# Molecular Characterization of Efflux-Mediated Macrolide Resistance Among Canadian Isolates of *Streptococcus pneumoniae*

**By**

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| | |
|---|---|
| ATCC | American Type Culture Collection |
| bp(s) | base pair(s) |
| CFU(s) | Colony Forming Unit(s) |
| DNase | Deoxyribonuclease |
| dNTP(s) | deoxy Nucleotide Tri-Phosphate(s) |
| HCL | Hydro Chloride |
| Kb | Kilo base |
| $MgCl_2$ | Magnesium Chloride |
| MHB | Mueller Hinton Broth |
| MIC | Minimum Inhibitory Concentration |
| $MIC_{90}$ | Minimum Inhibitory Concentration at which 90% of Isolates are Inhibited |
| Na0H | Sodium Hydroxide |
| $NaCl_2$ | Sodium Chloride |
| NCCLS | National Committee for Clinical Laboratory Standards |
| OD | Optical Density |
| orf(s) | open reading frame(s) |
| PCR | Polymerase Chain Reaction |
| RFLP | Restriction Fragment Length Polymorphism |
| RNase | Ribonuclease |
| rpm(s) | revolution(s) per minute |
| rRNA | ribosomal ribonucleic acid |
| TBE | TRIS Borate EDTA |

| | |
|---|---|
| THB | Todd Hewitt Broth |
| UV | Ultra Violet |
| YE | Yeast Extract |

## ABSTRACT

Efflux-mediated macrolide resistance in *S. pneumoniae* is due predominantly to the acquisition of the *mef*E gene and confers a low-level resistance to 14- and 15-membered macrolides such as erythromycin and clarithromycin. The genetic element carrying the *mef*E gene in *S. pneumoniae* has recently been characterized as macrolide efflux genetic assembly, mega. The majority of *S. pneumoniae* isolates possessing the *mef*E gene are inhibited by low concentrations, 1- 4μg/ml of macrolides. Although the $MIC_{90}$ of *S. pneumoniae* exhibiting an M-phenotype is usually low, 4μg/ml, the macrolide MICs can range from 0.5 to 64μg/ml. Recently a rightward shift in the macrolide MIC distribution has been reported. Increases in the macrolide MICs for *S. pneumoniae* exhibiting an M-phenotype can be attributed to higher *mef*E gene expression, multiple *mef*E gene copy number or the presence of dual efflux system.

The purpose of this thesis was to determine the molecular characteristics of the efflux pump gene *mef*E, in Canadian clinical isolates of *S. pneumoniae* with variable susceptibility to macrolides. The increase in the macrolide MICs for different isolates of *S. pneumoniae* was not found attributed to multiple *mef*E gene copy numbers as determined by Southern Blot Hybridization with *mef*E probe and *Eco*RI digestion. In light of the recent characterization of mega, the potential for dual efflux system in *S. pneumoniae* with increased MICs to macrolides has been hypothesized. However, no correlation indicative of the dual efflux system has been observed in Canadian isolates of *S. pneumoniae*. Interestingly, the expression of the efflux pump gene, *mef*E was found to increase with increasing MICs to macrolide as determined by relative RT-PCR,

indicating that higher *mef*E gene expression is correlated with lower susceptibility to macrolide antibiotics.

Further characterizing, efflux-mediated resistance in *S. pneumoniae* appeared to be inducible with 14- and 15-membered macrolides. In addition, although the predominating, mega was found not the only genetic element carrying macrolide efflux genes in Canadian clinical isolates of *S. pneumoniae* exhibiting an M-phenotype.

# Molecular Characterization of Efflux-Mediated Macrolide Resistance Among Canadian Isolates of *Streptococcus pneumoniae.*

## A. INTRODUCTION

### 1. *Streptococcus pneumoniae*

#### a. Identification and Morphology

*Streptococcus pneumoniae* (formerly called *Diplococcus pneumoniae*) is a facultative anaerobic (optimal growth in 5-7% $CO_2$ supplemented atmosphere), gram-positive, encapsulated coccus that has a characteristic lancet shape (rounded with pointed ends) and replicates in pairs (diplococcus) (39,41,95). It sometimes appears as short chains; in old cultures or purulent exudates, some of the organisms may stain gram-negative. Morphologically, pneumococcus has glistening colonies that are gray in color although dry, whitish color colonies are also sometimes observed (39, 41).

The organism is catalase-negative and therefore grows better in the presence of a source of catalase such as red blood cells and they are usually added as a supplement to broth or agar for optimal growth (39,41,84,95). Pneumococci produce pneumolysin (α-hemolysin), which breaks down hemoglobin into green pigment (41,84). Green zone of α-hemolysis surrounding colonies during growth on blood-agar plates is characteristic of *S. pneumoniae* and used in discrimination of *S. pneumoniae* from *S. pyogenes* (39,84,95). Growth of pneumococci is inhibited by ethyl hydrocupreine (Optochin) and organisms are lysed by autolysin-activated bile salts (41,95). The Optochin test is often used to discriminate *S. pneumoniae* from other α-hemolytic Streptococci. Thus, pneumococci are

identified in the microbiology laboratory by four reactions: (1) α-hemolysis of blood agar, (2) catalase negativity, (3) susceptibility to optochin, and (4) solubility in bile salts.

The capsule, visible in smears stained with methylene blue, consists of a complex polysaccharide that determines serologic type and contributes to virulence and pathogenicity of *S. pneumoniae* (39,84). The Neufeld quellung reaction, the best method for determining type, during which a capsule swells in the presence of type-specific rabbit antiserum, identified more than 90 different serotypes to date. In the American numbering system, serotypes are numbered 1-90 in the order in which they were identified (84). The more widely accepted Danish numbering system group's serotypes according to antigenic similarities. For example, Danish serotype 19 includes types 19F, 19A, 19B, and 19C (the letter F indicates the first member in the group to be identified, followed by A, B, C, etc.), which in the American system would be 19, 57, 58 and 59 respectively (84).

**b. Pathogenesis**

S. pneumoniae is an exclusively human pathogen, which is spread from person to person by aerosols (11,59,84,85,95). It colonizes the nasopharynx of up to 40% of healthy children and 5-10% of healthy adults (59). Carriage of a particular strain lasts 3-6 months depending on the serotype, and it is not uncommon for one individual to be colonized with multiple serotypes (11,59). It is poorly understood how S. pneumoniae trans-locates from the site of colonization, the nasopharynx to a site of infection, and causes invasive disease (85). However, invasive disease typically occurs not after prolonged colonization with a particular serotype but with acquisition of a new serotype

that occurs at an age dependent rate as frequently as every four months in infants (84,85,95).

The patients most susceptible to serious, invasive pneumococcal infections are the very young, the elderly, and those with lymphoma, Hodgkin's disease, multiple myeloma, splenectomy, other serious debilitating diseases or immunologic deficiencies, and sickle cell disease (84,95). Common respiratory viruses, notably influenza virus or chronic bronchitis damage the respiratory epithelium and may predispose to pneumococcal invasion (56,82).

The capsular polysaccharide plays a key role in allowing the establishment of infection (56,82). Over 90 different serotypes of *S. pneumoniae* have been identified on the basis of antigenic differences in their capsular polysaccharides (11,56,82). The capsule increases virulence by resisting opsonization and therefore preventing phagocytosis (56,59,82). The degree of this effect on virulence appears to be dependent on the composition of the capsule rather than its thickness (59). The organisms enter the site of infection in the face of impaired host defenses that normally include the cough reflex, ciliary movement, and secretary immunoglobulin (84,85). Once within the site of infection, they resist phagocytosis by macrophages, multiply, and induce an inflammatory response that impairs normal function (84,85). The best evidence that capsular polysaccharide is the primary virulence factor is that antibody to the capsular antigen is protective (84,85). Recovery from pneumococcal pneumonia in a non-immune individual is accompanied by a rise in capsular antibody titer, whereas presence of circulating antibodies prevents establishment of the disease (84,85). The most common serotypes of pneumococcus causing serious infections have been types 1, 3, 4, 7, 8, and

12 in adults and types 6,14,19, and 23 in infants and children but these patterns are slowly changing, in part, because of the wide use of polyvalent vaccine (81,84,85,95).

*S. pneumoniae* produces other factors that may play a role in virulence, including pneumolysin, an oxygen-sensitive toxin that is cytolytic for cells forming pores in leukocyte membranes. Pneumolysin also inhibits ciliary beating (56,82). Neuraminidase enzyme degrades surface structures of host tissue (56,82). The cell wall of pneumococci contains C substance, a teichoic acid that reacts with a certain serum proteins (C-reactive protein, CRP), resulting in the activation of some nonspecific host immune responses (56,82). Last but not least pneumococcus produces large amounts of IgA proteases that cleave mucosal IgA in attempt to combat type-specific antibody (56,82).

**c. Epidemiology**

S. pneumoniae is an important human pathogen that causes both mucosal and invasive infections worldwide (11,22,56). In certain population groups, such as the very young, the elderly, and those with co-morbid illness these infections can be particularly serious (11,56). Community-acquired pneumonia, CAP is the most frequent serious infection caused by pneumococci (56,59,80,81,82,84,85). Of all bacterial causes of CAP, S. pneumoniae is the most common organism (80,81). Globally, pneumococcal pneumonia is responsible for ~1.2 million deaths per year and nearly 40% of all pneumonia in children <5 years of age. S. pneumoniae causes about 50% of acute otitis media in infants and children (24,80,81). About 1/3 of children in most populations have an attack of acute pneumococcal otitis media in the first 2 years of life, and recurrent otitis media due to pneumococcus is common (59). It has been estimated that in the

United States alone there is approximately 7 million episodes of acute otitis media annually (59,80,81). A leading cause of bronchitis, and sinusitis, *S. pneumoniae* is also an important cause of septicemia, a frequent agent in bacteremia, and one of the three most common pathogens associated with bacterial meningitis (84,85).

Although sinusitis and otitis media are not generally life threatening, they cause significant discomfort and are a major cause of absenteeism from school and work and can have serious sequelae, such as hearing loss or meningitis if left untreated (84,85). Infections such as CAP and acute bacterial exacerbations of chronic bronchitis (AECB), along with meningitis and septicemia comprise the more serious infections caused by *S. pneumoniae* and are associated with greater morbidity and mortality (84,85). *S. pneumoniae* is also responsible for pneumococcal endocarditis and arthritis, which may occur as a rare complication of bacteremia (84,85).

### d. Macrolide Treatment of *S. pneumoniae*

Macrolide antibiotics have been known for more than five decades and since the introduction of erythromycin into the clinical practice, they have played a key role in treatment of community acquired respiratory tract pathogens, like *S. pneumococci* (4,7,8,47,67). Macrolide antibiotics represent a major alternative to the use of penicillins and cephalosporins for the treatment of community acquired respiratory illness such as community acquired pneumonia (CAP), most commonly caused by *S. pneumoniae* (7,8,17). Macrolide treatment of CAP was initially limited to patients with β-lactam allergy (47,8,128).

Agents with a broad spectrum of activity, macrolides, became the drugs of choice for the empiric treatment of community-acquired pneumonia as the importance of atypical pathogens in CAP was recognized (7,8). This followed a 1993 CAP treatment guidelines that were released recommending that macrolides be utilized as first-line treatment for patients younger than 60 years of age with no co-morbidity. As a result of these recommendations macrolides have been prescribed heavily over the past few years and many studies confirm that erythromycin and the newer macrolides, clarithromycin and azithromycin are the most often utilized agents as first-line therapy for adults in US and Canada with community-acquired pneumonia (7,8,17).

As a result of this new prescribing focus, rapid emergence of resistance to macrolide antimicrobials in *S. pneumoniae* has occurred (3,4,7,8,9). Many reports are now describing increasing macrolide resistance in pneumococci worldwide (20,21,35,37,52,61,96,97,119). The emergence of macrolide resistance in *S. pneumoniae* is a growing concern because of the importance of this pathogen in infections of the respiratory tract (3,4,47). Macrolide resistance in *S. pneumoniae* has increased during the 1990's to the extent that over 30% of clinical isolates are now resistant in some communities (47,50).

Telithromycin is the first of a new class of antibacterials, derived from erythromycin, the ketolides, which have been recently developed specifically to provide effective treatment for respiratory tract infections caused by macrolide-resistant *S. pneumoniae* (9,16,50,60,123). Telithromycin has undergone Phase II and Phase III clinical trails and the data support the use of it as a first line oral therapy for the treatment

of community-acquired respiratory tract infections caused by *S. pneumoniae* with reduced susceptibility to erythromycin A (16,116,123).

With the emergence of more and more macrolide-resistance mechanisms and *S. pneumoniae* with reduced susceptibility to macrolides, the ketolides may offer an attractive alternative to the currently used macrolides in treatment of community acquired respiratory infections (116).

## 2. Macrolide Antibiotics

### a. Characteristics and Chemistry

Macrolides are produced in nature by many actinomycete strains. A large lactone ring composed of 12 to 16 atoms is the main structural component of macrolide antibiotics (120). Clinically useful macrolides consist of a 14-, 15-, or 16-membered lactone ring, generally substituted with two or more neutral and/or amino sugars through glycosidic bonds (120). Hydroxyl or alkyl groups are also substituents of the lactone ring. The first macrolide to be used clinically from this group is the 14-membered ring macrolide erythromycin A (120). Erythromycin's spectrum of activity includes Gram-positive cocci (mainly staphylococci and streptococci) and bacilli, Gram-negative cocci, and atypical, intracellular pathogens (*Chlamydophila pneumoniae*, *Mycoplasma pneumoniae*, and *Legionella pneumoniae*) (120). However, the natural macrolide, erythromycin is limited clinically (120). Some of its limitations include, insufficient activity against *Haemophilus influenzae*, poor oral bioavailability, short serum half-life, high incidence of gastrointestinal (GI) adverse effects, phlebitis when administered parenterally and a significant number of drug-drug interactions (120). The creation of erythromycin

salts/esters such as, erythromycin stearate, erythromycin estolate and erythromycin ethylsuccinate has improved the acid stability of the drug, however frequent dosing is still required (120).

Structural modifications of the ketone at C-9, the hydrogen at C-8, the hydroxyl at C-6 and the diol at C-11 and C-12, the sites of the erythromycin's lactone ring that are particularly prone to acid degradation into inactive anhydroketal or spirochetal derivatives which are associated with the high incidence of GI adverse effects led to the production of newer macrolides (77,120). Roxithromycin, the first macrolide derivative was created by substitution of the ketone group at C-9 for an oxime group (77,120). This modification increased the acid stability but did not affect the *in vitro* activity (120). Addition of fluoride atom at the C-8 position of the lactone ring resulted in flurithromycin that similarly showed increased acid stability without having an effect on the *in vitro* activity (77,120).

The alkylation of the C-6 hydroxyl group resulted in the production of 6-O-methyl derivative of erythromycin named the clarithromycin. Gastrointestinal adverse effects are eliminated through this modification that prevents degradation to hemiketal and spirochetal metabolites through an increase in acid stability (120). More importantly the alkylation did not have a negative effect on the *in vitro* activity (120). Clarithromycin demonstrates *in vitro* activity equal to or greater than that of erythromycin against common respiratory pathogens such as *S. pneumoniae* (120). Clarithromycin also has an active metabolite, 14-hydroxy clarithromycin that has been reported to be "synergistic" with the parent compound and can enhance the activity of clarithromycin against *S. pneumoniae* and *H. influenzae* (120). The ability of a drug to demonstrate activity

against *H. influenzae* is particularly important as macrolides are often used an empiric treatment of community-acquired pneumonia often caused by *H. influenzae* and *S. pneumoniae.* This has been addressed in the Macrolide Antibiotic Treatment session.

Azithromycin is a clinically relevant 15-membered macrolide (azalide) (1,2,6). It is a semi-synthetic erythromycin derivative formed by the addition of methyl-substituted nitrogen at the C-9a position of the lactone ring (1,2,6). This modification not only prevents degradation of the drug in the gastric acid but also, significantly increases the activity against *H. influenzae* and other Gram-negative bacteria and atypical pathogens (1,2,6,120). It also leads to better tissue penetration and a significantly prolonged half-life compared to erythromycin (1,2, 6,120).

Josamycin, Kitasamycin, Spiramycin, Midecamycin, Rosaramicin, and Tylosin are examples of the naturally occurring 16-membered macrolides (120). They are secondary metabolites of *Streptomyces* species with the exception of Rosaramicin that is produced by *Micromonospora rosalia* (120). Some of the 16-membered macrolides have been chemically altered in order to increase their activity against and improve their pharmacokinetic properties (120). Examples of semi-synthetic 16-membered macrolides are Rokitamycin and Mikamycin that are produced though the acylation of the 3'-hydroxyl group of leucomycin and 9,3'-di-O-acetylation of midecamycin, respectively (120).

Recently, a new group of 14-membered macrolides, known as ketolides, has been developed (116,120,121). The main change that characterizes the ketolides is the replacement of the 3-L-cladinose moieties by a keto group (30,32,116,120,121). This replacement was achieved by removal of the cladinose from erythromycin to leave a 3-

hydroxyl group, which is further oxidized to form the 3-keto derivative (30,32,33). In addition to the main structural change, a methyl group is attached at the 6-O position as in clarithromycin to further improve acid stability by preventing hemiketalization (33,42,46,115). Removal of L-cladinose moiety in the absence of other modifications results in loss of antimicrobial activity (42,46). However, the loss of the cladinose can be compensated for by modifications at other positions of the macrolactone ring (42,46). This is the case for the ketolides, telithromycin, and ABT-773 both of which have a carbamate group at C11/C12 of their lactone ring (42,46).

**b. Mode of Action**

Macrolides are generally bacteriostatic agents, although some of these drugs may be bactericidal at very high concentrations (120,121). They exert their action by reversibly binding to the prokaryotic ribosome and blocking protein synthesis (29,91,92). Bacterial ribosomes (70S) are formed by a small, 30S subunit and a large, 50S subunit (107,120,121). The latter is composed of 23S rRNA and a minimum of 30 proteins (107,120,121). The secondary structure of 23S rRNA is folded due to base pairing and forms six domains numbered I to VI, while the tertiary structure of the 23S rRNA is maintained by its interactions with protein (107,120,121). Stoichiometric binding of erythromycin A to the 50S subunit causes inhibition of protein synthesis (107,120). The 30S subunit binds messenger RNA and begins the ribosomal cycle (initiation), whereas 50S subunit binds transfer RNA (tRNA) derivatives and controls elongation (107,120,121).

The ribosomal target for macrolides lies within the 23S rRNA at the peptidyl transferase center of the 50S subunit (107,120,121). Peptidyl transferase activity is associated with the central loop in 23S rRNA domain V, where macrolides make several contacts with the rRNA (107,120,121). Through chemical foot printing experiments the exact interaction of macrolides within the domain V of the ribosome has been pinpointed to nucleotides A2058 and A2059 (107,120,121). The binding of the macrolides also includes hairpin-35 of domain II of the rRNA. Stoichiometric (1:1) binding of macrolides to the ribosome indicates that domains II and V fold to lie in a close proximity in the tertiary structure of the rRNA and thereby form a single binding pocket (107,120,121). Ribosomal proteins L22 and L4 have also been shown to be part of the binding site of macrolides (107,120,121). The mechanism of action has been a matter of controversy for some time and the exact mechanism of action has not yet been elucidated, however many theories exist (107,120,121).

It has been proposed that macrolides inhibit protein synthesis by blocking elongation. The elongation cycle is composed of three phases: aminoacyl-tRNA binding, the transpeptidation reaction, and translocation (107,120,121). During the elongation cycle a new amino acid is added to a growing polypeptide that occupies the P site (peptidyl or donor site) of the ribosome (107,120,121). The other site of the prokaryotic ribosome is the site that accepts the new incoming aminoacyl-tRNA and is called the A site (aminoacyl or acceptor site) (107,120,121). The aminoacyl-tRNA bound to the A site initiates the transpeptidation reaction of the elongation cycle of protein synthesis. This reaction is catalyzed by the peptidyl transferase, located on the 50S subunit of the ribosome (107,120,121). The nucleophilic attack of the amino acid in the A site on the

C-terminal amino acid in the P-site results in a peptide chain growth by one amino acid that temporarily occupies the A-site (107,120,121). The final step of elongation is the translocation during which the peptidyl-tRNA moves from the A site to the P site, the ribosome moves one codon along the mRNA so that a new codon can be positioned in the A site, and empty tRNA leaves the P site (107,120,121). It is believed that 16-membered macrolides inhibit the peptidyl transferase reaction and the peptidyl transfer RNA (tRNA) translocation is blocked by 14-membered macrolides (107,120,121).

Recent studies show a marked increase in peptidyl-tRNA concentrations (i.e. immature peptides) in the presence of macrolides and this has led to the speculation that macrolides inhibit protein synthesis by stimulating the dissociation of peptidyl-tRNA from ribosomes during the translocation step of elongation (120,121). This may be a result of the weakening of the bonds between the ribosome and the peptidyl-tRNA (107,120,121).

Lastly, macrolides may inhibit the assembly of 50S ribosomal subunits (107,120,121). This theory was proposed by Champney and Burdine as a result of their experiments demonstrating that the addition of macrolides to bacterial cells caused a decrease in the specific activity ratio of 50S to 30S subunits suggesting the possibility of a secondary ribosomal target for the macrolides (120,121).

### c. Mechanisms of Resistance

#### i. Target-Site Modification

#### α. Methylation

Resistance to erythromycin in *S. pneumoniae* was first detected in 1967 in the United States and subsequently worldwide (3,4,518,27,65,128). The corresponding mechanism was rapidly identified as ribosomal methylation, which had been primarily reported as being responsible for erythromycin resistance in staphylococci (65,67,6970,71). Ribosomal modification by methylation was the first mechanism of resistance to erythromycin elucidated and remained unique for decades (70,71,121). It is secondary to the acquisition of an *erm* (erythromycin ribosome methylase) gene usually carried by transposable elements in pneumococci (70,71,121). This gene encodes a ribosomal methylase which di-methylates pneumococcal 23S rRNA at a single site, adenine at position 2058 (A2058, *Ecoli* numbering system) (67,69,70,71). As previously alluded to, the A2058 nucleotide is a key nucleotide for binding of erythromycin. The modification markedly reduces the affinity of erythromycin for its target, probably by preventing direct access to the target or by modifying the confirmation of the binding site (32,114). Cross-resistance to macrolide, lincosamide, and streptogramin B antibiotics, which gave its name to the $MLS_B$ resistance phenotype, is due to the overlapping binding sites of the drugs (32,67,69,70,71,113,121,122).

The *erm*B determinant, initially called *erm*AM, was first characterized on plasmid pAM77 in *S. sanguis* A1 isolated from dental plaque in 1978 (32,70,114). The gene is widely distributed, not only in *S. pneumoniae* but also is a variety of other streptococcal and enterococcal species, in enterobacteria, and in staphylococci, indicating easy

exchange of genetic information even between phylogenetically remote species (67,69,70,71,114). In pneumococci, the gene is borne by conjugative transposposon related to Tn*1545* that carries determinants that confer resistance to tetracycline and streptomycin, Tn*1545*-like elements, or a Tn*917*-like element that is part of a larger composite transposon, Tn*3872* (67,69,70,71,114). Both clonal spread of resistant strains and horizontal transfer of the element account for the high prevalence of the *erm*B gene in erythromycin resistant pneumococci in certain countries (67,69,70,71). Strains with different serotypes may have *erm*B gene carried on different elements (67,69).

Regulation of *erm*B expression and the $MLS_B$ phenotype has been studied extensively and is well characterized (67,69,70,71,114,121). The methylase encoded by *erm*B gene in *S. pneumoniae* may be constitutively or inducible synthesized (67,69,70,71,94,114,121,122,125). 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 being synthesized (67,69,70,94,125). However, when the resistance is inducible, *erm*B mRNA is synthesized, but in an inactive confirmation, and becomes active only in the presence of inducing macrolides (67,69,70,71,94,114,125). The mechanism of induction for *erm*B has not been thoroughly studied and a model inferred from the translation regulation of *erm*C in *S. aureus* has been proposed (67,69). Induction of the erythromycin ribosome methylase production occurs post-transcriptionally (67,69). Briefly, summarizing the model, the 5' end of the *erm*B gene presents a series of inverted repeats which are responsible for the lack of methylase synthesis in the absence of erythromycin (19,67,69,70,125). Fourteen pairs of repeats have been identified that could form alternative stem-loop structures by

base paring (67,69). These stem-loops sequester the ribosome's binding site and initiation codon for the methylase. As a result the methylase cannot be produced. Induction is related to the presence of sequences coding for a small leader peptide of 36 amino acids upstream of the methylase gene (67,69,70,71,125). In the presence of low concentrations of erythromycin, binding of the antibiotic to the ribosome translating the leader sequence causes the ribosome to stall (67,69,70,71). Stalling of the ribosome destabilizes the pairing of the inverted repeats and induces conformational rearrangements in the mRNA (67,69,70,71). As a result the initiation sequences for the methylase are unmasked, allowing synthesis to proceed by the ribosomes that are not in complexity with erythromycin or by those that are methylated (67,69,70,71). Methylation of the ribosomes in a strain that expresses inducible *erm*B might occur by transient rearrangements of the stem-loop structures, which would lead to the synthesis of a basal level of the methylase (67,69,70,71).

The inducing capacity of the macrolides depends on the antibiotic structure (69,67,70,71,125). The number of atoms in the lactone ring does not determine the inducting capacity and this has been clearly demonstrated with erythromycin, a 14-memberered macrolide and ketolide, a 14-membered erythromycin derivative (124). Erythromycin is a strong inducer for the production of erythromycin methylase, whereas ketolides are not (10,94,125). It has been elucidated that the lack of inducing ability of ketolides is related to the replacement of one of the erythromycin sugar, L-cladinose, by a keto group (10,94,125). In conclusion for *erm*B, the commercially available macrolides including the 14-, 15-, and 16-membered macrolides, lincosamides, and streptogramin B antibiotics are inducers of methylase synthesis (10,94,125). Inducible expression of the

*erm*B gene gives rise to a large variety of phenotypes (19,83,100,108). These phenotypes include high- or low-level resistance to erythromycin with susceptibility or resistance to clindamycin (100,108). The phenotypes and their correlation with the genotypes are still far from being understood (100,108). However, the degree to which these antibiotics are inducing the expression of the *erm*B varies, but in all instances it leads to cross-resistance to these antimicrobials (19,70,100,108,125).

