Evolution and Molecular Characterization of Clinical Respiratory Macrolide-Resistant *Streptococcus pneumoniae* in Canada

by

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Abstract

The purpose of this thesis was to molecularly characterize macrolide-resistant *S. pneumoniae* (SPN) isolates in Canada between 1998 and 2008. The characterization involved looking at the multi-drug resistant phenotype (MDR), the mechanisms of macrolide resistance, the genetic relatedness, the serotype distribution and PCV7 vaccine coverage as well as the determination of presence of pili-virulence factors. The hypothesis of the study was that macrolide-resistant SPN will growingly be MDR, genetically related, piliated and consisting of serotypes not found in PCV7 vaccine.

Over 1500 macrolide-resistant SPN isolates collected between 1998 and 2008 were studied. Macrolide-resistant isolates came from patients from all regions of Canada, and from all age groups. They came from slightly more males (60%) and slightly more in-patients (62%). Macrolide resistant SPN remained low at 8% during the first 4 years of the study, and started to increase reaching 22% by the end of the study in 2008 (p=0.001). Overall, the most common mechanism of resistance was efflux mediated by *mef*(A) (51%), followed by target site modification mediated by *erm*(B) (36%). The efflux mediated macrolide resistance in *S. pneumoniae* was predominantly due to the presence of subtype E (95%), which was resistant to more antibiotic classes, and was genetically and serotypically more diverse than the A subtype. Isolates carrying both *erm*(B) and *mef*(A) macrolide resistance genes increased overtime from 1% (1998) to 19% (2008) (p=0.002). Serotype distribution showed a decrease in PCV7 vaccine coverage from 67% to 31% (p=0.0072). Isolates with non-PCV7 serotypes increased overtime from 33% to 57% (p=0.0152).

Isolates with serotype 19A increased by 15% (p=0.005). They were found to be multi-drug resistant, carried both *erm*(B) and *mef*(A) subtype E macrolide resistance genes, and were genetically related. The presence of virulence factor pili-type 1 (PI-1) and pili-type 2 (PI-2) was found associated with these isolates, possibly contributing to its emergence. In conclusion, macrolide resistant SPN increased during the course of this study mostly due to emergence of multi-drug resistant, genetically related, piliated, 19A *S. pneumoniae*.

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Introduction

Part 1 *Streptococcus pneumoniae*

Taxonomy and Phenotypic Characterisation

Streptococcus pneumoniae are part of the genus Streptococcus (179). Like other members of the genus Streptococcus, they are Gram-positive, catalase-negative bacteria, but unlike the others, they produce capsule. Based on classical phenotypic differentiation, *S. pneumoniae* are alpha (α) - hemolytic streptococci belonging to the Mitis group within the viridians group Streptococci. *S. pneumoniae* are lancet-shaped cocci (elongated spheres with a slightly pointed outer curvature) usually seen as pairs of cocci (diplococci) but may also occur singly or in short chains (liquid media). Individual colonies are large, 0.5-2mm in diameter, having a characteristic central navel-like depression. They are grey or almost white in color, glistening and appear mucoid or moist. They are facultative anaerobic bacteria, incapable of respiratory metabolism, requiring 5% CO_2 levels for adequate growth. The temperature of 37^{o}C is optimum for its growth. They are considered fastidious and their complex nutritional requirements are usually provided by the addition of blood or serum to the growth medium. Glucose and other carbohydrates are metabolised through fermentative metabolism with lactic acid production as the end product. *S. pneumoniae* do not form spores and they are nonmotile. Most *S. pneumoniae* isolates are optochin susceptible, although optochin-resistant stains have been reported. They are also bile soluble (179).

Polysaccharide Capsule and Virulence

Since its discovery by Sternberg and Pasteur in 1881, the Gram-positive, encapsulated bacterium *S. pneumoniae* has been the most widely studied microorganism in medicine

(111). However, it was not until the 1920s, when Avery and coworkers made an observation of a type-specific soluble substance (CPS) in filtrates of pneumococcal cultures and in sera and urine of infected humans and rabbits, that the pneumococcal capsule was shown to be a polysaccharide (111). Of more importance was the finding that these pneumococcal polysaccharides were the first non-protein substances to be antigenic in humans. This discovery paved the way for the use of pneumococcal polysaccharides in vaccines today (111). It is now well accepted that a capsule composed of polysaccharide is an essential determinant of virulence in *S. pneumoniae* (111). The capsule interferes with the activation of the complement system, the essential element of host defence against pneumococci, by preventing complement C3b opsonisation and in turn, phagocytosis (111).

To date, 92 different serotypes have been described. They are distinguished by chemical differences in their CPSs, and on the ability of the immune system to recognize these structural differences and to respond with specific antibodies against the antigens of each different serotype. Two different systems of nomenclature exist for pneumococcal serotypes, the Danish system and the American system (111). The Danish system is based on cross-reactions between different serotypes, so that serologically cross-reactive types are assigned to a common serogroup, with individual serotypes within each group distinguished by a trailing letter. It is the system most widely used today (111). The American system, numbers serotypes sequentially; it does not recognize antigenic cross-reactivity among types and it is used less commonly.

Humoral Immunity

It is generally accepted that immunity to *S. pneumoniae* results from the development of antibodies against the type-specific capsular polysaccharides of the pneumococcus (116). This humoral immunity was demonstrated in the early 19th century, by showing that immunization of rabbits with killed *S. pneumoniae* protected them against subsequent challenge with viable *S. pneumoniae* (111). Subsequently it was shown that serum from immunized rabbits or from humans who recovered from pneumonia conferred protection. The efficacy of pneumococcal immunization was demonstrated in South African miners in the 20th century (129). Further studies discovered that the pneumococcal capsule made it resistant to phagocytosis and that antibody specific for capsular polysaccharide aided the killing of pneumococci. At the same time it was also recognized that different serotypes of pneumococci exist, and that immunization with a given serotype only protected against subsequent infection with the same serotype (116, 129).

Carriage and Infections

S. pneumoniae commonly colonizes the nasopharynx asymptomatically in healthy children and adults (104, 137). It is considered a constituent of the normal upper respiratory flora in humans (104). Although carried by adults, the main habitat of the pneumococcus is the nasopharynx of children. Carriage increases from birth, and is maximal at preschool age. It is estimated that 30-50% of children carry *S. pneumoniae* at any given time (137). The carriage rates decrease slowly and adults have much lower carriage rates (5%) than children (137). Children with young siblings and children associated with daycares acquire pneumococci earlier, and have more pneumococcal types and higher carriage rates than other children (137). Similarly, adults having close and frequent contact with children have higher carriage rates than others (137).

Nasopharyngeal colonization usually precedes pneumococcal infections (137). During carriage, adherence and subsequent colonization appears to be due to binding of the pneumococci to the disaccharide N-acetyl-glucosamine β1-3 galactose on pharyngeal epithelial cells (137, 194). Pneumococcus gains access to areas where it can manifest infection, either via direct extension or by lymphatic or hematogenous spread (150).

The pneumococcus is the most common cause of mild to moderately severe mucosal infections of the lower respiratory tract (pneumonia, particularly community-acquired pneumonia (CAP)), auditory canal (otitis media), upper respiratory tract (sinusitis), and large airway infections (bronchitis) (137, 150). A subset of these infections progress to cause severe pneumococcal disease with tissue invasion such as invasive pneumococcal disease (IPD) (bacteremia, septicaemia, and meningitis, necrotizing pneumonia) (150). Recently, hemolytic uremic syndrome (HUS) and empyema (collection of puss in the lung pleura) have been recognized as complications of IPD and pneumonia in children (24, 33, 44). In addition, *S. pneumoniae* is an important cause of septic arthritis, osteomyelitis, peritonitis, and endocarditis (150).

Risk Factors for Pneumococcal Disease

The risk factors for pneumococcal disease differ depending on the localization of the infection. Otitis media is extremely common in children. It accounts for most of their visits to the doctor and for the majority of their antimicrobial use (137, 150). It has been estimated that 84% of children have had one or more episodes of acute otitis media and 46% have had three or more episodes by the age of 3. Male gender, sibling history of recurrent otitis media, early occurrence of otitis media, and not being breastfed, are factors significantly shown to increase the risk of developing otitis media. Other factors

include association with daycare centres and recent infection with respiratory viruses (137, 150).

The risk factor for pneumococcal pneumonia and IPD are different from those of upper respiratory tract infections. Under normal conditions in the healthy host, anatomic and ciliary clearance mechanisms prevent clinical infection. However, clearance may be inhibited by chronic (chronic obstructive airway disease, smoking, allergies, bronchitis) or acute (viral infections, allergies) factors. In addition, conditions associated with decreased humoral immune response, such as alcoholic cirrhosis, diabetes mellitus, and asplenia, also predispose to pneumococcal disease. Specific genetic defects, such as antibody defects, complement defects, splenic dysfunction, combined immune defects, sickle cell disease, and nephritic syndrome predispose to IPD for life. Previously healthy persons may develop into a high risk group due to the acquisition of hematologic (HIV infection), oncologic (cancer), or medical problems (137, 150).

Invasive pneumococcal disease affects persons of selected races and ages, in certain social conditions and with specific underlying diseases at rates that greatly exceed those of the general population (150). The incidence of IPD has been shown to be higher among indigenous peoples of Alaska, and the Canadian Arctic, Inuits in Greenland, American Indians (white Mountain Apache and Navaho), Australian aboriginals, Maoris of New Zealand and Bedouins in Israel (137, 150). In the United States, IPD been shown to be more common among black Americans than among Caucasians (threefold), and among Native American populations in geographically diverse areas of Alaska to Arizona (five to 10-fold) than that of most of the United States. Certain genetic factors may contribute to the higher incidence of IPD among these aboriginal populations, however socioeconomic status of these people and that these groups often share, crowded living

spaces is a more likely factor. Children under the age of two, independent of race, experience ten-fold increases in the incidence of IPD compared to adult populations (137, 150).

Children under the age of 2 are part of the population at risk for pneumococcal disease mostly due to immaturity of their immune system. Their immature or inexperienced immune system manifests as the inability to produce antibodies to polysaccharide antigens of the pneumococcal capsule. This humoral defect is a result of compromised production of IgG_2, and lack of immunological memory. The risk for invasive pneumococcal disease in children declines with age due to maturation of the immune system and to an expanded repertoire of immunologic memory (137, 150). Infants under the age of 6 months may be relatively protected via transfer of maternal antibiotics during breastfeeding. However, IPD increases among premature, low birth weight infants under the age of 6 months, especially when older siblings are in the house. The elderly are also at an increased risk for IPD as their immune system function declines naturally with age. They are more also likely to have other underlying medical conditions which affect their immune system. Specific communities within the general population, such as those living in poverty, crowding, institutions, home care centres, pollution, and extreme stress, also have an increased risk for pneumococcal diseases (137, 150).

Epidemiology of Pneumococcal Disease

S. pneumoniae is a major cause of morbidity and mortality worldwide. Although all age groups may be affected, the highest rate of pneumococcal disease occurs in young children and in elderly populations (137, 150). Comparing with invasive disease, the non-invasive manifestations are usually less severe, but considerably more common.

Pneumococcal pneumonia is the most common clinical presentation of pneumococcal disease among adults. As many as 175,000 hospitalizations from pneumococcal pneumonia are estimated to occur annually in the United States. Pneumococci account for up to 25-50% of adult community-acquired pneumonia and 50% of hospital-acquired pneumonia. Pneumonia is a common bacterial complication of influenza and measles. The case-fatality rate is 5%–7% and may be much higher among elderly persons. In the US alone, pneumococcus is estimated to cause 40,000 deaths annually. In addition to pneumonia, more than 50,000 cases of pneumococcal bacteremia occur each year. Bacteremia occurs in about 25%–30% of adult patients with pneumococcal pneumonia (137, 150). The overall case-fatality rate for bacteremia is about 20% but may be as high as 60% among elderly patients. Pneumococci cause 13%–19% of all cases of bacterial meningitis in the United States with an estimated 3,000 to 6,000 cases occurring each year. One-fourth of patients with pneumococcal meningitis also have pneumonia. The case-fatality rate of pneumococcal meningitis is about 30% but may be as high as 80% among elderly persons.

Bacteremia without a known site of infection is the most common presentation of pneumococcal infection among children 2 years of age and younger, accounting for approximately 70% of invasive disease in this age group. Bacteremic pneumonia accounts for 12% to 16% of all invasive pneumococcal diseases among children 2-years-old or younger. With the decline of invasive *Heamophilus influenza* type B (Hib) disease, *S. pneumoniae* has become the leading cause of bacterial meningitis among children younger than 5 years of age in the United States. Before routine use of pneumococcal conjugate vaccine, children younger than 1 year of age had the highest rates of pneumococcal meningitis, approximately 10 cases per 100,000 population. In addition,

among children, pneumococci are a common cause of acute otitis media, and are detected in 28%–55% of middle ear aspirates (137, 150).

There was a significant burden of pneumococcal disease among children younger then 5 years of age, before the routine use of pneumococcal conjugate vaccine. An estimated 17,000 cases of invasive disease occurred each year, of which 13,000 were bacteremia without a known site of infection, and about 700 were meningitis. An estimated 200 children died every year as a result of invasive pneumococcal disease. Although not considered invasive disease, an estimated 5-7 million cases of acute otitis media occur each year among children younger than 5 years of age, causing a significant burden on the healthcare system (137, 150).

The impact of pneumococcal disease on young children is especially profound in developing countries. Unfortunately, there is a scarcity of information on disease burden in developing countries. However based on available data; pneumococcus causes over 1 million deaths in these nations (137, 150).

Overall, annual incidence rates of pneumococcal bacteraemia are 15-30 cases /100,000 population for all persons, 45-90 cases/100,000 population for persons ≥65 years of ageold, and >150 cases /100,000 populations for children ≤2 years of age (32). The overall rate of invasive pneumococcal disease among young children is much higher in the Untied States than in Europe, even though these regions are similar in socioeconomic status and access to healthcare. The annual incidence of IPD among children aged <5 in the US ranged from 72 to 103 cases per 100 000 children, whereas studies in the United Kingdom, Spain, Finland, Denmark and other European countries showed rates ranging from 10-24 cases per 100, 000 children per year (137, 150).

Serotypes and Pneumococcal Disease

Since its discovery in the 1920s, pneumococcal capsular polysaccharide has been studied extensively (111). Between 1920 and 1945, the chemical structure and antigenicity of the pneumococcal capsular polysaccharide, its association with virulence, and the role of bacterial polysaccharides in human disease were deduced. More than 80 of the 92 currently-known serotypes of pneumococci had been described by 1940 (111, 194). Although there are over 90 distinct serotypes of pneumococcal capsular polysaccharide, most human infections are caused by only 23 serotypes (5, 88, 129, 137). These serotypes are; 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F (5, 88, 129, 137). Unlike the larger number of serotypes that causes invasive disease in older children (>5 years) and adults, a relatively small number accounts for most of the invasive disease in young children (<5-years) "pediatric serotypes" around the globe. Serotypes 14, 6B, 19F, 18C, 23F, 4, 9V, 19A, 6A, 3, 7F, and 1 in decreasing order, are the 10 most common serotypes causing invasive pneumococcal disease in young children globally (5, 88, 129, 137). Serotypes, 14, 6B, 19F, 18C, 23F, 4, and 9V account for 80% of invasive diseases among children in North America (32). Clinically these seven serotypes are also associated with resistance; serotypes 6A, 6B, 9V, 14, 19F, and 23F are considered to be the most highly antibiotic resistant (47).

However, it is important to note that geographic differences do exist in terms of prevalence of certain serotypes between North America, Europe, Asia, and Africa (88). Particularly noteworthy is the relatively high proportion of serotype 1 invasive isolates from young children in a number of European countries (88). Serotype 1 has been shown to account for 7.2% to 9.5% of invasive isolates from German, Swedish, Italian

and Greek children and was the most important serotype in Slovenian children. A recent study from the Czech Republic showed a serotype 1 prevalence rate of 6% among invasive isolates from children less than 6-years-old. Studies from Denmark, Spain, and the United Kingdom have reported that serotype 1 accounted for a high percentage of invasive isolates throughout the 1980's. Recent studies from Asia, the Middle East and Africa also showed high proportions of serotype 1, ranging from 7% to 31% of invasive isolates isolated in Israel, Malaysia, and Mozambique (88). The high prevalence of serotype 1 in most European, Asian, Latin American, and African countries is in sharp contrast to the low prevalence of serotype 1 in the Untied States, Canada and Australia (2, 88).

An interesting study from Germany looking at serotype distribution of invasive pneumococcal disease during the first 60 days of life has been published recently (94). Leading serotypes among children in this study were 7F (14.8%), 1 and 14 (13.6% each), 3(8%), and 9V (6.8%). It was noted that serotype 7F was the most prevalent serotype among children younger than 60 days of age. The data from the German study indicate that the serotype distribution among children ≤60 days is partly deviant from that of children, and shows similarity to that of adults (94). In a study from Denmark, the proportion of serotypes 1, 3, 5, and 7F was higher among IPD from children aged <6 months than among children aged 6 months to <2 years (110). A US study looking at serotype distribution among neonates (≤30days) with pneumococcal infections showed serogroup 19 as the leading pneumococcal serogroup causing 32% of cases, followed by serogroup 9 (18%), serotype 3 and serogroup 18 (11% each), serotype 1, 6 and 14 (7% each) and serotype 5 and serogroup 12 (3.5%) each.

Treatment

Treatment of pneumonia infections is often empiric and takes into account the presenting illness, the spectrum of common pathogens, the probability of pneumococcal involvement, and the degree of drug resistance found in the patient's geographic area (51, 77). Treatment guidelines for management of pneumococcal infections take into account the type and/or site of infection, patient's age, patient's predispositions; such as co-morbidities, or immunosuppressing conditions, presence of various risk factors for drug-resistance; such as use of antimicrobials in the past three months, younger age, day-care attendance, prior hospitalization, nosocomial infection, severity of illness and probability of death, route of drug administration, and potential toxicity and costs (137). The 2007 US treatment guidelines (Infectious Diseases Society of America and American Thoracic Society) for community-acquired pneumonia in previously healthy adults included a macrolide, (azithromycin, clarithromycin, or erythromycin), or doxycycline (51, 139). In outpatients with co-morbidities or immunosuppressing conditions, or use of antimicrobial agents within the previous three months, or patients residing in a region with >25% macrolide resistance, the guidelines suggested using a macrolide in combination with a β-lactam, such as high dose amoxicillin or amoxicillin clavulanate, preferentially, or cefpodoxime, cefuroxime or intramuscular (IM) cefriaxone (139). Doxycycline could also be used as an alternative to macrolide in this setting. A respiratory fluoroquinolone, such as levofloxacin, moxifloxacin, or gemifloxacin, was also recommended as an alternative to a macrolide as first-line agents. These guidelines also applied to patients with community-acquired pneumonia who required hospitalization but not to an Intensive Care Unit. β-lactams such as amoxicillin rather than macrolides as first-line agents were recommended in other settings.

For out-patient children presenting with community-acquired pneumonia, high-dose amoxicillin, either alone or in combination with clavulanate was recommended. Daily IM ceftriaxone was recommended for those who did not tolerate oral β-lactams. Parenteral β-lactam agents, including penicillin G, cefotaxime and ceftriaxone were recommended for hospitalized children (139).

The initial treatment of meningitis for patients one month of age or older is a third generation cephalosporin such as ceftriaxone or cefotaxime, plus vancomycin. In patients over 50-years-old, ampicillin is added. Treatment is usually adjusted once the culture and susceptibility results are obtained. If the susceptibility results show a penicillin G susceptible strain (penicillin G; MIC $\leq$0.06μg/mL), vancomycin is discontinued, and treatment with cefotaxime or ceftriaxone is continued, or substituted with high-dose penicillin G. For strains with penicillin G MICs $\geq$ 0.12μg/mL, but susceptible to cefotaxime or ceftriaxone at their meningitis breakpoints ($\leq$ 0.5μg/mL), vancomycin may be discontinued. For isolates not susceptible to cefotaxime or ceftriaxone, treatment with these cephalosporins plus vancomycin are continued (139).

Treatment of acute otitis media (AOM), as recommended by the American Academy of Paediatrics and the American Academy of Family Physicians Subcommittee on Management of Acute Otitis Media, included initial treatment with amoxicillin or in the case of high temperature (>39^{o}C) or severe otalgia, amoxicillin/clavulanate.

The 2007 guidelines for sinusitis recommended treatment with high-dose amoxicillin-clavulanate, high-dose amoxicillin, cefpodoxime proxetil, cefuroxime axetil, or cefdinir. Patients with β-lactam hypersensitivity could be treated with trimethoprim-sulfamethoxazole, azithromycin, clarithromycin, or erythromycin(139).

Emergence of Penicillin Resistance and Its Significance

Shortly after the introduction of penicillin in 1940, the first strain of *S. pneumoniae* with decreased susceptibility to penicillin was produced in the laboratory, however, the first clinical isolate with reduced susceptibility did not appear until more than 20 years later (1965) in Boston (77, 81). Subsequently, throughout the 1960s, pneumococcal strains with moderate penicillin resistance were isolated in Australia and New Guinea (11, 81, 86, 87, 98, 103). By the 1970s, high-level penicillin resistant *S. pneumoniae* were detected in pediatric wards in South Africa and shortly after, resistant strains were identified in Europe, particularly in Spain (81, 103, 135). By the 1980s, penicillin-resistant pneumococci emerged in many countries (9, 77, 180). Today, the prevalence of penicillin-resistant pneumococci continues to increase worldwide.

The mechanism of penicillin resistance in pneumococci is due to alteration of the penicillin binding proteins (PBPs) that have reduced affinity for all β-lactam antimicrobials (10, 135). Therefore, the susceptibility is not only affected for penicillin but also for other β-lactams (98). The minimum inhibitory concentrations (MICs) of these drugs rise to different degrees in parallel with those of penicillin G. Cefotaxime, ceftriaxone, cefpirome, and cefepime are the parenteral cephalosporins that are the most active (have the lowest MICs) against penicillin- resistant pneumococcal strains (20, 98). Amongst oral β-lactams, amoxicillin is the most active against penicillin-resistant strains (20). As these β-lactams have retained good activity against penicillin-resistant pneumococci, they remain part of the treatment guidelines for pneumococcal infections as described above.

A baseline activity of penicillin G against wild-type *S. pneumoniae* was defined as MIC between 0.015μg/mL and 0.03μg/mL. This definition was based on the laboratory

finding that the vast majority of isolates during the first 20 years since penicillin introduction had this penicillin MIC(98). The first isolates with reduced penicillin susceptibility identified in Australia and New Guinea in the 1960s had MICs in the range of 0.12μg/mL and 1μg/mL. Subsequently, in the 1970s in South Africa, isolates were noted to have penicillin MICs in the range of 2μg/mL to 4μg/mL (11, 98, 103).

Up until 2008, the *in vitro* definition of penicillin resistance in *S. pneumoniae* (as defined by the Clinical and Laboratory Standard Institute (CLSI), formerly National Committee for Clinical Laboratory Standards (NCCLS)), was as follows: susceptible strains (MIC ≤ 0.06μg/mL of penicillin G), intermediate resistance: (MIC 0.12-1.0μg/mL of penicillin G) and high-level resistance (MIC ≥ 2.0μg/mL) (20, 40, 98, 201). It is important to note that these laboratory definitions of resistance to penicillin were established before the clinical relevance of this level of resistance was established or studied. It was noted early that these breakpoints had relevance for treatment of meningitis and for predicting susceptibility of penicillin-susceptible isolates to other β-lactams, but were not as useful for non-meningeal infections (98). Published data suggest that moderate penicillin-resistant pneumococci causing non-meningeal infections, such as bacteremia or pneumonia, have no therapeutic significance. There has not been a single report of bacteriologically-confirmed failure of intravenous penicillin therapy to eradicate pneumococcal pneumonia, although there have been reports of failure for other β-lactams that are less active and used orally against *S. pneumoniae* (81, 118). Some authors have suggested that the non-susceptible strains are best considered to be β-lactam challenged as this challenge can be overcome in many instances with appropriate dosing regimens (118). This idea followed from results of pharmacokinetic and pharmacodynamic studies that showed that the serum concentrations achieved with penicillin or related β-lactams

are several times higher than the MICs of the strains (98, 201). These studies also showed that pneumococcal meningitis poses a special therapeutic problem because the levels of penicillin achieved in the cerebrospinal fluid (CSF) are inadequate to kill penicillin-resistant pneumococci, including those with intermediate-resistance, therefore the breakpoints accurately predict treatment outcome for meningeal strains (20).

Since the publication of these pharmacodynamic studies, there have been several reports dealing with the treatment outcomes in response to β-lactam antibiotic therapy in penicillin-resistant pneumococcal meningitis, pneumonia and bacteremia cases. A prospective study of community-acquired pneumococcal infections in 207 South African children treated with penicillin or ampicilin at standard dosage was studied by Klugman *et al.* (119, 120) This study showed 14% mortality rate in children infected with penicillin-resistant pneumococcal infections, and 11% in children infected with penicillin-susceptible pneumococcal infections. A three-year study by Tan and colleagues showed that 18 out of 19 children with systemic infections caused by intermediate penicillin-resistant *S. pneumoniae* responded adequately to initial amoxicillin or cefuroxime therapy (188, 189). In another study by Friedland et al., 88% of children with penicillin-resistant infections and 93% of penicillin-susceptible infections improved by day seven of therapy (80). In a recent study of 504 adult patients with severe pneumococcal pneumonia treated with penicillin or ampicillin, there were no significant differences between patients infected with a penicillin-resistant strain and those infected with penicillin-susceptible strain in terms of mortality rate. These are just few examples of the many studies that concluded that despite escalation of penicillin resistance worldwide over the past three decades, no increases in the mortality rate or treatment failure has been noted and no significant differences in terms of mortality rates and response to β-lactam therapy

between infections caused by penicillin-resistant and penicillin-susceptible pneumococci exist. Treatment failures due to penicillin-resistant pneumococci causing bacteremia, otitis media, and pneumonia have been reported but so have they been reported for penicillin-susceptible pneumococci causing these infections (10, 77, 127). Therefore, factors independent of antimicrobial susceptibility of the infecting organism, such as host extremes of age, underlying immuno-suppressive conditions, diseases, and co-morbidities and possibly the virulence of the infecting organism, such as capsular type may reflect clinical failure (137).

As the relationship between penicillin resistance and treatment failure has not been convincingly established for these non-meningeal infections, penicillin was still recognized as a valuable treatment option. However, classification of penicillin MICs was confusing and led to inappropriate clinical applications (51, 81, 143). Even though most penicillin-resistance in non-meningeal infections can be overcome with appropriate dosing regimens, the laboratory report of penicillin-resistant strains steered physicians from using of penicillin as a therapeutic option. This situation has now largely been clarified by CLSI, with the development of clinically relevant susceptibility breakpoints for most β-lactams. These 2008 breakpoints are based on dosing regimen and site of infection, and they differ for meningeal and non-meningeal infections. In these new breakpoints, the susceptible penicillin G breakpoint remains at ≤0.06μg/mL for meningitis and for predicting the susceptibility to other β-lactams, while new susceptibility breakpoints for non-meningeal infections have been established at ≤2μg/mL susceptible; 4μg/mL intermediate, and ≥8μg/mL resistant (201). Application of these new breakpoints as shown in one study significantly increases the rates of susceptible *S. pneumoniae* to 92.6% from 50% (using the old breakpoints), significantly decreases the

rate of intermediate resistance to 7.1% from 18-20% (using the old breakpoints), and high-level resistance to 0.3% from 12-33% (using the old breakpoints) (98). These new breakpoints, supported by the long experience of years of a low incidence of treatment failure for non-meningeal infections despite increasing *in vitro* penicillin G resistance in pneumococcus should greatly facilitate appropriate reporting and use of penicillin G, thus sparing other antibiotics for use in other clinical situations.

Recently, results from the SENTRY Antimicrobial Surveillance Program indicate that penicillin susceptibility in ~15,000 *S. pneumoniae* has declined over the 12 years (1998-2009)(1). Using the penicillin susceptible breakpoint of ≤0.06μg/mL (old breakpoint) the susceptibility rate decreased from 71.6% in 1998 to 59.0% in 2009. This translates to over 40% penicillin resistance in pneumococci in 2009. Unfortunately, despite the revised breakpoint of ≤2.0μg/mL for penicillin susceptible *S. pneumoniae*, the data showed a decline from 96.8% in 1998 to 84.1% 2009 (1). Although using the revised breakpoints has reset the penicillin resistance rate to a lower level, penicillin non-susceptibility and possibly higher lever penicillin-resistance is on the rise.

Part 2 Macrolides

Role of Macrolides

The management of community-acquired pneumonia (CAP) over the past decade has been impacted by the increasing awareness of pathogens other than *S. pneumoniae* implicated in its aetiology (77). While *S. pneumoniae* remains the most common causative pathogen, *Haemophilus influenzae* and *Moraxella (Branhamella) catarrhalis*, as well as oral anaerobes, and to a lesser extent Gram-negative bacilli and *Staphylococcus aureus* have been traditionally associated with CAP. In addition, "atypical pathogens"

such as *Chlamydia pneumophila*, *Legionella* species, *Mycoplasma pneumoniae* have been recognized more recently. *Mycobacterium tuberculosis* and *Pneumocystis carinii (recently renamed Pneumocystis jiroveci)* have been identified as pathogens with increasing prevalence. Less common, but certainly present among the causative pathogens of CAP, are *Neisseria meningitidis*, *Streptococcus pyogenes*, *Streptococcus milleri*, *Coxiella burnetti*, and *Chlamydia psittaci*. In addition to *S. pneumoniae*, *Nocardia species* and *Mycobacterium* spp. (avium complex) have been shown to play a role in the aetiology of CAP especially among immunocompromised hosts.

In addition to bacterial pathogens, fungi and viruses also play a role in the aetiology of pneumococcal infections in both immunocompetent and immunocompromised hosts (77). In addition to all these pathogens, it is not uncommon to have multiple organisms concurrently or sequentially infecting a patient and causing CAP. It has been documented that influenza A or *C. pneumoniae* infections predispose to a secondary infection with *S. pneumoniae*.

Macrolides play a role in the management of CAP due to their wide *in vitro* activity against all key pathogens, both traditional and atypical, associated with CAP (77, 205, 213). This wide *in vitro* activity, along with oral formulation, makes macrolides an important agent for the empirical treatment of CAP (154). However, many observational studies of therapy for patients who require hospitalization have showed that better outcomes have been associated with antimicrobial treatments that have activity against atypical pathogens (154). Therefore, for both outpatients and inpatients, the option of using a macrolide, either in combination or as monotherapy, is one of the recommendations of many CAP guidelines (20). In addition, macrolides play a role in treatment of pneumococcal infections in patients allergic to penicillin.

Chemistry

The majority of naturally occurring macrolides are produced by *Streptomyces* species. Two macrolides, mycinamycin and rosaramycin, are produced by Micromonospora species (205, 213). The chemical structure of the macrolides includes a large lactone ring that varies in size from 12 to 16 atoms. In addition, one or more sugars, usually L-cladinose and D-desosamine, are attached to the lactone ring through a glycosidic covalent bond and the ring is substituted with hydroxyl or alkyl groups. Naturally occurring macrolides are unstable in gastric acid and have unfavourable pharmacokinetic properties that limit their use clinically. Semisynthetic derivatives of natural macrolides have been developed to overcome the clinical challenges of the natural macrolides. These synthetic modifications include esterification, salt formation and/or structural modification.

Macrolides are divided into four classes based on the number of atoms in the lactone ring. The characteristic feature of the 12-membered ring macrolides that distinguishes it from other macrolides is the presence of the ketone group at position C-7 and a desosamine moiety at the C-3 position via glycosidic bond. Methymycin and neomethymycin are naturally occurring 12-membered lactone ring macrolides. Attempts at creating semisynthetic 12-membered macrolides have been made, however they failed to produce clinical activity.

Erythromycin is the prototype for the 14-membered ring lactone macrolides. The lactone ring of erythromycin unlike that of 12-memerbered macrolides is substituted with two sugars, L-cladinose at position C-3, and D-desosamine at position C-5. The position of the L-cladinose sugar on the lactone ring is different than in the 12-membered macrolides. It's spectrum of activity includes Gram-positive cocci, such as *S.*

pneumoniae, *S. pyogenes*, *S. aureus* as well as atypical pathogens such as *M. pneumoniae*, *L. pnemophila* and *C. pneumoniae*. Although clinically effective and safe, erythromycin has been shown to decompose to inactive anhydroketal and spiroketal derivatives in gastric acid. These derivatives are associated with a high incidence of gastrointestinal (GI) adverse effects. In addition, erythromycin has been shown to have poor pharmacokinetic properties such as poor oral bioavailability and a short serum half-life that requires a frequent dosing regimen. Among other limitations is the development of phlebitis following parenteral administration and a significant number of drug-drug interactions. The adverse GI effects of erythromycin have been addressed with the creation of the erythromycin salts and esters, such as erythromycin stearate, erythromycin estolate, and erythromycin ethylsuccinate, however, the dosing regimen remained unchanged. Certain structural features of erythromycin have been shown to contribute to acid instability. These include the ketone at C-9, the hydrogen at C-8, the hydroxyl at C-6, and the diol at C-11 and C-12. Roxithromycin is the first semisynthetic 14-membered lactone ring macrolide that resulted from substitution of the ketone at position C-9 for an oxime group. This modification increased acid stability but did not appear to affect the *in vitro* activity of roxithromycin. Attempts to increase the *in vitro* activity by increasing the tissue concentration of the 14-membered semi synthetic macrolides was made by creation of dirithromycin which was produced through the combination of active drug with acetaldehyde but proved to be unsuccessful.

The alkylation of the C-6 hydroxyl group of erythromycin resulted in the production of a 14-membered lactone ring semisynthetic macrolide, clarithromycin. This modification eliminated the GI adverse effects as it prevented the creation of hemiketal and spiroketal metabolites. In addition to the improved GI adverse effects, clarithromycin has shown to

have *in vitro* activity equal to or greater than that of erythromycin against common respiratory pathogens, but it also demonstrated increased antibacterial activity against *H. influenzae*.

The addition of methyl-substituted nitrogen at the C-9a position of erythromycin has lead to the production a of the only 15-membered lactone ring macrolide, azithromycin. The addition of this basic nitrogen formed a chemical structure known as azalide, which is stable at low pH of the stomach thereby prevents the degradation of the drug into inactive metabolites responsible for the GI adverse effects. Azithromycin demonstrates an increased antibacterial activity not only against *H. influenzae* but also against other Gram-negative and atypical pathogens responsible for CAP. It has excellent tissue penetration and a very long serum half-life, allowing for once-daily dosing that optimizes patient compliance.

Among 16-membered lactone ring macrolides, the majority are natural; however two semisynthetic derivatives, rokitamycin and miokamycin have been developed which demonstrate increased activity against some resistant organisms and improved pharmacokinetic properties. These macrolides are available in certain countries or in veterinary practice (tylosin). In North America, three macrolides, erythromycin, clarithromycin, and azithromycin, have been used most extensively (205, 213).

Pharmacokinetics

Absorption, distribution, metabolism, and excretion are the pharmacokinetic attributes of a drug (206, 213). Absorption describes the bioavailability of a drug. The bioavailability for erythromycin base (25%), clarithromycin (55%), and azithromycin (37%) is low to moderate ranging form 25-55%. Erythromycin salts/esters, such as

erythromycin ethylsuccinate, estolate, and stearate depending on the formulation, have slightly increased bioavailability ranging from 45% to 80%. The food intake may affect the absorption of drugs. Food intake decreases the absorption of erythromycin base and erythromycin stearate because of increased gastric acid concentration, which leads to, increased drug degradation. Food intake decreases the absorption of azithromycin capsules and powder suspension, while it has no affect on tablet formulation. Food intake has no affect on the absorption of clarithromycin and erythromycin estolate. Other important parameters of absorption are the maximum concentration (C_{max}) that the drug can achieve and the time it takes to reach C_{max} (t_{max}), which describes the rate of absorption. Maximum concentration achievable by erythromycin base ranges from 0.3-0.9mg/L and it takes three to four hours after dosing to reach that. C_{max} and t_{max} for azithromycin is 0.4mg/ml and two hours, respectively. Clarithromycin‘s C_{max} ranges from 0.6-1.0mg/mL to 2.1-2.4mg/mL to 4.7mg/ml with increasing dose of 250mg to 500mg to 1000mg. The time to reach the C_{max} is two hours regardless of the dose (205, 213).

Distribution describes the localization of the drug in the body and it is dependent on the chemical structure of the drug. Macrolides are lipophilic in nature and have a low degree of ionization therefore, they have extensive penetration into tissues and fluids which results in a large volume of distribution. Concentrations of macrolides in respiratory tract tissues and fluids are in most cases higher than concurrent serum concentrations. This is an important attribute of macrolides in treating pneumonia, as antibiotic levels in extracellular fluid and alveolar lining fluid are probably more important than serum levels. In addition, macrolides enter host defence cells, particularly macrophages and polymorphonuclear leukocytes (PMNs). The exact mechanism of entry

of macrolides into the phagocytes is not known, however, it has been proposed that lipophilic nature and carrier-mediated transport mechanism may be involved. Upon entry into the cell, the majority of macrolides localize within lysosomes. The PMNs are believed to act as carriers in the transport of macrolides to the site of infection through chemotaxis. High concentrations of macrolide antibiotics within leukocytes amplify the effects of macrolides. First, during infection, leukocytes are attracted to sites of infection via chemotaxis, where the intracellular load of macrolides is delivered and released directly at the site of infection. Second, leukocytes directly phagocytose pathogens (*S. pneumoniae*), thereby exposing them to intracellular reserves of antimicrobial agents. In both cases, the result is to boost the concentration of antimicrobial in the vicinity of infecting pathogen well above the serum levels (205, 213).

Metabolism and excretion are two other aspects of pharmacokinetics. Erythromycin and clarithromycin display affinity for cytochrome P450 (CYP), therefore they are associated with potential drug interactions as their interactions with CYP interfere with the metabolism of a number of drugs. Azithromycin does not display affinity for CYP. The half-life ($t_{1/2}$) of the drug is an important aspect of metabolism and excretion. Erythromycin has $t_{1/2}$ of 2-3 hours, clarithromycin; 3-5 hours, and azithromycin; 40-68 hours. The longer the half-life, the less frequent the administration of the drug is required. Macrolide excretion is primarily through bile; however no dose adjustment is required for hepatic dysfunction. Renal excretion of macrolides is limited to clarithromycin, with 20-40% of the drug being excreted through this route. Dosage adjustment (decreased dose) is required for patients with renal impairment (205, 213).

Pharmacodynamics

Pharmacodynamic properties of the drug describe the relationship between the drug concentration and the antibacterial effect (206, 213). The antibacterial effect of macrolides is in turn dependent on the binding and interaction of the drug with its target (ribosomes). It is known that the bound drug has to occupy a critical number of binding sites; and that the antimicrobial activity associated with binding is estimated by measuring the serum levels. It is important to point out that because of the macrolide's unique pharmacokinetics, serum concentrations are not good predictors of macrolide activity. The drug also has to remain at the binding sites for long enough to exert its effect. Parameters such as Cmax and the area under the serum concentration-time curve (AUC) relative to the MIC (AUC/MIC) or time above the MIC (T/MIC) are used to predict antibacterial activity. Antibacterial activity may be either concentration dependent or concentration-independent. Concentration dependent drugs exert their killing by high concentrations of a drug at the site of infection. It has been estimated that concentration dependent drugs achieve bacterial death, clinical cure, and prevent the development of resistance when the ratio of Cmax to MIC (Cmax:MIC) is 10:1. Concentration independent drugs require the drug concentration to be above MIC for extended period of time (T/MIC) in order to exert its effect. Concentration independent drugs exhibit maximum efficacy when the serum concentrations are kept above the MIC for at least 40-50% of the administration interval. These agents achieve their peak antibacterial action at low serum concentrations with length of exposure playing a greater role. The pharmacodynamic properties of macrolides are difficult to classify. However, most experts suggest that the 14-membered macrolides, such as erythromycin and clarithromycin, are time-dependent, and concentration-independent, whereas the 15-membered macrolide azithromycin is concentration-dependent.

According to time-kill curves, erythromycin is bacteriostatic at the MIC and exhibits low bactericidal activity above the MIC, whereas clarithromycin is bacteristatic at MIC and rapidly bactericidal at higher concentrations (205, 213).

Other Properties of Macrolides - not antimicrobial and not PK/PD

Additional properties of macrolide antibiotics may influence the outcome of respiratory tract infection (134). It has been shown that erythromycin aids in mobilization of mucus and reduces bronchial hyperactivity in people with asthma. Clarithromycin has been demonstrated to normalize nasal mucus in patients with sinusitis and decrease sputum production. More importantly, macrolides have anti-inflammatory properties, which may play a role in attenuating chronic respiratory disorders (46). It has been shown that 300-600mg daily dose of erythromycin is beneficial for diffuse panbrochiolitis, a disease characterized by chronic sinusitis and brochiectasis. This daily dose is well below the MIC for the relevant infecting organisms; therefore these effects of macrolides are likely due to mechanisms other than antibacterial. Among patients with panbronchiolitis, treatment with erythromycin decreases or attenuates several inflammatory cells, number of neutrophils, and interleukins, leukotriene, and defensis. Macrolides modulate lymphocyte function.

Mechanism of Antimicrobial Action

Macrolide antibiotics are bacteriostatic agents that act on the large ribosomal subunit of bacteria and inhibit proteins (205, 213). The ribosome structure and contact points between the ribosome and erythromycin A were recently identified by crystallography studies. The bacterial ribosome is formed by a small, 30S subunit and a large, 50S

subunit (205, 213). The large subunit is composed of 23S ribosomal RNA (rRNA) and of a minimum of 30 proteins. The secondary structure of the 23S rRNA is folded due to base pairing and forms six domains numbered I to VI, while the tertiary structure is maintained due to its interactions with the proteins. The binding site of macrolides has been shown to be near the peptidyltransfrese centre of domain V of the 23S rRNA. Hairpin 35 in domain II has been shown to be in the vicinity of this binding site. The main sites of interaction of macrolides have been studied extensively by chemical footprinting, mutant analysis, and by crystallography. These studies have shown that the main site of interaction is within the domain II and V of the 23S rRNA. In the tertiary structure, domain II and V are in close proximity to each other and form a single binding pocket as has been shown by stoichiometric binding of macrolides. Chemical footprinting studies have elucidated the binding site of erythromycin and clarithromycin to be nucleotides A2058, A2059, G2505, and U2609 in domain V of the 23S rRNA as these sites bound by macrolides were protected from chemical modification (205, 213).

Through the high resolution X-ray crystal structure analysis of the 50S ribosomal subunit of *Deinococcus radiodurans* complexed with erythromycin, it was demonstrated that the 2'OH group of the desosamine sugar of the macrolide antibiotic forms three hydrogen bonds with adenines at positions 2058 and 2059 (*E. coli* numbering). The dimethylamino group of the desosamine sugar of the antibiotic appears to also interact with adenine at position 2505. The 6-OH of the lactone ring forms a hydrogen bond with adenine at position 2062 and the 11-OH and 12-OH forms a bond with uracil at position 2609. Cladinose sugar does not appear to be involved in interactions of macrolides with 23S rRNA. Although footprinting experiments have implicated adenine at position 752

of domain II in the binding of macrolides, no direct interaction has been shown between the two structures at least in the ribosome of *D. radiodurans* (205, 213).

The precise mechanism of action of macrolides has not been elucidated; however it is thought that macrolides inhibit protein synthesis by blocking elongation. The main action of macrolides is the stimulation of the dissociation of peptidyl-transfer RNA (tRNA) during translocation. Macrolides do not inhibit the pepidyltransferase activity but prevent the extension of the peptide chain by blocking the polypeptide exit tunnel. This causes early release of polypeptide resulting in an incomplete polypeptide chain. It has also been shown that macrolides inhibit ribosome assembly. Interaction of the hydroxyl group at position C-6 of erythromycin has been shown to interact with the lysine at position 63 of ribosomal protein L4 inhibiting the fixation of this protein on 23S rRNA and thereby preventing the ribosome assembly (205, 213).

Mechanism of Macrolide Resistance in *S. pneumoniae*

The diminution of the affinity of the antibiotic for its target is the most common mechanism shared by bacteria for becoming resistant to antimicrobial agents. This effect may result from enzymatic detoxification of the drug, often leading to changes in the actual drug structure that prevent it from binding to its target, or conversely, from the target modification so that the drug can no longer bind to it. A diminished access of the antimicrobial agent to its target is another common mechanism shared by many bacteria. This mechanism might be due to active efflux of the drug or decreased uptake of the drug. The resistance of *S. pneumoniae* to macrolides is due to modification of the ribosomal target by methylation or mutation and active efflux of the drug. Drug modification-detoxification has not been reported in this bacterium.

Target Modification by Ribosomal Methylation: The MLS_B Resistance Phenotype

Ribosomal methylation, which was previously identified responsible for erythromycin resistance in staphylococci, has been the first mechanism of resistance to macrolides elucidated in *S. pneumoniae* and has remained the only mechanisms known for decades (15). Ribosomal methylation in *S. pneumoniae* is due to acquisition of the *erm*(B) gene. The *erm*(B) determinant, initially called *erm*(AM), was first identified on plasmid pAM77 in *S. sanguis* isolated from dental plaque (126, 202). The gene is widely distributed not only in *S. pneumoniae* but also in other streptococcal and entercoccal species, as well as in enterobacteria and in staphylococci. Wide distribution of the *erm*(B) gene indicates easy exchange of genetic information even between phylogenetically remote species (59, 79). Unlike, the first discovered *erm(AM)* gene, the *erm*(B) gene in pneumococci is carried by conjugative transposon related to TN*1545*, Tn*1545*-like elements or a Tn*917*-like element that is part of a larger composite transposon, Tn*3872* (15). Transposition occurs from chromosome to chromosome of strains of *S. pneumoniae*. It has been shown that 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 *S. pneumoniae*.

The *erm*(B) gene encodes an erythromycin ribosome methylase which dimethylates pneumococcal 23S rRNA at a key binding site for macrolides: adenine at position 2058. This modification markedly reduces the affinity of erythromycin for its target by preventing direct access to the target or modifying the confirmation of the binding site. This modification leads to cross-resistance to structurally unrelated antibiotics: macrolides, lincosamides, such as clindamycin and streptogramin B, giving rise to the MLS_B phenotype due to the overlapping binding sites of these drugs (62-64).

Isolates carrying the *erm*(B) gene are clinically described as high-level macrolide resistant *S. pneumoniae* strains. Their macrolide MICs can range from 1->32µg/mL; however 90% of isolates are inhibited (MIC_{90}) at MICs greater than 32µg/mL. The MIC_{90}s for the other two groups of drugs to which these isolates are cross-resistant, the lincosamides and streptogramin B, are also greater than 32µg/mL. Isolates carrying the *erm*(B) gene are therefore described as high-level cross resistant to macrolides, lincosamides, and streptogramin B antibiotics and confer the MLS_B phenotype (124, 125). Although widely predominant, the *erm*(B) gene is not the only *erm* gene responsible for macrolide resistance. Rare cases of macrolide resistance due to *ermA* have been described in *S. pneumoniae* (184). In a 2001-2002 PROTEKT study, five isolates (0.2% of macrolide resistant strains) were found to carry *erm*(A). All five were resistant to all macrolides, and two were non-susceptible to clindamycin (107).

Regulation of *erm*(B) Gene Expression

The synthesis of erythromycin ribosome methylase encoded by the *erm*(B) gene can be inducible or constitutive (126, 173, 216). If resistance is constitutive, the strain is resistant to all 14-, 15- and 16-membered macrolides, lincosamides, and streptogramin B. The typical MLS_B phenotype is evident upon susceptibility testing. When resistance is inducible, the strain is generally susceptible to lincosamides and 16-membered macrolides, but resistant to 14- and 15-membered macrolides, such as erythromycin, clarithromycin, and azithromycin. However, inducible expression may give rise to a large variety of phenotypes, which include high and low-level resistance to erythromycin with susceptibility or resistance to clindamycin. The complexity of the inducible expression of the *erm*(B) gene has in addition been described as a result of various levels of basal

erm(B) *e*xpression which might be due to relaxed control of methylase synthesis (126, 173, 216). The mechanism of *erm*(B) induction has not been thoroughly studied; however a model has been proposed which was inferred from the translational regulation of *erm*(C) in *S. aureus* (124, 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. When resistance is inducible, *erm*(B) mRNA is synthesized in an inactive conformation, and becomes active only in the presence of inducers. Most members of the MLS_B group are inducers at various degrees of Erm(B) methylase production (124, 125).

The regulation of expression of the *erm*(C) determinant has been studied from the staphylococci plasmid pE194 and is explained by a translation attenuation mechanism. Through these studies, it was shown that adjacent to the *erm*(C) structural gene for the methylase there is an open reading frame encoding a 14 to 19-amino acid control peptide. Both *erm*(C) and the control peptide are co-transcribed in a single mRNA. Translation of this mRNA implies that the ribosomes recognize an initiation sequence for the two open reading frames. These sequences are called ribosome binding sites or Shine-Dalgarno (SD) sequences and are separated by a few base pairs from the initiation codons. At the 5`end of the *erm*(C) mRNA are four inverted repeats. In the absence of inducer, these inverted repeats fold into stem-loops that sequester the ribosome binding site and the initiation codon for the *erm*(C) methylase. This prevents the access of ribosomes and only the control peptide is translated. When the inducer is present, it is speculated that it binds to ribosomes, including those involved in the synthesis of the control peptide, and causes them to stall. The ribosome stalling probably induces conformational changes in the mRNA and causes displacement of the stem loop structure. This result in the SD

sequence for the mRNA of *erm*(C) to be free and accessible to the ribosomes and initiation of translation of the methylase can occur. It has been observed that the methylase is synthesized by ribosomes that are efficient for protein synthesis. Efficient ribosomes are the ones that are not complexed with erythromycin or that are methylated. Low level production of the methylase occurs in the absence of erythromycin as spontaneous and transitory rearrangements of mRNA occur, therefore it is possible to have some ribosomes that are methylated (124, 125).

Target Modification by Mutation

Studies with pneumococcal mutants have revealed domain II, domain V of the 23S rRNA, and L4 and L22 ribosomal protein as the structures participating in the binding of macrolides (34, 53, 56, 67, 147, 186, 187, 198). The clinical importance of mutations at these sites was recognized several years ago for microorganisms such as *Helicobacter pylori* and *Mycobacterium avium* but only recently for *S. pneumoniae*. Most target site mutations affect 23S rRNA. 23S rRNA is encoded by *rrl* gene, which is present in different copy numbers in different bacteria; four copies have been described in *S. pneumoniae.* Transformation experiments with mutated *rrl* genes have shown that the susceptibility of erythromycin decreases as the number of mutated *rrl* genes copies increases. In addition, these same studies have shown that high level erythromycin resistance can only be achieved when at least two copies are mutated. This finding may explain why resistance conferred by the 23S rRNA mutation is rare in pneumococcus in comparison to *H. pylori* or *M. avium*, which contain only one or two copies of the *rrl* gene.

In addition to the number of mutated *rrl* copies, the resistance phenotype varies according to the nature of the substituted base. Initial work depicting the mutations in the 23S rRNA and ribosomal proteins was performed on macrolide resistant *S. pneumoniae* mutants selected from susceptible strains by *in vitro* passage in azithromycin (187). These four mutants were resistant to 14- and 15-membered macrolides, but the resistance could not be explained by any known macrolide resistance determinants. The analysis of the 23S rRNA DNA sequences revealed changes in C26211A, C2611G, A2058G, and A2059G of the domain V of the 23S rRNA. These initial experiments also showed that, in addition to resistance to 14- and 15-membered macrolides, C2611A, C2611G, and A2058G mutations conferred resistance to streptogramin B antibiotics; C2611A, C2611G, A2058G, and A2059G conferred resistance to 16-membered macrolides; and A2058G and A2059G mutations conferred resistance to clindamycin in these *in vitro* selected mutants. In addition to mutations in the 23S rRNA, this preliminary work showed that mutations in the ribosomal protein L4 can be responsible for macrolide resistance in *S. pneumoniae* (187). Two mutants selected from *in vitro* passage in azithromycin showed no changes in the 23S rRNA but revealed changes in a highly conserved stretch of amino acids ($_{63}$KPWRQKGTGRAR$_{74}$) in ribosomal protein L4. A single amino acid change glycine to cysteine at position$_{69}$ ($G_{69}C$) was detected in one of the mutants, while the other had a 6-base insert, resulting in two amino acids, serine and glutamine (S and Q) being inserted between amino acids glutamine$_{67}$ and lysine$_{68}$ (Q_{67} and K_{68}). These changes resulted in a ≥4-fold increase in the MICs of 14- and 15-membered macrolides and streptogramin B. It was noted that these mutations did not significantly affect the MIC of lincosamides, as the MIC of clindamycin increased less than 2-fold comparing to the susceptible parent strain. The authors also noted that some of the

increases in the MICs of the 14- and 15-membered macrolides even though significant would not be significant to classifly these L4 mutants as macrolide resistant by the Clinical and Laboratory Standards Institute (CLSI) breakpoints. These initial experiments also investigated an important observation from past experiences with other bacteria that mutations in the 23S rRNA and ribosomal protein can alter growth rate and confer temperature sensitivity to growth. None of the *in vitro* selected 23S rRNA mutants showed changes in growth on different medium type, or in different temperatures. The doubling time remained the same between the mutant and its isogenic parent. On the other hand, the serine and glutamine insertion between amino acids glutamine$_{67}$ and lysine$_{68}$ in the conserved region of L4 protein had profound changes in the growth characteristics of its mutant. The mutant was unable to grow in certain temperatures (25^{o}C) and had a longer (110 min versus 60 min) doubling time at 35^{o}C than that of its isogenic parent. These experiments have demonstrated that the insertion mutation may have resulted in a protein that impacted the growth rate at 35^{o}C and viability at 25^{o}C (187).

Shortly after the initial studies demonstrating the possibility of 23S rRNA and ribosomal protein mutations to be responsible for macrolide resistance in *in vitro* selected mutants, clinical stains with unusual macrolide and lincosamide (ML) or macrolide and streptogramin B (MS_B) phenotypes that did not carry the typical macrolide resistance determinants that were being identified in Eastern Europe and North America (186). The ML (resistant to macrolides and lincosamides) strains were shown to carry A2059G mutations in three out of four 23S rRNA alleles. Again, as with the *in vitro* selected mutants, the susceptibility to macrolides and lincosamides decreased with increasing number of mutated *rrl* alleles. These isolates were resistant to all macrolides and were

16-fold more resistant to clindamycin than was the ATCC 49619 strain. As with the *in vitro* selected mutants, these isolates remained susceptible to streptogramin B antibiotics. The MS_B (resistant to macrolides and streptogramin B) strains did not have any changes in the *rrl* gene for any of the four alleles and the L22 ribosomal protein was wild type as well. However, L4 ribosomal protein of all the MS_B clinical isolates in this study demonstrated a 3 amino acid substitution $_{69}GTG_{71}$ to $_{69}TPS_{71}$ within the conserved region of the L4 protein. These strains were resistant to macrolide and streptogramin B but susceptible to lincosamides (186).

Since these initial studies, many other studies have been published describing their findings with mutations within the binding site for macrolide antibiotics that have been found to contribute to its resistance (56). A clinical isolate of *S. pneumoniae* isolated in France from a blood culture had a new resistance phenotype. This isolate was susceptible to 14- and 15-membered macrolides but resistant to 16-membered macrolide and streptogramin B (56). This resistance phenotype was due to an A2062C mutation in all four *rrl* alleles. L22 mutants were first described in macrolide-resistant mutants selected from parental strains of macrolide susceptible *S. pneumoniae* by serial passage in macrolides (34). Point mutations, G284A, C296A, and C278A in the *rpl*V gene encoding the L22 protein leading to a single amino acid change from glycine to aspartic acid at 95 (G95D), P99Q substitution, and a A93E substitution respectively were recently described in *S. pneumoniae*. In addition, L22 mutations were combined with L4 G71R mutation and with 23S rRNA C2611A mutation in few isolates (147). In this same study other mutations in the 23S rRNA were discovered namely, A2058U, C2610U, and C2611U as well as first mutation in domain II, a deletion of adenine at position 752 (34).

Mutation in domain V, domain II of the 23S rRNA, and in the key ribosomal proteins L4 and L22, have been identified among macrolide resistant *S. pneumoniae,* indicating these sites as the key contact regions for macrolide binding. Among all these mutations, mutations in domain V of 23S rRNA are the most prevalent (53, 198). Among these the most frequent substitutions are at positions A2058, A2059, and C6211 (53). The phenotype conferred by modification of the 23S rRNA target varies according to the mutated base. Change in the adenine at position 2058 for a G or U confers the MLS_B phenotype, defined as high level resistance to macrolide, lincosamides, and streptogramin B. The phenotype is therefore similar to that conferred by dimethylation of the A2058 by the Erm(B) methylase. The A059G mutation confers a high-level macrolide resistance and a moderate level resistance to clindamycin but does not affect the streptogramin B. It appears to be most common type of mutation in the domain V of the 23S rRNA in *S. pneumoniae*. The other less common 23S rRNA mutations, affecting the C2611 position, have been shown to have a weak impact on macrolide MICs but higher level resistance has been observed with streptogramin B antibiotics (53).

Active Efflux

It was not until the late 1990s, that erythromycin resistance was demonstrated to be present in the absence of cross-resistance to lincosamides and streptogramin B antibiotics in the pneumococcus (176, 183, 185, 203). Before the discovery of the M-phenotype, which is defined as resistance to 14- and 15-membered macrolides and susceptibility to 16-membered macrolides, lincosamides and streptogramin B antibiotics, erythromycin resistance was assumed to indicate cross-resistance to all the MLS_B antibiotics (176, 183, 185). Early studies have indicated that these strains did not carry the *erm*(B) methylase,

therefore resistance was not due to target methylation (176, 183, 185). These M-phenotype strains were shown to accumulate less erythromycin than susceptible strains, suggesting that erythromycin resistance might be due to an efflux pump. It was soon realized that the newly recognized M-phenotype was due to the presence of a membrane bound efflux protein, encoded by the *mef* genes, *mef*(A) and *mef*(E) (176, 183, 185).

Initially, *mef*(A) gene was identified in M-phenotype *S. pyogenes* and it was shown to encode a novel hydrophobic 44.2-kDa protein with homology at the amino acid level to other efflux proteins (37). Subsequently, *mef*(E) gene was indentified in *S. pneumoniae (185).* The initial work focused on elucidating the function of the *mef*(E) gene; to determine if *mef*(E) encoded the erythromycin determinant necessary for macrolide efflux in pneumococci (185). This was performed by comparing the isogenic stains of *S. pneumoniae* containing the functional, undisrupted *mef*(E) gene *mef*(E) with a disrupted, unfunctional, null *mef*(E) gene (*mef*(E)*::cat*). These experiments showed only *S. pneumoniae* containing the functional *mef*(E) gene to have reduced accumulation of C^{14} erythromycin, and *S. pneumoniae* containing the disrupted *mef*(E) gene was able to accumulate C^{14}erythromcin to the same extent as susceptible control strain (185). This confirmed that *mef*(E) had a role in mediating the efflux of erythromycin in *S. pneumoniae* isolates with the M-phenotype. These experiments were also used to evaluate the substrate specificity of *mef*(E) efflux pumps and have shown that it is specific for 14- and 15-membered macrolides only (185). Comparison experiments revealed that the newly identified *mef*(E) in *S. pneumoniae* is ~90% identical to the initially discovered *mef*(A) gene in *S. pyogenes (55)*. Due to degeneracy of the genetic code, the two genes share only 88% identity at the protein level (54, 55, 174). The 405-amino-acid protein encoded by the *mef*(E) gene has many of the same characteristics as the efflux pump

encoded by the *mef*(A) gene, however 48 mismatches were found. It is a hydrophobic protein with 12 putative membrane spanning regions (89). BlastX analysis of *mef*(E) encoded protein has shown homologies to other transport proteins or efflux proteins, including a putative transporter of ethambutol from a high-level ethambutol resistant mutant of *Mycobacterium smegmatis* and *cmr*, a transmembrane protein with homology to the major facilitator family from *Corynebacterium glutamicum* (89, 140).

It has also been shown that *mef*(E) encoded efflux pump uses proton motive force rather than ATP as part of the pump mechanism, as *mef*(E) containing strains accumulate significantly greater levels of erythromycin if the pump is inhibited by arsenate, an agent used to disrupt proton motive force (89, 140). The pump encoded by the *mef*(E) gene belongs to the major facilitator superfamily of efflux (MFS) proteins (31, 89, 140).

Since the homology of the *mef*(E) gene in *S. pneumoniae* and the *mef*(A) gene in *S. pyogenes* was very high, the two genes were assigned to the same gene class of macrolide resistance determinants, called *mef*(A) (172). Subsequently PCR assay using primers based on the conserved regions of the two genes was recommended. This led to *mef*(A/E) being detected by a PCR assay that did not distinguish between the two variants. However, the two variants were considered species specific; therefore if a *mef* gene was found in *S. pneumoniae* it was assumed to be *mef*(E) and if it was found in *S. pyogenes* it was assumed to be *mef*(A) (172).

Genes of the *mef*(A) class (*mef(*E) and *mef(*A)) have disseminated markedly and are being recognized in ever growing number of microbial species (12, 14, 115, 133, 181). At present, both the *mef*(A) and *mef*(E) genes have unambiguously been identified in five streptococcal species, where as *mef*(E) has been identified in five more streptococcal species and in nine nonstreptococcal species (115, 133). Among streptococcal species

found to carry both variants of *mef*(A) gene class are *S. pneumoniae, S. pyogenes, S. agalactiae, S. mitis,* and *S. oralis*. S. *salivarius, S. anginosus, S. intermedius*. Group C Strep are among the additional streptococcal species found only to carry *mef*(E) variant. Among non-streptococcal species found to carry *mef*(E) gene are *Enterococcus spp., Corynebacerium, Micrococcus, Staphylococcus aureus, hemolyticus, intermedius,* and others, *Neiserria gonorrhoeae*, Granilucatella adiacens, and Gemella haemolysins (115, 133). In addition to being identified in a variety of Gram-positive genera, it was demonstrated that the genes in the *mef*(A) gene class can be easily transferred between *S. pneumoniae* strains, from *S. pneumoniae* to *E. faecalis*, from viridans group streptococci to *S. pneumoniae*, and from *Enterococcus spp*., *C. jeikeium*, and *Corynebacterium spp*. *M. letues* to *E. faecalis*. The *mef*(E) gene was associated with DNase resistant mobility which suggested that the transfer of the efflux encoding gene among the four different genera is through conjugation via direct cell to cell contact (115, 133).

Plasmids were not detected in any of the donors and the transconjugants and *mef*(E) was detected in the chromosomal fractions in Southern blots, suggesting a chromosomal location. The presence of *mef*(E) gene as a normal silent resident in these bacteria was excluded as PCR with *mef*(E)-specific primers did not yield a product in macrolide-susceptible stains of pneumococci or enterococci (115, 133). Isolates carrying variant *mef*(E) or *mef*(A) of the *mef*(A) gene class, are clinically described as low-level macrolide resistant *S. pneumoniae* (55, 209). Typically their macrolide MICs are in the range of 1μg/mL to 32μg/mL, with 90% of isolates being inhibited (MIC_{90}) at a macrolide concentration of 4μg/mL. The MICs to lincosamides and streptogramin B antibiotics remain at ≤0.25μg/mL. Recently, a rightward shift has been observed where low-level, efflux mediated macrolide resistant strains are displaying higher than usual MICs (68).

