish-yellow zone of alpha (α) hemolysis because pneumococci produce pneumolysin that it can breakdown haemoglobin on blood agar. Colonies of pnuemococci are dome-shaped initially but collapse centrally owing to autolysis after the culture ages 24 hours to 48 hours. *S. pneumoniae* can be differentiated from other alpha-hemolytic *Streptococcus* species by its bile solubility and its susceptibility to optochin (55, 56).

# 2. ANTIGENIC STRUCTURE

## Capsule

Capsule is the major determinant of virulence. *S. pneumoniae* survives phagocytosis because of the antiphagocytic protection by its capsule (57). The capsular polysaccharride are soluble and have been called specific soluble substances (SSS). Free polysaccharide can protect viable organisms from phagocytosis by binding with opsonic antibodies. (58). The recognition of more than 90 distinct serotypes of capsule forms the basis of a pneumococcal serotype classification system and have been identified using a technique called the Quellung test or capsular swelling reaction (59, 60).

## Cell wall

The cell wall of *S. pneumoniae* attached to alternating subunits of *N*-acetylglucosamine and *N*-acetylmuramic acid are oligopeptide chains. The major component of the cell wall rich in choline is teichoic acid. The exposed teichoic acid is linked to the peptidoglycan layer and extends through the overlying capsule. This species-specific structure called the C polysaccharide that can be precipitated by a protein found in serum and produced during inflammatory diseases (60, 61). The serum protein is called C-reactive protein (CRP) which it is not an antibody. This is a useful diagnostic test for inflammatory disease. All humans have antibodies to cell wall antigens (62). These antibodies attach to cell wall and bind to the complement. This complex can activate the classic complement pathway. Winkelstein *et al.*(63) and Hummell *et al.*(64) found that C polysaccharide directly activated the alternate complement pathway. The C5a, interleukin-1 and tumor necrosis factor (TNF) were released, causing fever and other manifestations of sepsis (65).

## Pneumolysin

Pneumolysin is a cytoplasmic toxin similar to the streptolysin O in *S. pyogenes*. Pneumolysin is a 52.8-KD a polypeptide consisting of 470 amino acids and Owen RH. *et al.* (66) found that C-terminus of pneumolysin bound to cholesterol in the host cell membrane and created pores. This resulted in the leakage of intracellular solutes and an influx of water, leading to osmotic lysis of the cell. Pneumolysin is able to damage a wide range of eukaryotic cells. This activity can destroy the ciliated epithelial cells and phagocytotic cells (67). Houldsworth S. *et al.* (68) found that pneumolysin activates the classic complement pathway, resulting in the production of C3a and C5a. In turn, cytokines such as IL-1 and TNF-α were produced by the activated human leukocytes, leading to the further migration of inflammatory cells to the site of infection, fever and tissue damage.

## Autolysin

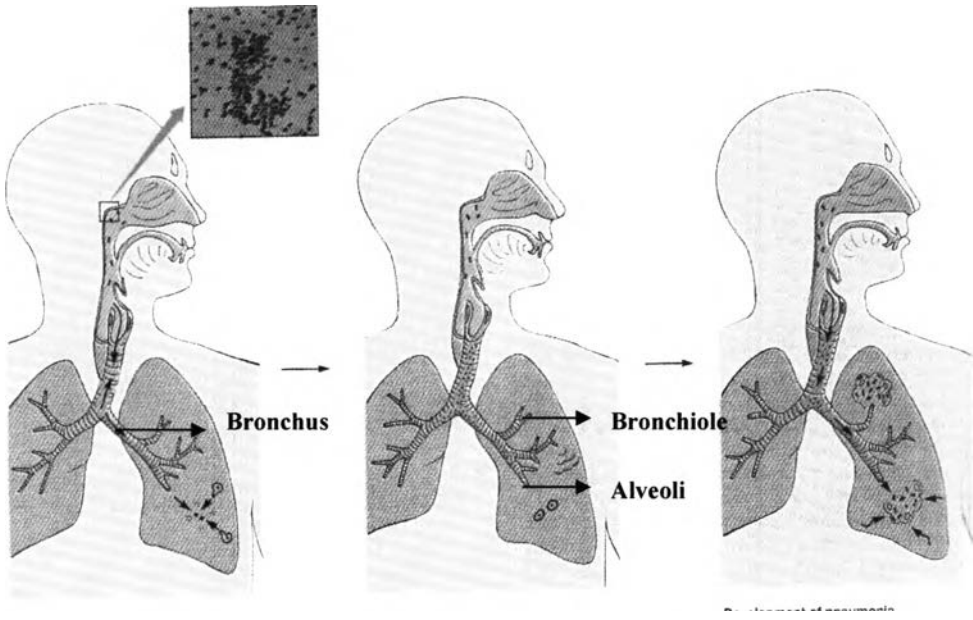
An autolytic enzyme, (an N-acetylmuramic acid L-alanine amidase, is presented in the cell wall and bind to a choline-containing teichoic acid attached to the peptidoglycan (69). The choline is unique to the cell wall of *S. penumoniae* and plays an important regulatory role in cell wall hydrolysis. Choline must be present for activity of the pneumococcal autolysin, during cell division. This enzyme is responsible for cellular autolysis that occurs at the end of log phase in pneumococci and for lysis of the cell wall in response to treatment. Its role in cell-wall turnover means that autolysin activity generates cell-wall breakdown products that are highly inflammatory. The cell-wall-degrading activity of autolysin also allows the release of intracellular toxins (e.g. pneumolysin) into the external medium.Older cells undergo spontaneous autolysin, producing colonies with dimpled center (70).

## Hyaluronidase

Hyaluronidase could facilitate pneumococcal invasion by degrading connective tissue. Pneumococcal strains with higher hyaluronidase activity breach the blood-brain barrier and disseminate more effectively (71). Volkova MO. *et al.*(72) showed that pneumococcal strains isolated from patients with meningitis and meningoencephalitis had significantly higher hyaluronidase activity than strains causing otitis media, indicating the importance of hyaluronidase in the pathogenesis of human pneumococcal meningitis.

# 3. PATHOGENICITY OF *STREPTOCOCCUS PNEUMONIAE*

**Figure 1 Predisposition to and the development of pneumococcal pneumonia (Lansing M. Prescott *et al.*, 2002).**




In the normal condition, *S. pneumoniae* was found in the respiratory tract. Healthy people commonly inhale microorganisms into the respiratory tract without serious consequences because of the host defenses are present there (Figure 1). However, disease usually occurs only in those individuals with predisposing factors such as viral infections of the respiratory tract, physical injury to the tract, alcoholism, or diabetes (60, 70). The mucosal epithelium of the nasopharynx is the primary site of

colonization. The initial colonization of the nasopharynx is mediated by the binding of the bacterial to epithelia cells by means of surface protein adhesions. Pneumonia is likely to occur when mucous containing a load of bacterial cells is aspirated from the nasopharynx into the lungs of susceptible individuals who have lost their defenses. The bacteria passing into the bronchioles and alveoli, the pneumococci multiply and produce the toxin pneumolysin that destroys ciliated epitheliun host cells, induce an overwhelming inflammatory response. The alveoli fill with blood cells and fluid and become inflamed. This is marked by the release of a torrent of edematous fluids into the lungs. In a form of pneumococcal pneumonia termed lobar pneumonia, this fluid accumulates in the alveoli along with red and white blood cells. The sputum is often rust colored because of blood coughed up from the lungs (60, 73).

Furthermore, *S. pneumoniae* can spread to the meninges and cause meningitis. It is even more common for this agent to gain access to the chamber of the middle ear by way of the eustachian tube and cause a middle ear infection called otitis media. This occurs readily in children under 2 years because of their relatively short eustachian tubes. It can also be transported in the blood stream to distal sites such as the brain (73-75).