The constitutive expression of the *erm*B is due to deletions, duplications, or point mutations in the control peptide sequence leading to derepressed production of the methylase (100,108,125).

**β. Mutation**

Recently, other mechanisms associated with unusual resistance phenotypes to $MLS_B$ antibiotics have been identified in clinical and as well laboratory isolates of *S. pneumoniae* (67,69,71). Study of a large number of *S. pneumoniae* mutants selected in the presence of various macrolides revealed that mutation of a variety of structures including domains V and II of the 23S rRNA and proteins L22 and L4, which are part of the binding site of macrolides, could be responsible for resistance to $MLS_B$ antibiotics (15,26,86,106,107,110). Mutations in the domain V of 23S rRNA were found to be the most frequent, in particular, substitutions of A2058, A2059, and C2611 (107,107,110). The phenotype conferred by modification of the 23S rRNA target varies according to the mutated base (107,110). Change of the adenine at position 2058 for a G or U conferred the $MLS_B$ phenotype similar to the one mediated by *erm*B (15,26,86). By contrast, the A2059G mutation confers a lower level resistance to macrolides, in particular,

clarithromycin and has been designated as ML phenotype since streptogramins B remain active (86,106,107,110). Substitution at position 2611 destabilizes the base pairing of the central loop and results in low-level resistance to 14-membered macrolides and clindamycin (86,106,107). Ribosomal mutation has been reported only recently in a few clinical isolates of *S. pneumoniae* (62,73,113,). Recently, A2059G mutation in the 23S rRNA has been described to occur *in vivo* during treatment of *S. pneumoniae* infection with macrolides and ultimately was responsible for clinical failure of macrolide treatment in a patient (62).

In addition to the mutated base and to its location in the 23S rRNA, differences in the number of mutated copies of 23S rRNA could lead to differences in the MIC. *S. pneumoniae* has four copies of the *rrl* gene for 23S rRNA, and transformation experiments with the mutated *rrl* gene have shown that susceptibility to erythromycin decreases as the number of the mutated gene copies increases (15,26,86). The incidence of resistance conferred by mutation in the 23S rRNA is low in comparison to other species such as *H. pylori* or *M. avium*, which contain only one or two copies of the *rrl* gene (67,69,86,108).

Various mutations in the *rplV* gene and *rplD* gene encoding L22 and L4 ribosomal proteins respectively have also been shown to play a role in macrolide resistance in *S. pneumoniae* (15,86). The mutations in the L22 protein confer resistance to streptogramin B and low-level resistance to macrolides, whereas clindamycin does not seem to be affected, $MS_B$ phenotype (15,86). Mutations in the L4 also generally confer $MS_B$ phenotype (15, 86,106). To date two types of *rplD* (L4) mutations have been described. The first type was identified in 16 isolates from Eastern Europe and contained

substitutions in three amino acids (69GTG71-69TPS71) displaying an $MS_B$ phenotype (15,86, 106). The second type, a six amino acid insertion (underlined) 71GREKGTGR72 was identified in a Canadian isolate (106). This isolate also displayed the $MS_B$ phenotype (106). A recent report of the emergence of the L22 protein mutant containing a six amino acid insertion during treatment with azithromycin of fatal pneumonia was published (129).

**ii. Efflux of the Antibiotic**

**α. *mef* Genes and Efflux Pumps**

Resistance to macrolides in the absence of resistance to lincosamides or streptogramin B has been recently described in *S. pneumoniae* and β-hemolytic (*S. pyogenes*) streptococci (18,44,67,69,103,132). This resistance is determined by the presence of a membrane bound efflux protein, encoded by the *mef* genes, *mef*A or *mef*E (104,105,111,123). The 405 amino acid protein encoded by *mef* genes, the MefA or MefE pump belongs to the major facilitator superfamily (MFS) class of membrane proteins (67,69,79,104,111). It is a hydrophobic protein with 12 transmembrane domains spanning the cytoplasmic membrane (49,67,69). The major facilitator uses the transmembrane electrochemical gradient (proton motive force) to actively transport macrolides out of the cell against their concentration gradient (49,67,69,104). The MefA or MefE pump seems to be specific to erythromycin and its derivatives and it sufficiently effluxes 14- and 15-membered macrolides out of the cell (79,104,111). 16-membered macrolides, lincosamides, and streptogramin B are not substrates of the pump, and these agents remain active (67,69). Resistance to 14- and 15-membered macrolides such as

erythromycin and clarithromycin combined with susceptibility to 16-membered macrolides such as josamycin, lincosamides such as clindamycin and also streptogramin B antibiotics, defines the M resistance phenotype in *S. pneumoniae* (67,69,71).

Of the two variants of *mef* gene, *mef*A was originally found in *S. pyogenes*, and *mef*E was originally found in *S. pneumoniae* (105). *mef*A and *mef*E genes are 90% identical at the nucleotide level and were assigned to the same class, A: *mef*A and the MefA protein of macrolide resistance determinants (105). In subsequent studies, *mef* detected by PCR could not distinguish between the two variants (25). Both *mef*E and *mef*A have recently been found in *S. pneumoniae* and are known to be transferable (25,76).

### β. Characteristics of the Genetic Elements Carrying *mef* Genes

The genetic element carrying the efflux gene *mef*A in *S. pneumoniae* was characterized and published by a group from Italy in the fall of 2000 (98). The *mef*A gene from a clinical isolate of *S. pneumoniae* exhibiting the M-phenotype, resistant to macrolides was found to be part of the 7,244-bp chromosomal element (transposon) Tn*1207.1*(98). Sequence data analysis showed the presence of 8 open reading frames (ORFs), of which the first five have the same direction of transcription, while *orf*6, *orf*7, and *orf*8 are oriented opposite to the others. Tn*1207.1* integrates at a specific site in *S. pneumoniae* chromosome, the *cel*B gene that is responsible for competence (98). Integration of the transposon causes a 1,947-bp deletion in the *S. pneumoniae* chromosome and results in loss of competence (98).

The identity of the gene homologous to the ORFs of Tn*1207.1* was determined at the protein level, with the help of BLAST software by the same researchers (98). No

homologue was found for *orf*1 and *orf*3. *orf*2 was found to be homologous to site-specific recombines of other genetic elements of gram-positive bacteria (98). *mefA* was identified as *orf*4 of the Tn*1207.1*(98). *orf*5 was found 119 bp downstream of the *mef*A stop codon and showed approximately 40% homology to *msr*SA and *vga*A, genes of the ABC transporter superfamily that mediate resistance by encoding an macrolide and streptogramin specific efflux pump in *S. aureus* (98). *orf*6, *orf*7, and *orf*8 are homologous to 3 ORFs of the pneumococcal conjugative transposon, Tn*5252* (98). No function is currently known for the gene products of *orf*6 and *orf*7 (98). The gene product of *orf*8 is highly homologous to pneumococcal UV-resistance protein, however it was shown to be slightly shorter in length than normal Tn*1207.1* and therefore is considered a defective transposon (98). In addition to being transcribed in the opposite direction to the first five ORFs, *orf*6, *orf*7, and *orf*8 were shown to overlap as well (98).

Following the characterization of *mef*A-containing element, Tn*1207.1*, the genetic structure of a novel *mef*E-containing chromosomal insertion element in *S. pneumoniae* was defined by a group of researchers in United States (43). The element that carries *mef*E gene in *S. pneumoniae* was designated as macrolide efflux genetic assembly (mega) (43). The mega insertion element was found to be smaller than Tn*1207.1* with a size of 5,532-bp and 5 ORFs (43). Sequence data analysis showed that the first 944-bp genomic sequence at the 5' end of mega contained no predicted open-reading frame (ORF) or homology to nucleotide or amino acids entries in the Gen Bank database (43). The first ORF began at position 1125 and was designated as *mef*E (43). Interestingly, a 180-bp region immediately preceding *mef*E was identical to the sequence preceding *mef*A in Tn*1207.1*. Similar to the finding in Tn*1207.1* a predicted adenosine triphosphate-binding

cassette homologous to *msrS*A was found immediately 3' of *mef*E (43). These two first ORFs of the element form an operon composed of *mef*E and *mel* and unlike in Tn*1207.1* were shown to be co-transcribed (43). In addition, the defined 119bp intergenic region between *mef*A and *orf*5 in Tn*1207.1* was shown to vary between *mef*E and *mef* of mega. In the 3' end of *mel*, two overlapping ORFs (ORF3 and ORF4) were found, which were oriented opposite to *mef*E and *mel* (43) The last ORF of mega ORF5 was also found oriented opposite to *mef*E and *mel* but was not overlapping with the other two (43). These three ORFs had significant homology to the predicted proteins of ORFs 11,12, and 13 of Tn5252. In fact, the 5 ORFs of mega and Tn*1207.1* ORFs 4-8 were shown to have more than 90% identity at the nucleotide level (43). Unlike ORF8 in Tn*1207.1,* ORF5 of mega was not truncated at the 3' end, however both 5' and 3' ends of the mega element had imperfect inverted repeats that were also unique (43).

Both *mef*A and *mef*E were identified as single copy genes (43,98). However, unlike, *Tn1207.1*, the mega element does not insert into the chromosome at a specific site but was found in more than 4 distinct sites in the *S. pneumoniae* chromosome (98). The chromosomal competence gene, *cel*B was not the target for insertion of mega (43,98). Gay and Stephens further designated mega elements as Class I-IV mega depending where and how it inserts in the chromosome (43). Many changes were found to occur in the chromosome at the site of insertion, certain insertions caused a 99bp deletion in the region between *mef*E and *mef* of mega and those were designated as Class II and IV mega inserts (43). Some site of insertions did not have an effect on the region between *mef*E and *mel* and those were designated as Class I and Class III (43).

### 3. Mechanisms of Action of Ketolides

The ketolides are a new class of macrolides specifically designed to combat respiratory tract pathogens that have acquired resistance to macrolides (117,121,122). Ketolides have a mechanism of action similar to that of macrolides (117,121,122). They inhibit bacterial protein synthesis by interacting close to the peptidyl transferase site of the 50S ribosomal subunit within domains II and V of the 23S rRNA (117,121,122). Chemical foot printing experiments defined nucleotides A2058 and A2059 in domain V of the RNA as the main site of interaction of ketolides with the ribosome (117,121,122). Although both macrolides and ketolides protect these bases from chemical modifications, the ketolides have been shown to display a higher affinity than macrolides for forming interactions with the ribosomes (16,42,46). This increased affinity is due to structural characteristics of ketolides in particularly the 11, 12-carbamate side chain of telithromycin that forms additional interaction at A752 in domain II of the 23S rRNA (16,33,46). Chemical foot printing experiments have shown that telithromycin protects A752 in domain II from chemical modification implying that ketolide with 11,12-carbamate side chain interacts directly with the base of A752 (16,33,42,46,122). Base substitution at position A752 reduces the binding of ketolides, but not macrolides, reinforcing the adenine at position A752 as an important secondary contact site for ketolides (16,33,42,46,122). This additional contact presumably enables the ketolides to retain activity against bacteria that have base modifications in domain V (16,33,42,46,116,122).

Similarly to macrolides, ketolides also demonstrate a significant inhibitory effect on the formation of 50S ribosomal subunits (16,121,122). In one study higher

concentrations of ketolides inhibited protein synthesis to such an extent that the formation of the 30S ribosomal subunit was impaired (16). Recently it has also been shown that ketolides accumulate at a greater rate than macrolides in bacterial cells. These results confirm ketolides as very potent inhibitors of protein synthesis (16).

The development of ketolides as agents to overcome various mechanisms of macrolide resistance has been a success for clinical isolates of *S. pneumoniae* (12,23,38,51,102,115). It has been shown that ketolides remain active against *S. pneumoniae* strains expressing efflux resistance *mef*E/*mef*A either because of their high intrinsic activity and/or tight ribosomal binding or because they are poor substrates for efflux pump (12,16,23,33,38,51,102,115). The exact mechanism of ketolide action against strains with efflux pump remains to be determined (122). Ketolides retain activity against *S. pneumoniae* that have base modifications mediated by *erm*B gene (12,23,38,51). It has been shown that the L-cladinose moiety of macrolides contributes to the strong induction effect of macrolides, leading to expression of the $MLS_B$ phenotype (67,69,70,71,94,114,121,122,125). Favorably, because of the 3-keto-group substitution of the L-cladinose sugar, ketolides lack inducibility properties of $MLS_B$ phenotype (10). Ketolides not only have clinical activity against strains inducible resistant to macrolides and also those that express *erm*B constitutively (12,23,38,51,102,115).

Point mutations in the 23S rRNA of domain V affect ketolide binding but macrolides are affected to a much greater extent (16,33). The most common mutation of A2058 and A 2059-G confers resistance to $MLS_B$ antibiotics, but do not affect telithromycin activity (12,23). Changes in the ribosomal protein, L4 have also been shown to affect ketolide binding (86). A recently identified 6 amino acid insertion in L4 conferred ketolide

resistance (86). Small deletions or insertions in the same region of L4 confer macrolide resistance without giving cross- resistance to ketolides (86).

In vitro studies have shown that exposure of *S. pneumoniae* to ketolides can select for resistant mutants (68,122). However, the selection of resistant mutants occurred at mutational frequencies much lower than those obtained for macrolides and resulted in only a slight increase in the minimum inhibitory concentration (MIC) values (68,122). The extent to which ketolides are able to select for resistance remains to be determined (68,122).

In conclusion ketolides have been shown to be very effective against macrolide-resistant *S. pneumoniae* regardless of the mechanism of resistance (117,121,122). Mechanistically they offer many advantages over macrolides in treating respiratory tract infections (68,117). They have been shown to have greater affinity for bacterial ribosomes (16). Also unlike macrolides they display some affinity for methylated ribosomes (16). Ketolides were shown to accumulate in macrolide-sensitive *S. pneumoniae* at a higher rate than erythromycin (16). They are poorer substrates for efflux pumps in comparison to macrolides (16,33). They are less likely to select for resistant mutants (68). Lastly ketolides lack inducibility properties of $MLS_B$ phenotype in *S. pneumoniae* (10). All these properties make ketolides very useful agents in the treatment of respiratory tract infections caused by *S. pneumoniae*, however a potential for the development of clinical resistance exists (117). Clinical resistance to ketolides could occur via mutations in domain II of the 23S rRNA in strains that already express modified domain V either by erythromycin methylase enzyme or by ribosomal RNA and/or protein mutations.

## 4. Prevalence of Macrolide Resistance in *Streptococcus pneumoniae*

Macrolide antibiotics are used extensively for the treatment of infections of the lower respiratory tract infections such as community-acquired pneumonia (7,8,9). The most common cause of community-acquired pneumonia is *S. pneumoniae* and this is the pathogen most frequently associated with poor outcome and mortality (80). Because of the serious nature of CAP, *S. pneumoniae* susceptibility testing has become increasingly important as macrolide resistance has occurred (109,112). Macrolide resistance in *S. pneumoniae* is widespread, however the prevalence of resistant strains varies geographically and temporally (50). Changing patterns of antimicrobial resistance in *S. pneumoniae* have prompted several surveillance initiatives in recent years (23,35,36,37,52,61,64,75,97,99,101,119,120).

The "Alexander Project " was established in 1992 to monitor the susceptibility of the major lower respiratory tract bacterial pathogens to a variety of antimicrobials and to identify trends in the development of resistance over time (37). For the first 4 years of the study, 10 European and 5 USA centers were monitored (37). Recently the study was expended to include centers located in Central and South America, the Middle East, South Africa, Hong Kong and other European countries not included previously (37). The 1996 and 1997 data from this international study of bacterial pathogens from community-acquired lower respiratory tract infections showed the overall rate of macrolide resistance for isolates of *S. pneumoniae* at 16.5 and 21.9%, respectively (37). Of the European centers included from the beginning of the Alexander Project, France and Spain were established as areas with high rates of *S. pneumoniae* macrolide resistance (37). The macrolide resistance rate in France during 1996 and 1997 study

period was reported at 40.6 and 45.9%, respectively (37). During the same study period macrolide resistance in Spain was reported at 19.1 and 32.6%, respectively (37). Belgium and Italy are other two countries with macrolide resistance exceeding 20% (37). Germany and Switzerland are considered countries with lower level (>10%) of macrolide resistance (37). Out of all the centers included in this study, Hong Kong showed the highest rate of macrolide resistance where over 70% of *S. pneumoniae* isolates were resistance to macrolides (37). This longitudinal surveillance study of resistance in *S. pneumoniae* revealed that significant changes do occur in just a single year and supports the recently proposed need for surveillance at least on annual basis, if not continuously (96).

The 1997-1998 resistance surveillance study of *S. pneumoniae* isolated in Asia and Europe performed by MRL Pharmaceutical Services, Cypress, CA, USA confirmed France and Spain as the European centers with high rates of macrolide resistance, at 57% and 36.6%, respectively (97). Of all the other European centers included in this study, the order of decreasing rates of macrolide resistance was for Italy (23.5%), Germany (9.2%) followed by UK (8.7%) (97). Similarly to the "Alexander Project ", this study found, Asian countries in particularly Japan and China as the countries with very high rates of macrolide resistance in *S. pneumoniae*, at 66.1% and 71.8%, respectively (37,97).

Recently the first report of a new worldwide surveillance study, PROTEKT, Prospective Resistant Organism Tracking and Epidemiology for the Ketolide Telithromycin was published (35). This study monitors resistance in Europe including countries such as Austria, France, Germany, Italy, Poland, Portugal, Spain, Sweden, Switzerland, Turkey, and UK, Asia with countries such as Hong Kong, Japan and South

Korea, Latin America, including Argentina, Brazil and Mexico, Australia and North America, including USA and Canada (35).

The worldwide prevalence of macrolide resistance in *S. pneumoniae* identified in this study was 31.8% (35). By geographic location, Asia was identified as the region with highest rate of macrolide resistance at 79.2% (35). Of the 3 countries in this region, South Korea had the highest rate of macrolide resistance at 86.2% (35). The prevalence of macrolide resistance in Latin America was 17.6% with Mexico having the highest rate of 28.6% and Brazil the lowest of 7.8% (35). In Australia, macrolide resistance in *S. pneumoniae* was found to be moderate at 12.3% (35).

The prevalence of macrolide resistance amongst isolates of *S. pneumoniae* from North America and Europe was similar at 22.4 and 23.5%, respectively, however the prevalence varied significantly by country in the two regions (35). In accordance with the other surveillance studies, France was identified as the country with the highest rate of macrolide resistance at 58%, however Italy superseded Spain with a rate of 44.6% versus 35.2% (35). The lowest rates were observed for Sweden (4.7%), Portugal (7.2%) and Switzerland (9.6%) (35). Amongst the North American countries, the macrolide resistance rate in USA was found to be double that in Canada, at 30% and 16.3%, respectively (35).

In addition to the global surveillance studies, there are several North American Studies (52,75,101,119). Among these is the SENTRY Antimicrobial Surveillance Program out the University of Iowa, College of Medicine (Iowa City, IA, USA) established in 1997. However, the SENTRY study also extends to a global surveillance network including Europe, Latin America, and the Asia-Pacific region so it is not an exclusively North

American Surveillance Study (52). Recently, the 1999-2000 report of the antimicrobial resistance among clinical isolates of *S. pneumoniae* in the United States was published as part of SENTRY indicating overall macrolide resistance rate in the USA of 26.2% (101. Comparison of 3 national antimicrobial (10.3% in 1994-1995 and from 19.2% in 1997-1998) resistance surveillance studies revealed that the macrolide resistance among isolates of *S. pneumoniae* in the US has increased significantly in the last 6 years (28,101). It is interesting to note that the rate of macrolide resistance among clinical isolates of *S.pneumoniae* varies by geographic location from a low of 6.1% in New Hampshire to a high of 53.7% in North Carolina (28). Other states with higher macrolide resistance included Texas (49.1%), D.C. (45%), Ohio (38.5) and Florida (33.3%) among others (28).

An ongoing national Canadian Respiratory Organism Surveillance Study, CROSS representing all regions in Canada was initiated by researchers in our laboratory, Dr. Hoban and Dr. Zhanel in 1997 (50,119,120). The goal of the study is to monitor and report resistance patterns of common respiratory tract pathogens to clinical and experimental antimicrobial agents. Recently published, macrolide resistance in *S. pneumoniae* in Canada over the last five years has increased from 7.9% in 1997 to 11.1% in 2002 (120). The increase in the national resistance rate appears to be attributed to local increases in certain provinces (120). In particular, macrolide resistance rate among clinical isolates of *S. penumoniae* in Manitoba has increased from 1.6 % in 1997 to 8.9 % in 2002 (120). Similarly, a 7.2 % increase during the same study period was observed in the Maritimes (120). A 3.1 % and 1.6 % increase in the macrolide resistance rate was observed in Quebec and Saskatchewan, respectively (120). Interestingly, macrolide

resistance rate of 12.9 % in 1997 has dropped to 7.2 % by 2002 in British Columbia (120). Macrolide resistance rate of approximately 9 and 10% has remained unchanged for two out of eight Canadian provinces, Alberta, and Ontario, respectively (120).

## 5. Prevalence of Macrolide Resistance Mechanisms in *S. penumoniae*

The two major mechanisms of resistance to macrolide antibiotics in *S. pneumoniae*; target site modification and active efflux of the drug have been described in great detail earlier in the Introduction. Briefly, the first of the two major mechanisms, the target site modification is due to the acquisition of the *erm*B gene that encodes erythromycin methylase enzyme that di-methylates the ribosome's binding site (67,69,71,108). This di-methylation leads to high-level resistance to macrolides, lincosamides and streptogramin B compounds and confers the $MLS_B$-phenotype (67,69). The second major mechanism of resistance, the active efflux is due to the acquisition of *mef*A or *mef*E genes that code for an efflux pump protein that has sufficient energy to efflux 14- and 15-membered macrolides out of the cell (104,111). *S. pneumoniae* isolates possessing the *mef*E or *mef*A genes usually have low-level resistance to 14- and 15-membered macrolides with complete susceptibility to 16-membered macrolides, lincosamides and streptogramin B antibiotics (M-phenotype) (104,111).

The prevalence of the $MLS_B$ and M-phenotypes among macrolide resistant *S. pneumoniae* isolates varies both geographically and temporally (21,50,57,66,78,87). In the United States and Canada, the M-phenotype predominates, whereas in Europe, Asia, and Australia and in South Africa the $MLS_B$-phenotype predominates (50). In the United States the predominance of the M-phenotype is very evident with a majority of studies

reporting more than 60% of macrolide resistant *S. pneumoniae* with the M-phenotype and some studies report few isolates with the $MLS_B$-phenotype (101). In a recent US study 61% macrolide-resistant *S. pneumoniae* contained the efflux pump whereas 32% contained the ribosomal methylase enzyme (101). In Canada, based on our own study and that of others the efflux mechanism is still the predominant form of macrolide resistance however the $MLS_B$-phenotype follows closely (50,75). In a recent study based in Toronto, 43.5% of macrolide resistant *S pneumoniae* exhibited the $MLS_B$-phenotype and 55.8% exhibited the M-phenotype. The prevalence of the $MLS_B$ and M-phenotypes among macrolide resistant isolates of *S. pneumoniae* identified in our lab during the 1999 and 2000 was 46.5 and 48.8%, respectively (75).

The general consensus among the European countries is that the target site modification due to *erm*B gene is the predominant form of macrolide resistance in *S. pneumoniae* and some countries such as Italy and Belgium for example report that 90% of their macrolide resistant isolates exhibit the $MLS_B$-phenotype and harbor the *erm*B gene (66,78). This exclusive presence of the $MLS_B$-phenotype among macrolide resistant *S. pneumoniae* is not present in all European countries (127). In Germany for example, the ratio of 43.4% to 56.1% for $MLS_B$ to M-phenotype mimics that in Canada (127).

**6. Definition of Macrolide Resistance**

In 1996, *in vitro* the breakpoints for erythromycin for *S. pneumoniae* as per the National Committee for Clinical Laboratory Standards (NCCLS) were changed from >1 to < 4μg/ml (intermediate) and ≥4μg/ml (resistant) to 0.5μg/ml (intermediate) and ≥1μg/ml (resistant) (126). For *S. pneumoniae*, *in vitro* resistance to clarithromycin is

defined exactly as for erythromycin ≥1μg/ml and azithromycin resistance, the breakpoint is ≥2μg/ml (126).

### 7. Clinical Significance of Macrolide Resistant *S. pneumoniae*

Bacterial resistance presents therapeutic dilemmas to clinicians and microbiologists worldwide (40,72,90). Drug-resistant *S. pneumoniae* is a growing concern because of the importance of this pathogen in infections of the respiratory tract in infants and children (20,40,47,60). Worldwide *erm*B and *mef*E/A account for >90% of macrolide resistance among pneumococci, but the prevalence of these genes varies considerable among countries or regions as has been described earlier. The erythromycin MICs range usually between 1 and 8μg/ml among *S. pneumoniae* isolates containing the efflux pump gene, *mef*E or *mef*A, whereas among the isolates with *erm*B gene, MICs typically exceed 128μg/ml (126). It has been suggested that the MIC differences between the *erm*B and *mef*E/A mutants are likely to be important *in vivo*, however many experts believe that the clinical impact of macrolide resistant *S. pneumoniae* is limited (126). Unfortunately, data assessing the impact of macrolide resistance on clinical outcomes are sparse (126). Based on the *in vitro* MICs, clinical failures can be expected with *erm*B strains, and concentrations of macrolides achieved in tissues or at sites of infection may override *mef*E/A mechanism (126). In addition to the differences in the level of macrolide resistance between *erm*B and *mef*E/A-mediated resistance, the cross-resistance to two other completely distinct groups of antibiotics, the lincosamides and the streptogramin B may make *erm*B-mediated resistance more significant clinically than *mef*E/A-mediated (126). Nevertheless macrolide treatment failure has been reported in macrolide-resistant

*S. pneumoniae* regardless of the mechanism of resistance including with *S. pneumoniae* harboring the 23S ribosomal RNA mutations and ribosomal protein mutations (40,55,62,73,113,126,129). Current rates of macrolide resistance at least in North America do not warrant moving away from this class of antimicrobials for most patients with CAP (126). However, as macrolide-resistant *S. pneumoniae* become endemic in communities, additional treatment failures can be expected making macrolides dangerous drugs to be used empirically for treatment of respiratory tract infections in particularly CAP.