Tn*1207.1*

Tn*1207.1* was the first *mef*-carrying genetic element described (174). It was found to be integrated into the chromosome of clinical stain of *S. pneumoniae* exhibiting M-phenotype resistance and it carried a *mef*(A) gene which was considered typical of *S. pyogenes*. The element is 7,244bp in size and contains 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 other. Homology searchs were performed on all the *orfs*. Orf2 was found to be homologous to site-specific recombinases of genetic elements in Gram-positive bacteria. Orf5 immediately downstream (119bp downstream) of *mef*(A) was found to be a homolog of the macrolide-streptogramin B resistance determinant *mrs*(A)/*vga*(A) in *S. aureus* and might be involved in determining macrolide and streptogramin B resistance in *S. pneumoniae/ S. pyogenes.* The *msr*(A) and *vga*(A) genes, unlike the *mef*(A) gene class, are putative members of the ATP-binding cassette (ABC) superfamily. *orf*6, *orf*7, and *orf*8 were found to be homologous to 3 ORFs of the pneumococcal conjugative transposon Tn*5252*. *orf*8 is homologous to a UV resistance protein, but no proposed function for *orf*6 and *orf*7 was made. The integration site of Tn*1207.1* in the *S. pneumoniae* chromosome was found to be specific into the *cel*(B) gene. The *cel*(B) gene encodes a DNA binding protein and its disruption impairs the capability of Tn*1207.1*-carrying strains to become competent of genetic transformations. The integration causes a 1,947-bp deletion in the pneumococcal genome.

Tn*1207.1*, which carries the *mef*(A) gene in *S. pneumoniae,* is considered a defective tansposon, as it is truncated on the 3'end when compared to Tn*1207.3*, the original *mef*(A) carrying transposon in *S. pyogenes* (165). The Tn*1207.3* is a big 52kb conjugative transposon and DNA sequencing showed that the 7,244bp at the left end of Tn*1207.3* was

identical to Tn*1207.1*. Tn*1207.3* integrates at a single specific site, the coding sequence of *com*(EC), within the *S. pyogenes* chromosome. A transfer from *S. pyogenes* to *S. pneumoniae* has been demonstrated. It was noted that direct cell to cell contact was required for gene transfer. There is speculation that Tn*1207.3* might be a bacteriophage as it seems to behave like one in *S. pyogenes* matings. However, it behaves as a conjugative transposon in interspecies matings, complicating the understanding of the mechanism by which Tn*1207.3* is exchanged among bacteria (165). The exact mechanism of the exchange of this genetic element among bacteria is not known and further work will help to understand the reasons of the success of this genetic element.

MEGA (Macrolide Efflux Genetic Assembly)

The structure of the genetic element carrying the *mef*(E) gene was determined in 2001 (83). This novel *mef*(E) containing chromosomal insertion element was designated as the macrolide efflux genetic assembly (mega). The mega element is 5.5- or 5.4Kb in size and the *mef*(E) gene was located on the 5'end. The element has five open reading frames (ORFs). The first two ORFs form an operon composed of *mef*(E) and a predicted adenosine triphosphate-binding cassette homologous to *msr*(A) and are co-transcribed. The region between *mef*(E) and *orf*2 designated as intergenic region was shown to vary in size from 119bp to 20bp. The mega element with the intergenic region of 119bp was further designated as Class I insert and the mega element with the 99bp deletion in the intergentic region was designated as Class II insert. The element inserts in more than four distinct locations in the pneumococcal chromosome with certain locations causing the deletion of the 99bp in the intergenic region (83).

Dual *mef*(E) and *msr*(D) *(mel)* Efflux Pump

It is now well established that the genetic elements that carry *mef*(A) and *mef*(E) in *S. pneumoniae* also carry an open reading frame that is homologous to *msr*(A) in *S. aureus.* *msr*(A) -like homolog in mega and in Tn*1207.1* was believed to be a part of the efflux system and its function was studied by Daly et al in 2004 (50). The *msr*(A) -like homolog was given a name *msr*(D), initially being called *mel* in mega. The *msr*(D), gene was shown to be always present whenever *mef*(A) or *mef*(E) was found. This gene was not found alone or in non-*mef*, macrolide-resistant strains. The function of *msr*(D), was studied in the absence of Mef efflux pump to evaluate the contribution of *msr*(D), to efflux of macrolides in *S. pneumoniae*. The *msr*(D), was cloned and inserted into macrolide-susceptible *S. pneumoniae.* Transformants exhibited the efflux phenotype as indicated by the 64-fold increase in macrolide MICs over those of the parents strain. The MICs of clindamycin, streptogramin and 16-membered macrolides remained unchanged. Based on this study *msr*(D), gene was shown to be sufficient to confer efflux phenotype in *S. pneumoniae*. To observe the differences between the three different efflux determinants, *mef*(A), *mef*(E) and *msr*(D), *mef*(A) and *mef*(E) genes were also cloned in the absence of *msr*(D). The transformants carrying the *mef*(E) and *mef*(A)genes exhibited the typical efflux phenotypes, with MICs similar to those for the donor strains. The transformants carrying the *msr*(D), gene exhibited efflux phenotype, but the macrolides MICs were lower than the MICs for the donor strain. This suggested that the *msr*(D), gene was not solely responsible for macrolide efflux in *S. pneumoniae* (50).

Recently a study by Ambrose et al. confirmed that macrolide efflux in *S. pneumoniae* is mediated by a dual efflux pump (*mef* and *mel*) (6). This was demonstrated by independent deletion mutants of both *mef*(E) and *mel* and by expression studies.

Independent deletion mutants of both *mef*(E) and *mel* demonstrated a significantly reduced MICs for macrolides compared to those of the parent strains; however, this reduction in MICs was even more pronounced when both *mef*(E) and *mel* were mutated. The expression of *mef*(E) in *mel* deletion mutants was increased more than 10-fold; however *mel* expression in *mef*(E) deletion mutants remained unchanged. Both Mef(E) and Mel efflux pumps appear to be necessary for macrolide resistance and are predicted to interact to drive the efflux of macrolides (6).

Differences in *mef*(E)- and *mef*(A)-Carrying *S. pneumoniae*

Although the two genes display high DNA homology and conserved role of the macrolide efflux in *S. pneumoniae*, a number of marked differences between *mef*(E) and *mef*(A)-carrying strains exist (55). In addition to the differences in the genetic element carrying the two genes and the differences in the dissemination of those elements among bacteria, other differences exist between bacteria carrying the *mef*(A) and *mef*(E) gene. It has been demonstrated that the majority of *mef*(A)-carrying *S. pneumoniae* are of serotype 14 and they appear by PFGE to be very similar. They belong to the internationally disseminated clone England^{14}-9. Unlike *mef*(A), *mef*(E) carrying isolates were shown to be of various serogroups; most common in decreasing order were 19, 6, 14, and 23 in one study and were shown to be unrelated by PFGE (55). In addition, susceptibility profiles of the *mef*(A)-carrying isolates are different from those carrying *mef*(E) The majority of *mef*(A)- carrying *S. pneumoniae* are resistant to 14 and 15-membered macrolides, such as erythromycin, clarithromycin, and azithromycin, but are susceptible to other antibiotics such as penicillin, tetracycline, and chloramphenicol. The majority of *mef*(E) carrying isolates are resistant to 14- and 15-membered macrolides as

well as to penicillin, tetracycline, and chloramphenicol. Interestingly, despite susceptibility to other drugs, *mef*(A) carrying isolates were shown to have higher MICs to 14- and 15-membered macrolides than isolates carrying the *mef*(E) gene (55).

Emergence of Dual *mef*(A) and *erm*(B) Resistance in *S. pneumoniae*

The majority of macrolide resistant *S. pneumoniae* isolates carry either the *mef*(A) gene class or the *erm*(B) gene. In the early 2000s, reports of isolates carrying both of these macrolide resistance genetic determinants were emerging (57, 66, 70, 72, 73, 105, 107, 121, 142, 193, 199). A 2001 publication from South Africa, described a prevalence of both *mef*(A) and *erm*(B) genes in 30.5% of macrolide resistant *S. pneumoniae* (142). The isolates harbouring both resistance genes showed high level resistance to macrolides and clindamycin, such as that described for the *erm*(B) genotype. In addition, all were multiply resistant, showing high-level penicillin resistance as well as resistance to chloramphenicol, tetracycline, and trimethoprim-sulfamethoxazole. The 36 isolates in this study were identified in four cities throughout South Africa, and were shown to belong to a single multiply resistant 19F clone closely related to a Taiwanese clone. At the time when these multiply resistant isolates were reported from South Africa, 3.3% of macrolide-resistant strains were reported from the US and were shown to carry both genes and were related to the same 19F multi-drug resistant clone. It was quickly realized that this new clone was circulating and contributing to the increases in macrolide resistance at least in South Africa, however hypotheses were being drawn that *S. pneumoniae* carrying both *erm*(B) and *mef*(A) might represent the emergence of a global clone. A 1999-2000 PROTEKT study of 1043 macrolide-resistant *S. pneumoniae* isolates from 25 countries identified the presence of dual *erm*(B) and *mef*(A) genotype

among macrolide resistant strains in Mexico (1.8%), Japan (3.3%), Hungary (3.3%), Canada (3.5%), USA (12.4%), and South Korea (38.3%) (72). Most were multi-drug resistant, demonstrating high-level resistance to penicillin G, tetracycline, the macrolides, clindamycin, and trimethoprim-sulfamethoxazole. In addition one of these isolates was fluoroquinolone (FQ) resistant and another was resistant to quinopristin-dalfopristin. Most of these dual strains belonged to serotype 19F (predominantly) or 19A (few), and a few were clonal complexes. All were shown to carry the mobile genetic element Tn*1545* and *mega* and were negative for Tn*1207.1*. Sequence analysis of *mef*(A) gene class confirmed *mef*(E) as the variant present along with the *msr*(A) gene. Tetracycline resistance was mediated by *tet*(M) gene. The mechanism of FQ resistance was not known. Molecular characteristics of the dual *mef*(A) and *erm*(B) strains from South Korea isolates from the PROTEKT study were published in a 2003 study and showed that 76% belonged to serogroup 19 and 68% were related by PFGE (72). Subsequently, the evolution of erythromycin resistant *S. pneumoniae* with both *erm*(B) and *mef*(A) was described in 2004 (121). It was suggested that the putative evolutionary pathway of erythromycin resistant clones containing both *erm*(B) and *mef*(A) genes involved the introduction of *erm*(B) gene into Taiwan 19F-14 *mef*(A) containing strain. The 2001-2002 PROTEKT US study described macrolide resistant *S. pneumoniae* with both *erm*(B) and *mef*(A) at a rate of 12.2% in the USA (69). The prevalence of the dual *mef*(A) and *erm*(B) strains varied from Arkansas and Rhode Island (0%) to South Dakota (32.9%). In 11 states, mostly North-central, dual *erm*(B) and *mef*(A) genotype was the second most prevalent after *mef*(A) resistance mechanism (69). The subsequent year 2002-2003 of PROTEKT US showed a further increase in the prevalence of *erm*(B) and *mef*(A) carrying *S. pneumoniae* to 16.4% (107). The marked increase from 9.7% (2000) to 16.4%

(2003) in the prevalence of dual *erm*(B) and *mef*(A) carrying *S. pneumoniae* in the USA coincided with a decrease from 68.8% (2000) to 63.9% (2003) in the prevalence of *mef*(A) (107). An interesting study looking at erythromycin- and clindamycin-resistant *S. pneumoniae* isolates from patients in tertiary care community hospitals in Ohio, was published in 2006 and it demonstrated that 71% of these typically *erm*(B) carrying isolates carried both *erm*(B) and *mef*(A) (193). Year five of the PROTEKT (2003-2004) study identified the high prevalence of multi-drug resistant *erm*(B) and *mef*(A) *S. pneumoniae* among macrolide resistant *S. pneumoniae* in Australia (21.6%), China (21.6%), South Africa (46.4%), South Korea (40.8%), USA (29.6%) and Venezuela (27.3%)(66). Canada was shown to have a low rate of 3.8% during this study. The prevalence of *erm*(B) and *mef*(A) isolates ranged from 0% to 17.6% for other countries. Furthermore, this study showed that the global prevalence of *erm*(B) and *mef*(A) genotype among patients 0 to 2 years of age was significantly higher than the frequency among patients aged 3-14, 15-64 and >64. The increase of *erm*(B) and *mef*(A) *S. pneumoniae* among patients 0 to 2 years of age increased from 35.5% (PROTEKT 2005) to 38.6% (PROTEKT 2006) (66, 106). Up until 2004, serotype 19A made up a small percentage of dual *erm*(B) and *mef*(A) carrying isolates, the majority were of serotype 19F. Other serotypes; 14, 23F, 6B, 34, 46 were also present in a very small number of isolates. However, by 2004, the 19A serotype *S. pneumoniae* containing both *erm*(B) and *mef*(A) macrolide resistance genes increased relative to 19F (71). In 2000, the ratio of serotype 19A to 19F was 7.8% to 86.7% and in 2004, was 45.5% to 51.7% of all dual *erm*(B) and *mef*(A) carrying *S. pneumoniae* (71). Year 6 (2005-2006) of the PROTEKT study showed similar results; 69.2% of isolates containing both *erm*(B) and *mef*(A) were serotype 19A and only 26.8% were serotype 19F. An interesting observation was made

regarding the susceptibility to amoxicillin, where overall 72.8% of erythromycin resistant *S. pneumoniae* and over 90% of isolates harbouring one gene were susceptible to this agent, the susceptibility rate decreased to less than 10% among isolates carrying both *erm*(B) and *mef*(A) .

Emergence of Macrolide Resistance

In vitro susceptibility testing of an organism to specific antimicrobials is performed by the microbiology laboratory. The organism is reported as susceptible (S), intermediate (I), or resistant (R) to a tested antimicrobial based on susceptibility breakpoints developed by the Clinical and Laboratory Standards Institute, CLSI. The isolate is considered S to erythromycin and clarithromycin when the MIC is ≤0.25μg/ml; I when the MIC is equal to 0.5μg/ml and R when ≥1.0μg/ml. The azithromycin breakpoints are S, ≤ 0.5μg/ml; I, 1μg/ml and R, ≥ 2μg/ml. Susceptibility and resistance to azithromycin, clarithromycin can be predicted by using erythromycin (40, 134).

Erythromycin was introduced into clinical practice in 1952 as the first macrolide. Bacterial resistance to erythromycin was initially reported in staphylococci in 1956, only a few years after its introduction into clinical practice. The first report of erythromycin resistant *S. pneumoniae* in the United States was in 1967, and subsequently was seen worldwide (58). As macrolides became the empiric treatment of choice during the 1990's because of concerns of β-lactam resistance and because macrolides were effective against the emerging atypical pathogens, macrolide resistance began to be noticed. The development of newer semi-synthetic macrolides, such as clarithromycin and azithromycin, resulted in much more widespread use of these agents.

As these advanced macrolides began to be used widely for the empiric treatment of community-acquired respiratory tract infections, microbiological surveillance programs, such as PROTECT, TRUST, SENTRY, and the Alexander Project began reporting a rise in macrolide resistance (68, 75, 76, 90). Macrolide resistance among strains of *S. pneumoniae* has escalated dramatically within the past decade worldwide, but the prevalence rates are highly variable among countries. The Alexander Project is an international surveillance program established in 1992 which tracks resistance rates from multiple laboratories in North and South America, Europe, Asia, South Africa, and Mexico (76). The PROTEKT, Prospective Resistant Organism Tracking and Epidemiology for the Ketolide Telithromycin study was initiated in 1999 (75). It was designed to evaluate the activity of telithromycin, a new ketolide antibacterial against *S. pneumoniae* and other common respiratory tract pathogens and to compare its activity with that of other antimicrobial agents. In addition, this study analysed mechanisms of antimicrobial resistance. It is an international study, including Asia, Latin America, Middle East, North America, Europe, South Africa and Australia. PROTEKT US was established 2000 as a sister project of PROTEKT and it focused on the United States (68). The SENTRY Antimicrobial Surveillance Program was established in 1997. It was designed to analyze the predominant pathogens present in the hospitals as well as in the community, and also to monitor their antimicrobial resistance patterns (90).

Trends of Macrolide Resistance in North America

Throughout the 1970s and 1980s, few cases of macrolide resistance in *S. pneumoniae* were described in the literature. In a Centre for Disease Control and Prevention (CDC) survey conducted between 1979 and 1986, only 0.3% of more than 5000 isolates of

pneumococci were resistant to macrolides (43). During the late 80s (1988-1989), macrolide resistance remained low, at 1-1.5% (134).

By the early 1990s macrolide resistance began to emerge. A large surveillance study during 1991 and 1992 cited rates of erythromycin resistance between 2.2%-3.7% among children older than four and younger than two, respectively. By 1992-1993, overall rates of resistance to erythromycin were at 3.5%. In a separate study during the same time, macrolide resistance of 5% was noted. By 1993-1994, macrolide resistance rates of 10.4%-11.5% were cited in the United States. During 1994, a survey from Metropolitan Atlanta, 15% of pneumococci studied showed resistance to macrolides. By 1999, the same study noted a resistance rate of 31% (134). Data from TRUST, Tracking Resistance in the United States Today, which surveys more over 400 medical centres in over 40 states, reported rates of macrolide resistance among *S. pneumoniae* of 18% in the 1996 to 1997 respiratory season (191). Rates from the same study a year later noted a resistance rate of 21% to 22% among pneumococci (192). Data from this and other US studies confirmed geographic variation in the range of 3.5% to 46.9% in the prevalence of pneumococcal erythromycin resistance across the many US states. The highest prevalence of erythromycin resistance exists in the South-central region and the lowest in the Southwest region.

Rates of macrolide resistance from SENTRY, another US study showed a resistance rate of 13% among respiratory isolates of *S. pneumoniae* in 1997 and 19% among all *S. pneumoniae* in 1999 (60, 61). Taking only respiratory isolates into consideration, the SENTRY study reported a macrolide resistance rate of 12% in the US (60). Overall in the US, macrolide resistant *S. pneumoniae* made up less than 7% of isolates during the early 1990s, however the prevalence of macrolide resistance in the US continued to

increase throughout the 1990s and early 2000s, rising to 10% by 1995, 26% by 2000 and over 30% by 2002. Erythromycin resistance then appeared to remain stable for a few years. Then PROTEKT 2005-2006 reports increases in macrolide resistance from 30% to 35%, indicating a first significant increase since the study began 2000 (106).

Macrolide resistance rates among pneumococci isolates from five centers participating in the Alexander Project during 1993 and 1996 study years increased from 2.2% to 13.9%.

A recent publication describing the US experience over 12 years (1998-2009) showed a decline in macrolide susceptible *S. pneumoniae* from 82.2% (1998) to 60.8% (2009) (109). At the same time a marked decline was observed for clindamycin susceptibility from 96.2% (1998) to 79.1% (2009).

In Canada, several surveillance studies from 1993-1998 found lower rates of macrolide since, ranging form 2.5% to 9.3% (214). The Canadian macrolide resistance rate in the SENTRY study during the 1997 and 1998 respiratory season was between 13% and 15% (108). A recent study indicated that macrolide resistance is approaching 20% (207).

Global Trends of Macrolide Resistance

Globally, macrolide resistance rates among strains of *S. pneumoniae* have escalated dramatically throughout the 1990s, but the prevalence rates are highly variable among countries. Reports from the Alexander Project, an international surveillance, in the late 1990s, showed an overall prevalence of erythromycin resistance in *S. pneumoniae* of around 16.5% (76). Among 10 European countries, 23% of pneumococci isolated exhibited macrolide resistance, but the variation among centres was great, ranging from 0% to 48%. France, Spain, Italy and Belgium showed the highest prevalence of macrolide resistance, while Germany, Czech Republic, Switzerland, and Austria had the

lowest prevalence of macrolide resistance (29, 76). During this study macrolide resistance increased significantly from 16.5-21.5% (76). In just one year, resistance to erythromycin increased from 41 to 46% in France, from 22% to 31% in Belgium, from 19% to 33% in Spain, and from 24% to 30% in Italy. Although prevalence of macrolide resistance was lower in Germany, a significant increase from 2.7% to 6.5% was observed. Similarly, an increase from 6.4 to 15.8% in Switzerland was noted. Rates of macrolide resistance at other centers in the Alexander Project were highly variable as well. In Hong Kong, a representative Asian country, macrolide resistance increased by 10% during the 1996 and 1997 study year and was highest worldwide at 78%. Resistance rates of 31.4% were noted in Mexico, 3.7% for Saudi Arabia, and 3.2% in Brazil.

A high level of macrolide resistance was reported for Japan (67%) and for China (73%) showing that globally, Asia is the region of highest macrolide resistance (100). Data from PROTEKT 1999-2004, further confirmed Asia as a region of high macrolide resistance which ranged from 77.6% in 1999 to 81.4% in 2004 in Japan(95).

A separate surveillance study of respiratory tract isolates of *S. pneumoniae* from 10 European Countries in 1994-1995 sited macrolide resistance rates ranging from 0% to 35%. France, Spain, Hungry, and Italy were identified as countries of highest macrolide resistance (>15%). In Spain, the incidence of macrolide resistance in one hospital in Barcelona, increased from 0% in 1979 to 9.4 % in 1990 and furthermore to 34% in 1997. Similarly, in Italy, erythromycin resistance among pneumococci rose from 6%-7% in 1993 to 23% and 33% in 1996 and 1997, respectively in two separate studies. In France, macrolide resistance was first detected in 1976 and it escalated dramatically during the 1980s. A nationwide study of more than 8000 isolates from 31 French hospitals documented a significant rise in macrolide resistance from 19% in 1984 to 26% in 1990.

27.5% of pneumococci were reported as macrolide resistant by 1992. By the late 1990s, 45% of *S. pneumoniae* isolates were resistant to macrolides. In Hungry, macrolide resistance was 49% in the 1980s. Nationwide studies in Europe indicate that by the mid 1990s, macrolide resistance rates exceeded 20% in Spain, Greece, Slovakia, Bulgaria, and Rumania.

A 1996-1997 study of 11 Asian countries noted highly variable rates of macrolide resistance, ranging from 3% in Malaysia to 89% in Taiwan. In Hong Kong, erythromycin resistance rose from 0% to 42.4% in 1993. During the same study, 37% of pneumococci in Seoul, Korea were noted to be erythromycin resistant. Macrolide resistance rates exceeded 66% during a 1997-1998 study surveying Japan and China.

In some countries, macrolide resistance remained uncommon or low during the late and early 1990s. Although the first multi-drug resistant *S. pneumoniae* was detected in South Africa in the 1970s, macrolide resistance has remained low, detected in only 2.3% of blood and CSF isolates during 1987 and 1990. Reports from the early 1990s still report a macrolide resistance rate of less than 3% in South Africa. In addition to South Africa, throughout the 1990s, among the countries known for low-level macrolide resistance were Denmark, Norway, Finland, Germany, Austria, England, Portugal and Israel. However, a 1992-1996 study from South Africa showed a macrolide resistance rate of 19.6%.

Trends in Macrolide Resistance Mechanisms

Globally, the most common mechanism of resistance among erythromycin resistant *S. pneumoniae* obtained during the 2001-2004 PROTEKT (Prospective Resistant Organism Tracking and Epidemiology for the Ketolide Telithromycin) study was *erm*(B), 55%,

followed by *mef*(A) gene class, 30% (66, 72, 74, 75). 12% of all erythromycin resistant *S. pneumoniae* carried both *erm*(B) and *mef*(A) genes. The distribution of resistance genotypes differed considerably between countries. Methylase mediated [*erm*(B)], resistance predominated among many European countries, whereas efflux [*mef*(A)], mediated resistance was the most common genotype among macrolide resistance isolates in North American countries (66, 72, 74, 75, 107). Belgium, France, Italy, Poland, and Spain were identified as European countries with predominating *erm*(B) genotype, ranging from 55.8% to 91.5% (66, 72). In North America, *mef*(A) genotype was present in 57.7% of Canadian macrolide resistant isolates, and 55.2% in American isolates. Greece and Germany were unique among the European countries as *mef*(A) was more common than *erm*(B) at 66.2%, and 56.5%, respectively (75, 170, 171). A recent study from Greece, which analyzed macrolide resistance rates and mechanisms in a 20-year period (1985-2004) identified *mef* genes as the predominant macrolide resistance determinant. (49). Furthermore, it was the *mef*(E) variant that was more common, with *mef*(A) variant being present in 25.6% of macrolide resistant isolates and observed for the first time in 1995 (49). In Germany, the most common macrolide resistance gene is *mef*(A) (27). All representative Asian countries in the PROTEKT study identified *erm*(B) as the predominating genotype (75). Dual e*rm*(B) and *mef*(A) isolates were particularly common in China (21.6%), South Africa (4.4%), South Korea (40.7%), and USA (29.6%). A 2002 publication describing the prevalence of erythromycin resistant genotypes in *S. pneumoniae* isolated between 1997 and 1999 in New Zealand showed that the most predominating was dual *mef*(E) and *erm*(B) genotype, present in 62.1% (22).

Efflux mediated resistance is due to two variants, *mef*(A) and *mef*(E) of the *mef*(A) gene class (174). The distribution of the two variants of the *mef*(A) gene class varies

temporally. In North America, *mef*(E) is the predominant gene encoding efflux pump in macrolide resistant *S. pneumoniae* (82, 83, 209). The initial study describing the differentiation of *mef*(A) genes class into *mef*(A) and *mef*(E) from Italy showed that the *mef*(A) variant predominates among macrolide resistant *S. pneumoniae* in that country (55). Other European studies that followed confirmed these initial results (13, 27, 28, 122, 153, 156). More recent data from Italy however, showed that although *mef*(A) still predominates at 55.6% there was an emergence of *mef*(E) variant, being present in 44.4% of macrolide resistant isolates (145). In Finland, *mef*(E) was cited as the most common efflux gene, present in 44% of macrolide resistant strains, while *mef*(A) was only present in 6% of isolates (166). The distribution of the two variants *mef*(A) and *mef*(E) was studied recently among M phenotype isolates in Spain and *mef*(E) variant as the predominating type being present at 90% of isolates, with *mef*(A) being present at 10% of isolates (12).

Risk Factors for Macrolide Resistance in *S. pneumoniae*

As the majority of initial antimicrobial therapy is empirical, it is of value for clinicians to be able to predict which patients are at increased risk for infection with resistant strains of *S. pneumoniae*. Many studies have been published describing the risk factors for macrolide resistance as being either personal or population based (134, 136). Personal risk factors include patients' overall health, extreme age; younger than 5 and older that 65, history of recent antibiotic use, history of recent hospitalization, residence in a chronic care facility or personal care home, residence with a child in a daycare facility, middle ear or sinus infection, underlying immuno-suppression, and multiple co-morbidities; chronic

obstructive pulmonary disease, cardiac disease, diabetes, cancers and renal disease (134, 136).

Population risk factors deal more with geography and drug usage within a specific region. The high-prevalence of macrolide resistance in a given geographical region increases the risk of having an infection with macrolide resistant *S. pneumoniae* and has been described as the main population risk factor (134, 136). This main population risk factor may be influenced by other factors. These include being insured or from affluent population, overuse or inappropriate use of macrolides, use of long acting-macrolides, low or sub inhibitory doses, and use of low potency macrolides. Some geographical regions are known to have higher levels of resistance than others. The geographical resistance trends may be driven by selective usage patterns. Several population based studies have noted correlations between the prevalence of macrolide resistance among *S. pneumoniae* and overall macrolide consumption in a given region or country (118). The relationship of antimicrobial resistance among *S. pneumoniae* to antimicrobial consumption was assessed by Granzio et al. and colleagues in Spain over 19 years (85).

A significant relationship was found between erythromycin resistance and macrolide consumption. The prevalence of high level penicillin resistance was also found to be predictive of erythromycin resistance (135). The erythromycin resistance was found to be linked to consumption of long-acting macrolides. Some suggest that macrolide resistance is more likely to develop with the longer-acting macrolides due to suboptimal pharmacodynamics. A recent study from Finland compared the regional macrolide resistance rates with the regional use of all macrolides and of azithromycin specifically (25, 118). This study confirmed the findings of others that there exists a positive connection between macrolide resistance in *S. pneumoniae* and total macrolide

consumption. A relationship between macrolide resistance and azithromycin use had previously been noticed in Spain, Germany, and Israel. The reasons why long acting macrolides, such as azithromycin have been proposed to select resistance more effectively than other macrolides have been explained using a model of selective window (118, 158, 197). This model describes that when an antimicrobial has a low maximum concentration (low C_{max}) and a long half-life ($t_{1/2}$ ~35-40h); there is a long selective window which is more likely to promote resistance than antimicrobials with a shorter selective window (118, 158, 197). It has been shown that azithromycin can persist in the infected tissues for several weeks at sub-inhibitory concentrations, therefore it has very long selection window (158, 197). It is known that prolonged exposure at sub-therapeutic levels is more likely to lead to resistance (118, 158, 197). There is also evidence that the use of less potent antibiotics might increase resistance as these agents are not able to eliminate low level resistant isolates, such as those conferred by the *mef*(A) gene organisms (118). Research has shown that azithromycin and erythromycin, which are less potent than clarithromycin are more likely to be associated with treatment failures due to low-level macrolide resistant *S. pneumoniae* (25). There is also evidence that the use of lower potency macrolides, especially azithromycin might increase the prevalence of multi-drug resistant *S. pneumoniae* as these multi-drug resistant clones might not be eradicated.

The greatest limitation of studies which attempt to show a relationship between antibiotic use and antibiotic resistance is that the consumption figures used are often based on the amount of antibiotics sold. The figures of antibiotics sold may not directly reflect the amount that is consumed by the patients, as they may fill the prescription but may never take the drugs. Another problem is that inventories of sold or filled prescriptions are often used to estimate the amount of antimicrobial use in a given area,

however it is possible that the drug is used by a person who lives outside the region where the drugs were purchased (35, 127, 132).

Clinical Significance of Macrolide Resistance

With the increasing worldwide frequency of *S. pneumoniae* resistant to macrolides, concerns have been raised with respect to the treatment guidelines for community-acquired infections which have advocated macrolides as first line therapy (51, 154). Questions arise as to whether the *in vitro* susceptibility results of macrolides against *S. pneumoniae* are relevant with respect to clinical efficacy and outcomes, and whether macrolides should continue to be the initial treatment of choice for pneumococcal infections. With the increasing trend of macrolide use and increasing macrolide resistance the question that needs to be addressed is whether these *in vitro* results correlate with a negative impact on clinical efficacy. There has been significant controversy concerning this question and arguments can be made for and against the relevance of *in vitro* macrolide resistance. Some authors have suggested that the current treatment guidelines that recommend macrolides for pneumococcal infections, particularly CAP, need to be re-evaluated, while other authors consider macrolide resistance to be of little clinical concern (43).

One of the main reasons behind the arguments against the relevance of *in vitro* macrolide resistance stems from the pharmacokinetic and pharmakodynamic properties of macrolides *in vivo* (43). Macrolides have a high degree of tissue penetration and accumulation in the infected pulmonary tissue, therefore many argue that the MIC breakpoints which are established based on serum levels greatly underestimate the concentration of the drug that is achieved at the site of pulmonary infection (43). An

isolate might be resistant to macrolides based on the current CLSI MIC breakpoints, but the levels of macrolide that reach the infection site might be higher than the MIC of the bacteria at that site therefore its eradication is still possible making the resistant phenotype based on the current definition not relevant (43). The favourable pharmacokinetic and pharmacodynamic properties of macrolides are supported by a paucity of studies describing that the current macrolide resistance trends are translating into clinical failures (43, 134). Some argue that these pharmacokinetic and pharmacodynamic properties hold true for low-level macrolide resistance, such as that conferred by the *mef*(A) carrying isolates with MICs in the range of 1-32µg/mL (134).

A recent study from Toronto which identified a macrolide treatment failure in 3.5% of 1696 episodes of pneumococcal bacteremia confirmed that macrolide treatment failures were more common in patients infected with resistant isolates displaying an macrolide MIC of ≥1µg/mL than in those infected with susceptible isolates displaying an MIC of 0.5≤µg/mL(52, 118), however, this study did not show an increasing treatment failure with increasing macrolide MICs; in fact it showed that the risk of treatment failure is constant through to highest MIC values (128µg/mL) once the MIC of ≥1µg/mL is reached. Therefore this study questions the favourable pharmacokinetics/pharmacodynamics of macrolides in the treatment of pneumococcal bacteremia caused by low-level macrolide resistant *S. pneumoniae* (52, 118). Relevance of *in vitro* low and high level macrolide resistance has also been described by Lonks et al. (130). The uptake of macrolides by white blood cells at the site of infection has been thought to contribute to additional extracellular release of the agents as well as exposure of organism to high intracellular concentration which further supports the irrelevance of macrolide resistance (8, 43).

There are several arguments in favour of the clinical relevance of *in vitro* macrolide resistance. It was recognized early by Austrian and Gold that even in the face of appropriate antimicrobial therapy, there was a high mortality rate due to pneumococcal infections (17). They reported a mortality rate of 13% in their study, during the time (1952 to 1962) when there was no resistance to antimicrobials (17, 26). Results from a more recent study (1995-1997) by Feikin et al. showed a mortality rate of 12% in patients living in an environment of 18% macrolide resistance rate (26). These authors believe that there is constancy in pneumococcal pneumonia mortality rates despite a rise in drug resistance; therefore they feel that macrolide resistance does not seem to be clinically relevant. It has been estimated that 10-15% of hospitalized patients will fail therapy despite appropriate antimicrobial regimen and no resistance (26). As there will always be anecdotal instances of treatment failure because of the intrinsic pathogenicity of *S. pneumonaie*, it is not so much macrolide or antibiotic resistance but *S. pneumoniae* infection itself that is clinically relevant.

Another important argument against the clinical relevance of macrolide resistance or drug resistance altogether is that many feel that the drug resistance might be overestimated due to the nature of many surveillance studies. In the US, many of the surveillance studies used to estimate antibiotic resistance are from centres which serve patients with poorer general health, patients of extreme ages, recent antibiotics use, recent hospitalization, residence in a home care centre, and immunocompromised status, therefore they are not reflective of patients treated in the community hospital or in the outpatients setting.

As mentioned before, macrolides became the empiric treatment of choice in light of increasing penicillin resistance among *S. pneumonaie*. However, *in vitro* laboratory data

suggests that macrolides do not provide adequate coverage of penicillin resistant pneumococci because macrolide resistance, especially high-level macrolide resistance (high MICs), is more common among such strains (43, 93). High-level macrolide, *erm*(B) mediated resistant strains have MICs typically higher than the macrolide concentration achievable at the site of infection and therefore make high-level macrolide resistance clinically relevant and likely to result in clinical failure (93).

Penicillin resistance is less common among low-level, *mef*(A) gene class mediated macrolide resistant isolates, and therefore they provide adequate coverage for those isolates (93). Pharmacokinetic studies have shown the macrolide concentration at the pulmonary site of infection is higher than the MIC of typical *mef*(A)-carrying *S. pneumoniae* therefore the clinical significance of these low-level macrolide-resistant isolates might be irrelevant. However, it has been observed that these low-level macrolide resistant strains are becoming more resistant (having higher MICs) and the required concentration to eradicate the bacteria at the site of infection might no longer be achievable (68, 207, 208).

Although macrolide resistance is higher among penicillin resistant *S. pneumoniae*, it is important to note that macrolide resistance is increasing independent of penicillin resistance as well. Aside from the mechanism of macrolide resistance which influences whether the pharmacokinetic and pharmacokinetic properties of the drugs are enough to overcome resistance, there is the issue of increasing use of macrolides (43). Since the introduction of new, longer acting macrolides, clarithromycin and azithromycin, macrolide use has been on the rise. In the United States, an increase of 320% was noted between 1993 and 1999 (93), in Canada a 30% increase was reported during 1995 and 1998 study(43, 91). It has been proposed that the use of these newer macrolides may be

the most significant factor associated with the increases in macrolide resistance (19, 43). From a clinical point of view, there have been cases of patients with breakthrough bacteraemia and clinical failures of patients with documented pneumonia while being treated with newer macrolides (78, 97, 113, 200). These clinical failures might have been due to development of high level macrolide resistance while being treated with newer macrolides.

In addition, there is the concern with the increasing description of concomitant *mef*(E) and *erm*(B) mediated resistance in *S. pneumoniae.* These isolates display the MLS_B phenotype and the macrolide MICs needed at the site of infection are not clinically achievable. Plus the increasing awareness of the non-typical macrolide resistance, mutations in the 23S rRNA, and ribosomal proteins, L4 and L22 often with high macrolide MICs support the clinical relevance of macrolide resistance.

Some authors argue that even taking the most optimal pharmacokinetic and pharmocodynamic parameters into consideration, the increasing prevalence of higher MICs for macrolide-resistant pneumococci with the M-phenotype, and the increasing global prevalence of the more highly resistant MLS_B phenotype *S. pneumoniae,* are sufficient cause for concern and are likely to be clinically relevant.

Part 3. Prevention and Vaccines

Prevention of pneumococcal disease is a public health concern because of the high impact of the disease and because of the increasing treatment problems due to antimicrobial resistance (32, 111, 116, 129). Early vaccines were crude whole-cell preparations (111). Shortly after the discovery of the antigenicity of the polysaccharide capsule, the first attempt at polysaccharide vaccines was made in the early 1940s (111).

However, the discovery of penicillin decreased the interest in further vaccine development and the introduction of the 6-valent polysaccharide vaccine was not met with great enthusiasm(111). By the 1960s, vaccines were again of interest as the mortality from pneumococcal disease was high despite appropriate antimicrobial therapy. The first commercial vaccine; 14-valent polysaccharide vaccine was introduced in 1977, and was replaced in 1983 by a vaccine that is still used today; the 23-valent polysaccharide vaccine (PPV-23) (Pneumovax II) and Pnu-Immune 23 (2002)). In addition to PPV-23, two other vaccines, 7-valent pneumococcal conjugate vaccine, PCV7 (Prevnar®) and 13-valent, PCV-13 are currently available in North America (111).

23-Valent Polysaccharide Vaccine (PPV-23)

The 23-valent polysaccharide vaccine, Pneumovax is manufactured by Merck & Co, Whitehouse Station, NJ. In the USA, it was licensed in 1977 and introduced into clinical practice in 1983 and it is still available today (111). Another PPV-23 vaccine, licensed in 1979 known as Pnu-Imune was manufactured by Wyeth until 2002 (111). It contained CPS from serotype 17A rather than 17F and had different dosage formulations, and used thimerosal as a preservative. Pneumovax vaccine contains 25µg of purified CPS from 23 serotypes and uses phenol as a preservative. The serotypes (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F,and 33F) in the vaccine were chosen to contain CPS from serotypes that are the most common cause of invasive pneumococcal disease (111). These serotypes have been estimated to cover over 90% of invasive pneumococcal disease in the USA (111).

The PPV-23 is recommended for all people age 65-years or older, adult cigarette smokers, and adults with chronic pulmonary diseases such as asthma, emphysema, and

chronic obstructive pulmonary disease. The vaccine is recommended for all residents of nursing homes or personal care homes to prevent the spread of IPD among institutionalised persons. This vaccine is indicated for people two-years or older who are at increased risk for pneumococcal disease (111). This includes patients with chronic illnesses: chronic heart, lung, kidney or liver disease; brain or spinal fluid leaks (CSF leaks); alcoholism and diabetes mellitus. Persons with a weakened immune system due to cancer, long term kidney failure, nephritic syndrome, organ or bone marrow transplantation, AIDS, chemotherapy or radiation therapy are also at higher risk. It is also recommended for people with splenic dysfunction or anatomic asplenia. PPV-23 is also recommended for children with these risks factors who are older than two, but younger than five, as a supplementary vaccine following the administration of PCV7. Pneumococcal polysaccharide vaccines have a number of limitations.

Although the pneumococcal polysaccharide vaccine is effective against invasive infections caused by pneumococcus in adult patients, immunization has not been protective in infants and young children (111). The reason for the lack of protection from IPD in these age groups is because polysaccharides are not sufficiently immunogenic. Therefore, the polysaccharide vaccine is not recommended for children younger than two years of age, and this has been the most important limitation of this vaccine as children in this age group experience the highest incidence of IPD. The pneumococcal polysaccharides are T-cell independent immunogens (thymus-independent type 2 antigens), therefore there is a poor antibody response (restricted to IgG), and poor immunological memory (poor generation of B cells) and failure to stimulate antibody response in children under the age of two, who do not have fully developed B-cell mediated immunity (111). Although it is recommended for adults and children over the

age of two with certain chronic and immunosuppressive conditions, the vaccine confers only limited protection against IPD to these patients. A second dose is recommended five years after the first dose for these persons. A further drawback of the polysaccharide vaccine is its failure to reduce mucosal carriage of *S. pneumoniae*, and to protect against mucosal infections, such as non-bacteremic pneumonia and limit the spread of resistant strains (111).

Pneumococcal Conjugate Vaccines

Immunogenicity of the polysaccharide vaccines has been enhanced by coupling polysaccharides to proteins (32, 111, 116). This led to the production of conjugate vaccines. This coupling of polysaccharides to a protein results in a switch from a thymus-independent to a thymus-dependent immune response. This way conjugate vaccine posses T cell dependent properties, such as immunogenicity in early infancy, stimulation of high-level IgG antibodies, and enhanced immunologic memory responses (32, 111, 116). Excellent results with the widespread use of *Haemophilus influenzae* type b (Hib) conjugate vaccines have encouraged manufacturers in the development of pneumococcal conjugate vaccines (116). Based on the experience obtained from Hib vaccines, pneumococcal conjugate vaccines were expected to be efficacious against invasive disease, reduce the mucosal carriage of pneumococcus, diminish the spread of resistant strains and reduce the burden caused by mucosal infections such as pneumonia and otitis media (116).

PCV7 or Prevnar® 7

A 7-valant protein-polysaccharide (PCV7) vaccine has been developed by Wyeth (subsidiary of Pfizer) for use in children. This first conjugate pneumococcal vaccine, known also as Prevnar®7 has been used in the United States since 2000. In Canada, the vaccine was approved in 2001, however was initially only available for private purchase. It was offered to high-risk children at the end of 2002 by government sponsored programs in some provinces (Quebec). By the fall of 2004, PCV7 was offered by most provinces through a broader government sponsored routine immunization program to all children under the age of two, and high risk children under the age of five. As with all vaccinations, the PCV7 vaccination schedule was mandated at a provincial level and the dose schedule varied slightly by province and depending on the age of the child at first dose. Typically, however, PCV7 is administered at three doses, eight weeks apart (2, 4, 6 months) followed by one booster dose at 12-15 months of age. The vaccine is indicated for the prevention of invasive pneumococcal disease in infants and children (2 to 23-months-old). Vaccination of high risk (same risks as for PPV23) older children aged 24 to 59-months may be beneficial and therefore indicated, however the antibody response may be lower due to compromised immune system in comparison to children without those risks. These children typically receive just one dose of PCV7.

The 7-valent vaccine contains CPSs from the seven most common serotypes (4,6B, 9V, 14, 18C, 19F, and 23F) causing invasive pneumococcal disease in this age group. It has been estimated that PCV7 covers 80% of serotypes causing IPD in this age group(111, 116). The serotypes contained in the PCV7 vaccine have also been traditionally associated with high antibiotic resistance. In addition, some serotypes included in the vaccine, like 6B and 19F, were thought to confer cross-protection to other serotypes

within the group. Therefore, the vaccine was thought to protect against serotype 6A and 19A as well and these strains were considered vaccine related.

The vaccine is manufactured as a liquid preparation for intramuscular injection. Each 0.5mL dose is formulated to contain 2µg of each 4, 9V, 14, 18C, 19F, and 23F and 4µg of 6B serotypes individually conjugated to diphtheria CRM_{197} protein (in total ~20µg of the carrier is used) and 0.125mg of aluminum phosphate as adjuvant. CRM_{197} is a nontoxic variant of diphtheria toxin isolated from cultures of *Corynebacterium diphtheriae* strain C7 (β197). One of the limitations of Prevnar®7, like all vaccinations, is that is may not elicit protection in all individuals. In addition to being at higher risk for acquiring IPD, vaccination in an immuno-compromised host may result in a lower antibody response compared to healthy individuals. More importantly, Prevnar®7 will not protect against *Streptococcus pneumoniae* serotypes that are not in the vaccine or serotypes unrelated to those in the vaccine. It will also not protect against other microorganisms. This vaccine does not treat active infection. In light of the newer PCV vaccine, PCV7 (Prevnar® 7) vaccination will most likely be replaced by its successor, PCV13 (Prevnar®13).

PCV13 (Prevnar® 13)

A new 13-valent protein conjugate vaccine has been developed by Wyeth (Pfizer) and licensed in early 2010 in the US, and subsequently in Canada. It is essentially the same vaccine as PCV7 except it helps protect against six additional strains of pneumococcal bacteria**.** The additional serotypes from which PCV13 helps protect are 1, 3, 5, 6A, 7F and 19A. Therefore, PCV13 is indicated for active immunization for the prevention of IPD caused by 13 strains of *S. pneumoniae* with the following serotypes: 1, 3, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F. Prevention of otitis media (ear infection due to 7

serotypes of *S. pneumoniae* (4, 6B, 9V, 14, 18C, 19F and 23F) is also an indication of PCV13 vaccination. No efficacy data for prevention of otitis media for new 6 serotypes present in PCV13 is available. PCV13 is recommended for children six-weeks to five-years old (before their sixth birthday). Typically PCV13 is administered in four doses at 2, 4, 6, and 12-15 months. Transition schedules from PCV7 to PCV13 have been published to guide physicians in terms of administration of PCV13 in children who have been started or completed the whole four dose series of PCV7. Children under two who were started on PCV7 should be switched to PCV13 on their next scheduled dose. Children who have completed their four doses of PCV7 and are under the age of six should receive a supplemental PCV13 dose at least eight weeks after the last PCV7 dose to protect them against the additional 6 serotypes that were not part of PCV7. The best efficacy of PCV13 is obtained with four doses, therefore the extent of protection with PCV13 in children in the above categories is not known.

Limitations of Prevnar® 13 are the same as those outlined for Prevnar®7, with the exception that it offers protection against more serotypes of *S. pneumoniae*. Based on clinical trials it is expected that the protection from otitis media will be lower than protection of IPD.

Consequence of PCV7 Vaccination

Reduction of PCV7 Serotypes, PCV7 Related Antibiotic Resistance and Disease Burden

The implementation of the 7-valent pneumococcal conjugate vaccination program has been a remarkable public health success story. Over the last 10 years, many studies have been published evaluating the burden of invasive pneumococcal disease as well as

serotypes and antibiotic susceptibilities among invasive isolates prior and post PCV7 implementation (23, 42, 45, 47, 84, 102, 114, 128, 161, 162, 169, 190). Many of the studies came to the same conclusion; the burden of invasive disease was significant and relatively constant prior to the introduction of PCV7 in 2000, and post vaccination there was a precipitous decrease in the incidence of invasive disease. Unexpectedly, PCV7 has not only been found remarkably effective in reducing invasive pneumococcal disease among vaccinated children but as well among unvaccinated children and adults. This unexpected effect of the vaccine on groups which were not targeted (older children and adults) by the vaccine was due to vaccine's ability to reduce the carriage rate of PCV7 vaccine serotypes in the nasopharynx of children. By reducing the carriage rate of pneumococci in vaccinated children, the pneumococcal transmission from young children to older children and adults was disrupted and resulted in herd immunity effect. All these studies showed that majority of isolates causing IPD prior to the implementation of PCV7 vaccine in 2000 were caused by the serotypes covered by the PCV7 vaccine, the vaccine serotypes (VS) and therefore it is not surprising that the subsequent reductions in VS were being noted following the implementation of the vaccination. The reduction of vaccine serotypes led to the reduction of invasive isolates and hence decreased the IPD burden.

Another unexpected effect of the PCV7 vaccination, although not to the same extent as on the IPD, was the observed decrease in frequency of VS otitis media and other non-invasive diseases. In addition, as majority of the VS were antibiotic resistant, the reduction of these antibiotic resistant serotypes indirectly reduced the prevalence of antibiotic resistance. The effect of the PCV7 was described in a recent study looking at changes in serotypes and antibiotics susceptibilities in invasive *S. pneumoniae* in Cleveland prior (1979-1999) and post PCV7 (2000-2004) vaccination (101, 102). The

overall incidence of invasive disease decreased 61% post PCV7. The decrease in incidence of invasive disease was higher in children (79%) than in adults (20%). Among children, the highest decrease of 84% was observed for children younger than five-years-old compared with a 23% decrease in children 5 to 18-years-old. A similar decrease of the incidence of IPD pre and post vaccination was seen among the two adult age groups; 19-65 and older than 65. Since the introduction of PCV7, an overall 76% decrease of isolates with vaccine serotypes was noted. The highest decrease (92%) was among children younger than five-years-old. Among the seven serotypes all but 9V decreased 59-95% overall, and 81-100% in children younger than five-years-old (101, 102). A follow up study by the same group looking at period 1999-2007 indicates a 27% decrease in adult invasive isolates and 69% decrease in pediatric invasive isolates consistent with their prior study. This translates to an overall decrease in pneumococcal isolates of 38.4% (reduction of pneumococcal disease). An overall decrease in vaccine serotypes of 92.6% was noted. There was a decrease of 93% and 99% among adult and pediatric invasive vaccine serotypes, respectively. In this followed up study, the authors evaluated the incidences of non-invasive isolates and reported a decrease in incidence of VS of 78.2% and 88.9% pre-and post-vaccination among adults and children, respectively, confirming the remarkable success of PCV7 not only on IPD but also on non-invasive isolates (101, 102).

These positive effects of the vaccine did not last, and three years after the introduction of vaccine, vaccine to non-vaccine serotype replacement and serotype switching, emergence of novel serotypes, plus increased antibiotic resistance and multidrug resistance, and novel diseases processes were being described. In addition, certain vaccine related

serotypes, those thought to be covered by the vaccine started to emerge and increase following the PCV7 implementation.

Increase or Emergence of Vaccine-Related (VR) 19A and 6C

When serotypes 6B and 19F were selected for inclusion in PCV7 vaccine, it was thought that antibody responses to structurally similar serotypes would be cross-reactive with polysaccharides from 6A and 19A (101). The cross-reactivity with serotype 19A has been very limited as documented by many studies by the emergence of this serotype following the PCV7 implantations. Although, in the Cleveland study described above, VR serotypes decreased 5% overall and 34% in children <5 years of age, serotype 19A increased 87% overall, and 91% in children <5 years of age (102). In the follow up study, the overall increase in the vaccine related serotypes among both adult and pediatric isolates has been solely attributed to the increase of single serotype 19A which alone increased over 400% post PCV7 implementation (101).

The VR serotype 6A decreased 72% in the first and 46% in the follow up Cleveland study post vaccination (101, 102). Other studies have shown similar results. However some studies have failed to show the expected reduction in the 6A serotype following the PCV7 introduction (152). The failure to observe the lack of reduction in the 6A serotype was mostly in studies which did not differentiate between the newly described 6C serotype which is cross-reactive with 6A (99, 152, 159, 160). In these studies, the decreased incidence of 6A serotype was countered in part by an increase or emergence of serotype 6C, resulting in a lack of change in the incidence of 6A serotype. In the Cleveland study, the authors note an emergence of serotype 6C in 2002 which doubled by 2007, however the numbers were still too low to affect the incidence rate of the 6A

serotype (101). Since the discovery of the 6C serotype, many studies are being published looking retrospectively at the presence of 6C serotype pre and post PCV7 to evaluate the impact of the vaccine on its prevalence. Many are looking at the 6A:6C ratios before and after the implementation of the vaccine, and it is evident that 6C serotype existed prior to PCV7 implementation, but at very low rates and it gradually become more prevalent after 2000 to rates that exceeded the rates of 6A in some studies by 2008(152). In one study, the incidence of 6A and 6C serotypes among invasive and non-invasive isolates of *S. pneumoniae* was 9.6% and 0.6% prior to 2001 and 8% and 2.2% in 2004 and 2.9% and 8.7% in 2007 respectively (152). This study clearly demonstrated that the 6C serotype made up the majority of previously not differentiated 6A serotype after the introduction of PCV7 vaccine. The emergence of the 6C serotype was observed among both invasive and non-invasive isolates, although many studies point out that 6C is more common among non-invasive adult and pediatric isolates than among those, which are invasive. Therefore, PCV7 introduction has led to the expected reduction in serotype 6A diseases but it appears to have been replaced by serotype 6C.

Emergence of Non-Vaccine (NV) Serotypes Following the Introduction of PCV7 Vaccination

During the 1979-2004 Cleveland study, the incidence of NV serotypes was shown to decrease 64% (102), however during the 1999-2007 follow-up study an increase of 18% in was noted (101), suggesting the affect of the PCV7 vaccination on the increase/emergence of NV serotypes. The increase in the NV serotypes was mostly attributed to increase in few serotype/serogroups. These included serogroup 15 and serotype 22F and 35B (101). Among the serogroup 15, serotype 15A increased the most,

with serotype 15C following closely and serotype 15B the least(101). In addition to these NV serotypes, studies from Europe (Portugal) describe the emergence of serotype 1 and 7F following the implementation of the PCV7 vaccine, stating that these serotypes along with 19A have become the leading causes of pediatric invasive pneumococcal infections (2). In addition serotype 3 has been on the rise in Europe.

Effect on the Antibiotic Resistance

Antibiotic susceptibility (macrolide, penicillin and clindamycin) has been affected by the introduction of PCV7 vaccination program. In the Cleveland 1979-2004 study, penicillin resistance rate of 36.3% in 2000 declined to 15% a few years following the implementation of PCV7, however by 2003 it was back to 33.3% and by 2004 it was 41% (102). Similarly, macrolide resistance initially declined from 38% to 19.2% from 2000 to 2002 and by 2003 it was as high as 50% (102). Along with increase in macrolide resistance, isolates became less susceptible to clindamycin(102). The increase in observed resistance was attributed to increased resistance among emerging NV or VR serotypes. A recent study evaluated the antibiotic susceptibly among NV serotypes and concluded that penicillin resistance rates increased from 12.7% to 16.1%; penicillin intermediate rates increased 20%-31.5%; erythromycin resistance increased from 21.2% to 31.6% post the introduction of vaccination among these isolates (71). In addition, multi-drug resistance (resistance to $\geq$2 antimicrobials) increased since the introduction of PCV7 in these isolates. Multidrug resistance (resistance to penicillin, amoxicillin, macrolide, clindamycin and T/S) was strongly associated with serotype 19F before 2000 and with serotype 19A after 2000. So the increase in the 19A serotype among non vaccine serotypes has contributed to an increase in not only the prevalence of macrolide and

penicillin resistance, but also to the prevalence of multi-drug resistance. A major finding of that study was the increase in the prevalence of antimicrobial resistance among NV serotype *S. pneumoniae*; resistance to penicillin, macrolides, T/S, amoxicillin-clavulanate, and multi-drug resistance all increased (71). This study evaluated the respiratory tract isolates, therefore the clinical significance of this increased resistance as it relates to IPD in children is difficult to determine as the authors point out.

Serotype Switch and Serotype Replacement as it Relates to 19A Serotype

Since the implementation of PCV7 there were speculations that any serotype specific vaccine that is of limited valency may affect the nasopharyngeal carriage of pneumococci, but it was not known with what consequences. Reports from the Centers for Disease Control and Prevention (CDC) which have been monitoring the invasive pneumococcal disease through its Active Bacterial Core (ABC) surveillance since 1995 indicate that vaccine-escape strains began to emerge in 2003 in the USA (21). Since the capsule is the principle invasive disease determinant and is the target of serotype-specific prevention of disease by vaccination, it is not surprising that PCV7 vaccination had an effect on the biology of the pneumococcal capsule. The two effects that have been described are serotype replacement and serotype switching (30, 195). Serotype replacement refers to a decrease in the prevalence of vaccine serotype pneumococci in the nasopharynx accompanied by a corresponding increase in non-vaccine serotype pneumococci. The replacements of vaccine serotype pneumococci by non-vaccine serotype pneumococci may not be a concern unless the non-vaccine serotypes are able to cause an invasive disease (30, 195). Serotype switching is the second major vaccine-related concern and it refers to an exchange of genes encoding one type of capsule via transformation and recombination for the genes encoding a different type of capsule.

Capsular switching between vaccine serotypes has been described a long time ago, but only recently has serotype switching from vaccine to non-vaccine serotype been described. This novel type of capsular switch is of particular concern as it contributes to serotype replacement and allows for the possibility of vaccine escape mutants with high levels of pathogenicity and antimicrobial resistance.

Serotype 19A has been the most commonly identified emerging, non-vaccine serotype since the introduction of PCV7. Its emergence was initially described in the US, but now is being reported in Europe as well (21, 30, 146, 163, 168, 175, 195). Post vaccination surveillance indicates that the invasive disease in children younger than five-years-old is now predominantly due to non-vaccine (or vaccine-related serotype) 19A. The increase in prevalence of 19A invasive disease was initially reported among Native Alaskan children. A great effort has been put into understanding the mechanisms driving its emergence. There are many hypotheses why serotype 19A emerged since PCV7 introduction. First is expansion of a single pre-existing clone of serotype 19A. Genetic characterisation of 19A strains has shown that a clone known as ST199 existed before vaccination and simply may have expanded to fill the ecological niche left by the vaccination. Secondly, more than one clone may have been introduced into the population. The third hypothesis is that a successful clone previously associated with a vaccine serotype may have undergone a recombinational switch to serotype 19A (21, 30, 146, 163, 168, 175, 195).

Emergence of New Disease Processes

Complications of Otitis Media

Mastoiditis

A variety of suppurative complications of acute otitis media comprise mastoiditis. These complications can be acute (periosteitis) or chronic (coalescent) and primarily affect children <2-years-old. With the advent of antibiotics, the incidence rate of mastoiditis has been low. *S. pneumoniae* is the most common cause of mastoiditis among children. Before PCV7, the predominant strain associated with these complications was serotype 19F, which accounted for 57% of the isolates in one study. Following the PCV7 implementation, an unexpected and concerning increase in the incidence of pneumococcal mastoiditis has been noted. In one study between 2000 and 2006, 15 cases of pneumococcal mastoiditis occurred and five were 19A (33%) (155). The following year 2006-2007, all 14 cases identified were serotype 19A (100%). In addition, to all 19A being multidrug resistant and majority being genetically related, the masteoiditis caused by 19A serotype were shown to be more complex, resulting in subperiosteal abscess which often required surgical intervention compared to the pre vaccine 19F mastoiditis. Although the incidence of otitis media has been affected by the implementation of PCV7 vaccine, a rise in pneumococcal mastoiditis mostly due to vaccine escape serotype 19A has been observed since 2000 (155).

Complications of IPD

Empyema

Empyema is a complication of pneumonia in pediatric patients, referred to as pediatric pneumococcal emypema (PPE). Following the introduction of PCV7 vaccine a dramatic increase in pediatric empyemas was also observed. In one study in Utah, more than one third of pediatric pneumonia was complicated by empyema and PPE is now the most common form of IPD (33). The majority of PPE are due to serotypes 1, 3, 7F, and 19A

(non-vaccine serotypes) and many cases occur in children older than the current recommend age for immunization. PPE was present prior to PCV7 vaccination and was consistently associated with serotype 1. Increased rates of PPE since the implementation of PCV7 vaccine have been associated with serotype 3, 7F, and 19A (33).

Hemolytic Uremic Syndrome (HUS)

Hemolytic uremic syndrome (HUS) is an uncommon but serious complication of IPD in children (24, 44, Bender, #401) HUS is a common cause of acute kidney injury in children and is associated with chronic liver morbidity. It is most commonly associated with infection by E. coli 0157. However, *S. pneumoniae* has been recognized as a common cause of nonenteropathic HUS in children. *S. pneumoniae* associated HUS (SP-HUS) has been shown to develop most commonly in individuals with empyemas(44). As the incidence of empyemas increased following the implementation of PCV7 vaccine so did the frequency of SP-HUS. Relatively little is known about the serotypes associated with SP-HUS but data in five cases from Atlanta prior to 2000 showed that all were PCV7 serotypes (14, 23F and 6B). In 2007, a report of SP-HUS from United Kingdom showed 19A as the predominant *S. pneumoniae* serotype (24). In Utah,post introduction of vaccine six out of seven cases were non-vaccine seroypes (1, 3,7F, and 22F) (24). Serotype 1, 3, and 7F was associated with complicated pneumonia and 22F with complicated meningitis. Serotype 3 was most strongly correlated with HUS as serotype 3 is most frequently identified in empyema cases (24).

Meningitis

Although the rate of IPD since the introduction of PCV7 vaccine has declined in children, a case of meningitis due to uncommon serotype has been recently (2009) reported in a young child. Non-vaccine serotype 13 was associated with this case. This case illustrates that non-vaccine serotypes remain a concern in IPD (4).

Emergence of Piliated *S. pneumoniae*

Recently, pili were described in Gram-positive bacteria, including *S. pneumoniae* mediating critical host-bacterial interactions, such as adherence to the epithelium and interaction with extracellular matrix proteins and increasing virulence (3, 18, 65, 96, 148, 149, 151, 167, 177, 178, 210). The first type of pilus described in *S. pneumoniae* now called type 1 pilus is encoded by a *rlrA* pathogenicity islet including genes for three structural proteins, *rrgABC*, three sortases, *srtBCD*, and a regulator, *rlrA*. It has been suggested that the type 1 pneumococcal pilus mediates host-bacterial interactions as an adhesion, a pro-inflammatory stimulus, and a virulence factor (167).

Prior to PCV7 vaccination, the prevalence of type 1 pilus among *S. pneumoniae* was approximately 25% and it was mostly in the vaccine serotypes (167). Initially there was a decrease in the prevalence of the type 1 piliated *S. pneumoniae* in direct correlation with the decrease of vaccine serotypes following the PCV7 implementation. However, in the years 2004-2007, type 1 piliated isolates re-emerged. The piliated *S. pneumoniae* mostly re-merged among non-vaccine serotypes, where its presence increased from 8.9% to 12% and to 30.1% in 2001, 2004, and 2007, respectively. The distribution of the piliated S. *pneumoniae* was found to be clonal and mostly in 19A serotype. Type 1 pili were also present among *S. pneumoniae* with 35B serotype, another non vaccine serotype that has emerged since the introduction of PCV7 vaccine (167). The second type of pilus in *S.*

pneumoniae is called pilus islet 2 or PI-2 (18, 210). It is smaller than the first, encoding 5 genes. PI-2 has been shown to be present in 3.6% of invasive *S. pneumoniae* in 1999, prior to the PCV7 vaccine implementation in a US study from Atlanta. However, after 2000, PI-2 started to emerge being present in 23% of invasive strains, 40% of those were of serotype19A and serotype 7F (210). Other non-vaccine serotypes that were associated with PI-2, although in small numbers in this study were serotype 1, and 11A.

B. Rationale of the Study

S. pneumoniae is an important pathogen implicated in respiratory tract infections, particularly community-acquired pneumonia. It is associated with high mortality and high morbidity. Macrolide antibiotics are among the first-line agents recommended for the empiric treatment of community-acquired pneumonia. In light of the recommendations, macrolides have been used extensively and this has resulted in increasing incidence of macrolide-resistant pneumococcal isolates. Macrolide resistance, particularly high-level resistance and multidrug resistance have complicated the treatment of pneumococcal infections. In Canada, the resistance to macrolide has been on the rise over the last decade. As macrolides continue to be used in Canada, it is likely to affect the resistance.