## 4. DISEASES

### Pneumococcal pneumonia

Normally, 10-30% of healthy individuals carry one or more serologic types of *S. pneumoniae* in the throat. Pneumococcal pneumonia develops when encapsulated pneumococci, the virulent form, are inhaled into the alveoli of a susceptible host, multiply rapidly, and cause an inflammatory response. The bacteria are resistant to phagocytosis because their capsules interfere with the action of C3b, the fraction of complement responsible for opsonization. Serum and phagocytic cells pour into the air sacs of the lung, causing difficulty breathing. This increase in fluid produces abnormal shadows on chest X-ray films of patients with pneumonia. Sputum contains pus, blood, and many pneumococci (76).

About 60-80% of all respiratory disease known as pneumonia are caused by *S. pneumoniae* (60). *S. pneumoniae* is a leading cause of pneumonia in all ages (particularly the young and old). It provokes intense inflammation and exudates formation. The blocking of the bronchioles and alveoli by consolidation of inflammatory cells and products is evident (77). The an estimated 150,000 to 300,000 people in the United States contract this form of pneumonia annually, and between 13,000 to 66,000 deaths result (78).

## Meningitis

*S. pneumoniae* can spread into the central nervous system after bacteremia, infections of the ear or sinuses. Proinflammatory cytokines (TNF-α, IL-1, IL-6) were released by mononuclear cells within the central nervous system (CNS) such as ependymal cells, astrocytes and macrophages/microgia. These cytokines trigger a complex cascade of inflammatory mediators, which in concert regulate the various arms of the inflammatory response (79). The cytokines released induce an opening of the tight junctions between brain capillary endothelial cells associated with enhanced pinocytotic activity, allowing an influx of serum components, notably chemotactic complement factors (80). The most important consequence of this inflammatory reaction in the CNS is increased intracranial pressure, which results from cerebral oedema, increased cerebral blood volume and alterations of CSF hydrodynamics.This increased intracranial pressure, plays a significant role in reducing cerebral blood flow, resulting in ischaemic necrosis and neuronal loss from energy failure (81, 82). Several vasoconstrictive mediators such as endothelin and reactive oxygen intermediates (NO production) that is important mechanism by which brain damage occurs.

*S. pneumoniae* causes meningitis in all age groups and is the most common cause of bacterial meningitis in middle-aged and elderly populations and in patients with dural defects. Meningitis accounts for about 10% of invasive pneumococcal infections. Its incidence in developed countries is about 1.5 per 100,000, rising to 8 per 100,000 in children below the age of 5 years (83). In the United States, *S. pneumoniae* is the most frequent cause of bacterial meningitis. Based on data from a population-based, multistate active surveillance system, the estimated annual incidence of

pneumococcal meningitis in the United States is 1.1 cases per 100,000 population (or ~ 3000 cases), with an estimated case-fatality rate of 21% (84).

## Otitis media

Otitis media, an inflammation of the middle ear associated with fever earache, and abnormalities of hearing, is most commonly caused by the pneumococcus. The bacteria from the nasopharynx enter the middle ear through the eustachian tube. Seventy percent of pneumococci isolated from the middle-ear cavity of infants and children with acute otitis media were of one of the seven serotypes 1, 3, 6, 14, 18, 19, or 23 (85).

## Sinusitis

*S. pneumoniae* is a common cause of acute infections of the paranasal sinuses. When infection extends from the nose into one of the sinuses and sets up an inflammation there. (86). Diseases that obstruct drainage can result in a reduced ability of the paranasal sinuses to function normally. The sinus ostia may become occluded, leading to mucosal congestion. The mucociliary transport system becomes impaired, leading to more stagnation of secretion and epithelial damage, followed by decreased oxygen tension and subsequent bacterial growth.

## Bronchitis

Bronchitis or tracheobronchitis may be a primary manifestation of infection or a result of spread from upper respiratory tissues. It is characterized by cough, variable fever, and sputum production, which is often clear at the onset but may become purulent as the illness persists (87). Chronic bronchitis is a result of long-standing damage to the bronchial epithelium. A vicious cycle of recurrent infection may evolve, leading to further damage and increasing susceptibility to pneumonia (88).

## 5. TRANSMISSION

*S. pneumoniae* is an exclusively human pathogen and in most case the source of human disease is endogenous present in the respiratory tract. Person-to-person aerosol transmission occurs from extensive, close contact. Daycare center attendance and crowed living conditions (persons, nursing homes and homeless shelters) are associated with an increased transmission rate, but casual contact is usually not. The most common predisposing factors are viral infections of the respiratory tract, physical injury to the respiratory tract from inhaling toxic or irritating substances, including anesthetic gases, prolonged immobilization in bed which may result in accumulation of fluids in the lungs, alcoholism, increasing age, diabetes, and immunodeficiency diseases such as Hodgkin's diseases, sickle cell anemia and AIDS.

## 6. EPIDEMIOLOGY

Infections with *S. pneumoniae* cause significant morbidity and mortality. Community-acquired pneumonia (CAP) still remains one of the most important causes of death, especially in older adults and those with co-morbid diseases, despite advances in modern medicine (89, 90). Most infections are caused by endogenous spread from the colonized nasopharynx to distal site (e.g., lungs, sinuses, ears, blood, meninges). In the most severe forms of pneumonia, the alveoli, or air sacs, in the lungs may be destroyed or filled with fluid so that a person no longer can breathe (2). In the United States, pneumonia is the sixth leading cause of death, and the number one cause of death from infectious disease (91). Because pneumonia is not a reportable illness, information about its incidence is based on crude estimates, but it appears that up to 5.6 million cases of community-acquired pneumonia (CAP) occur annually, and as many as 1.1 million of these require hospitalization (92). In the outpatient, the mortality rate of pneumonia remains low, in the range of <1-5% but among patients with CAP who require hospitalization, the mortality rate averages 12% overall and approaches 40% in those who are most ill and who require admission to the intensive care unit (93-97). Breiman RF. *et al.* (98) found that invasive penumcoccal infecton is relatively common in neonates and children below the age of 2 years (160 per

100,000), the incidence falling sharply in adolescent and young adult years (5 per 100,000), to rise again in populations over the age of 65 years (70 per 100,000).

*S. pneumoniae* also can migrate from the nasal passages into the ear region, where it causes several million cases of otitis media or middle ear infection (99, 100), meningitis (101, 102), bacteremia (103) and other infectious processes. Pneumococcal pneumonia occurs in all age groups (104), but it is more common in people over 40 years of age (105, 106). *S. pneumoniae* meningitis affects mainly young children, especially those who are 2 years old or younger (83, 104, 107). Asymptomatic carriage of penumococci in the throat or nasopharynx is widespread, with carriage rates being especially high in children.

In Europe, community-acquired pneumonia (CAP) is a common condition affecting about 1/1,000 of the adult population per year. It occurs when bacteria enter the alveolar spaces of the lung initiating an inflammatory response which leads to the clinical features of cough, sputum production, breathlessness and sometimes chest pain and haemoptysis. Studies in Spain (108, 109), Finland (110) and England (111) have suggested frequencies of 1.6, 2.6, 4.7 and 9 cases per 1000 of the general adult population per year. The frequency of the condition is age-related with the highest rates in the very young and very old. A study from Finland found rates of 36 of 1000 in those aged < 5 yrs falling to 4.4 of 1000 in the 15–29 age group and rising again to 34.2 of 1000 in those aged >74 yrs (110). Of those in the community, between 8% (112) and 51% (108) are admitted to hospital and between 4% and 15 % of such patients will die. Its frequency, morbidity and mortality are the reasons why CAP is such an important disease. Almirall J. *et al.* studies to epidemiology of CAP for adults in Spain from December 1993 to November 1995, found that pneumonia is substantially more common in winter and affects more males (n=140) than females (n=110). It is commoner amongst older persons, the annual incidence of pneumonia that requires hospitalization of those older than 64 years is 1.12-3.16 cases per 1,000, for those aged 40-64 years it is 0.54 cases per 1,000 persons (109).