**8. Hypotheses and Thesis Objective**

The efflux-mediated resistance in *S. pneumoniae* is due to the acquisition of the efflux pump genes, *mef*E or *mef*A and confers an M-phenotype that is resistance to 14- and 15-membered macrolides and susceptibility to lincosamides and type B streptogramin molecules (50). Until recently *S. pneumoniae* isolates exhibiting an M-phenotype were generally considered as isolates of low-level macrolide resistance with the $MIC_{90}$ of 8 μg/ml or sometimes as low as 4 μg/ml (50). However, although the $MIC_{90}$ is low for majority of clinical M-phenotype *S. pneumoniae*, interestingly, the range of MICs is increasing (0.5-64μg/ml). Recently, a rightward shift in the macrolide MIC distribution has been reported for *S. pneumoniae* by a few investigators (44,54), with higher MICs observed overtime. Such changes in the MIC distribution for macrolides can lead to therapeutic challenges as isolates become more resistant especially in North America where efflux-mediated resistance to macrolides dominates.

The objective of my thesis was to study efflux-mediated resistance in Canadian clinical isolates of *S. pneumoniae* with variable susceptibilities to macrolides (Clarithromycin MICs; 0.5–64 μg/ml). More specifically, the goal was to determine the cause of the decreased susceptibility to clarithromycin as depicted by higher MICs in M-phenotype clinical isolates of *S. pneumoniae.* Several hypotheses of the variable MICs to macrolide among M-phenotype *S. pneumoniae* isolates were postulated.

First, I hypothesize that the decreased susceptibility to macrolides might be due to higher expression of macrolide efflux gene, *mef*E. Higher expression of the efflux pump gene might lead to higher expression of the efflux pump protein that would be able to pump out higher amounts of the macrolide antibiotics and therefore raise the amount of antibiotic needed to inhibit the efflux mechanism of resistance. In addition, I hypothesized that changes in the promoter region of the *mef*E gene might lead to better polymerase binding and therefore contribute to higher transcription of the *mef*E gene. Second hypothesis; multiple copy number of the *mef*E gene may also contribute to decreased susceptibility of *S. pneumoniae* to macrolide antibiotics. Similarly to increased *mef*E gene expression, multiple copy number of the efflux pump gene might contribute to increased amounts of efflux pump protein that would pump out more antibiotics giving *S. pneumoniae* a selective advantage in the presence of higher concentrations of the macrolide antibiotic. Structural changes in the efflux pump protein might make the protein more efficient or more specific and thus able to pump out more antibiotics leading to greater resistance or higher MICs. Therefore my third I hypothesis was that that structural changes in the efflux pump protein might be responsible resistance to higher contractions of macrolide antibiotics. In addition, the degree of inducibility of efflux-

mediated resistance in *S. pneumoniae* has been studied as it has been recently hypothesized to be inducible by erythromycin and its derivatives (67,69,124).

To meet the objective and prove the hypotheses of my thesis several experiments incorporating many techniques were implemented.

- To determine whether *mef*E gene expression is responsible for higher MICs to macrolides, Northern Blot Hybridization and Relative Real Time RT-PCR experiments were utilized to quantitate the mRNA of the efflux pump gene *mef*E in isolates with variable susceptibility (0.5-32μg/ml) to clarithromycin.
- To investigate changes indicative of a stronger, better Polymerase binding promoter of the *mef*E gene, the upstream region of the efflux pump gene was sequenced from isolates with variable MICs to clarithromycin.
- To elucidate the presence of the multiple copy number of the *mef*E gene in isolates with decreased susceptibility to macrolide antibiotics, Southern Blot Hybridization was implemented.
- To determine structural changes within efflux pump protein, the entire DNA nucleotide sequence of the *mef*E gene encoding the efflux pump protein was sequenced and analyzed.

## B. MATERIALS AND METHODS

### 1. Bacterial Isolates

#### a. Isolate Selection

Twenty-seven isolates of *S. pneumoniae*, selected from more than 3000 isolates obtained as part of an ongoing Canadian Respiratory Organism Susceptibility Study (CROSS), were tested (50,119,120). Selection of isolates was based on clarithromycin and clindamycin MIC (as determined by the National Committee for Clinical Laboratory Standards [NCCLS]- recommended broth microdilution method) (109). Twenty-four macrolide-non-susceptible (clarithromycin MIC; 0.5-32μg/ml, clarithromycin susceptible ≤ 0.25μg/ml, intermediate 0.5μg/ml and resistant ≥1μg/ml) and clindamycin-susceptible (MIC≤ 0.25μg/ml) isolates were randomly chosen to represent a *S. pneumoniae* study group exhibiting the M-phenotype with various susceptibilities to clarithromycin (103). In addition, two macrolide-resistant (clarithromycin MIC; ≥64μg/ml) *S. pneumoniae* isolates exhibiting an $MLS_B$-phenotype, (clindamycin MIC; ≥32μg/ml) and one macrolide-susceptible (clarithromycin MIC ≤ 0.25μg/ml) clinical isolate of *S. pneumoniae* were included as controls. All the organisms have been submitted to or isolated by the Department of Clinical Microbiology, Health Sciences Centre, Winnipeg, Manitoba, Canada between August 28, 1997 and June 9, 1999 (50). Study isolates were obtained from eleven different medical centers widely distributed throughout Canada (50). Sources of the isolates consisted mostly of sputum samples, as well as eye, endotracheal tube, lung aspirate and tracheal specimens (50). A reference strain of *S. pneumoniae* (ATCC 49619) was included as a control for antibiotic susceptibility testing as well as for the PCR amplification methods.

**b. Isolate Identification**

The species identity of all selected isolates was confirmed on the basis of Gram stains and colony morphology, pattern of growth on sheep blood agar plates, and the results of the sodium deoxycholate solubility (Bile Solubility) and ethylhydrocupreine hydrochloride test (Optochin Susceptibility) as suggested in the Manual of Clinical Microbiology published by the American Society for Microbiology (95).

**2. Antibiotics**

Antibiotics were obtained as laboratory grade powders from their respective manufactures: erythromycin and clarithromycin, (Abbott, Abbott Park, Illinois), azithromycin (Pfizer, Groton, Connecticut), telithromycin (Aventis, Romainville, France), and clindamycin (Pharmacia and Upjohn, Michigan.). Antibiotics were reconstituted according to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines. The activity of all pure antibiotics was confirmed using susceptible *Staphylococcus aureus* ATCC 29213 and *S. pneumoniae* ATCC 49619 control strains.

**3. Determination of MICs**

**a. Broth Microdilution**

Following two subcultures from a frozen stock, susceptibility of each *S. pneumoniae* isolate to erythromycin, clarithromycin, azithromycin, telithromycin, and clindamycin, was determined using the NCCLS M7-A4 broth microdilution method (131). In this method, each final-panel well volume was 100µl, with a bacterial inoculum of 5 X $10^5$ CFU/ml (131). Panels were read following 20 to 24 h of incubation at 35°C in ambient air

(131). The MIC was defined as the lowest concentration of antibiotic inhibiting visible growth (131). Colony counts were performed periodically to confirm inocula. All susceptibility testing was performed in duplicate using cation-adjusted Mueller-Hinton broth supplemented with 5% lysed horse blood.

**b. E-Test**

E-Test was performed according to the instructions of the manufacturer (AB Biodisk, Solna, Sweden). In brief, test strip is placed aseptically on a Mueller-Hinton agar plate, supplemented with 5% sheep blood, which had been inoculated with a swab dipped into bacterial suspension with the turbidity equivalent to that of a 0.5 McFarland standard. The inoculated organism will not grow where the concentration of the drug is above the inhibitory level (109,112). Because of the linear concentration of the antimicrobial agent on the strip, an elliptical area of no growth develops (109,112). Following a 20-24 hour incubation at 35°C in 5% $CO_2$, the MIC of each *S. pneumoniae* was read directly from the strip where the ellipse of growth inhibition intercepts the strip.

**c. Disk Diffusion**

In accordance with the 2001 NCCLS guidelines, 2μg clindamycin and 15μg each of erythromycin, clarithromycin, and azithromycin filter disk (Becton-Dickinson Microbiology Systems, Cockeysville, MD) were used to screen for resistance (111,130). Antibiotic impregnated disks were applied aseptically to Mueller-Hinton agar plates, supplemented with 5% sheep blood, which had been inoculated with a swab dipped into a bacterial suspension with a turbidity equivalent to that of a 0.5 McFarland standard

(111,130). As the antimicrobial agent diffuses through the agar, the concentration drops logarithmically creating a concentration gradient surrounding the disk (109,130). Inoculated bacteria grow on the areas of agar that contain sub-inhibitory concentrations of the drug until a thick carpet of growth is visible (109,111,130). In the areas of the agar that contain inhibitory concentrations of the antimicrobial agent no growth occurs (109,111,130). The end result is a circular area of no growth surrounding the disk (109,111,130). The diameter of this circular area, the zone of inhibition, is measured in millimeters (109,130). The zone size is inversely proportional to the MIC, the larger the zone the lower the MIC (109,111,130). Following a 20-24 hour incubation at 35°C in 5% $CO_2$, the zone of inhibition was measured (109,111,130). Isolates with a zone of inhibition smaller or equal to 15, 15, 16, and 13mm were considered resistant to clindamycin, erythromycin, clarithromycin, and azithromycin, respectively, while clindamycin, erythromycin, clarithromycin, and azithromycin zone of inhibition greater than or equal to 19, 21, 21, and 18mm identified isolates as susceptible (130).

**4. Polymerase Chain Reaction (PCR)-Based Detection of *mef*A/E Gene**

**a. DNA Isolation**

*S. pneumoniae* cultures were grown overnight on Trypticase soy agar with 5% lysed horse blood, and two to five colonies were re-suspended in 1 ml of sterile saline. Following centrifugation at 13,000 rpm for 10 min, supernatants were removed, and the resulting bacterial pellets were re-suspended in 300µl of lysis buffer containing 0.1M NaOH, 2.0M NaCl, and 0.5% SDS. Cell suspensions were then boiled for 15 min, and 200µl of 0.1M Tris-HCL (pH 8.0) was added. For extraction of genomic DNA, 500µl of

phenol-chloroform-isoamyl alcohol (25:24:1) was added, and the mixture was centrifuged at 13,000 rpm for 10 min: 1ml of cold (-20°C) anhydrous alcohol was then added, and DNA was precipitated at –80°C for a minimum of 30 min. The precipitated DNA was collected by centrifugation at 13,000 rpm for 15 min at 4°C, and the pellets were allowed to air dry for no less than half an hour. Pellets containing purified DNA were subsequently re-suspended in sterile distilled water. The concentration and purity of DNA was determined by measuring the absorbance at 260nm ($A_{260}$) and 280nm ($A_{280}$) in a spectrophotometer. An absorbance of 1 unit at 260nm corresponds to 50µg of DNA per ml of water ($A_{260}$=1=50µg/ml). The ratio between the readings taken at 260nm and 280nm ($A_{260}/A_{280}$) provides an estimate of the purity of DNA. DNA preparations with a ratio of $A_{260}/A_{280}$ greater than 1.6 were adjusted to a final concentration of 20ng/µl and were used as templates in all PCR experiments.

**b. PCR Reaction**

Amplification reaction was carried out in 50µl of mix containing 25µl of 2X master mix [2X GeneAmp® PCR buffer II (Applied Biosystems), 6mM $MgCl_2$ (Applied Biosystems), 0.4mM dNTPs (Invitrogen)], 2.5µl of 10µM primer mix [*mef*AE-1 + *mef*AE-2], 0.5µl of 5U/µl AmpliTaq Gold® DNA polymerase (Applied Biosystems), 17µl $H_2O$ and 5µl of DNA template (20ng/µl). The primers used were synthesized with an Oligo 1000 DNA Synthesizer (Beckman), DNA Core Facility (Health Canada, Winnipeg, Manitoba). The concentration of each primer was determined by measuring the absorbance at 260nm ($A_{260}$). Optical density at 260nm divided by (0.01 x # of bases in a given primer) x dilution factor was employed to express the concentration in µmoles.

Primers used for the amplification of *mef*A/E were based on previously published primers: 5'- AGT ATC ATT AAT CAC TAG TGC-3' and 5'-TTC TTC TGG TAC TAA AAG TGG-3' (PCR product, 346bp) and are listed in Table 1 (50,103,105). Amplification of *mef*A/E was performed using a Perkin-Elmer GeneAmp® PCR system 9700 and consisted of initial AmpliTaq Gold® activation at 95°C for 10 min, 30 cycles at 95, 53, 72°C for 1 min each, and a final extension at 72°C for 10 min. One negative control (comprised of the identical reaction mixture with sterile distilled water in place of the template DNA), one positive control (clinical isolate #15353), two *erm*B-positive isolates (clinical isolate #12778, 12767), one susceptible clinical isolate (#1333), and one laboratory reference *S. pneumoniae mef*A/E-negative ATCC 49619 isolate were included in each PCR reaction. To verify the reproducibility of PCR of *S. pneumoniae*, isolates were tested under the same conditions in triplicate.

### c. PCR Product Detection

The amplified products were run in 1% agarose gels (Gibco-BRL) made with 0.5X Tris-Borate-EDTA (TBE) buffer at 100V either containing ethidium bromide or stained after and were visualized under UV light. A 100bp DNA ladder (Gibco-BRL) was used as a molecular weight marker.

## 5. Induction of *mef*-Mediated Resistance in *S. pneumoniae*

Phenotypic inducibility of the efflux-mediated macrolide resistance in *S. pneumoniae* isolates, if it occurs, would be seen as an increase in the MIC of the test drug following

incubation of the isolate with sub-inhibitory concentration of that drug. To test this hypothesis the following study was designed.

**a. Growth of Culture**

Following two subcultures from frozen stock, an initial inoculum of 5 $x10^4$ to 5 $x10^5$ CFU/ml of each strain was prepared in a final volume of 60ml of Todd Hewitt Broth, THB (Difco, Detroit, MI) supplemented with Yeast Extract, YE (Difco, Detroit, MI) (0.5%v/v). Cells were grown in screw-cap bottles in a shaker bath at 37°C for 17-19 hours.

**b. Induction of M-phenotype**

Induction studies were performed twice with each isolate by diluting the overnight growth 1:100 so that the initial concentration of the bacteria was within the $1.5x10^5$-$1.5x10^6$ CFU/ml range before the antibiotic is added. 1/8x the MIC (sub-inhibitory concentration) of erythromycin, clarithromycin, azithromycin, clindamycin, and telithromycin antibiotic was then added and cells were grown in a shaker bath at 37°C for 3-4 hours.

**c. Result Interpretation**

Cultures were sampled pre- and post-induction and erythromycin, clarithromycin, azithromycin, clindamycin and telithromycin MICs were determined by E-test (AB Biodisk, Solna, Sweden) as described in Section 3b. All E-test susceptibility testing was performed in duplicate and the mean MIC was determined.

## 6. Distinction Between *mef*A and *mef*E

### a. Polymerase Chain Reaction (PCR)

The amplification of the *mef*A *or mef*E genetic determinant from each of the 28 *S. pneumoniae* isolates was performed using the previously described primer set: *mef*A/E-1 and *mef*A/E-2 (50). The presence of the upstream (*orf*2 and *orf*3) flanking region of the *mef*A gene was determined using specifically designed primer sets *orf*2-A and *orf*2-B or *orf*3-DN and *mef*A-UP respectively. Both primer sets are specific for Tn*1207.1* (98). The downstream (*orf*5) flanking region of the *mef*A gene was detected using the specifically designed primer set *mef*A-DN and *orf*5-UP also based on the recently published nucleotide sequence of Tn*1207.1* (98). The presence of the upstream region of the *mef*E gene was determined using a primer set *mef*A-UP and *orf*1-UP based on the recently published mega (macrolide efflux genetic assembly) nucleotide sequence (43).

Primer sequences, source (Tn*1207.1* or Mega) and annealing temperature are shown in Table M1. PCR amplification reactions consisted of initial denaturation at 95°C for 10 min, 30 cycles at 95°C, specific annealing temp, and 72°C for 1 min each, and a final extension at 72°C for 10 min.

### b. Differentiation Between *mef*A and *mef*E

To discriminate between *mef*A and *mef*E, PCR-Restriction Fragment Length Polymorphism, PCR-RFLP analysis was performed using a previously described protocol (25). Briefly, the *mef*-3 and *mef*-4 primer pair was used to generate a 1743-bp PCR product. The amplicon was then digested with *Bam*HI or *Dra*I restriction enzyme. In *mef*A there is one *Bam*HI site, so restriction generates two fragments of **1340** and

**403bp,** respectively while in *mef*E there are no *Bam*HI site restriction sites (25). Restriction of the *mef*A with *Dra*I yields two fragments of **1493** and **250bp**, respectively, while restriction of *mef*E yields three fragments of **782**, **711** and **250bp**, respectively (25).

| Primer pair | Primer sequence (5'- 3') | Position in Tn*1207.1* (Mega) 5'- 3' | PCR product size (bp) | Reference | Annealing Temp C° |
|---|---|---|---|---|---|
| *mef*A/E-1 | AGT ATC ATT AAT CAC TAG TGC | 3310-3330 (1181-1201) | | | |
| *mef*A/E-2 | TTC TTC TGG TAC TAA AAG TGG | 3655-3635 (1526-1506) | 346 | Tn1207.1 Mega | 53 |
| *mef*A-up | ATG GCA CTA GTG ATT AAT G | 3333-3315 | | Tn1207.1 | |
| *orf*3-DN | GAT TCA CAT TCC TGA AGA T | 2353-2371 | 1000 | Tn1207.1 | 59 |
| *orf*2-A | AGA TTT CAT TAG TAT TTA CCG | 1280-1300 | | Tn1207.1 | |
| *orf*2-B | TCA AGT ACA ACA AAA TCC CTC | 1966-1946 | 700 | Tn1207.1 | 58 |
| *mef*A-DN | TCT TTG CTG ATA GAA TCG | 4356-4373 | | Tn1207.1 | |
| *orf*5-UP | AAT TGC TCA ACA CCT AGC | 4874-4857 | 400-500 | Tn1207.1 | 58 |
| *mef*A-up | ATG GCA CTA GTG ATT AAT G | 3333-3315 | | Tn1207.2 | |
| *orf*1-UP | TGA GGT TGA GTT AGA AAA TCC | (562-582) | 600 | Mega | 55 |
| *mef*-3 | GCG TTT AAG ATA AGC TGG CA | 2973-2992 (828-847) | | | |
| *mef*-4 | CCT GCA CCA TTT GCT CCT AC | 4715-4696 (2586-2567) | 1743 | Tn1207.1 Mega | 57 |

**Table 1. Oligonucleotide Primers used in Polymerase Chain Reactions and PCR-Restriction Fragment Length.**

## 7. Northern Blot Hybridization

The complete system for Northern Blotting, the NorthernMax- Gly system supplied by Ambion, was utilized. The NorthernMax-Gly kit uses glyoxal/dimethylsulfoxide (DMSO) instead of formaldehyde to denature the RNA. This eliminated the need for a fume hood and the gels were poured and run without the exposure to noxious fumes normally associated with formaldehyde. BrightStar Psoralen-Biotin nonisotopic labeling and BrightStar BioDetect kits (Ambion) were utilized in preparation of the hybridization probe, signal generation and detection. Manufactures instructions were closely followed and only modifications are discussed.

### a. Bacterial Growth

Following two sub-cultures from a frozen stock, isolates were grown in a final volume of 10 ml of Todd Hewitt Broth (Difco) supplemented with 0.5% Yeast Extract (Difco) v/v in ambient air without shaking at 37°C. Each isolate was grown to mid-log and an optical density of approximately 0.3-0.4 at a wavelength of 580nm was reached.

### b. RNA Protection

In order to ensure accurate gene-expression analysis, it is important that the RNA analyzed truly represents the in vivo gene expression of the bacterial cells. During isolation and handling of RNA, enzymatic degradation and the very short half-life of bacterial RNA can result in a reduction or loss of many transcripts. RNA stabilization before cell lysis using RNAprotect Bacteria Reagent (Qiagen) allows more time for the

efficient disruption of cell, while it ensures that the bacterial RNA expression profiles are not being affected by degradation. RNA protection was performed according to the instructions of the manufacturer (Qiagen). In short, 1 ml of bacterial culture grown to mid-log in Todd Hewitt Broth (as described above) was added to a reaction tube containing 2 ml of RNAprotect Bacterial Reagent. Following a short, vigorous mixing for 5s, and a 5-minute incubation at room temperature, bacteria were pelleted by centrifugation for 10 min at 5000x g. Cell pellets were stored at –20°C until RNA isolation or used immediately.

**c. RNA Isolation**

RNA isolation from all samples was aided by the use of RNeasy Mini Kit (Qiagen). In short, this kit utilizes an enzymatic (lysozyme) digestion of the cell wall followed by addition of lysis buffer as a disruption method. Homogenization was accomplished by vortex. Manufactures instructions were closely followed throughout the isolation procedure. Enzymatic digestion of the cell wall with lysozyme at a concentration of 10mg/ml instead of the recommended 3mg/ml and incubation time of 15 min instead of 5 min was the only modification from the manufactures protocol.

**d. Quantification of Total RNA**

The concentration and purity of RNA was determined by measuring the absorbance at 260 nm ($A_{260}$) and 280nm ($A_{280}$) in a spectrophotometer. An absorbance reading of 1 unit at 260nm corresponds to 40μg of RNA per ml. The ratio between the readings taken at 260nm and 280nm (A260/A280) provides an estimate of the purity of RNA. Pure

RNA has an A260/A280 ratio of 1.8 –2.1. Throughout all the experiments RNA outside of this range were discarded.

**e. DNA Contamination**

All RNA preparations that were determined to be of good quantity and purity were treated with DNA-free (Ambion), according to manufactures instructions to remove contaminating DNA.

**f. Integrity**

Following DNase treatment, the integrity of total RNA purified with RNeasy kit was checked by denaturing-agarose gel electrophoresis and ethidium bromide staining of 23S and 16S ribosomal RNA, (rRNA) bands. RNA of good integrity contains un-degraded 23S and 16S ribosomal RNA visible on the gel as sharp, distinct bands 2.9Kb and 1.5Kb in size, respectively. In addition the 23S rRNA bands should be present at approximately twice the amount of the 16S rRNA. If the ribosomal bands were not sharp, but appeared as a smear, the RNA under analysis has suffered major degradation during its preparation and was not used as a RNA template in further application such as Northern Blotting or RT-PCR.

**g. Gel Preparation**

Performed exactly in accordance with the NorthernMax- Gly user instructions.

**h. Preparation of Sample RNA**

During this step, the RNA sample is mixed with equal volume of Glyoxal Load Dye. For large sample volumes, one-half the volume of Glyoxal Load Dye was used and the time of incubation was increased to one hour from the normally recommended half an hour.

20 µg of total RNA from each sample was used. Millennium RNA ladder (Ambion) was used as a molecular weight marker. Two µl of the RNA marker was utilized in each experiment.

**i. Electrophoresis**

RNA samples were run on an 1% denaturing-agarose gel at 100V until the bromphenol blue dye front corresponding to approximately 500 nt has migrated almost to the bottom of the gel.

**j. Transfer of RNA to the Membrane**

Transfer was performed using a Turboblotter™ (Schleicher & Schuell). The assembly of the Turboblotter and Blotting Stack was set up according to manufactures instructions. Ambion's BrightStar-Plus membranes were utilized in all Northern Blotting experiments.

**k. Cross-Linking the RNA**

Following the transfer of RNA to the membrane, a commercial UV crosslinker, was used to covalently cross-link the RNA to the membrane.

**l. Pre-Hybridization and Hybridization**

Blots were allowed to pre-hybridize in the ULTRAhyb buffer for at least one hour at 42° in a hybridization oven before the addition of the labelled *mef*E probe.

**m. Preparation of the Probe**

BrightStar Psoralen-Biotin Labelling Kit (Ambion) was used to nonisotopically label the *mef*E probe. 346bp *mef*A/E-1 and *mef*A/E-2 PCR products amplified in several reactions from a positive control strain were used as a *mef*E DNA probe in all Northern Blotting experiments. Each PCR reaction was pooled together and adjusted to a final *mef*E probe concentration of 50ng/µl. In total, 500ng of *mef*E probe in a final volume of 10 µl was labelled. To ensure the kit is performing well, Ambion provides unlabeled and labeled control DNAs. Labeling of the *mef*E probe along with the unlabeled control DNA provided by the company was performed in accordance with the instructions provided in the user manual. At the end of the labelling reaction the final concentration of the labelled *mef*E probe is 5ng/ul. Following the labelling, both DNAs were subjected to a biotin detection procedure and the results were compared with the Ambion labelled DNA. Ten-fold dilutions of biotinylated samples from 1 ng/ul down to 1fg/ul were made and one µl aliquot of each dilution was spotted on a dry positive charged membrane, autocross-linked using commercial cross-linker. Following the cross-linking the biotin was detected using BrightStar BioDetect nonisotopic detection system (Ambion).

**n. Hybridization**

Following the pre-hybridization, the labelled *mef*E probe was added directly to the ULTRAhyb buffer and was allowed to hybridize overnight.

**o. Washing**

Following the overnight hybridization, the blot was washed once with a low stringency buffer and twice with high stringency buffer according to the instructions.

**p. Signal Generation and Detection**

Signal generation and detection was performed using BrightStar BioDetect system from Ambion in accordance with the instructions of the manufacturer. It takes 2-4 hours for CDP-Star to reach peak in light emission and the blot was exposed to the film for 3 hours.

**8. Southern Blot Hybridization**

**a. Culture Growth**

Following two subcultures from a frozen stock, (isolates were taken out of a frozen stock onto a MHB agar plate grown for 24 hr at 37° in $CO_2$ re-subbed next day) were grown overnight in 10 ml of Todd Hewitt Broth supplemented with 0.5% Yeasts Extract in ambient air without shaking at 37°C.