This points out to the rationale of the study. It is important to perform surveillance studies that monitor the resistance. It is also important to molecularly characterize resistant isolates in order to understand their mechanisms of resistance, genetic relatedness and spread. In light of increasing antibiotic resistance, limited number of novel antimicrobials being developed by pharmaceutical industry, and high mortality and

morbidity associated with pneumococcal infections, vaccination has become an important way of preventing pneumococcal infections. Therefore, in addition to monitoring the resistance and its mechanism, it is important to analyze the serotype distribution among *S. pneumoniae* in Canada to ensure efficacy of the current pneumococcal vaccines and to guide the development of new vaccine formulations.

A great deal of *S. pneumoniae* research looking at the mechanisms of macrolide-resistance, the genetic relatedness, and the serotyping has been done worldwide. This study is the first longitudinal study in Canada, which characterised and tied together the macrolide resistance mechanisms, the serotypes, and the genetic relatedness of clinical macrolide-resistant *S. pneumoniae*. Furthermore, this study is unique as it focused on clinical respiratory isolates and not on invasive isolates, which are described more commonly in the literature. This study described work not done before in Canada on the efflux containing isolates, the discrimination between the two variants A and E of the *mef*(A) gene and the *mef*(A)-E gene expression. In addition, this is the first study in Canada, looking at the prevalence of the virulence factors, Pili-type 1 and Pili-type 2 among macrolide-resistant *S. pneumoniae* and showing its presence among MDR isolates. Theses studies in addition to Canada having a low pre-existing macrolide resistance, low population density, restricted antibiotic use and implementation of vaccination program provided a great rationale for performing this work and are unique to Canada.

C. Hypothesis

The hypothesis of this research is that macrolide-resistant *S. pneumoniae* will growingly become more multi-drug resistant, genetically related, piliated, and consisting of serotypes not found in the PCV7 over the 10-year study period.

D. Thesis Objectives

The thesis objective was to molecularly characterize the clinical respiratory macrolide-resistant *S. pneumoniae* over a 10-year period. The macrolide-resistant *S. pneumoniae* were characterised for susceptibility to other agents, particularly penicillin, clindamycin, and trimethoprim/sulfamethoxazole, in order to determine a multi-drug resistance (MDR) phenotype. This was performed using a broth microdilution technique. The macrolide-resistant isolates were molecularly characterised for macrolide resistance mechanisms and for genetic relatedness. This was performed by PCR and by pulsed-field gel electrophoresis (PFGE), respectively. Further characterization involved serotyping of macrolide-resistant *S. pneumoniae* isolates and was performed using Quellung reaction. The serotyping data was used to assess the prevalence of serotypes found in the PCV7 vaccine, as well as to determine the emergence of serotypes not included in the PCV7 vaccine. This allowed studying the vaccine coverage overtime. Lastly, the molecular characterisation of the macrolide-resistant *S. pneumoniae* involved detection of virulence factors, Pili-type 1 and Pili-type 2 and was performed by PCR detecting the key regions in the gens encoding both pili.

In order to fulfil the objective of this research project isolates for this study had to be obtained from two studies. The reasons for doing that were strictly do with the fact that one of the studies ended in 2006. The two studies were designed with different objectives, one looking at mostly respiratory organisms, and the other at all organisms in Canadian hospitals. As such, both studies had different design in terms of isolate collection; however the way they provided the source of *S. pneumoniae* isolates was similar and therefore adequate for the use in this thesis.

E. Material and Methods

Bacterial Isolates

Lower respiratory tract (LRT) isolates of *S. pneumoniae* for this thesis were obtained from two studies, the Canadian Respiratory Organism Susceptibility Study (CROSS)(91) and the Canadian Ward study (CANWARD)(211). Both studies were conducted at the Department of Clinical Microbiology, Health Sciences Centre, and Winnipeg, Manitoba, Canada.

CROSS Study

The Cross study was initiated in September of 1997 and ended in December of 2006(91). Prior to 2003, the CROSS study year was from November 1st of one year to October 31st of the following year. Commencing in 2003, the CROSS study year was from January 1st to December 31st (followed the calendar year). CROSS included 25 health care centres in 9 out of 10 provinces. It was designed to assess the prevalence of antimicrobial resistance in key respiratory tract isolates such as, *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* in Canada. For *S. pneumoniae*, only lower respiratory tract isolates were accepted; outer and inner ear and eye isolates were not included in the study. The study also included respiratory tract *S. pyogenes* isolates (throat and nasal) and bacteremic *S. pneumoniae* isolates. Each year specific objectives were set out for collection of these isolates. Each site was asked to collect isolates: consecutive, one per person, identified as significant respiratory tract pathogens, not dependent on age. During 1998 and 2005, the goal was to obtain 100 *S. pneumoniae*, 50 *H. influenzae*, 20 *M.*

catarrhalis, and 20 *S. pyogenes*. In addition, all bacteremic *S. pneumoniae* were collected. Starting in 2006, CROSS only included *S. pneumoniae* and each site was asked to collect 100 lower respiratory tract isolates and all bacteremic isolates each year. Each centre was asked to record specific information about collected isolates: such as date of collection, patient's age, gender, specimen source, in/out patient status, β-lactamase result for *H. influenzae* and *M. catarrhalis* if available(91).

CANWARD Study

The CANWARD study is a national population based surveillance system, designed to assess the pathogens associated with respiratory, skin and soft tissue, urinary and bacteremic infections in Canadian hospital patients as well as to determine the prevalence of antimicrobial resistance in these pathogens (211). It was initiated in January 2007 and it is on going(211). Since its initiation 10 to 15 sentinel hospital sites in Canada participated in the study, representing 7 out of 10 Canadian provinces. The collection period was from January 1st to December 31st each year between 2007 and 2009 and from January 1st to November 30th in 2010. During each year of the study objectives were set out for collecting specific isolates and they differed slightly from year to year. The objectives were divided into: lower respiratory tract infections (LRT), wound infections, urinary infections, and blood infections. Each site was asked to identify the isolates using their own criteria and only send clinically significant isolates. CANWARD study excluded isolates from eye/ear/nose swabs, surveillance swabs, genital tract specimens, gastrointestinal specimens, anaerobes, fungi and yeast (with the exception of Candida spp. from blood in 2007). In 2007 and 2008, 200 and 150 consecutive isolates, respectively (100/75 (2007/2008) outpatient and 100/75(2007/2008) inpatient) from lower

respiratory tract infections were requested. During these two years, a minimum of 50 isolates, either from outpatients or inpatients of *S. pneumoniae* from lower respiratory tract were requested. If the target of 200/150 LRT isolates was met, however the minimum of 50 *S. pneumoniae* was not collected, the sites were asked to continue collecting only *S. pneumoniae* until the target of 50 for each site was achieved. In 2009 and 2010, each site was asked to collect 100 consecutive LRT isolates without the regard for whether they were from outpatients or inpatients as well as no minimum requirement for *S. pneumoniae* was set out. Each year of the study 50 isolates causing wound infections were requested from each site, with the first two years asking to provide 25 consecutive isolates from outpatients and 25 from inpatients and the last two years having no such requirement. In 2007 and 2008, 100 consecutive isolates causing urinary tract infections, 50 from outpatients and 50 from inpatients were part of the study objective from each site. In 2009 and 2010, 50 consecutive urinary isolates regardless of whether from outpatients or inpatients were requested. The final objective was for isolates causing blood infections. The collection of blood isolates differed in that there was a request to collect specific consecutive number of blood isolates monthly by each site instead of yearly. In 2007 and 2008, 30 and 20 isolates causing blood infections were requested from each site per month for a total of 360 and 240 per year per site of with a further request to have 15 (180 per site per year) and 10 (120 per site per year), respectively from outpatients and inpatients. In 2009 and 2010, 15 consecutive isolates regardless of whether from outpatient or inpatient per site per month for a total of 180 per site per year were requested. Each centre was asked to record specific patient information for each collected isolate. This information included the city, province, collection date, patient's age, patient's gender, in or out- patient information, specimen source and ward type(211).

Isolate Identification

The identity of each *S. pneumoniae* isolate received as part of the study was confirmed by Gram stain, colony morphology, α-hemolysis on Trypticase Soy Agar (TSA) plate supplemented with 5% sheep blood (SBA) and the results of Bile Solubility and Optochin Tests as recommended in the Manual of Clinical Microbiology (179). Following the identification, *S. pneumoniae* isolates were inoculated into skim milk and stored at -80^{o}C.

Bile Solubility

The bile solubility test was used to aid in the differentiation of *S. pneumoniae* from other α-hemolytic streptococci as they share similar colony morphology, Gram stain and hemolysis pattern on blood supplemented TSA plates which makes the identification based on growth characteristics often difficult (36, 138). The test utilizes bile salts (2% sodium deoxycholate), which indirectly induce lysis of the *S. pneumoniae* by activating pneumococcal enzyme autolysin which acts on the call wall of the pneumococcus.

Procedure

The test was carried out in 96 well plates. Bacterial suspension equivalent to 1 or 2 McFarland standard was made in 1.0 to 2.0 mL saline. Each isolate used two wells (ideally one below the other). To one well, 100µl of saline (control) was added and to the well below, 100µl of 2% deoxycholate (test) was added. Subsequently 100µl of the bacterial suspension was added to the control and test wells. The test was carried out at room temperature for 15 min to 2 hours; however a positive reaction was usually evident after 15min incubation. In cases where the clearing did not occur within the first 15 min, incubation at 37^{o}C was used. A positive test was indicated by clearing of a turbid

suspension in 2% deoxycholate (test) and lack of this clearing in the 0.85% saline (control) tube. The test was negative if the cells in the test well did not lyse (no clearing).

Optochin Test

Susceptibility to optochin (ethlyhydrocupreine hydrochloride) is a simple and reliable method of differentiating *Streptococcus pneumoniae* from other alpha-hemolytic streptococci (36, 138). The optochin test detects an organism's susceptibility to the chemical optochin. The chemical tests the fragility of the bacterial cell membrane and causes *S. pneumoniae* to lyse due to changes in surface tension. The optochin test is widely used in the form of filter paper discs, impregnated with 5 μg ethylhydrocupreine hydrochloride, which are applied directly to inoculated plates before incubation. Some "viridans" streptococci may produce a small zone of inhibition, ie <14mm. Occasional strains of optochin resistant *S. pneumoniae* have been reported. In cases where an alpha-hemolytic streptococcus is found to be resistant to optochin or produce a small zone, a bile solubility test should be carried out for confirmation.

Procedure

The test was carried out on a blood agar plate. First the plate was streaked with the isolate to be tested. Next the optochin disk was placed in the centre of the inoculum. Subsequently, the plates were incubated at 35°C to 37°C in CO_2 for 18-24 hours. Following the incubation the zones of inhibition were examined. A positive test was indicated by a zone of inhibition of ≥14mm in diameter indicating it is a *S. pneumoniae*. A negative test was indicated by a no zone of inhibition indicating that it was not a *S. pneumoniae*. Sometimes the zones of inhibition were < 14mm which complicated the interpretation.

Determination of MICs

Antimicrobial Preparation

Antimicrobials were obtained as laboratory grade powders from their respective manufactures. Antimicrobials were reconstituted and stored according to the CLSI Methods for Dilution and Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically, M7-A6 (38, 41). The activity of the antimicrobials was confirmed using *S. pneumoniae* ATCC® 49619, Staphylococcus aureus ATCC® 29213, and *Pseudomonas aeruginosaa* ATCC® 27853.

Broth Microdilution

Following two subcultures form frozen stock on SBA, the antimicrobial susceptibilities of the *S. pneumoniae* isolates were tested by the broth microdilution method according to the CLSI guidelines outlined in the Methods for Dilution and Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically, M7-A6 document (38, 41). The test was carried out in custom-designed and in-house made 96-well microtiter plates containing doubling antimicrobial dilution in 100µl of cation-adjusted Mueller-Hinton broth plus 2-5% vol/vol lysed horse blood.

Procedure

First bacterial suspensions were made in sterilized water and adjusted to a McFarland turbidity standard of 0.5, which is equivalent to 1-2 x 10^8 CFU/mL. Panels were inoculated to a final bacterial inoculum of 5 x 10^5 CFU/mL and incubated for 22- 24 hours at 35^{o}C in ambient air. The lowest concentration of an antimicrobial that completely inhibited visible growth was recorded as the MIC. The MICs of erythromycin-resistant *S. pneumoniae* were conducted in triplicate on separate days to

ensure reproducibility. Colony counts were performed to make sure the inoculums were in the correct concentrations.

E-Test

E-tests were performed according to the manufacturer's instructions (AB Biodisk, Solna, Sweden). Briefly, the procedure involved preparing a 0.5 McFarland standard for each isolate (sub-cultured twice from frozen stock) to be tested in Mueller Hinton broth and subsequently inoculating it onto Mueller Hinton agar containing 5% lysed sheep blood. The plates were allowed to dry (3-5 minutes, but no longer than 15 minutes) prior to the application of the E-test strip. The E-tests were applied to the inoculated surface of the plate aseptically and were incubated for 20-24 hours at 35^{o}C in 5% CO_2. Macrolide and clindamycin MICs were read at the point of intersection between the ellipse of growth inhibition and the MIC scale on the E-test strip. E-test was only used in cases where a discrepancy occurred between macrolide or/and clindamycin broth microdilution MIC value and genotype as an additional confirmatory test.

Disk Diffusion (Kirby Bauer method)

Disk diffusions were preformed according to CLSI M100-S20 guidelines for zone diameter and MIC interpretative standards for *S. pneumoniae* Table 2G(41). Briefly, the procedure involved preparing an inoculum of 0.5 McFarland by directly suspending bacterial colonies in saline. Cultures taken from the freezer were subcultured twice before performing Disk diffusion. Subsequently, this suspension was used to inoculate a Mueller Hinton plate supplemented with 5% sheep's blood. The plates were allowed to air dry (3-5 minutes, but no longer than 15 minutes) prior to the application of the disks.

15µg macrolide and 2µg clindamycin disks were applied aseptically and the plates were incubated for 20-24 hours at 35^{o}C in CO_2. Following the incubation the zones of inhibition were measured as judged by an unaided eye including the diameter of the disk. The isolates were recorded as resistant, intermediate or susceptible to macrolide and clindamycin by interpreting the zone of inhibition. An isolate was recorded susceptible to erythromycin, clarithromycin and azithromycin when the zone of inhibition was ≥21mm, ≥21mm, and ≥18mm; intermediate 16-20mm, 17-20mm, and 14-17mm; and resistant ≤15mm, ≤16mm, and ≤13mm in diameter, respectively (38, 41). An isolates was recorded as susceptible to clindamycin when the zone of inhibition was ≥19mm, intermediate 16-18mm, and resistant ≤15mm in diameter. Disk diffusion was only used in cases where discrepancy occurred between macrolide or/and clindamycin broth microdilution MIC and genotype as an additional confirmatory test.

D-Test

Macrolide resistant *S. pneumoniae* isolates may have constitutive or inducible resistance to lincosamides (clindamycin)(38, 39). Inducible clindamycin resistance was detected using the disk approximation test with a clindamycin disk placed 12mm away from an erythromycin disk, edge to edge, following the procedure described above for disk diffusion susceptibility testing. A bacterial suspension was prepared in sterile saline equivalent to a 0.5 McFarland standard. Subsequently, a Mueller Hinton agar plate supplemented with 5% sheep's blood was inoculated and allowed to dry prior to the application of the disks. A special template for the placement of the clindamycin and erythromycin disks was available to aid in correctly distancing the two disks. A plate was placed on a template which allowed for accurate 12mm spacing between the two disks.

2µg clindamycin and 15µg erythromycin impregnated disks were aseptically applied to the surface of the agar plate at the correct locations. The plates were incubated for 20- 24 hours at 35^{o}C in CO_2. After incubation the D-Test was interpreted by measuring the diameters of the zones of complete inhibition with a ruler according to the CLSI M100-S19 guidelines. Organisms that showed a flattening of the clindamycin zone adjacent to the erythromycin disk in the shape of the letter D (referred to as a "D zone") have inducible clindamycin resistance. All isolates which were resistant to macrolides but susceptible to clindamycin based on broth microdilution MICs and had an *erm*(B) gene mediated macrolide resistance as determined by PCR were screened for inducible clindamycin resistance. Organisms positive for the D-test (D zone positive) would be clinically recorded as clindamycin resistant even though they were susceptible based on broth microdilution.

DNA Isolation

S. pneumoniae isolates were grown overnight on SBA before the genomic DNA was isolated. The method used was that described by Ubukata *et al. (196)*. This method involved preparation of a bacterial suspension by emulsifying a small loopful of bacterial in a lysis buffer (below) and subsequent lysis by incubating the cell suspensions at 60^{o}C for 10min followed by a 5min incubation at 94^{o}C in a Perkin-Elmer GeneAmp® PCR System 9700.

***S. pneumoniae* Lysis Buffer/ per isolate**

1 M Tris-HCl (pH 9.0)	3 µl

Proteinase K (25 mg/ml)	0.24 µl
Tween 20	0.0675 µl
Nonidet P-40	0.0675 µl
10x PCR Buffer (15 mM $MgCl_2$)	3 µl
dH_2O	23.625 µl

Procedure

S. pneumoniae cultures were plated for overnight growth on blood agar plates. Subsequently, a small amount of growth was suspended in 30 µl of lysis buffer. Next, the suspension was transferred to a 0.2ml PCR tube and allowed to lyse in a Perkin-Elmer GeneAmp PCR system by incubating it at 60°C for 10 minutes and at 94°C for 5 minutes. The resultant lysates were used as DNA templates for all PCR reactions

Determination of Macrolide Resistance Determinants

Amplification of *mef*(A/E) and *erm*(B) macrolide resistance determinants was conducted by PCR using previously described method(182). The amplification reaction consisted of 10µL of 5X PCR buffer, 3µL of 25mM $MgCl_2$, 1µL of 10mM dNTPs, 1.0µL of each primer, 0.25µL of Taq DNA polymerase, 5µL of the DNA template, and sterile water to a final volume of 50µL according to the Promega usage information sheet. The thermocycler conditions are described below and the reactions were conducted with a Perkin-Elmer GeneAmp PCR system 9700.

Primer Sets

erm(B): 5’-GAAAAGGTACTCAACCAAATA-3’ [forward primer]

5’-AGTAACGGTACTTAAATTGTTTAC-3’ [reverse primer]

PCR product, 639 bp

mef(A/E): 5’-AGTATCATTAATCACTAGTGC-3’ [forward primer]

5’-TTCTTCTGGTACTAAAAGTGG-3’ [reverse primer]

PCR product, 348 bp

Positive Controls: *erm*(B) positive isolate #15359

mef(E)*/ mef*(A) positive isolate # 13353

50 µl PCR Reaction

Component	Final Volume	Final Concentration
5X GoTag® Flexi PCR buffer	10 µL	1X
$MgCl_2$ (25mM)	3 µL	1.5mM
dNTPs (10mM)	1 µL	0.2mM
Forward primer (100µg/ml)	1 µL	1.0 µM
Reverse primer (100µg/ml)	1 µL	1.0 µM
GoTaq® DNA Polymerase (5U/µL)	0.25 µL	1.25U
dH_2O	28.75µL	
DNA template	5 µ	

Thermal Cycling Parameters

Initial denaturation: 94^{o}C - 2 minutes

Denaturation: 94^{o}C - 1 minute

Annealing: 53°C - 1 minute

Extension: 72°C - 1 minute

Cycles: 25x-30x

Final extension: 72°C - 10 minutes

Agarose Gel electrophoresis

The amplified DNA fragments were visualized by electrophoresis in a 2% agarose gel made with 0.5X Tris-Borate-EDTA (TBE) and containing ethidium bromide. Gels were run for 45min at 110V/cm and bands were visualized under UV transilluminator. For PCR reactions containing the 5X Green GoTag® Flexi Buffer, the PCR products were leaded into the gel directly after amplification without the addition of tracking dye. A 123bp ladder was used as a molecular weight standard.

Discrimination of *mef*(A) Gene Class into *mef*(E) and *mef*(A)

PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)

To discriminate between A and E subclasses of the *mef*(A) gene PCR-RFLP analysis was performed using a previously described protocol(55). DNA lysates were prepared as described above, a 1743bp DNA fragment was amplified by PCR using two primers, *mef*-3 (5'-GCGTTTAAGATAAGCTGGCA-3') and *mef*-4 (CCTGCACCATTTGCTCCTAC-3') using slightly adapted method shown below. The amplified DNA fragments were visualized by electrophoresis as described above. 1kb ladder was used as a molecular weight marker.

The amplicon was then digested with *Bam*HI or the *Dra*I restriction enzyme. Restriction digestion was carried out as described in the usage information sheet from Amersham Pharmacia Biotech for 2 hours at 37^{o}C in a total volume if 20µL containing 10µL of the PCR product, 2uL of 10X restriction buffer and 5U of enzyme. The digestion products were run on a 2% agarose gel containing ethidium bromide and visualized under the UV transilluminator.

In *mef*(A) there is one *Bam*HI site, so the restriction generates two fragments of 1,340bp and 403bp, while in *mef*(E) there are no *Bam*HI restriction sites. Restriction of *mef*(A) with *Dra*I yields two fragments of 1,493bp and 250bp, while restriction of *mef*(E) yields three fragments of 782, 711, and 250bp respectively. The amplified DNA fragments were visualized by electrophoresis as described above. 1kb ladder was used as a molecular weight marker(55).

50 µl PCR Reaction

Component	Final Volume	Final Concentration
5X GoTag® Flexi PCR buffer	10 µL	1X
$MgCl_2$ (25mM)	3 µL	1.5mM
dNTPs (10mM)	1 µL	0.2mM
mef-3 primer (100µg/ml)	1 µL	1.0 µM
mef-4 primer (100µg/ml)	1 µL	1.0 µM
GoTaq® DNA Polymerase (5U/µL)	0.25 µL	1.25U
dH_2O	28.75µL	
DNA template	5 µL	

Thermal Cycling Parameters

Initial denaturation:	94°C - 2 minutes
Denaturation:	94°C - 1 minute
Annealing:	57°C - 1 minute
Extension:	72°C - 1 minute
Cycles:	25x-30x
Final extension:	72°C - 10 minutes

Mulitplex PCR for Discrimination Between *mef*(E), *mef*(A) Subclasses of the *mef*(A) Gene Along with Amplification of *erm*(B)

Multiplex PCR assay was developed to detect *erm*(B), *mef*(E), and *mef*(A) gene simultaneously reducing the need for PCR-RFLP analysis (144, 157). The amplification reaction consisted of 10µL of 5X PCR buffer, 8µL of 25mM $MgCl_2$, 1µL of 10mM dNTPs, 0.2- 0.5 .0µL of primers, 0.25µL of Taq DNA polymerase, 5µL of the DNA template, and sterile water to a final volume of 50µL according to the Promega usage information sheet. The thermocycler conditions are described below and the reactions were conducted with a Perkin-Elmer GeneAmp PCR system 9700. All four positive controls were run each time along with water and a negative (macrolide-susceptible control). The amplified DNA fragments were visualized by electrophoresis in a 2% agarose gel made with 0.5X Tris-Borate-EDTA (TBE) and containing ethidium bromide. Gels were run for 45min at 110V/cm and bands were visualized under UV transilluminator. For PCR reactions containing the 5X Green GoTag® Flexi Buffer, the

PCR products were leaded into the gel directly after amplification without the addition of tracking dye. A 123bp ladder was used as a molecular weight standard. Controls were run at the same time.

Primers

EB1 5'-GAA AAA GTA CTC AAC CAA ATA-3'

EB2 5'-AGT AAT GGT ACT TAA ATT GTT TAC-3'

OM10 5'-AGC ATT GGA ACA GCT TTT CA-3'

mef(A) 5'-ATT TTG CCG TAG TAC AGC C-3'

mef(E) 5'-TAC ATG CTT TTC GAA GCC-3'

PCR reaction final volume -50µl

Component	Final Volume	Final Concentration
5X GoTag® Flexi PCR buffer	10 µL	1X
$MgCl_2$ (25mM)	8 µL	4.5mM
dNTPs (10mM)	1 µL	0.2mM
EB1 and EB2 primers (100µg/ml)	0.5 µL each	0.5 µM
OM10, *mef*(E) and *mef*(A) primers (100µg/ml)	0.25 µL each	0.25 µM
GoTaq® DNA Polymerase (5U/µL)	0.25 µL	1.25U
dH_2O	24µL	
DNA template	5µl	

Expected PCR product size:

erm(B) – 639bp

mef(E) – 519bp

mef(A) – 318bp

Positive controls *erm*(B)-positive isolate #3361

mef(E)-positive isolate #18404

mef(A)- positive isolate #12300

erm(B)/*mef*(E) double positive isolate # 28086

Thermal Cycling Conditions

Initial denaturation:	94^{o}C - 2 minutes
Denaturation:	94^{o}C - 1 minute
Annealing:	50^{o}C - 1 minute
Extension:	72^{o}C - 1 minute
Cycles:	25x-30x
Final extension:	72^{o}C - 10 minutes

Detection of Macrolide Resistance Mechanism in Isolates Negative for *erm*(B) and *mef*(A) Genetic Determinants

Amplification of the 23S rRNA and ribosomal proteins

The DNA was extracted as described above. Amplification of the four *S. pneumoniae* 23S rRNA alleles was performed using a previously published method (67, 187). The key regions within the 23S rRNA, both domain II and domain V, as well as the ribosomal

proteins L4 and L22 were amplified by PCR using primers shown below(34). The amplification reaction contained 23µl of Platinum® PCR SuperMix High Fidelity (Invitrogen by life technologies) 0.5µL of each primer [10nM], (synthesized by DNA Core facility, National Microbiology Laboratory) and 1µL of DNA template to a final volume of 25µL. The amplified DNA fragments were visualized by agarose gel electrophoresis as described before.

Primers

***rrl* (23S rRNA domain II) product size -273bp**

forward 5'- CGGCGAGTTACGATTATGATGC-3'

reverse 5'- CTCTAATGTCGACGCTAGCC-3'

***rrl* (23S rRNA domain V)**

region 1 product size- 144bp

forward 5'- CTGTCTCAACGAGAGACTC-3'

reverse 5'- CTTAGAACTCCTACCTATCC-3'

region 2 product size- 439bp

forward 5'- GTATAAGGGAGCTTGACTG-3'

reverse 5'- GGGTTTCACACTTAGATG-3'

***rplD* (L4) product size- 720bp**

forward 5'- AAATCAGCAGTTAAAGCTGG-3'

reverse 3'- GAGCTTTCAGTGATGACAGG-3'

***rplV* (L22) product size - 420bp**

forward 5'- GCAGACGACAAGAAAACACG-3'

reverse 5'- GCCGACGACGCATACCAATTG-3'

Thermal Cycling Conditions

Initial denaturising:	94°C - 3 minutes
Denaturation:	94°C - 0.5 minute (30 sec)
Annealing:	51-61°C - 0.5 minute (30 sec)
rrl domain region 2	51°C
*rpl*D	54°C
rrl domainV region 1	57°C
rrl domainII	59 °C
*rpl*V	61°C
Extension:	72°C - 0.5 minute (30 sec)
Cycles:	30x
Final extension:	72°C - 10 minutes

Purification and Quantification of DNA Template for Sequencing

PCR products for the genes encoding the 23S rRNA and ribosomal proteins were purified using QIA quick spin column PCR purification kit (Qiagen, Mississauga, ON, Canada) according to the instructions provided by the manufacturer. The QIA quick system uses a simple bind-wash-elute procedure. Binding buffer was added directly to the PCR sample and the mixture was applied to the QIA quick spin column. The binding buffer contains a pH indicator, allowing easy determination of the optimal pH for DNA binding. Nucleic acids adsorb to the silica-gel membrane in the high-salt conditions provided by the buffer. Impurities were washed away and pure DNA was eluted with a

small volume of low-salt buffer provided or water, ready to use in all subsequent applications such as sequencing. Quantification of the DNA in the purified PCR samples was performed with a NanoDrop 2000 micro volume spectrophotometer (Thermo Scientific), which allows the use small volumes (1μL) and calculates concentration and purity of the DNA sample.

Sequencing Reaction

Sequencing reactions were performed by the National Microbiology Laboratory (NML) DNA Core facility using ABI PRISM BigDye Terminator Cycle Sequencing Ready reaction Kit (Applied Biosystems, Foster City, CA). The thermocycler conditions are described below and the reactions were conducted with a Perkin-Elmer GeneAmp PCR system 9700. The DNA facility was provided with 100ng of purified PCR product for each sequencing reaction. Sequencing of the 23S rRNA regions and L4 and L22 ribosomal proteins was carried out in forward and reverse directions using the same primers just at lower concentration [1nM] as for the initial PCR.

Typical Sequencing Reaction

Premix 4μL

Primer [1nM] 1.6μL

dH_2O QS to 10μL

Template 100ng (volume variable depending on the sample)

Thermo cycler conditions

96^{o}C 10 seconds

50^{o}C 5 seconds

60^{o}C 4 minutes

4^{o}C - ∞

Sequencing Product Purification by Ethanol/Sodium Acetate Precipitation

Following the sequencing reaction the sequencing products were purified by ethanol/sodium acetate as recommended in the ABI PRISM BigDye Terminator Cycle Sequencing Ready Kit information sheet (Applied Biosystems, Foster City, CA). Purified sequencing products were reconstituted in 15µL of Template Suppression Reagent for analysis on the DNA Analyzer 3730X (Applied Biosystems, Foster City, CA, U.S.A.) as recommended by Applied Biosystems.

Sequence Analysis

Sequence analysis was conducted on the DNA Analyzer 3730X by the DNA care facility at the NML.

Sequence Alignment

DNA sequences of both strands obtained by the DNA Analyzer 3730X were edited, assembled, and aligned with published sequences obtained from all strains using software, Laser Gene from DNAStar, Inc. (Madison, WI, U.S.A.)

Pulsed Field Gel Electrophoresis (PFGE)

The genetic relatedness of *S. pneumoniae* was studied by PFGE adapted from Louie *et al*. 1999. J. Infect. Dis. 179:892-900 and McEllistrem *et al*. 2000. J. Clin. Microbiol. 38:351-353 (131, 141).First step to PFGE was preparation of the genomic DNA, followed by restriction digestion, and subsequently electrophoresis.

Plug preparation

Each *S. pneumoniae* isolate already grown once was subcultured onto two plates of TSA supplemented with 5% sheep's blood and grown overnight. Using a loop, the colonies from both plates were suspended in 2 ml of cell suspension buffer (10 mM Tris-HCl [pH 7.2], 20 mM NaCl, 50 mM EDTA [pH 8.0]) to an optical density of 2.4-2.5 (disposable plastic cuvette) at 560 nm. After the optimal optical density was achieved, 100µl of the adjusted bacterial suspension was combined with 100µl of of 1.6% low-melt agarose (InCert® agarose; FMC BioProducts, Rockland, ME). Next the suspension was mixed by pipetting up and down several times. Immediately after the mixture was dispensed into disposable plug molds (Bio-Rad Laboratories, Hercules, CA) (approximately 100 µl per plug) and plugs were allowed to solidify for approximately 10-15 minutes at room temperature or 5 minutes at 4°C. the plugs were next transfered into a 1.5 ml centrifuge tube containing 1 ml of lysis solution (10 mM Tris-HCl [pH 7.2], 50 mM NaCl, 50 mM EDTA [pH 8.0], 0.5% Brij-58, 0.2% deoxycholate, 0.5% sarcosyl, 1 mg/ml of lysozyme, 20 µg/ml of RNase) and incubated in a 37°C water bath for a minimum of 1 hour. The lysis solution was aspirated and 1 ml of ESP solution (250 mM EDTA [pH 9.0], 1% sarcosyl, 200 µg/ml Proteinase K) was added and allowed to incubate in a 50°C water bath for a minimum of 1 hour to overnight. Again the ESP

solution was aspirated or poured off leaving transparent very fragile plugs. After this the plugs were rinsed with 1 ml of TE buffer (10 mM Tris-HCl [pH 7.5], 0.1 mM EDTA [pH 7.5]) then washed at least 4 additional times with 1 ml of TE buffer for a minimum of 30 minutes each wash. All washes were conducted at 37°C. Following the final wash, the plugs were stored in 1 ml of fresh TE buffer at 4°C for up to six months.

Restriction Endonuclease Digestion

The restriction enzyme / buffer mixture was prepared using 135µl of sterile distilled water, 15µl of 10X NEBuffer 4 (New England Biolabs, Mississauga, ON) and 25U (1.25 µl of 20000U/ml stock) of *Sma*I enzyme. 150ul was used per isolate. To this ½ of the plug was added and were allowed to digest at room temperature for a minimum of 2 hours.

Electrophoresis

2000 ml of 0.5X TBE buffer was prepared by diluting 10X TBE with distilled water. 1% Seakem® Gold (FMC BioProducts, Rockland, ME) agarose gel was prepared in this 0.5X TBE (100 ml for short gel, 150 ml for long gel). The gel was allowed to solidify at room temp for 1 hour. The 0.5X TBE buffer was used in the electrophoresis chamber. After digestion was completed the enzyme/buffer mixture was aspirated and the plugs were melted at 65-69°C for 20 minutes. The 30µl of melted plugs were loaded in the well of the agarose gel and allowed to solidify at room temp for 5 min. Lambda ladder was used as a molecular weight marker. The restriction fragments were resolved in a contour-clamped homogeneous electric field apparatus (CHEF DRIII; Bio-Rad Laboratories, Hercules, CA) with the following parameters: initial switch time 2 seconds, final switch time 30 seconds, Voltage 200 V, 6 V/cm, included angle 120, and run time of 18.5

hours. Following the run the gel was stained with SYBR® Green (Molecular Probes, Eugene, OR) and visulazed under UV light.

PFGE Pattern Analysis

PFGE profiles were scanned and digitized with the Gel Doc 1000 System (BioRad Laboratories, Hercules, CA, U.S.A.) and analyzed using BioNumerics TM (Applied Maths, Austin Texas, U.S.A.). Dendrograms were calculated by the unweighted pair group method with arithmetic average (Band Tolerance: 1% and Dice Coefficient 1%). Isolates were defined as genetically indistinguishable, related, or genetically unrelated if their PFGE profiles differed by 0, 1-3, or ≥4 bands, respectively.

Serotyping

Quellung Reaction

S. pneumoniae isolates were serotyped in-house based on capsular polysaccharide antigens by the Quellung reaction following standard methodology described by Austrian R et al. in 1976 (16). Briefly, the method involves testing each strain with Pool sera A through I until a positive reaction is obtained. Subsequently, the strain was tested with the Type and Group sera for a known Pool, until a positive reactions obtained. Strains belonging to a Group were further tested with factors sera to identify the Type. Type-specific antisera were obtained from the Statens Serum Institute (Copenhagen, Denmark).

Procedure

First a bacterial suspension was made from a ¼ plate fresh overnight subculture of *S. pneumoniae* in saline or PBS buffer. Next 2- 3µL spots of the bacterial suspension

were pipeted onto a glass slide. Using a sterile technique 3µL of antisera from a chosen Pool was pipeted onto one of the bacterial suspension spots on the glass slide and mixed well. The most frequently occurring serotypes based on experience indicated what pool order was tested. Coverslip was placed on each spots and the slides were examined with a phase contrast microscope under oil immersion. Positive reaction was characterized by a "hairline" demarcation outlining the capsule. Positive reaction is due to in situ immuno-precipitation which leads to a change in the refractory index, and the capsule may appear beige or gray-white in colour. A negative reaction did not show defined capsulation.

Molecular Serotyping

Molecular serotyping is a PCR based method for identifying a capsular serotype in *S. pneumoniae*. It was performed based on the CDC protocol http://www.cdc.gov/ncidod/biotech/files/pcr-US-clinical-specimens-March2010.pdf (36). All the primer concentrations and conditions used were as outlined in the protocol. The method involved subjecting each isolate to be tested to 8 different multi- PCR reactions. All reactions contained an internal control designed to amplify the capsular polysaccharide gene, *cps*A and primers for 5 different serotypes. In addition there was a separate reaction called reaction 6C which is designed to differentiate between 6A and 6C serotype. All isolates which tested positive for serogroup 6 in reaction 1 were subjected to reaction 6C to further differentiate into 6A or 6C.

Analysis of PCV-7 and PCV13 Vaccine Coverage

Vaccine coverage was assessed by grouping pneumococcal serotypes into three groups as follows: PCV7 serotypes - 4, 6B, 9V, 14, 18C, 19F and 23F, PCV7-Related - 6A, 9A,

9L, 9N, 18A, 18F, 19B, 19C, 23A, AND 23B, Non-PCV7- all other serotypes plus 19A, PCV13 serotypes- PCV7 + 1, 3, 5, 6A, 19A and 7F, PCV13-Related - 9A, 9L, 9N, 18A, 18F, 19B, 19C, 23A, AND 23B, Non-PCV13 -all other serotypes.

Pili- type 1 (PI-1) and pili type 2 (PI-2) Determination

The DNA templates were prepared using the crude method described above.

The detection of PI-1 and PI- 2 in *S. pneumoniae* was carried out by PCR using described methodology and primers (210). The presence or absence of PI-1 and PI-2 was ascertained by performing two PCR reactions, one designed at the flanking genes Rlr_up and Rlr_dn, pepT and hemH, respectively and the other at PI-1 and PI-2 specific genes, Rlr_SrtC and Rlr_SrtD and sipA_up and sipA_dn, respectively.

Primer Sets

PI-1 Absence

Rlr_up_F (Forward) 5' CTTCCACGAAGTTCTTTCAATGG3'

Rlr_do_R (Reverse) 5' GTCTTAGAATATCATGGTTTACGTGC 3'

PI-2 Absence

pepT_F (Forward) 5' TAAGAAGCGGTCCAAGAGATTTGG 3'

hemH_R (Reverse) 5' AATAATGGGGCTCCAAAATCAAGC 3'

These primers are designed against the flanking genes of the PI-1 and PI-2; therefore the primers detect the absence of the PI-1 (band). The PI-1 is a 14 kb and PI-2 is a 7- kb mobile genetic element that when inserted is too big the give a PCR product (no band).

PI-1 Presence

Rlr_srtC_F (Forward) 5’ GGGGAAGATTATGCGACCTT 3’

Rlr_srtD_R (Reverse) 3’ GCTTGGCTCTGCACGGTGCC 3’

PI-2 Presence

sipA_up_F (Forward) 5’ CTCTAGGAGGGATCTTCTTTATCATC 3’

sipA_do_R (Reverse) 3’ CTACAGCCGTGTTCGATTGTCC 3’

These primers are designed against a *srtC/D* (sortase) gene within the PI-1 therefore always present when the PI-1 is present.

Two PCR tests were performed together on each strain. For all strains one of the two PCR resulted in the PCR product.

PI-1 Detection

50 µl PCR Reaction

DNA template	**5 µl**
Master Mix	
5X PCR buffer (Promega)	10 µl
$MgCl_2$ (25mM) *	3-6 µl
dNTPs (10mM Promega)	1 µl
Forward primer (100µg/ml)	1 µl
Reverse primer (100µg/ml)	1 µl
Taq DNA polymerase (Go Taq Promega)	0.25 µl
dH_2O	25.75-28.75µl

*** 3µl for Rlr_up_F/ Rlr_do_R and 6µl for Rlr_srtC_F/ Rlr_srtD_R**

Thermal Cycling Parameters

Initial denaturation:	95°C - 2 minutes
Denaturation:	95°C - 0.5 minute
Annealing:	55°C - 1 minute *
Extension:	72°C - 1 minute
Cycles:	30x
Final extension:	72°C - 5 minutes

* Annealing time of 30-45 sec worked well for Rrl_up/Rrl_do PCR

PI-2 Detection

50 µl PCR Reaction

DNA template	**5 µl**
Master Mix	
5X PCR buffer (Promega)	10 µl
$MgCl_2$ (25mM) (1.5 mM final)	3 µl
dNTPs (10mM Promega)	1 µl
Forward primer (100µg/ml)	1 µl
Reverse primer (100µg/ml)	1 µl
Taq DNA polymerase (Go Taq Promega)	0.25 µl
dH_2O	28.75µl

Thermal Cycling Parameters

Initial denaturation:	95^{o}C - 2 minutes
Denaturation:	95^{o}C – 0.5 minute
Annealing:	55^{o}C – 0.5-0.75 minute *
Extension:	72^{o}C - 1 minute
Cycles:	30
Final extension:	72^{o}C - 5 minutes

*Annealing of 30sec and 45 sec gave the same results

Statistical Analysis

Statistical significance was analyzed by univariate analysis (chi-square (X^2), t-tests or fisher exact tests) and by full factorial multiple regression or logistic regression, depending on the variable in question. EpiInfoStats Cals 2 statistical program was used.

F. Results

Part I Characterization of Canadian Clinical Macrolide-Resistant *S. pneumoniae* Isolates

Demographic data of Macrolide-Resistant *S. pneumoniae*

Between November 1997 and December 2008 inclusive, over 12,000 respiratory tract *S. pneumoniae* isolates were collected as part of CROSS and CANWARD. The annual macrolide resistance, represented as clarithromycin resistance, of all isolates collected is shown in Figure 1. The macrolide resistance remained stable near 8% for the first four years (1998-2001) of the study; however, it gradually started to increase thereafter, reaching 15% by 2003 and 21% at the end of the study in 2008 (p=0.0001).

One thousand five hundred and eighteen respiratory *S. pneumoniae* isolates were determined to be macrolide-resistant (clarithromycin MIC ≥1.0μg/mL) during the 1998 and 2008 collection period. The isolates were collected from geographically diverse regions of Canada. These regions were BC/AB (British Columbia and Alberta), SK/MB (Saskatchewan and Manitoba), ON (Ontario), QC (Quebec), and the Maritime provinces (Nova Scotia, New Brunswick, and Prince Edward Island). The regional distribution of macrolide-resistant *S. pneumoniae* isolates is shown in Figure 2a. Of all the macrolide resistant isolates collected during the 1998 and 2008 study, 10% (152) came from the Maritime region, 20% (304) came from BC/AB region, 22% (334) from ON region, and 24% from each QC (364) and SK/MB (364) regions. Regional distribution of macrolide-resistant isolates by year is also shown in Figure 2a. Between 1998 and 2008 study, changes were noted for the BC/AB and ON regions. In 1998, 43% (30/71) of macrolide resistant isolates were obtained from the BC/AB region, however in 2008, 9% (8/84)

were from the BC/AB region. In 1998, of all the macrolide-resistant isolates obtained, 6% (4/70) came from the ON region, however by 2008; the ON region contributed 30% (25/84) of macrolide-resistant isolates. No major changes for the other three regions, SK/MB, QC and the Maritime were noted during the study.

The distribution of macrolide-resistant *S. pneumoniae* by age group is shown in Figure 2b. Overall, the majority of macrolide-resistant isolates were obtained from adult (18-64) population (46%) and from elderly (≥65) (35%) population. Eighteen percent of macrolide-resistant isolates were obtained from the pediatric (0-17) age group. Among the pediatric age group, 8% was from those younger than 2 years of age (<2), while those 2-4 years of age and those 5-17 years of age each contributed 5% to macrolide-resistant isolates. The percentage of macrolide-resistant *S. pneumoniae* from the elderly (≥65) population and from children 2-4 and 5-17 years years of age remained relatively the same at 34%, 4% and 4% in 1998 and at 36%, 5% and 6% in 2008. The percentage of macrolide-resistant *S. pneumoniae* from adult (18-54) population, and from pediatric population younger than 2 years of age changed from 46% to 54% and from 8% to 0% between 1998 and 2008, respectively.

The distribution of macrolide-resistant *S. pneumoniae* by gender is shown in Figure 2c. Overall, 60% of macrolide-resistant isolates were obtained from male patients, while 40% were from female patients. This ratio of 60:40 male to female was maintained throughout each year of the study, Figure 2c.

The distribution of macrolide-resistant *S. pneumoniae* by patient status is shown in Figure 2d. Overall, 60% of macrolide-resistant *S. pneumoniae* were from inpatients and 40% were from outpatients. The percentage of macrolide-resistant *S. pneumoniae* from inpatients varied from a low of 49% in 2007 to a high of 73% in 1998. Similarly the

percentage of outpatients varied from a low of 27% in 1997 to a high of 50% in 2007, however each year more inpatient isolates were obtained than outpatient.

Figure 1. Macrolide (represented as clarithromycin) resistance among Canadian clinical respiratory tract *S. pneumoniae* per year (1998-2008) collected by CROSS and CANWARD studies. The number of isolates obtained each year was as follows: 1998 (1155), 1999 (1268), 2000 (1522), 2001 (1425), 2002 (1556), 2003 (1283), 2004 (1300), 2005 (1231), 2006 (1168), 2007 (445), and 2008 (405).

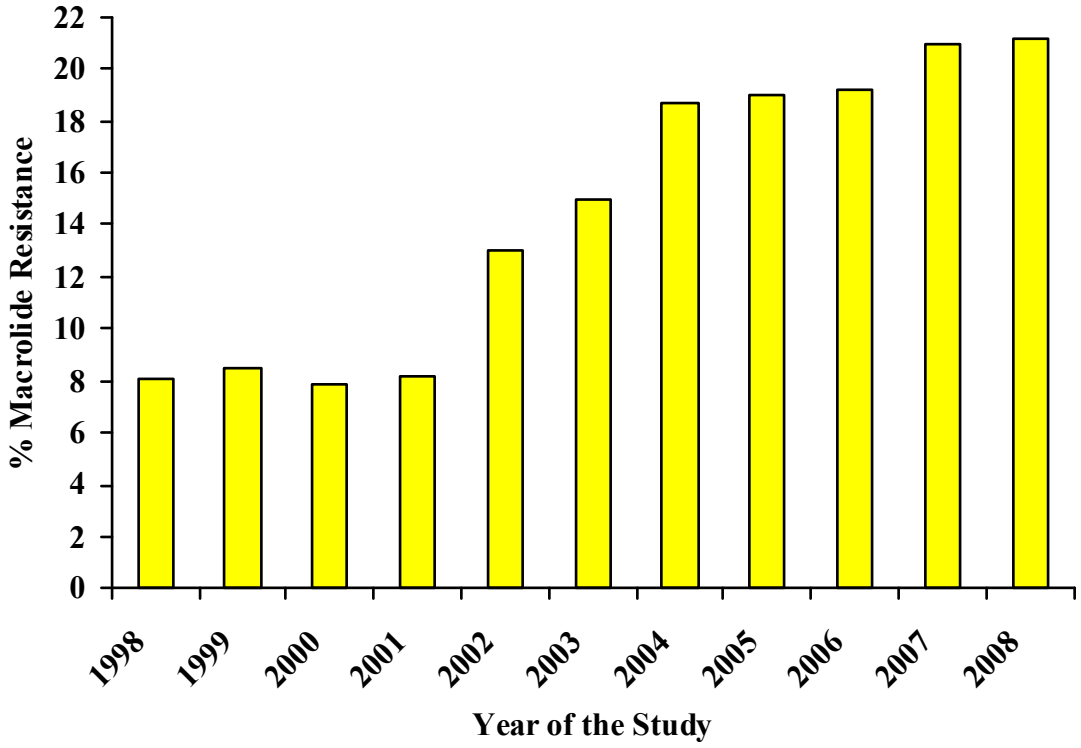


Figure 2. Distribution of the 1518 macrolide-resistant *S. pneumoniae* by (a) region overall and by year, (b) age group overall and by year, (c) gender overall and by year and (d) patient status overall and by year. The number of macrolide resistant *S. pneumoniae* each year was as follows: 1998 (n=70), 1999 (n=131), 2000 (n=81), 2001 (n=120), 2002 (n=159), 2003 (n=147), 2004 (n=173), 2005 (n=233), 2006 (n=223), 2007(n=97), and 2008 (n=84).

Fig. 2a.

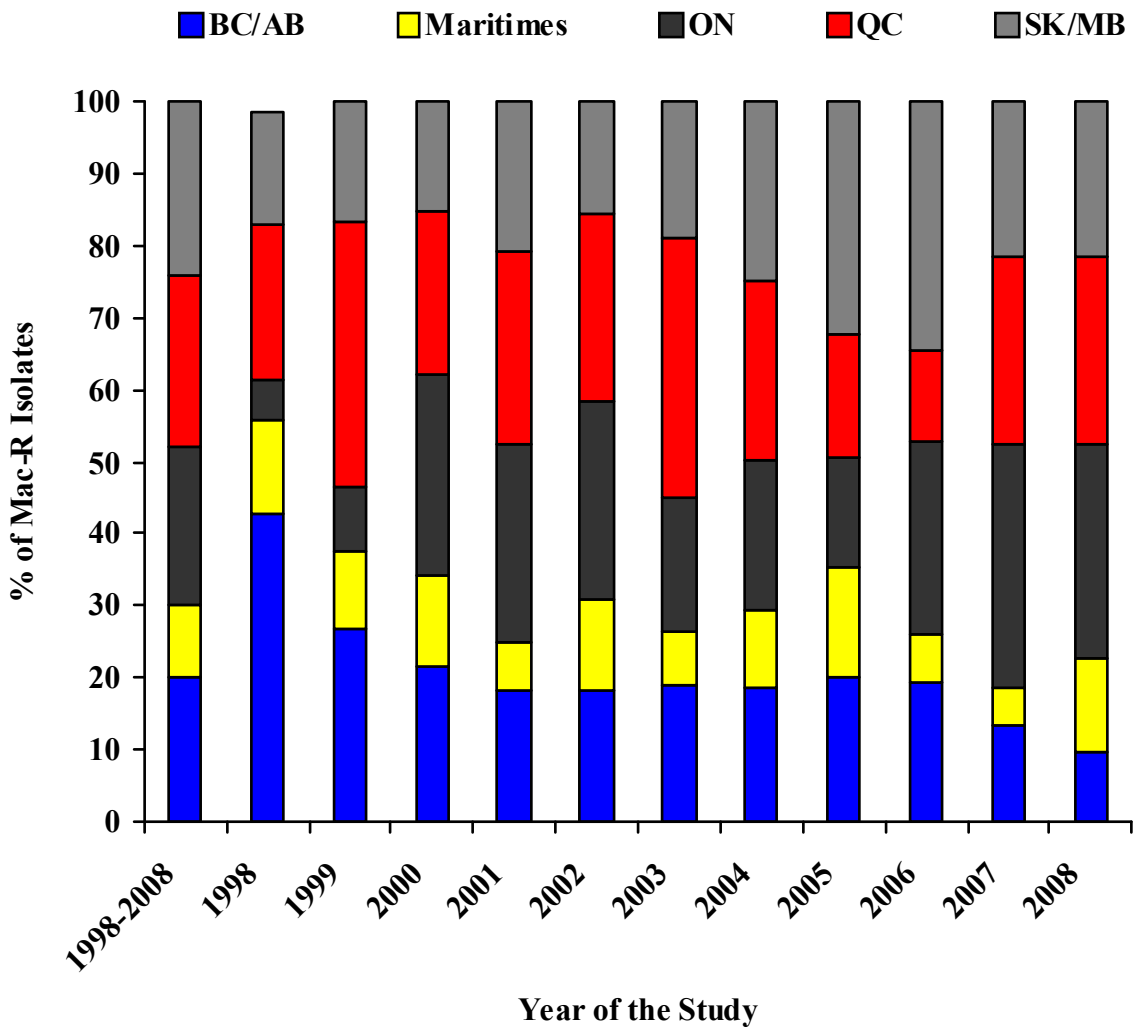

BC/AB
Maritimes
ON
QC
SK/MB
% of Mac-R Isolates
100
90
80
70
60
50
40
30
20
10
0
1998-2008
1998
1999
2000
2001
2002
2003
2004
2005
2006
2007
2008
Year of the Study

Fig. 2b.

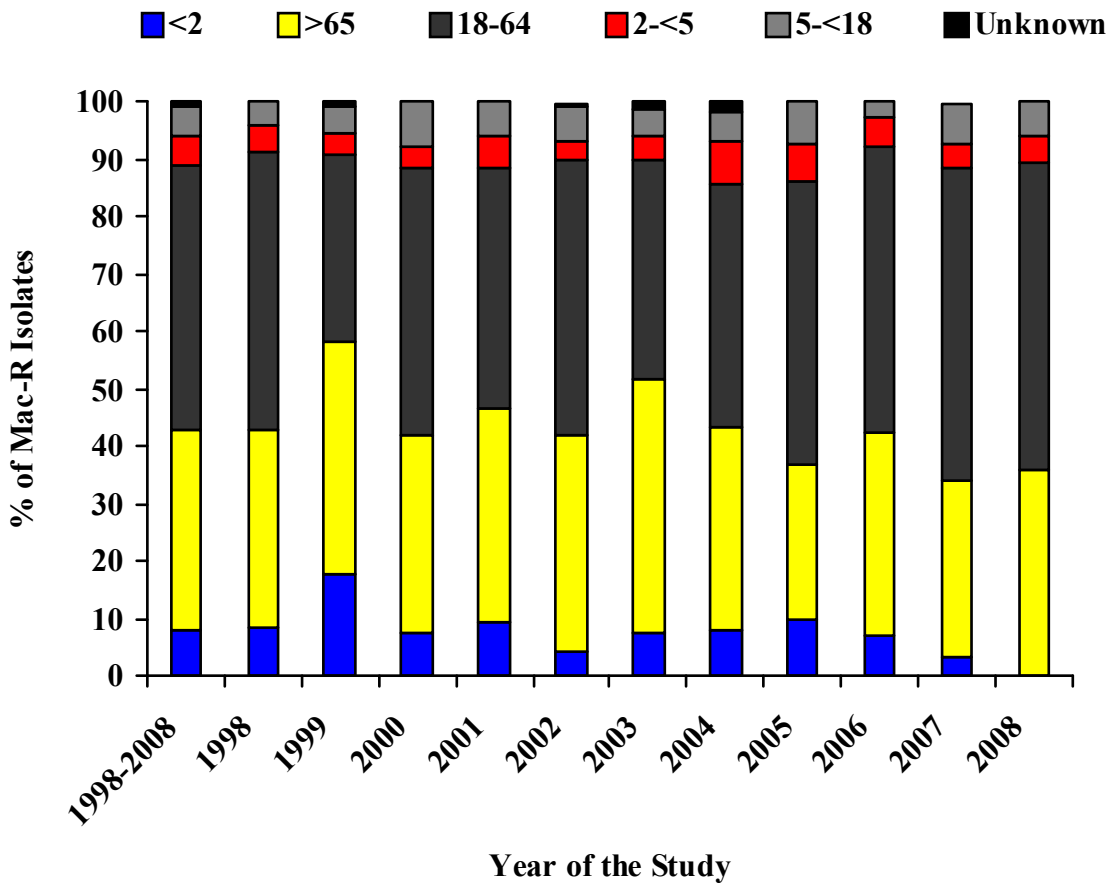

<2
>65
18-64
2-<5
5-<18
Unknown
% of Mac-R Isolates
100
90
80
70
60
50
40
30
20
10
0
1998-2008
1998
1999
2000
2001
2002
2003
2004
2005
2006
2007
2008
Year of the Study

Fig. 2c.

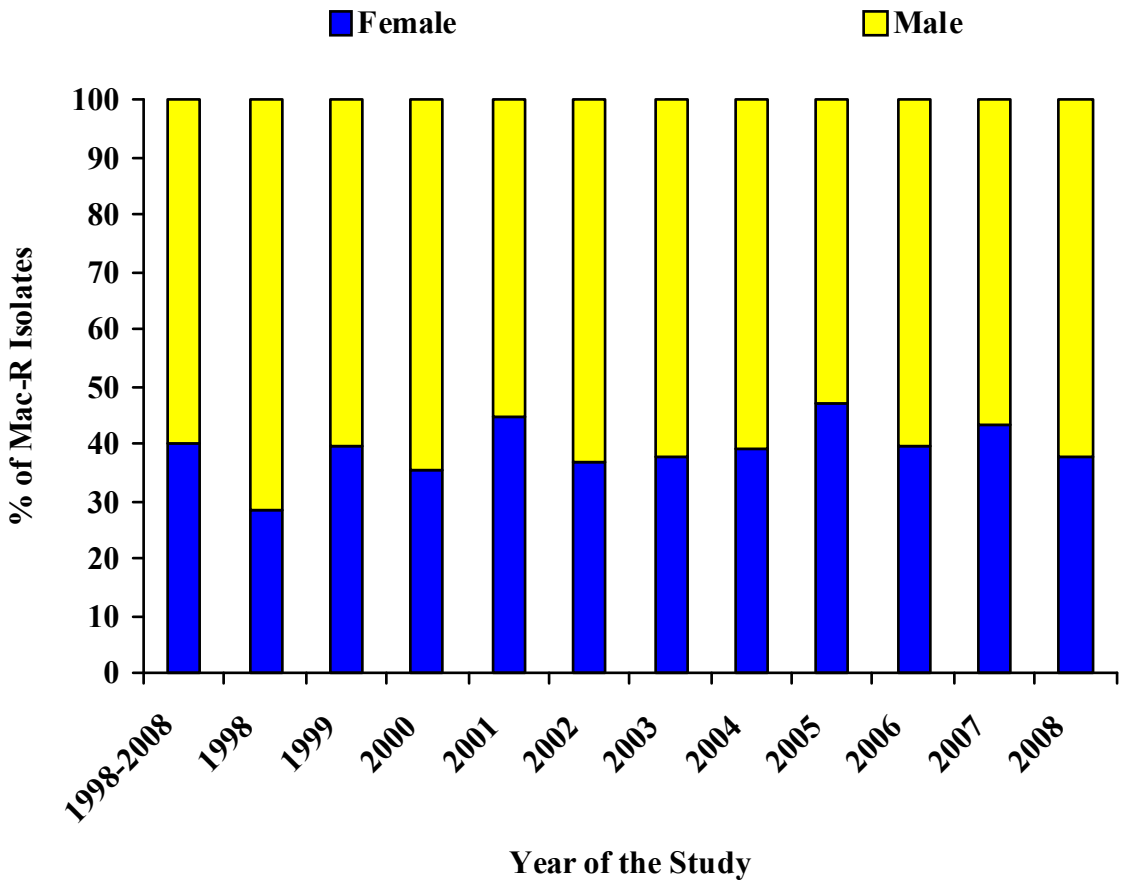
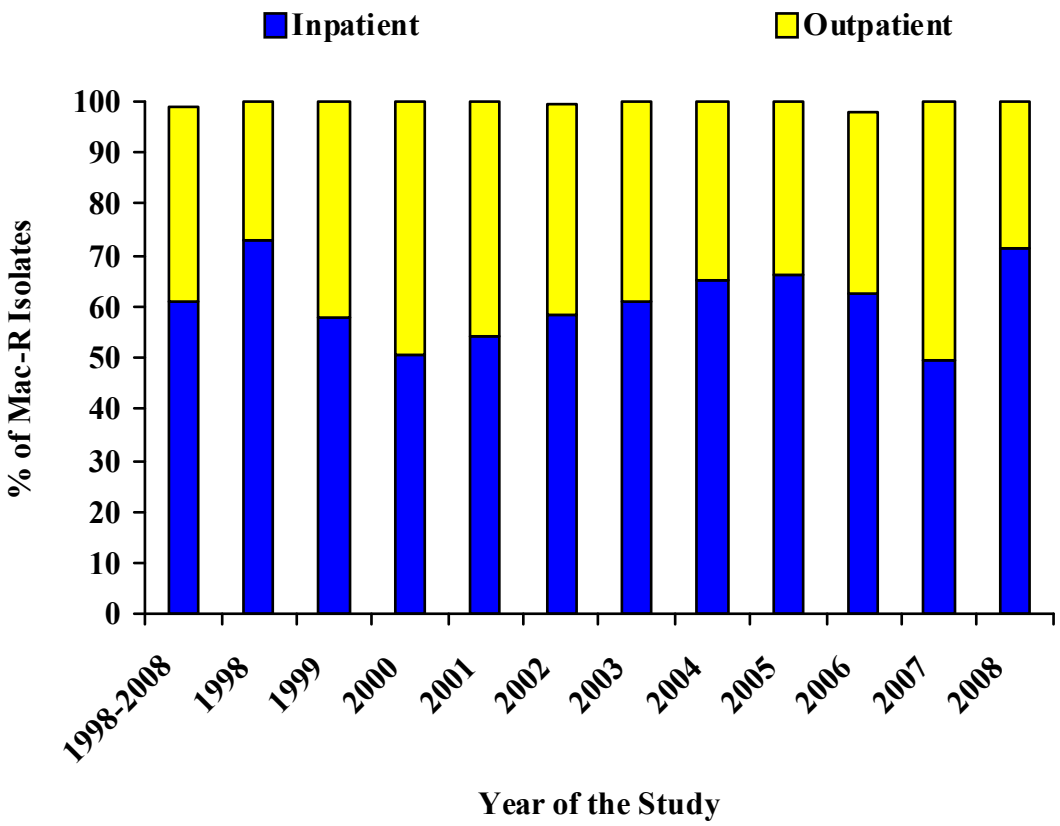

Female
Male
% of Mac-R Isolates
100
90
80
70
60
50
40
30
20
10
0
1998-2008
1998
1999
2000
2001
2002
2003
2004
2005
2006
2007
2008
Year of the Study

Fig. 2d.


Inpatient
Outpatient
% of Mac-R Isolates
100
90
80
70
60
50
40
30
20
10
0
1998-2008
1998
1999
2000
2001
2002
2003
2004
2005
2006
2007
2008
Year of the Study

Mechanisims of Macrolide Resistance in *S. pneumoniae*

Among a total of 1518 macrolide-resistant *S. pneumoniae* isolates, 51% (776) carried *mef*(A) gene, 36% (546) carried *erm*(B) gene, 8% (116) carried both *mef*(A) and *erm*(B) genes, and 5% (80) did not carry either gene, Figure 3. Isolates carrying *mef*(A) gene ranged from 43% (2001) to 59% (2007) and they decreased from 56.8% (1998) to 50% (2008) (p=0.037). Isolates carrying *erm*(B) gene ranged from 27% (2005) to 51% (2001) and they decreased from 41.2% (1998) to 27.4% (2008) (p=0.015). The presence of both *mef*(A) and *erm*(B) genes among macrolide-resistant *S. pneumoniae* varied from 0.8% (1999) to 19% (2008) and it increased from 0.8% (1998) to 19% (2008) (p=0.001). Isolates which did not carry either *mef*(A) or *erm*(B) genes varied from 0.8% (1999) to 11% (2005) and they increased from 08% (1998) to 3.6% (2008) (p=0.0365).

Figure 4a-4d shows the distribution of macrolide resistance genotypes by age, region gender and patient status, respectively. Figure 4a shows the distribution of isolates carrying various macrolide-resistance genotypes by age. Among all age groups, adult (18-64) population contributed the highest proportion of macrolide resistant isolates regardless of genotype. The proportion of isolates from the adult age group ranged from 44% (*mef*(A)) to 49% (neither *mef*(A) nor *erm*(B)). Elderly (≥65) population contributed the second highest proportion of macrolide resistant isolates regardless of genotype ranging from 34% (*mef*(A)) to 44% (no *mef*(A) and no *erm*(B)). The highest proportion (21%) of pediatric isolates was identified among *mef*(A) carrying isolates and the lowest (7.6%) among isolates which did not carry either gene. Among the pediatric age group the highest proportion (9.3%), those under the age of 2 was identified among *mef*(A) carrying isolates. Pediatric age group 2-4 was most commonly (8.6%) identified among isolates carrying both *mef*(A) and *erm*(B).

The highest proportion (30%), of isolates carrying *mef*(A) genotype was obtained from SK/MB region, Figure 4b. The highest proportion of *erm*(B) carrying isolates was obtained from QC region (38%). SK/MB was also the region which contributed the highest proportion (31%) of isolates carrying neither *mef*(A) nor *erm*(B) gene. Isolates carrying both *mef*(A) and *erm*(B) genes were most commonly isolated from ON region (29%), Figure 4b. Figures 4c and 4d depict the distribution of macrolide resistance genotypes by gender and by patient status, respectively. Male gender and inpatient patient status predominated among macrolide-resistant *S. pneumoniae* regardless of genotype.