In Asia 2008, ANSORP (Asian network for surveillance of resistant pathogens) study epidemiology of CAP in adult patients in Asian countries. By performed a prospective observational study of 955 cases of adult CAP in 14 hospitals in eight

Asian countries (South Korea, China, Taiwan, Singapore, Viet nam, Philippines, Hong Kong and India). The overall mortality rate in 777 patients was 7.3%, and nursing home residence, mechanical ventilation, malignancy, cardiovascular diseases, respiratory rate > 30/min and hyponatraemia were significant independent risk factors for mortality by multivariate analysis ($P < 0.05$). *Streptococcus pneumoniae* (29.2%) was the most common isolate, followed by *Klebsiella pneumoniae* (15.4%), *Haemophilus influenzae* (15.1%) *Mycoplasma pneumoniae* (11.0%) and *Chlamyophila pneumoniae* (13.4%). Only 1.1% was positive for *Legionella pneumophila* (113).

In Thailand 1999, lower respiratory tract infections accounted for 9,500-150,000 cases each year and resulted in 800-1,500 deaths. The incidence of pneumonia was highest among children below five years of age. Mortality rate of pneumonia was 30.5% among all hospitalized patients and 42.7% in those over 60 years of age (114).

In northern Canada from 1999-2005, The crude annual incidence rate for *S. pneumoniae* was highest in 2001 (38.4/100,000 population) and lowest in 2005 (17.3/100,000 population). The incidence of *S. pneumoniae* was highest among adults ≥65 years of age. The most common primary clinical finding for invasive *S.pneumoniae* was pneumonia (64.5%), followed by bacteremia/septicemia (21.5%) (115).

## 7. LABORATORY DIAGNOSIS

### MICROSCOPY

Gram stain of specimens is a rapid way to diagnose pneumococcal disease. *S. pneumoniae* characteristically appear as lancet-shaped, gram-postive diplococci surrounded by an unstained capsule (70, 116).

### CULTURE

The specimens were inoculated onto an enriched nutrient medium supplemented with blood. After overnight incubation, typical pneumococcal colonies

are round and glistening with entire edges, transparent and about 1 mm in diameter. They are surrounded by 2-mm zone of alpha-hemolysis (70, 116).

## IDENTIFICATION

**1. Catalase test.** *S. pneumoniae* does not produce the enzyme catalase and can not reduce diatomic oxygen to hydrogen peroxide or superoxide. These catalase-producing organism will catalyze the breakdown of hydrogen peroxide ($H_2O_2$) to oxygen and water. Oxygen is released from the hydrogen peroxide in the form of bubbles. If bubbles or froth forms, the organism is said to be *catalase-positive* if not, the organism is *catalase-negative* (116).

**2. Bile solubility**. Surface-active agents, such as bile, bile salts, act on the cell wall of pneumococci and bring about lysis of the cell. Isolates of *S. pneumoniae* are lysed rapidly when a few drops of 10% sodium deoxycholate solution are added on an isolated colony. Most colonies of *S. pneumoniae* are dissolved within a few minutes whereas other alpha-hemolytic streptococci are not lysed by bile (117).

**3. Optochin susceptibility**. *S. pneumoniae* can also be identified from its susceptibility to optochin (ethyl-hydrocupreine dihydrochloride). The optochin is placed in the middle of the inoculum. A zone of inhibited bacterial growth is seen around the optochin disk after overnight incubation. (118).

## 8. TREATMENT OF *S. PNEUMONIAE* INFECTION

*S. pneumoniae* is an important cause of both invasive and noninvasive infections in all age groups throughout the world (119-121). The drug of choice for treating infections caused by *S. pneumoniae* has been penicillin G. However, the first penicillin resistant *S. pneumoniae* (PRSP) was isolated in 1967, and since then penicillin-resistant strains have steadily become more common (122). In a study of 30 medical centers in the United States, more than 23% of *S. pneumoniae* isolates obtained between 1994 and 1995 were resistant to penicillin (123, 124). Worldwide,

incidence rates of PRSP ranging from 33% to nearly 80% have been reported (125-128).

Macrolides and chloramphenicol have been used to treat cases involving PRSP, and case involving *S. pneumoniae* that is resistant to multiple antibiotics are treated with vancomycin (129). Erythromycin are now associated with significant rates of resistance (6, 8, 130, 131). Macrolides are used to treat infections such as respiratory tract and soft tissue infections. The antimicrobial spectrum of macrolides is slightly wider than that of penicillin, and therefore macrolides are a common substitute for patients with a penicillin allergy. Beta-hemolytic streptococci, pneumococci, staphylococci and enterococci are usually susceptible to macrolides. Unlike penicillin, macrolides have been shown to be effective against *Mycoplasma*, *Mycobacterium*, some *Legionella* and *Chlamydia* species (6, 7, 130, 131). However, pneumococci nonsusceptible to the macrolides have been rapidly increasing, and could develop during therapy, leading to treatment failure (4, 8, 132).

## 9. MACROLIDES

Macrolides have a common form by a large lactone ring. Erythromycin is a mixture of antibiotics that includes erythromycin A. Erythromycin is produced from a strain of the actinomycete *Saccharopolyspora erythraea*, formerly known as *Streptomyces erythraeus*. Erythromycin is one of the macrolide antibiotics which consist of a large ring to which sugars are attached. In structure, this macrocyclic compound contains a 14-membered lactone ring with ten asymmetric centers and two sugars (L-cladinose and D-desoamine) (Figure 2-3). Erythromycin, clarithromycin, azithromycin and dirithromycin are the macrolides currently available for clinical use in the United States. The 15-membered ring resulting from a nitrogen insertion. The structural modifications of erythromycin A result in improved pharmacokinetic profiles and better tolerance, but cross-resistance between members of this class of antimicrobial agents was still observed. The 16-membered ring (spiramycin, rokitamycin, josamycin, midecamycin and miocamycin) are also available in a few countries (10, 133, 134). Erythromycin has an antimicrobial spectrum similar to or

slightly wider than that of penicillin and is often used for people that have an allergy to penicillins. For respiratory tract infections, it has better coverage of atypical organisms, including mycoplasma and legionellosis. It is also used to treat outbreaks of chlamydia, syphilis, acne and gonorrhea. Although erythromycin is still a very useful drug, the newer macrolides have a lower incidence of side effects than erythromycin. The newer macrolides produce less gastrointestinal irritability, are more stable in gastric acid, are better absorbed from the gut, have better tissue penetration and possess longer half-lives, permitting once- or twice daily administration (135, 136).