**b. DNA Isolation**

One ml of the overnight culture was centrifuged for 5 min at > 10 000 rpm to pellet

the cells. Next, 600µl of PBS solution containing 5mg/ml of lysozyme was added. Following a 15 minute incubation at 37°C, 60µl of 10% SDS was added, mixed by pipeting up and down few times and incubated at 65°C until the solution became clear. The incubation of 65°C was not longer than 5 min. For extraction of genomic DNA, 600µl of phenol-chloroform-isoamyl alcohol (25:24:1) was added, the mixture was mixed gently until a homogenous solution formed, then it was centrifuged for 3 min in microfuge and the top layer containing the DNA was transferred to a new tube. The phenol-chloroform-isoamyl extraction was performed twice and it was followed by a single extraction with 600µl of chloroform-isoamyl (24:1). To precipitate the DNA, 600µl of ice-cold isopropanol was added, mixed gently by inverting the reaction tube. The precipitated DNA was collected by centrifugation at 13,000 rpm for 15 min at 4°C subsequently the pellets were washed with ice cold 70% ethanol and allowed to air dry for no less than half an hour. Pellets containing purified DNA were subsequently re-suspended in sterile distilled water. The concentration and purity of DNA was determined by measuring the absorbance at 260nm ($A_{260}$) and 280nm ($A_{280}$) in a spectrophotometer. An absorbance of 1 unit at 260nm corresponds to 50ug of DNA per ml of water ($A_{260}$=1=50ug/ml). The ratio of 1.7 or above, between the readings taken at 260nm and 280nm determined purity of the isolated DNA.

**c. Restriction Endonuclease Digestion**

*Eco*R1 Endonuclease was used to restrict the genomic DNA in all isolates. It was chosen based on the restriction map of the mega element. *Eco*RI recognises a GAATTC sequence and creates sticky ends upon cutting. The restriction reaction was carried out in

a final volume of 50µl containing 5µl of a 10x concentrate NE buffer for EcoR1 enzyme (New England Biolabs Inc.), 1 µl of 20 000 U/ml *Eco*R1 enzyme (New England Biolabs Inc.), 20 µl of DNA template (1µg/ul), and 24µl of sterile water for 2 hours at 37°C water bath. Following a 2-hour restriction digestion subsequent aliquot of *Eco*R1 was added and reaction was carried out for another 2 hours to ensure complete digestion of the genomic DNA.

**d. Gel Electrophoresis**

The digested products were run on a 0.8% agarose gels (Gibco-BRL) prepared with 0.5X TAE buffer at 64V overnight (17hours). A 1kb extension (Invitrogen) DNA ladder was used as a molecular weight marker. The ethidium stained products were visualized by UV light to ensure complete digestion.

**e. Gel Processing**

**α. Depurination**

Fragments greater than 15 KB are difficult to transfer to the blotting membrane. Depurination with 250 mM HCl takes the purines out, fragmenting the DNA into smaller fragments. Depurination was carried out in a container that allowed free movement of the gel using excess amount of the dupurination solution, until the bromophenol blue dye has turned yellow, approximately 20 minutes with gentle agitation at room temperature. The depurinated gel was than rinsed with distilled water.

### β. Denaturation and Blotting

It is necessary to denature (double stranded DNA into single stranded DNA; only single stranded DNA can transfer) the DNA before it is transferred to a membrane. Alkali transfer buffer (1.5M NaCl and 0.5M NaOH) has denaturing properties and DNA was efficiently denatured while it was being transferred to a membrane without the need for a separate denaturation step. Alkali transfer also automatically binds single stranded DNA to a membrane eliminating the need for UV cross-linking of the DNA to a membrane after transfer. Transfer was performed using a Turboblotter$^{TM}$ (Schleicher & Schuell) and the assembly of the Turboblotter and Blotting Stack was set up according to manufactures instructions. Hybond -N+ (Amersham Pharmacia Biotech) membrane was used in all blotting experiments.

### f. Processing the Blot

Following the disassembly of the blotting apparatus, the membrane was placed in 5x SSC and rinsed for 1 minute with gentle agitation to remove any remaining agarose gel. Pre-hybridization, hybridization, probe labelling and detection was performed using the ECL direct labelling and detection system (Amersham Life Sciences) according to the instructions of the manufacturer. Comments to the outlined procedure are made when they differed from the manufacture's instructions.

### g. Pre-Hybridization and Hybridization Buffer Preparation

A specifically optimized hybridization buffer (Gold hybridization buffer) is included with the ECL direct labelling and detection system. The formulation of this buffer

includes 6 M urea and, therefore, the stringency of hybridization, can be controlled only by altering the salt concentration. The sodium chloride concentration of 0.5M was optimal

for *mef*E probe.

**h. Pre-Hybridization**

Blots were allowed to pre-hybridize for 60 minutes before the addition of the labelled *mef*E probe at 42°C in a hybridization oven.

**i. Preparation of Labelled Probe**

346bp *mef*AE-1 and *mef*AE-2 PCR product amplified in several reactions from a positive control strain was used as a *mef*E probe in Southern Blotting experiments. The probe was purified, quantitated and stored at a concentration of 10ng/ul until labelling. The labelling was performed exactly as described in the ECL system. The total amount of DNA that was labelled and used in hybridization was 200ng instead of the recommended 100ng.

**j. Hybridization**

Following pre-hybridization, the labelled *mef*E probe was added to the pre-hybridization buffer and allowed to hybridize for 17 to 20 hours at 42°C in a hybridization oven.

### k. Stringency Washes

Following overnight hybridization the membrane was washed twice with 0.5xSSC primary wash buffer without urea for 10 min at a temperature of 55°C to remove any un-hybridized *mef*E probe. Primary wash was followed by one 15- minute 2xSSC secondary wash before detection.

### l. Signal Generation and Detection

Signal detection was performed in accordance with the instructions of the manufacturer. A brief 60 seconds exposure to the film was followed by a 15- minute exposure to generate the desired signal.

## 9. Real-Time RT- PCR

Relative real-time RT-PCR was utilized to study the *mef*A/E gene expression in the study isolates after unsuccessful detection of mRNA of *mef*A/E gene in these isolates with Northern Blot Hybridization (13,14,48,45). A relative quantification was based on the relative expression of a target gene (*mef*A/E) versus a reference gene. The glucose kinase (*gki*) gene was used as a reference gene (34). TaqMan One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems) was used throughout these experiments.

### a. Primer and Probe Design

Following the rules outlined in the TaqMan One-Step RT-PCR Master Mix Reagents Kit the forward and reverse primers, along with the probes for the target and the reference gene were designed based on *mef*E gene and *gki* gene bank sequence, respectively. The primers were synthesised by the DNA CORE facility (Health Canada, Winnipeg,

Manitoba). TaqMan probe consists of an oligonucleotide with a 5'-reporter dye and a 3'-quencher dye. The fluorescent reporter dye, HEX or FAM was covalently linked to the 5'end of the *mef*E and *gki* oligonucleotide, respectively (Synthegen). Each of the reporters was quenched by TAMRA dye at the 3'end. All the primers and probes are presented in a Table 2 below.

**b. Preparation of RNA Template**

Bacterial growth, RNA protection and isolation, quantification and purification for RT-PCR were performed as described for Northern Blotting.

**c. RT-PCR**

The co-amplification of the *mef*E and *gki* genes was carried out in 50µl mix containing 25µl of TaqMAn One-Step RT-PCR 2X Master Mix without UNG (Applied Biosystems), 2.5 µl of *mef*E forward and reverse primer mix ([10µM]), (*mef*E-RT-1 + *mef*E-RT-2) 2.5 µl of forward and reverse *gki* primer mix ([10µM]), (SpglK-RT-1 + SpglK-RT2), 0.125µl of HEX labelled *mef*E and FAM labelled *gki* probe ([100µM each]) (Synthegen), 1.25ul of 40X Multiscribe and RNase inhibitor, 3.5µl of dNTPs ([100µM]) (Applied Biosystems) and 3.5µl of $MgCl_2$ ([25mM]) (Applied Biosystems), 1.5ul of AmpliTaq Gold ([5U/ul]) (Applied Biosystems), 5µl of water and 5µl of RNA template (20ng/ul). The final concentration of dNTPs and $MgCl_2$ is 175µM and 1.75mM more, respectively than recommended in the protocol. The actual concentrations of the components of the 2X Master Mix are not known so it is not possible to determine the actual final concentrations. The probe to primer ratio of 1:2 was optimal for the co-

amplification of *mef*E and *gki*. Co-amplification of *mef*E and *gki* was performed using a Mx4000, Multiplex Quantitative PCR System (Stratagene) and consisted of initial 30 min Reverse Transcription at 48°C, followed by AmpliTaq Gold® activation at 95°C for 10 min, and 40 cycles of PCR at 95°C (denaturation), 58°C (Annealing) for 30sec and 1 min, respectively. Negative control, comprised of the identical reaction mixture with sterile distilled water in place of the template RNA is included with each run. All RNA samples are also used as templates in a reaction that contains all the components of the above described mixture but no Reverse Transcriptase (RT). Any signal detected in the absence of the RT must be from the amplification of the DNA that is contaminating the RNA sample. Each RNA sample was run in triplicate.

**d. Data Analysis**

Complete instructions of the data analysis are provided with the Mx4000, Multiplex Quantitative PCR System (Stratagene) and were followed when analyzing the data. Briefly, the *gki* was the normalizer and before beginning the analysis of the expression of the *mef*E gene, the expression the *gki* was normalized throughout all the samples. Because relative and not absolute quantification was employed the *mef*E signal was expressed as that relative to the *gki* signal in each sample. Relative quantification corrects for the minute differences in the quantity of the starting template.

**e. Statistical Analysis**

Pearson correlation was utilized to study the degree of linear relationship between the clarithromycin MIC and the relative *mef*E gene expression. An analysis of variance

(ANOVA) was than employed to determine the degree of statistical difference between the different levels of *mef*E gene expression. All the statistical tests were performed with the 2001 version of NCSS statistical program.

## 10. Sequencing

A 1945bp nucleotide sequences encompassing the upstream and downstream intergenic regions of *mef*E and *mel* genes were amplified from all 23 M-phenotype *S. pneumoniae* isolates and were sequenced using overlapping primers. In addition, the same 1945bp nucleotide sequence was amplified and sequenced from isolate number 18, identified as containing the *mef*A variant of *mef* residing on transposon, Tn*1207.1* and was compared to the published Tn*1207.1* sequence. The purpose of the sequencing was three-fold. The first goal of the sequencing was to determine significant differences in the potential promoter of the *mef*E gene. The second goal was to determine the *mef*E gene sequence homology and the third to further sub-classify the mega element as class I or class II inserts.

### a. Design of the Sequencing Primers

The principal of sequencing a bigger DNA fragment, such as 1945bp is based on a proper design of the primers so that they can produce overlapping sequences. Six primers were utilized in the sequencing of this 1945bp DNA fragment. Three primers were in the forward (5' to 3') and 3 were in the reverse (3' to 5') direction. The complete DNA sequences of each of the primers used in the sequencing reactions are presented in Table 2.

**b. Preparation of Bacterial Lysates for Sequencing**

The extraction of the DNA for sequencing from each of the 24 M-phenotype *S. pneumoniae* isolates was performed exactly as described in Section IV part a, of the Material and Methods.

**c. PCR Protocol for the Sequencing of the Promoter Region of *mef*E**

An approximately 650bp region upstream of the *mef*E gene of mega genetic element was amplified using *orf*1-up and *mef*A-Up primers, shown in Table M2 following exactly the protocol outlined in Section IV part b, of the Material and Methods. Annealing temperature was 54°C.

**d. PCR Protocol for the Sequencing of the *mef*E Gene and *mef*E-*mel* Intergenic Region**

A 1480bp region encompassing the upstream and downstream intergenic region of *mef*E and *mel* was amplified using *mef*E-UP and *mef*E-DN2 primers, shown in Table 2 following the previously described protocol in Section IV part b of the Materials and Methods. Annealing temperature was 54°C.

**e. Preparation of the DNA Template for Sequencing**

Following the PCR reaction the amplified products were run on a 1% agarose gel and visualized with UV light following the ethidium bromide staining as described in section IV part c of the Materials and Methods to confirm the PCR product of approximately the right size. PCR products were subsequently purified with Microcon microconcentrators

(Millipore, Bedford, MA) according to manufacture's instructions. Following the purification, the concentration of each PCR product was adjusted to a final concentration of 50μg/ml as needed for sequencing.

### f. Sequencing of the Promoter Region of *mef*E

The *mef*A-up and *orf*1-Up primers used in the PCR were also used for sequencing of the upstream region of the 23 M-phenotype *S. pneumoniae* isolates containing *mef*E. The primers utilized for sequencing were diluted to a final concentration of [1μM]. All the primers are presented in Table 2 of the Materials and Methods

### g. Sequencing of the Downstream Region of *mef*E

In addition the to *mef*E-UP and *mef*E-DN2 primers used in the PCR, 2 other primers, one from the RT-PCR experiment, *mef*E-RT1 and one newly designed, *mef*E-MID were implemented in sequencing of all 24 M-phenotype *S. pneumoniae* isolates including the 1 containing *mef*A. As described above each primer was adjusted to a final concentration of 1μM. All the primers are presented in Table M2 of the Materials and Methods.

### h. Sequencing

The correct concentrations and volumes of DNA templates and sequencing primers were supplied to the sequencing facility, DNA CORE, at the National Microbiology Laboratory in Winnipeg, Manitoba. Five μl of DNA template at [50μg/ml] and 5μl of each primer at [1μM] were needed for each sequencing reaction and were supplied as requested.

### i. Sequence Analysis

Utilizing Lasergene's (DNA Star Inc., Madison, WI) Seqman II module, individual sequence fragments from the 4 sequencing reactions for each of the 24 M-phenotype *S. pneumoniae* isolates and *mef*A and 2 with *mef*E sequencing reactions for each 23 M-phenotype *S. pneumoniae* isolate with *mef*E only were arranged into contig and analysed. The alignment of the efflux pump gene encompassing region from each *S. pneumoniae* isolates and comparison with the published mega nad Tn1207.1 elements carrying the *mef*E and *mef*A, respectively was performed using Lasergene's MEG align module.

| Primer | Primer sequence ( 5' - 3') | Position in (Mega) 5'- 3' | Reference |
|---|---|---|---|
| *mef*A-up | ATG GCA CTA GTG ATT AAT G | (1205-1186) | Mega |
| *orf*1-UP | TGA GGT TGA GTT AGA AAA TCC | (562-582) | Mega |
| *mef*E-UP | GCCTATAATGCTATTCAAAAT | (1066-1086) | Mega |
| *mef*E-DN2 | TACTAAACCAATACGGTCATA | (2569-2548) | Mega |
| *mef*E-RT1 | AGCTACCTGTCTGGATGATT | (1420-1439) | Mega |
| *mef*E-MID | ATAGAAATATGCACAGGCGTT | (1900-1880) | Mega |
| *mef*E-RT2 | TCGTTAGCTGTTCTTCTGGT | (1536-1517) | Mega |
| *mefE*-RT-PCR probe | HEX-ACCCCAGCACTCAATGCGGTTACAC | (1482-1506) | Mega |
| *Spglck*-RT1 | CATCGATAATGATGCCAACGT | - | Gene bank |
| *Spglck-RT2* | AGTACCGAGTGTCATAAAGAC | - | Gene bank |
| *Spglck*-RT-PCR probe | FAM-GCACCCATCCAGCGCTCACCAA | - | Gene bank |

**Table 2. Oligonucleotide Primers used in Sequencing and Real-Time RT-PCR.**

## C. RESULTS

To determine the characteristics of the *mef*E/A gene in isolates with variable susceptibility to clarithromycin 24 clinical isolates of *S. pneumoniae* were chosen from a collection of more than 3000 isolates obtained between 1997 and 1999 as part of an ongoing annual Canadian Respiratory Organism Surveillance Study (CROSS). Selection of isolates was based on clarithromycin and clindamycin MIC (as determined by the National Committee for Clinical Laboratory Standards [NCCLS]- recommended broth microdilution method). The antibiotic profile of a clarithromycin and clindamycin as well of a telithromycin were initially determined by broth microdilution as part of CROSS. Following the selection, the MICs to erythromycin, clarithromycin, azithromycin, clindamycin, and telithromycin were confirmed in triplicate by broth microdilution, E-Test and disc diffusion. Genotypic confirmation of the M-phenotype was determined molecularly by *mef*A/E gene PCR amplification.

First, the effect of sub-inhibitory concentration of each of the antibiotic on the MIC of that antibiotic was studied for each isolate to determine phenotypically the inducibility of the *mef*E/A gene expression. Second, the genetic element carrying the *mef*E/A genes among *S. pneumoniae* isolates was studied by PCR of the various regions of the two elements and also by PCR-Restriction Fragment Length Polymorphism. Third, to assess whether higher *mef*E gene expression might be responsible for lower susceptibility to clarithromycin, Northern Blot Hybridization and Relative Real-Time RT-PCR were employed. The Southern Blot Hybridization with the *mefE* probe and *Eco*RI digestion consisted of the 4$^{th}$ experiment and was utilized to assess the presence of multiple *mefE* gene copies in isolates with decreased susceptibility to clarithromycin. Lastly, a 1945bp

nucleotide region encompassing the upstream and downstream intergenic region of *mef*E and *mel* of mega was amplified and sequenced using overlapping primers to identify nucleotide sequence changes within the potential promoter region of the *mef*E gene, as well as to identify the *mef*E gene nucleotide sequence homology and to further sub-classify the recently identified mega insertions in these clinical isolates of *S. pneumoniae*.

**Part I. Antibiotic Susceptibility Testing of *S. pneumoniae***

The susceptibility to erythromycin, clarithromycin, azithromycin, clindamycin and telithromycin for each of the 28 isolates was tested by broth microdilution and E-Test. The disc diffusion method was utilized to confirm the M-phenotype. The susceptibility to the different antibiotics was performed in triplicate and the mean MICs for the broth microdilution and E-Test along with the diffusion size for the disc diffusion method is shown in Table 3. Broth microdilution MICs for the three different macrolides were within $\pm$ one dilution of each other for the majority of isolates. The broth microdilution MIC distribution for the three macrolides was slightly different but the ranges remained very close at 1- 32μg/ml for both erythromycin and azithromycin and 0.5-32μg/ml for clarithromycin (Table 4). The $MIC_{90}$ for both erythromycin and clarithromycin was 16μg/ml while for azithromcyin it was 32μg/ml (Table 4). Comparing the clarithromycin broth microdilution MICs with E-Test clarithromycin MICs revealed that E-Test MICs were on average 4 dilutions higher than broth microdilution MICs, however the MIC correlation between isolates was maintained. Broth microdilution MICs for clindmycin were 100% in concordance with the clindamycin E-Test MICs $\pm$ 1 dilution. In accordance with NCCLS zone diameter interpretive standards, all isolates identified by broth microdilution and E-Test as susceptible and resistant to clindamycin were 100% in concordance with the zone size of $\geq$19 and $\leq$16mm, respectively, thus distinguishing between the M-phenotype and the $MLS_B$-phenotype very clearly. Similarly isolates identified as clarithromycin susceptible, intermediate and resistant were 100% in concordance with the clarithromcyin zone interpretive standards of $\geq$21, 17-20 and $\leq$16mm, respectively. Within the clarithromycin-resistant isolates the disc diffusion zone

sizes directly correlated with the broth microdilution MICs and one dilution difference in the clarithromycin MIC was distinguishable with disc diffusion method. The erythromycin and clindamycin MIC for *S. pneumoniae* 49619 by all methods was consistently within the proposed quality control range of 0.25-1μg/ml.

**Table 3. Susceptibility of 28 Study Isolates to Various Macrolides, Ketolide and Clindamycin Performed with NCCLS approved Broth Microdilution and Disc Diffusion method and confirmed with E-Test.**

| Isolate # | | Microdilution MIC[a] (μg/ml) | | | | | Disc Diffusion Size (mm) | | E-Test MIC (μg/ml) | |
|---|---|---|---|---|---|---|---|---|---|---|
| Stock | Experimental | ERY | CLR | AZI | CLI | TEL | CLR | CLI | CLR | CLI |
| 12810 | 1 | 1 | 0.5 | 2 | 0.12 | 0.015 | 17 | 20 | 4 | 0.19 |
| 12862 | 2 | 1 | 1 | 1 | 0.12 | 0.004 | 16 | 26 | 2 | 0.023 |
| 11183 | 3 | 2 | 2 | 4 | 0.12 | 0.03 | 16 | 21 | 4 | 0.023 |
| 12809 | 4 | 2 | 8 | 2 | 0.12 | 0.12 | 9 | 20 | 24 | 0.19 |
| 10725 | 5 | 1 | 0.5 | 1 | 0.12 | 0.015 | 17 | 21 | 4 | 0.023 |
| 11599 | 6 | 1 | 0.5 | 1 | 0.12 | 0.008 | 17 | 23 | 3 | 0.094 |
| 11692 | 7 | 2 | 2 | 1 | 0.12 | 0.12 | 13 | 25 | 8 | 0.125 |
| 12780 | 8 | 4 | 4 | 1 | 0.12 | 0.008 | 15 | 19 | 6 | 0.023 |
| 12815 | 9 | 2 | 1 | 2 | 0.12 | 0.015 | 18 | 23 | 3 | 0.032 |
| 12880 | 10 | 4 | 1 | 4 | 0.12 | 0.008 | 13 | 26 | 12 | 0.19 |
| 10733 | 11 | 2 | 2 | 4 | 0.12 | 0.015 | 16 | 22 | 3 | 0.032 |
| 11430 | 12 | 4 | 4 | 8 | 0.12 | 0.008 | 14 | 23 | 8 | 0.047 |
| 12808 | 13 | 2 | 1 | 2 | 0.12 | 0.12 | 10 | 19 | 8 | 0.023 |
| 12848 | 14 | 4 | 2 | 8 | 0.12 | 0.015 | 12 | 26 | 12 | 0.023 |
| C | 15 | 32 | 32 | 64 | 0.12 | 0.12 | 7 | 24 | 96 | 0.023 |
| 12629 | 16 | 4 | 4 | 8 | 0.12 | 0.008 | 10 | 23 | 16 | 0.032 |
| 12751 | 17 | 4 | 4 | 4 | 0.12 | 0.015 | 14 | 25 | 8 | 0.016 |
| 12300 | 18 | 4 | 8 | 8 | 0.12 | 0.015 | 9 | 24 | 24 | 0.023 |
| B | 19 | 16 | 16 | 32 | 0.12 | 0.12 | 8 | 25 | 32 | 0.023 |
| F | 20 | 16 | 16 | 32 | 0.12 | 0.12 | 8 | 25 | 64 | 0.023 |
| A | 21 | 16 | 16 | 32 | 0.12 | 0.12 | 8 | 24 | 32 | 0.25 |
| D | 22 | 16 | 16 | 16 | 0.12 | 0.12 | 8 | 21 | 32 | 0.032 |
| E | 23 | 16 | 16 | 32 | 0.12 | 0.12 | 8 | 21 | 32 | 0.047 |
| 12767 | 24 | >64 | >64 | >64 | >32 | 0.06 | 6 | 6 | >256 | >256 |
| 12778 | 25 | >64 | >64 | >64 | >32 | 0.06 | 6 | 6 | >256 | >256 |
| 1333 | 26 | 0.25 | 0.25 | 0.25 | 0.12 | 0.004 | 22 | 24 | 0.19 | 0.016 |
| 49619 | 27 | 0.25 | 0.25 | 0.25 | 0.12 | 0.002 | 24 | 26 | 0.125 | 0.19 |
| 15353 | 28 | 4 | 4 | 8 | 0.12 | 0.25 | 14 | 24 | 8 | 0.047 |

**Footnote:**

[a]ERY, Erythromycin; CLR, Clarithromycin; AZI, Azithromycin; CLI, Clindamycin; TEL, Telithromycin. Breakpoints for Broth Microdilution (in μg/ml) per NCCLS guidelines, unless otherwise noted, are as follows: Erythromycin: susceptible, $\leq$ 0.25; intermediate, 0.5; resistant, $\geq$ 1; Clarithromycin: susceptible, $\leq$ 0.25; intermediate, 0.5; resistant, $\geq$ 1; Azithromycin: susceptible, $\leq$ 0.5; intermediate, 1; resistant, $\geq$ 2; Clindamycin, susceptible, $\leq$ 0.25; intermediate, 0.5; resistant, $\geq$1. Telithromycin does not have established breakpoints. Breakpoints for Disc diffusion (in millimeters, mm) per NCCLS guidelines, unless otherwise stated are as follows: Clarithromycin (15 μg disc content): susceptible, $\geq$ 21; intermediate, 17-20; resistant, $\leq$ 16; Clindamycin (2 μg disc content): susceptible, $\geq$ 19; intermediate, 17-18, resistant, $\leq$ 16.

| *S. pneumoniae* | N (%) | Microbroth MIC[a] (μg/ml) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ERY | | CLR | | AZI | | CLI | | TEL | |
| | | $MIC_{90}$[b] | Range | $MIC_{90}$ | Range | $MIC_{90}$ | Range | $MIC_{90}$ | Range | $MIC_{90}$ | Range |
| M-phenotype | 24 (86) | 16 | 1-32 | 16 | 0.5-32 | 32 | 1-64 | 0.12 | 0.12 | 0.12 | 0.004-0.25 |
| $MLS_B$-phenotype | 2 (7) | >64 | >64 | >64 | >64 | >64 | >64 | >32 | >32 | 0.06 | 0.06 |
| Susceptible | 2 (7) | 0.25 | 0.06- 0.25 | 0.25 | 0.03-0.25 | 0.25 | 0.06-0.25 | 0.12 | 0.12 | 0.008 | 0.002-0.008 |

[a]ERY, Erythromycin; CLR, Clarithromycin; AZI, Azithromycin; CLI, Clindamycin; TEL, Telithromycin. Breakpoints for Broth Microdilution (in μg/ml) per NCCLS guidelines, unless otherwise noted, are as follows: Erythromycin: susceptible, ≤ 0.25; intermediate, 0.5; resistant, ≥ 1; Clarithromycin: susceptible, ≤ 0.25; intermediate, 0.5;
resistant, ≥ 1; Azithromycin: susceptible, ≤ 0.5; intermediate, 1; resistant, ≥ 2; Clindamycin, susceptible, ≤ 0.25; intermediate, 0.5; resistant, ≥1. Telithromycin dose not have established breakpoints.
[b]MIC of an antibiotic at which 90% of isolates is inhibited.

**Table 4. Antibiotic Susceptibility of 28 Study *S. pneumoniae* Isolates to Macrolides, Clindamycin and Telithromycin Antibiotics.**

**Part II. Polymerase chain reaction (PCR)-based detection of *mef*A/E gene**

The antibiotic susceptibility testing confirmed 24 out of the 28 isolates, numbered 1-23 and 28 as those of M-phenotype. Two out of the 28 isolates, numbered 24 and 25 were confirmed as those of an $MLS_B$-phenotype and the remaining two isolates, numbered 26 and 27 were confirmed as susceptible. Following the susceptibility testing, the 28 isolates of *S. pneumoniae* were subjected to Polymerase Chain Reaction (PCR) to detect the presence of the efflux pump gene, *mef*A/E, and to confirm genotypically the M-phenotype. Figure 1 shows the results of *mef*A/E PCR. As described in the Material and Methods section the expected size of the *mef*A/E PCR product is 346bp long. The *S. pneumoniae* isolate numbered 28 has previously been confirmed to carry *mef*A/E gene and was serving as a positive control in all the PCR reactions.