Antibiotic susceptibility profile among isolates with different macrolide resistant genotypes is shown in Figure 5a-5d. The penicillin susceptibility profile among all macrolide resistant *S. pneumoniae* was as follows: penicillin-resistant 26%, penicillin intermediate 38%, and penicillin susceptible 36%, Figure 5a. Twenty one percent and 25% of all *erm*(B) and *mef*(A) carrying isolates were penicillin resistant, respectively. Among isolates carrying both *mef*(A) and *erm*(B) genes, 67% were penicillin resistant. Penicillin resistance rate of 11% was found among isolates carrying neither *mef*(A) nor *erm*(B) gene. The trimethoprim/sulfamethoxazole (T/S) susceptibility profile is shown in Figure 5b. Among all macrolide resistant *S. pneumoniae*, 34% were T/S resistant, 17% were T/S intermediate, and 49% were T/S susceptible. T/S resistance rate of 32% was found among isolates carrying either *mef*(A) or *erm*(B) genotypes. Among isolates carrying both *erm*(B) and *mef*(A), 73% were resistant to T/S. T/S resistance rate of 16% was identified among isolates not carrying either *mef*(A) or *erm*(B)gene. Figure 5c shows the doxycycline susceptibility profile. Among all macrolide resistant isolates, 21% were doxycycline resistant, 16% were doxycycline intermediate, and 62% were doxycycline

susceptible. Among *erm*(B) carrying isolates 37% were shown to be doxycycline resistant, while isolates carrying *mef(A)* or carrying both *mef*(A) and *erm*(B) were 13% and 14% doxycycline resistant, respectively. Five percent of isolates carrying neither *mef*(A) nor *erm*(B)were resistant to doxycycline. Clindamycin susceptibility prolife is shown in Figure 5d. Among all macrolide resistant isolates 40% were clindamycin resistant, 2% were clindamycin intermediate and 58% were clindamycin susceptible. Among isolates carrying *mef*(A) gene 2% were clindamycin resistant and 98% were clindamycin susceptible, while isolates carrying *erm*(B) gene were 89% clindamycin resistant and 9% clindamycin susceptible. Isolates carrying *mef*(A) and *erm*(B)genes were 87% resistant to clindamycin. Twenty percent clindamycin resistance was identified among isolates carrying neither *mef*(A) nor *erm*(B) genes.

Table 1a-1e shows the MIC_{50} and MIC_{90} as well the ranges of penicillin, trimethoprim/sulfamethoxazole, doxycycline, macrolides, erythromycin, azithromycin, and clarithromycin, as well as ketolides, cethromycin and telithromycin for all macrolide-resistant *S. pneumoniae* as well as by various genotypes. Serotype distribution among various genotypes is shown in Figure 6a-6d. The 10 most common serotypes found among isolates carrying the *erm*(B) gene in descending order were: 6B, 23F, 19F, NT, 15A, 19A, 6A, 33F, 11A and 9V, Figure 6a. The 10 most common serotypes found among isolates carrying the *mef*(A) gene in descending order were: 19F, 6B, 14, 6A, NT, 23F, 12F, 9V, 15B and 15C, Figure 6b. The 10 most common serotypes found among isolates carrying neither *mef*(A) nor *erm*(B) genes in descending order were: 6B, 23F, 19F, 3, 11A, 9N, 14, 19A, 22F and 33F, Figure 6c. The 10 most common serotypes found among isolates carrying both *mef*(A) and *erm*(B) genes in descending order were: 19F, NT, 19A, 6B, 14, 6A, 23F, 9V, 11A and 15C, Figure 6d.

Figure 3. Incidence of macrolide resistance genotypes among macrolide resistant *S. pneumoniae* during the 1998 and 2008 study. Macrolide resistant *S. pneumoniae* defined as clarithromycin resistant according to CLSI breakpoint of ≥1µg/mL.

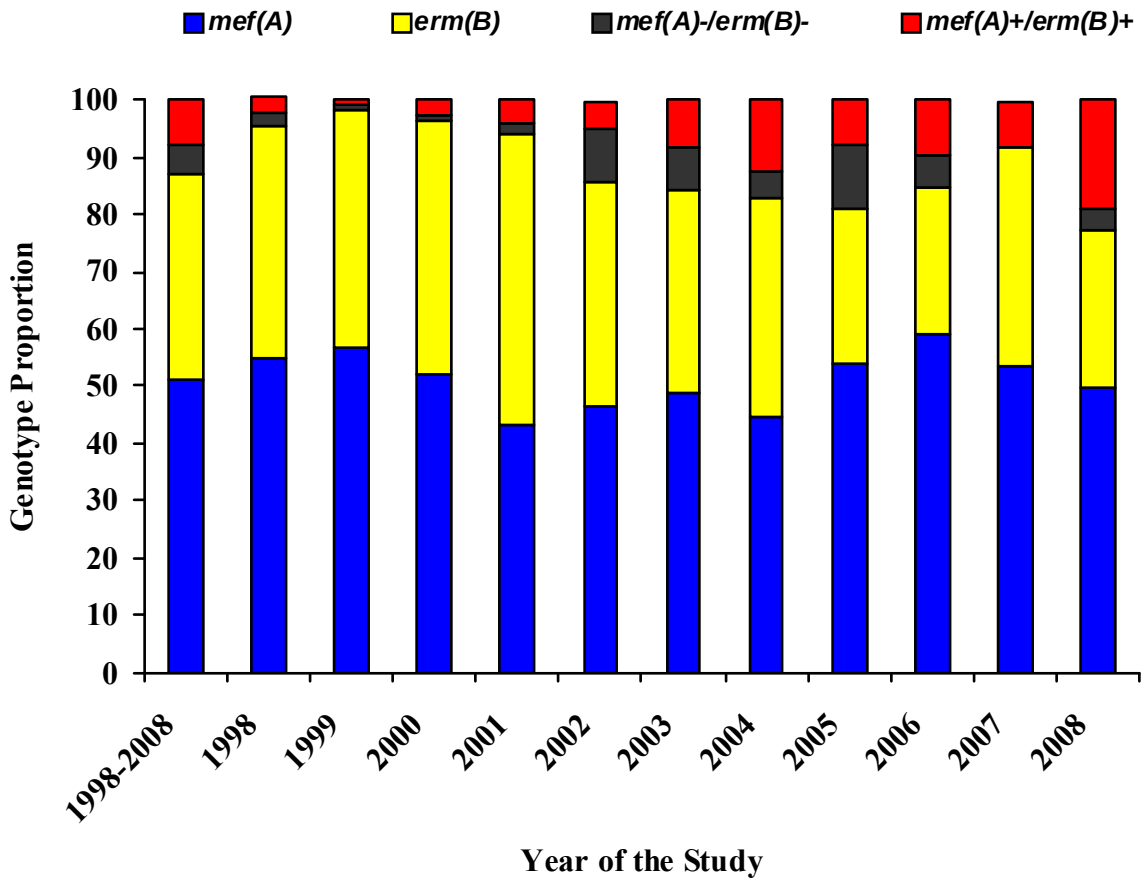


Figure 4. Prevalence of macrolide resistance genotypes by (a) age group (b) region (c) gender (d) patient status during the 1998 and 2008 study.

Fig. 4a.

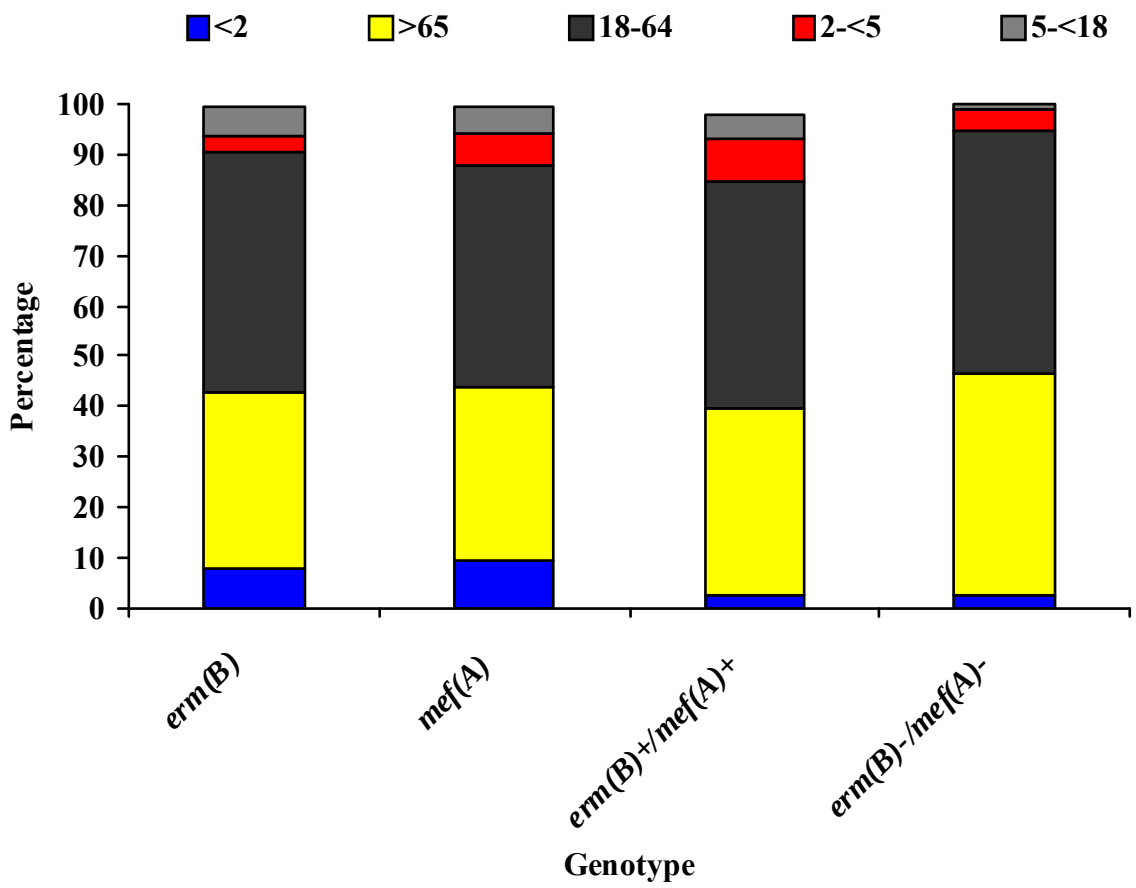

<2
>65
18-64
2-<5
5-<18
100
90
80
70
60
50
40
30
20
10
0
Percentage
erm(B)
mef(A)
erm(B)+/mef(A)+
erm(B)-/mef(A)-
Genotype

Fig. 4b.

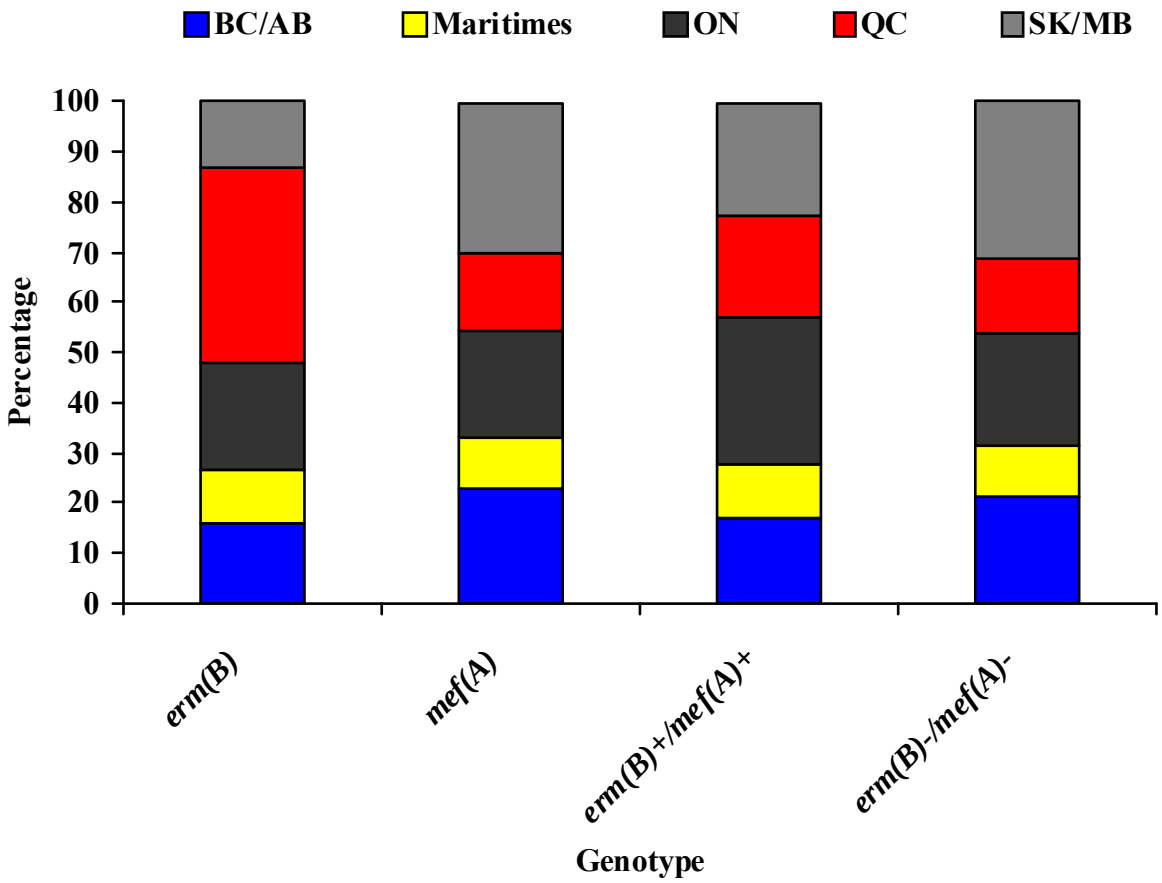

BC/AB
Maritimes
ON
QC
SK/MB
100
90
80
70
60
50
40
30
20
10
0
Percentage
erm(B)
mef(A)
erm(B)+/mef(A)+
erm(B)-/mef(A)-
Genotype

Fig. 4c.

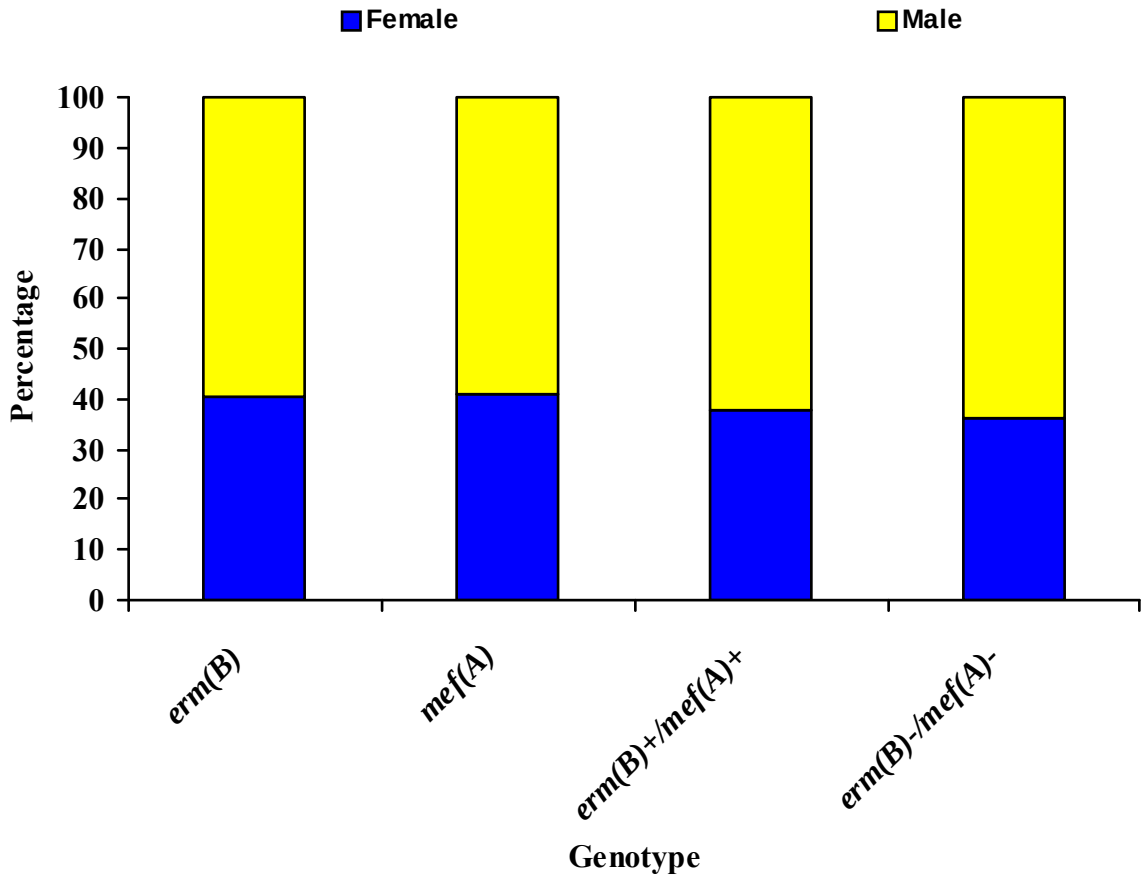

Female
Male
100
90
80
70
60
50
40
30
20
10
0
Percentage
erm(B)
mef(A)
erm(B)+/mef(A)+
erm(B)-/mef(A)-
Genotype

Fig. 4d.

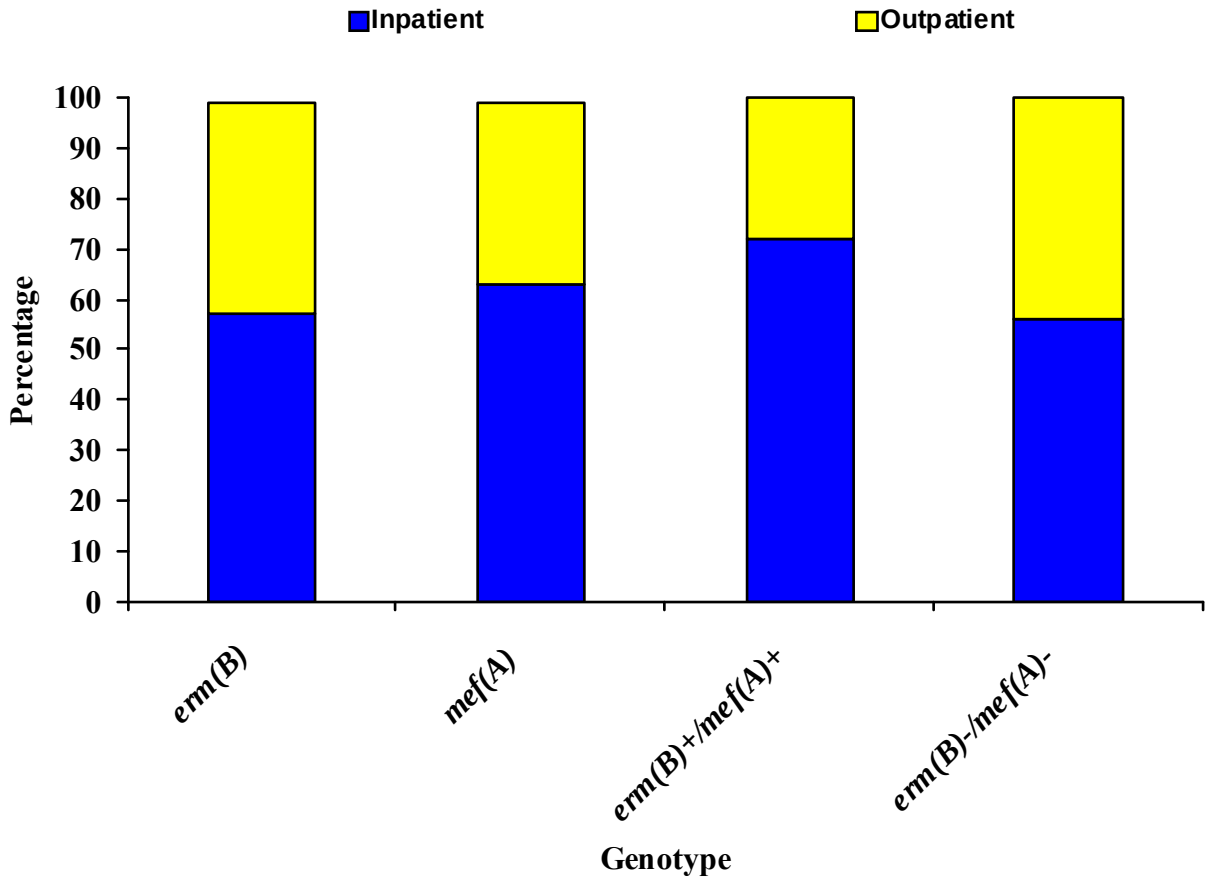

Inpatient
Outpatient
100
90
80
70
60
50
40
30
20
10
0
Percentage
erm(B)
mef(A)
erm(B)+/mef(A)+
erm(B)-/mef(A)-
Genotype

Figure 5. Penicillin (Pen) (a), Trimethoprim/sulfamethoxazole (T/S) (b), Doxycycline (Dox) (c), Clindamycin (Cd) (d) susceptibility profile among the isolates with different macrolide resistance genotypes during the 1998 and 2008 study. Isolates were defined as Sensitive (S), Intermediate (I), and Resistant (R) based on CLSI interpretative breakpoints as follows: Pen (oral penicillin V): S ≤0.06µg/mL, I 0.12-1µg/mL, R ≥ 2µg/mL; T/S: S ≤0.5/9.5µg/mL, I 1/19-2/38µg/mL, R ≥4/76µg/mL; Dox (Tetracycline): S ≤2µg/mL, I 4µg/mL, R ≥8µg/mL; Cd: S ≤ 0.25µg/mL, I 0.5µg/mL, R ≥1µg/mL.

Fig. 5a.

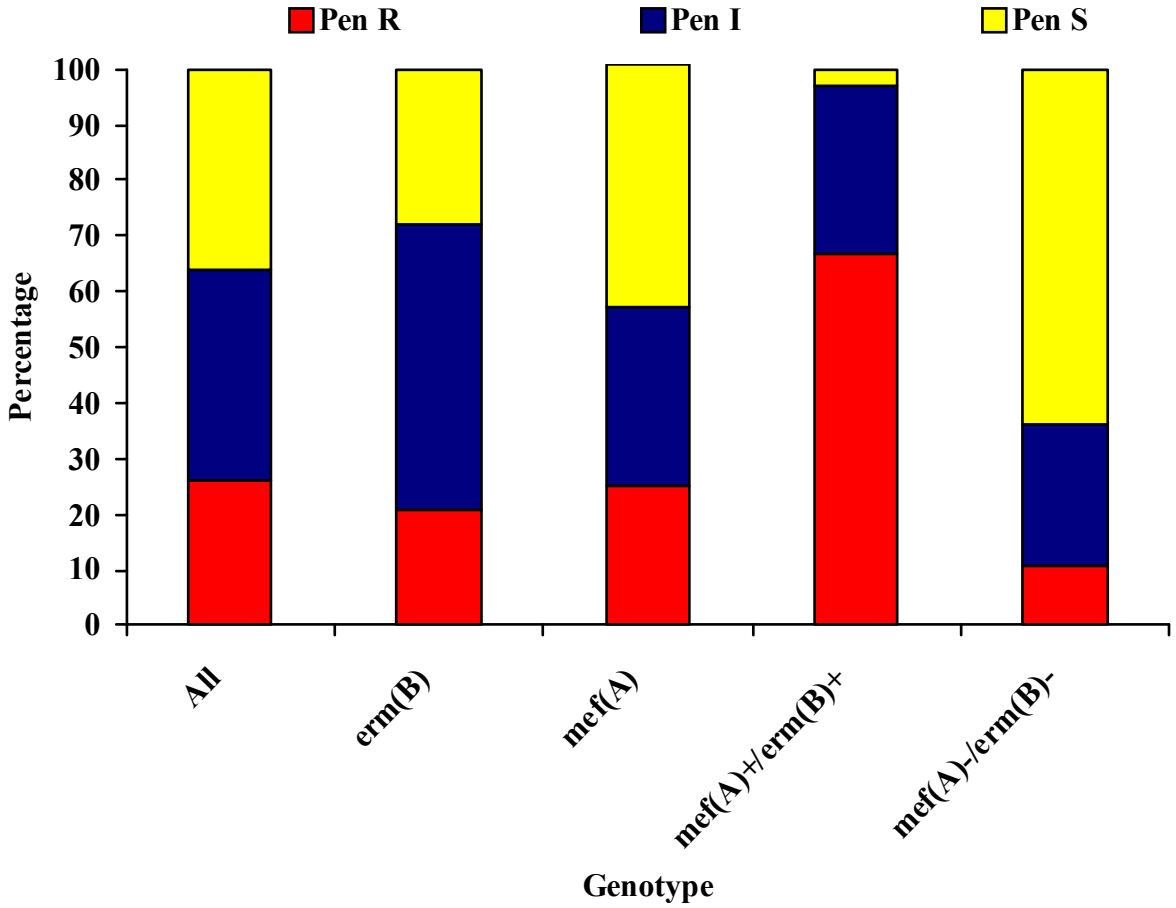

Pen R
Pen I
Pen S
100
90
80
70
60
50
40
30
20
10
0
Percentage
All
erm(B)
mef(A)
mef(A)+/erm(B)+
mef(A)-/erm(B)-
Genotype

Fig. 5b.

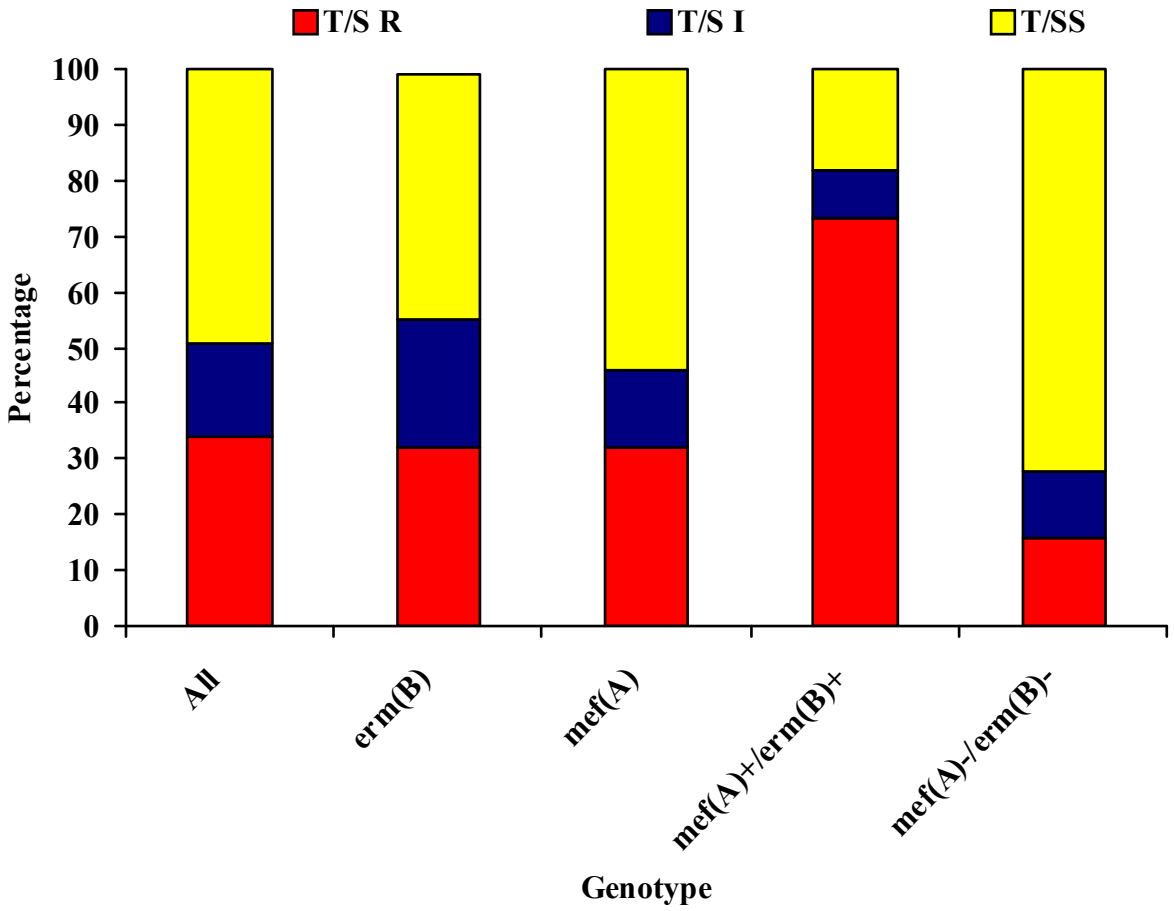

T/S R
T/S I
T/SS
100
90
80
70
60
50
40
30
20
10
0
Percentage
All
erm(B)
mef(A)
mef(A)+/erm(B)+
mef(A)-/erm(B)-
Genotype

Fig. 5c.

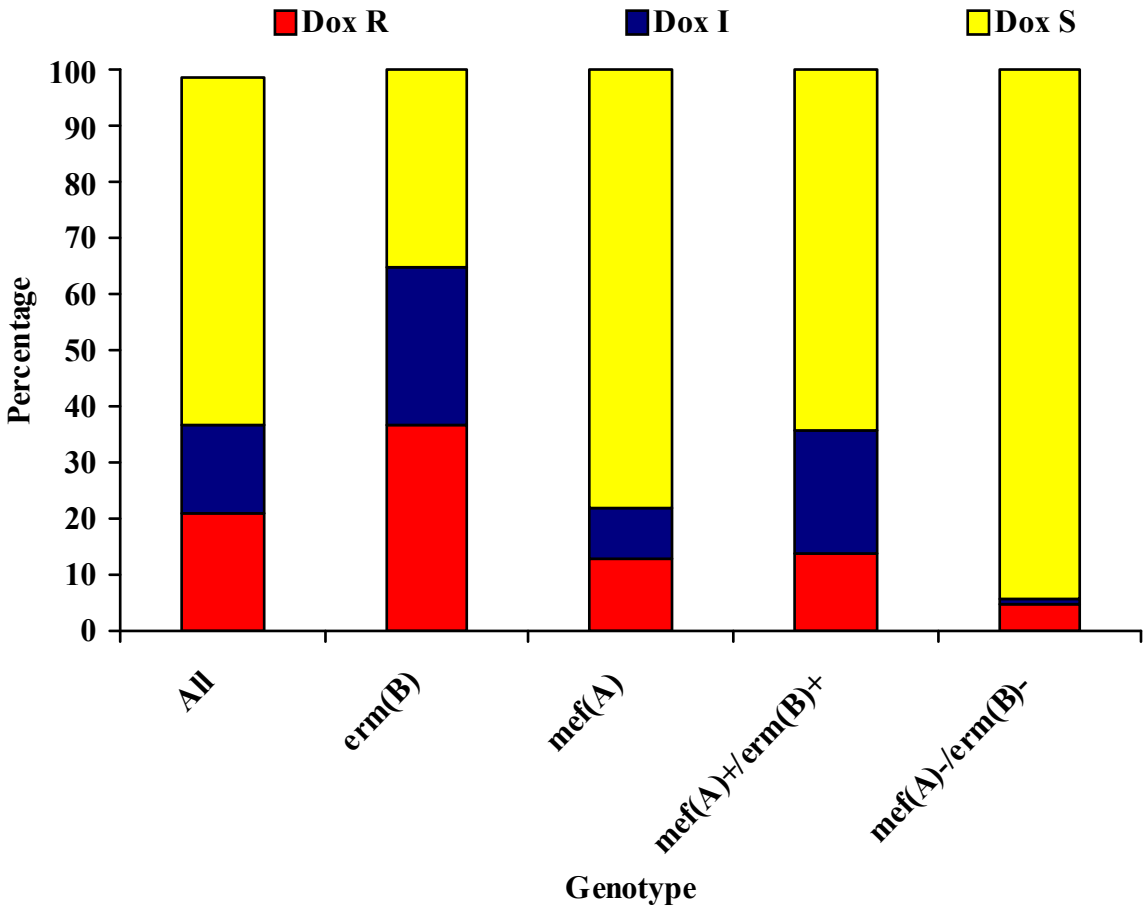

Dox R
Dox I
Dox S
Percentage
100
90
80
70
60
50
40
30
20
10
0
All
erm(B)
mef(A)
mef(A)+/erm(B)+
mef(A)-/erm(B)-
Genotype

Fig. 5d.

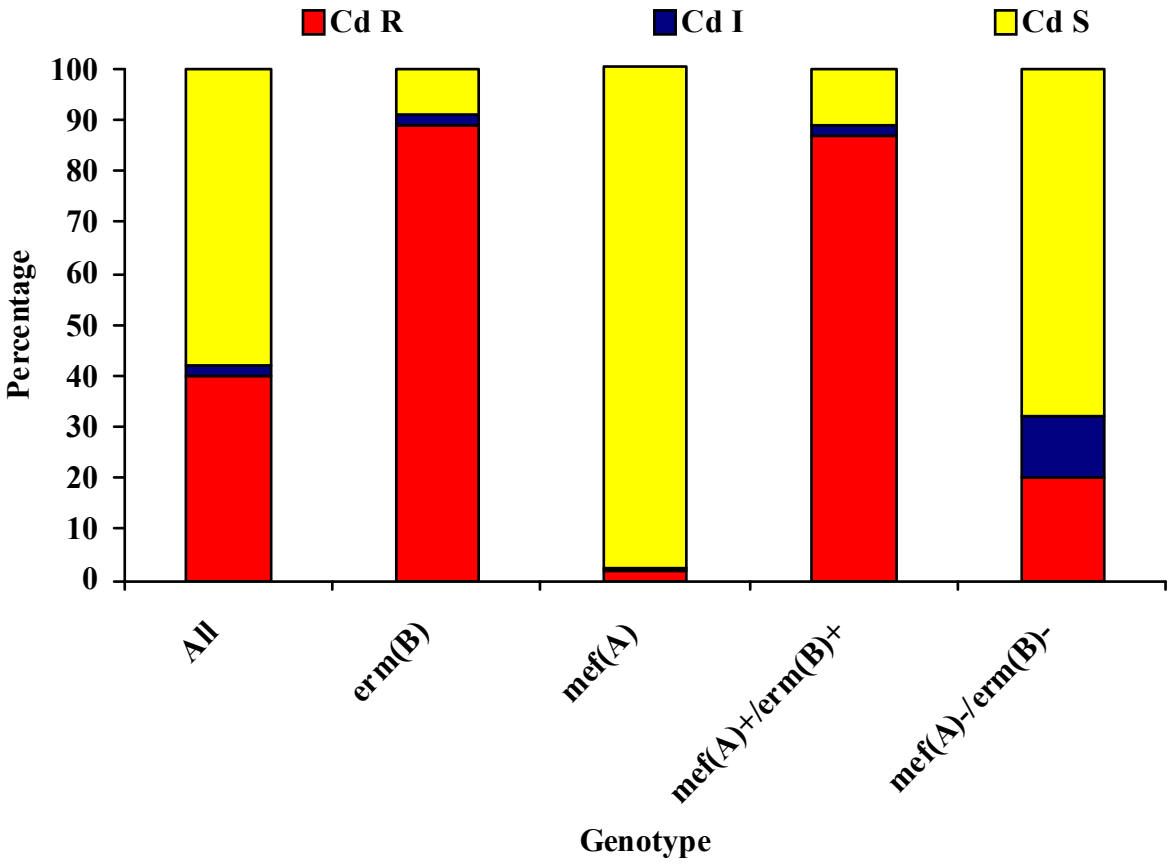

Cd R
Cd I
Cd S
100
90
80
70
60
50
40
30
20
10
0
Percentage
All
erm(B)
mef(A)
mef(A)+/erm(B)+
mef(A)-/erm(B)-
Genotype

Table 1. MIC_{50}, MIC_{90}, range for penicillin (Pen), trimethoprim/sulfamethoxazole (T/S), Doxycycline (Dox), Erythromycin (Ery), Clarithromycin (Clar), Azithromycin (Azi), Clindamycin (Cd), Telithromycin (Tel), and Cethromycin (Cethro), against (a) all, (b) *erm*(B) genotype , (c) *mef*(A) genotype, both *mef*(A) and *erm*(B) genotype and neither *mef*(A) nor *erm*(B) genotype.
Isolates were defined as Sensitive (S), Intermediate (I), and Resistant (R) based on CLSI interpretative breakpoints as follows: Pen (oral penicillin V): S ≤ 0.06µg/mL, I 0.12-1µg/mL, R ≥ 2µg/mL; T/S: S ≤ 0.5/9.5µg/mL, I 1/19-2/38µg/mL, R ≥ 4/76µg/mL; Dox (Tetracycline): S ≤ 2µg/mL, I 4µg/mL, R ≥8 µg/mL; Ery and Clar: S ≤ 0.25ug/mL I 0.5ug/mL, R ≥ 1ug/mL; Azi: S ≤ 0.5ug/ml, I 1ug/mL, R ≥ 2ug/mL; Cd: S ≤ 0.25µg/mL, I 0.5µg/mL, R ≥1µg/mL, Tel: S≤ 1µg/mL, I 2µg/mL, R ≥4µg/mL, and Cethro (Telithromycin).

Table 1a. All macrolide-resistant S. *pneumoniae* (n=1518).

Antibiotic	MIC_{50}	MIC_{90}	Range (µg/mL)			S	I	R
Pen	0.25	4	0.03	-	8	35.9%	38.0%	26.1%
T/S	1	8	0.12	-	16	48.8%	16.7%	34.5%
Dox	1	16	0.25	-	32	62.4%	16.1%	21.4%
Ery	8	64	1	-	64	0.0%	0.0%	100.0%
Clar	4	64	1	-	64	0.0%	0.0%	100.0%
Azi	8	64	1	-	64	0.0%	9.4%	90.6%
Cd	0.12	16	0.12	-	16	57.7%	1.7%	40.6%
Tel	0.03	0.25	0.001	-	4	99.8%	0.1%	0.0%
Cethro	0.015	0.06	0	-	2	100.0%	0.0%	0.0%

Table 1b. All *erm*(B) carrying macrolide-resistant *S. pneumoniae* (n=546).

Antibiotic	MIC_{50}	MIC_{90}	Range (µg/mL)			S	I	R
Pen	0.25	4	0.03	-	8	27.8%	50.8%	21.4%
T/S	1	8	0.12	-	16	44.4%	23.1%	32.5%
Dox	4	16	0.25	-	32	34.8%	28.3%	36.9%
Ery	64	64	1	-	64	0.0%	0.0%	100.0%
Clar	64	64	1	-	64	0.0%	0.0%	100.0%
Azi	64	64	1	-	64	0.0%	2.8%	97.2%
Cd	16	16	0.12	-	16	9.0%	1.8%	89.1%
Tel	0.008	0.03	0.001	-	4	99.2%	0.4%	0.2%
Cethro	0.008	0.03	0	-	2	100.0%	0.0%	0.0%

Table 1c. All *mef*(A) carrying macrolide-resistant *S. pneumoniae* (n=776).

Antibiotic	MIC_{50}	MIC_{90}	Range (µg/mL)			S	I	R
Pen	0.12	4	0.03	-	8	43.6%	31.8%	24.6%
T/S	0.5	8	0.12	-	16	53.8%	14.1%	32.1%
Dox	0.25	8	0.25	-	32	77.9%	8.6%	13.5%
Ery	4	4	1	-	64	0.0%	0.0%	100.0%
Clar	2	4	1	-	64	0.0%	0.0%	100.0%
Azi	4	8	1	-	64	0.0%	14.2%	85.8%
Cd	0.12	0.12	0.12	-	16	97.9%	0.5%	1.6%
Tel	0.06	0.25	0.002	-	1	100.0%	0.0%	0.0%
Cethro	0.03	0.06	0.001	-	2	100.0%	0.0%	0.0%

Table 1d. Macrolide-resistant *S. pneumoniae* carrying both *mef*(A) and *erm*(B) (n=116).

Antibiotic	MIC_{50}	MIC_{90}	Range (µg/mL)			S	I	R
Pen	4	8	0.03	-	8	2.6%	29.8%	67.5%
T/S	8	16	0.12	-	16	18.6%	8.8%	72.5%
Dox	2	16	0.25	-	32	64.3%	21.7%	13.9%
Ery	64	64	1	-	64	0.0%	0.0%	100.0%
Clar	64	64	1	-	64	0.0%	0.0%	100.0%
Azi	64	64	1	-	64	0.0%	4.3%	95.7%
Cd	16	16	0.12	-	16	11.2%	1.7%	87.1%
Tel	0.12	0.5	0.002	-	2	99.1%	0.9%	0.0%
Cethro	0.03	0.06	0.002	-	0.25	100.0%	0.0%	0.0%

Table 1e. Macrolide-resistant *S. pneumoniae* carrying neither *mef*(A) nor *erm*(B) (n=80).

Antibiotic	MIC_{50}	MIC_{90}	Range (µg/mL)			S	I	R
Pen	0.06	2	0.03	-	4	63.3%	25.3%	11.4%
T/S	0.25	4	0.12	-	16	72.1%	11.8%	16.2%
Dox	0.25	0.5	0.25	-	32	93.8%	1.3%	5.0%
Ery	16	64	1	-	64	0.0%	0.0%	100.0%
Clar	4	64	1	-	64	0.0%	0.0%	100.0%
Azi	64	64	1	-	64	0.0%	13.0%	87.0%
Cd	0.12	4	0.12	-	16	67.5%	12.5%	20.0%
Tel	0.015	0.25	0.001	-	2	97.5%	1.3%	0.0%
Cethro	0.03	0.5	0.001	-	2	100.0%	0.0%	0.0%

Figure 6. The top 10 serotypes among (a) *erm*(B) (b) *mef*(A) (c) neither *erm(B)* nor *mef*(A) and (d) both *erm*(B) and *mef*(A) carrying *S. pneumoniae* during the 1998-2008 study. PCV7 serotypes are shown in red color. Non-PCV7 serotypes are shown in yellow.

Fig. 6a.

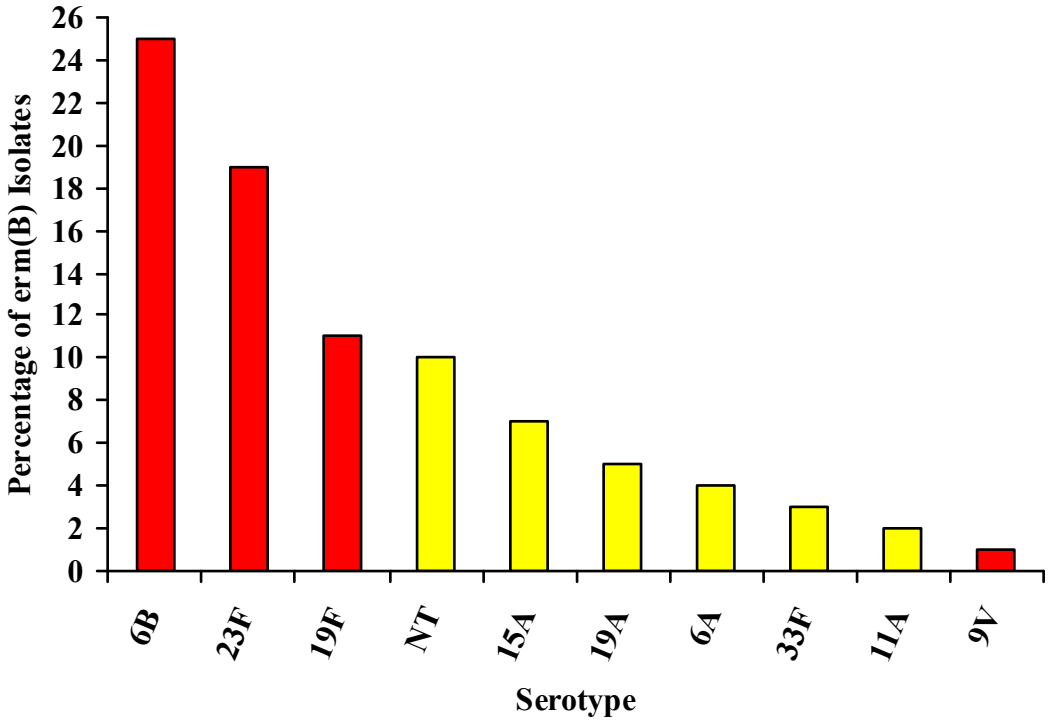

Percentage of erm(B) Isolates
26
24
22
20
18
16
14
12
10
8
6
4
2
0
6B
23F
19F
NT
15A
19A
6A
33F
11A
9V
Serotype

Fig. 6b.

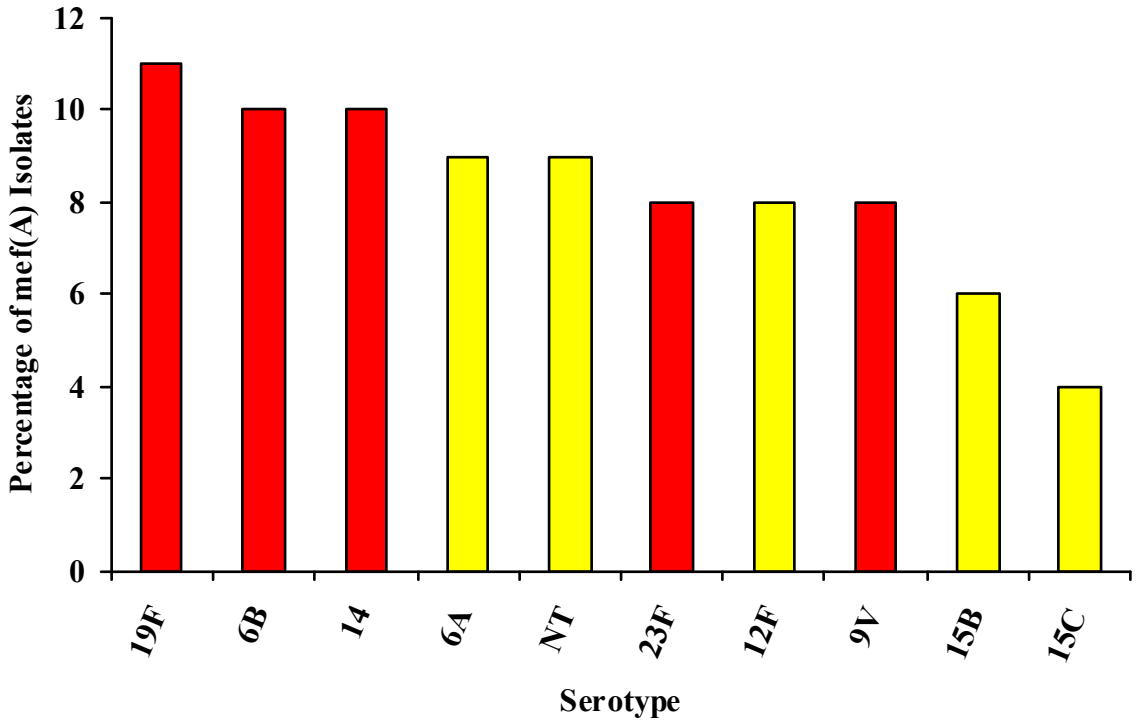

Percentage of mef(A) Isolates
12
10
8
6
4
2
0
19F
6B
14
6A
NT
23F
12F
9V
15B
15C
Serotype

Fig. 6c.

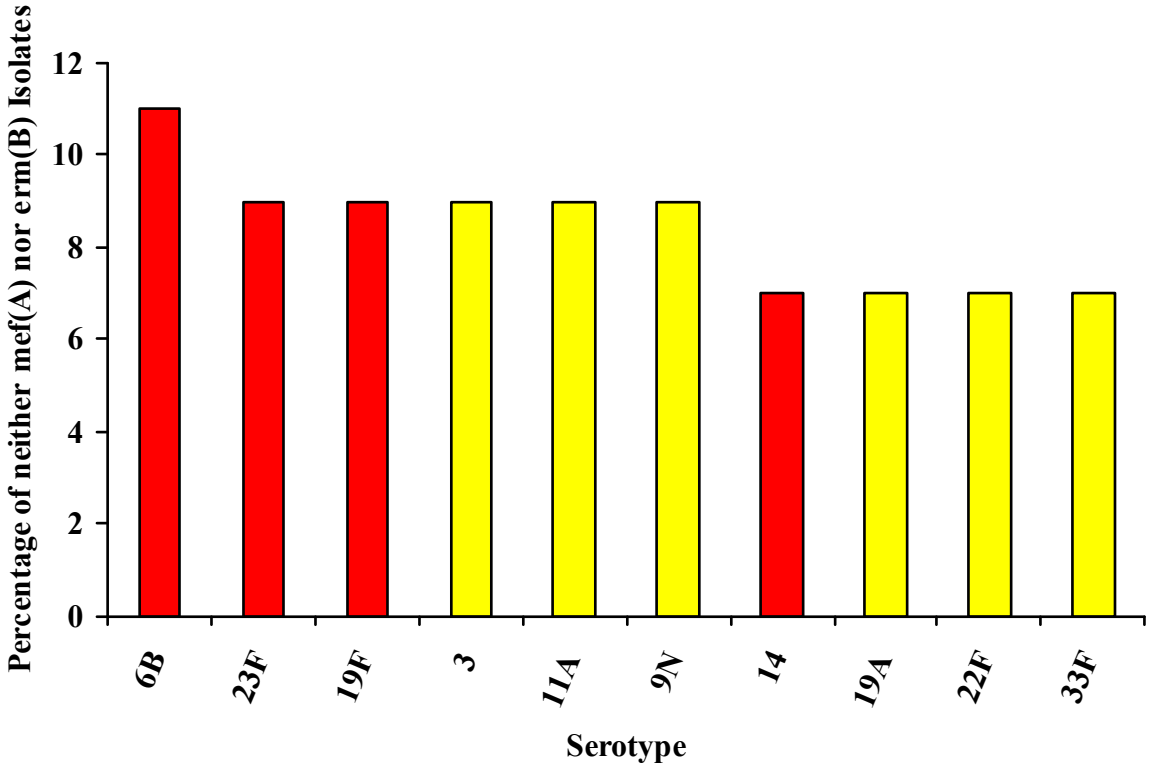
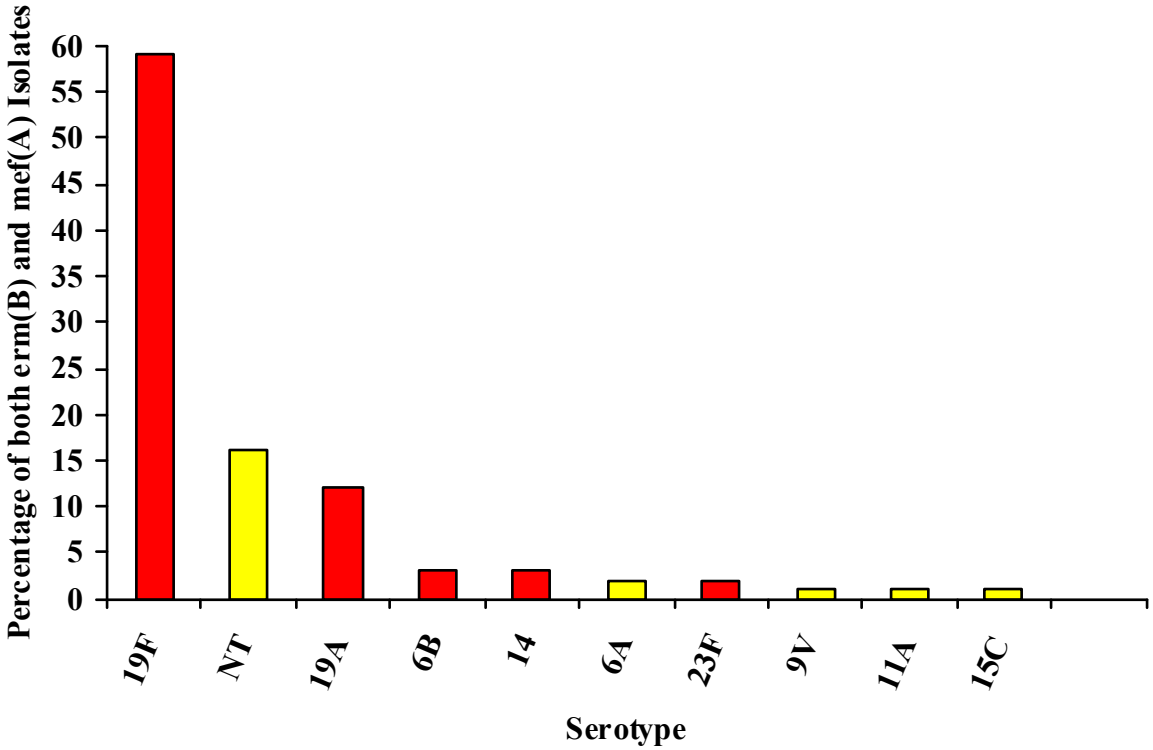

Percentage of neither mef(A) nor erm(B) Isolates
12
10
8
6
4
2
0
6B
23F
19F
3
11A
9N
14
19A
22F
33F
Serotype

Fig. 6d.


Percentage of both erm(B) and mef(A) Isolates
60
55
50
45
40
35
30
25
20
15
10
5
0
19F
NT
19A
6B
14
6A
23F
9V
11A
15C
Serotype

Discrimination of *mef*(A) Class Gene into *mef*(A)-*A* and *mef*(A)-*E* and Their Characteristics

Figure 7 shows the incidence of two subclasses of the *mef*(A) gene, *mef*(A)-class A and *mef*(A)- class E. In total, 95% (133/140) of isolates with *mef*(A) gene tested had the *mef*(A) -class E gene and 5% (7/140) had the *mef*(A)-class A gene. The prevalence of the *mef*(A)-class E varied from 88% (24) to 100% (29) between 1998 and 2002. The prevalence of the *mef*(A)-class A varied from 0% (0) to 11% (3) between 1998 and 2002.

Figure 8a-8d shows the distribution of *mef*(A)-class A and *mef*(A)-class E by age, region, gender and patient status. The isolates carrying *mef*(A)-class E were identified most commonly (42%) among the adult (18-64) population, followed by elderly population (37%) and pediatric population (21%), Figure 8a. The isolates carrying *mef*(A)-class A were identified among adult (50%) population, followed by elderly (33%) population and pediatric population (17%). Pediatric isolates carrying *mef*(A)-class A gene were only from those <2 (17%), whereas the pediatric isolates carrying the *mef*(A)-class E gene were identified among <2 (13%), 2-4 age group (6%) and 5-7 age group (2%), Figure 8a. Both *mef*(A)-class A and *mef*(A)-class E carrying isolates were found most commonly from BC/AB region at 34% and 32%, respectively, Figure 8b. Both *mef*(A)-class A and *mef*(A)-class E carrying isolates were found more commonly among male gender at 67% and 57%, respectively, Figure 8c. Fifty-seven percent and 50% of *mef*(A)-class E and *mef*(A)-class A carrying isolates were found among inpatients, Figure 8d.

Penicillin, T/S, and doxycycline antibiotic susceptibility profile for isolates carrying *mef*(A)-class A and *mef*(A)-class E is shown in Figure 9. Among isolates carrying the *mef*(A)-class E, 59% were penicillin non-susceptible and 61% were T/S non-susceptible.

Among isolates carrying the *mef*(A)-class A gene, all (100%) were susceptible to penicillin and T/S. Doxycycline non-susceptibility rate of 39% and 17% was identified among *mef*(A)-class E and *mef*(A)-class A, respectively.

Serotype distribution among isolates carrying *mef*(A)-class A and *mef*(A)-class E is shown in Figure 10. Thirteen (12F, 19F, 23F, 14, 6B, 9V, 6A, 11A, 15C, 18C, 9A, 19B, 34 and non-typeable) serotypes were identified among *mef*(A)-class E, while 1 serotype (14) was identified among *mef*(A)-class A. The most common serotypes among *mef*(A)-class E were; 12F and 19F both present at 13%, followed by 23F and 14 both present at 12%. Twenty percent of *mef*(A)-class E isolates were non-typeable.

The genetic relatedness among of *mef*(A) class E and *mef*(A) class A is shown in figure 11. There were 19 clusters, containing 2 to 11 isolates, which accounted for 47% (63/133) of the *mef*(A) class E *S. pneumoniae* isolates. Among the 19 clusters, 13 (68%) demonstrated cluster-specific serotypes. Isolates with these 13 clusters belonged to serotype 6B (3 clusters), 6A (2 clusters), 12F (2 clusters), 23F (2 clusters), 11A (1 clusters), 9V (1 cluster), 18C (1 cluster) and 14 (1 cluster). Among the 7 *mef*(A) class A isolates, one major cluster was found containing 6 out of the 7 isolates, making up 86% of *mefA* class A *S. pneumoniae*. All *mef*(A) class A isolates belonged to serotype 14.

Figure 7. Incidence of two subtypes, *mef*(A)-*E* and *mef*(A)-*A* among 140 *mef*(A) gene carrying macrolide-resistant *S. pneumoniae* from 1998 and 2002 study years.

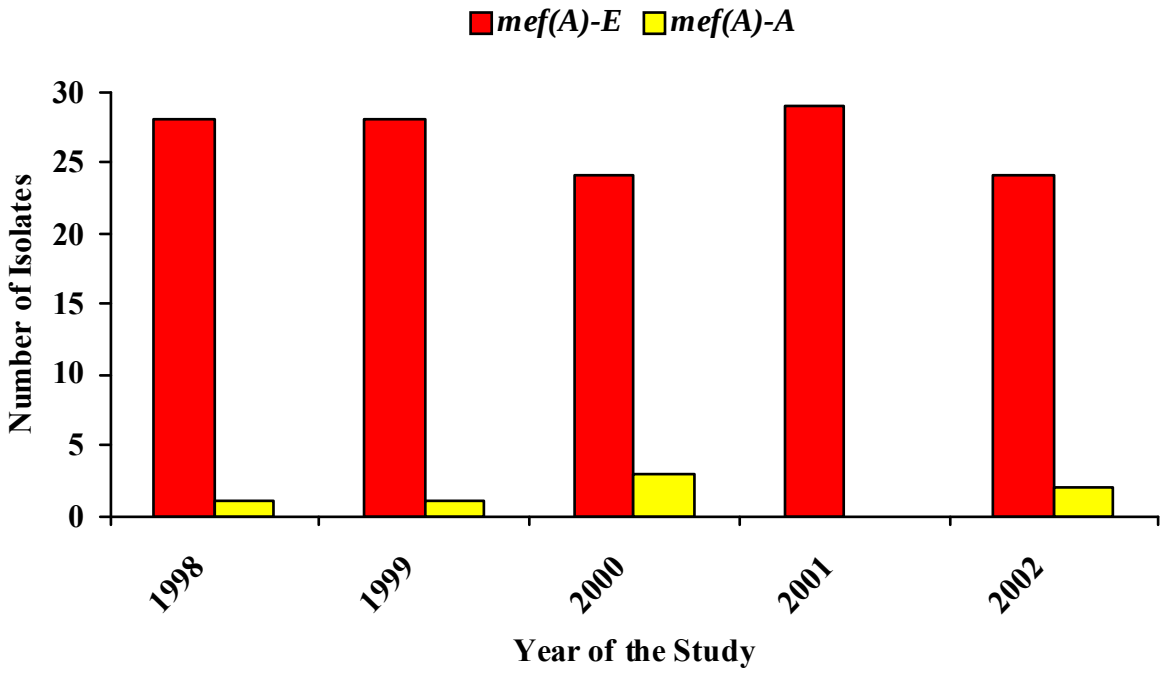


Figure 8. Prevalence of the two subtypes of the *mef*(A) gene class, *mef*(A)-*E* and *mef*(A)-*A* by (a) age group (b) region (c) gender and (d) patient status among the 140 *S. pneumoniae* selected during the 1998 and 2002 years.

Fig. 8a.

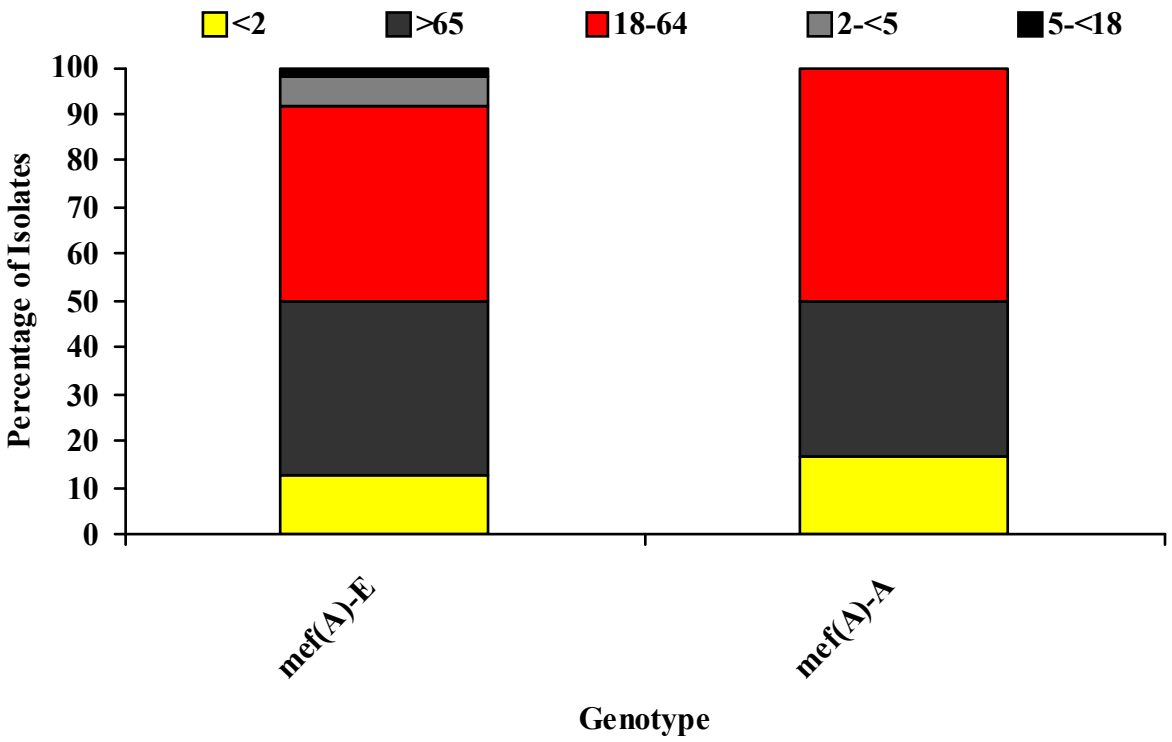

<2
>65
18-64
2-<5
5-<18
100
90
80
70
60
50
40
30
20
10
0
Percentage of Isolates
mef(A)-E
mef(A)-A
Genotype

Fig. 8b.