**Figure 2 Group of macrolides (Douthwaite S. *et al.*, 2001).**

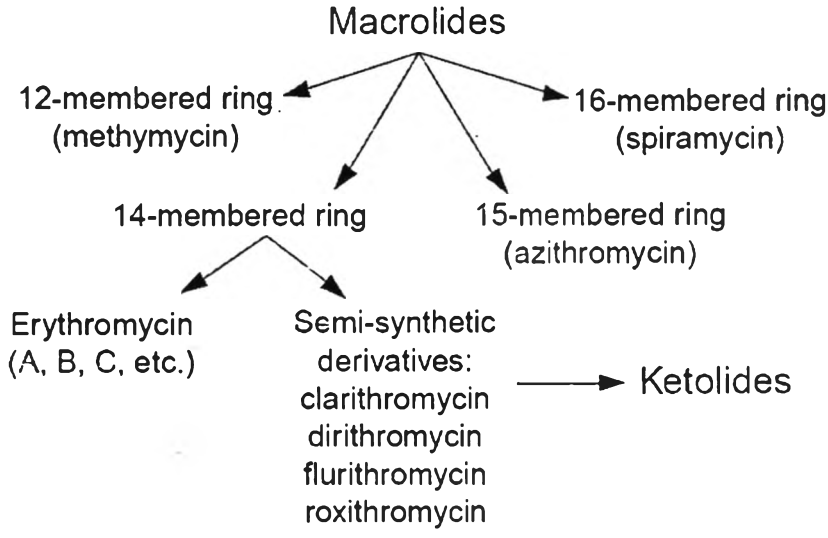
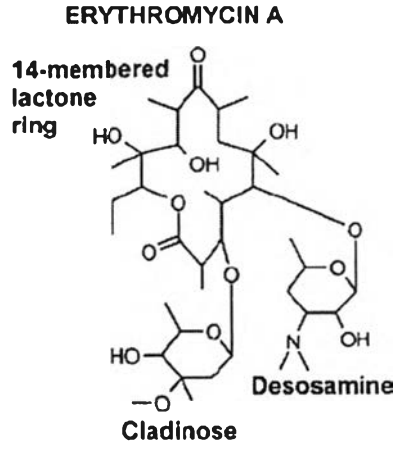
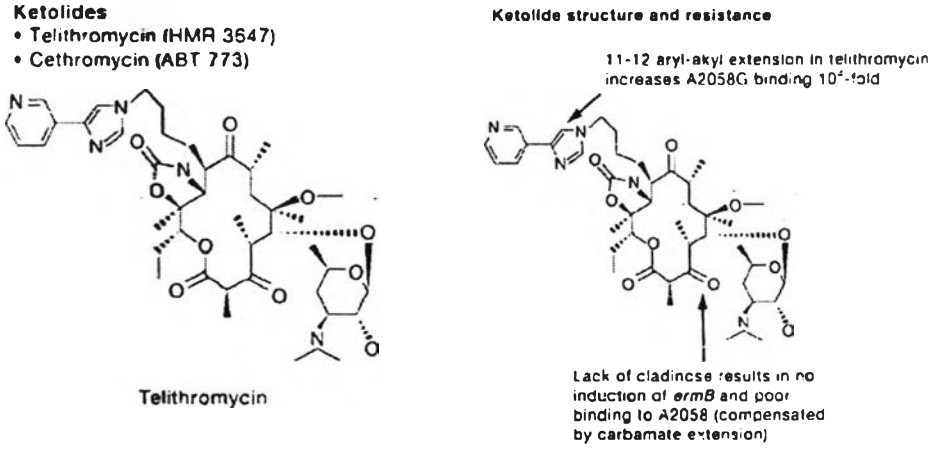



**Figure 3 Structure of erythromycin (Tenson T.,2003).**



The recently developed ketolides telithromycin and ABT773 are derived from clarithromycin and have two major modifications, replacement of L-cladinose by a keto group and an 11- to 12-carbamate extension with an arylakyl modification in telithromycin (Figure 4). In most circumstances, the ketolides are uniquely active, retaining activity despite the emergence of resistance to macrolides, lincosamides and streptogramin B agents (137, 138).

**Figure 4 Structure of ketolides (Edelstein PH., 2004).**

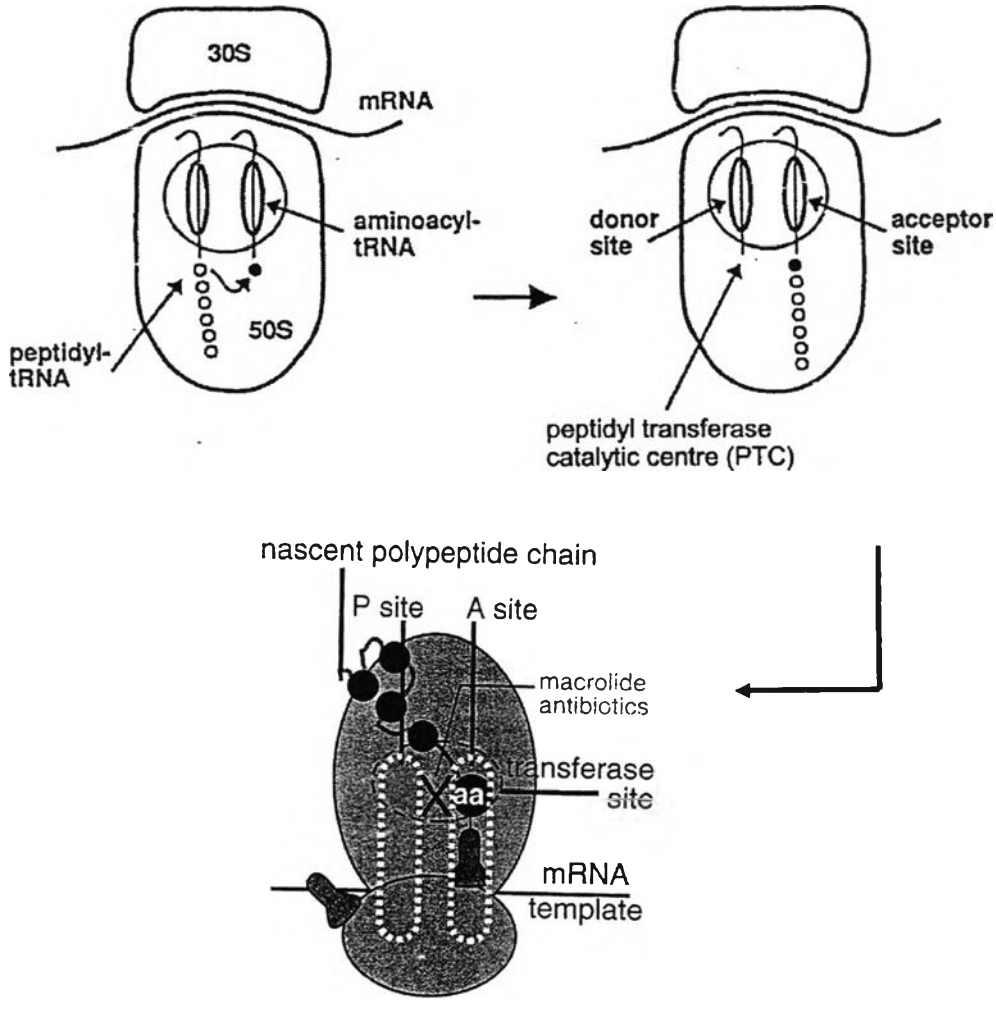



## MECHANISM OF ACTION OF MACROLIDES

All the macrolides have a mechanism of action identical to that of erythromycin. Erythromycin binds in a specific manner to the 50S subunit of the bacterial ribosome. It does not bind to mammalian 80S ribosomes and this accounts in part for its selective toxicity. Like chloramphenical, erythromycin can inhibit protein synthesis on ribosomes from mammalian mitochondria. Competition experiments indicate that the erythromycin binding site on the 50S ribosome subunit overlaps or interacts with the binding sites for chloramphenicol and the lincomycin. The binding of [$^{14}$C]chloramphenicol to bacterial ribosomes is prevented by erythromycin and lincomycin, but the binding of [$^{14}$C]erythromycin is not inhibited by chloramphenicol or lincomycin. The binding of [$^{14}$C] lincomycin is inhibited by erythromycin. Erythromycin prevents the binding of chloramphenicol to isolated 70S ribosomes but

not ribosomes in polysome form. If peptidyl-tRNA is removed from polyribosomes, however, chloramphenicol binding is inhibited by erythromycin. This suggests that the erythromycin binding site is in the peptidyl-tRNA binding region (P site) on the 50S ribosome subunit (Figure 5). The binding site of erythromycin is composed of domain V sequences near the peptidyltransferase center, where the polypeptide chain is synthesized. It is not completely clear whether erythromycin inhibits protein synthesis by inhibiting peptide bond formation or by interfering with the subsequent translocation step. Several studies both *in vivo* and *in vitro* strongly suggest that erythromycin inhibits the translocation step. The presence of erythromycin on the 50S subunit may inhibit translocation by preventing the proper association of the peptidyl-tRNA with its binding site after formation of the peptide bond (10, 134, 139-143).