The M-phenotype was verified genotypically by the presence of a positive *mef*A/E. PCR product in all 23 Study isolates, numbered 1-23, and the results were identical to the positive control isolate 28, while no *mef*A/E PCR product was detected as expected from susceptibility data in four isolates, numbered 24,25,26,27. All 28 isolates were also subjected to *erm*B gene detection by PCR to verify the absence of this resistance gene in all M-phenotype isolates and to confirm its presence in the $MLS_B$-phenotype isolates numbered 24,25. All M-phenotype study isolates including the *mef*A/E positive control isolate numbered 28 did not amplify the *erm*B gene product as expected, however such amplification was present for isolates 24 and 25 (Results Not Shown). Both *mef*A/E and *erm*B PCR did not yield a positive result as expected for a susceptible clinical isolate 26 and for susceptible laboratory reference isolates 27. All PCR results are summarized in Table 5.

**Figure 1. The Polymerase Chain Reaction for the Detection of the Efflux Pump Gene, *mef*A/E in 28 Study Isolates of *S. pneumoniae.*** *mefA/E* gene PCR product size is 346bp in length. 100bp DNA ladder in Lane 1, 20, 21,and 33 was used for sizing. Lanes 2-19 contain isolates 1-18, respectively and lanes 22-31 contain isolates 19-28 respectively. For actual stock numbers of each isolate refer to Table 3. Lane 32 contains water as control.

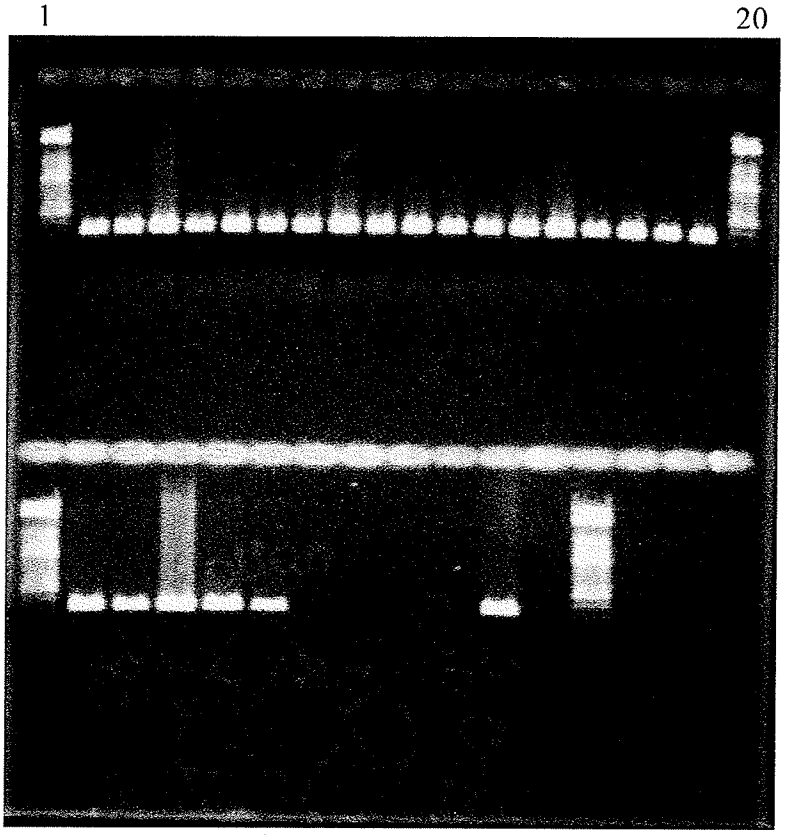


**Table 5. The *mef*E/A and *erm*B PCR Results and Susceptibility of 28 Study Isolates to Various Macrolides, Ketolide, and Clindamycin.**

| Isolate # | | PCR Result | | Microdilution MIC[a] (μg/ml) | | | | |
|---|---|---|---|---|---|---|---|---|
| Stock | Experimental | *mef*A/E | *erm*B | ERY | CLR | AZI | CLI | TEL |
| 12810 | 1 | + | - | 1 | 0.5 | 2 | 0.12 | 0.015 |
| 12862 | 2 | + | - | 1 | 1 | 1 | 0.12 | 0.004 |
| 11183 | 3 | + | - | 2 | 2 | 4 | 0.12 | 0.03 |
| 12809 | 4 | + | - | 2 | 8 | 2 | 0.12 | 0.12 |
| 10725 | 5 | + | - | 1 | 0.5 | 1 | 0.12 | 0.015 |
| 11599 | 6 | + | - | 1 | 0.5 | 1 | 0.12 | 0.008 |
| 11692 | 7 | + | - | 2 | 2 | 1 | 0.12 | 0.12 |
| 12780 | 8 | + | - | 4 | 4 | 1 | 0.12 | 0.008 |
| 12815 | 9 | + | - | 2 | 1 | 2 | 0.12 | 0.015 |
| 12880 | 10 | + | - | 4 | 1 | 4 | 0.12 | 0.008 |
| 10733 | 11 | + | - | 2 | 2 | 4 | 0.12 | 0.015 |
| 11430 | 12 | + | - | 4 | 4 | 8 | 0.12 | 0.008 |
| 12808 | 13 | + | - | 2 | 1 | 2 | 0.12 | 0.12 |
| 12848 | 14 | + | - | 4 | 2 | 8 | 0.12 | 0.015 |
| C | 15 | + | - | 32 | 32 | 64 | 0.12 | 0.12 |
| 12629 | 16 | + | - | 4 | 4 | 8 | 0.12 | 0.008 |
| 12751 | 17 | + | - | 4 | 4 | 4 | 0.12 | 0.015 |
| 12300 | 18 | + | - | 4 | 8 | 8 | 0.12 | 0.015 |
| B | 19 | + | - | 16 | 16 | 32 | 0.12 | 0.12 |
| F | 20 | + | - | 16 | 16 | 32 | 0.12 | 0.12 |
| A | 21 | + | - | 16 | 16 | 32 | 0.12 | 0.12 |
| D | 22 | + | - | 16 | 16 | 16 | 0.12 | 0.12 |
| E | 23 | + | - | 16 | 16 | 32 | 0.12 | 0.12 |
| 12767 | 24 | - | + | >64 | >64 | >64 | >32 | 0.06 |
| 12778 | 25 | - | + | >64 | >64 | >64 | >32 | 0.06 |
| 1333 | 26 | - | - | 0.25 | 0.25 | 0.25 | 0.12 | 0.004 |
| 49619 | 27 | - | - | 0.25 | 0.25 | 0.25 | 0.12 | 0.002 |
| 15353 | 28 | + | - | 4 | 4 | 8 | 0.12 | 0.25 |

**Footnote:**

[a]ERY, Erythromycin; CLR, Clarithromycin; AZI, Azithromycin; CLI, Clindamycin; TEL, Telithromycin. Breakpoints for Broth Microdilution (in μg/ml) per NCCLS guidelines, unless otherwise noted, are as follows: Erythromycin: susceptible, $\leq 0.25$; intermediate, 0.5; resistant, $\geq 1$; Clarithromycin: susceptible, $\leq 0.25$; intermediate, 0.5; resistant, $\geq 1$; Azithromycin: susceptible, $\leq 0.5$; intermediate, 1; resistant, $\geq 2$; Clindamycin, susceptible, $\leq 0.25$; intermediate, 0.5; resistant, $\geq 1$. Telithromycin does not have established breakpoints.

**Part III. Induction of *mef*-mediated resistance in *S. pneumoniae***

Following the confirmation of efflux, as the sole mechanism of resistance in study isolates (1-23) of *S. pneumoniae*, the inducibility of expression of efflux-mediated resistance was tested phenotypically. Isolate number 28 was not included in this experiment. Table 6 shows the MICs of different antibiotics as determined by E-Test before and after induction with sub-inhibitory concentrations of each antibiotic. Fold increase in MIC was calculated by dividing the MIC after induction by the MIC before induction and it is reported for each isolate in Table 6. Two susceptible isolates (26 and 27) were included as negative controls.

Figures 2 to 6 represent fold increase in erythromycin, clarithromycin, azithromycin, clindamycin and telithromycin MICs following induction in 23 M-phenotype *S. pneumoniae* isolates. 4-fold increase in the MIC following the induction was considered as significant. Efflux-mediated resistance in *S. pneumoniae* appeared to be inducible by all macrolide antibiotics tested and was expressed at higher levels. Of all the macrolides, clarithromycin appeared to be the strongest inducer of *mef*A/E gene expression where MICs of 78% (18/23) of M-phenotype *S. pneumoniae* isolates showed a significant ($\geq$4) fold increase following exposure to clarithromycin (Figure 3). The other two macrolides tested, erythromycin and azithromycin showed the same degree of induction where MICs of 57% (13/23) of M-phenotype *S. pneumoniae* isolates showed a significant fold following exposure to erythromycin and azithromycin (Figure 2 and 4). Efflux-mediated macrolide resistance in *S. pneumoniae* appeared not to be inducible by telithromycin and clidamycin where MICs of all isolates of M-phenotype *S. pneumoniae* showed non-

significant (<4) fold increase following exposure to clindamycin and telithromycin (Figure 5 and 6).

Figure 7 shows the clarithromycin MIC distribution for 23 M-phenotype and 2 susceptible *S. pneumoniae* isolates before and after induction. As expected the clarithromycin MIC distribution before and after induction for the 23 M-phenotype *S. pneumonaie* isolates has changed while it has remained the same for the 2-susceptible *S. pneumonaie* isolates. Figures 8 and 9 show the clindamycin and telithromycin MIC distribution data, respectively, for the 23 M-phenotype and 2-susceptible *S. pneumoniae* isolates before and after induction. As can be seen from Figure 8 and 9, induction with clindamycin and telithromycin has no effect on MIC distribution for 23 M-phenotype and also for the 2 susceptible *S. pneumoniae* isolates.

Table 7 depicts the $MIC_{90}$ values for erythromycin, clarithromycin, azithromycin, clindamycin and telithromycin antibiotics before and after induction in 23 M-phenotype and 2-susceptible *S. pneumoniae* isolates. The average erythromycin $MIC_{90}$ value of 64μg/ml before induction increased 4-fold to 256μg/ml following the induction in all the M-phenotype *S. pneumoniae* isolates. The azithromycin $MIC_{90}$ value of 96μg/ml before induction increased 3-fold to 256μg/ml following induction. Six-fold increase from 32μg/ml to 196μg/ml in the $MIC_{90}$ value was observed following the induction with clarithromycin. As expected, no significant changes in the $MIC_{90}$ values before and after induction with clindamycin and as well with telithromycin were observed. Similarly, no significant changes in the $MIC_{90}$ values were observed for the 2-susceptible *S. pneumoniae* isolates (Table 7).

| Isolate # | | E-Test MIC (μg/ml) [a] | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ERY | | | CLR | | | AZI | | | CLI | | | TEL | | |
| Stock | Experimental | BI[b] | AI[c] | FOLD[d] | BI | AI | FOLD | BI | AI | FOLD | BI | AI | FOLD | BI | AI | FOLD |
| 12810 | 1 | 4 | 8 | 2 | 4 | 12 | 3 | 24 | 96 | 4 | 0.094 | 0.094 | 1 | 0.19 | 0.25 | 1.3 |
| 12862 | 2 | 6 | 16 | 3 | 2 | 12 | 6 | 24 | 32 | 1 | 0.064 | 0.125 | 2 | 0.125 | 0.25 | 2 |
| 11183 | 3 | 4 | 16 | 4 | 4 | 12 | 3 | 16 | 64 | 4 | 0.047 | 0.047 | 1 | 0.125 | 0.38 | 3 |
| 12809 | 4 | 12 | 48 | 4 | 24 | 128 | 5 | 32 | 256 | 8 | 0.047 | 0.094 | 2 | 0.75 | 1.5 | 2 |
| 10725 | 5 | 4 | 16 | 4 | 4 | 24 | 6 | 24 | 192 | 8 | 0.023 | 0.047 | 2 | 0.125 | 0.25 | 2 |
| 11599 | 6 | 8 | 16 | 2 | 8 | 24 | 3 | 32 | 48 | 2 | 0.094 | 0.19 | 2 | 0.5 | 0.75 | 1.5 |
| 11692 | 7 | 4 | 16 | 4 | 3 | 16 | 5 | 32 | 256 | 8 | 0.064 | 0.094 | 1 | 0.19 | 0.38 | 2 |
| 12780 | 8 | 24 | 32 | 1 | 6 | 24 | 4 | 48 | 128 | 3 | 0.064 | 0.125 | 2 | 0.25 | 0.25 | 1 |
| 12815 | 9 | 3 | 12 | 4 | 3 | 12 | 4 | 96 | 256 | 3 | 0.023 | 0.047 | 2 | 0.5 | 1 | 2 |
| 12880 | 10 | 12 | 64 | 5 | 12 | 48 | 4 | 64 | 256 | 4 | 0.047 | 0.094 | 2 | 0.5 | 1 | 2 |
| 10733 | 11 | 16 | 32 | 2 | 3 | 24 | 8 | 16 | 96 | 6 | 0.047 | 0.047 | 1 | 0.38 | 0.75 | 2 |
| 11430 | 12 | 12 | 96 | 8 | 8 | 64 | 8 | 96 | 256 | 3 | 0.023 | 0.047 | 2 | 0.38 | 0.5 | 1.3 |
| 12808 | 13 | 16 | 94 | 6 | 8 | 24 | 3 | 16 | 48 | 3 | 0.094 | 0.125 | 1 | 0.19 | 0.38 | 2 |
| 12848 | 14 | 12 | 64 | 5 | 12 | 64 | 5 | 48 | 256 | 5 | 0.25 | 0.25 | 1 | 0.25 | 0.75 | 3 |
| C | 15 | 64 | 256 | 4 | 32 | 256 | 6 | 128 | >256 | 2 | 0.094 | 0.25 | 3 | 0.19 | 0.25 | 1.3 |
| 12629 | 16 | 32 | 48 | 2 | 16 | 24 | 2 | 48 | 256 | 5 | 0.023 | 0.047 | 2 | 0.75 | 1 | 1.3 |
| 12751 | 17 | 24 | 32 | 1 | 8 | 32 | 4 | 24 | 192 | 8 | 0.094 | 0.19 | 2 | 0.5 | 0.5 | 1 |
| 12300 | 18 | 24 | 128 | 5 | 24 | 196 | 8 | 64 | 256 | 4 | 0.19 | 0.25 | 2 | 0.19 | 0.5 | 2.6 |
| B | 19 | 64 | 256 | 4 | 32 | 196 | 6 | 64 | >256 | 4 | 0.19 | 0.25 | 2 | 0.125 | 0.25 | 2 |
| F | 20 | 128 | 256 | 2 | 64 | 256 | 4 | 128 | >256 | 2 | 0.064 | 0.125 | 2 | 0.25 | 0.38 | 1.5 |
| A | 21 | 64 | 256 | 4 | 32 | 196 | 6 | 64 | >256 | 4 | 0.19 | 0.25 | 2 | 0.125 | 0.25 | 2 |
| D | 22 | 128 | 256 | 2 | 32 | 256 | 6 | 128 | >256 | 2 | 0.064 | 0.125 | 2 | 0.125 | 0.25 | 2 |
| E | 23 | 128 | 256 | 2 | 64 | 256 | 4 | 128 | >256 | 2 | 0.094 | 0.19 | 2 | 0.38 | 0.38 | 1 |
| 1333 | 26 | - | - | - | <0.25 | <0.25 | - | - | - | - | 0.032 | 0.047 | - | <0.008 | <0.008 | - |
| 49619 | 27 | - | - | - | <0.25 | <0.25 | - | - | - | - | 0.047 | 0.047 | - | <0.008 | <0.008 | - |

**Table 6. Susceptibility of 23 *mef*-positive Study Isolates to Various Macrolides, Telithromycin and Clindamycin Before and After Induction.**

**Footnote:**
[a]ERY, Erythromycin; CLR, Clarithromycin; AZI, Azithromycin; CLI, Clindamycin; TEL, Telithromycin.
[b]MIC as determined by E-Test in μg/ml before induction with sub-inhibitory concentration of a given antibiotic.
[c]MIC as determined by E-Test in μg/ml after induction with sub-inhibitory concentration of a given antibiotic.
[d]Fold increase in the MICs before and after induction. Expressed as MIC after induction/MIC before induction.

| *S. pneumoniae* | N (%) | Experimental condition | E-Test MIC[a] (μg/ml) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | ERY | | CLR | | AZI | | CLI | | TEL | |
| | | | $MIC_{90}$[b] | Range | $MIC_{90}$ | Range | $MIC_{90}$ | Range | $MIC_{90}$ | Range | $MIC_{90}$ | Range |
| M-phenotype | 23 (92) | Before Induction | 64 | 3-128 | 32 | 2-64 | 96 | 16-128 | 0.19 | 0.023-0.25 | 0.5 | 0.125-0.75 |
| | | After Induction | 256 | 8-256 | 196 | 12-256 | 256 | 32-256 | 0.25 | 0.047-0.25 | 1 | 0.25-1.5 |
| Susceptible | 2 (8) | Before Induction | - | - | 0.25 | 0.25 | - | - | 0.047 | 0.032-0.047 | 0.008 | 0.008 |
| | | After Induction | - | - | 0.25 | 0.25 | - | - | 0.047 | 0.047 | 0.008 | 0.008 |

[a]ERY, Erythromycin; CLR, Clarithromycin; AZI, Azithromycin; CLI, Clindamycin; TEL, Telithromycin.
[b]MIC before and after induction with sub-inhibitory concentration of antibiotics as determined by E-Test at which 90% of isolates are inhibited.

**Table 7. Phenotypic Induction of Macrolide Resistance in 23 *mef*-positive and 2 Susceptible Isolates of *S. pneumoniae*.**

**Figure 2. Fold Increase in Erythromycin MIC Following Induction.**

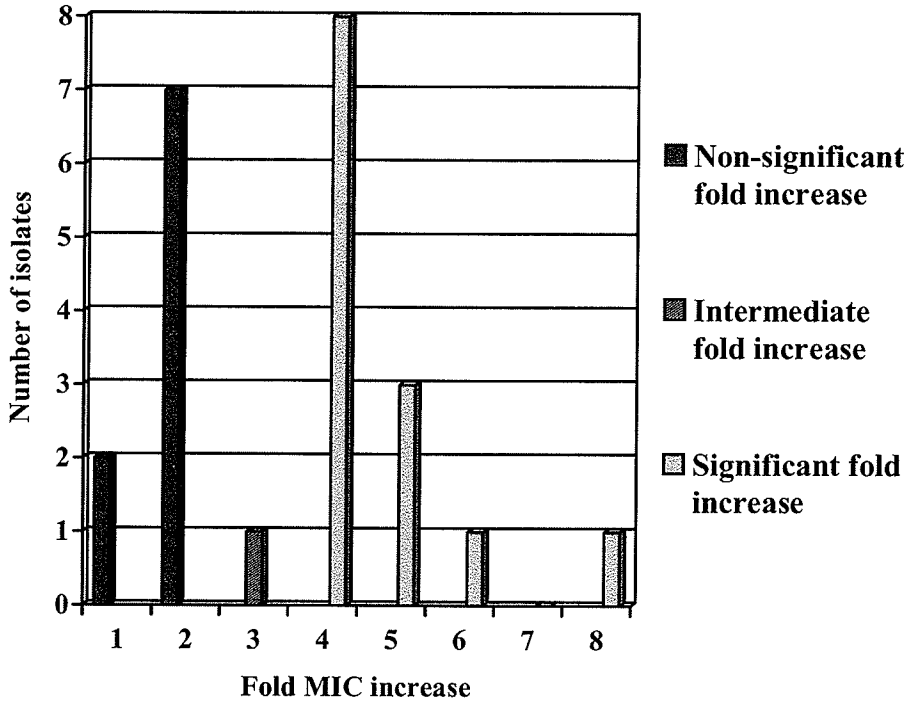


**Figure 3. Fold Increase in Clarithromycin MIC Following Induction.**

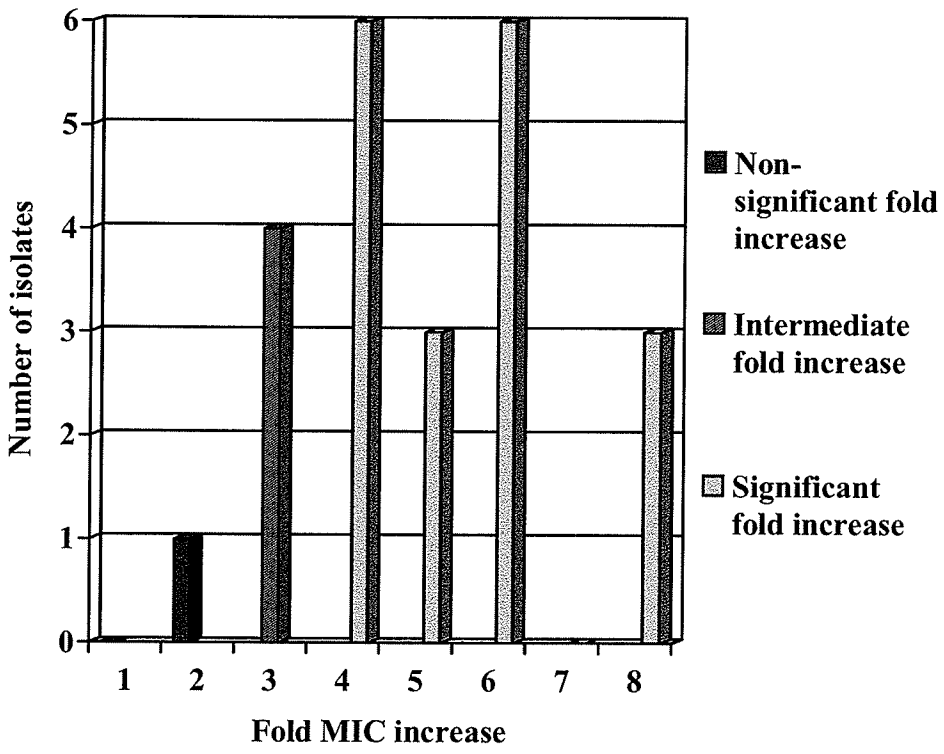


**Figure 4. Fold Increase in Azithromycin MIC Following Induction.**

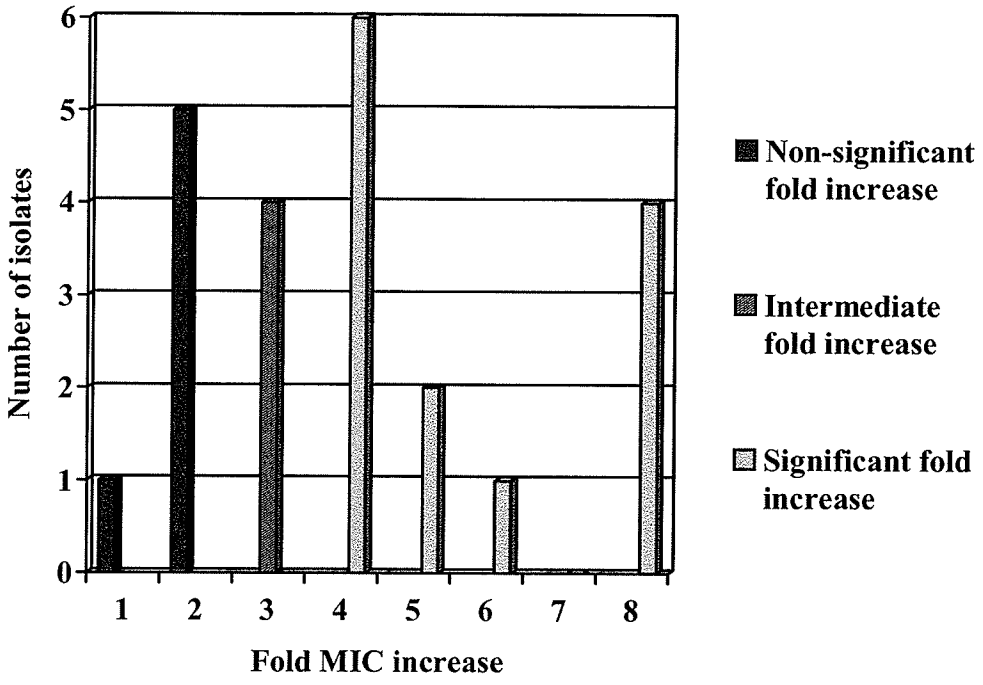


**Figure 5. Fold Increase in Clindamycin MIC Following Induction.**

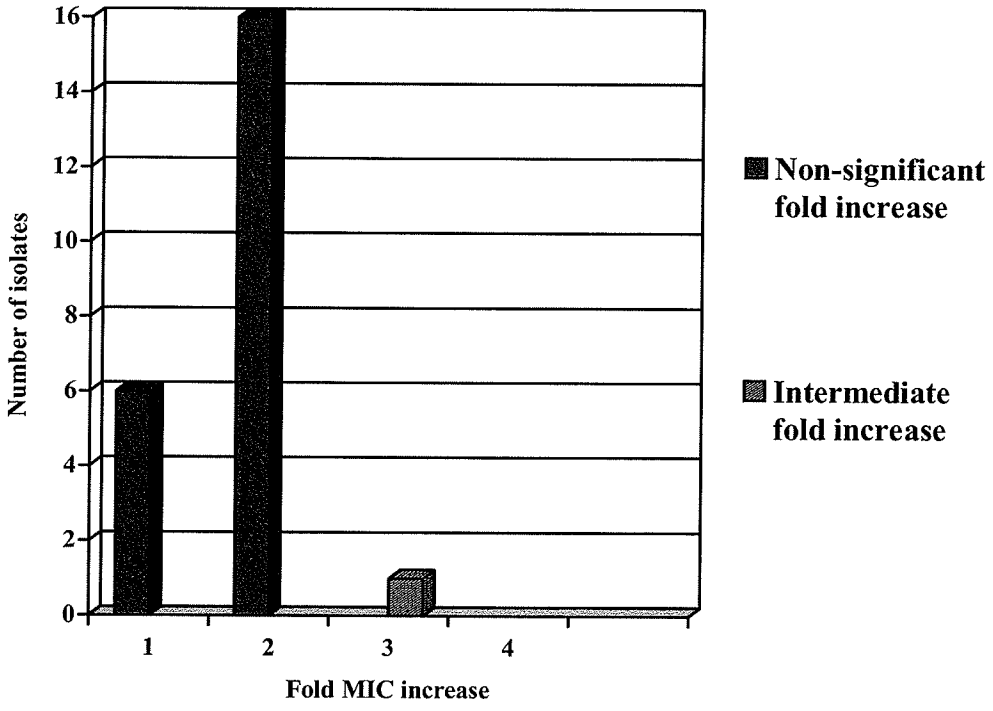


**Figure 6. Fold Increase in Telithromycin MIC Following Induction.**

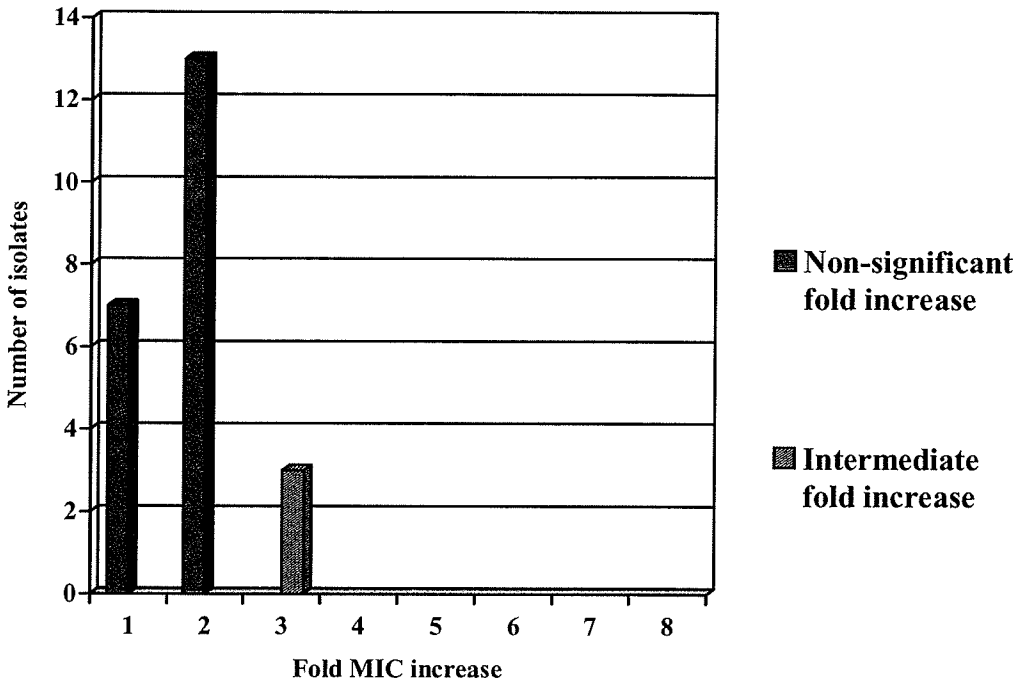


**Figure 7. The Clarithromycin MIC Distribution for 23 *mef*-Positive Isolates of *S. pneumoniae* Before and After Induction.**