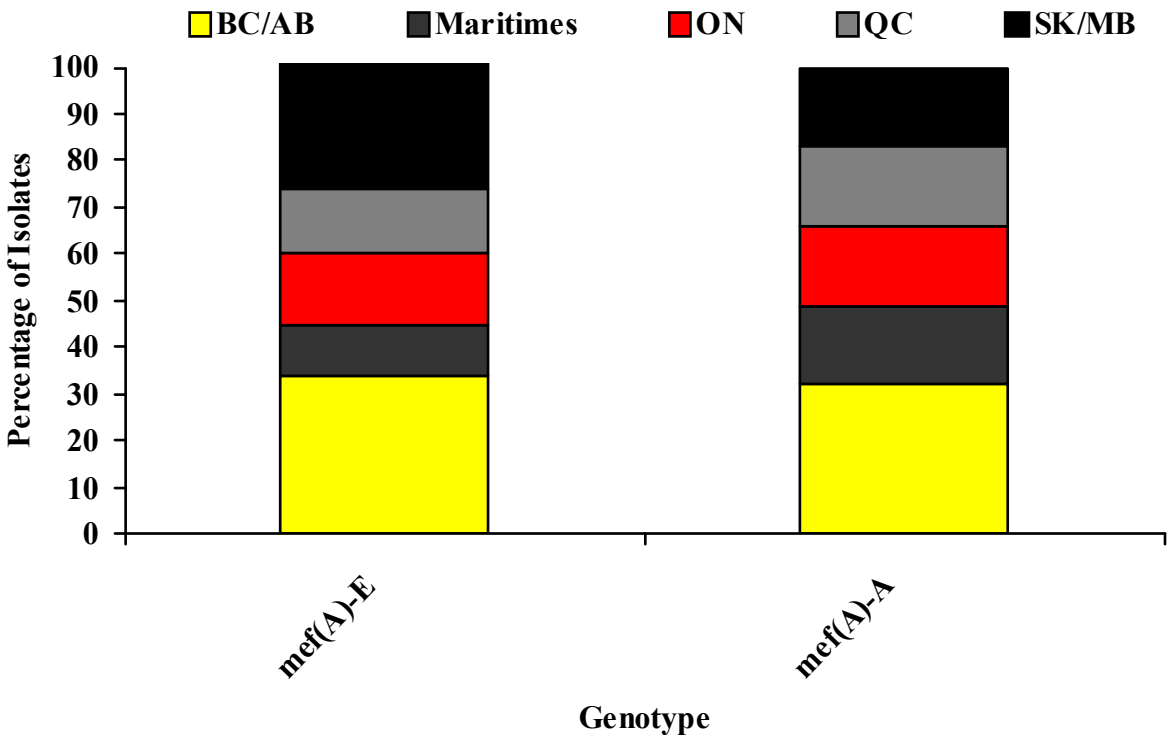

BC/AB
Maritimes
ON
QC
SK/MB
Percentage of Isolates
100
90
80
70
60
50
40
30
20
10
0
mef(A)-E
mef(A)-A
Genotype

Fig. 8c.

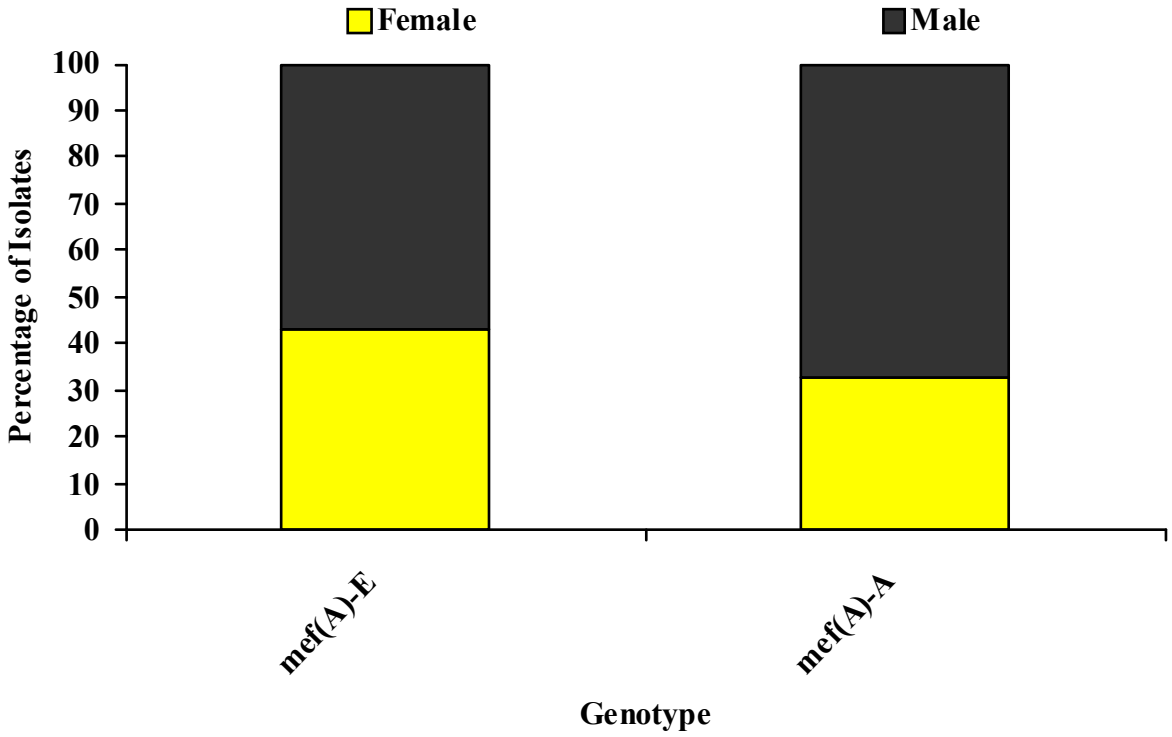

Female
Male
100
90
80
70
60
50
40
30
20
10
0
Percentage of Isolates
mef(A)-E
mef(A)-A
Genotype

Fig. 8d.

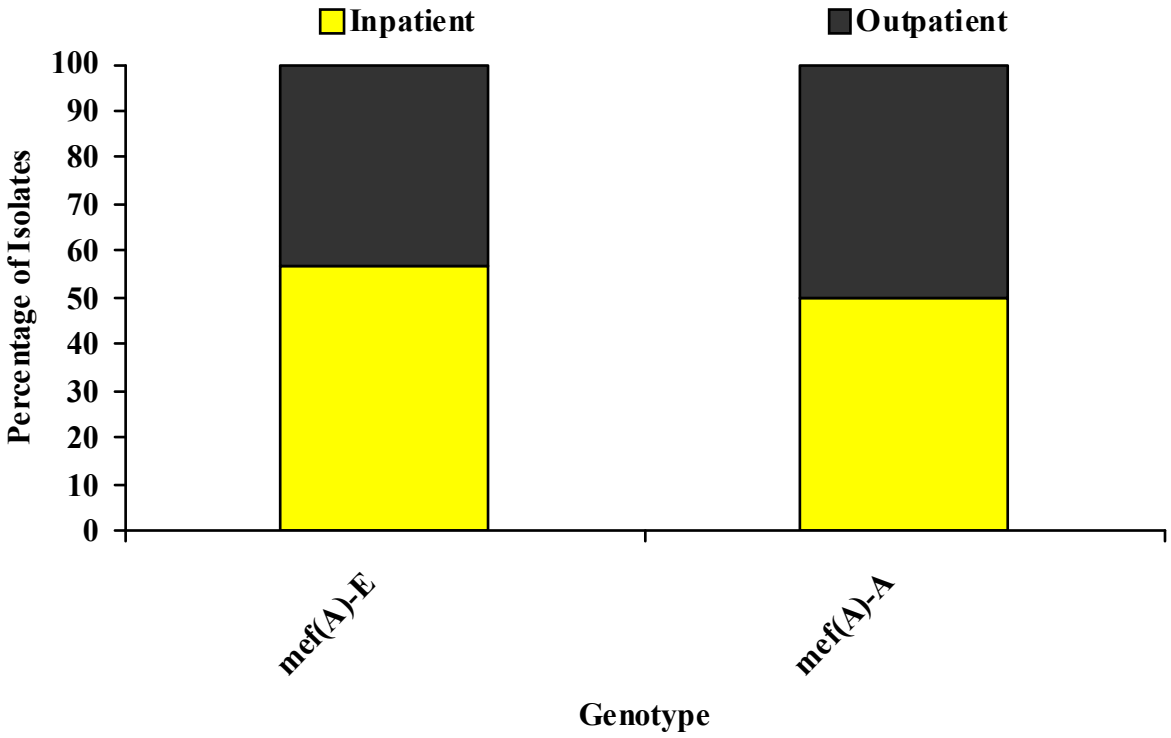

Inpatient
Outpatient
100
90
80
70
60
50
40
30
20
10
0
Percentage of Isolates
mef(A)-E
mef(A)-A
Genotype

Figure 9. Penicillin (Pen), Trimethoprim/sulfamethoxazole (T/S) and Doxycycline (Dox) susceptibility profiles among the isolates with *mef*(A)-*E* and *mef*(A)-*A* subclasses of the *mef*(A) gene. Isolates were defined as non-susceptible (NS) based on CLSI interpretative breakpoints as follows: Pen NS (oral penicillin V) 0.12- ≥ 2µg/mL; T/S NS: 1/19- ≥4/76µg/mL; Dox (Tetracycline) NS: 4- ≥8µg/mL.

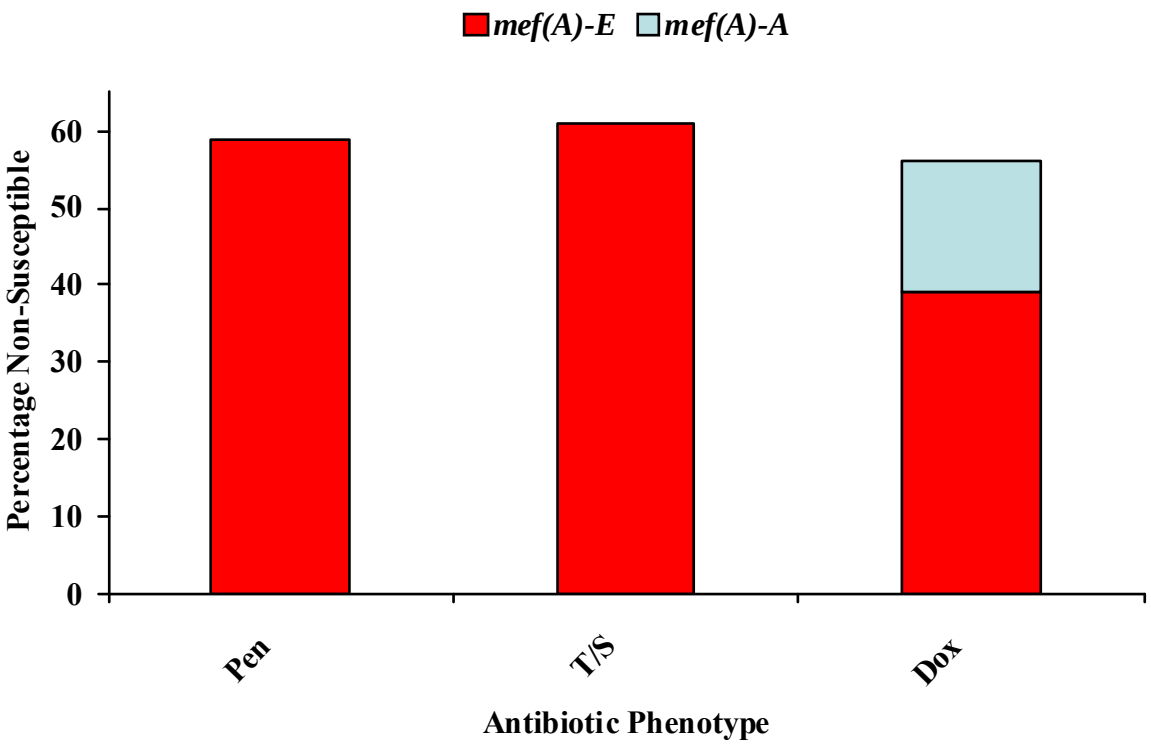


Figure 10. Serotype distribution among *S. pneumoniae* isolates with *mef*(A)-*E* and *mef*(A)-*A* subclasses of the *mef*(A) gene.

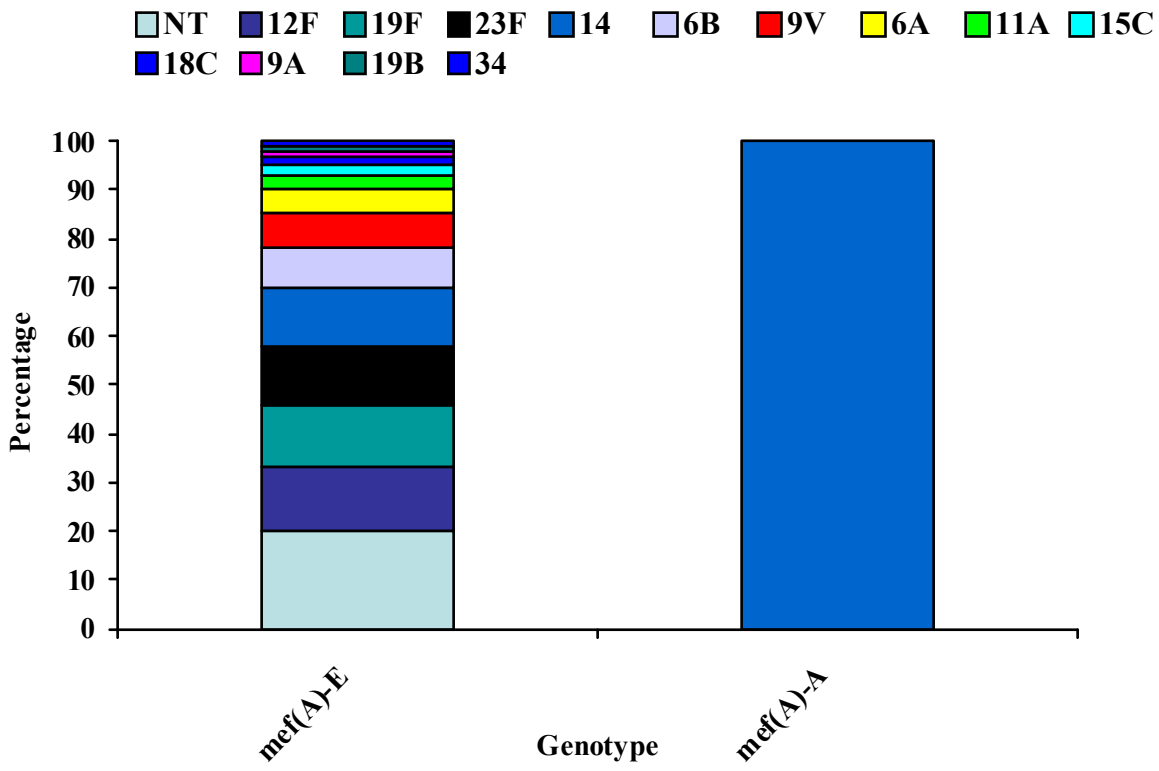
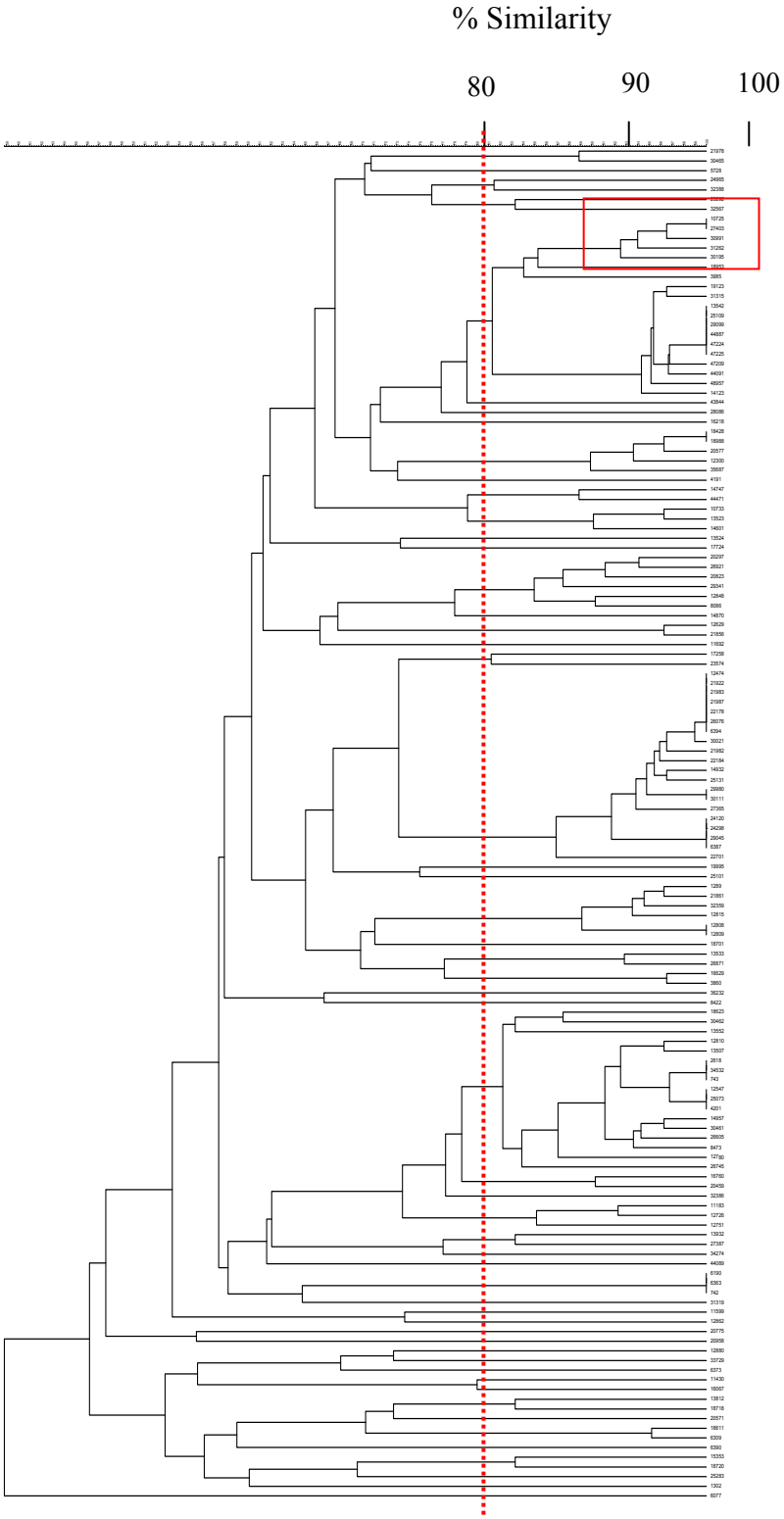


Figure 11. Dendrogram depicting genetic relatedness of the *mef*(A) class A and *mef*(A) class E isolates on the basis on PFGE results. PFGE was conducted with *Sma*I digestions. 80% similarly is indicated with a dashed line. Cluster containing the *mef*(A) class A variant is boxed.

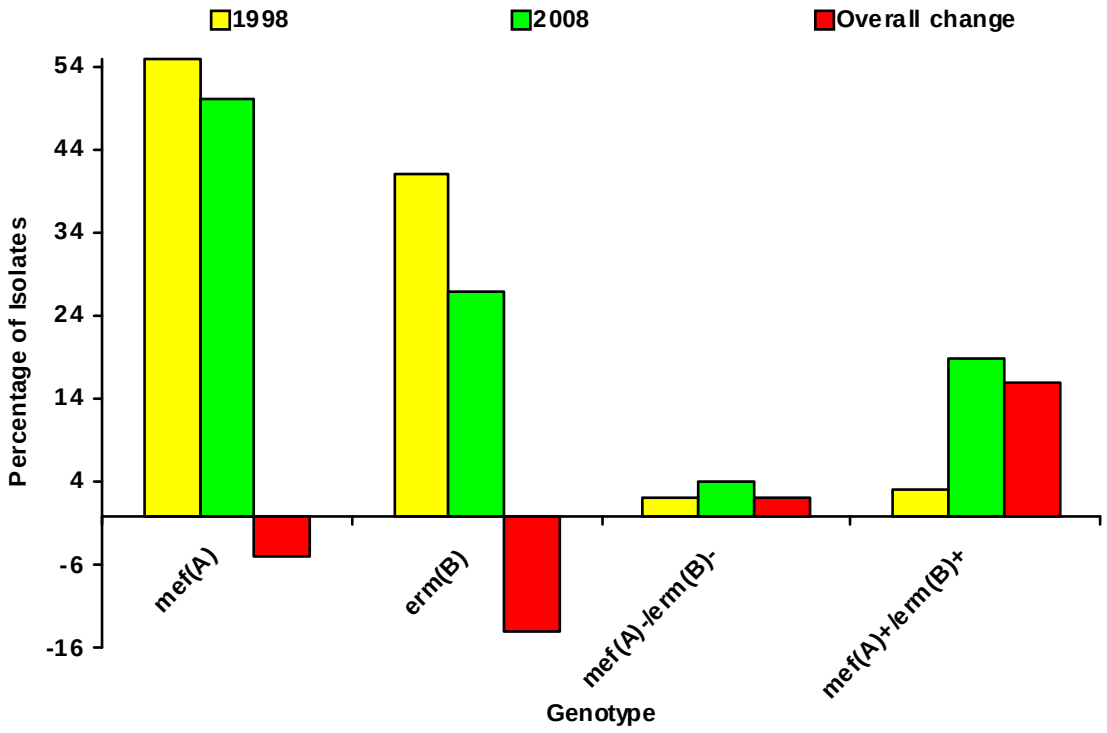
Fig.11.



Changes in the Prevalence of Macrolide Resistance Genotypes

Figure 12 shows the prevalence of macrolide resistance genotypes in 1998 and 2008 as well as the change between these two study years. Isolates carrying the *mef*(A) gene decreased 5%, from 55% (1998) to 50% (2008). Isolates carrying the *erm*(B) gene decreased 14%, from 41% (1998) to 27% (2008). Four percent increase was observed among isolates carrying neither *mef*(A) nor *erm*(B) gene from 2% (1998) to 4% (2008). Isolates carrying both *mef*(A) and *erm*(B) genes increased by 16% from 3% (1998) to 19% (2008).

Figure 12. Prevalence of macrolide resistance genotypes in the first year (1998) and the last year (2008) of the study as well as the overall change in the prevalence between these two years of the study.



Incidence and Characterization of Macrolide Resistant Isolates Carrying both *mef*(A) and *erm*(B) Genes

Figure 13 shows the percentage and the number of isolates carrying both *mef*(A) and *erm*(B) genes during each year of the study. In total, 116 isolates carrying both *mef*(A) and *erm*(B) genes were identified during this study. The percentage of these isolates varied from 0.8% (1999) to 19% (2008). The number of these isolates varied from 1 (1998) to 22 (2003, 2006).

Serotype distribution among *mef*(A) and *erm*(B) carrying isolates is shown in Figure 14. Eleven different serotypes were identified: 14, 11A, 15C, 19A, 19F, 22F, 23F, 33F, 6A, 6B, 9V, and non-typeable. The percentage of each serotype is shown in Figure 14. The 10 most common serotypes are shown in Figure 6d and they are in descending order: 19F (68), NT (18), 19A (14), 6B (3), 14 (3), 6A (2), 23F (2), 9V (1), 11A (1) and 15C (1). The number of isolates with each serotype is shown in the parenthesis. The most common serotype was 19F (59%), followed by 19A (12%). All other serotypes ranged in incidence from 1% to 3%. Sixteen percent of isolates carrying both *mef*(A) and *erm*(B) genes were non-typeable, Figure 6d.

Figure 15 shows the emergence of serotype 19A among macrolide resistant isolates carrying both *mef*(A) and *erm*(B) genes. Serotype 19A was first identified among these isolates in 2004 at 0.5% of all *mef*(A) and *erm*(B) carrying isolates. Subsequently, it was identified in 1% (2005), 4% (2007) and 8% (2008) of all *erm*(B) and *mef*(A) carrying isolates.

Among the 116 *mef*(A) and *erm*(B) carrying isolates, 68 were serotype 19F and 14 were serotype 19A. The genetic relatedness among 82 isolates carrying *mef*(A) and *erm*(B) and having either 19A or 19F serotype is presented in the dendrogram in Figure

16. There were 4 clusters (≥80% genetic relatedness) numbered 1-4 on the figure. Cluster number 1 contained 46 isolates, 39 with serotype 19F and 7 with serotype 19A. Cluster number 2 contained 6 isolates, 2 with serotype 19F and 4 with serotype 19A. Cluster 3 contained 24 isolates, 17 with serotype 19F and 7 with serotype 19A. Cluster 4 contained 2 isolates both serotypes 19F. Together all four clusters contained 95% (78/82) of 19F or 19A, *erm*(B) and *mef*(A) carrying isolates.

Antibiotic susceptibility of these genetically related *mef*(A) and *erm*(B) carrying serotype 19A or serotype 19F isolates showed a Pen non-susceptibility rate of 100%, (Pen R of 85.4%, Pen I of 14.6%); T/S non-susceptibility rate of 97.1% (T/S R of 95.7%, T/S I of 1.4%); Dox non-susceptibility rate of 30.5% (Dox R of 2.4%, Dox I of 28%) and Cd non-susceptibility rate of 97.6% (Cd R 96.3% , Cd I of 1.2%).

Figure 13. Emergence of isolates containing both *mef*(A) and *erm*(B) genes of macrolide resistance during the 1998 and 2008 study. Isolates are shown as the number and the percentage of a total isolates collected each year of the study. The number of macrolide resistant *S. pneumoniae* each year was as follows: 1998 (n=70), 1999 (n=131), 2000 (n=81), 2001 (n=120), 2002 (n=159), 2003 (n=147), 2004 (n=173), 2005 (n=233), 2006 (n=223), 2007(n=97), and 2008 (n=84).

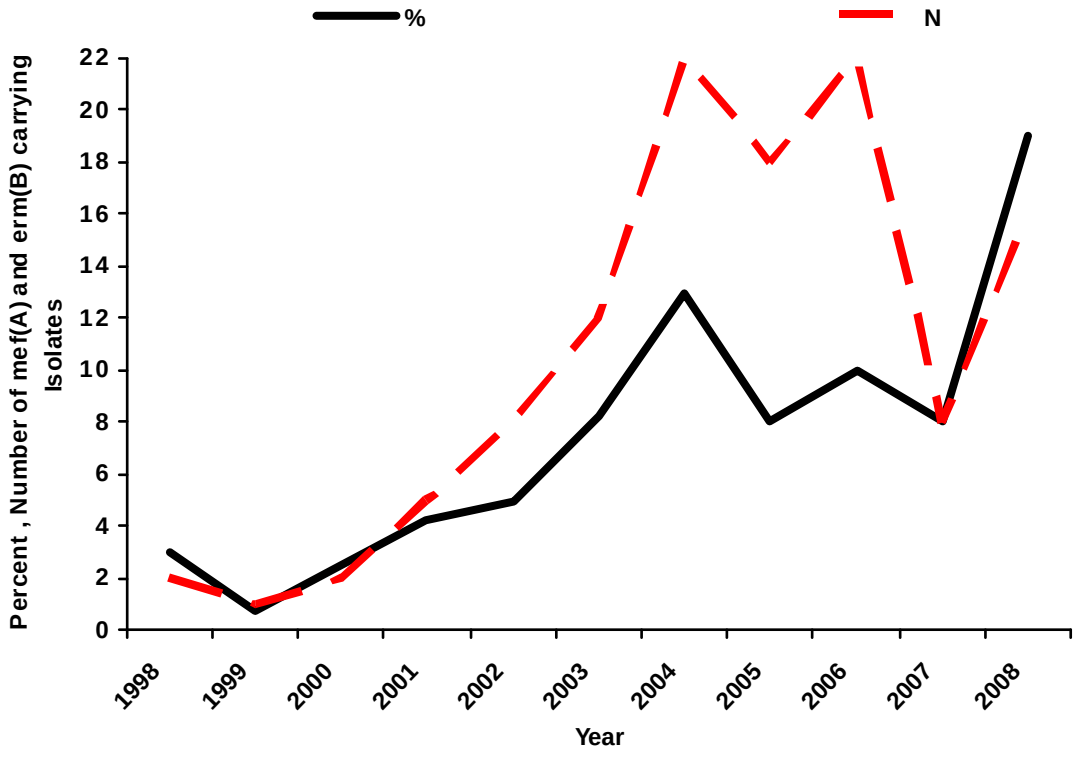


Figure 14. Serotype distributions among *S. pneumoniae* isolates carrying both *mef*(A) and *erm*(B) macrolide resistance genes during the 1998 and 2008 study.

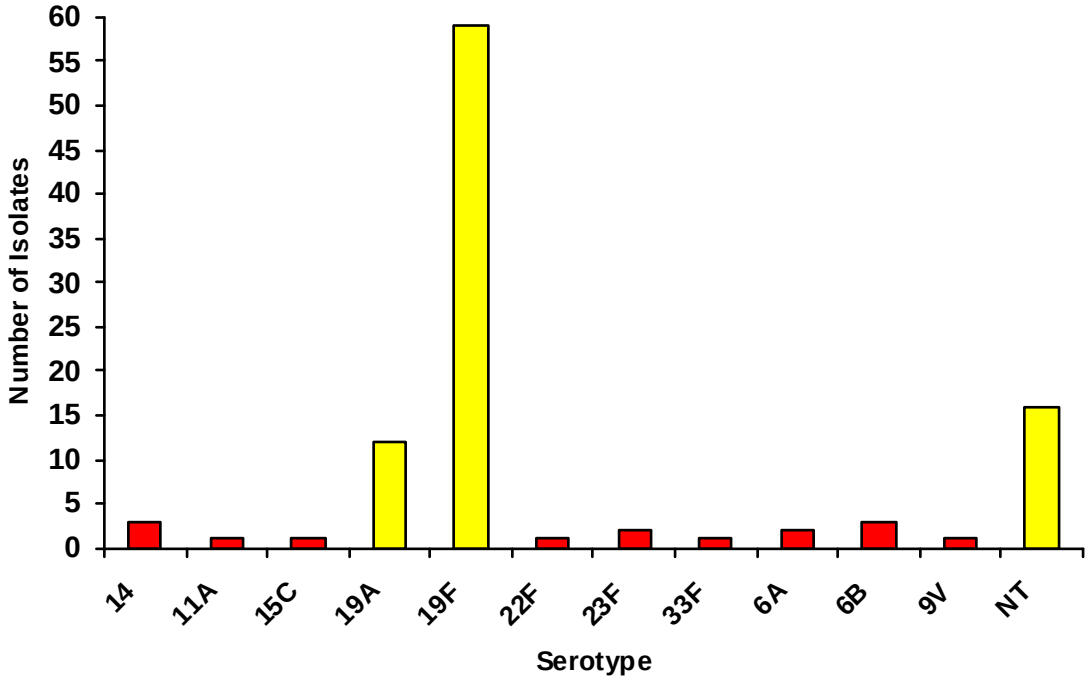


Figure 15. Emergence of serotype 19A among *S. pneumoniae* isolates carrying both *mef*(A) and *erm*(B) macrolide resistance genes. Serotype 19A is shown as a proportion of 19F and also other serotypes.

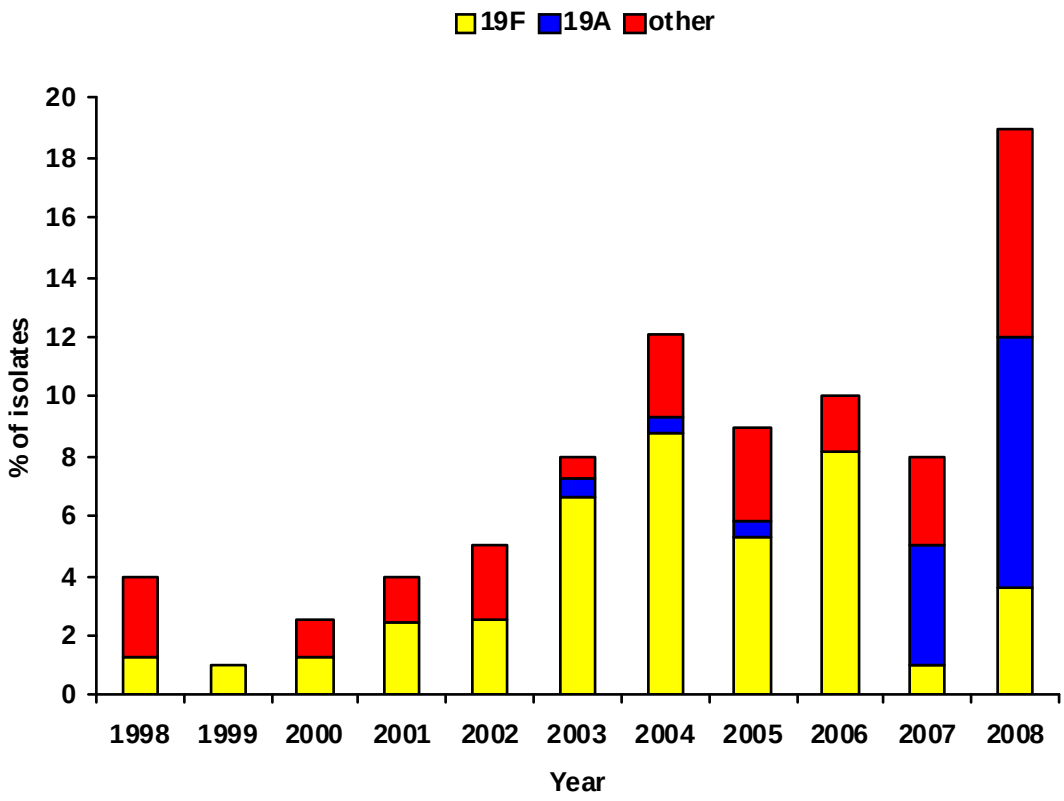
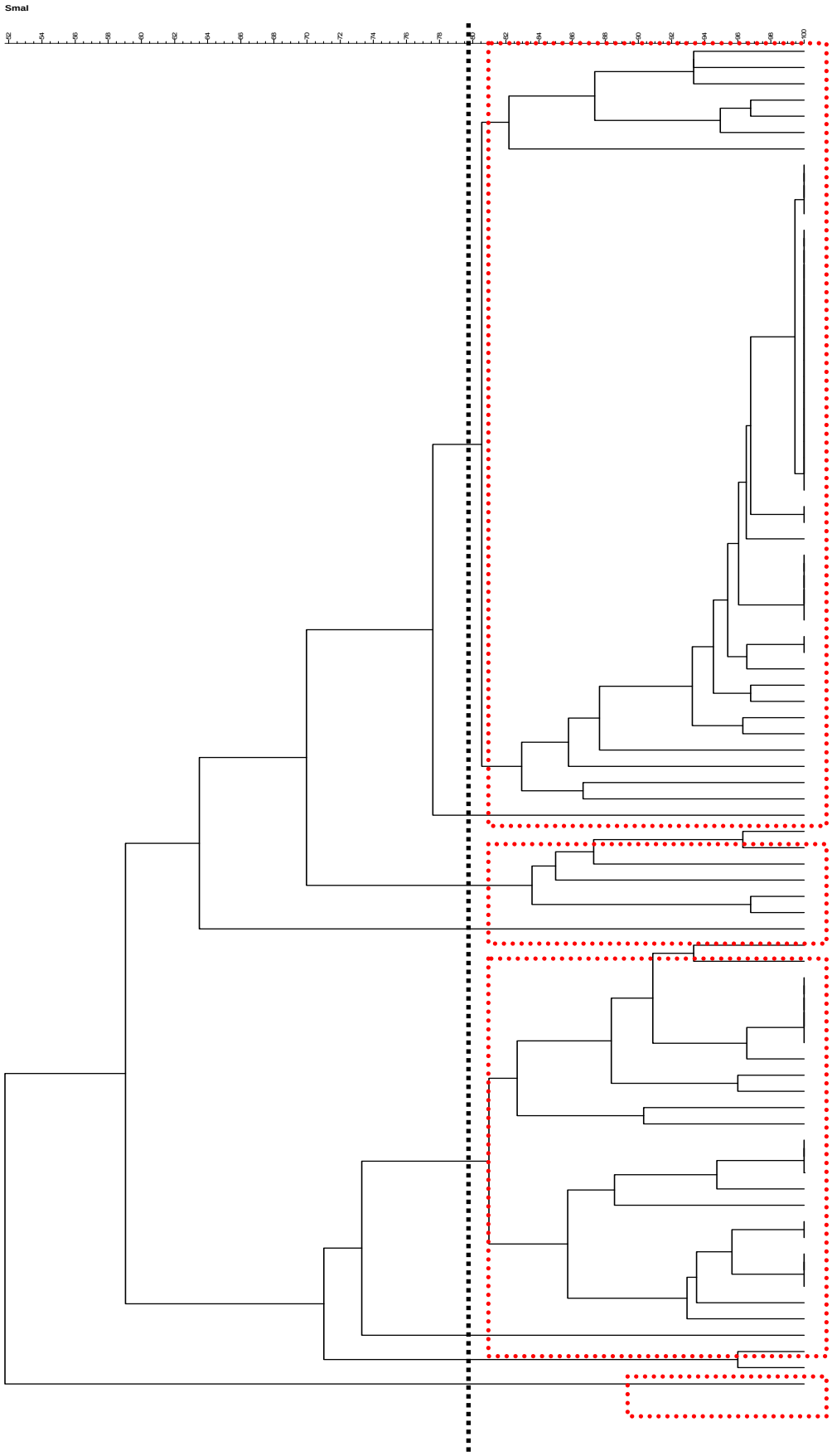


Figure 16. Dendrogram depicting genetic relatedness of the 82 (19F or 19A) *mef*(A) and *erm*(B) carrying *S. pneumoniae* isolates.


SmaI
1
2
3
4

Characterization of Macrolide-Resistant *S. pneumoniae* Isolates with Neither *mef*(A) nor *erm*(B) Genes

Out of the total of 1518 macrolide-resistant isolates, 80 did not carry either *mef(A)* or *erm*(B) genes. These isolates were further characterized to determine possible mutations in the ribosomal proteins, L4 and L22, and ribosomal rRNA. In total 53/80 (66%) isolates were identified as having ribosomal protein and/or ribosomal RNA mutation. Thirty-seven out of the 80 isolates (46%) had mutations within the 23rRNA in at least one allele, 8/80 isolates (10%) had combination of L4 and 23rRNA mutation and 8/80 (10%) had an L4 mutation alone. The results of the isolates with mutations are shown in table 3. Among 23rRNA mutation, A2059G was the most common mutation, followed by and A2058G; however A2059C and A2058T were also identified. Among L4 mutations, the most common was S20N (15 isolates). Eight out of the 15 S20N L4 mutants had an additional 23S RNA in at least one allele. One isolate had an E30K mutation with no additional 23S rRNA mutation. No mutations in the ribosomal protein L22 was found. Erythromycin MICs among isolates with L4 mutation S20N only, ranged from 1-16μg/mL, while those with combination of L4 protein S20N and at least one 23S rRNA allele mutation ranged from 16- ≥ 64μg/mL. Clindamycin MICs ranged from 0.12-4μg/mL regardless of whether the L4 S20N mutants had additional 23S rRNA mutation or not. The isolate with L4 E30K had a macrolide MIC in the range of 1-4μg/mL, and clindamycin MIC of 0.12μg/mL.

Table 2. Susceptibility profile for the 53 isolates having a ribosomal protein and/or ribosomal rRNA mutation among macrolide resistant *S. pneumoniae* isolates identified during the 1998-2008 study. Penicillin (Pen), Trimethoprim/sulfamethoxazole (T/S), Doxycycline (Dox), Erythromycin (Ery), Clarithromycin (Cla), Azithromycin (Azi), Clindamycin (Cd). Isolates were determined to be susceptible S, intermediate I, and resistant R based on CLSI breakpoints.

Antimicrobial	MIC_{50}	MIC_{90}	Range (µg/mL)			S	I	R
Pen	0.06	2	0.03	-	4	63.3%	25.3%	11.4%
T/S	0.25	4	0.12	-	16	72.1%	11.8%	16.2%
Dox	0.25	0.5	0.25	-	32	93.8%	1.3%	5.0%
Ery	16	64	1	-	64	0.0%	0.0%	100.0%
Cla	4	64	1	-	64	0.0%	0.0%	100.0%
Azi	64	64	1	-	64	0.0%	13.0%	87.0%
Cd	0.12	4	0.12	-	16	67.5%	12.5%	20.0%

Table 3. Ribosomal protein L4 and L22 as well as ribosomal 23S rRNA mutations among the macrolide resistant *S. pneumoniae* containing neither *mef*(A) nor *erm*(B) macrolide resistance genes.

		MICμg/mL							Mutations					
Year	**Stock #**	**Pen**	**T/S**	**Dox**	**Ery**	**Clar**	**Azi**	**Cd**	**L22**	**L4**	**18S**	**23S**	**30S**	**91S**
1999	13502	0.03	4	0.25	4	1	1	0.12	WT	None	None	A2059G	A2058G	A2058G
2000	21059	0.03	4	0.25	4	4	1	0.12	WT	None	None	A2059G	A2058G	A2059G
2001	23448	0.03	0.25	0.25	8	4	16	0.25	WT	None	None	A2059C	A2058G	A2059G
2001	27938	2	16	0.25	8	8	64	0.5	WT	None	None	A2059G	A2059G	A2059G
2002	30481	0.06	0.25	0.25	64	64	64	16	WT	None	None	A2058G	A2058G	A2058G
2002	30611	2	16	0.25	8	8	64	0.5	WT	None	None	A2059G	A2058G	A2058G
2002	31652	0.03	0.25	0.25	64	16	64	1	WT	S20N	None	A2058G	A2058G	A2058G
2002	33364	0.03	0.25	0.25	64	8	32	0.12	WT	None	A2058G	A2059G	A2058G	A2058G
2002	33700	0.03	0.25	0.25	64	16	64	0.25	WT	None	A2059G	A2058G	A2058G	A2058G
2002	34567	0.03	0.12	0.25	64	32	8	0.12	WT	None	A2059G	None	None	None
2002	34597	0.03	0.25	0.25	1	2	64	0.12	WT	None	None	None	A2059G	None
2002	35149	0.06	0.25	0.25	32	8	64	0.5	WT	None	None	A2059G	None	A2058G
2002	36231	1	8	1	2	1	1	0.12	WT	None	A2059G	None	None	None
2002	43850	0.06	0.12	0.25	64	64	64	4	WT	None	A2058G	A2058G	A2058G	A2058G
2002	44077	0.5	0.12	0.25	64	64	64	0.12	WT	S20N	A2058G	None	None	None
2003	47803	0.03	0.25	0.25	4	1	4	0.12	WT	None	A2058G	None	None	A2058G
2003	49001	0.03	0.25	0.25	64	32	64	2	WT	None	A2058G	A2058G	A2058G	A2058G
2003	49766	0.03	0.12	0.25	4	4	64	0.12	WT	None	None	A2059G	A2058G	A2059G
2003	49767	0.03	1	0.25	16	2	64	0.25	WT	None	None	A2059G	A2058G	A2059G
2003	50063	0.06	2	0.25	32	2	64	0.25	WT	S20N	None	None	None	A2059G
2003	50111	0.03	1	0.25	16	1	16	0.12	WT	S20N	None	A2058G	A2059G	A2059G
2003	50420	0.12	0.12	0.25	4	1	2	0.12	WT	E30K	None	None	None	None
2003	51126	0.03	0.25	0.25	64	8	64	0.12	WT	S20N	A2058G	A2059G	A2058G	A2058G
2004	52936	0.06	0.12	0.25	64	16	32	0.5	WT	None	A2059C	A2059C	A2059C	A2059C
2004	53562	0.5	4	0.25	64	8	64	0.12	WT	None	A2059G	A2059G	A2059G	None
2004	54239	0.06	0.12	0.25	32	4	64	0.5	WT	None	A2059G	A2059G	A2059G	A2059G

		MIC ug/mL							Mutations					
Year	**Stock #**	**Pen**	**T/S**	**Dox**	**Ery**	**Clar**	**Azi**	**Cd**	**L22**	**L4**	**18S**	**23S**	**30S**	**91S**
2004	55430	0.12	0.25	0.25	8	1	2	0.12	WT	None	None	A2059G	None	None
2004	55931	0.03	0.12	0.25	64	8	64	0.5	WT	None	None	A2059G	A2058G	None
2004	56283	0.03	4	0.25	32	2	64	0.25	WT	S20N	A2058G	A2058G	A2059G	A2059G
2004	56745	0.03	0.12	0.25	64	64	64	4	WT	S20N	A2059G	None	A2058G	A2058G
2005	58046	0.03	1	0.25	64	16	64	0.12	WT	S20N	A2059G	A2059G	A2059G	A2059G
2005	59280	1	0.12	0.25	64	8	64	0.12	WT	None	A2059G	A2059G	None	None
2005	59601	0.06	0.12	0.25	16	4	64	0.25	WT	None	A2059G	A2059G	None	None
2005	59636	1	0.12	0.25	32	4	64	0.12	WT	None	A2059G	A2059G	None	None
2005	59863	0.06	0.12	0.25	1	1	8	0.12	WT	None	None	None	A2059G	None
2005	60139	2	2	0.25	64	64	64	8	WT	None	None	A2058G	A2058G	A2058G
2005	60482	2	2	0.25	64	16	64	0.5	WT	None	A2059G	A2059G	None	None
2005	60496	0.03	0.12	4	16	2	2	1	WT	S20N	None	None	None	None
2005	62071	0.03	0.12	0.25	8	2	64	0.12	WT	None	A2059G	A2059G	None	None
2005	63551	0.03	0.12	0.25	4	2	4	0.12	WT	S20N	None	None	None	None
2005	63693	0.03	0.12	0.25	32	2	64	0.12	WT	None	None	None	None	None
2005	64257	0.5	0.12	0.25	8	4	1	4	WT	S20N	None	None	None	None
2005	65835	0.03	0.12	0.25	16	8	64	0.5	WT	None	A2058T	A2058T	A2058T	None
2005	66330	0.03	0.25	0.25	32	8	64	1	WT	None	None	A2059G	None	None
2006	67091	0.06	0.12	0.25	64	8	64	0.5	WT	None	None	None	A2059G	None
2006	67127	0.03	0.12	0.25	32	8	64	0.5	WT	None	None	A2059G	A2059G	None
2006	67289	0.06	0.12	0.5	16	2	64	0.12	WT	None	A2059G	A2059G	A2059G	None
2006	67634	0.03		0.25	1	1	2	0.12	WT	S20N	None	None	None	None
2006	68048	0.12	0.12	0.25	1	1	1	0.12	WT	S20N	None	None	None	None
2006	68119	0.06	0.12	0.25	16	4	64	0.25	WT	None	None	None	A2059G	A2059G
2006	68224	0.06	0.12	0.25	4	1	4	0.12	WT	S20N	None	None	None	None
2006	69050	0.03	0.25	0.25	16	2	64	0.12	WT	None	A2059G	A2059G	A2059G	None
2008	81790	2	4	1	8	4	1	0.25	WT	S20N	None	None	None	None

Inducible Clindamycin Resistance

Twenty-seven out of 542 (5%) *erm*(B) carrying *S. pneumoniae* isolates tested positive for double disk diffusion for erythromycin and clindamycin test (D-test). The demographic and antibiotic susceptibility profile for these isolates is shown in Table 4. These isolates were from all regions of Canada: BC/AB (6), SK/MB (4), ON (4), QC (8), and Maritime provinces (5). Majority of isolates were from adult (18-64), 17/27 (63%) and elderly (≥65), 7/27 (26%). Female to male ratio was 17:10, and outpatient to inpatient ratio was 13:14.

Among all these isolates erythromycin MICs ranged from 1-64µg/mL and clindamycin MICs ranged from 0.12-0.5µg/ml. Erythromycin and Clindamycin MIC_{50} and MIC_{90} were 16µg/mL and 64µg/mL and 0.25µg/mL and 0.5µg/mL, respectively. None of these isolates were resistant to clindamycin, 11.1% were intermediate and 88.9% were susceptible by broth microdilution antimicrobial susceptibility testing. The susceptibility profile for penicillin, T/S, and doxycycline is shown in Table 5.

Table 4. Demographic and antimicrobial susceptibility data for the 27 inducible clindamycin resistant *S. pneumoniae* identified during the 1998-2008 study. Penicillin (Pen), Trimethoprim/sulfamethoxazole (T/S), Doxycycline (Dox), Erythromycin (Ery), Clarithromycin (Cla), Azithromycin (Azi), Clindamycin (Cd). Isolates were determined to be susceptible S, intermediate I, and resistant R based on CLSI breakpoints.

Demographic Data						Antibiotic susceptibility profile MIC µg/mL							
Year	**#**	**Region**	**Age**	**Sex**	**In/Out**	**Pen**	**T/S**	**Dox**	**Ery**	**Clar**	**Azi**	**Cd**	**genotype**
1999	15080	QB	≥65	M	In	0.12	0.12	8	16	16	32	0.25	*erm*(B)
2000	18080	QB	18-64	F	Out	0.5	4	8	16	64	64	0.25	*erm*(B)
2001	23725	QB	18-64	F	Out	1	2	16	16	16	16	0.12	*erm*(B)
2002	31259	QB	≥65	M	Out	0.03	2	0.25	64	64	64	0.12	*erm*(B)
2002	31716	Maritime	18-64	F	Out	0.5	16	1	8	8	4	0.12	*erm*(B)
2002	32537	QB	18-64	F	Out	4	1	4	64	64	64	0.12	*erm*(B)
2002	35161	ON	≥65	F	Out	0.12	0.5	0.25	1	64	64	0.5	*erm*(B)
2002	39528	BC/AB	18-64	F	In	0.25	8	4	16	16	8	0.25	*erm*(B)
2002	39531	BC/AB	18-64	F	In	0.25	8	4	16	16	8	0.25	*erm*(B)
2002	44000	QB	5-17	F	Out	2	4	4	16	16	32	0.25	*erm*(B)
2003	47813	QB	18-64	M	Out	2	4	1	16	16	4	0.25	*erm*(B)
2003	49895	ON	≥65	F	Out	0.06	4	1	64	16	64	0.25	*erm*(B)
2004	52567	Maritime	U	F	In	0.03	0.12	8	64	16	64	0.12	*erm*(B)
2004	53567	SK/MB	18-64	M	Out	1	0.12	0.25	8	1	4	0.5	*erm*(B)
2004	56233	QB	≥65	M	In	2	4	8	64	8	64	0.25	*erm*(B)
2005	58660	ON	18-64	F	In	0.06	1	1	16	16	4	0.25	*erm*(B)
2005	58840	BC/AB	18-64	F	Out	0.5	8	4	64	64	64	0.12	*erm*(B)
2005	61299	Maritime	≥65	F	In	0.25	1	1	64	64	64	0.5	*erm*(B)
2005	64009	BC/AB	18-64	M	In	0.03	0.25	0.5	2	1	8	0.12	*erm*(B)
2005	65434	SK/MB	18-64	F	In	0.12	0.25	2	64	64	64	0.12	*erm*(B)
2005	65814	Maritime	18-64	F	In	0.25	8	2	16	16	8	0.25	*erm*(B)
2005	65876	BC/AB	18-64	F	In	0.5	0.25	8	16	16	2	0.12	*erm*(B)
2006	68642	ON	18-64	M	Out	0.06	0.12	8	64	64	64	0.25	*erm*(B)
2006	68872	BC/AB	≥65	M	Out	0.5	0.5	16	64	64	64	0.25	*erm*(B)
2007	73838	SK/MB	18-64	M	In	0.03	0.25	4	16	16	16	0.25	*erm*(B)
2008	82586	SK/MB	5-17	F	In	0.03	0.25	1	16	8	16	0.12	*erm*(B)
2008	83963	Maritime	18-64	M	In	0.25	8	4	16	16	16	0.25	*erm*(B)

Table 5. Susceptibility profile for the 27 inducible clindamycin resistant *S. pneumoniae* isolates identified during the 1998-2008 study. Penicillin (Pen), Trimethoprim/sulfamethoxazole (T/S), Doxycycline (Dox), Erythromycin (Ery), Clarithromycin (Cla), Azithromycin (Azi), Clindamycin (Cd). Isolates were determined to be susceptible S, intermediate I, and resistant R based on CLSI breakpoints.

Antibiotic	MIC_{50}	MIC_{90}	Range (µg/mL)			S	I	R
Pen	0.25	2	0.03	-	4	29.6%	55.6%	14.8%
T/S	1	8	0.12	-	16	44.0%	20.0%	36.0%
Dox	4	8	0.25	-	16	44.4%	25.9%	29.6%
Ery	16	64	1	-	64	0.0%	0.0%	100.0%
Cla	16	64	1	-	64	0.0%	0.0%	100.0%
Azi	32	64	2	-	64	0.0%	0.0%	100.0%
Cd	0.25	0.5	0.12	-	0.5	88.9%	11.1%	0.0%

Part II: Serotype Distribution

Forty one different serotypes were found among the 1071 serotyped macrolide resistant *S. pneumoniae*. These serotypes were: 3(10), 4(1), 6A(67), 6B(158), 6C(7), 7F(1), 8(1), 9A(7), 9L(1), 9N(6), 9V(48), 11A(30), 12B(1), 12F(39), 14(83), 15A(40), 15B(30), 15C(21), 15F(1), 16F(1), 17F(1), 18B(1), 18C(7), 19A(44), 19B(1), 19F(171), 20(1), 21(1), 22F(10), 23A(3), 23B(2), 23F(122), 29(2), 31(1), 33(1), 33B(10), 33F(20), 34(4), 35A(4), 35B(10) 35F(4), and non-typeable (NT)(102). The number of isolates with each serotype is indicated in the parentheses, while the percentage is shown in Figures 17a and 17b. Figure 17a shows the top 20 serotypes found among macrolide-resistant SPN, while Figure 17b shows the remaining 21 serotypes. Serotype 19F was the most prevalent serotype making up 16% of all macrolide resistant SPN, followed by serotype 6B (15%) and serotype 23F (11%).

Figure 17. Percentage of the (a) top 20 serotypes and (b) the remaining 21 serotypes found among 1071 macrolide resistant *S. pneumoniae* serotyped during 1998 and 2008 CROSS and CANWARD studies. Serotypes included in the PCV7 vaccine are designated as PCV7 serotypes and are indicated in red color. Non-PCV7 serotypes are shown in yellow.

Fig. 17a.

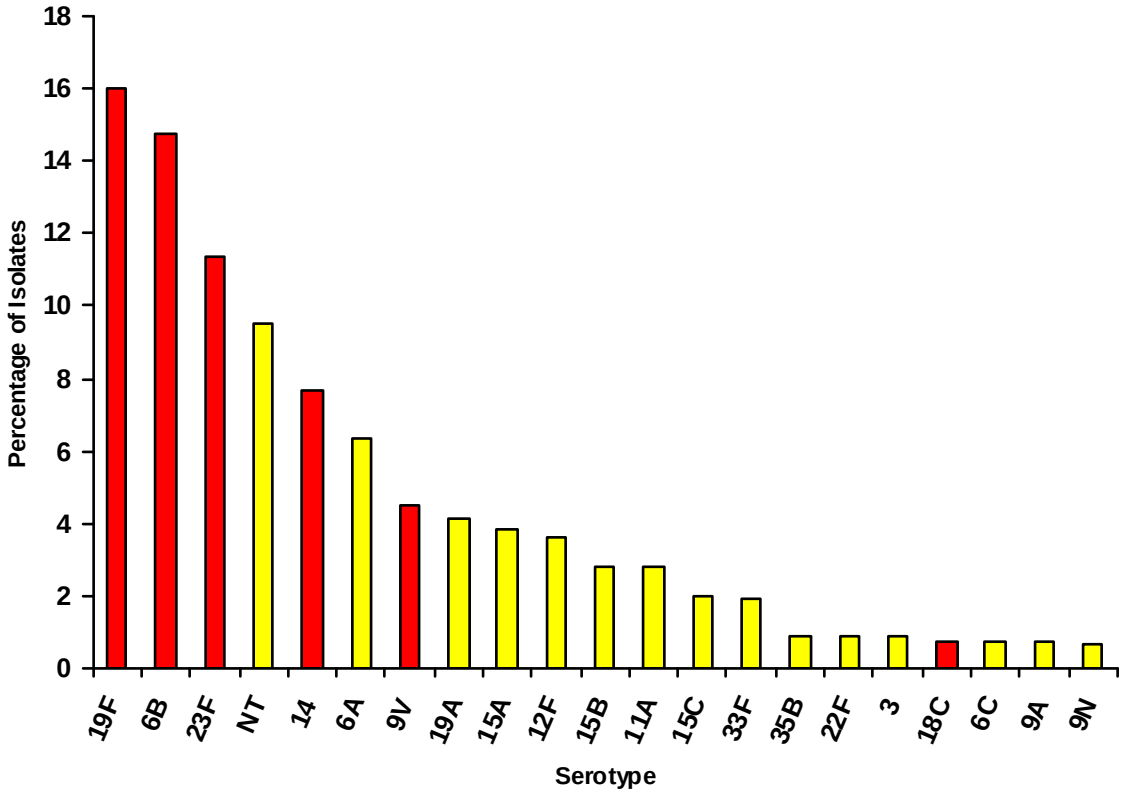
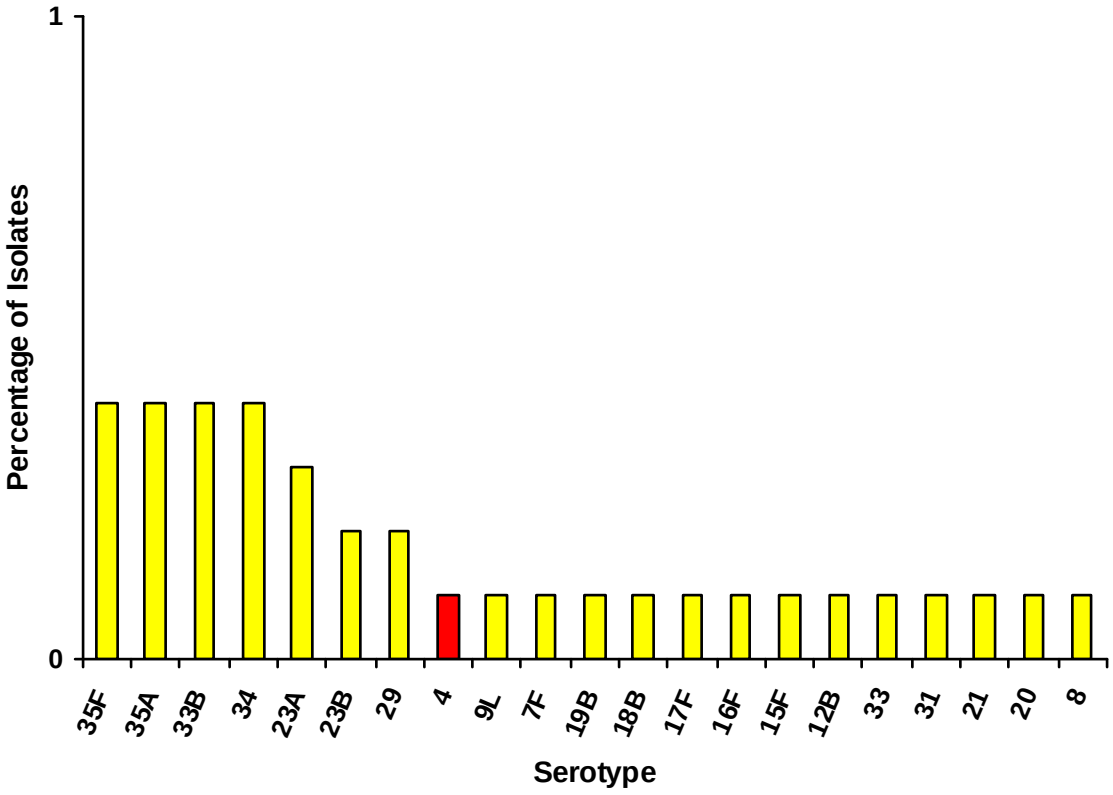

Percentage of Isolates
18
16
14
12
10
8
6
4
2
0
19F
6B
23F
NT
14
6A
9V
19A
15A
12F
15B
11A
15C
33F
35B
22F
3
18C
6C
9A
9N
Serotype

Fig. 17b.


1
0
Percentage of Isolates
35F
35A
33B
34
23A
23B
29
4
9L
7F
19B
18B
17F
16F
15F
12B
33
31
21
20
8
Serotype

Distribution and Characterization of PCV7 serotypes

Five hundred and eighty two isolates (54%) had PCV7 serotype (4, 6B, 9V, 14, 18 C, 19F, and 23F) and are shown in red in Figures 17a and 17b. All of the PCV7 vaccine serotypes except serotype 4 and 18C were among the top 10 serotypes found among macrolide resistant SPN. Changes in the number of the PCV7 serotypes by year are shown in Figure 18. Most PCV7 serotypes were found to varying degrees in each year of the study. Serotype 18C was not found in 2001, 2002, 2006, and 2007, while serotype 4 was only found in 2004 and not in any other year of the study.

Isolates with PCV7 serotypes were predominantly found among adult (18-64) and elderly (≥65) populations, Figure 19a. All isolates with PCV7 serotypes were found among all five age groups with the exception of isolates with PCV7 serotype 18C which were found only among adult (18-64) and elderly (≥65), population. Among pediatric isolates with PCV7 serotypes the majority came from the <2 age group with the exception of isolates with serotype 9V which were more common among age group 2-4 and 5-17.

Distribution of isolates with PCV7 serotypes by region is shown in Figure 19b. Isolates with PCV7 serotypes were isolated from all the five regions of Canada with the exception of isolates with serotype 18C which were predominantly from the Maritime region. Distribution of isolates with PCV7 serotypes among different macrolide resistance genotypes is shown in Figure 19c. Isolates with serotype 6B and 23F were more common among *erm*(B) carrying isolates, while 9V and 14 were more common among *mef*(A) harboring isolates. Isolates with serotype 18C were predominantly found among *mef*(A) containing isolates. Isolates with serotype 19F were most commonly found among isolates containing both *mef*(A) and *erm*(B).

Distribution of isolates with PCV7 serotypes by gender and patient status is shown in Figures 19d and 19e, respectively. Male gender and inpatient patient status were more common among isolates with PCV7 serotypes.

Percentage of isolates with PCV7 serotypes at the beginning of the study in 1998 and at the end of the study in 2008 as well as overall change between these two study years is shown in Figure 20. Isolates with 5 (6B, 9V, 14, 19F, and 23F) of the 7 PCV7 serotypes showed a decrease in the range of 5% (6B) to 16% (19F) from 1998 to 2008. Prevalence of isolates with serotype 18C remained unchanged, and isolates with serotype 4 were not assessed as only one isolate was identified throughout the study.

The prevalence of PCV7 serotypes in 1998 and in 2008 as well as overall change between these two years of the study by age group is shown in Figure 21. A decrease in the prevalence of all PCV7 serotypes was found among age groups: <2 (12%), 2-4 (2%), and ≥65 (7%). The prevalence of PCV7 serotypes increased among age groups: 5-7 (9%) and 18-64 (14%). Figures 22a - 22e show changes in the prevalence of individual PCV7 serotypes: 6B, 9V, 14, 19F and 23F by age group between 1998 and 2008 study year. Among isolates with serotype 6B, decreases of 16% and 33% were noted among age groups: ≥65 and <2 and increases of 16% and 33% were noted among age groups: 18-64 and 5-17, Figure 22a. Among isolates with serotype 9V, a decrease of 33% and an increase of 33% were noted for age groups: ≥65 and 18-64, respectively, Figure 22b. Among isolates with serotype 14, a decrease of 10% and 25% among age groups: 18-64 and <2, respectively and an increase of 35% among age group ≥65 years old, was observed, Figure 22c. Figure 22d shows a decrease in isolates with serotype 19F for age groups: <2 (8%) and 2-4 (8%) and ≥65 (21%), respectively and an increase in isolates with serotype 19F of 5% among age group 5-17 and of 32% among age group 18-64.

Figure 22e shows a decrease of isolates with serotype 23F of 10% among age group 18-64 and increase of 10% among age group ≥65.