**Figure 5 Peptide bond synthesis catalysed by the peptidyl transferase center of the 50S ribosome subunit (Courvalin P. *et al.*, 2002 and Cocito C. *et al.*, 2002).**

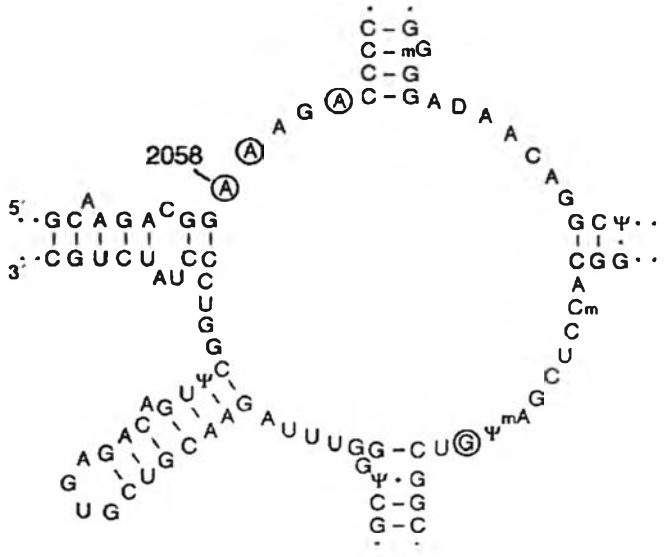


## 10. MECHANISM OF MACROLIDE RESISTANCE

There are two main mechanisms of resistance to macrolides, The first mechanism described is based on ribosomal protection due to acquisition of the *erm* (B) gene. The *erm* (B) positive isolates are resistant to macrolides, lincosamides, and streptogramin B ($MLS_B$ phenotype), is due to the overlapping binding sites of these drugs and exhibit high-level resistance to erythromycin (MICs above 64 μg/ml). The *erm* (B) gene encodes a ribosomal methylase which dimethylates pneumococcal 23S rRNA of 50S ribosomal subunit at a single site, adenine at position 2058 (*Escherichia coli* numbering) (Figure 6). These modification precisely reduces the affinity of erythromycin for its target, probably by preventing direct access to the target or by modifying the conformation of the binding site (14, 52, 133, 144).

More recently, erythromycin resistance in clinical isolates of *S. pneumoniae* harboring the *erm* (A) subclass *erm* (TR) gene has been described mostly in *Streptococcus pyogenes* (145) such as in Italy (146), France (147), Spain (148) and Canada (149), but it has been described reported in pneumococci from Greece (150) and Spain (19). The streptococcal *erm* (B) gene is associated with conjugative transposons of the Tn*916*-Tn*1545* family that also confer resistance to tetracycline [by the *tet* (M) gene] and/or kanamycin [by the *aph (3')-III* gene] (151, 152). In Europe, South Africa and Asia, the *erm* (B) predominant the macrolide-resistant *S. pnemoniae* more than North America (17, 19, 28, 151, 153, 154).

**Figure 6 Structure of domainV of 23S rRNA (Courvalin P. *et al.*, 2002).**

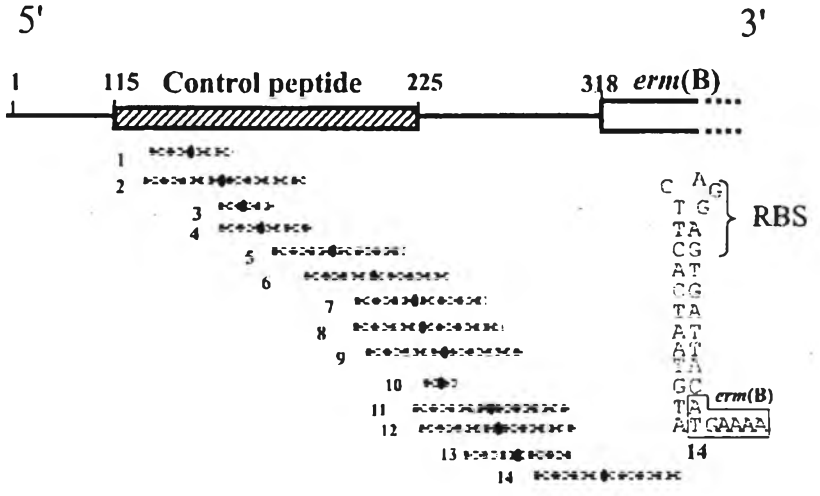



## Regulation of *erm* (B) expression and the $MLS_B$ resistance phenotype

Ribosome modification involving a ribosome methylase, associated with the *erm* (B) gene (erythromycin ribosome methylase). The *erm* (B) gene is associated with high-level resistance to macrolides, lincosamides, and streptogramin B ($MLS_B$ phenotype). Expression of $MLS_B$ resistance can be constitutive ($cMLS_B$) or inducible ($iMLS_B$) (155). Inducible resistance was indicated by blunting of the clindamycin zone on the side closest to the erythromycin disk resulting in a D-shaped zone. When expression is constitutive, the *erm* (B) mRNA is active and its translation by the ribosomes allows constitutive methylation of the ribosomes, probably while they are synthesized. If resistance is inducible, mRNA of *erm* (B) was synthesized but in an active form and becomes active form only in the presence of inducing macrolides. The 5' end of *erm* (B) presents a series of inverted repeats which are responsible for the lack of methylase synthesis in the absence of erythromycin. Inducible mRNA had a repeat fourteen base pairs and form the alternative stem-loop structures (Figure 7), the number 1 to 14 indicate inverted repeated with their symmetry axes and RBS is ribosome binding site. However, these stem-loops sequesters the ribosome binding site and initiation codon for the methylase. Thereby, the methylase can not be produced, since the initiation motifs for translation of the enzyme are not accessible to the ribosomes. These induction, to relate the presence of sequences coding for a small

leader peptide of 36 amino acid upstream from the *erm* (B) gene. The low concentrations of erythromycin, binding to a ribosome translating the leader peptide causes the ribosome to stall, in turn destabilizing the pairing of the inverted repeats and inducing form rearrangments in the mRNA. Therefore, the *erm* (B) mRNA is active, and its translation by the ribosomes allows methylation of the ribosomes (10).