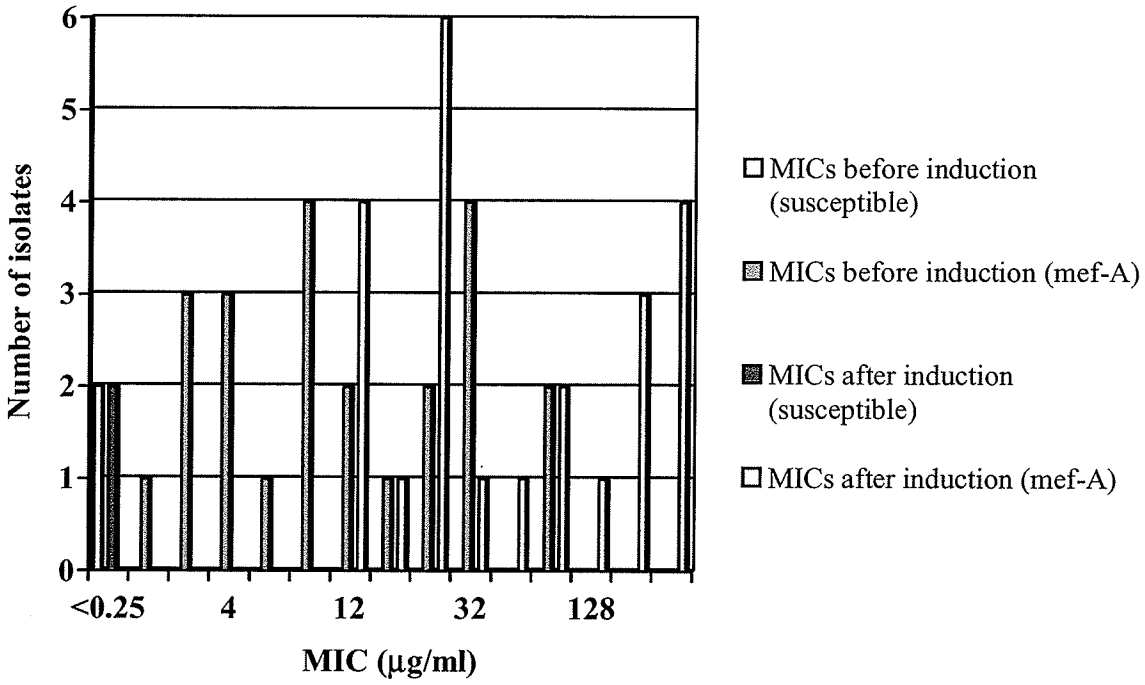


**Figure 8. Clindamycin MIC Distribution for 23 *mef*-Positive Study Isolates of *S. pneumoniae* Before and After Induction.**

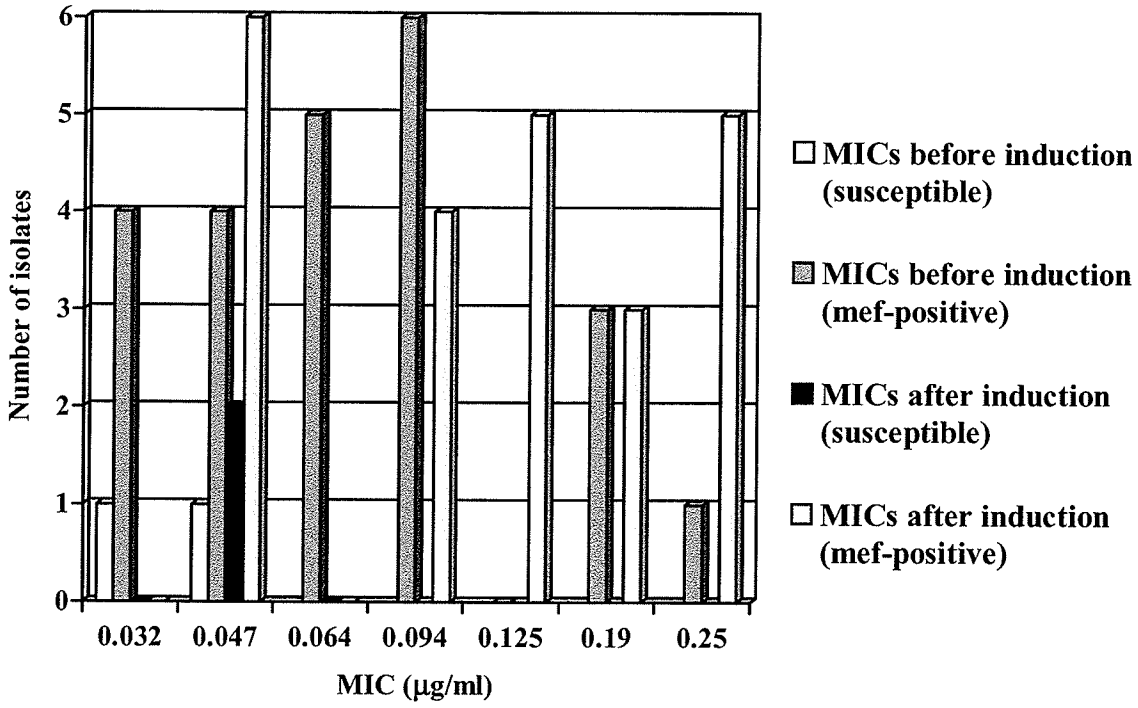
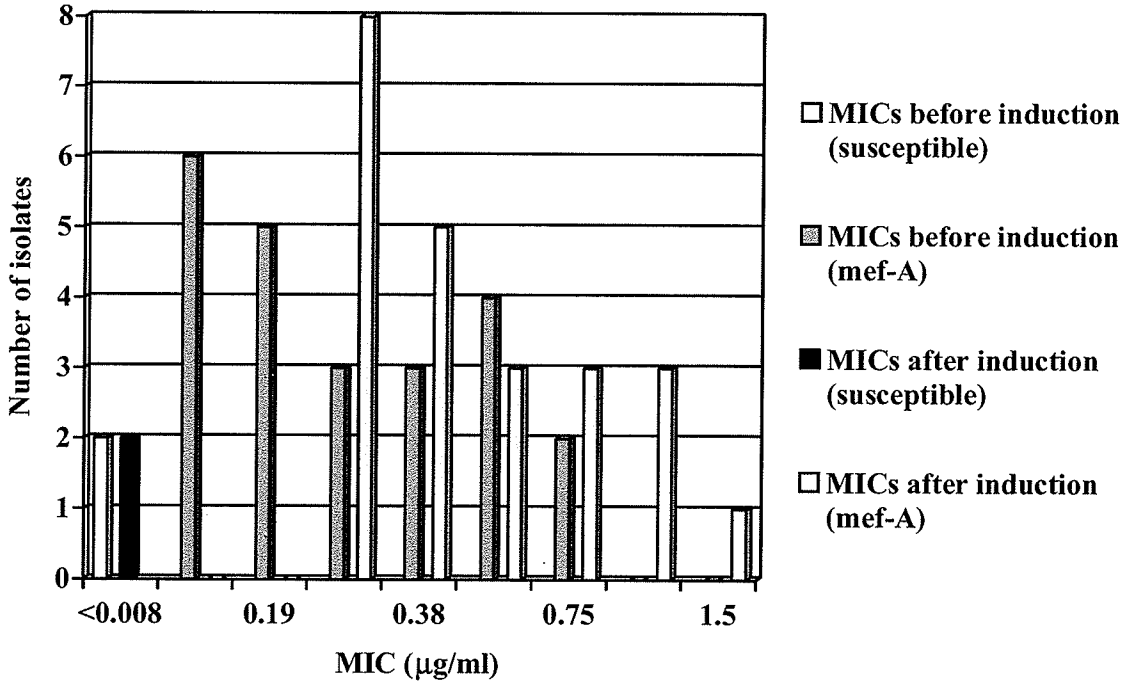


**Figure 9. Telithromycin MIC Distribution for 23 *mef*- positive Study Isolates of *S. pneumoniae* Before and After induction.**



## Part IV. Distinction between *mef*A and *mef*E variants

### a. Polymerase Chain Reaction (PCR)

The results of the PCR amplification of Tn*1207.1* and Mega elements in 24 M-phenotype isolates of *S. pneumoniae* numbered, 1-23 and 28 exhibiting *mef*E or *mef*A genotype, are summarized in Table 8. More specifically, Table 8 depicts all the primer pairs that were utilized in the distinction, by PCR and also by PCR-Restricted Fragment Length Polymorphism (described below) between of *mefA* and *mefE* variants of the efflux pump gene in *S. pneumoniae*. For the complete description of this experiment please refer to the Materials and Methods Distinction Between *mefE* and *mef*A section.

In all the 24 isolates of *S. pneumoniae* exhibiting the M-phenotype, the efflux pump gene, *mef* was detected by a PCR assay (*mef*A/E-1/*mef*A/E-2) that did not distinguish between the two variants (Figure 1, Table 8). The two variants of the *mef* gene were distinguished by 3 separate PCRs and confirmed by 1. The 3 PCRs utilized in the distinction were based on the published nucleotide sequence of the Tn*1207.1* genetic element carrying the *mefA* variant. The 2 of the 3 reactions designed to detect the upstream region of *mef*A, the open reading frame, *orf*2 and *orf*3 respectively, yielded a positive result for only 1 (number 18) of the 24 M-phenotype *S. pneumoniae* isolates (Table 8). Figure 10 depicts the agarose gel electrophoresis and ethidium bromide staining of the PCR product of the open reading frame 3, *orf*3 of transposon Tn*1207.1* carrying *mef*A variant of the efflux pump gene, *mef* in 1 (number 18) of the 24 M-phenotype *S. pneumoniae* isolates. The same isolate, number 18 showed a positive PCR result for detection of the *orf*2 (Figure not shown). The third of the 3 distinguishing PCRs designed to detect the downstream inter-genic region of the *mef*A/E and *orf*5 or *mel*

in Tn*1207.1* or mega, respectively yielded a positive results for all of the 24 M-phenotype *S. pneumoniae* isolates (Table 8). The results of this PCR are shown in Figure 11. As can be seen from Figure 11, however, the PCR of the intergenic region between *mef*A/E and the *orf*5/*mel* resulted in the approximately 500bp expected positive PCR product in 13 of the 24 M-phenotype *S. pneumoniae* isolates, including the control isolate numbered 28. For the remaining 11 isolates this PCR product was approximately 100bp smaller in size (Figure 11). No size differences in the intergenic region are known to exist in the Tn*1207.1* genetic element.

The results of the 3 different PCRs based on the Tn*1207.1* nucleotide sequence determined the *mef*A variant of the *mef* genes as the less probable of the two to confer macrolide resistance in the 23 M-phenotype *S. pneumoniae* isolates. To confirm *mef*E as the *mef* gene variant in the 23 of the 24 M-phenotype *S. pneumoniae* isolates, a PCR to detect the upstream region of the *mef*E gene in mega was designed. The 23 of the 24 M-phenotype isolates previously negative by PCR for *orf*2 and *orf*3 amplified a product of the expected size (Table 8, Figure12). Correspondingly, isolate number18, previously PCR positive for *orf*2 and *orf*3, yielded a negative results for the upstream region of *mef*E (Table 8, Figure 12).

### b. PCR-Restriction Fragment Length Polymorphism

A PCR product was generated in all of the 24 M-phenotype *S. pneumoniae* isolates, using previously described primer set (*mef*-3/*mef*-4) that does not distinguish between *mef*E and *mef*A variants (Table 8). This 1743bp amplicon was digested with *Bam*HI and *Dra*I restriction enzymes to generate a restriction pattern based on which the

differentiation of *mef*E and *mef*A was made (Table 9). Restriction of the amplicon with *Bam*HI resulted in a generation of two fragments of 1340bp and 403bp, respectively for only 1, numbered 18 of 24 M-phenotype *S. pneumoniae* isolates, while the remaining 23 isolates were not affected by this restriction. Restriction of the amplicon with *Dra*I resulted in the generation of 3 fragments of 782, 711, and 250 or 151 bp, respectively for 23 of the 24 M-phenotype *S. pneumonaie* isolates previously unaffected by *Bam*HI restriction. More specifically, out of these 23 isolates, 11 had a restriction pattern with fragments of 782, 711 and 151 bp as that for isolates 6, 9 and 13 in Figure 13, while the remaining 13 had the other fragment restriction pattern of 782, 711 and 250bp as that for isolates 1, 4, 12, 15, and 23 in Figure 13. The PCR restriction fragment length polymorphism results are in 100% concordance with the results from the PCR for the detection of the intergenic region of *mef*E/A and *orf*5/*mel.* Restriction of the amplicon with *Dra*I also resulted in a generation of two fragments of 1493 and 250bp, respectively for the 1 isolate, numbered 18 previously restricted with *Bam*HI into two fragments. Figure 13 depicts the restriction pattern of the 1743bp amplicon with *Bam*HI and *Dra*I of 8 representative *S. pneumonaie* isolates, numbered 1, 4, 6, 9, 12, 13, 15 and 23 containing the *mef*E variant and the one, numbered 18 containing *mef*A.

**Table 8. PCR Amplification of Tn*1207.1* and Mega Elements in *S. pneumoniae* Study Isolates Exhibiting *mef*E or *mef*A Genotype.**

| Primer Sets | Number of Positive PCR Reactions N=24 |
|---|---|
| *mef*(AE)-1/*mef*(AE)-2 | 24 |
| *mefA*-UP/*orf*(3)-DN | 1 |
| *orf*(2)-A/*orf*(2)-B | 1 |
| *mefA*-DN/*orf*(5)-UP | 24 |
| *orf*(1)-UP/*mefA*-UP | 23 |
| *mef*-3/*mef*-4 | 24 |

**Figure 10. The Polymerase Chain Reaction for the Detection of the Open Reading Frame 3, *orf*3 of Transposon Tn*1207.1* carrying *mef*A Variant of the Efflux Pump Gene, *mef* in 28 Study Isolates of *S. pneumoniae.*** *orf*3 gene PCR product size is 1000bp in length. 100bp DNA ladder in Lane 1, 20, 21,and 35 was used for sizing. Lanes 4-19 contain isolates 1-16, respectively and lanes 22-33 contain isolate 17-28 respectively. For actual stock numbers of each isolates refer to Table 1.Lane 34 contains water as control. Lane 2 and 3 not used.

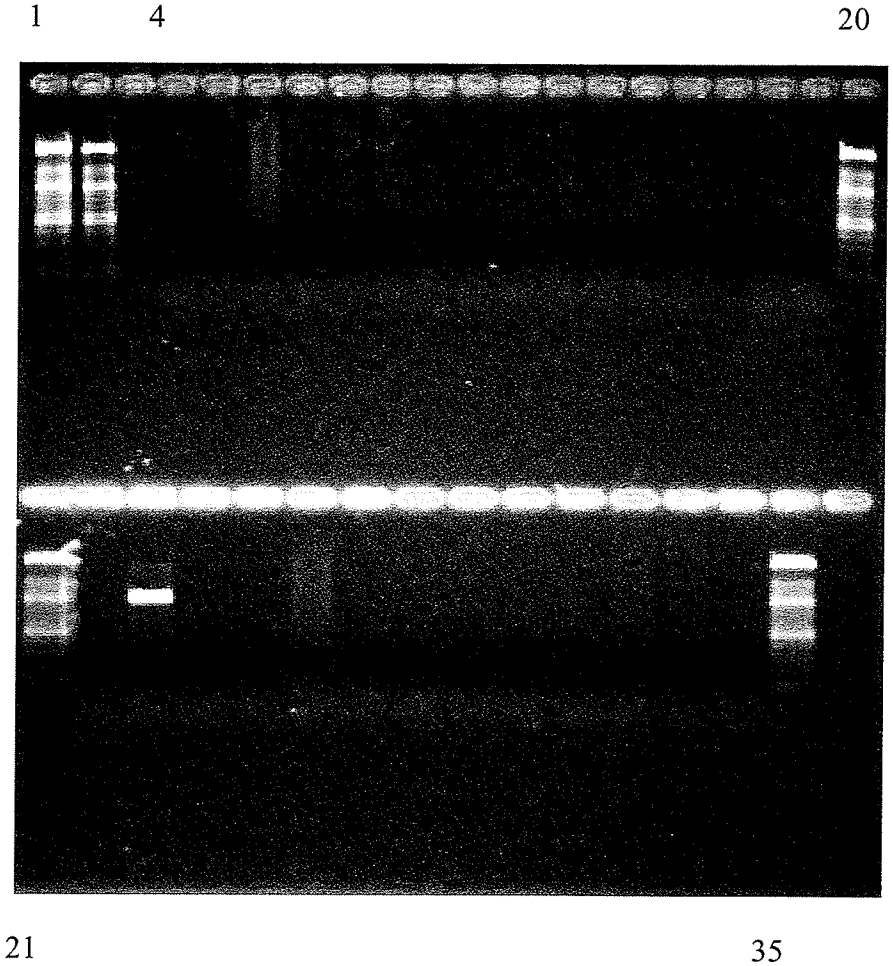
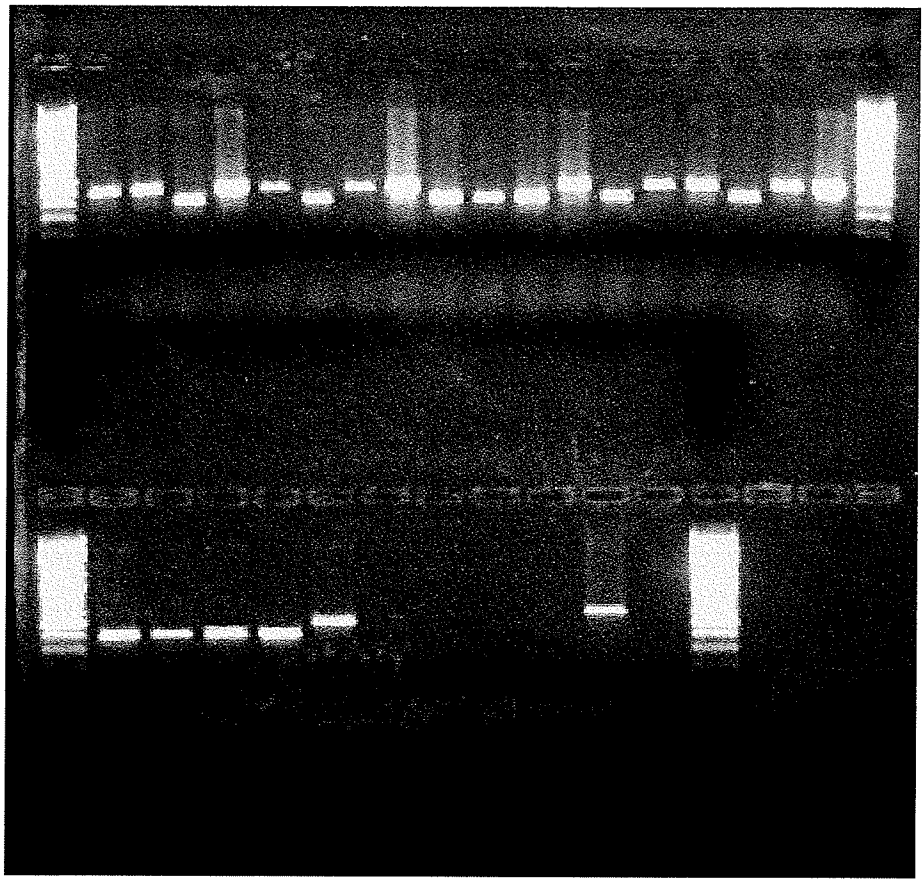


**Figure 11. The Polymerase Chain Reaction for the Detection of the Efflux Pump Gene, *mef*-Open Reading Frame 5, *orf*5 in 28 Study Isolates of *S. pneumoniae.*** *mef-orf*5 intergenic region PCR product is 400 and 500bp in length. 100bp DNA ladder in Lane 1, 20, 21,and 33 was used for sizing. Lanes 2-19 contain isolates 1-18, respectively and lanes 22-31 contain isolates 19-28 respectively. For actual stock numbers of each isolate refer to Table 1. Lane 32 contains water as control.

1 20



21 33

**Figure 12. The Polymerase Chain Reaction for the Detection of the Efflux Pump Gene, *mef*E promoter region in 28 Study Isolates of *S. pneumoniae.*** *mef*E gene promoter PCR product size is 600bp in length. 100bp DNA ladder in Lane 3, 20, 21,and 35 was used for sizing. Lanes 4-19 contain isolates 1-16, respectively and lanes 22-33 contain isolates 17-28 respectively. For actual stock numbers of each isolate refer to Table 1. Lane 34 contains water as control.