Figure 23 shows the prevalence of isolates with PCV7 serotypes pre (1998-2000), during (2001-2004) and post (2005-2006) PCV7 vaccine introduction in Canada as well as overall change between pre and post vaccine introduction periods. Isolates with 6 (6B, 9V, 14, 18C, 19F, and 23F) out of the 7 serotypes showed an overall decrease in the range of 2% (18C) to 11% (23F).

Figure 18. Prevalence of 582 PCV7 (4, 6B, 9V, 14, 18C, 19F, and 23F) serotypes during the 1998-2008 CROSS and CANWARD studies by year.

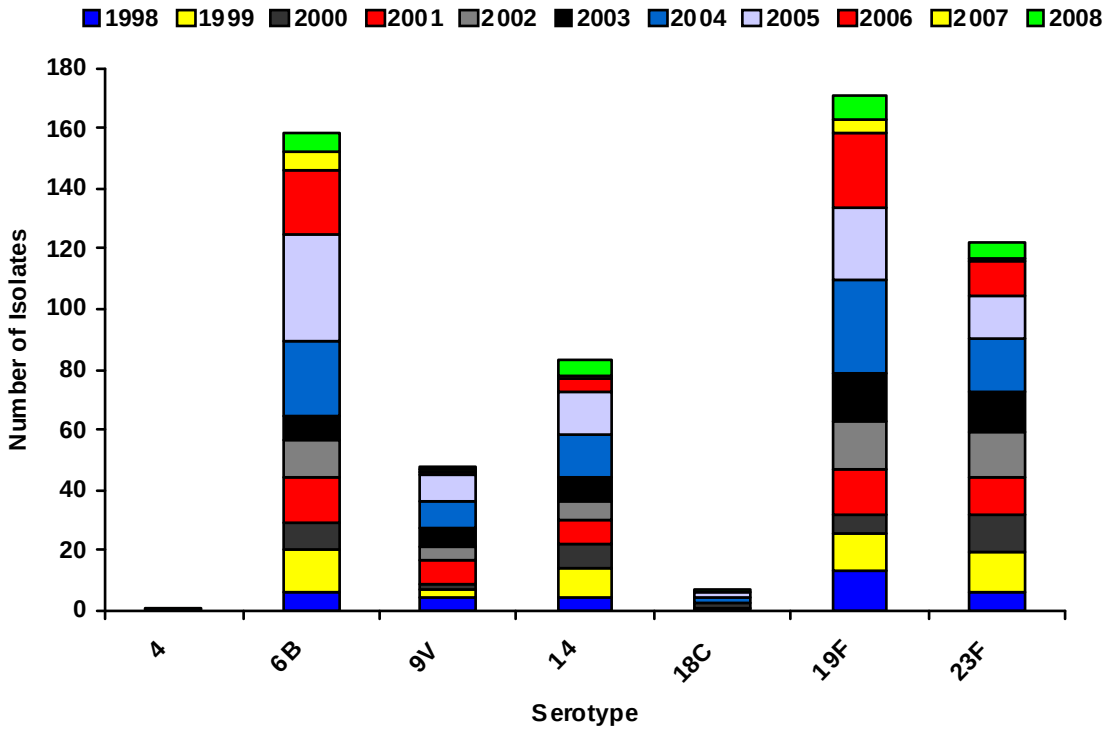


Figure 19. Prevalence of 582 PCV7 (4, 6B, 9V, 14, 18C, 19F and 23F) serotypes by (a) age group (b) region (c) genotype (d) gender (e) patients status. Age groups are as follows: pediatric: <2, 2-4, 5-17, adult: 18-64, and elderly: ≥65. Regions are as follows BC/AB (British Columbia and Alberta), SK/MB (Saskatchewan and Manitoba), ON (Ontario), QC (Quebec), and the Maritime provinces (Nova Scotia, New Brunswick, Prince Edward Island). Patient status defined as inpatient or outpatient.

Fig. 19a.

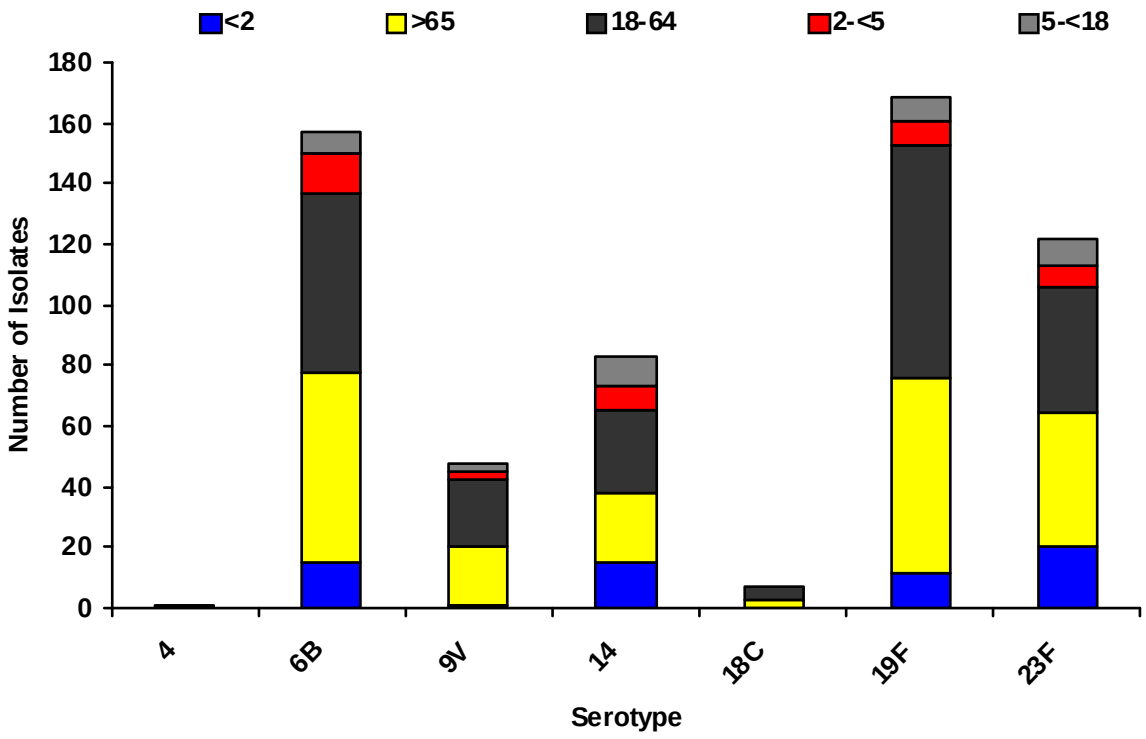

<2
>65
18-64
2-<5
5-<18
Number of Isolates
180
160
140
120
100
80
60
40
20
0
4
6B
9V
14
18C
19F
23F
Serotype

Fig. 19b.

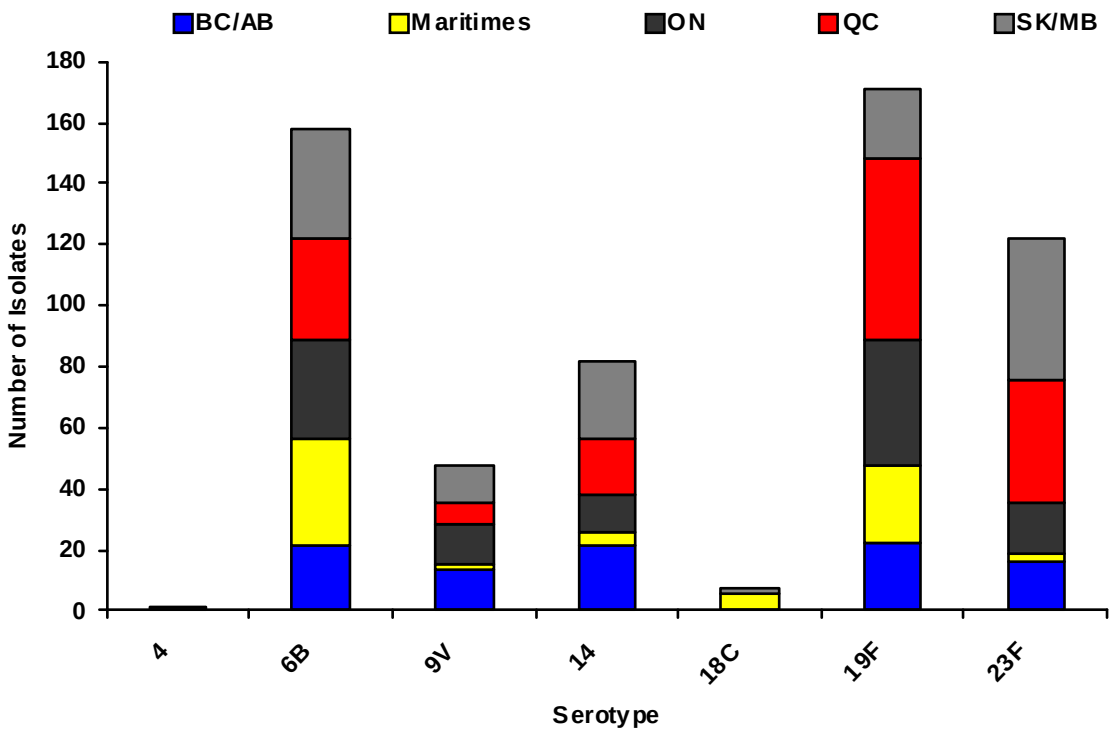

BC/AB
Maritimes
ON
QC
SK/MB
Number of Isolates
180
160
140
120
100
80
60
40
20
0
4
6B
9V
14
18C
19F
23F
Serotype

Fig. 19c.

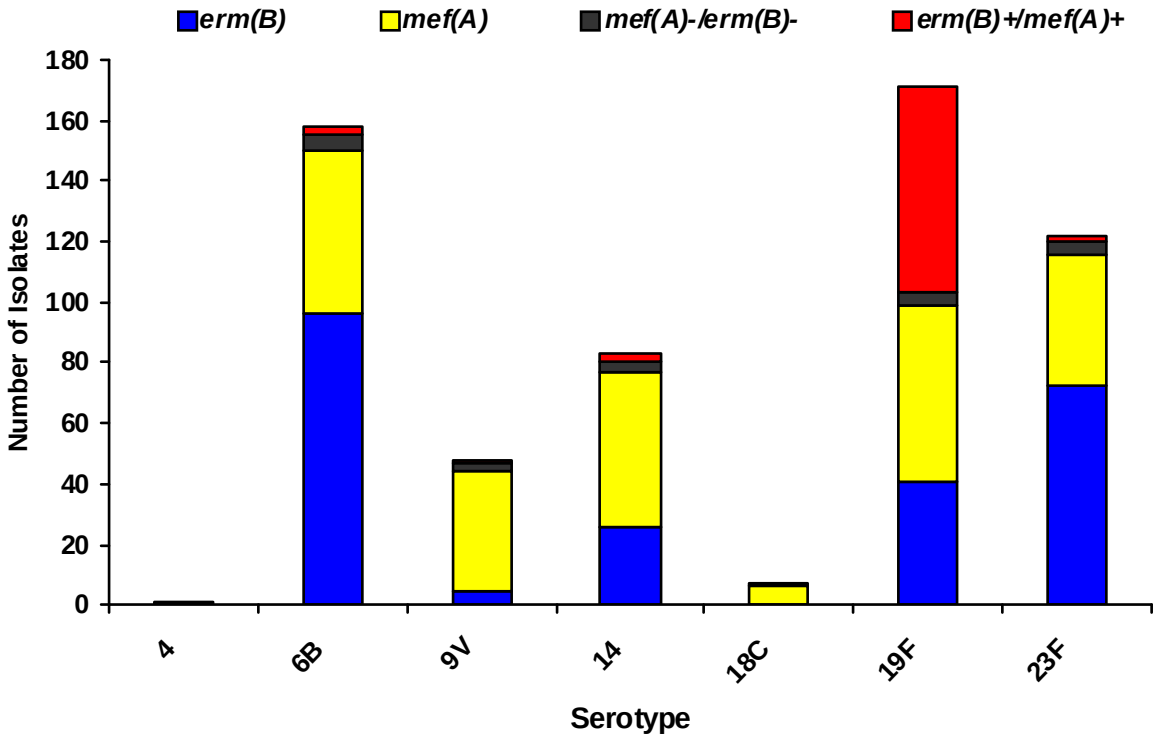

erm(B)
mef(A)
mef(A)-/erm(B)-
erm(B)+/mef(A)+
Number of Isolates
180
160
140
120
100
80
60
40
20
0
4
6B
9V
14
18C
19F
23F
Serotype

Fig. 19d.

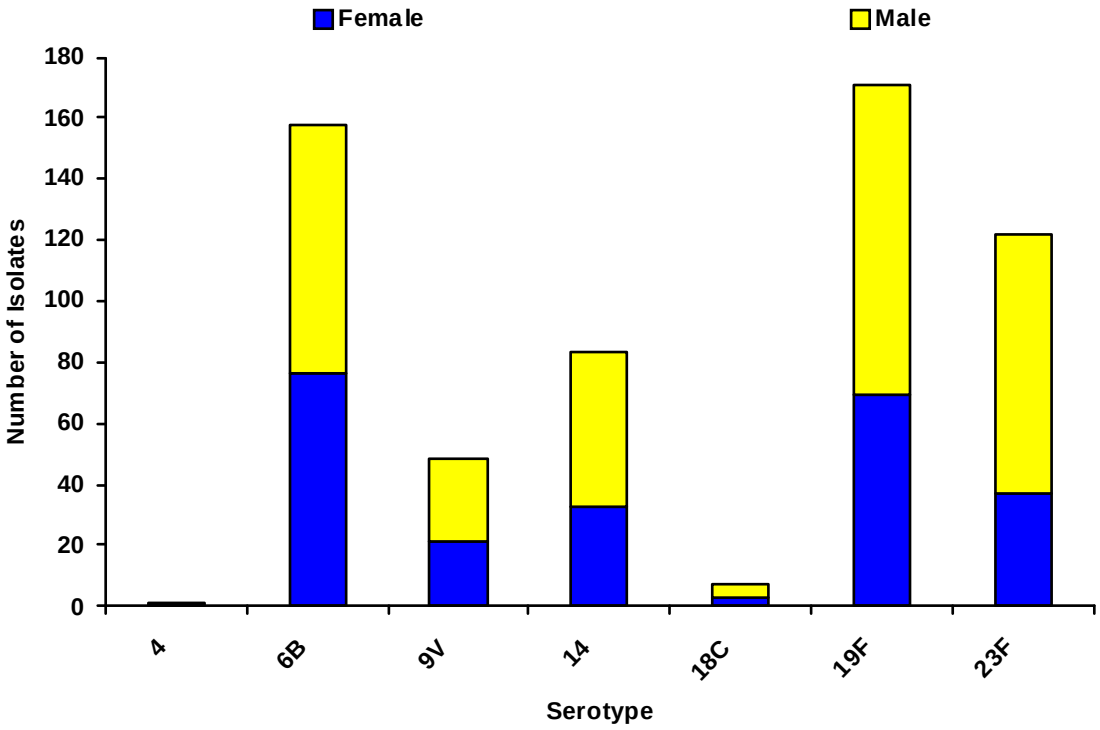

Female
Male
180
160
140
120
100
80
60
40
20
0
Number of Isolates
4
6B
9V
14
18C
19F
23F
Serotype

Fig. 19e.

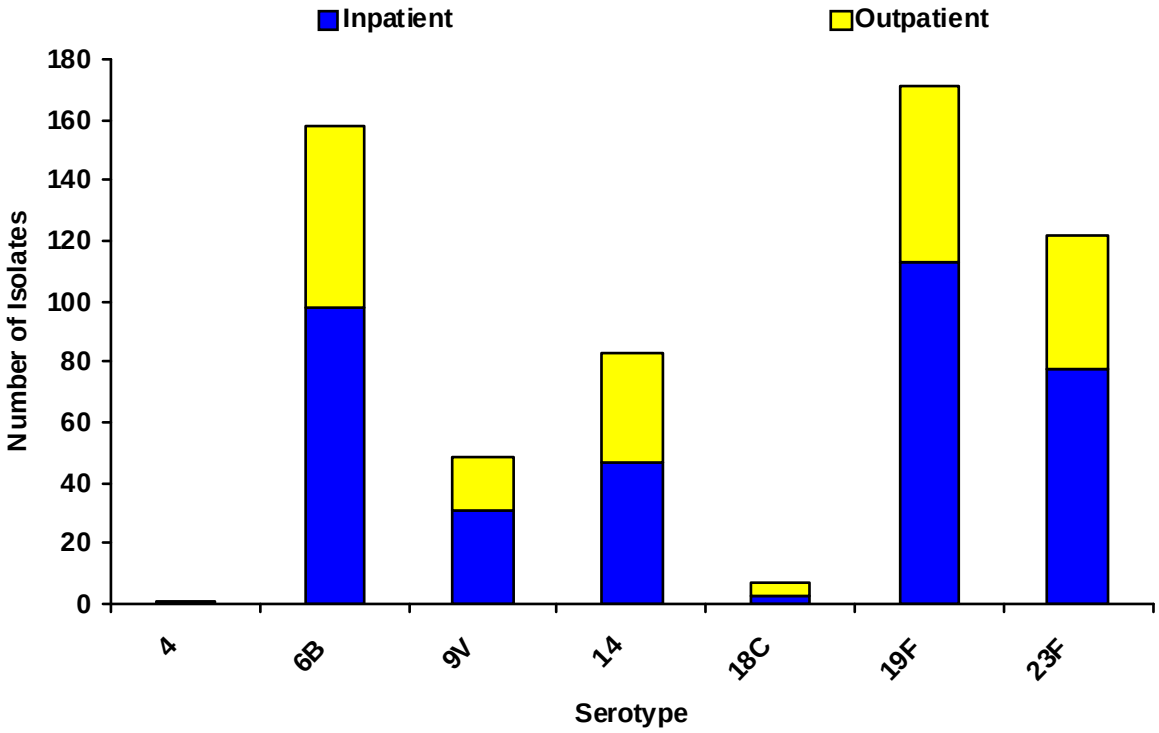

Inpatient
Outpatient
Number of Isolates
180
160
140
120
100
80
60
40
20
0
4
6B
9V
14
18C
19F
23F
Serotype

Figure 20 Prevalence of the PCV7 (6B, 9V, 14, 18C, 19F, and 23F) serotypes in the first year of the study (1998) and the last year (2008) of the study as well as the overall change between these two years of the study. Overall change defined as the percent increase or decrease between these two years.

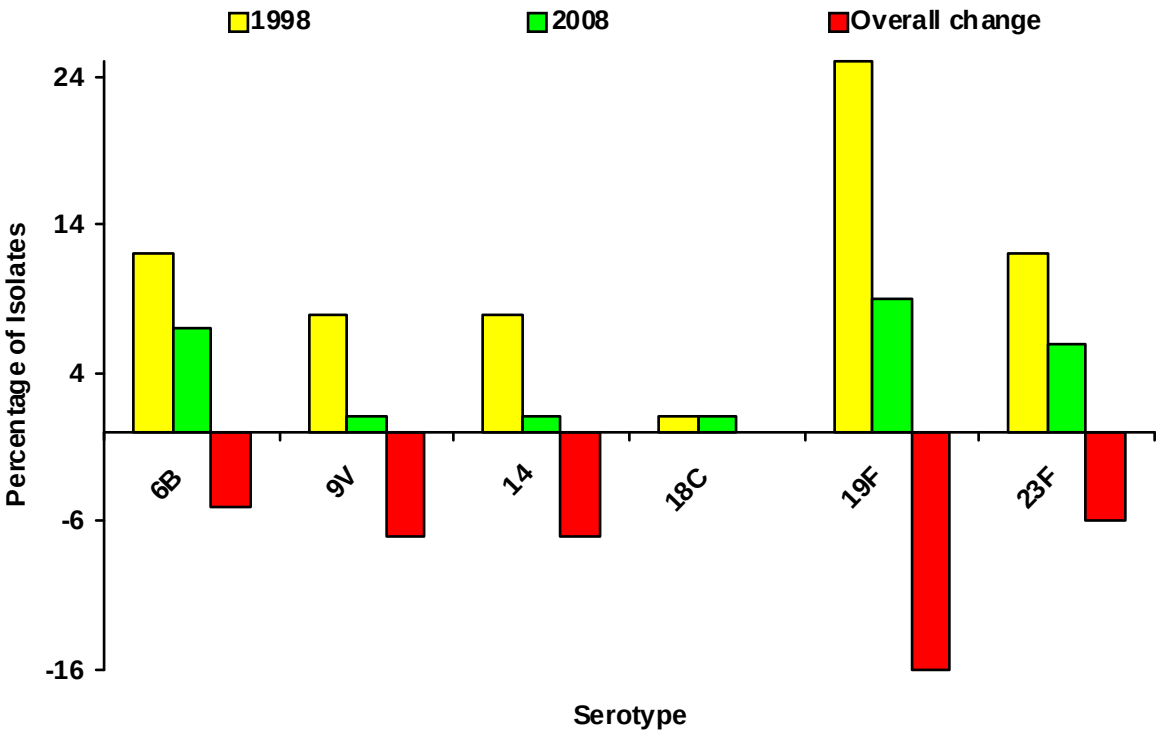


Figure 21. Prevalence of the PCV7 serotypes in the first year of the study (1998) and the last year of the study (2008) as the overall change between these two years among different age groups. Age groups defined as: pediatric <2, 2-<5, and 5-<18, adult 18-64 and elderly ≥ 65 years old.

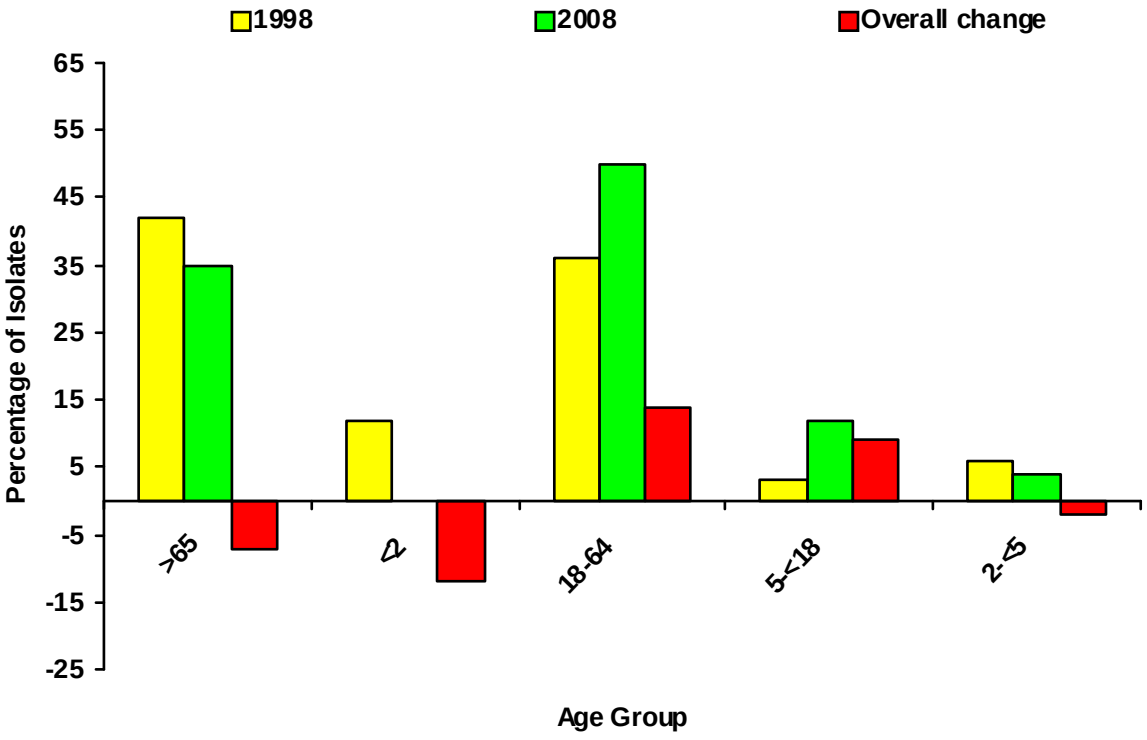


Figure 22. Prevalence of (a) 6B (b) 9V (c) 14 (d) 19F and (e) 23F serotype in the first year of the study (1998) and the last year of the study (2008) as well as the overall change between these two years among different age groups.

Fig. 22a.

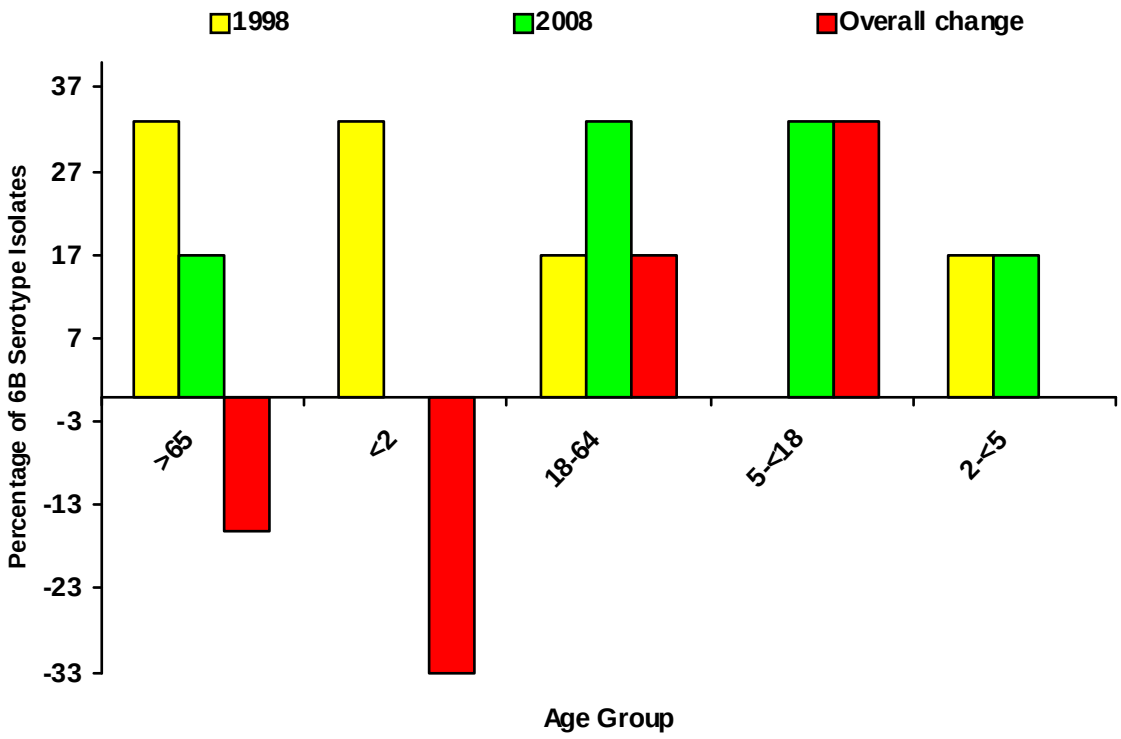

1998
2008
Overall change
37
27
17
7
-3
-13
-23
-33
Percentage of 6B Serotype Isolates
>65
<2
18-64
5-<18
2-<5
Age Group

Fig. 22b.

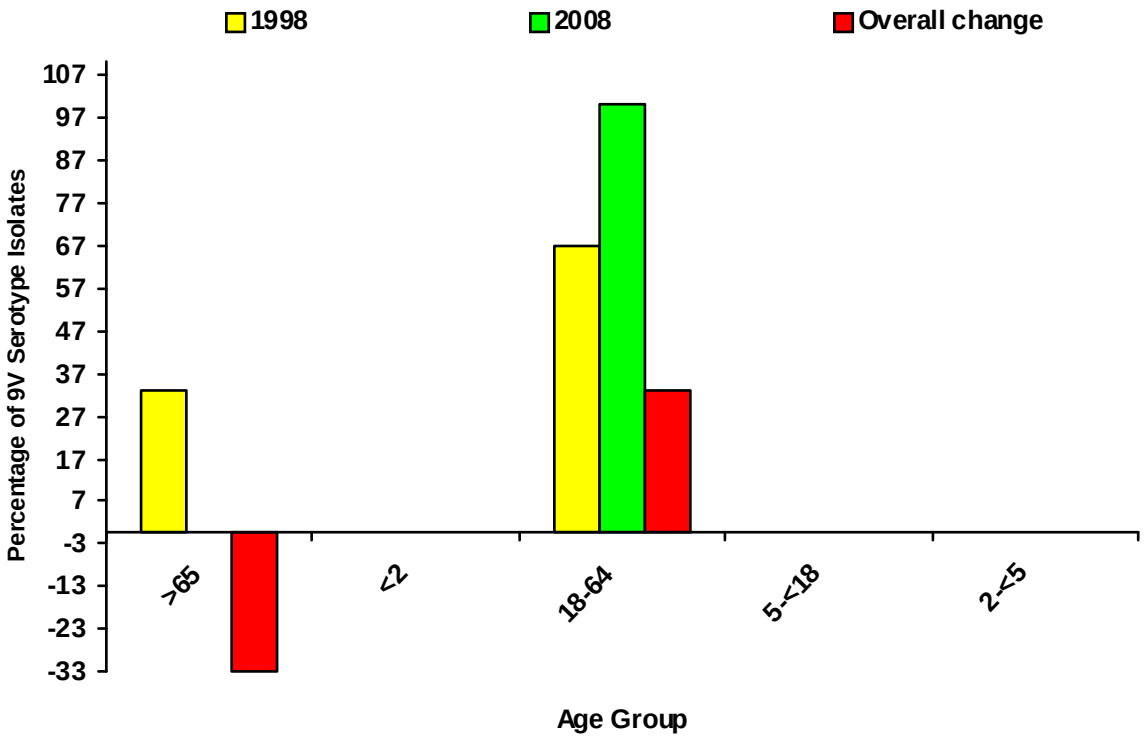

1998
2008
Overall change
107
97
87
77
67
57
47
37
27
17
7
-3
-13
-23
-33
Percentage of 9V Serotype Isolates
>65
<2
18-64
5-<18
2-<5
Age Group

Fig. 22c.

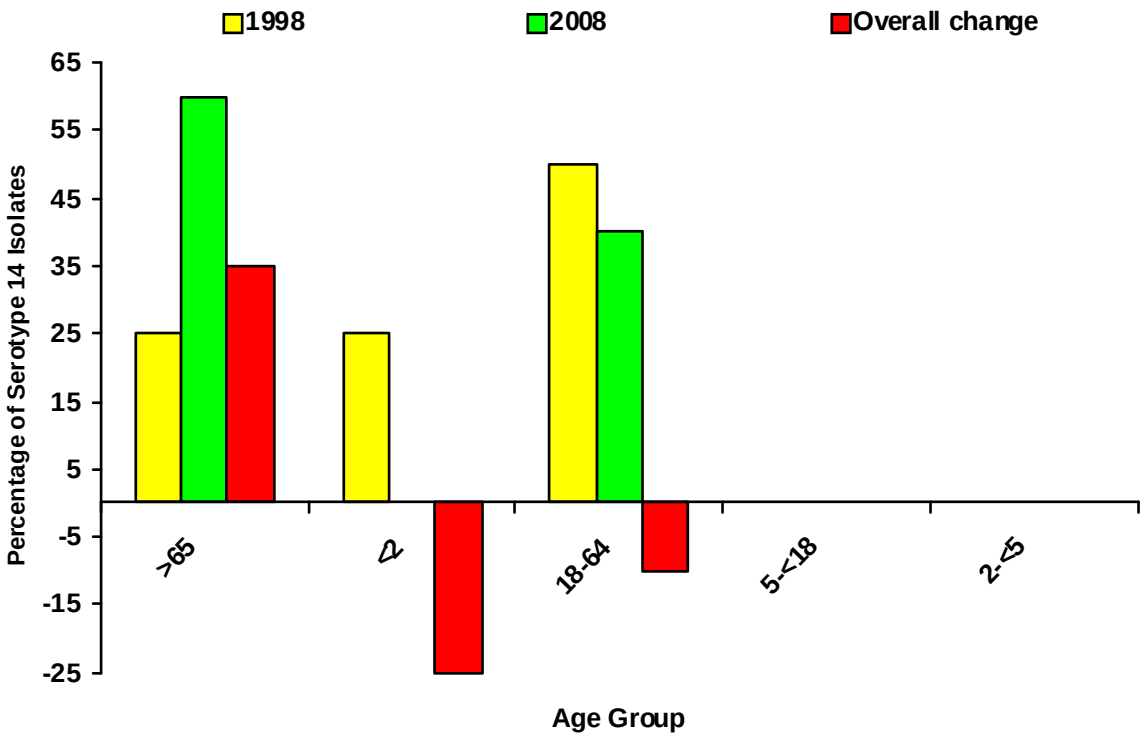

1998
2008
Overall change
65
55
45
35
25
15
5
-5
-15
-25
Percentage of Serotype 14 Isolates
>65
<2
18-64
5-<18
2-<5
Age Group

Fig. 22d.

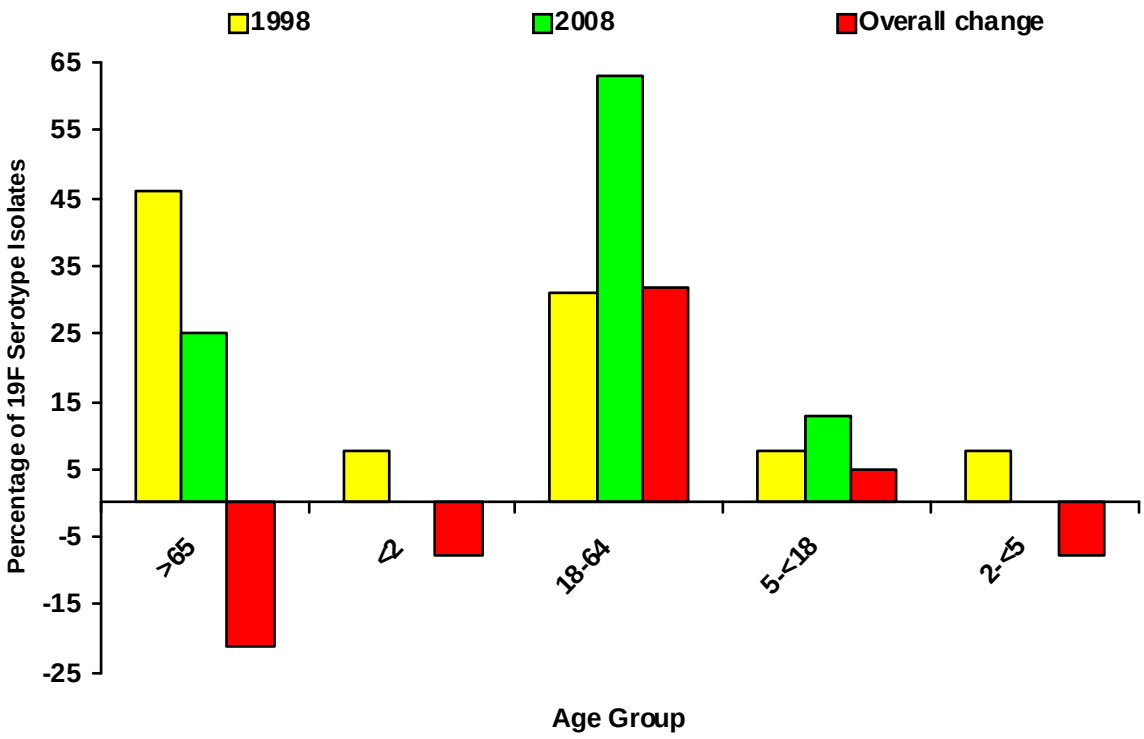

1998
2008
Overall change
65
55
45
35
25
15
5
-5
-15
-25
Percentage of 19F Serotype Isolates
>65
<2
18-64
5-<18
2-<5
Age Group

Fig. 22e.

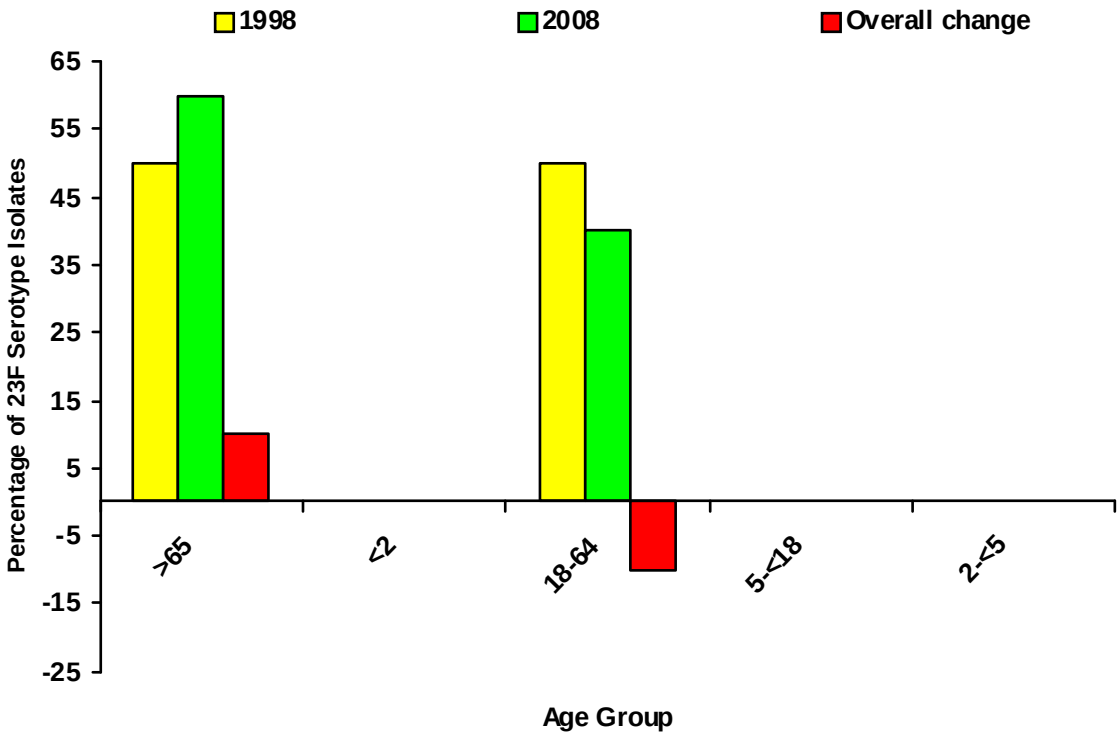
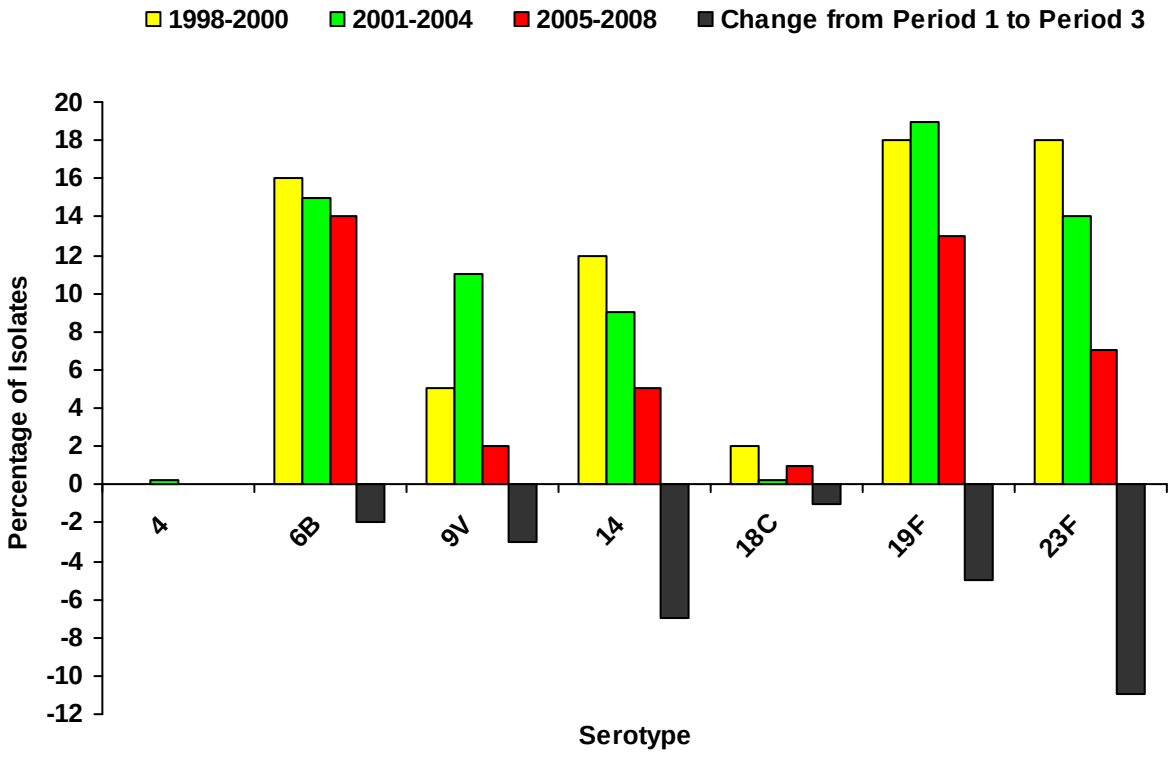

1998
2008
Overall change
65
55
45
35
25
15
5
-5
-15
-25
Percentage of 23F Serotype Isolates
>65
<2
18-64
5-<18
2-<5
Age Group

Figure 23. Prevalence of PCV7 serotypes (4, 6B, 9V, 14, 18C, 19F and 23F) pre (1998-2000), during (2001-2004) and post (2005-2008) PCV7 vaccine introduction in Canada as well as overall change between the pre and the post periods.



Distribution and Characterization of Serotypes not Found in the PCV7 Vaccine

Serotypes 6A, 11A, 12F, 15A, 15B, 15C, 19A and 33F were among the top 20 serotypes found among macrolide resistant SPN, Figure 17a. These serotypes were not covered by the PCV7 vaccine and therefore were labeled as emerging (non-PCV7) serotypes throughout this thesis. The number of isolates with these emerging serotypes during each year of the study is shown in Figure 24. The number of isolates with non-PCV7 serotypes increased from 4/51 (8%) in 1998 to 36/84 (43%) in 2008. The number of different non-PCV7 serotypes present each year also increased from 2 serotypes in 1998 to 7 serotypes in 2008. Years 2004 to 2007 contained all 8 emerging non-PCV7 serotypes.

The number of isolates with serotype 6A varied by year, ranging from 2 (2000, 2002) to 13 (2004) isolates. It was first identified in 1999, contributing 8.1% (6/74) to macrolide resistant SPN, reaching its peak at 12.3% (12/97) in 2007, however leveled off at 8.3% (7/84) in 2008, Figure 24. Isolates with serotype 6A were identified predominantly from an adult population (18-64), ON region, those with male gender, and an inpatient patient status and *mef*(A) genotype, Figures 25a-25e.

The number of isolates with serotype 11A varied by year ranging from 1 (1999, 2003) to 6 (2004, 2005) isolates. It was first identified in 1999 at a percentage of 1.4% (1/74) and reached its high at 5.2% (5/97) in 2007 and was present at 3.6% (3/84) by the end of the study in 2008, Figure 24. Isolates with 11A serotype were equally prevalent among adult (18-64) and the elderly (≥65) populations, mostly identified from ON region, male gender, inpatient patient status and *mef*(A) genotype, Figures 25a-25e..

The number of isolates with serotype 12F varied by year, ranging from 1 (2006, 2008) to 8 (2005). Serotype 12F was first identified in macrolide resistant SPN in 1998 at 3.9%

(2/51), reached its high at 8.9% (7/78) in 2001 and declined to 1.2% (1/84) in 2008, Figure 24. These isolates were predominantly identified among the adult population (18-64), in BC/AB and SK/MB regions, those with male gender, and inpatient patient status and found solely among *mef*(A) genotype, Figures 25a-25e..

The number of isolates with 15A serotype ranged from 1 (2000, 2006) to 10 (2005). It was first identified in 1998 at 3.9% (2/51) reached its high at 8.3% (7/84) in 2008, Figure 24. Isolates with 15A serotype were equally prevalent among adult (18-64) and elderly (≥65) population, mostly from QC region, male gender, inpatient patient status and predominantly among *erm*(B) genotype, Figures 25a-25e..

The number of isolates with 15B serotype ranged from 3 (2008) to 10 (2005), Figure 24. It was first identified in 2004 at 2.3% (4/173) and reached its high at 7.2% (7/97) in 2007 and leveled off at 3.6% (3/84) in 2008. It was equally found among adult (18-64) and elderly (≥65) population, BC/AB and SK/MB regions, male gender, inpatient patient status and solely among *mef*(A) genotype, Figures 25a-25e..

The number of isolates with 15C serotype ranged from 1 (2001, 2002, 2004, 2005) to 8 (2006). It was first identified in 2001 at 1.3% (1/78), reaching its high at 7.2% (7/97) in 2007, and leveling off at 2.3% (2/84) in 2008. These isolates were predominantly found among adult (18-64) population, SK/MB region, female gender, inpatient patient status, and *mef*(A) genotype, Figures 25a-25e..

The number of isolates with 19A serotype ranged from 1 (1999, 2006) to 13 (2008). It was first identified in 1999 at 1.4% (1/74) and reached its high at 15% (13/84) in 2008, Figure 24. It was predominantly identified among adult population (18-64), equally from BC/AB, SK/MB, ON, and QC regions, male and female gender, inpatient and outpatient

patient status and both among *erm*(B) and dual *erm*(B) and *mef*(A) genotypes Figures 25a-25e..

The number of isolates with serotype 33F ranged from 4 (2004) to 6 (2005). It was first identified in 2005 at 2.3% (4/173) and reached its high at 6% (5/97) in 2007, Figure 24. Isolates with this serotype were equally identified among adult (18-64) and elderly (≥65) populations, QC region, male gender, inpatient patient status, and *erm*(B) genotype, Figures 25a-25e..

Figure 24. Prevalence of the emerging serotypes (6A, 11A, 12F, 15A, 15B, 15C, 19A, and 33F) during 1998 and 2008 study by year. Emerging serotypes defined as serotypes found among the top 20 serotypes (Fig. 16a) not covered by the PCV7 vaccine during the study.

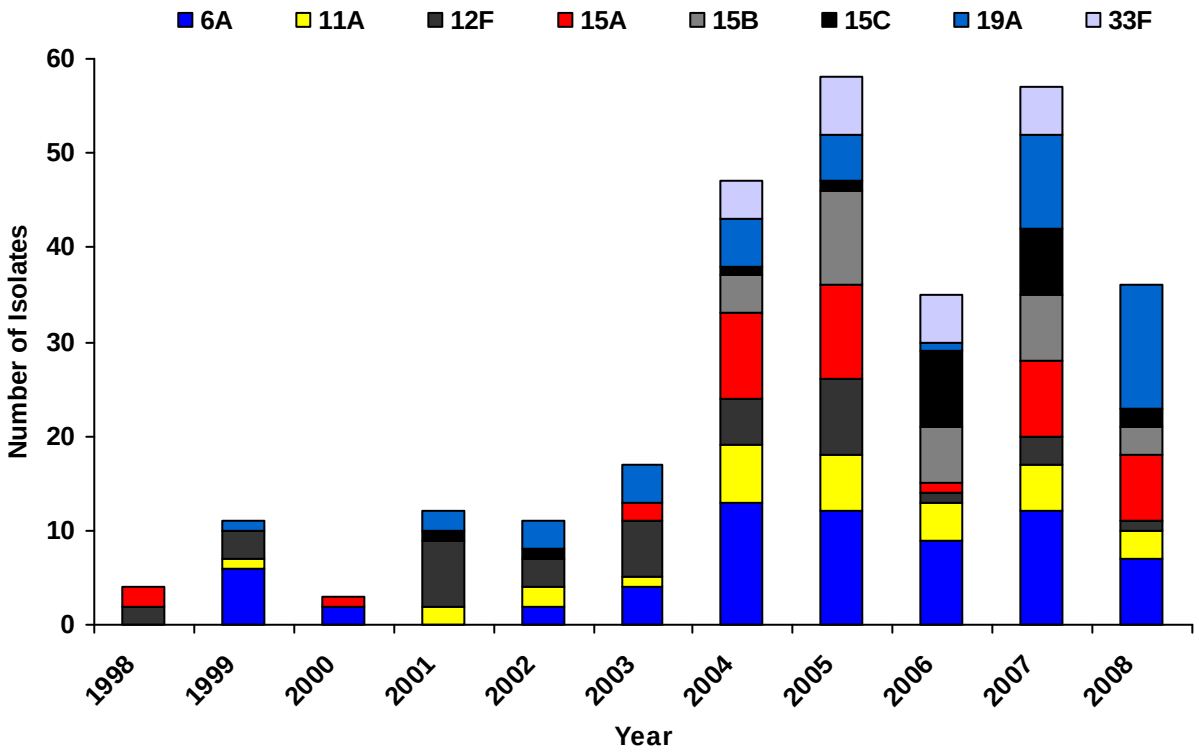


Figure 25. Prevalence of the emerging serotypes (6A, 11A, 12F, 15A, 15B, 15C, 19A, and 33F) by (a) age groups (b) region (c) genotype (d) gender and (e) patient status.

Fig. 25a.

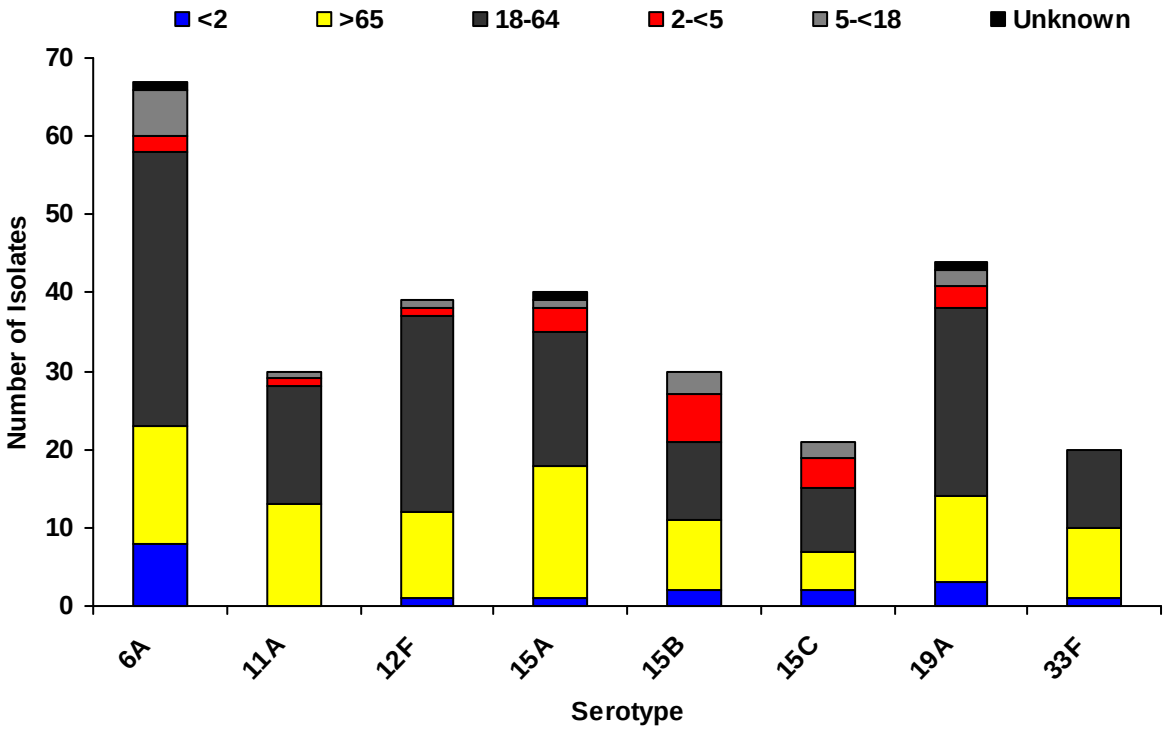

<2
>65
18-64
2-<5
5-<18
Unknown
70
60
50
40
30
20
10
0
Number of Isolates
6A
11A
12F
15A
15B
15C
19A
33F
Serotype

Fig. 25b.

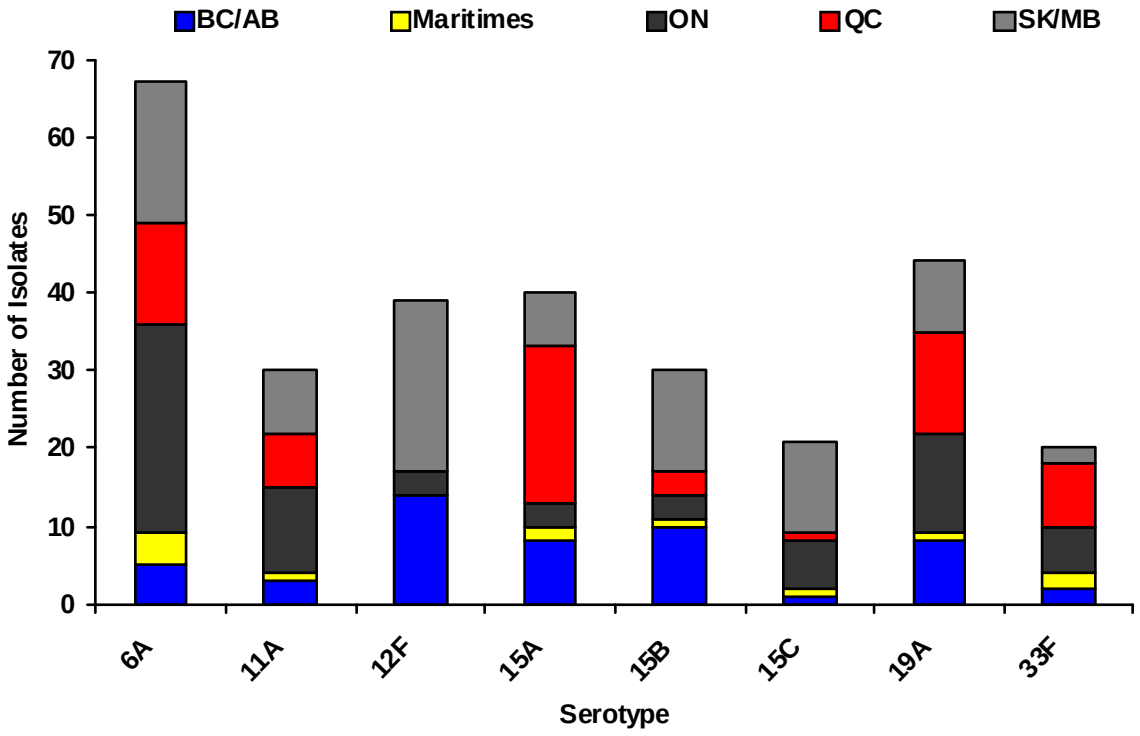

BC/AB
Maritimes
ON
QC
SK/MB
70
60
50
40
30
20
10
0
Number of Isolates
6A
11A
12F
15A
15B
15C
19A
33F
Serotype

Fig. 25c.

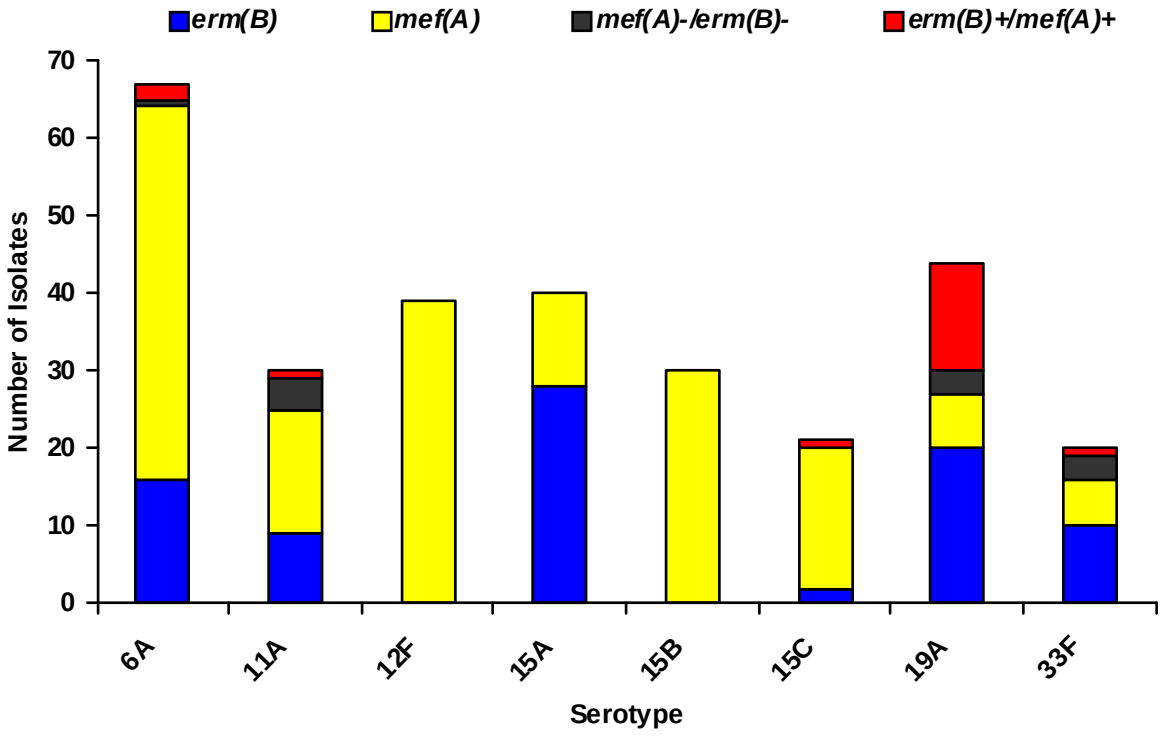

erm(B)
mef(A)
mef(A)-/erm(B)-
erm(B)+/mef(A)+
Number of Isolates
70
60
50
40
30
20
10
0
6A
11A
12F
15A
15B
15C
19A
33F
Serotype

Fig. 25d.

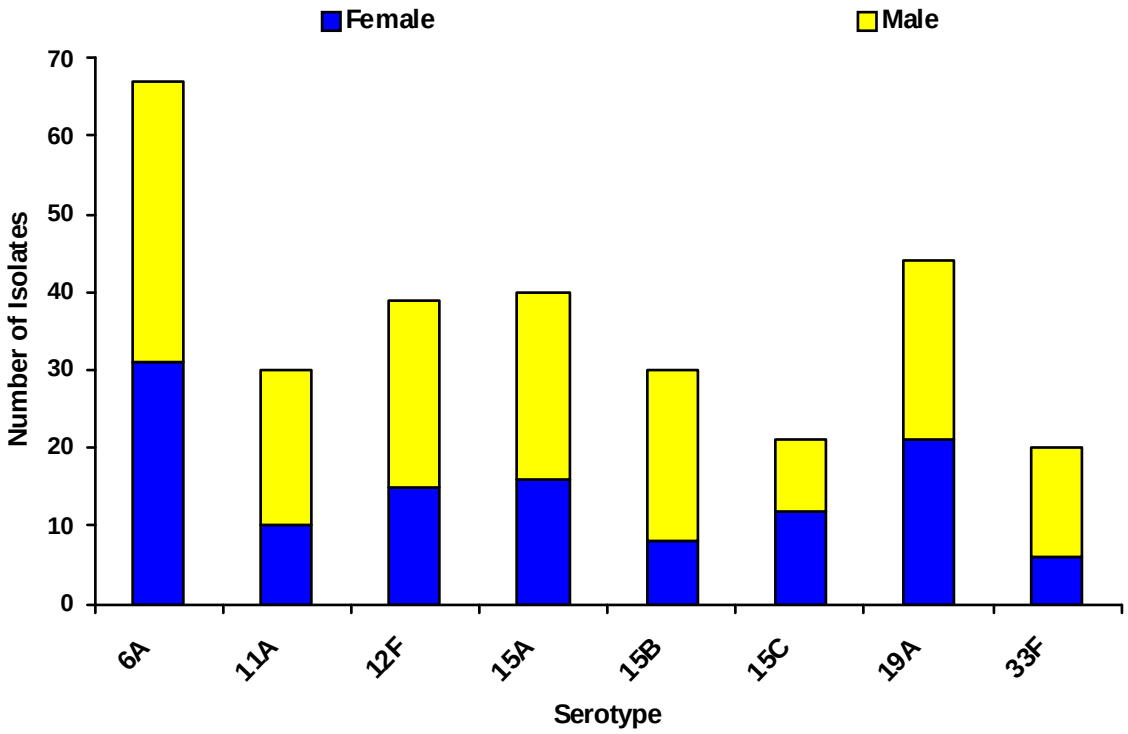
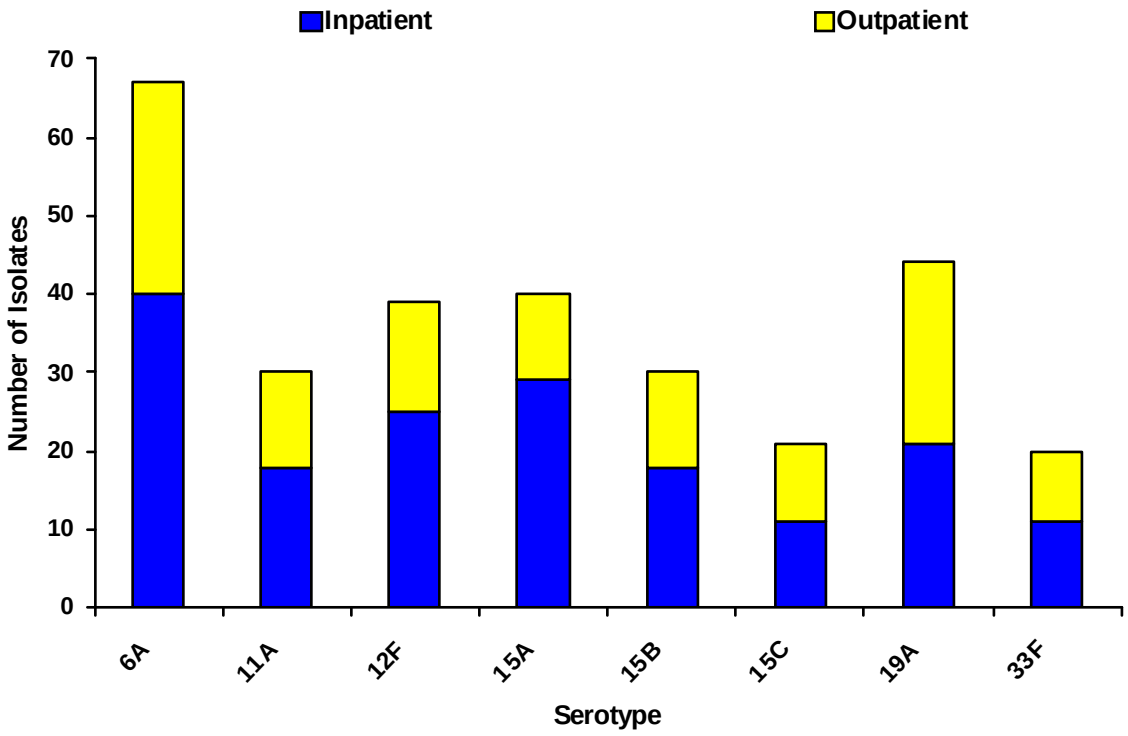

Female
Male
70
60
50
40
30
20
10
0
Number of Isolates
6A
11A
12F
15A
15B
15C
19A
33F
Serotype

Fig. 25e.