**Figure 7 Schematic representation of the structure of the mRNA from the inducible *erm* (B) gene. (Courvalin P. *et al.*, 2002).**



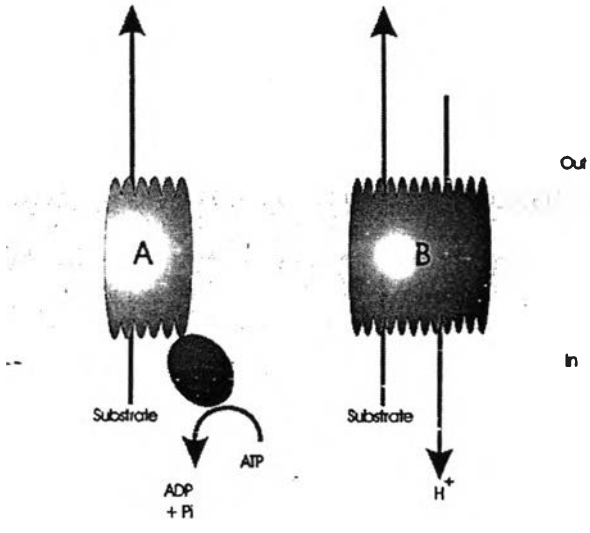

The second mechanism is active efflux due to the acquisition of the *mef* gene was identified in isolates expressing a low-level resistance to erythromycin (MICs ranged from 1 to 32 μg/ml). The *mef* genes specific for 14- and 15-membered macrolides but susceptible to 16-membered macrolides, lincosamides and streptogramin B. Since 16-membered macrolides, lincosamides, and streptogramin B antibiotic are not substrates of the pump. The phenotype conferred by these pumps was designated as M phenotype. Mef pumps belong to the Major facilitator superfamily of transporters with 12-transmembrane spanning domains in the cytoplasmic membrane, driven the proton motive force (Figures 8-9). The level of resistance provided by the *mef* genes lower than the *erm*-mediated resistance (10, 27, 49).

Mef proteins exist in both Gram positive bacteria and Gram negative bacteria as well as in fungi and mammalian (tumour) cells (156, 157). Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) can inhibit efflux effect in *S. pneumoniae* by destroy

the proton gradient of bacterial transcytoplasm membrane, causing transport proteins to lose energy supply and eventually leading to increasing accumulation of drug concentration (158, 159).

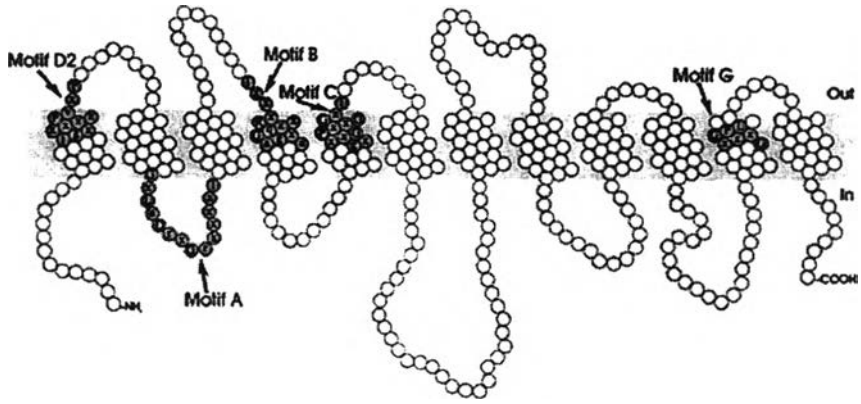
Recently, there were several reports of clinical strains of *S. pneumoniae* that do not contain either *erm* (B) or *mef* gene. These isolates had changes in 23S rRNA genes (A to G at position 2059 *E. coli* numbering) or changes in a highly conserved region of ribosomal protein L4 ($_{69}$ GTG $_{71}$ to TPS mutation or a 6-amino-acid L4 insertion, $_{69}$ GTG*REKGTG*RAR) which can confer a macrolide (M) and linconsamide (L)-resistant (ML) phenotype or macrolide (M) and streptogramin$_B$ ($S_B$)-resistant ($MS_B$) phenotype.

**Figure 8 Schematic representation of the two major classes of multidrug transporters (Putman M. *et al.*, 2000).**



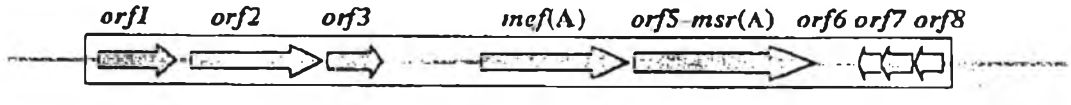

A : ABC-type multidrug transporters utilize the free energy of ATP hydrolysis to pump drugs out of the cell, B : Secondary multidrug transporters mediate the extrusion of structurally unrelated drugs in a coupled exchange with protons or sodium

**Figure 9 Structural model for the 12-TMS multidrug transporters of the MFS. (Putman M. *et al.*, 2000).**




There are two subclasses of the *mef* (A/E) gene : The *mef* (A) gene was first identified in *Streptococcus pyogenes* (26) while *mef* (E) was found in *Streptococcus pneumoniae*(12). The two *mef* genes showed 90% DNA identity and were regarded as a single gene class designated *mef* (A) (40). Although *mef* (A) and *mef* (E) are 90% identical at the nucleotide level, they are characterized by major differences (42). Recently, the genetic elements carrying *mef* (A) and *mef* (E) were first characterized in *S. pneumoniae*. The *mef* (A) gene has been shown to be part of a 7.2 kb defective transposon (Tn*1207.1*) that carries eight open reading frames (ORFs), of which the first 5 have the same direction of transcription, while *orf*6, *orf*7, and *orf*8 are oriented opposite to the others (Figure 10). Between *orf*3 and *mef* (A) there is an intergenic region with a high potential for the formation of hairpins. *mef* (A) is the fourth and *orf2* encodes a resolvase/invertase (43).

**Figure 10 Structure of the chromosomal genetic element Tn*1207.1*, which is 7,244-bp long (GIANNI POZZI G. *et al.*, 2000).**



In 2001, Gay & Stephens described a 5.4 or 5.5 kb genetic element containing a *mef* (E) gene called MEGA (macrolide efflux genetic assembly) (Figure 11). The sequence immediately 3' of *mef* (E) contained a 1,464-bp ORF with the same orientation (Figure 12). In MEGA, the open reading frame (ORF) sequence 3' of the *mef* (E) gene was designated *mel* gene which a 119-bp intergenic region was between *mef* (E) and *mel* gene. This region contained a consensus Shine-Dalgarno sequence upstream from the predicted start codon for *mel*. The *mel* gene ORF is also a homologue of the macrolide and streptogramin B resistance protein A (*msrA*) in stapylococci, which encodes an ATP-binding cassette to provide the energy for efflux (49, 51, 160-163). The *mef* (E) and *mel* are co-transcribed, which suggests that both are required for efflux (52). As well as of downstream from *mef* (A) lies a gene that putatively *msrA* gene in *Staphylococcus epidermidis* encodes an ATP binding cassette transporter, most commonly involved in the uptake of nutrients that requires a periplasmic binding protein (160, 163).