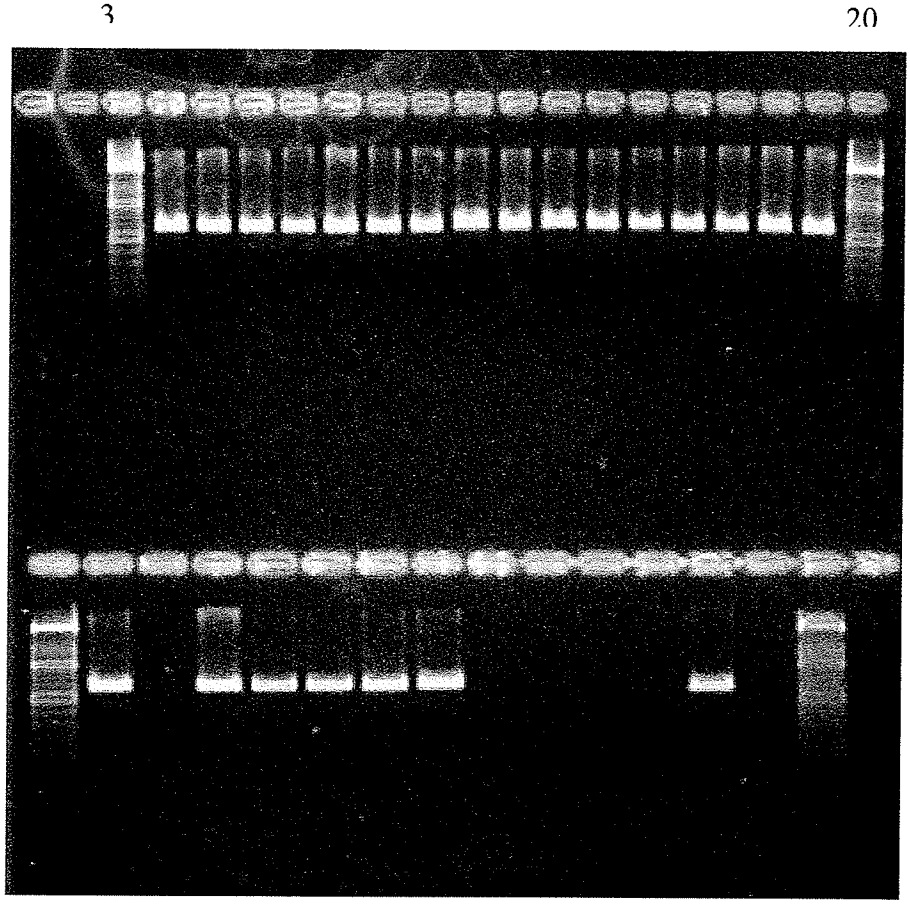
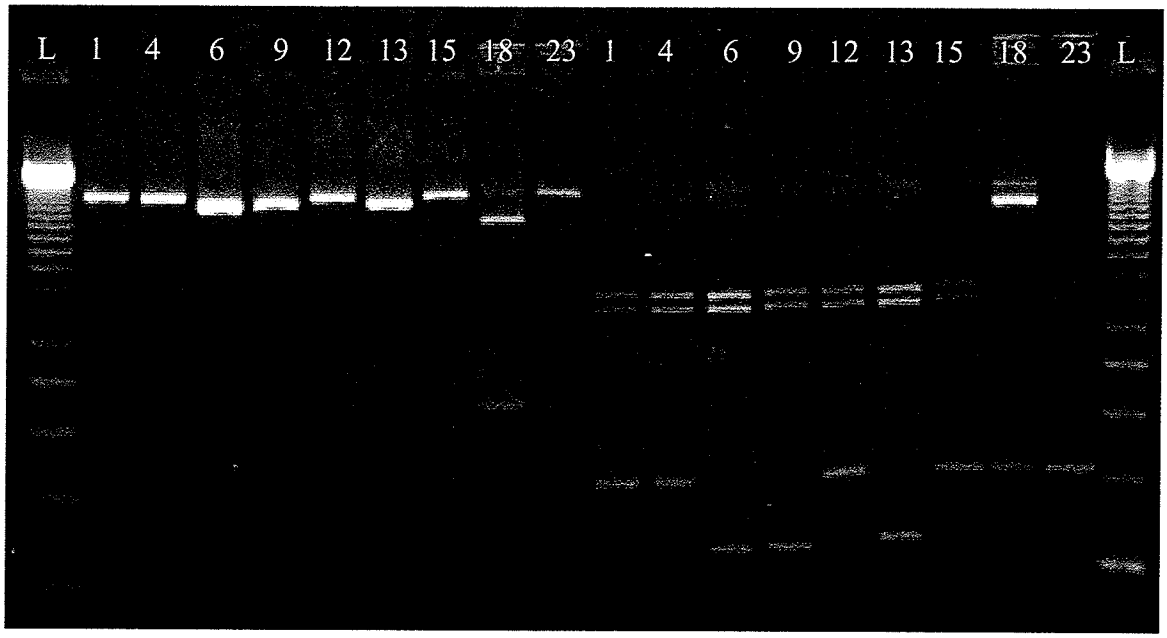


**Table 9. PCR-Restriction Fragment Length Polymorphism for 24 *mef*A/E-Positive Study Isolates.**

| ***Mef* Gene Variant** | **# Of Restriction Fragments** | | **Number of Isolates with Restriction Pattern** |
|---|---|---|---|
| | *Bam*HI | *Dra*I | |
| *mefA* | 2 | 2 | 1 |
| *mefE* | 1 | 3 | 23 |

**Figure 13. PCR-Restriction Fragment Length Polymorphism as performed in discrimination between *mef*E and *mef*A for all 24 *mef*-positive *S. pneumoniae* isolates.** PCR-Restriction Fragment Length Polymorphism analysis was performed using a previously described protocol as described in Material and Methods. Lane L, 1Kb DNA ladder, Lane 1 to 18, sequence of isolates labeled with Experimental Research Number, 1,4,6,9,12,13,15,18,23 restricted with *Bam*HI, and same sequence, 1,4,6,9,12,13,15,18,23, restricted with *Dra*I.

*Bam*HI Digest[a] *Dra*I Digest[b]




[a]In *mef*A there is one *Bam*HI site, so restriction generates two fragments of 1340 and 403bp, respectively, while in *mef*E there is no *Bam*HI sites and the 1743bp amplicon remains undigested.
[b]Restriction of the *mef*A with *Dra*I yields two fragments of 1493 and 250bp, respectively, while restriction of *mef*E yields three fragments of 782,711 and 250 or 151bp, respectively.

## Part V. Northern Blot Hybridization

### a. RNA Template

Figure 14 depicts the denaturing-agarose gel electrophoresis and ethidium bromide staining of the total RNA extracted with the RNeasy Mini Kit (Qiagen) used as a template in the Northern Blotting experiments and also in the RT-PCR experiments. The figure depicts the 23S and 16S ribosomal bands 2.9 and 1.5kb in size, respectively. In addition the 23S ribosomal RNA, (rRNA) band is present at approximately twice the amount of the 16S rRNA.

### b. Probe Labeling

Following a successful isolation of RNA for Northern Blotting experiments, the level of sensitivity for detection of a *mef*- biotinylated DNA probe was confirmed. To ensure correct size and composition of the selected DNA to be used as a probe in Northern Blotting Experiments the *mef* DNA probe was tested twofold. First, the ability of the label to incorporate into the nucleic acid was compared with the DNA provided by the company that ideally incorporates the label. Second, the limit of sensitivity for detection of the *mef* DNA probe was compared to the company's labeled DNA probe by carrying out a simultaneous detection. Figure 15 depicts the level of sensitivity for detection of a *mef* DNA, the unlabeled and the labeled DNA probe. The efficiently labeled DNA probe should be detectable at a 1pg. All three DNAs were equally detectable at this amount and the limit of sensitivity of 100fg was in accordance with the manufactures instructions. The same steps were taken with labeling of the housekeeping gene, glucose kinsase to ensure it as proper for use in Northern Blotting experiments (Figure not shown).

### c. Gene Expression

Despite successful isolation of RNA and proper labeling of the target and the housekeeping probe no signal was detected following hybridization and washing for either of the genes. Efflux pump gene, *mef* expression in *S. pneumoniae* isolates with an M-phenotype was not successfully studied with the Northern Blot Hybridization. Troubleshooting of every step from the amount of RNA used, through transfer of the RNA to the membrane to hybridization salt and temperature conditions, washing and detection resulted in no signal generation of not only the *mef* gene but also the housekeeping, glucose kinase gene.

**Figure 14. Representative Sample of Total Genomic RNA Isolated from 28 Study Isolates of *S. pneumoniae* using the Quiagen Kit as used for Northern Blotting and Real-Time RT-PCR.** Lane 4, 1 Kb RNA ladder (Ambion), Lanes, 1-3 and lanes 5-7, RNA preps from samples 1,3,5, and 16,20,22, respectively. For actual stock numbers of each isolates refer to Table 1. 23S and 16S ribosomal RNA bands are 2.9 and 1.5Kb in size and 23S rRNA should be present in twice the amount of the 16S rRNA.

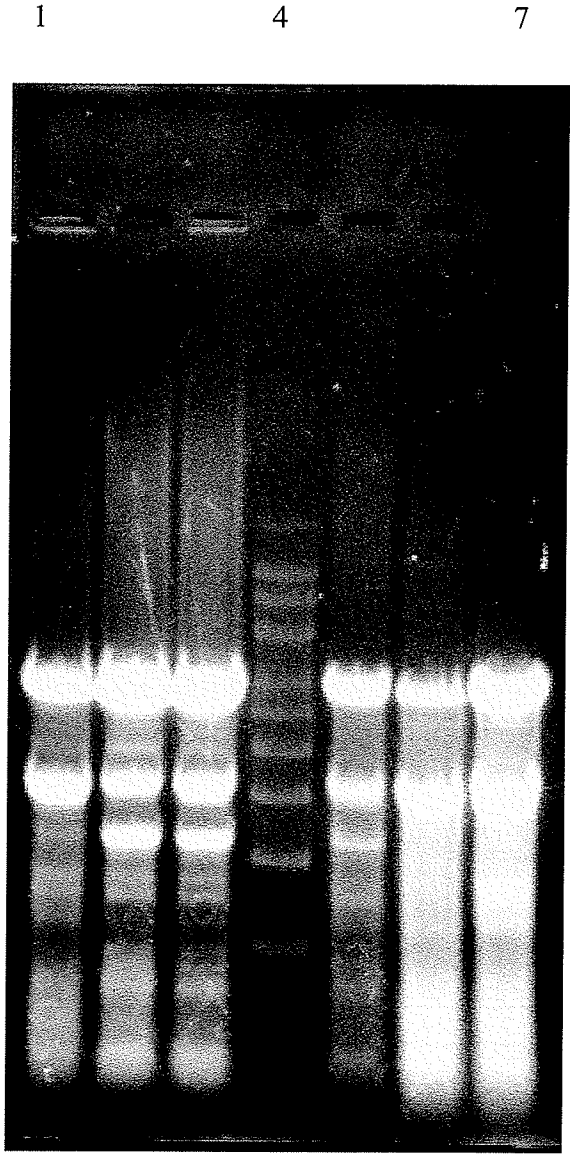
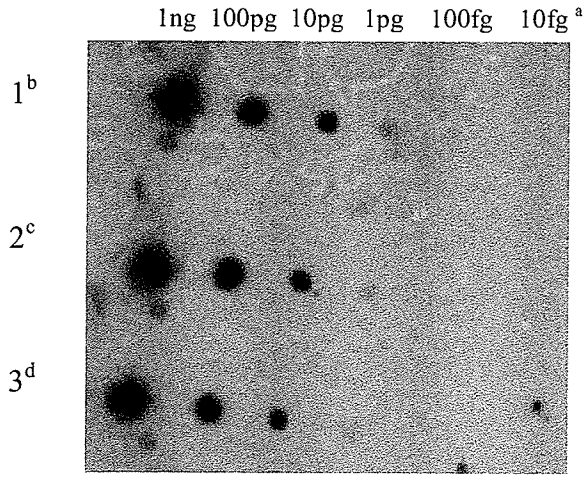


**Figure 15. The Limit of Sensitivity for Detection of Biotinylated *mef*-DNA Probe.** 1 µl aliquots of biotinylated samples diluted 10-fold from 1ng/ul down to 1fg/ul were carefully spotted on a dry positively charged membrane. The nucleic acids were cross-linked with UV and the biotin was detected using the Ambion's BrightStar BioDetect Nonisotopic Detection Kit. A 45 min exposure to X-ray film reveled the sensitivity limit of 100fg for *mef*–probe and is in accordance with the expected manufactures results. For more details of labeling and detection please refer to Northern Blotting section of the Materials and Methods.




[a]dilution series of a diotinylated DNA probe
[b]Ambion Psoralen-Biotin labeled control DNA
cAmbion Psoralen-Biotin unlabeled control DNA
[d]Psoralen-Biotin *mef* DNA probe

## Part VI. Southern Blot Hybridization

The Southern Blot Hybridization with *mefE* probe and *Eco*RI digestion was utilized to assess the presence of multiple *mefE* gene copies in isolates with decreased susceptibility to clarithromycin. Success of the Southern Blot Hybridization depended on many factors. The isolation of intact, undegraded DNA, complete restriction enzyme digestion and the efficient transfer of the *Eco*RI digested DNA to the membrane were very important steps of the Southern Blotting. The complete protocol describing details of every step of this experiment is described in the Materials and Methods, Southern Blot Hybridization section.

### a. DNA Isolation

Figure 16 depicts the integrity of the isolated total DNA following an RNase treatment of 5 representative samples, numbered 2,5,11,17 and 22. High molecular weight compact bands with no smearing are indicative of intact un-degraded total genomic DNA and were essential to a successful Southern Blot. All of the 23, (numbered 1-17,19-23 and 28) M-phenotype *S. pneumoniae* isolates containing the *mef*E variant of the efflux pump gene, *mef* were analyzed. Isolated number 18, containing the *mef*A variant of the efflux pump gene, *mef* was not studied.

### b. *Eco*RI digestion

Figure 17 depicts the gel electrophoresis and ethidium bromide staining of *Eco*RI restricted DNA of the 5 representative isolates, numbered 2,5,11,17 and 22. Digestion of

the genomic DNA with *Eco*RI resulted in progressively smaller bands visible under UV light following ethiduim bromide staining.

**c. Southern Blotting**

Figure 18 depicts the Southern Blot hybridization with *mef*E probe and *Eco*RI digestion of the 5 representative samples, numbered 2,5,11,17 and 22. Southern blot hybridizations using a *mef*E probe and *Eco*RI digestion of all 23 M-phenotype *S. pneumoniae* isolates containing *mef*E indicated that *mef*E was present as a single band. Southern blot hybridization also revealed that *mef*E gene inserts in more than 4 distinct sites within the pneuococcal genome as indicated by 5 different band sizes. Table 10 shows the sizes of the bands and the number of isolates with each band type found in the 23 M-phenotype *S. pneumoniae* isolates. The band sizes were difficult to size exactly and only approximate sizes are available. Southern blot hybridization with *mef*E probe and *Eco*RI digestion identified 5 distinct bands sizes of 2800 for 1 isolate, 3100 for 3 isolates, 4000 for 2 isolates, 8000 for 3 isolates and $\geq$10000 bp for majority of isolates, 14 (Table 10).

**Figure 16. Un-Degraded Genomic DNA Following RNase Treatment from 5 Study Isolates of *S. pneumoniae* as needed for Southern Blotting.** For complete isolation procedure refer to the Southern Blotting Hybridization in Material and Methods. Lane 1 and 7, 1Kb extension ladder, Lanes 2-6, contain isolates 2,5,11,17,22, respectively. For actual isolates numbers refer to Table 1.

1 7

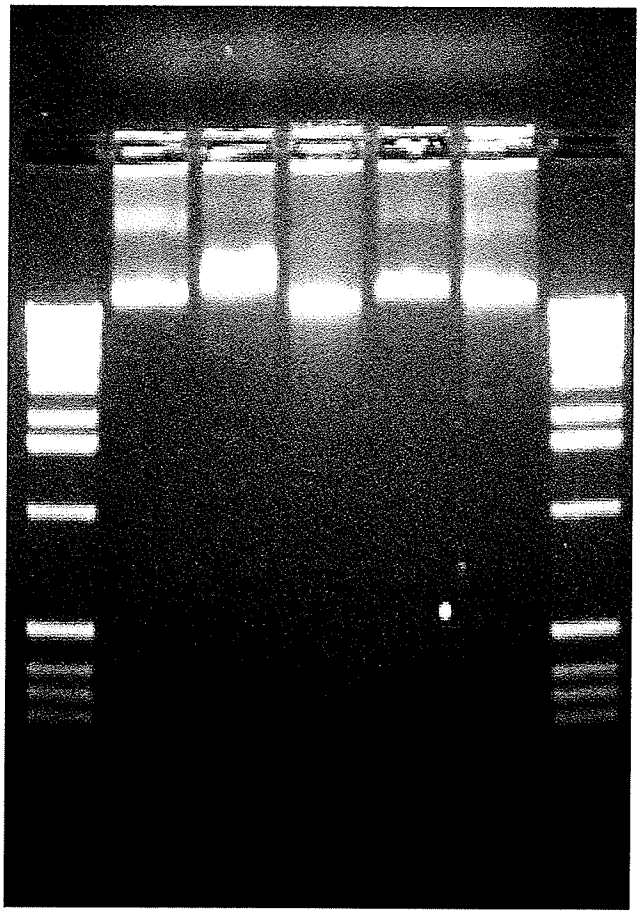


**Figure 17. *Eco*RI Digest of Genomic DNA from 5 Study Isolates of *S. pneumoniae.***

Lane 1 and 7, 1Kb extension ladder, Lanes 2-6, contain isolates 2,5,11,17,22, respectively. For actual isolates numbers refer to Table 1.

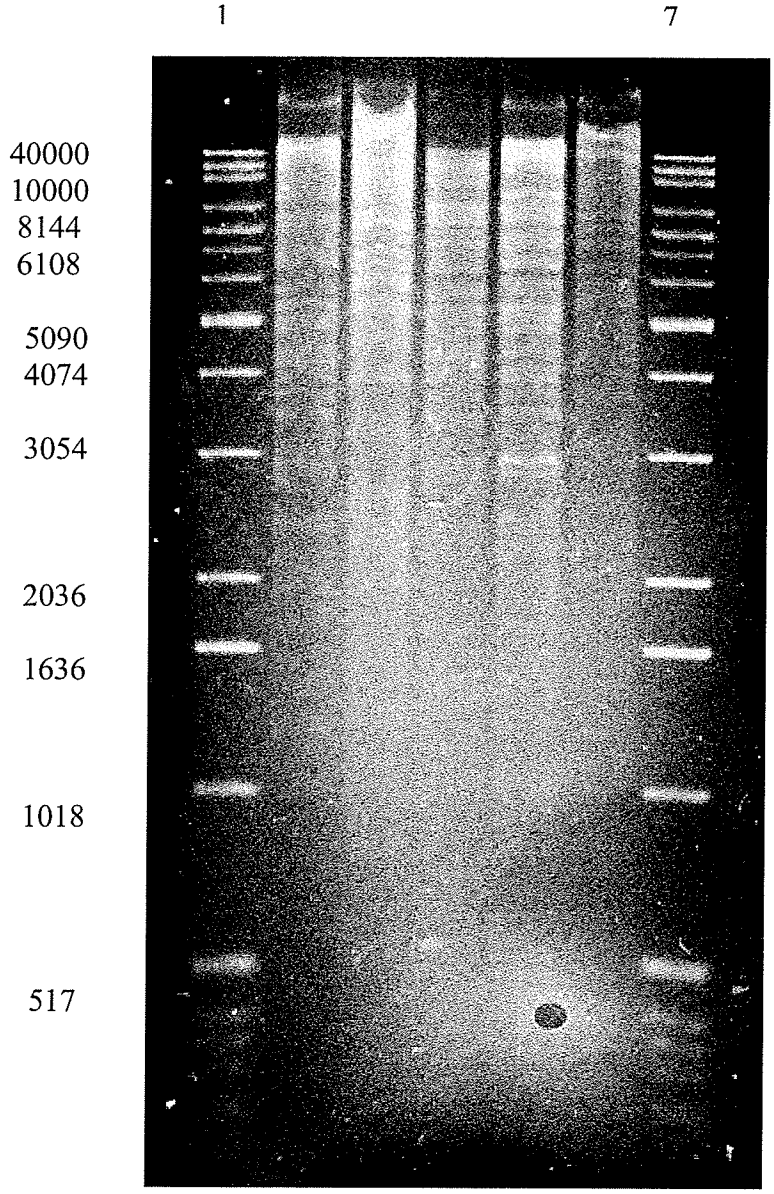


**Figure 18. Southern Blot Hybridization with *mef*E probe and *Eco*RI digest of 5 Study Isolates of *S. pneumoniae.*** Genomic DNA (Figure 16) was digested to completion with *EcoRI* (Figure 17) as described in the materials. Lane 1 and 7, 1Kb extension ladder, Lanes 2-6, contain isolates 5,2,11,17,22, respectively. For actual isolates numbers refer to Table 1.

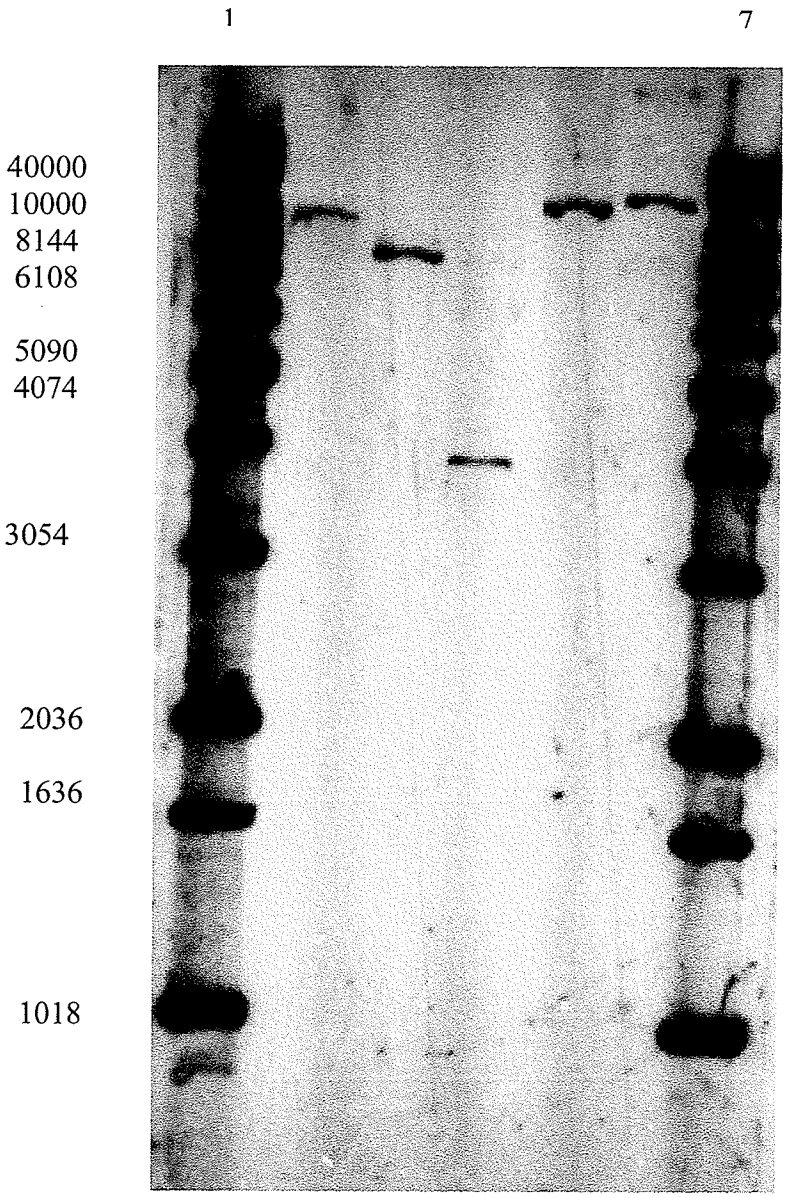


**Table 10. Southern Blot Hybridization with *mef*E Probe and *Eco*R1 Digestion for 23 Study Isolates of *S. pneumoniae*.** Isolate with the *mef*A variant of the Efflux Gene was not included in Southern Blot experiments.

| Approximate Band Size (bp)[a] | # Of Isolates with the Band Size |
|---|---|
| 2,800 | 1 |
| 3,100 | 3 |
| 4,000 | 2 |
| 8,000 | 3 |
| ≥10,000 | 14 |

[A] bands were not sized exactly

## Part VII. RT-PCR

### a. Raw data analysis

Southern Blot hybridizations with *mef*E probe and *Eco*RI digestion identified the *mef*E gene as a single copy gene in 23 M-phenotype isolates of *S. pneumoniae*. The presence of multiple *mefE* gene copies in isolates with decreased susceptibility to clarithromycin was not detected. Relative Real-Time RT-PCR was employed to study the *mef*E gene expression after unsuccessful attempts with Northern Blot Hybridization. For complete details of this experiment please refer to the Material and Methods, RT-PCR section. Table 9 shows the differences in the relative expression of efflux pump gene, *mef*E in 23 M-phenotype *S. pneumoniae* isolates with various susceptibilities to clarithromycin. In addition 1 isolate with the $MLS_B$-phenotype, number 24 and 2 susceptible, 1 clinical, number 26 and 1 laboratory, number 27 were included as controls. The isolate previously identified as containing *mef*A variant of the efflux pump gene, *mef*, numbered 18 was not included in these experiments. Each relative expression value is the mean of 3 replicas. Analysis of the results in Table 9 reveals that the relative expression of the *mef*E versus glucose kinase, *gki* increases as the susceptibility to clarithromycin deceases, (MIC increases). No presence of the *mef*E gene was identified by RT-PCR for the isolates with the MLS-phenotype and as well as for the two susceptible isolates as expected.

Figure 19 depicts the bar graph of the relative expression of *mef*E as a function of clarithromcyin MIC. Of the 23 *mef*E-positive isolates tested isolate, isolate number 6 was excluded from the analysis. The mean value of the relative expression of *mef*E for each of isolates with the same MIC to clarithromycin was calculated and indicates that the relative expression of *mef*E increases in isolates with decreased susceptibility to

clarithromycin. The most significant difference of 700-fold in the relative expression of *mef*E was between the isolate with the highest MIC and the lowest MIC. Comparing the mean relative expression of *mef*E gene in isolates with the clarithromycin MIC of 2ug/ml, 4ug/ml, 8ug/ml to the relative expression of *mef*E in the isolate with clarithromycin MIC of 32ug/ml reveals that the relative *mef*E expression approximately 20-fold, 6-fold and 4-fold lower, respectively (Figure 19).

**b. Statistical Analysis**

The analysis of the data obtained from the real-time RT-PCR experiments revealed differences in the relative expression of the *mef*E gene. The Pearson Correlation and the ANOVA statistical tests were utilized to assess whether the increasing expression of the *mef*E gene in isolates with increasing clarithromycin MICs is statistically significant.

First the Pearson Correlation was applied to the data to assess the degree of linear relationship between the relative expression of the *mef*E gene and the clarithromycin MICs. The Pearson Correlation coefficient has an absolute value between 0 and 1 with 1 indicating a perfect linear relationship and 0 meaning no linear relationship exists. When two variables increase or decrease proportionally (as one variable increases, the other variable increases; and when one variable decreases, so does the other), a positive correlation between them exists (direct relationship). When one variable increases when the other decreases proportionally, there is a negative correlation (inverse relationship). The Pearson Correlation coefficient of 0.9476 for the analysis of the degree of linear relationship between the expression of the *mef*E gene and the clarithromycin MICs indicated a nearly perfect linear relationship between these two variables.

When doing statistical analysis of two interdependent variables, such as the relative expression of the *mef*E gene, and the clarithromycin MIC, it is important to determine whether any differences observed between them are statistically significant. An analysis of variance (ANOVA) studies the effect of independent variable (clarithromycin MIC) on a dependent variable (the relative expression of *mef*E gene) and was utilized as a statistical tool. The relative *mef*E expression values of the 7 groups of isolates (based on the clarithromycin MICs of 0.5, 1, 2,4, 8, 16 and 32) were compared using the Multiple Comparison Analysis, part of ANOVA and revealed differences between different groups as statistically significant. The most statistically significant difference was observed for the group with the MIC of 32μg/ml. The relative expression of *mef*E in this group was statistically significant from all the other groups of isolates. The relative expression of *mef*E for the group with the MIC of 16μg/ml was statistically significant from all other groups except the group with the MIC of 8μg/ml. On the other hand comparing the relative expression of *mef*E for the group with the MIC of 8μg/ml revealed significance in all except the group with the MIC of 16 and 4μg/ml. The relative expression of *mef*E for three groups with the lowest MICs, 0.5, 1 and 2μg/ml were determined as statistically signicifant from all the other groups, such as 4,8,16 and 32μg/ml but were not statistically from each other.