Inpatient
Outpatient
Number of Isolates
70
60
50
40
30
20
10
0
6A
11A
12F
15A
15B
15C
19A
33F
Serotype

Changes in the Prevalence of Emerging Serotypes pre and post PCV7 Vaccine Introduction, Antibiotic Susceptibility and Genetic Relatedness

Figure 26 shows the percentage of isolates with the emerging serotypes in the first year (1998) and the last year of the study (2008). It also shows the overall change between these two years. Isolates with seven (6A, 11A, 15A, 15B, 15C, 19A, 33F) of the 8 emerging serotypes showed an overall increase in the prevalence between 1998 and 2008. This increase ranged from 0.2% for isolates with serotype 6A to 17.1% for isolates with serotype 33F. Isolates with serotype 19A increased by 14.1% from 1998 to 2008. Isolates with serotype 12F showed a decrease in prevalence between 1998 and 2008 of 2.7%.

Figure 27 analyses the prevalence of the emerging serotypes in pre (1998-2000), during (2001-2004) and post (2005-2008) PCV7 vaccine introduction periods. It also shows the overall changes in the prevalence between pre and post periods. The overall changes in the prevalence of emerging serotypes ranged from 3% (serotypes; 11A, 15A, and 33F) to 5% (serotypes; 15B and 19A). No change was noted for isolates with serotype 12F.

Figure 28 shows the percentage of isolates in the pre (1998-2000) and the post (2005-2008) PCV7 vaccine introduction periods by age group. The prevalence of isolates with the emerging serotypes increased among elderly (≥65), and the pediatric 5-17 and 2-4 age groups by 18%, 6%, and 3%, respectively.

Figures 29a -29h show the prevalence and the overall change for the pre and post PCV7 periods for isolates with the emerging serotypes by different age groups. Isolates with serotype 6A and 11A increased predominantly among elderly (≥65) population by

25% and 40%, respectively figures 29a and 29b. Isolates with serotype 12F and 33F increased predominantly among adult population (18-64) by 32% and 63% respectively, Figures 29c and 29h. Isolates with serotype 15A increased among elderly (≥65) (9%) and among all pediatric age groups; <2 (4%), 2-4 (4%), and 5-17 (4%), Figure 29d. Isolates with serotypes 15B, 15C and 19A increased among all five age groups, however most predominantly among elderly (≥65) by 31%, 28%, and 24%, respectively and adult (18-64) population by 38%, 33%, and 60%, respectively, Figures 29e, 29f, and 29g.

Figure 30a-30d shows the antimicrobial susceptibility profile for penicillin, T/S, doxycycline and clindamycin among the emerging serotypes. Penicillin resistance rate of 8%, 12%, 24% and 52% was detected among isolates with serotypes 15A, 6A, 11A, and 19A, Figure 30a. T/S resistance rate of 5%, 5%, 14%, 24% and 53% was detected among isolates with serotype 15A, 15C, 6A, 11A, and 19A, Figure 30b. Doxycycline resistance was detected among isolates with serotypes; 12F (3%), 15C (5%), 33F (5%), 6A (9%), 11A (10%), 19A (12%) and 15A (55%), Figure 30c. Clindamycin resistance was detected among isolates with emerging serotypes; 15C (9%), 6A (24%), 11A (30%), 33F (55%), 15A (68%), and 19A (73%), Figure 30d.

The genetic relatedness among the isolates with emerging serotypes is shown in dendrograms in Figures 31 a-h. The dendrogram depicting the genetic relatedness of the isolates with serotype 6A is shown in Figure 31a. There were 7 clusters, numbered 1-7 on the figure, with 3 (3), 5 (1), and 8 (1) isolates per cluster observed among these isolates. In addition there were 8 clusters containing 2 isolates each. Among these isolates there were the 7 isolates with serotype 6C that are described in more detail later. Overall the clusters contained 61% of all 6A/6C (65) typed isolates. The dendrogram depicting the genetic relatedness among isolates with 11A serotype is shown in Figure

31b. There were 6 genetic clusters, numbered 1-6 in the figure, with 2 (3), 3 (2), and 6 (1) isolates per cluster observed among these isolates. Overall the 6 clusters contained 18 of the 29 typed 11A isolates, making up 62% of these isolates. The genetic relatedness among emerging isolates with serotype 12F is shown in Figure 31c. The overall genetic relatedness among these isolates was 61%. There was 1 major genetic cluster, numbered 1 on the Figure containing 31 of the 36 typed 12F serotype isolates, making up 92% of all 12F serotype isolates. A second cluster, numbered 2 in the Figure with 2 isolates was also observed. The genetic relatedness among isolates with 15A serotype is shown in Figure 31d. In total 39 isolates were typed. The overall genetic relatedness among these isolates was 46%. There were 5 genetic clusters, numbered 1-5 in the Figure with 2 (1), 3 (1), 7 (2), and 8 (1) isolates per cluster, making up 69% (27) of all 15A serotype isolates. Figure 31e shows the genetic relatedness among isolates with 15B serotype. The overall genetic relatedness among all these isolates was 60%. There were 2 major clusters with 9 and 12 isolates in each, making up 78% (21/27) of all 15B serotype isolates. Figure 31f shows the genetic relatedness among isolates with serotype 15C. The overall genetic relatedness of 42% was observed among these isolates. Among the 20 typed isolates 10 were contained in 2 clusters, numbered 1 (7) and 2 (3), making up 50% of all serotype 15C isolates. The genetic relatedness among isolates with 19A serotype is shown in figure 31g. There were 10 clusters, numbered 1-10 in the Figure, with 2 (6), 3 (1), 4 (1), 5 (1), and 8 (1) observed among these isolates. Together isolates within these clusters made up 50% (22) of 19A serotype isolates. Figure 31h shows the genetic relatedness among isolates with serotype 33F. Overall the genetic relatedness among the 19 typed isolates was 52%. One major cluster, numbered 1 in the Figure, containing 10 isolates and 2 other clusters, numbered 2 and 3 containing 3 and 2 isolates were observed among these

isolates. Overall these clusters contained 15 of the 19 typed 33F isolates, making up 79% of all 33F serotype isolates.

Figure 26. Prevalence of emerging serotypes (6A, 11A, 12F, 15A, 15B, 15C, 19A, and 33F) in the first year (1998) and the last year (2008) of the study, as well as the overall change between these two years of the study.

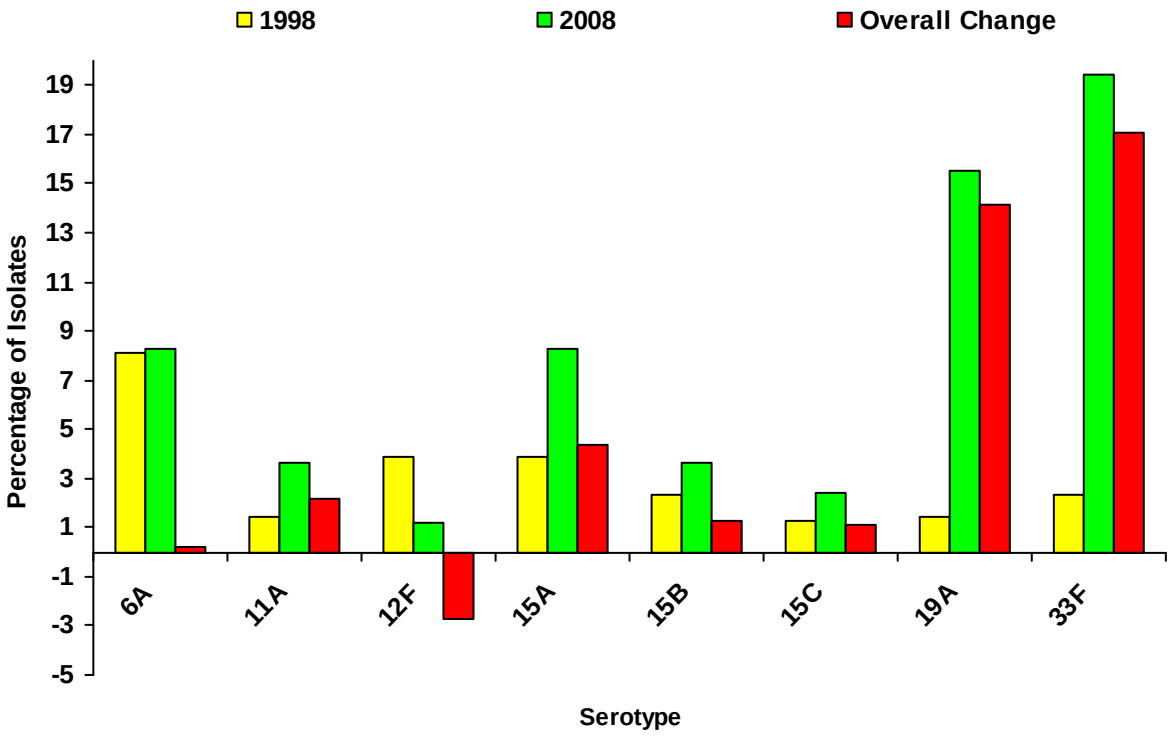


Figure 27. Prevalence of the emerging serotypes (6A, 11A, 12F, 15A, 15B, 15C, 19A, and 33F) pre (1998-2000), during (2001-2004) and post (2005-2008) PCV7 vaccine introduction in Canada as well as overall change between the pre and the post periods.

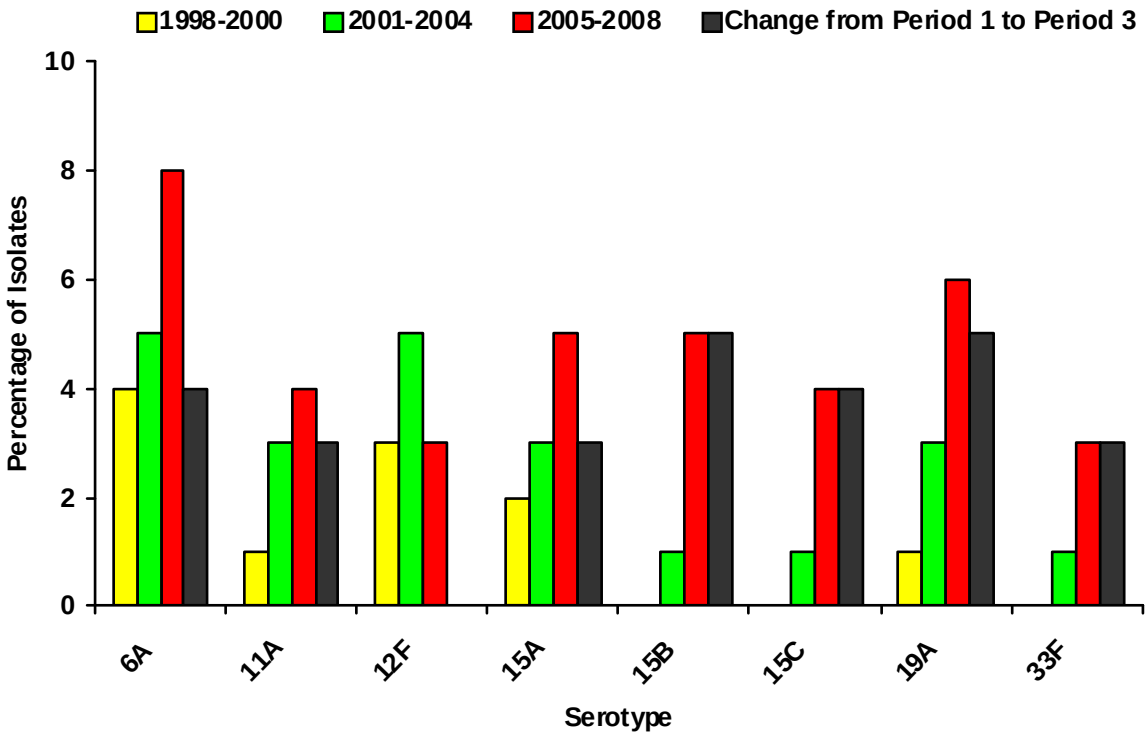


Figure 28. Prevalence of the emerging serotypes (6A, 11A, 12F, 15A, 15B, 15C, 19A, and 33F) in the pre (1998-2000) and the post (2005-2008) PCV7 vaccine introduction periods in Canada as the overall change between these two periods among different age groups. Age groups defined as: pediatric <2, 2-<5, and 5-<18, adult 18-64 and elderly ≥ 65 years old. Period 1 defined as 1998-2000 and period 3 defined as 2005-2008.

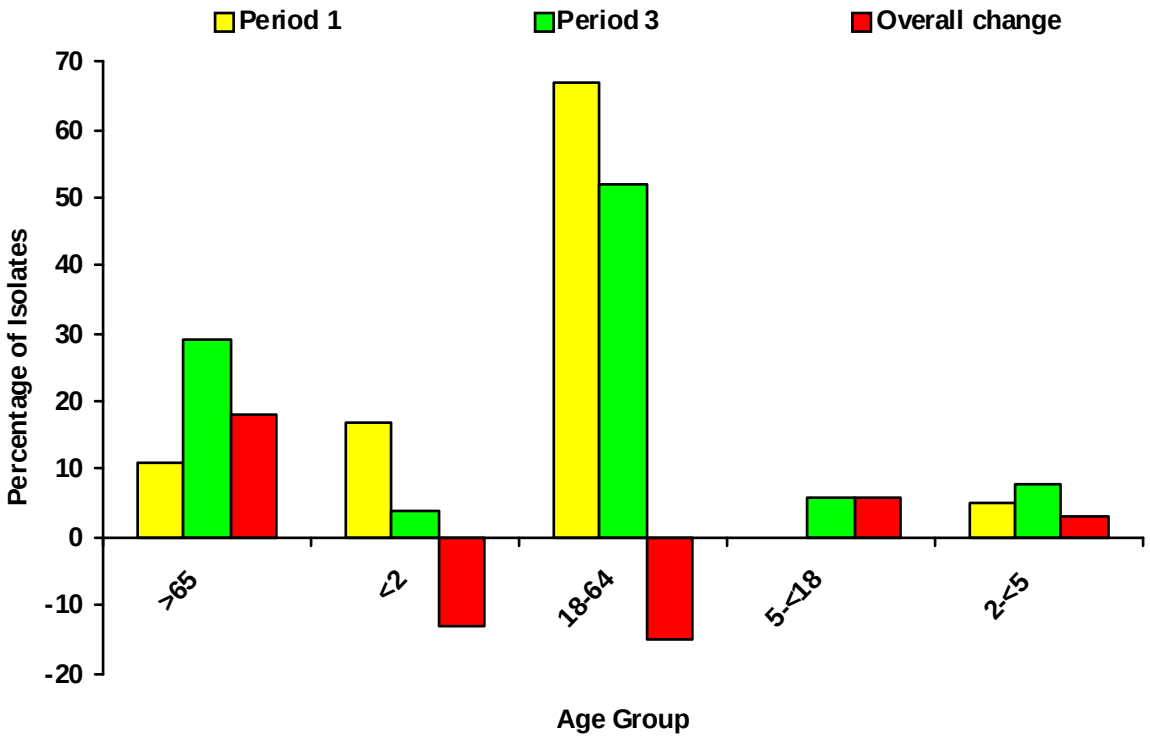


Figure 29. Prevalence of the emerging serotype (a) 6A (b) 11A (c) 12F (d) 15A (e) 15B (f) 15C (g) 19A and (h) 33F during the pre (1998-2000) and the post (2005-2008) PCV7 vaccine introduction periods in Canada as well as the overall change between these two periods among different age groups.

Fig. 29a.

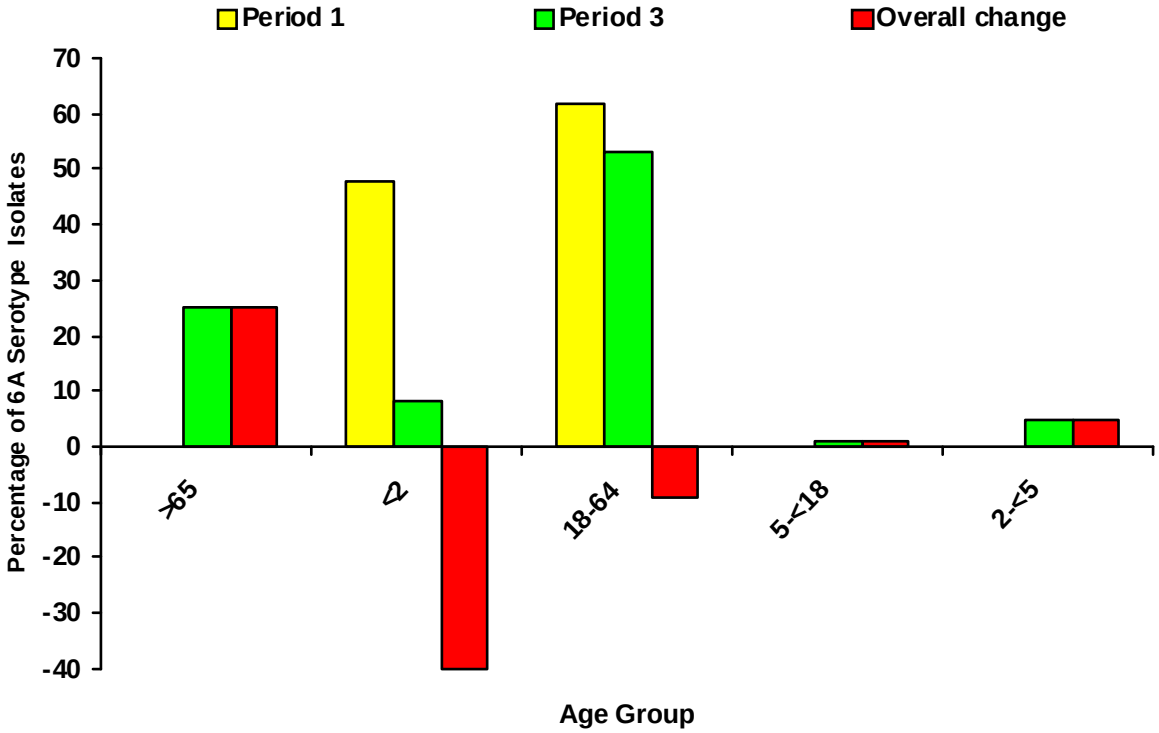

Period 1
Period 3
Overall change
Percentage of 6A Serotype Isolates
70
60
50
40
30
20
10
0
-10
-20
-30
-40
>65
<2
18-64
5-<18
2-<5
Age Group

Fig. 29b.

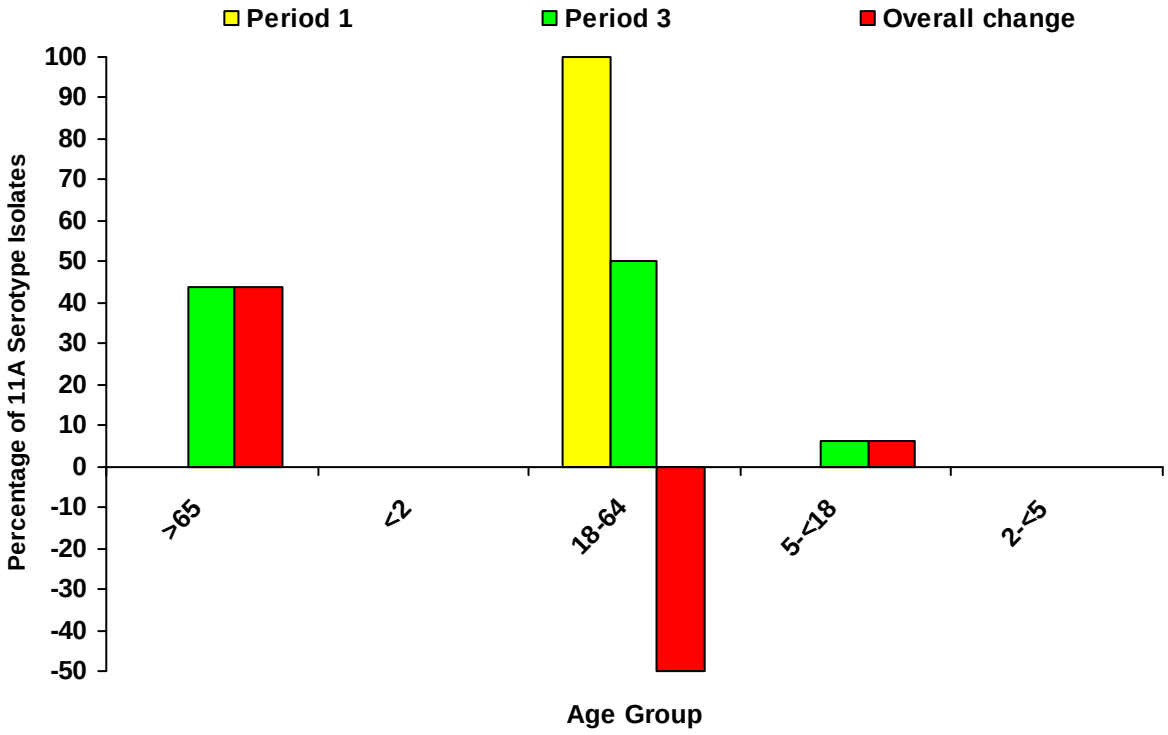

Period 1
Period 3
Overall change
Percentage of 11A Serotype Isolates
100
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
>65
<2
18-64
5-<18
2-<5
Age Group

Fig. 29c.

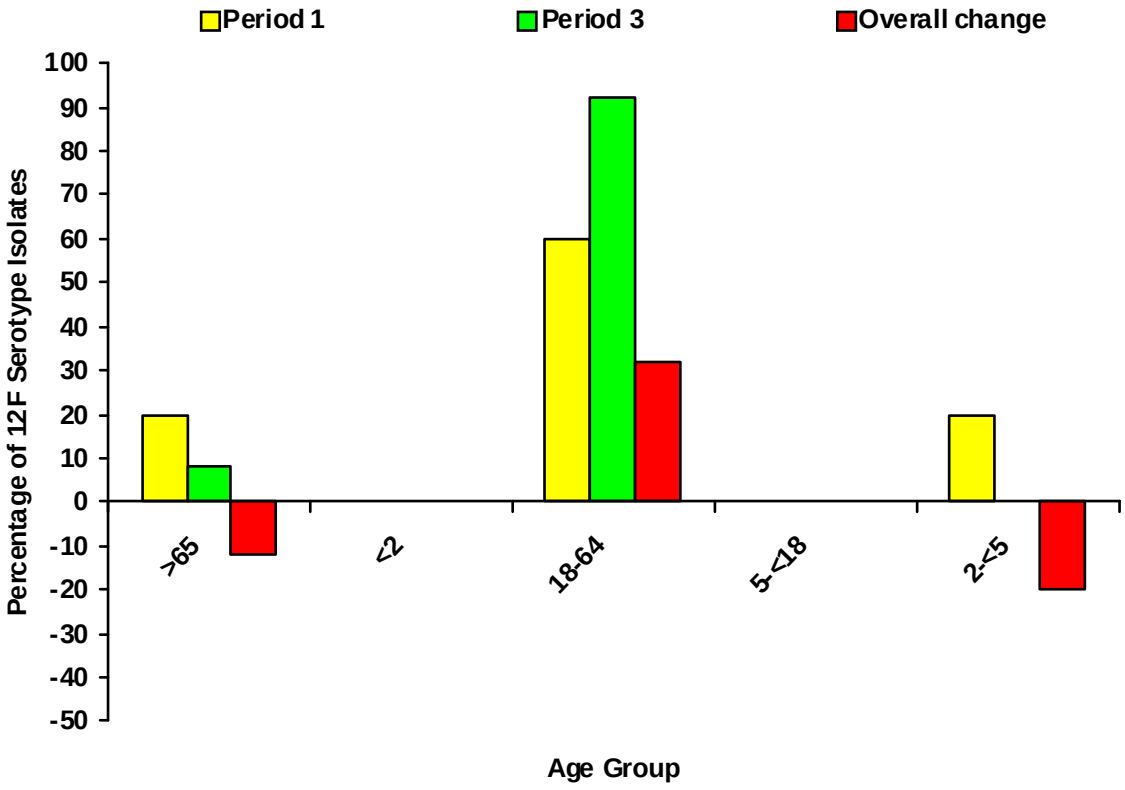

Period 1
Period 3
Overall change
Percentage of 12F Serotype Isolates
100
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
>65
<2
18-64
5-<18
2-<5
Age Group

Fig. 29d.

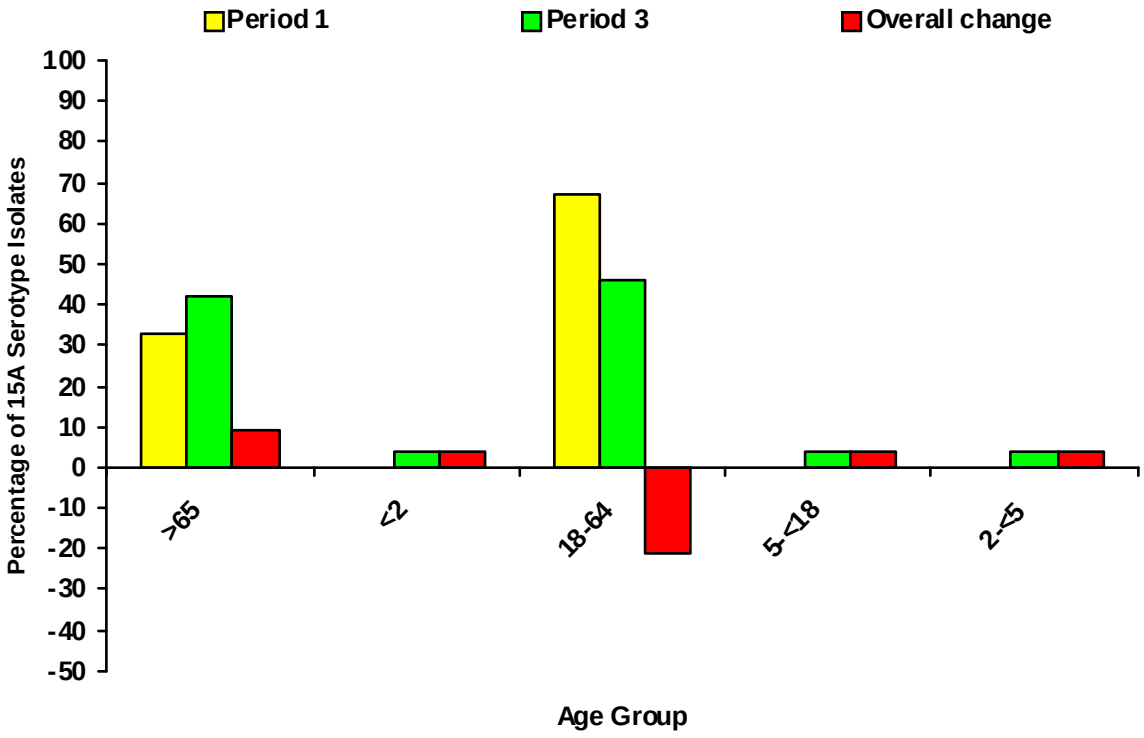

Period 1
Period 3
Overall change
Percentage of 15A Serotype Isolates
100
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
>65
<2
18-64
5-<18
2-<5
Age Group

Fig. 29e.

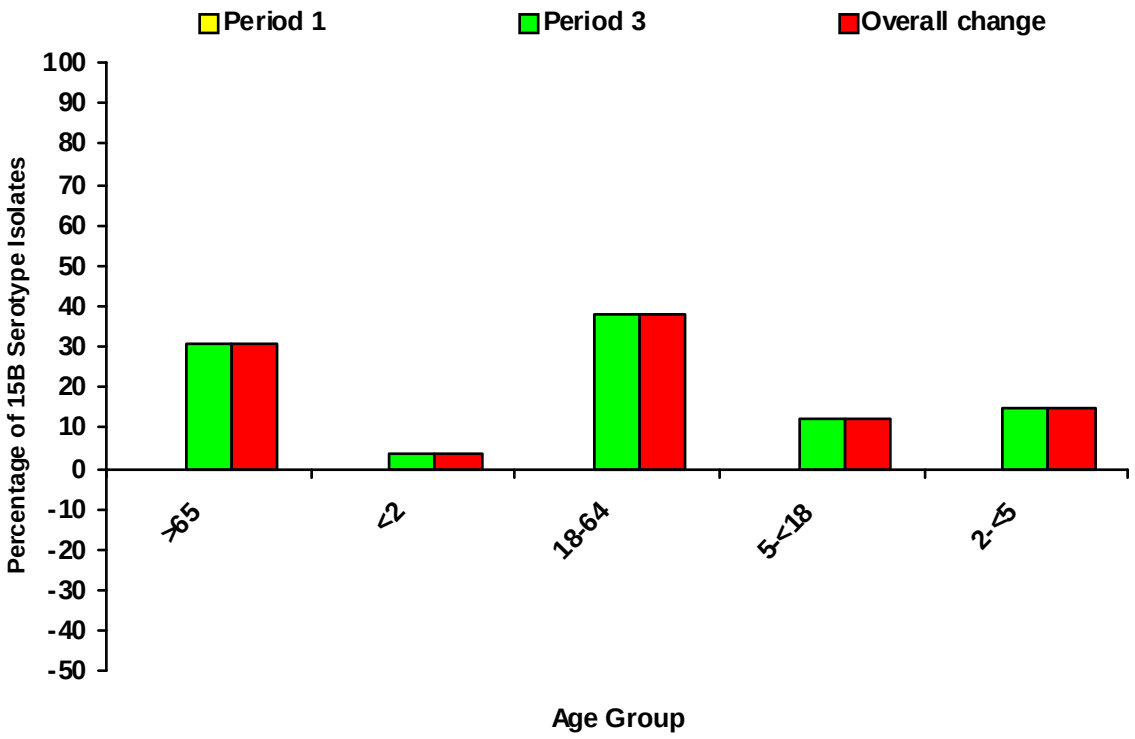

Period 1
Period 3
Overall change
Percentage of 15B Serotype Isolates
100
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
>65
<2
18-64
5-<18
2-<5
Age Group

Fig. 29f.

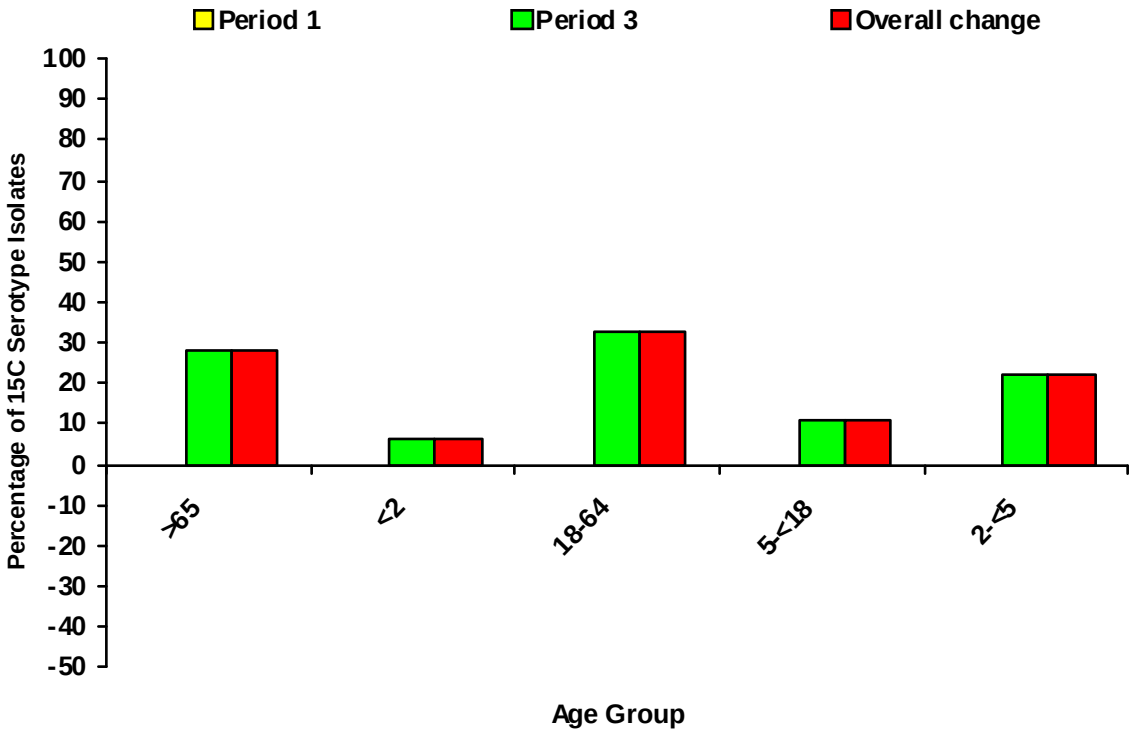

Period 1
Period 3
Overall change
Percentage of 15C Serotype Isolates
100
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
>65
<2
18-64
5-<18
2-<5
Age Group

Fig. 29g.

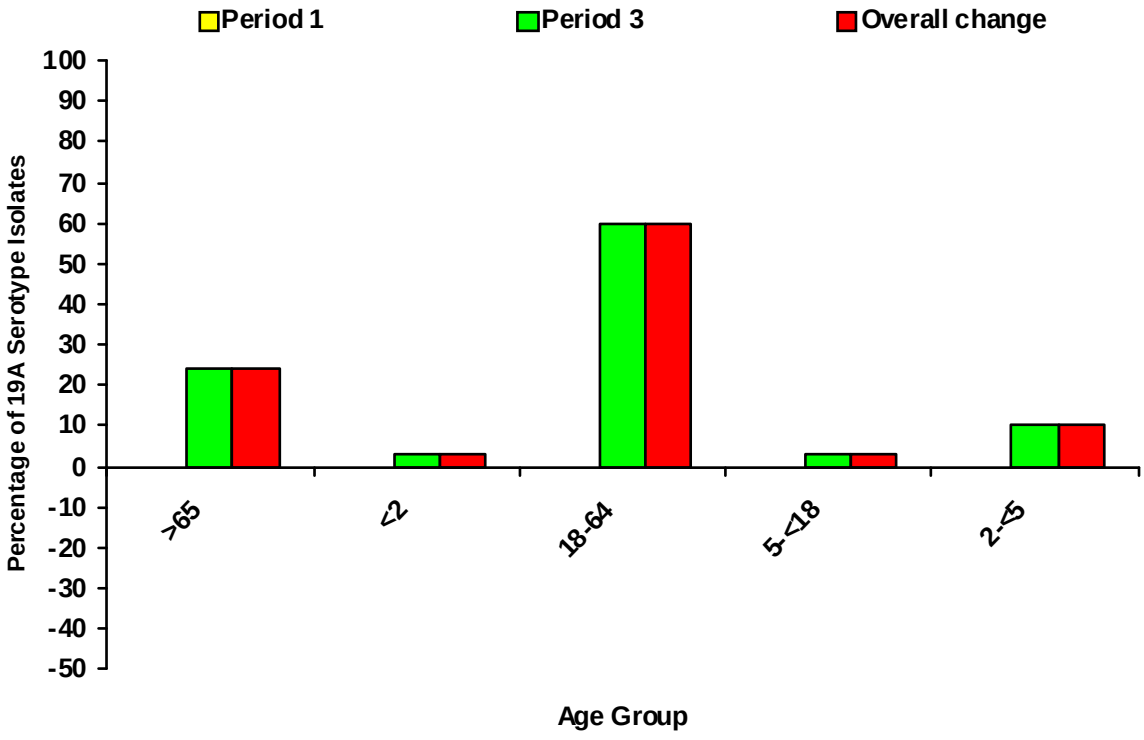

Period 1
Period 3
Overall change
100
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
Percentage of 19A Serotype Isolates
>65
<2
18-64
5-<18
2-<5
Age Group

Fig. 29h.

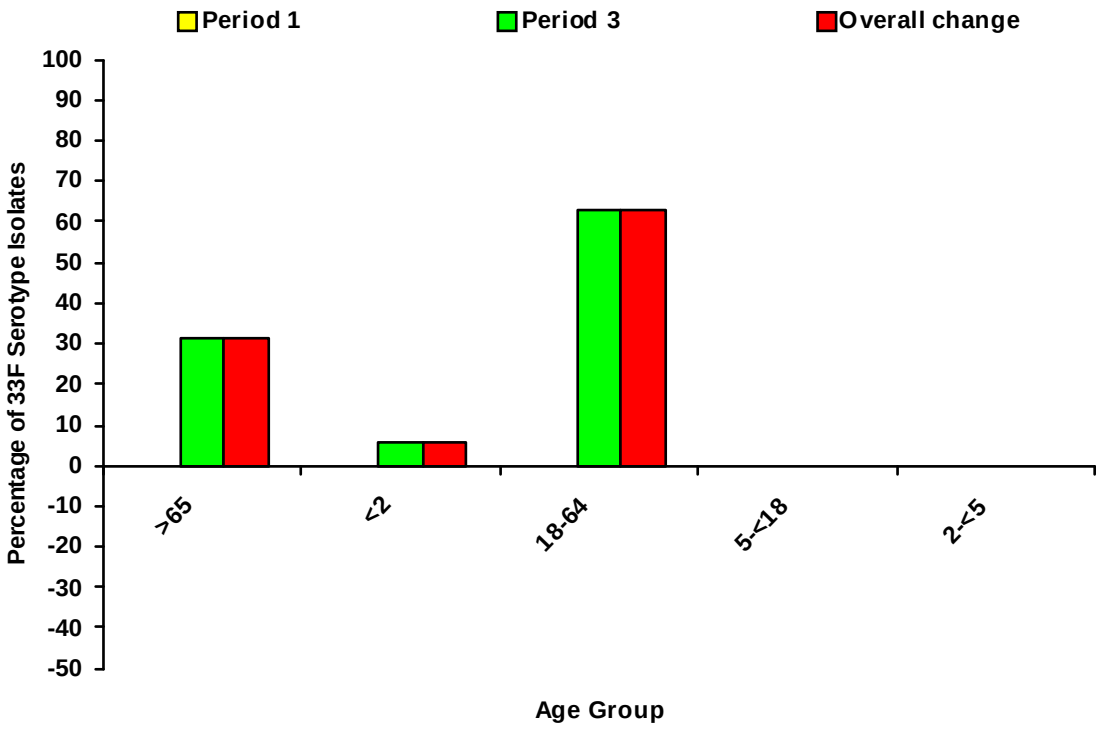

Period 1
Period 3
Overall change
100
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
Percentage of 33F Serotype Isolates
>65
<2
18-64
5-<18
2-<5
Age Group

Figure 30. Penicillin (Pen) (a), Trimethoprim/sulfamethoxazole (T/S) (b), Doxycycline (Dox) (c) and Clindamycin (Cd) (a) susceptibility profile among the emerging serotypes (6A, 11A, 12F, 15A, 15B, 15C, 19A, and 33F) during the 1998 and 2008 study. Isolates were defined as Sensitive (S), Intermediate (I), and Resistant (R) based on CLSI interpretative breakpoints as follows: Pen (oral penicillin V): S ≤0.06µg/mL, I 0.12-1µg/mL, R ≥ 2µg/mL; T/S: S ≤0.5/9.5µg/mL, I 1/19-2/38µg/mL, R ≥4/76µg/mL; Dox (Tetracycline): S ≤2µg/mL, I 4µg/mL, R ≥8µg/mL; Cd: S ≤ 0.25µg/mL, I 0.5µg/mL, R ≥1µg/mL.

Fig. 30a.

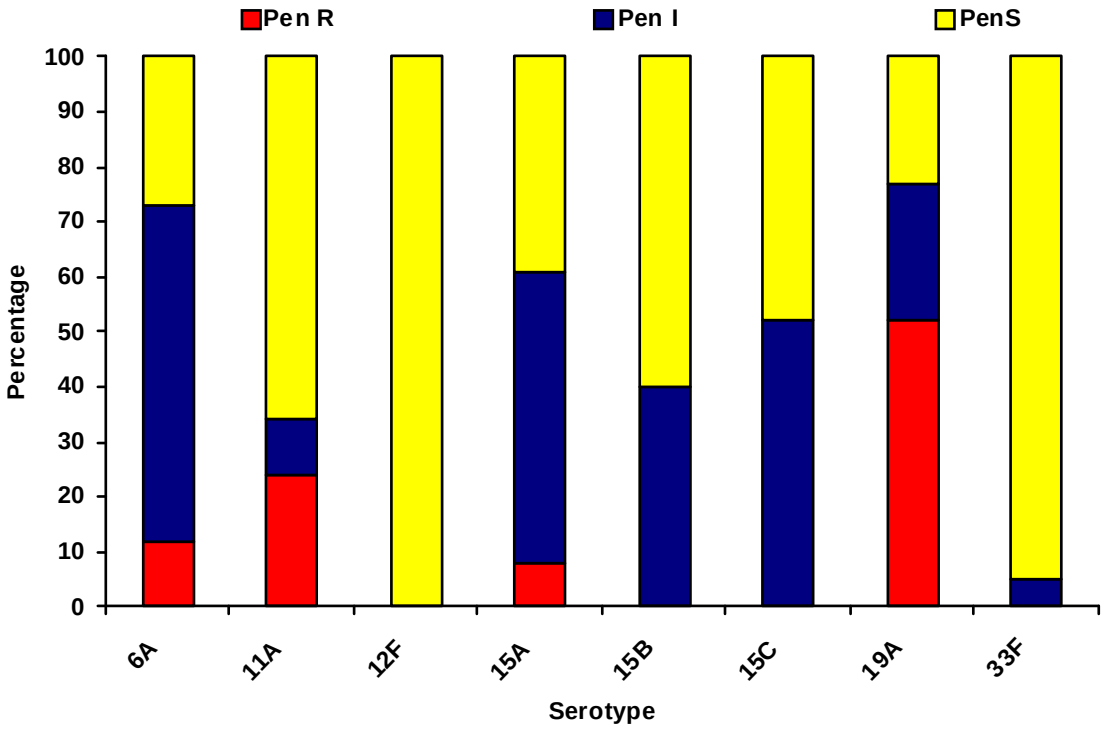

Pen R
Pen I
PenS
100
90
80
70
60
50
40
30
20
10
0
Percentage
6A
11A
12F
15A
15B
15C
19A
33F
Serotype

Fig. 30b.

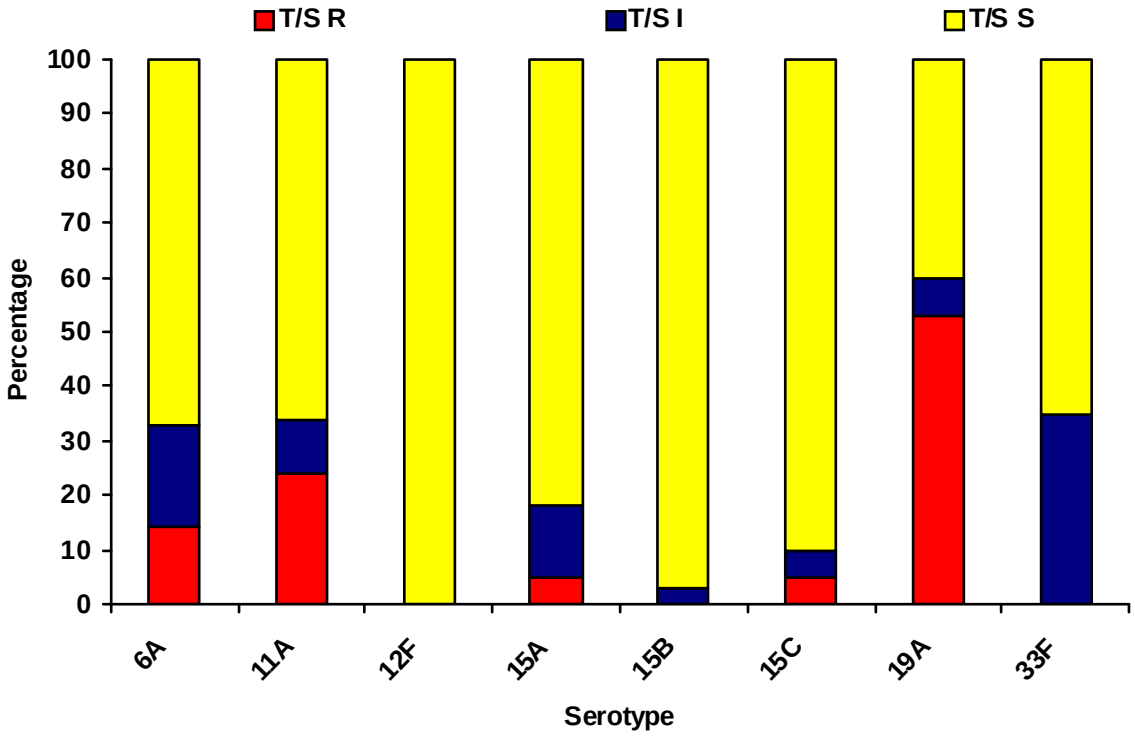

T/S R
T/S I
T/S S
100
90
80
70
60
50
40
30
20
10
0
Percentage
6A
11A
12F
15A
15B
15C
19A
33F
Serotype

Fig. 30c.

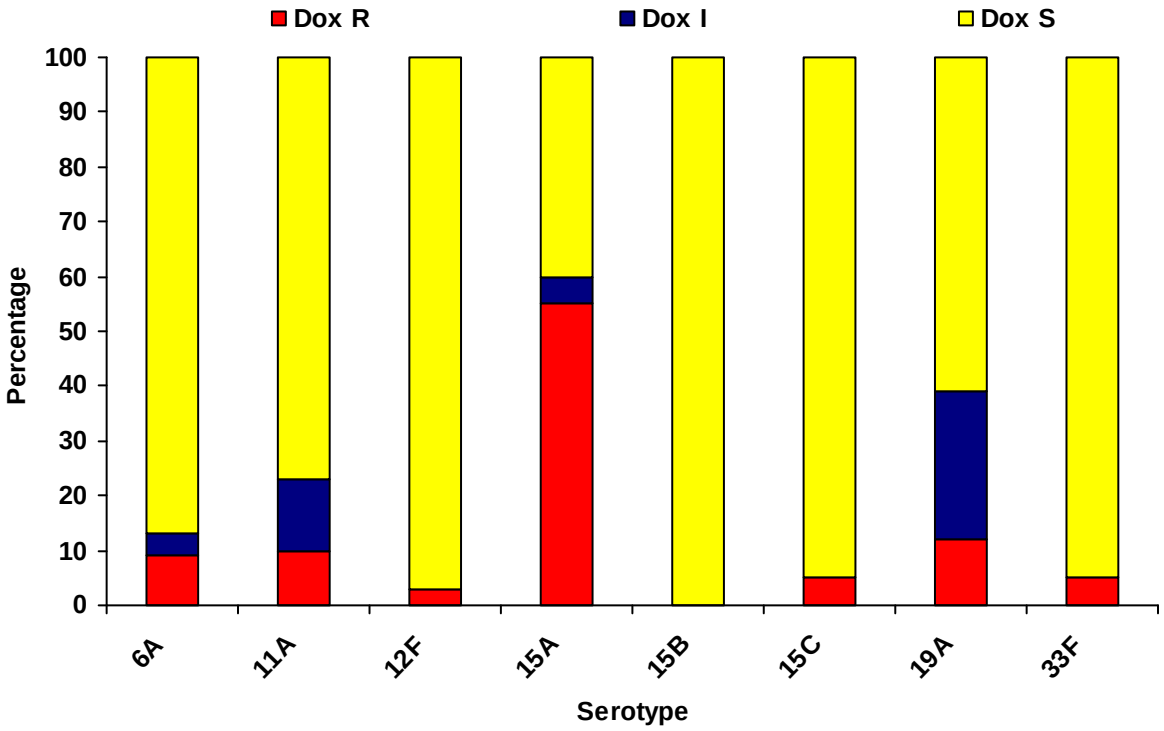

Dox R
Dox I
Dox S
100
90
80
70
60
50
40
30
20
10
0
Percentage
6A
11A
12F
15A
15B
15C
19A
33F
Serotype

Fig. 30d.

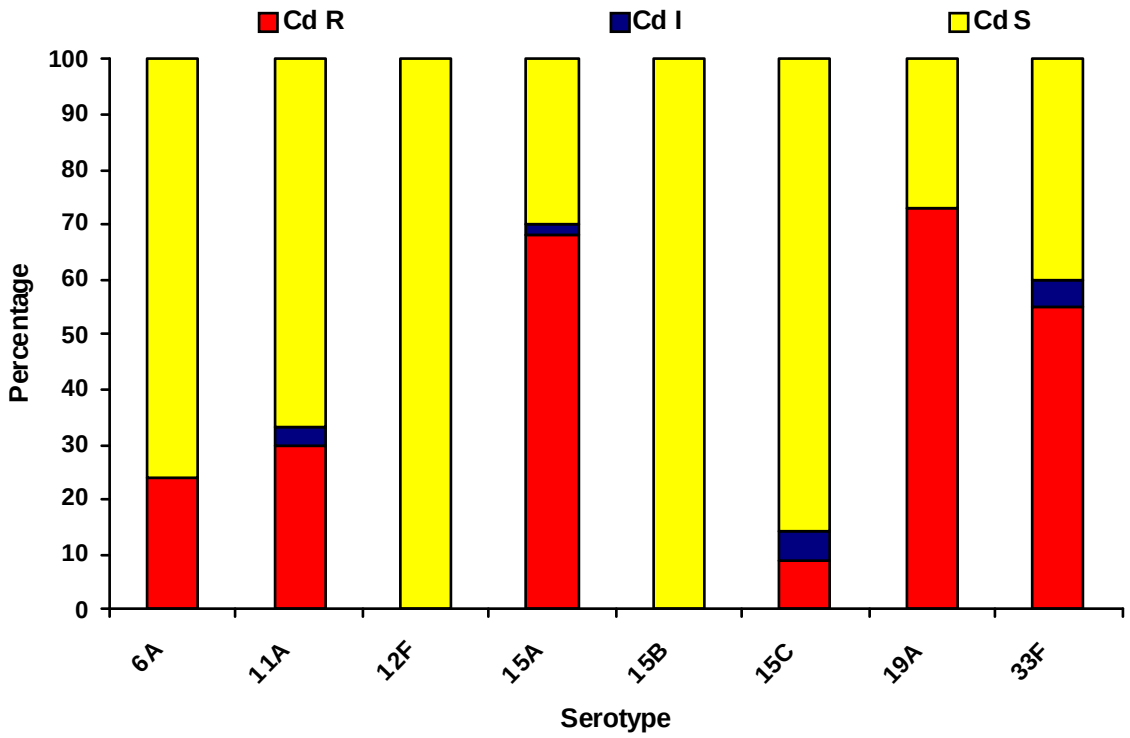

Cd R
Cd I
Cd S
Percentage
100
90
80
70
60
50
40
30
20
10
0
6A
11A
12F
15A
15B
15C
19A
33F
Serotype

Figure 31. Dendrogram depicting genetic relatedness of the emerging macrolide resistant *S. pneumoniae* with serotype (a) 6A, (b) 11A, (c) 12F, (d) 15A, (e) 15B, (f) 15C, (g) 19A, and (h) 33F isolates on the basis on PFGE results. PFGE was conducted with *Sma*I digestions. 80% similarly is indicated with a dashed line. Clusters are boxed with dotted lines and cluster numbered as indicated.

Fig. 31a.

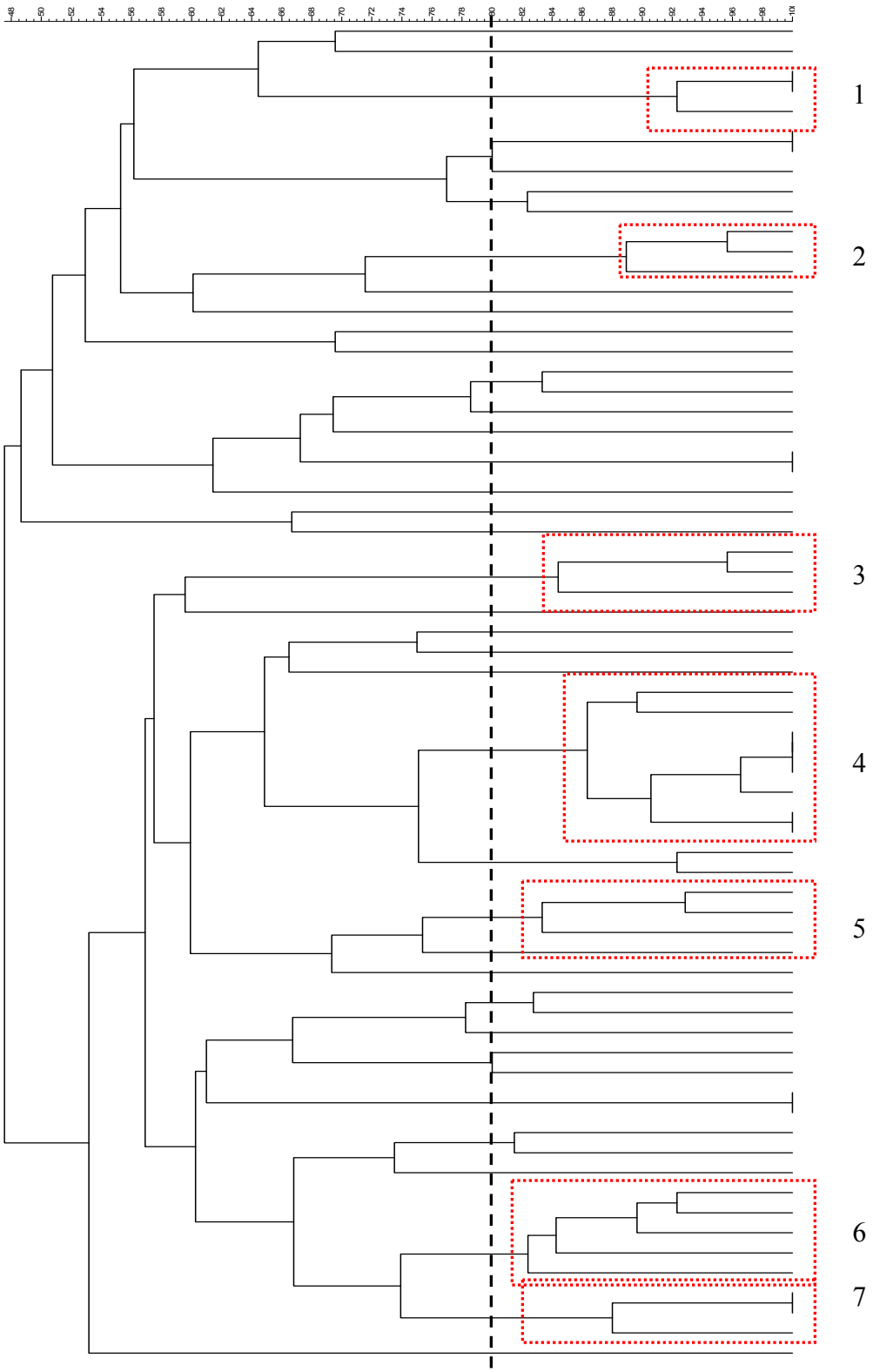


Fig. 31b.

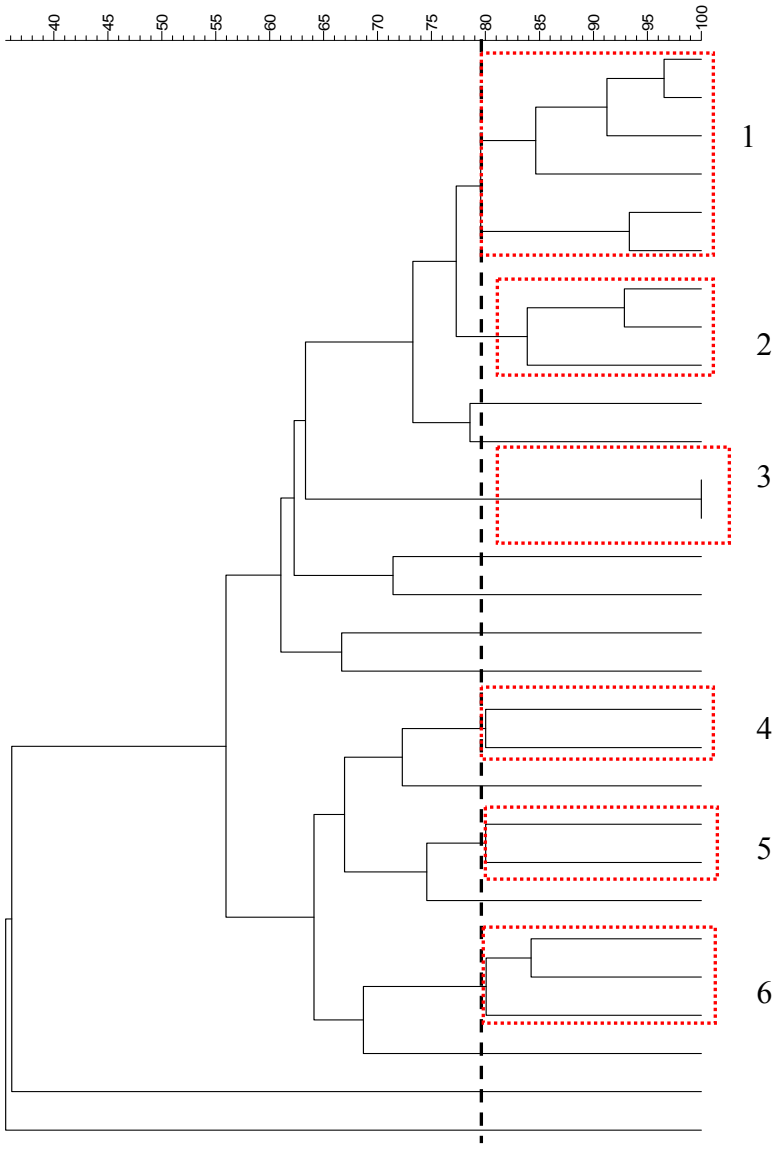


Fig. 31c.

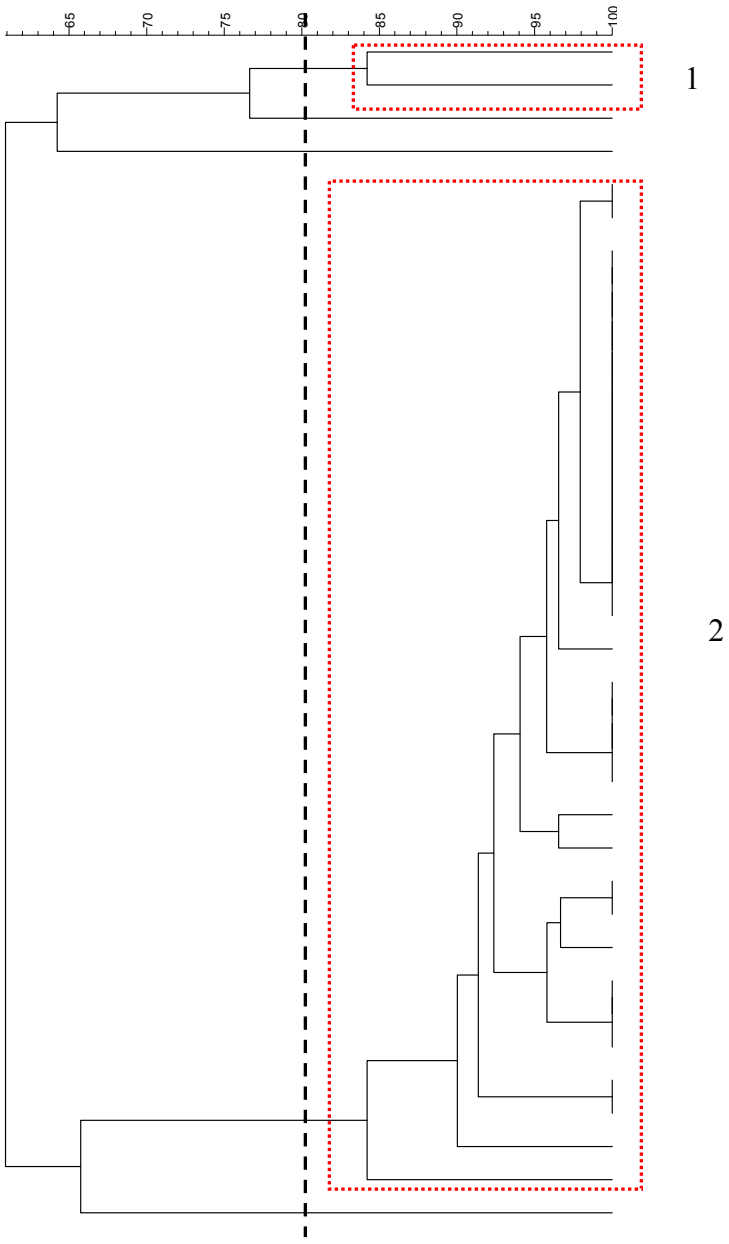


Fig. 31d.

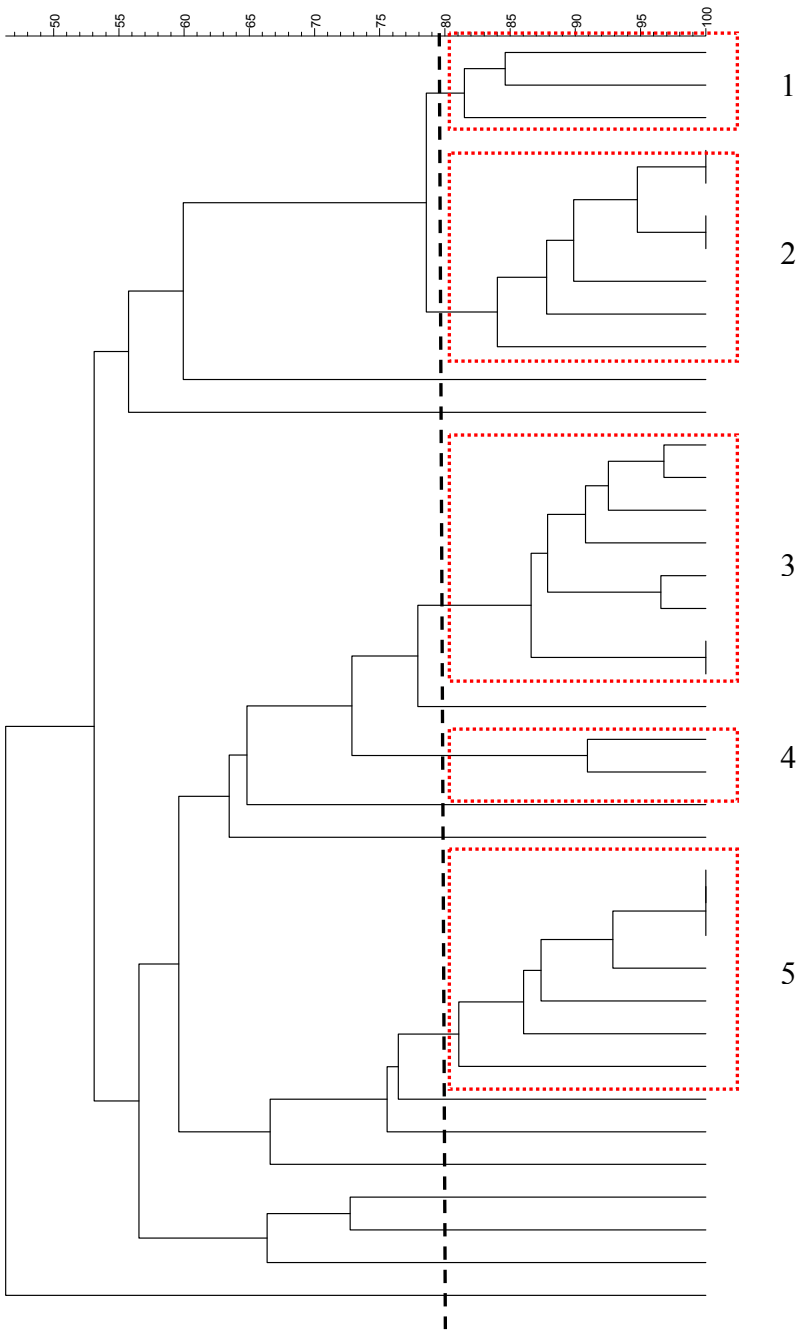


Fig. 31e.