**Figure 11 Structure of the MEGA element which is 5,500-bp long (Gay K. *et al.*, 2001).**

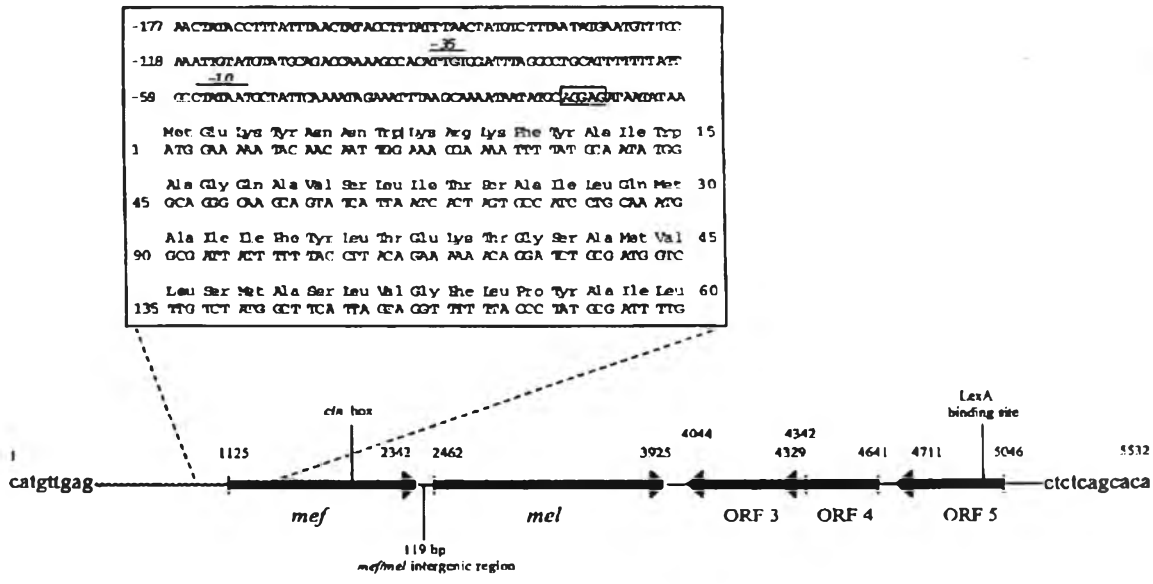
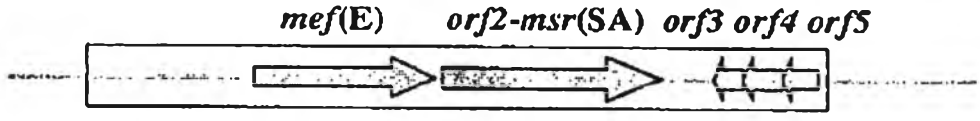


**Figure 12 Structure of the chromosomal genetic element *mef* gene, which is 1,218 bp long (Grosso M. Del., 2002).**




The mega element, was found to be inserted in a Tn*916*-like element, in a sequence homologous to orf6 of Tn*916*. The integration of the mega element into a Tn*916*-like transposon generates a new composite element of approximately 23.5 kb that designated Tn*2009* (Figure 13). Tn*2009* carries determinants for tetracycline and erythromycin resistance and is apparently transferred by transformation to pneumococci (164). The new composite element of approximately 26.3 kb, carrying *mef* (E), *tet* (M), and *erm* (B), was found in *S. pneumoniae* and designated Tn*2010* (Figure 14) (50).

**Figure 13 Structure representation the Tn*2009*, composed of the mega element inserted into a Tn*916*-like transposon (Pantosti A., 2004).**

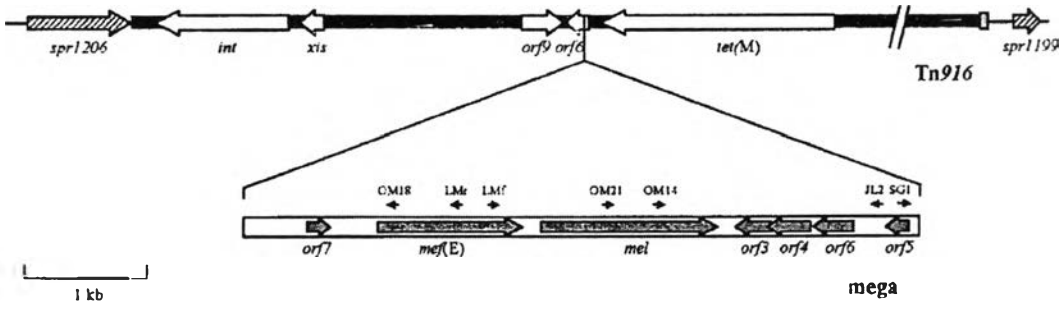
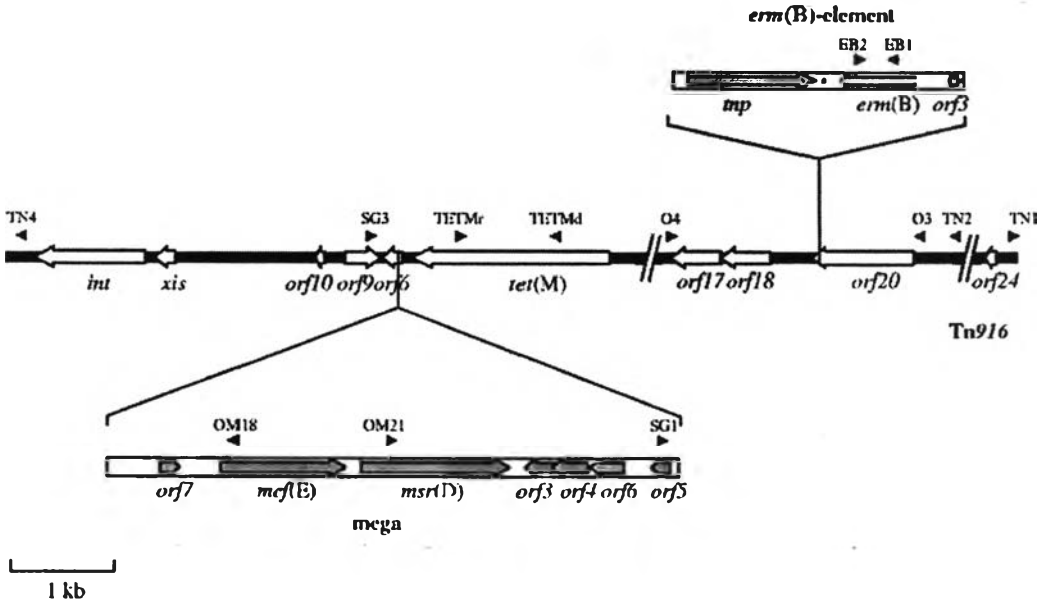


**Figure 14 Structure representation the Tn*2010*, composed of the mega element and *erm* (B) gene inserted into a Tn*916*-like transposon (Pantosti A., 2006).**




Of the two variants of the *mef* gene, *mef* (A) was originally found in *S. pyogenes* (26) and *mef* (E) was originally found in *S. pneumoniae* (12). *mef* (A) and *mef* (E) are 90% identical at the nucleotide level and did not distinguish between the two variants (40). However, the two variants were considered species specific, if a *mef* gene was found in *S. pneumoniae*, it was generally assumed to be *mef* (E). However, *mef* (A) was shown to be present in macrolide-resistant *S. pneumoniae* isolates such as study by Oster P. *et al.* (165) in 1999 from Italy reported that macrolide-resistant *S. pneumoniae* carried *mef* (A). Therefore, the *mef* (A) located in *S. pyogenes* could be transferred into *S. pneumoniae* whereas the *mef* (E) genes also located in *S. pneumoniae* could be transferred into *S. pypgenes* recipients. Furthermore, the *mef* (A) gene may also be widespread among gram-negative such as *Acinetobacter junii* (45, 166), *Neisseria gonorrhoeae* isolates (45). In addition, a study by Ijo K.K. *et al.* (167) found that the *mef* gene identified in gram-negative 13 genera carried *mef* (A) gene, including *Acinetobacter* spp, *Citrobacter* spp, *Enterobacter* spp, *Escherichia* spp, *Klebsiella* spp, *Morganella* spp, *Pantoeae* spp, *Providencia* spp, *Pseudomonas* spp, *Proteus* spp, *Ralstonia* spp, *Serratia* spp. *and Stenotrophomonas* spp. All of *mef* genes have been able to horizontal transfer to a variety of recipients.

Recently, a new variant designated *mef* (I) has been described in two pneumococcal isolates from Italy (168) showed that the *mef* gene of these two isolates did not match with the *mef* (E) gene of the mega element (93.6% homology) and which exhibited comparable homology (91.4%) to the *mef* (A) gene of the Tn*1207.1* transposon, was designed *mef* (I).