**Table 11. The Differences in the Expression of the Efflux Pump Gene, *mef*E in S. *pneumoniae* with Various Susceptibilities to Clarithromycin.**

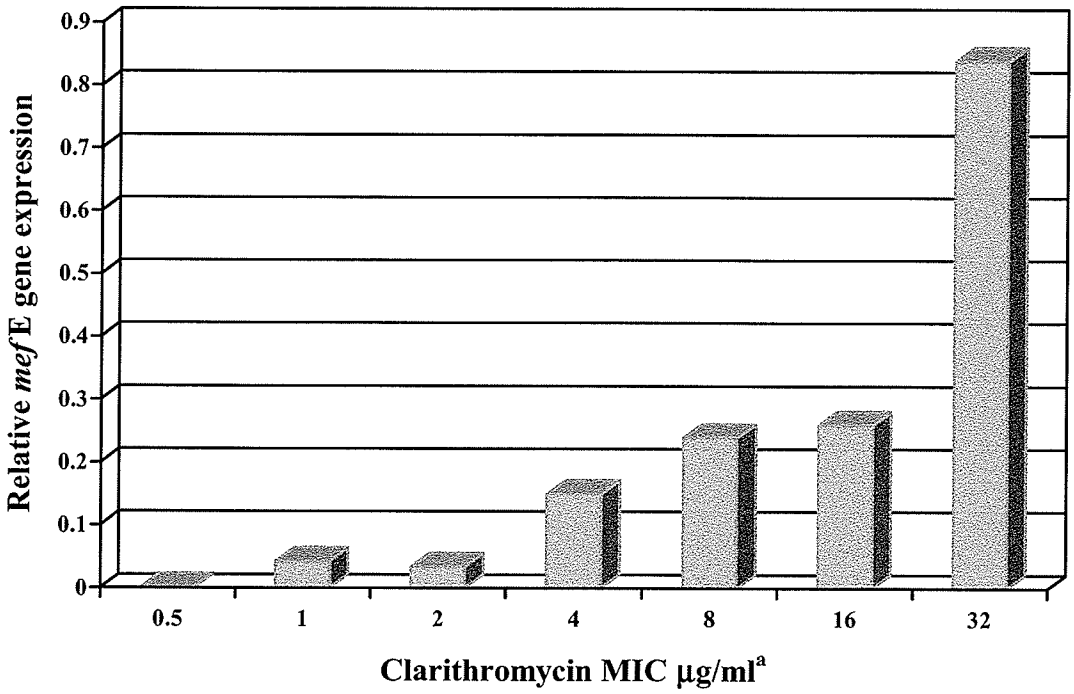
| Isolate # | | PCR Result | | Microbroth MIC[a] µg/ml | | Relative *mef* Quantity |
|---|---|---|---|---|---|---|
| Stock | Experimental | *mef*A/E | *Erm*B | CLR | CLI | |
| **12810** | 1 | + | - | 0.5 | 0.12 | 8.53E-04 |
| **12862** | 2 | + | - | 1 | 0.12 | 9.66E-02 |
| **11183** | 3 | + | - | 2 | 0.12 | 4.95E-02 |
| **12809** | 4 | + | - | 8 | 0.12 | 2.10E-01 |
| **10725** | 5 | + | - | 0.5 | 0.12 | 1.52E-03 |
| **11599** | 6 | + | - | 0.5 | 0.12 | 1.79E+00 |
| **11692** | 7 | + | - | 2 | 0.12 | 6.69E-02 |
| **12780** | 8 | + | - | 4 | 0.12 | 1.66E-01 |
| **12815** | 9 | + | - | 1 | 0.12 | 3.63E-02 |
| **12880** | 10 | + | - | 1 | 0.12 | 2.74E-02 |
| **10733** | 11 | + | - | 2 | 0.12 | 1.99E-02 |
| **11430** | 12 | + | - | 4 | 0.12 | 1.18E-01 |
| **12808** | 13 | + | - | 1 | 0.12 | 5.92E-03 |
| **12848** | 14 | + | - | 2 | 0.12 | 1.34E-03 |
| **C** | 15 | + | - | 32 | 0.12 | 8.35E-01 |
| **12629** | 16 | + | - | 4 | 0.12 | 1.40E-01 |
| **12751** | 17 | + | - | 4 | 0.12 | 1.66E-01 |
| **12300** | 18 | + | - | 8 | 0.12 | N/A[c] |
| **B** | 19 | + | - | 16 | 0.12 | 1.87E-01 |
| **F** | 20 | + | - | 16 | 0.12 | 1.87E-01 |
| **A** | 21 | + | - | 16 | 0.12 | 2.78E-01 |
| **D** | 22 | + | - | 16 | 0.12 | 3.10E-01 |
| **E** | 23 | + | - | 16 | 0.12 | 3.13E-01 |
| **12767** | 24 | - | + | >64 | >32 | No.ct[b] |
| **12778** | 25 | - | + | >64 | >32 | N/A |
| **1333** | 26 | - | - | 0.25 | 0.12 | No.ct |
| **49619** | 27 | - | - | 0.25 | 0.12 | No.ct |
| **15353** | 28 | + | - | 8 | 0.12 | 2.71E-01 |

[a]CLR, Clarithromycin; CLI, Clindamycin; Breakpoints for Broth Microdilution (in µg/ml) per NCCLS guidelines, unless otherwise noted, are as follows: Clarithromycin: susceptible, $\leq 0.25$; intermediate, 0.5; resistant, $\geq 1$; Clindamycin, susceptible, $\leq 0.25$; intermediate, 0.5; resistant, $\geq 1$.

[b]no message detected; i.e. no *mef* gene present

[C]not tested

**Figure 19. The Mean Relative Expression of the Efflux Pump Gene, *mef*E in 23 M-phenotype *S. pneumoniae* Isolates with Various Susceptibility to Clarithromycin.**




[a]Clarithromycin Breakpoints for Broth Microdilution (in µg/ml) per NCCLS guidelines, unless otherwise noted, are as follows: Clarithromycin: susceptible, ≤ 0.25; intermediate, 0.5; resistant, 1.0.

## Part VIII. Sequencing

The results obtained from the real-time RT-PCR revealed that the expression of the efflux pump gene, *mef*E in M-phenotype *S. pneumoniae* isolates increases with the increasing MIC of clarithromycin antibiotic. 1945bp nucleotide sequences encompassing the upstream and downstream intergenic regions of *mef*E and *mel* genes were amplified and sequenced using overlapping primers from all 23 M-phenotype *S. pneumoniae* isolates containing *mef*E variant of the *mef* gene and compared with each other and with the published sequence of mega. In addition, the same 1945bp nucleotide sequence was amplified and sequenced from isolate number 18, identified as containing the *mef*A variant residing on transposon, Tn*1207.1* and was compared to the published Tn*1207.1* sequence.

### a. Analysis of the Upstream Region of the *mef*E gene in *S. pneumoniae*

A 542bp upstream region of *mef*E in the 23 M-phenotype *S. pneumoniae* isolates contained 24 nucleotide changes, including a 16bp deletion153 bp upstream and a single T-C substitution 30bp upstream of the putative *mef*E start site where all 23 isolates differed from the published mega sequence. In addition to these changes, 8 isolates showed a single base pair substitution in this region. Analysis of a putative –10 and –35 regions of all isolates did not reveal any changes. This does not support the hypothesis that a higher *mef*E gene expression is due to greater binding affinity of the DNA polymerase to the promoter. All the changes are presented in Table 12.

### b. Analysis of the *mef*E gene and *mef*A gene in *S. pneumoniae*

A 1217bp nucleotide sequence of the *mef*E gene differed by a single A-T substitution 165bp upstream of the putative *mef*E start site in 3 isolates from the published mega sequence. No other changes were observed. The 1217bp nucleotide sequence of the isolate containing *mef*A, number 18, and the published Tn*1207.1* sequence were 100% identical. However, the two genes, *mef*E and *mef*A were found to differ at 122 nucleotide positions. All the changes are presented in Table 12.

### c. Analysis of the *mef*E and *mel* intergenic region of mega

A119bp intergenic region between *mef*E and *mel* contained a 99bp deletion in 11 of the 23 M-phenotype *S. pneumoniae* isolates. The intergenic region in these isolates was only 20bp long. The deletion found by sequencing for the 11 isolates numbered 3,6,9,10,11,13,16,19,20,21 and 22 is in 100% concordance with the results obtained by PCR of the intergenic region of *mef* and *orf*5/*mel* during the distinction between *mef*E and *mef*A variants of the efflux pump gene (Figure 11). The remaining 12 isolates did not contain the 99bp deletion in this region and were 100% in concordance with the published mega sequence available at that time. The *mef*A and *orf*5 intergenic region in isolate number 18 was also sequenced and was not found to contain the deletion. The results were consistent with the published Tn*1207.1* sequence. All the changes are presented in Table 12.

**Table 12. Nucleotide Sequence Differences found in the 23 M-Phenotype *S. pneumoniae* Isolates and the Published mega Sequence.**

| Position Relative to *mef*[a] | Change | # Of Isolates with Change |
|---|---|---|
| -30 | T - C | 23 |
| -52 | T - G | 23 |
| -60 | Deletion of T | 23 |
| -75 | A - T | 23 |
| -78 | T - G | 23 |
| -79 | A - G | 23 |
| -153 | 16bp deletion | 23 |
| -360 | G - T | 5 |
| -361 | T- A | 1 |
| -372 | A - C | 2 |
| +165 | A - G | 3 |
| +1234 | 99bp deletion | 11 |

## E. DISCUSSION

### Part I. The Genetic Elements Carrying the Efflux Pump Genes, *mef* in *S. pneumoniae*

Until recently, ABC transporters encoded by the plasmid borne *msr*(A) genes in *Staphylococcus* species were the only characterized efflux proteins conferring acquired macrolide efflux (67,79). *Staphylococci* appear to have an efflux system with specificity for 14- and 15-membered macrolides and type B streptogramin molecules but not for lincosamide antibiotics (MS-phenotype) (67,79). This efflux system has been studied extensively and it appears to be multi component system, involving 3 genes, two encoding ATP-binding proteins and the third, the putative pump. Like all ABC transporters it requires energy in the form of ATP. The expression of the MsrA efflux protein has been shown to be inducible with erythromycin and other 14- and 15-membered macrolides, however not with streptogramin B (67,79). Therefore the strains are resistant to streptogramin B only after induction with erythromycin. Clindamycin is neither an inducer nor a substrate for the pump, and thus the strains are fully susceptible to this antibiotic.

First described by Sutcliffe et al. (1996) in clinical *Streptococcus pyogenes* strains the M-phenotype, that is resistance to 14- and 15-membered macrolides but susceptibility to lincosamides and streptogramin B antibiotics, is now the most predominant phenotype in North America among the clinical isolates of macrolide resistant *S. pneumoniae* (67,79,103). Interestingly, the recent rapid increase in macrolide resistance among *S.*

*pneumoniae* isolates in the United States has been identified as due to the emergence of the M-phenotype in this species (44). In this first report Sutcliffe et al. described that mechanism of M resistance present in *Streptococcus pneumoniae* was not mediated by target modification, as isolated ribosomes from the pneumococcal strain bearing the M-phenotype were fully susceptible to erythromycin and the presence of the *erm* methylase was excluded with primers specific for an *erm* consensus sequence. The presence of the macrolide efflux determinant was confirmed by the reduced uptake and incorporation of radiolabeled erythromycin into the cells in the absence of efflux pump inhibitor. Based on the extended knowledge of the macrolide efflux system in Staphylococci Sutcliffe et al. concluded that the *S. pneumoniae* strains bearing the M-phenotype contained an efflux system distinct from the efflux system described for erythromycin-resistant staphylococci. Until the discovery of the M-phenotype of resistance among clinical *S. pneumoniae* isolates, the National Committee for Clinical Laboratory Standards (NCCLS) for streptococci did not recommend clindamycin or streptogramin B as the primary set of antibiotics to be tested by the clinical laboratories, primarily because resistance to erythromycin in the past has signaled cross-resistance to these antibiotics ($MLS_B$-phenotype) (103). The value in testing clindamycin susceptibility in *S. pneumoniae* as part of the primary evaluation has been recognized as meaningful and is now recommended by the NCCLS.

The molecular cloning and functional analysis of a novel macrolide-resistance determinant, *mef*A, from *S. pyogenes* has been published by Clancy et al. (132) not long after the discovery of the M-phenotype by Sutcliffe et al. (132). Soon after the cloning and sequencing of the *mef*A gene in *S. pyogenes*, *mef*E was identified in *S. pneumoniae*

and it was shown to be necessary for the erythromycin-resistant M phenotype in *S. pneumoniae* by Tait-Kamradt et al. in 1997 (104). Introduction of the *mef*E gene into an erythromycin-susceptible pneumococcal strain rendered the strain erythromycin-resistant, phenotypically, M resistant, and able to efflux erythromycin. Also during this study, the two *mef* gene ( *mef*A *and mef*E) sequences were compared and were found to be closely related 90% homologous (104). Based on high nucleotide sequence homology (90%) and similarly high amino acid homology (91%) the two efflux pump genes, *mef*A and *mef*E, were assigned to the same class, *mef*A of macrolide resistance determinants by Roberts et al at the end of 1999 (93).

The *mefA* gene from a clinical isolate of *S. pneumoniae* exhibiting the M-phenotype of resistance to macrolides was found to be part of the 7,244bp chromosomal element Tn*1207.1*, that contains 8 open reading frames, one of which is a putative site-specific recombines (98). This was the first report describing the element that carries the efflux pump gene in Streptococcus spp. As a result of the recommendations by Roberts et al. (93) to assign the 2-*mef* genes into one class: *mef*A one could have considered that the two genes *mef*E and *mef*A were carried on the same Tn*1207.1* genetic element. Knowing the sequence of the genetic element carrying the efflux pump gene has provided the backbone for the experiments that lead to the elucidation that the Tn*1207.1* genetic element does not appear to be the primary element carrying the efflux pump gene in my study isolates. As shown in Figures 10, 11, 12 of the results section the PCR amplification of the many parts of the Tn*1207.1* element determined that the macrolide efflux pump gene is carried on this element for only 1 isolate out of the 24 that were

studied. The results of this experiment were very unexpected but the possibility of a new discovery was very exciting.

At around the same time a novel *mefE* containing chromosomal insertion element designated as mega (macrolide efflux genetic assembly) was found to be the predominating element carrying the efflux pump gene in clinical isolates of *S. pneumoniae* from US by Gay et al. the following year (43). The nucleotide sequence of the mega element became available and enabled the design of a PCR amplification that allowed the confirmation of mega as the primary element of the 23 out of the 24 isolates that I studied. Furthermore, the results obtained from the PCR amplification downstream of the efflux pump gene in the study isolates did not agree with the expected results published by Santagati et *al* (98), however they were in complete agreement with the results obtained for mega element and allowed to further designate the isolates as Class I mega or Class II mega inserts (43). At this point it was clear that the macrolide efflux gene in the 24 *S. pneumoniae* isolates studied is carried by two genetic elements and that the mega element which was found as the sole genetic element in *S. pneumoniae* isolates from US is the predominating (23/24) element in Canadian isolates of *S. pneumoniae* with the M-phenotype. These experiments not only conclude that the efflux pump gene in Canadian isolates of *S. pneumoniae* are carried on different genetic elements but that the two variants of the *mef* gene, *mefA* and *mefE* are present in the Canadian pneumococcal population.

Del Grosso concluded at the beginning of 2002 that macrolide efflux genes, *mefA* and *mefE* are carried by different genetic elements, Tn*1207.1* and mega, respectively in *S. pneumoniae* by showing that both gene variants were present in *S. pneumoniae* isolates in

Italy (25). In Italy as in other European countries the M-phenotype is not very common in clinical *S. pneumoniae* isolates occurring at less than 20% (25,123). Although carriage of *mef*E has been considered typical of *S. pneumoniae*, interestingly the majority of the *S. pneumoniae* bearing the M-phenotype in Italy were found to carry *mefA* and *mefE* was found only in few isolates. The *mefE* variant of the *mef* gene was also previously shown to be carried by viridans group Streptococci, while *mefA* has been identified in *S. pyogenes*. The presence of the *mefE* and *mefA* genes in Canadian isolates of *S. pneumoniae* and also those in Italy may suggest that there is a great opportunity in the nasopharynx for these elements to be transferred from both species to *S. pneumoniae*. The presence of *mefA* in Italian isolates of *S. pneumoniae* has been speculated to be a consequence of the high prevalence of macrolide-resistant *mefA*-carrying *S. pyogenes* isolates (123). Whether or not the prevalence of the *mefE* gene in viridans group *Streptococci* contributes to its presence in macrolide-resistant *S. pneumoniae* isolates remains to be determined.

As suggested by Del Grosso et al (25) the two genetic elements were distinguished by PCR-restriction fragment length polymorphism analysis and the results confirmed the Tn*1207.1* in 1 isolate and mega in 23 of the 24 *S. pneumoniae* isolates as the genetic element carrying the efflux pump gene in Canadian *S. pneumoniae* isolates. In addition to the PCR and the PCR-RFLP analysis, sequencing of a 1945bp fragment encompassing the upstream region and the downstream region of the *mef* gene revealed the presence of two genetic elements. The 1945bp DNA sequence from each of the isolates was compared with the available Tn*1207.1* and mega sequence. Although the isolate with the Tn*1207.1* was identical to the published Tn*1207.1* sequence, the sequence of mega in my

study isolates contained many nucleotide changes from the published mega sequence. A 542bp upstream region of *mef*E contained 24 nucleotide changes, including a 16bp deletion 153bp upstream and a single T-C substitution 30bp upstream of *mef*E where all 23 isolates differed from mega. Recently, the mega genetic element of *S. pneumoniae* was found to be present in erythromycin-resistant *S. salivarius* (133). The upstream region of *mef*E in this species also was found to contain a 16-bp gap that eliminates a direct repeat in the mega of *S. pneumoniae* (133). This is the first report of the pneumococcal mega in oral streptococci and the mechanism of dissemination of the mega remains to be established, however it may suggest that the mega element in *S. pneumoniae* disseminated from *S. salivarius*. A 1217bp sequence of *mef*E showed a single A-T substitution 165bp downstream of the putative *mef*E gene start site in 3 isolates, indicating that the nucleotide sequence of *mef*E is conserved among different isolates of *S. pneumoniae*. A 99bp deletion was observed in 11 out the 23 isolates in the intergenic region of *mef*E and *mel*, the *msr*A homolog which is consistent with Class II mega insert described by Gay et al (43).

**Part II. Macrolide efflux gene, *mef*E expression increases with decreased susceptibility to macrolides in Canadian isolates of *S. pneumoniae*.**

The efflux-mediated resistance to macrolides in *S. pneumoniae* is the most predominating mechanism of resistance in North America (50,67,69). In fact the increasing rate of macrolide resistance in the US in the last few years has been linked to the recent emergence of efflux-mediated resistance (44). Once considered a mechanism conferring a low-level macrolide resistance now shows a rightward shift in the MIC distribution, with higher MICs for isolates with the efflux-mediated phenotype observed overtime.

The goal of the thesis was to study the macrolide efflux pump gene expression in isolates variably resistant to macrolides. Clarithromycin was considered as the representative of the macrolides. Twenty-three isolates of *S. pneumoniae* with variable susceptibility to clarithromycin, ranging from 0.5μg.ml to 32μg/ml were tested. To determine the cause of the rightward shift in the MIC distribution and to learn as much as possible about the *mef*E gene in these variably susceptible isolates *mef*E gene inducibility, copy number and mRNA levels were tested. Knowing that the *mef*E gene carried on mega is the predominating macrolide efflux pump gene in Canadian isolates of *S. pneumoniae* provided the backbone for all the other experiments.

Unlike the resistance due to *msr*A macrolide system in *Staphylococcus*, the efflux-mediated resistance in *Streptococcus* has not been studied extensively. Recently, it has been hypothesized that the efflux-mediated resistance in *S. pneumoniae* conferred by *mef*E gene is inducible by erythromycin and its derivatives and is expressed at

moderately higher levels (67,71,124). Through the experiments outlined in the results section of this thesis the efflux-mediated resistance in *S. pneumoniae* appeared to be inducible by all macrolide antibiotics (erythromycin, clarithromycin, azithromycin) tested and was expressed at higher levels. Of all the macrolides, clarithromycin was shown to be the strongest inducer of the *mef*E gene expression, where 78% of the M-phenotype *S. pneumoniae* isolates showed a significant (>4) fold increase in its MIC following exposure to subinhibitory concentration of clarithromycin. MICs of 57% of M-phenotype *S. pneumoniae* isolates showed a significant (>4) fold increase following exposure to erythromycin and azithromycin indicating equal inducing ability of the two macrolides. As expected MICs of all M-phenotype *S. pneumoniae* isolates showed a non-significant (<4) fold increase following exposure to clindamycin or telithromycin. The results from these experiments concluded that efflux–mediated macrolide resistance in *S. pneumoniae* is inducible by all 14 and 15-membered macrolides, while it is not inducible by clindamycin and telithromycin. The induction of the *mef*E gene indicates some level of gene expression regulation and it needs to be studied more. It is not possible to exclude the possibility that the pump interacts with other proteins.

Following the inducibility study the Southern Blot Hybridization experiments using *mef*E probe and *Eco*RI digestion revealed that *mef*E gene in the 23 isolates of *S. pneumoniae* was present as a single copy. This excluded the possibility of multiple macrolide efflux genes in isolates with higher MIC to clarithromycin. The Southern Blot Hybridization experiments also showed that the *mef*E gene inserts into the pneumococcal chromosome at least in 5 different locations as bands of 5 different sizes lit up following hybridization with the *mef*E probe. The results for these experiments were consistent

with the previous findings by Gay and Stephens indicating the *mef*E as a single copy gene in their isolates (43). They also found *mef*E in more than 4 distinct sites within the pneumococcal chromosome.

Through the work of Gay and Stephens it is known that the mega elements carrying the efflux pump gene, *mef*E in *S. pneumoniae* also carries another putative efflux pump, a homologue to macrolide-streptogramin B efflux pump gene, *msr*A in *Staphylococcus* (43). The two genes are 119bp apart in some isolates while in others 99bp deletion causes them to be co-transcribed. A dual efflux system that is co-transcribed has been hypothesized to be responsible for the increase in the MICs to macrolides in some M-phenotype *S. pneumoniae* isolates (43). Analysis of the clarithromycin MICs for the isolates with the 99bp deletion did not reveal the possibility of a dual efflux system in Canadian isolates of *S. pneumoniae*. In fact some of the isolates without the 99bp deletion were the ones that were considered of higher clarithromycin resistance (MICs: 16 and 32μg.ml) and some with the deletion had clarithromycin MICs of 0.5, 1 and 2μg/ml.

Many cellular decisions concerning survival, growth and differentiation are reflected in altered patterns of gene expression and the ability to quantitate transcription levels of specific genes has always been central to any research into gene function (13,14). Four methods are in common use for the quantification of transcription: northern blotting and *in situ* hybridization, RNAse protection assays and the reverse transcription polymerase chain reaction (13,14). A fifth method, cDNA arrays, is still limited in its use due to cost considerations (13,14). Northern analysis is the only method providing information about the mRNA size, alternate splicing, and the integrity of RNA samples (13). Those reasons among others made Northern analysis the method of choice however after

numerous attempts no *mef*E mRNA nor the housekeeping gene glucose kinase (*gki*) mRNA was detected. RT-PCR is an *in vitro* method for enzymatically amplifying defined sequences of RNA and permits the analysis of different samples in the same experiment (13,14,45,48,89). It is the most sensitive method and the most flexible of the quantification methods and can be used to compare the levels of mRNAs in different sample populations, to characterize patterns of gene expression (13,14,89). The fluorescence-based real-time RT-PCR was utilized to study the expression of the macrolide efflux pump gene *mef*E in *S. pneumoniae* isolates variably susceptible to clarithromycin. The potential of combining several dyes allowed synchronous scanning of the target gene, *mef*E and the housekeeping gene, glucose kinase. The expression level of the *mef*E gene was qunatitated relative to the glucose kinase gene mRNA level. Higher expression of macrolide efflux pump gene, *mef*E was shown to be associated with higher MICs to macrolide antibiotics. A shown in Figure 19 the mean relative expression of the *mef*E gene increased with increasing MIC to clarithromycin. Statistical analysis indicated that there is a direct linear relationship between the expression of the efflux gene and the clarithromycin MIC with the $P< 0.005$. No previous studies have been published describing the expression of the macrolide efflux pump gene, *mef*E in *S. pneumoniae* to my knowledge. Expression studies of the efflux pump gene *pmr*A in flouroquinolone resistant isolates of *S. pneumoniae* have been described in the literature and they showed a similar direct correlation between the level of expression and the MIC to the antibiotic (134). Finally, it must be remembered that real-time RT-PCR quantities steady state mRNA levels and therefore quantification of mRNA levels tells the researcher nothing about either transcription levels or mRNA stability. What this means is that the mRNA

levels may not reflect the levels of protein produced by the cell, as for many regulation occurs at the post-transcriptional stage. Therefore, the observed increase level of *mef*E gene expression in isolates with decreased susceptibility to clarithromycin may or may not indicate the actual amount of the protein. MefE efflux pump protein analysis would have to be performed to confirm variable amounts of the efflux protein in isolates with different susceptibility to clarithromycin. The only conclusion that can be drawn from the RT-PCR experiments is that the increased levels of *mef*E mRNA are present in isolates with different clarithromycin susceptibility but whether this increase in *mef*E mRNA level translates to increased amount of the MefE efflux pump protein remains to be determined.

## F. CONCLUSION

In conclusion, macrolide efflux in Canadian isolates of *S. pneumoniae* is due to the acquisition of *mef*E gene that is carried on the macrolide efflux genetic assembly, mega element. The *mef*A gene carried on Tn1207.1 element has been shown to mediate M-phenotype of resistance in some Canadian isolates, however it is not very common. Resistance has been shown to be inducible by 14- and 15-membered macrolides but not by other macrolides, clindamycin and telithromycin. The increased MICs to clarithromycin were not found to be due to multiple copies of the *mef*E genes as Southern Blot Hybridization determined the *mef*E gene to be a single copy gene that inserts in $\geq$4 distinct sites within the pneumococcal chromosome nor where they due to presence of a dual efflux pump system. The expression of the efflux pump gene, *mef*E in Canadian clinical isolates of *S. pneumoniae* increased with the increasing MIC of macrolide antibiotics as determined by RT-PCR.

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