## 11. OTHER MECHANISM OF MACROLIDE RESISTANCE AMONG *S. PNEUMONIAE*

*S. pneumoniae* was resistant to 14-, 15-, and 16-membered ring macrolides and to streptogramin B and were susceptible, intermediate, or low-level resistant to clindamycin (lincosamide). These strains did not carry *mef* (A/E) or *erm* (B) resistance genes (169). $MLS_B$ and M phenotypes were also described in *erm* (B)-negative and *mef* (E)-negative strains, indicating the presence of novel genes or allelic variants of already identified genes. Tait-Kanradt *et al.* in 2000 decribed mutations in the peptidyl transferase loop of the 23S rRNA and ribosomal protein L4 as a cause of a new resistance type in pneumococci (47, 170). However, mutations in 23S rRNA and the L4 ribosomal protein also seem to confer macrolide resistance in at least (171-173).

In Finland, Pihlajamaki M. *et al.* showed mutations in domain V of the 23S rRNA or ribosomal protein L4 at position A2059C, A2059G, C2611G and $_{69}GTG_{71}$-to-$_{69}TPS_{71}$ substitution. They were resistant to marolides and streptogramin B but susceptible to lincosamides and no acquired macrolide resistance genes (174). In Germany, Reinert R.R. *et al.* showed that multiple mutations in the 23S rRNA (at position A2058G, A2059, G211A, T389C and A2937G), mutations in ribosomal protein L4 (at position A197V and R95H) and L22 (at position C117T) accounted for macrolide resistance in pneumococci (175).

## 12. PREVALENCE OF MACROLIDES-RESISTANT *S. PNEUMONIAE*

The rapid development of macrolide resistance in *S. pneumoniae*, especially over the last decade, is of major clinical concern. Established in 1999, the PROTEXT (Prospective Resistant Organism Tracking and Epidemiology for the Ketolide Telithromycin) worldwide surveillance study to monitor longitudinally the susceptibility of common respiratory pathogens from patients with community-acquired respiratory tract infections (CARTI) to current and new antimicrobials. The results from 2,371 isolates collected during 2000 by North America centers. Overall, 21.3% of pneumococci (n=687) were penicillin G-resistant (Canada, 10.3%; USA, 32.6%) and corresponding rates o erythromycin resistance were 16.3% and 31.5% but telithromycin inhibited all penicillin- and erythromycin-resistant isolates at ≤1 μg/ml (29). In 2000-2001 PROTEKT US study, of the 31% (n=3,133) erythromcin-resistant *S. penumoniae* isolates collected during year 1 of sutdy, five resistance genotypes were *mef* (A) 70.9% (n=2,157), *erm* (B) 17.4% (n=530) , *mef* (A) *erm* (B) 10.0% (n=304) and *erm* (TR) 0.2% (n=5). The second year of the PROTEKT US study (2001-2002), 68.7% *mef* (A), 16.8% *erm* (B) and 12.2% *mef* (A) *erm* (B) (30).

Interestingly, in 2008 PROTEKT US have shown that the predominant mechanism of pneumococcal macrolide resistance in the USA is mediated by *mef* (A). However, the latest data presented (during 2000-2004) here confirm that the prevalence of the *mef* (A) genotype is decreasing and that clones expression both *mef* (A) *erm* (B) genes are increasing in prevalence (34).

In 2004, Asian Network for Surveillance of Resistant Pathogens (ANSORP) had a report erythromycin resistance in *S. pneumoniae* isolates from 10 Asian countries during 1998-2001, a total of 555 erythromycin-resistant *S. pneumoniae*. The percentage of isolates resistant to erythromycin were 59.3% (n=329). Vietnam had the highest prevalence 88.3%, followed by Taiwan 87.2%, Korea 85.1%, Hong Kong 76.5% and China 75.6%. This is in contrast to India (1.5%) and Sri Lanka (10.3%), which showed the lowest prevalence rates. In most countries, $MIC_{90}$ of erythromycin

among pneumococcal isolates were 128 μg/ml or higher, except among isolates from Thailand and India (4 and 0.12 μg/ml), respectively.

The prevalence of macrolide resistance amongst *S. pneumoniae* in different geographic areas in each country is shown in Table 1. The *erm* (B) gene predominant in Europe ans South Africa (such as 67.9% Greece, 86% Spain, 90.9% Belgium 46.7% Canada 92.4%Tunisia and 83.3% South Africa) (150, 176-178). It confers high-level resistance (MIC >512 μg/ml) whereas *mef* (A) and *mef* (E) are found less often. In contrast, most macrolide-resistant penumococcal strains in North America harbor *mef* (A) gene, which confers constitutive low-level resistance (MIC <64 μg/ml) (34, 35). However, some European countries such as Germany, Norway, Finland and Austria have reported an increasing incidence of the efflux mechanism, similar to rates in North America centres (17, 36-39).

**Table 1 Prevalence of antibiotic resistance amongst *S. pneumoniae* in different geographic areas.**

| REPORT Country /region | Year (collected) | No. of test isolates | Percentage of erythromycin resistance % | $MIC_{50}$ / $MIC_{90}$ of erythromycin resistance | Percentage of penicillin resistance % | Mechanism of erythromycin resistance % | | | | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | *mef* (A) | *erm* (B) | *mef* (A)+ *erm* (B) | Ribosomal mutation and other | |
| PROTEKT US | 2000-2001 | 10,103 | 31.0 | 0.12 / 16 | 12.5 | 68.8 | 16.9 | 9.7 | 4.6 | (34) |
| PROTEKT US | 2001-2002 | 10,012 | 27.9 | ≤0.06 / 16 | 14.2 | 67.3 | 16.5 | 12 | 4.2 | (34) |
| PROTEKT US | 2002-2003 | 10,886 | 29.2 | 0.12 / 64 | 15.3 | 63.9 | 16.5 | 16.4 | 3.2 | (34) |
| PROTEKT US | 2003-2004 | 8,494 | 29.1 | ≤0.06 / 64 | 20.0 | 65.7 | 16.6 | 13.9 | 1.4 | (34) |
| Canada | 1998-2004 | 865 | 100 | 2 / 4 | ND | 46.7 | 42.9 | 5.8 | 4.6 | (35) |
| Germany | 1992-2004 | 3,845 | 11.2 | ND | ND | 63.5 | 35.6 | 0.45 | 0.45 | (179) |
| Norway | 2001-2002 | 2,200 | 2.72 | ND | ND | 60.0 | 40.0 | 0 | 0 | (17) |
| Finland | 2002 | 1,007 | 21.5 | 8 / 64 | ND | 50.0 | 41.0 | 2.0 | 6.0 | (37) |
| Austria | 2001-2003 | 160 | 10.0 | 0.125 / 0.25 | 4.4 | 58.33 | 41.64 | 0 | 0 | (39) |
| South Africa | No data | 40 | 100 | 8 / > 64 | ND | 16.7 | 83.3 | 0 | 0 | (21) |
| Greece | 1997-1999 | 781 | 18 | 0.78 / 3.12 | ND | 29.2 | 67.9 | 0 | 2.9 | (150) |
| Tunisia | 1998-2004 | 100 | 34 | 8 / 12 | 68 | 12 | 86 | 2 | 0 | (176) |
| Belgium | 1993-1997 | 100 | 33 | 2 / >512 | ND | 9.1 | 90.9 | 0 | 0 | (177) |
| Spain | 1999-2000 | 203 | 38.91 | 0.03 / >64 | 75 | 5 | 92.4 | 1.3 | 1.3 | (178) |
| Korea | 2002-2006 | 235 | 80 | 4 / ≥128 | 67.2 | 10.1 | 42.6 | 47.3 | ND | (180) |