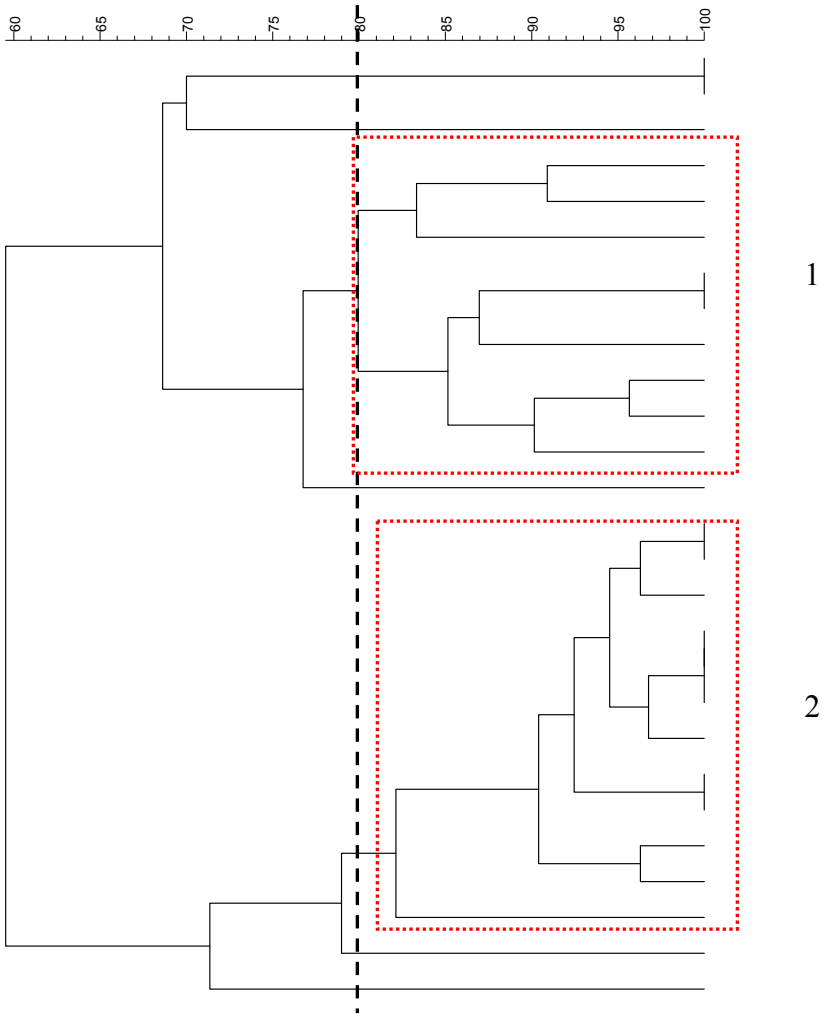


Fig. 31f.

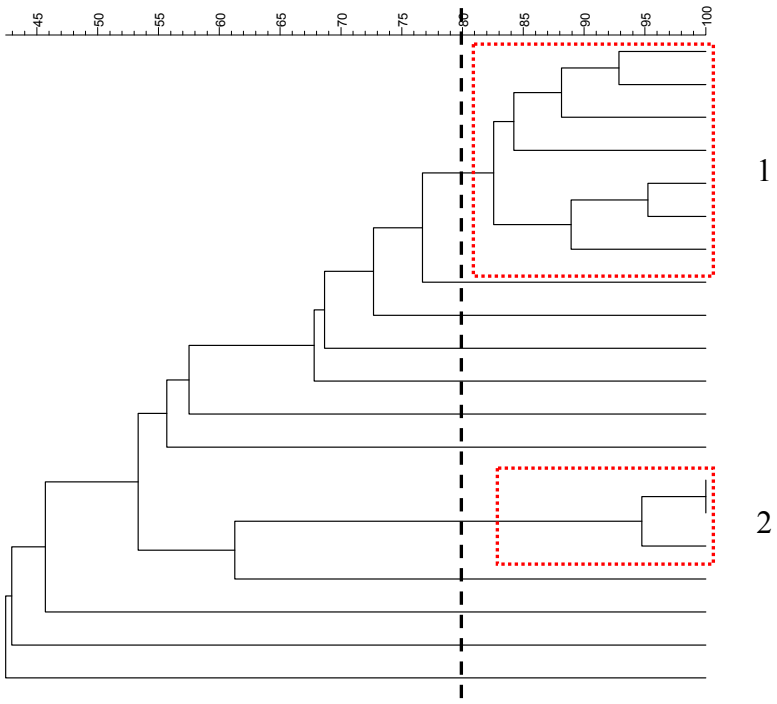


Fig. 31g.

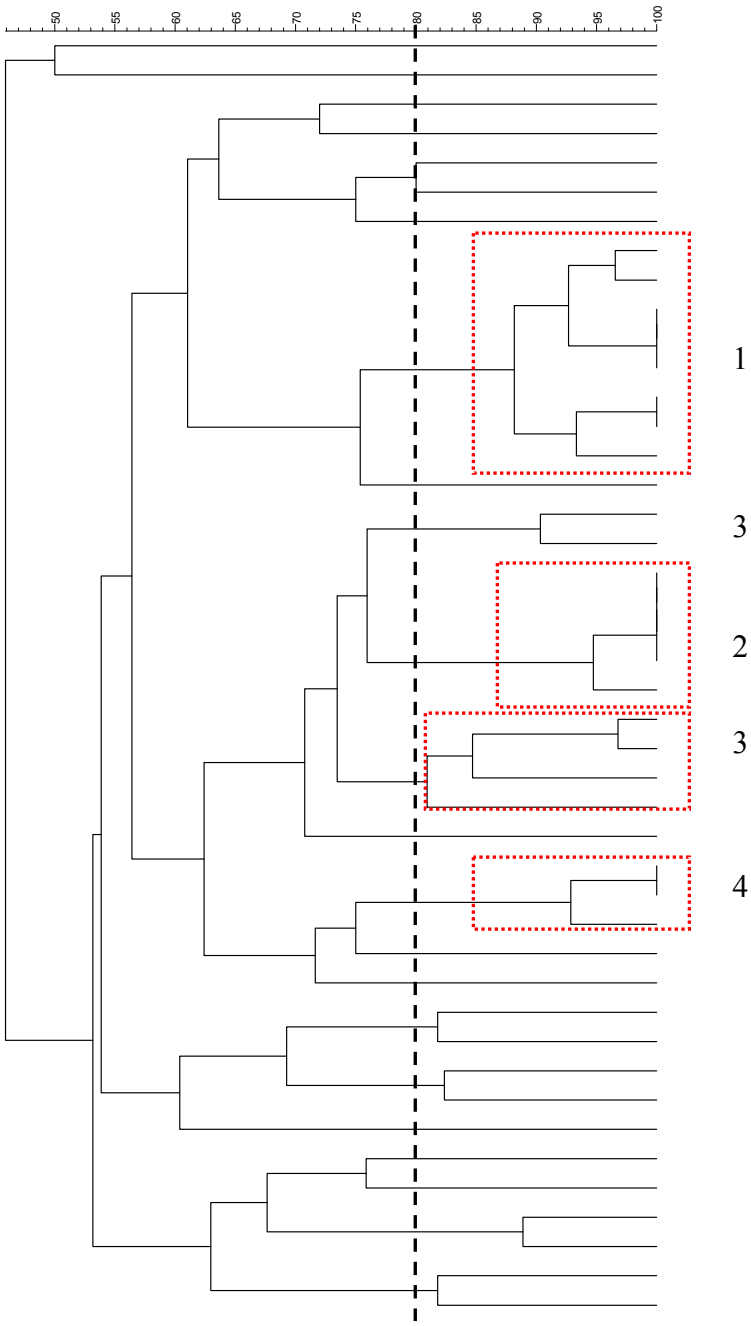
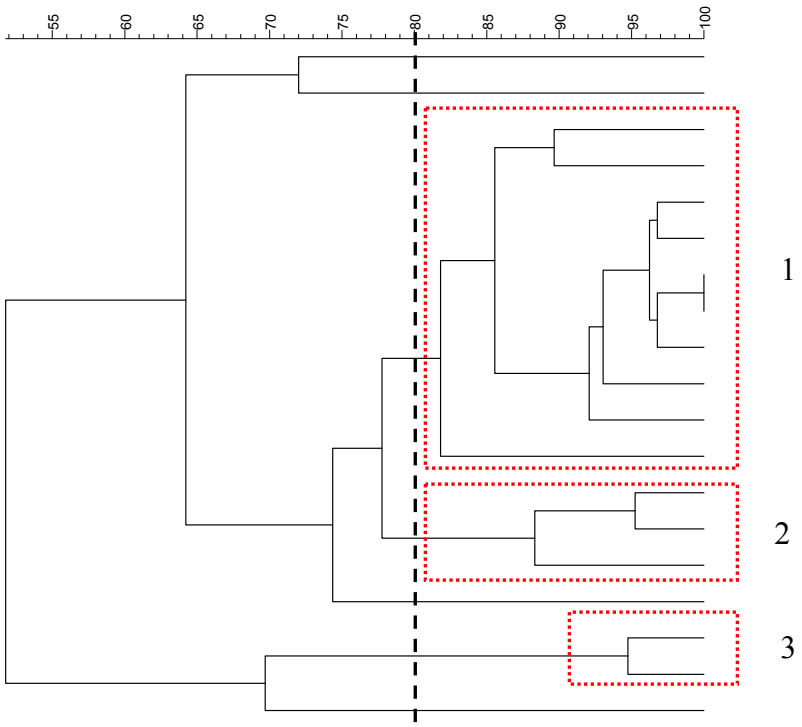


Fig. 31h.



Increasing Prevalence of Multidrug Resistant Serotype 19A Isolates

Figure 32 shows the percentage and the number of isolates with 19A serotype during each year of the study. These isolates increased from 1/74 (1.4%) in 1999, the first year identified to 13/84 (15.5%) in 2008, the last year of the study and overall increase by 14.1% from 1998 to 2008, Figures 12, 17. Isolates with serotype 19A increased predominantly among adult (18-64) and elderly (≥65) populations but also 3% (<2, 5-17) to 10% (2-4) increase among pediatric populations was noted, Figure 17.

Antibiotic profile of the total 44 19A serotype isolates identified during this study is shown in Figure 33. Out of all 19A isolates 77% (34) were non-susceptible to penicillin, 61% (27) were non-susceptible to T/S, 34% (15) non-susceptible to doxycycline, 73% (32) were non-susceptible to clindamycin. In addition, 77% (56) of isolates were identified as non-susceptible to both erythromycin and penicillin (multidrug resistant MDR2) and 61% (27) were non-susceptible to erythromycin, penicillin and T/S (MDR3).

Figure 32. Emergence of 19A serotype represented as percentage and number of the total isolates each year during the 1998 and 2008 study. The number of macrolide resistant *S. pneumoniae* each year was as follows: 1998 (n=70), 1999 (n=131), 2000 (n=81), 2001 (n=120), 2002 (n=159), 2003 (n=147), 2004 (n=173), 2005 (n=233), 2006 (n=223), 2007(n=97), and 2008 (n=84).

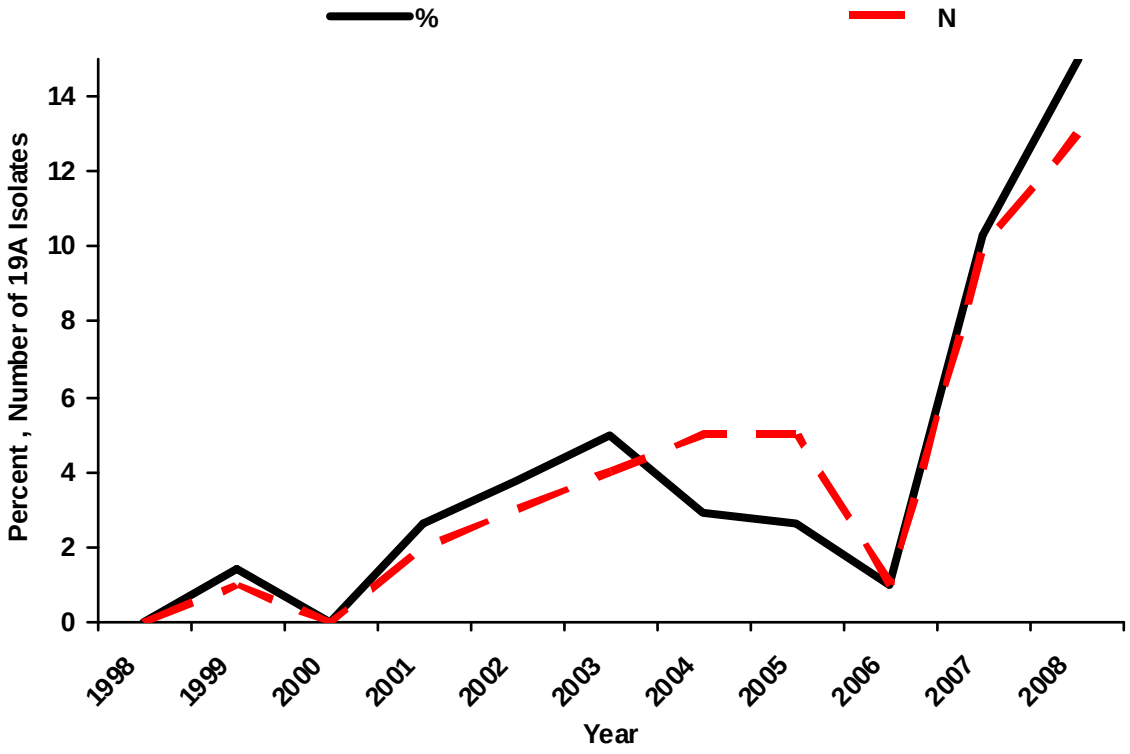
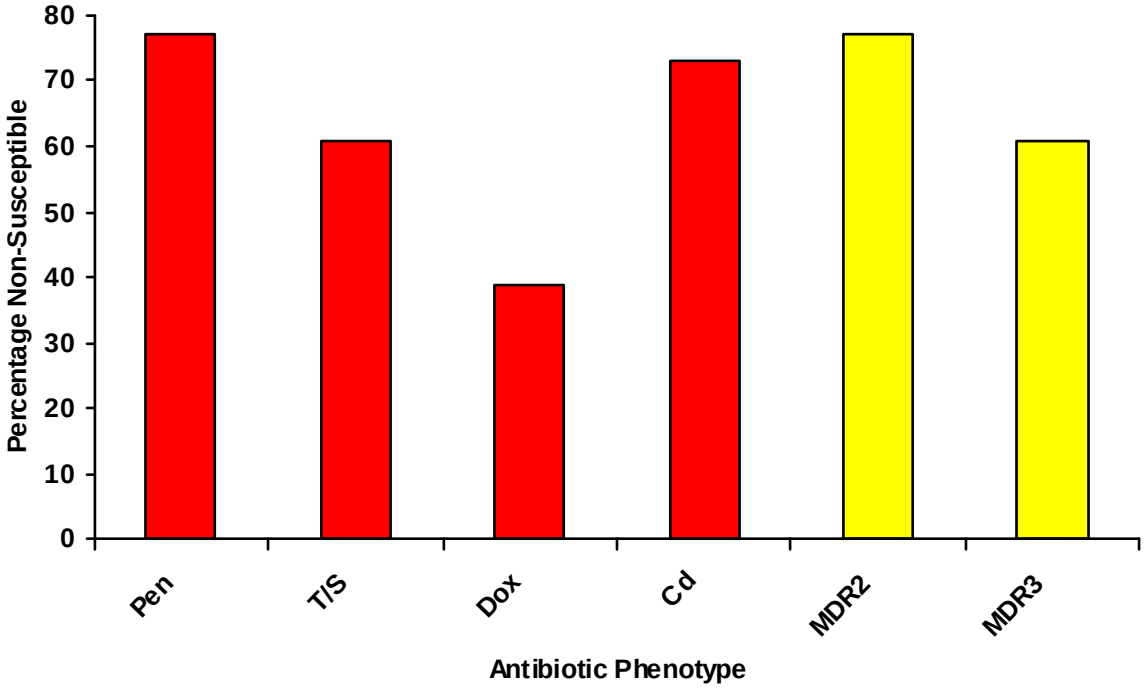


Figure 33. Antibiotic susceptibility profiles for the emerging 19A serotype isolates during the 1998 and 2008 study. Isolates represented as percent non-susceptible according to CLIS breakpoints as follows: Pen (oral penicillin V) 0.12- ≥2µg/mL; T/S 1/19-38 - ≥4µg/mL; Dox (tetracycline) 4- ≥8µg/mL and Cd 0.5 - ≥1µg/mL. Multidrug resistant 2 MDR2 is defined as non-susceptible to macrolide (clarithromycin) 0.5- ≥1µg/mL and pen (oral penicillin V) 0.12- ≥2µg/mL. Multidrug resistant 3 (MDR3) is defined as non-susceptible to macrolide (clarithromycin) 0.5- ≥1µg/mL, pen (oral penicillin V) 0.12- ≥2µg/mL and T/S 1/19-38 - ≥4µg/mL.



Emergence and characterization of serotype 6C

Figure 34, shows the number of serotype 6A and 6C isolates during each year of the study. In total, 67 (6A) and 7 (6C) isolates were identified during the study. Serotype 6C was first identified in 2003 in 2/80 (3%) and subsequently in 2006 in 1/111 (2%), in 2007 in 1/97 (1%) and in 2008 in 2/84 (2%) macrolide resistant SPN isolates.

Comparison of serotype 6A isolates with serotype 6C isolates in terms of age group, region, genotype, gender, and patient status is shown in Figures 35a-35e. Fifty two percent (34) of isolates with serotype 6A were found among adult (18-64) population and 43% (3) of isolates with serotype 6C were found among elderly (≥65) population, Figure 35a. Isolates with 6A serotype were predominantly identified from ON region (40%), while those with 6C were predominantly from Maritime region (43%), Figure 354b.

Isolates with 6A serotype were predominantly of *mef*(A) genotype (72%); however they were present among all other genotypes as well, while isolates with 6C serotype isolates were solely of *mef*(A) genotype, Figure 35c. Male gender predominated among serotype 6C (86%) isolates while an equal male to female ratio was present among serotype 6A isolates, Figure 20d. No difference among patient status was noted, Figure 35e.

Antibiotic susceptibility profile for serotype 6A and serotype 6C isolates is shown in Figure 36. Among isolates with serotype 6A, 77% percent were penicillin non-susceptible (penicillin intermediate and resistant, CLSI breakpoint 0.12- ≥2μg/ml), 61% were penicillin intermediate (CLSI breakpoint of 0.12-1μg/ml) and 12% were penicillin resistant (CLSI breakpoint ≥2μg/ml). Among isolates with serotype 6C, 57% were penicillin intermediate and 0% was penicillin resistant. Among isolates with serotype 6A, 33% were T/S non-susceptible, 9% were doxycycline non-susceptible, and 24% were

clindamycin non-susceptible. Eighty percent of serotype 6C isolates were susceptible to T/S, 100% were susceptible to doxycycline and clindamycin.

The genetic relatedness of the 7, 6C serotype isolates is shown in Figure 37. Overall all 6C serotype isolates were 65% genetically related. Seventy-one percent (5/7) of the 6C serotype isolates were part of two clusters, numbered 1 and 2 showing 80% genetic relatedness.

Figure 34. Emergence of isolates with serotype 6C during the 1998 and 2008 study.

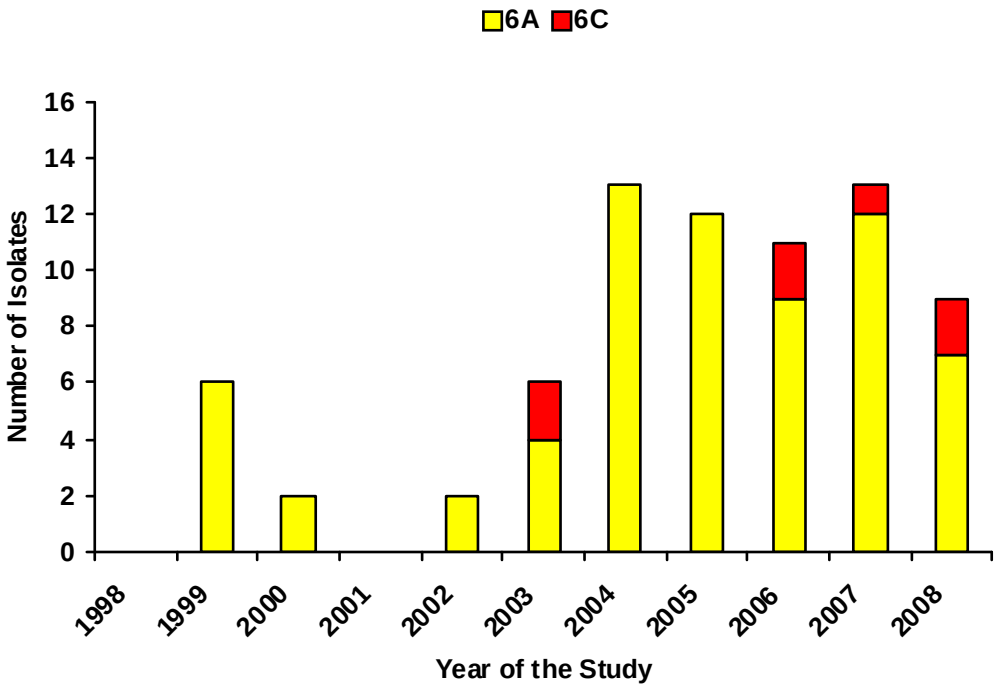


Figure 35. Prevalence of isolates with 6A versus 6C serotype by (a) age group (b) region (c) genotype (d) gender and (e) patient status. Five age groups are as follows: pediatric: <2, 2-4, 5-17, adult: 18-64, and elderly: ≥65 and 5 regions are as follows BC/AB (British Columbia and Alberta), SK/MB (Saskatchewan and Manitoba), ON (Ontario), QC (Quebec), and the Maritime provinces (Nova Scotia, Newfoundland, New Brunswick, Prince Edward Island). Patient status defined as outpatient or inpatient.

Fig.35a.

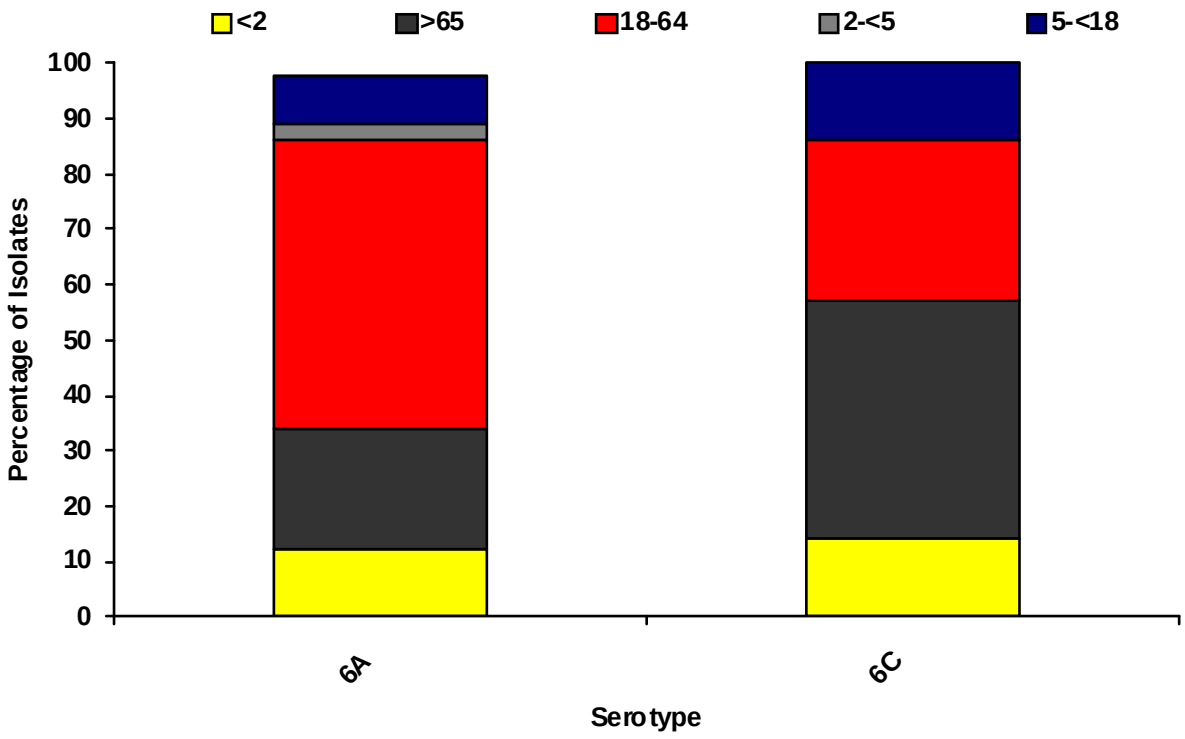

<2
>65
18-64
2-<5
5-<18
100
90
80
70
60
50
40
30
20
10
0
Percentage of Isolates
6A
6C
Serotype

Fig 35.b.

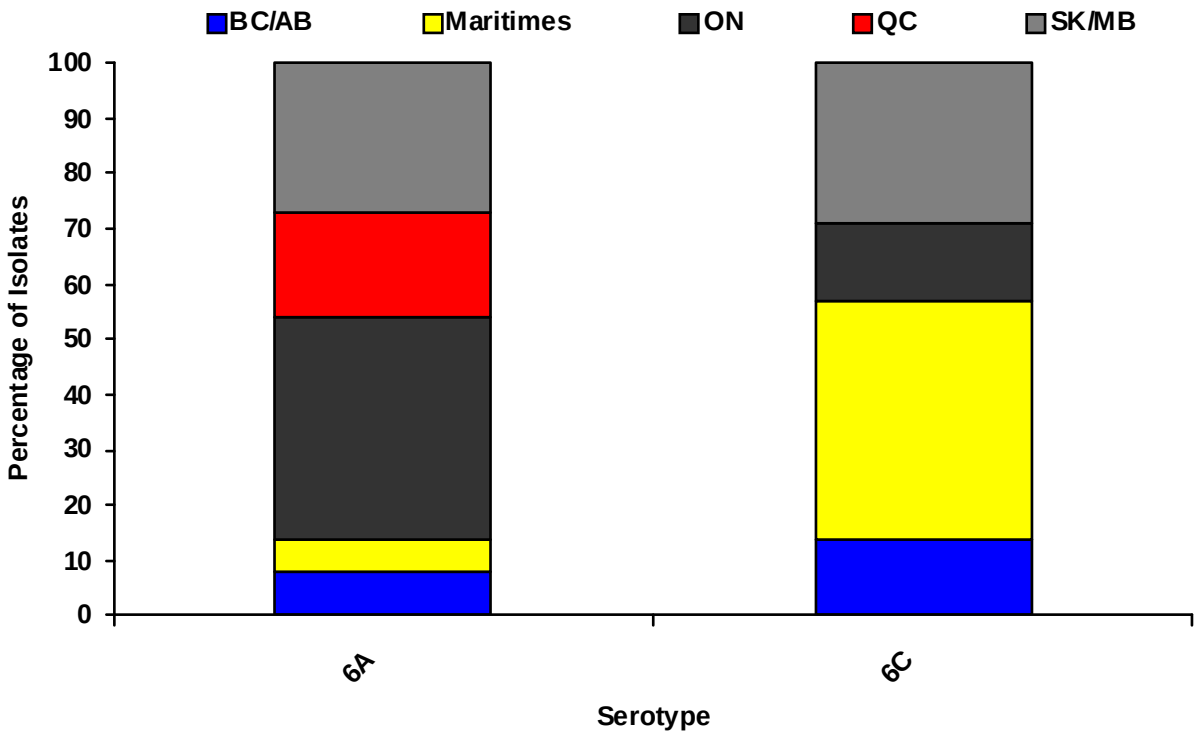

BC/AB
Maritimes
ON
QC
SK/MB
100
90
80
70
60
50
40
30
20
10
0
Percentage of Isolates
6A
6C
Serotype

Fig. 35c.

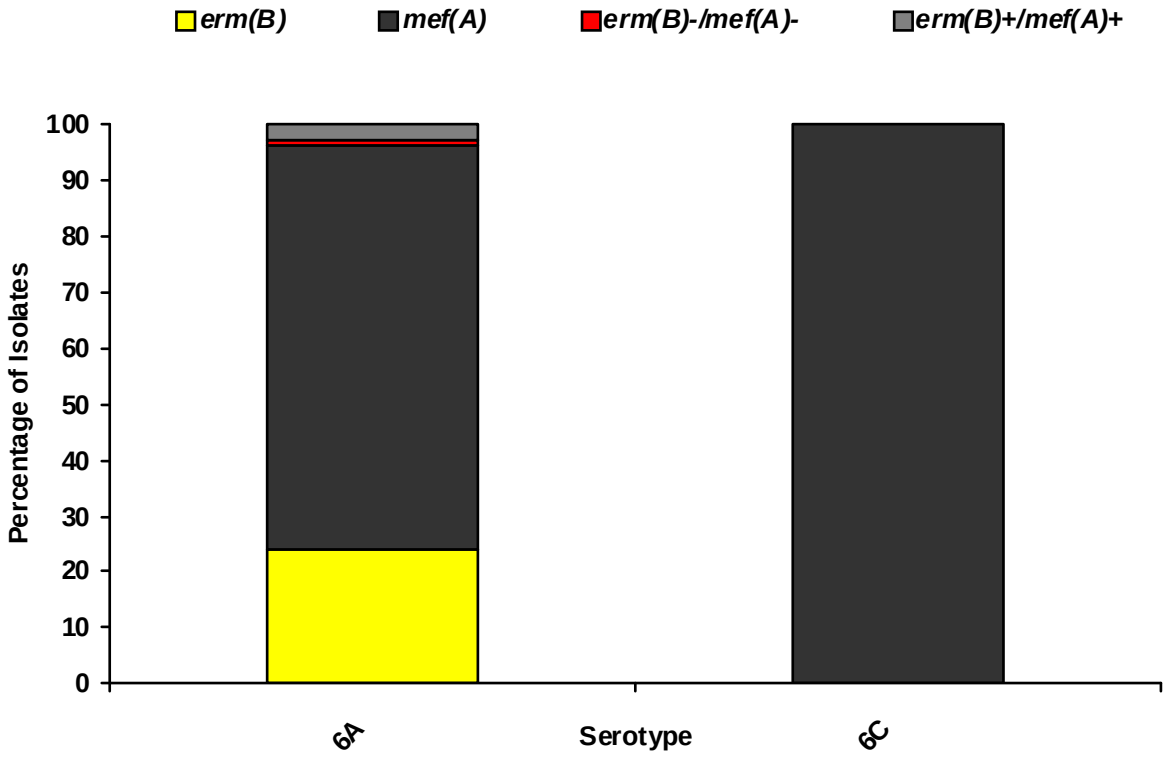

erm(B)
mef(A)
erm(B)-/mef(A)-
erm(B)+/mef(A)+
Percentage of Isolates
100
90
80
70
60
50
40
30
20
10
0
6A
Serotype
6C

Fig. 35d.

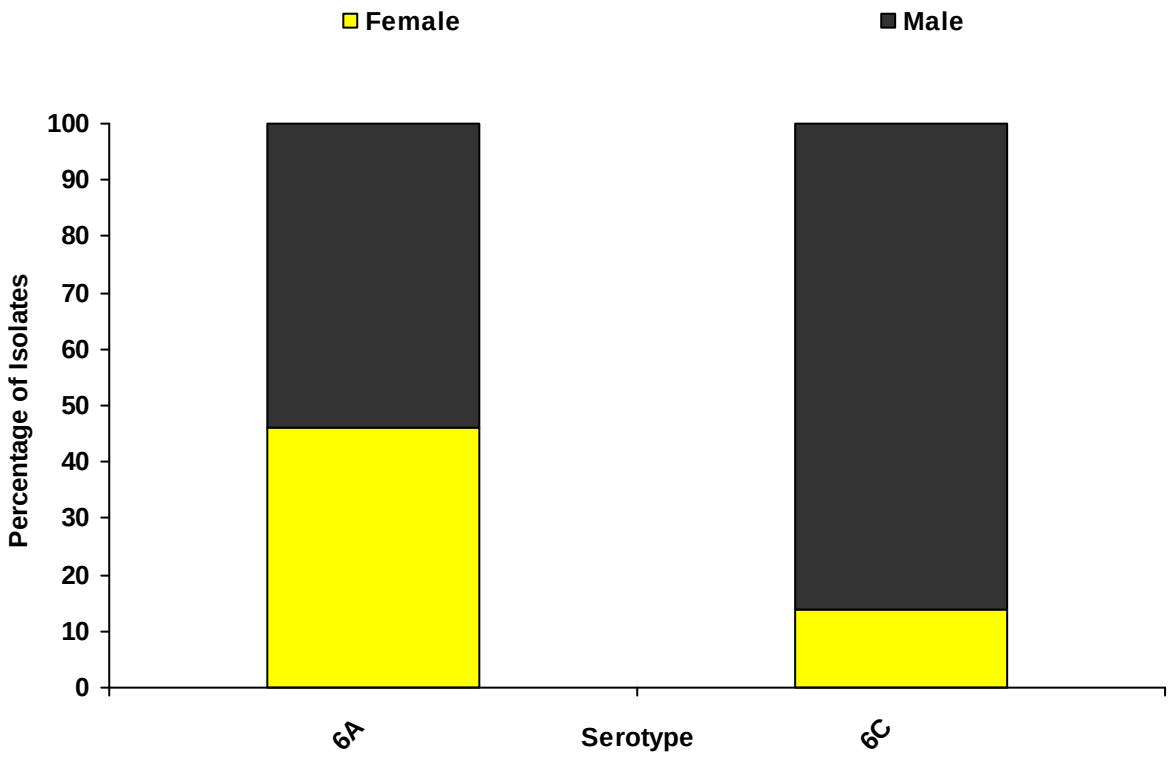

Female
Male
100
90
80
70
60
50
40
30
20
10
0
Percentage of Isolates
6A
Serotype
6C

Fig. 35e.

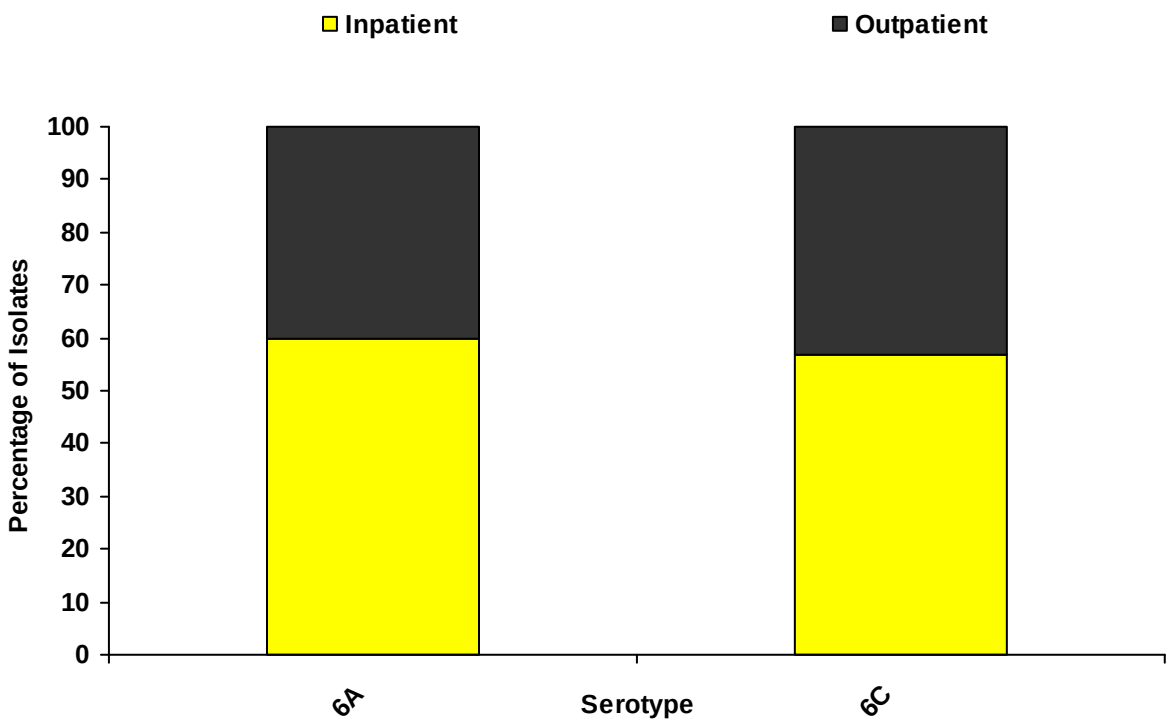

Inpatient
Outpatient
Percentage of Isolates
100
90
80
70
60
50
40
30
20
10
0
6A
Serotype
6C

Figure 36. Antibiotic susceptibility profiles for isolates with 6A versus 6C serotype during the 1998 and 2008 study. Isolates represented as percent non-susceptible according to CLIS breakpoints as follows: Pen I +R (oral penicillin V) 0.12-≥2µg/mL, Pen I (oral penicillin V) 0.12-1µg/mL, Pen R ≥2µg/mL; T/S 1/19-38 - ≥4µg/mL; Dox (tetracycline) 4- ≥8µg/mL and Cd 0.5 - ≥1µg/mL.

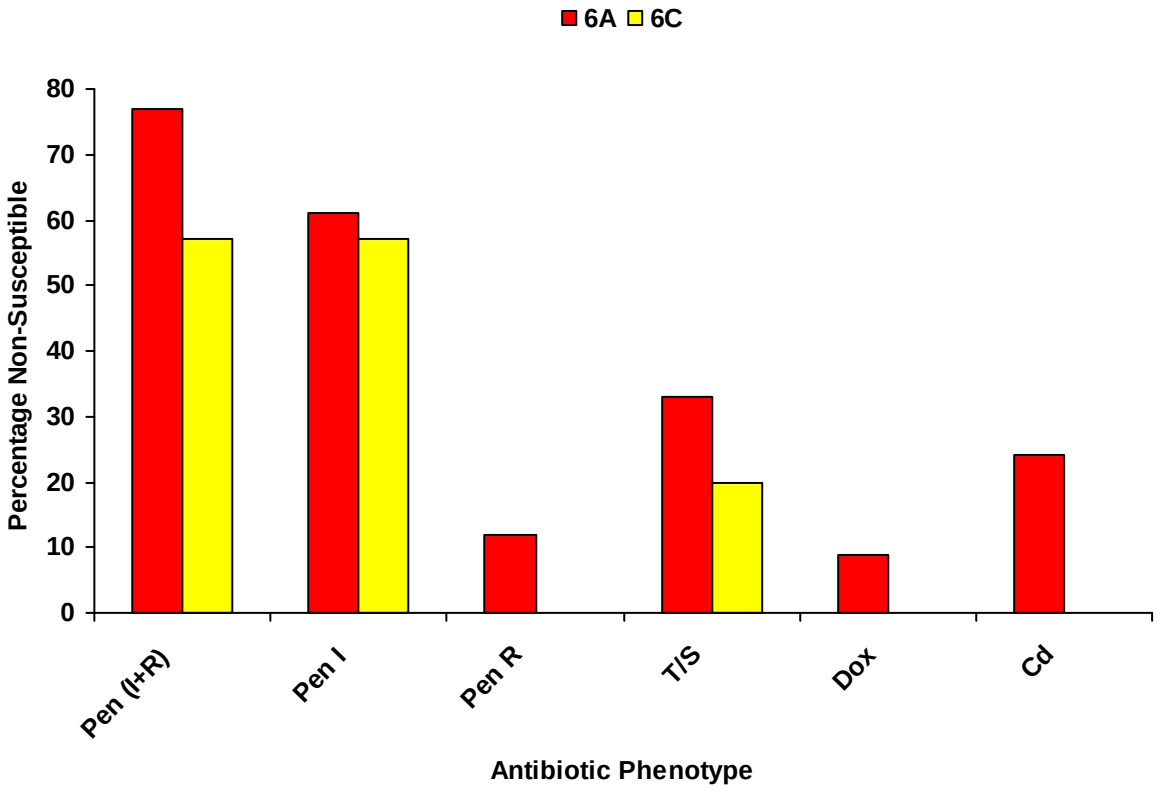
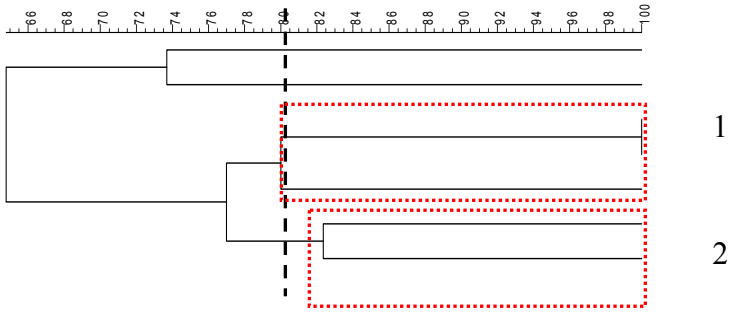


Figure 37. Dendrogram depicting genetic relatedness of the emerging macrolide resistant *S. pneumoniae* with serotype 6C. PFGE was conducted with *Sma*I digestions. 80% similarly is indicated with a dashed line. Clusters are boxed with dotted lines and cluster numbered as indicated.



PCV7 Vaccine Coverage

Figure 38 depicts the PCV7 vaccine coverage for all serotyped macrolide resistant SPN during the study. Fifty-seven percent of isolates were covered by the vaccine, 36% were not covered and 7% were identified as possibly covered. In addition, Figure 38 shows the PCV7 vaccine coverage by year. The percentage of isolates covered by the vaccine ranged from a high of 74% (2000, 2001) to a low of 18% (2007). It decreased from 67% to 31% between 1998 and 2008 study (p=0.0072). The percentage of isolates not covered by the PCV7 vaccine ranged from a high of 65% (2007) to a low of 18% (1999). It increased from 33% in 1998 to 57% in 2008 (p=0.0152. The percentage of isolates possibly covered by the PCV7 vaccine ranged from a low of 0% (1998, 2001, and 2004) to a high of 17% (2007). The percentage of isolates possibly covered by the PCV7 vaccine increased from 7% in 1998 to 12% in 2008 (p=0.362). PCV7 vaccine coverage by genotype is shown in Figure 39. The coverage ranged from a low of 47% [neither *mef*(A) nor *erm*(B)] to a high of 66% [both *mef*(A) and *erm*(B)] SPN. The coverage was 64% for *erm*(B) carrying isolates and 50% for *mef*(A) carrying isolates.

Antibiotic susceptibility among isolates with non-PCV7 serotypes and with PCV7 serotypes pre (1998-2000), during (2001-2004) and post (2005-2008) PCV7 vaccine introduction is shown in Tables 2a and 2b. Among 464 isolates with non-PCV7 serotypes, 52 (11%) were obtained pre (1998-2000), 151 (33%) during and 261 (56%) post PCV7 vaccine introduction. Among 582 isolates with PCV7 serotypes, 125 were obtained pre (1998-2000), 260 during (2001-2004) and 197 post (2005-2008) PCV7 vaccine introduction. Among isolates with PCV7 serotypes, penicillin non-susceptibility increased by 4.7%. This increase was mostly due to increase in penicillin resistance by

11.3%, while penicillin intermediate isolates decreased by 6.6% between the pre (1998-2000) and the post (2005-2008) PCV7 vaccine introduction periods. Clindamycin resistance among isolates with PCV7 serotypes increased 3.1% during the pre and the post PCV7 vaccine introduction periods. T/S and Dox non-susceptibility decreased 11% and 27%, respectively. Among isolates with non-PCV7 serotypes, penicillin non-susceptibility increased 4.6% (resistant by 4.4% and intermediate by 0.2%) between the pre and the post PCV7 vaccine introduction periods. Doxycycline and Clindamycin non-susceptibility decreased, but increases in the intermediate resistance of 4% and 0.8%, respectively were noted.

Retrospective PCV13 coverage is shown in Figure 40. The percentage of isolates covered by the vaccine was 68%, not covered was 30% and possibly covered was 2%. The percentage of isolates covered by the PCV13 vaccine ranged from a low of 50% (2007) to a high of 81% (2000). The PCV13 vaccine coverage decreased 11% from 69% in 1998 to 58% in 2008. The percentage of isolates not covered by the vaccine ranged from a low of 17% (1999) to a high of 50% (2007). It increased from 31% in 1998 to 40% in 2007, an increase of 9%. The percentage of isolates possibly covered by the vaccine ranged from a low of 0% (1998, 2000, 2001, and 2007) to a high of 3% (1999, 2002, and 2006). It changed from 2% to 1% between 1998 and 2008. The PCV13 vaccine coverage by genotype is shown in Figure 41. The coverage ranged from a low of 60% [neither *mef*(A) nor *erm*(B)] to a high of 80% [both *mef*(A) and *erm*(B)] SPN. The PCV13 coverage for *erm*(B) carrying isolates was 62% and the coverage for *mef*(A) isolates was 73%.

Figure 38. PCV7 vaccine coverage of all 1071 serotyped macrolide resistant *S. pneumoniae* as well as changes in the coverage by year during the 1998-2008 study.

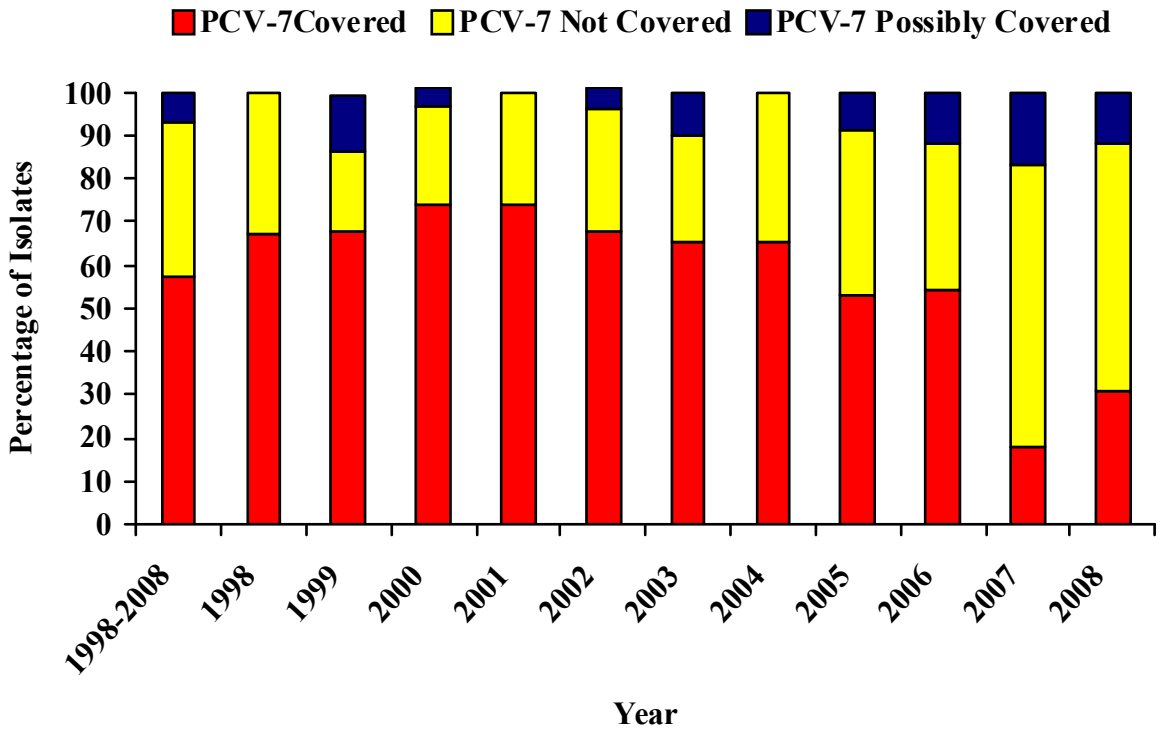


Figure 39. PCV7 vaccine coverage of all serotypes 1071 macrolide resistant *S. pneumoniae* by genotype during the 1998-2008 study.

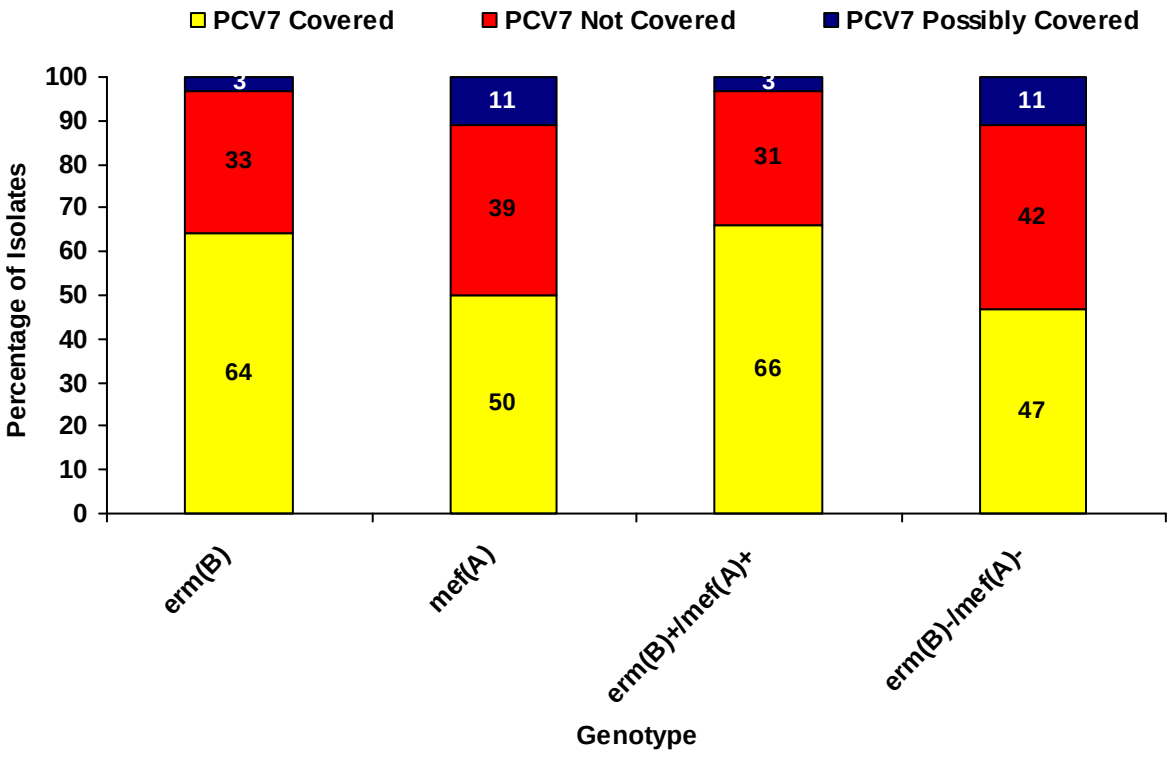


Figure 40. Retrospective coverage of new PCV13 vaccine of all 1071 serotyped macrolide resistant *S. pneumoniae* as well as the changes in the coverage by year during the 1998-2008 study.

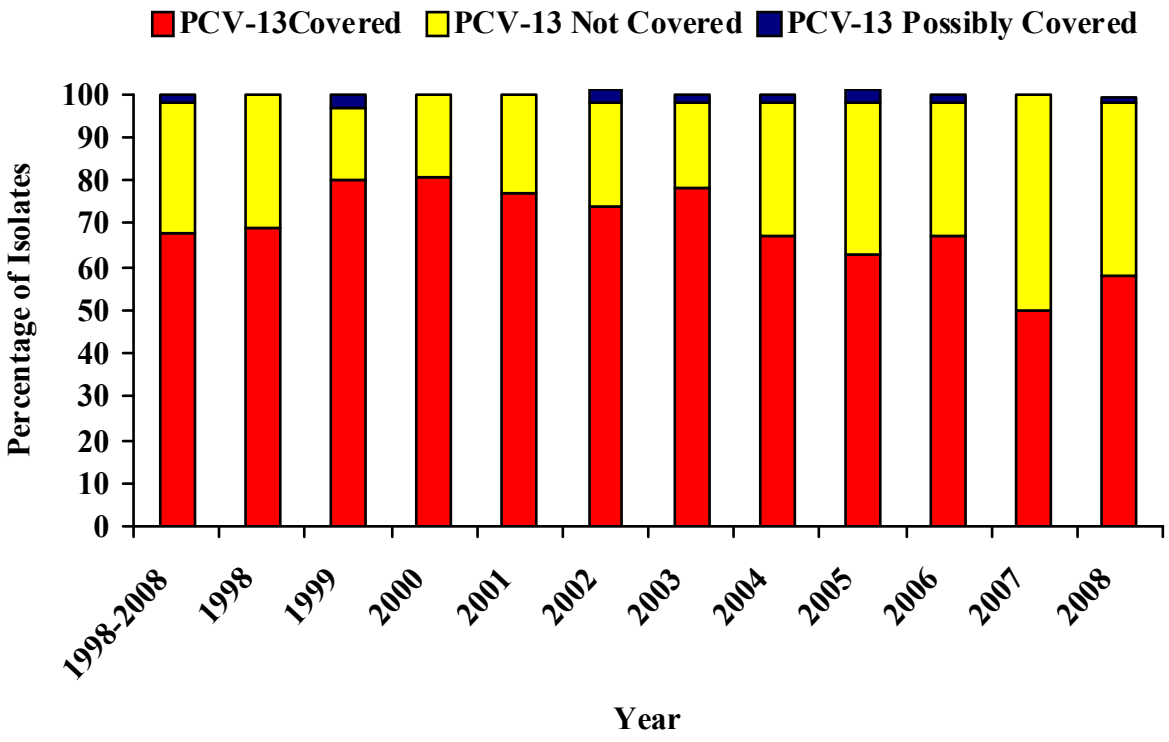


Figure 41. Retrospective PCV13 vaccine coverage of all serotypes 1071 macrolide resistant *S. pneumoniae* by genotype during the 1998-2008 study.

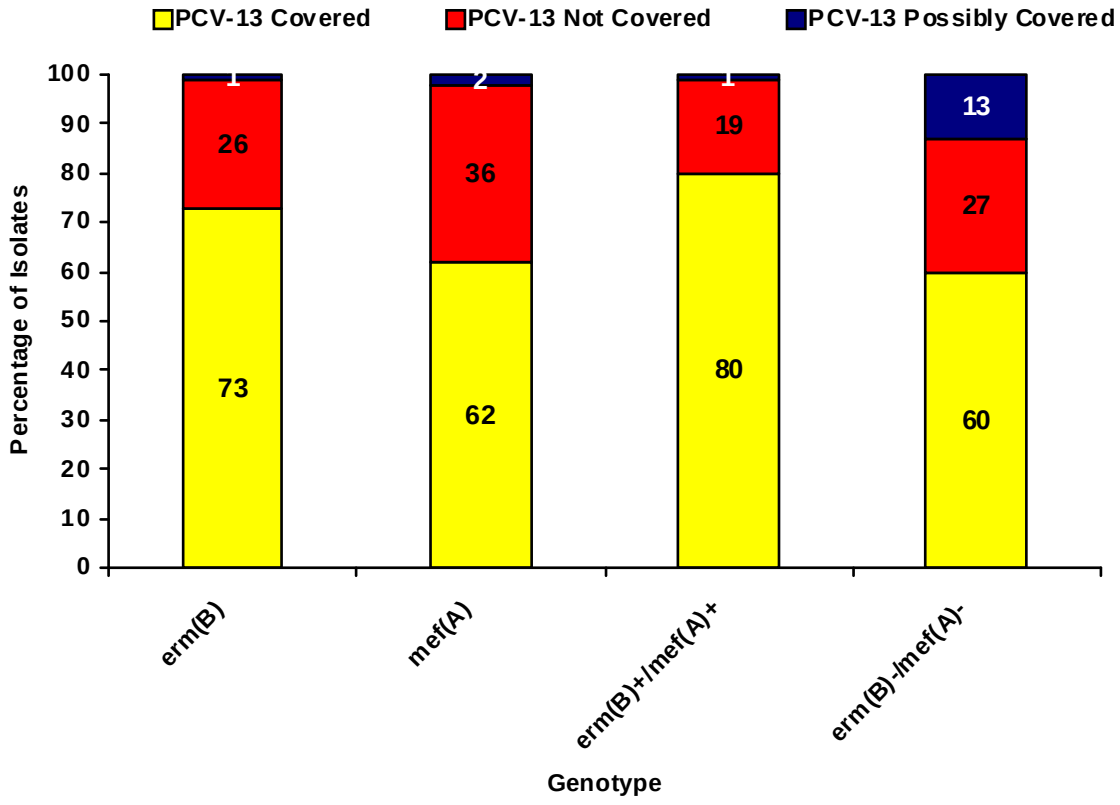


Table 6. Antimicrobial resistance among macrolide-resistant *S. pneumoniae* with (a) non-PCV7 vaccine serotypes and (b) PCV7 vaccine serotypes collected during the CROSS and CANWARD studies.

Table 6a.

	No. (%) of Resistant Isolates			
Antimicrobial	**1998-2000 (n=52)**	**2001-2004 (n=151)**	**2005-2008 (n= 261)**	**Change***
Pen				
R	3 (5.9)	35 (23.2)	26 (10.3)	4.4
I	21 (41.2)	49 (33.1)	107 (41.4)	0.2
I+R	24 (47.1)	84 (56.3)	136 (51.7)	4.6
T/S				
R	16 (31)	33 (22)	42 (16.2)	-14.8
I	6 (11.9)	19 (13)	23 (8.8)	-3.1
I+R	22 (42.9)	68 (45)	65 (25)	-17.9
Dox				
R	20 (38.1)	32 (21.2)	39 (14.9)	-23.2
I	3 (4.8)	16 (10.6)	23 (8.8)	4
I+R	22 (42.9)	48 (31.8)	63 (23.7)	-19.2
Cd				
R	19 (36.5)	56 (37.1)	94 (35.6)	-0.9
I	0	6 (4)	3 (0.8)	0.8
I+R	19 (36.5)	62 (41.1)	94 (36.4)	-0.1

*Comparison of years 1998-2000 and years 2005-2008

Table 6b.

Antimicrobial	No. (%) of Resistant Isolates 1998-2000 (n=125)	2001-2004 (n=260)	2005-2008 (n= 197)	Change *
Pen				
R	36 (29.3)	164 (62.7)	81 (40.6)	11.3
I	48 (39.1)	65 (25)	65 (32.5)	-6.6
I+R	84 (68.4)	229 (87.7)	24 (73.1)	4.7
T/S				
R	66 (53.4)	146 (56.3)	81 (41.3)	-12.1
I	26 (20.7)	55 (20.7)	43 (21.9)	1.2
I+R	92 (74.1)	201 (77)	124 (63.2)	-10.9
Dox				
R	60 (47.7)	36 (13.5)	20 (9.6)	-38.1
I	24 (18.9)	73 (27.7)	59 (29.9)	11
I+R	84 (66.6)	109 (41.2)	79 (39.5)	-27.1
Cd				
R	62 (49.6)	140 (53.8)	102 (51.8)	2.2
I	2 (1.6)	4(1.5)	5 (2.5)	0.9
I+R	64 (51.2)	144 (55.3)	107 (54.3)	3.1

*Comparison of years 1998-2000 and years 2005-2008

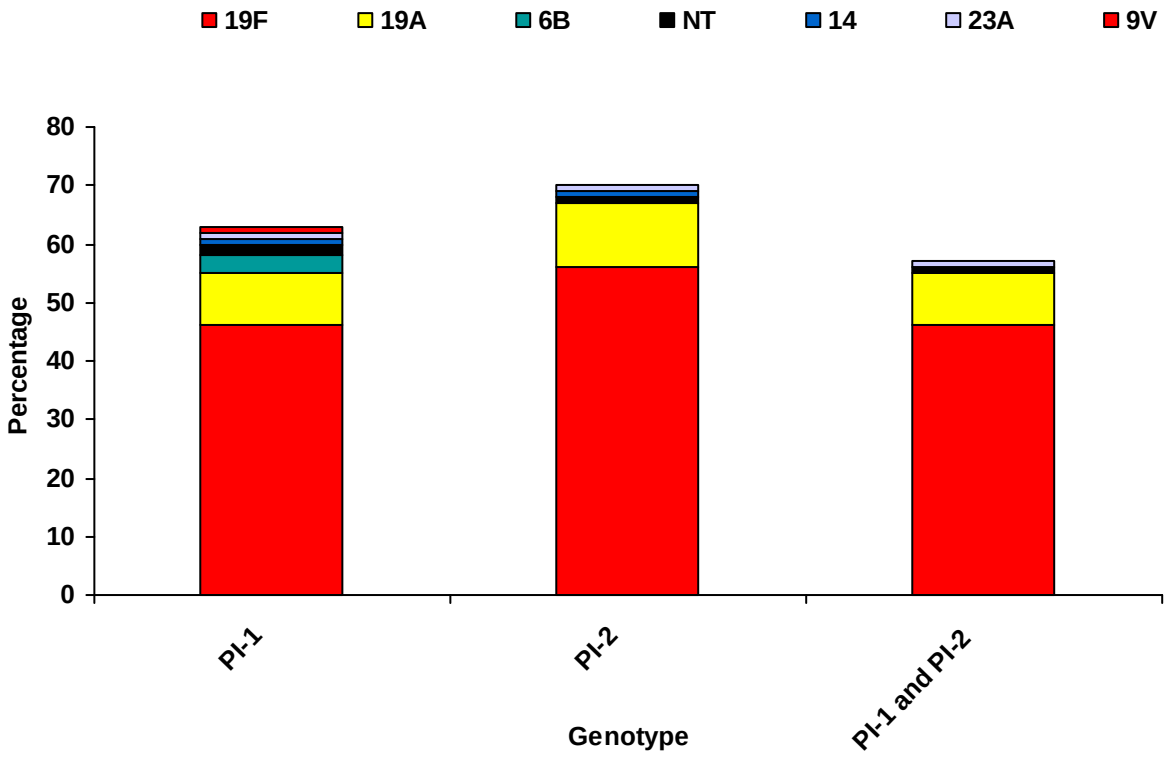
Part III: Pili Virulace Factors

Prevalence and Characterization of Virulence Factor Pili-1(PI-1) and Pili-2 (PI-2) Among both *erm*(B) and *mef*(A) Carrying SPN

Among the 116 *erm*(B) and *mef*(A) carrying isolates, 62% (72/116) carried PI-1 and 70% (81/116) carried PI-2, Figure 42. Fifty-seven (66/116) percent of isolates carried both types of pili.

The serotype distribution among PI-2 carrying isolates was as follows: 19F (65), 19A (11), 14 (1), 23A (1), NT (1). The serotype distribution among PI-1 and both PI-1 and PI-2 carrying isolates was: 19F (53), 19A (11), 6B (3), NT (2), 23A (1), and 9V (2) and 19F (53), 19A (11), 23A (1), and NT (1), respectively.

Figure 42. Differences in serotype distribution among dual *mef*(A) and *erm*(B) PI-1, PI-2 and both Pili-1 and Pili-2 carrying macrolide-resistant *S. pneumoniae*.



Presence of PI-1 and PI-2 Among Isolates Carrying both *erm*(B) and *mef*(A) 19F and 19A and Among Other 19F and 19A SPN

In total, 215 19F or 19A SPN isolates were assessed for the presence of PI-1 and PI-2. The presence of PI-1 was found among 124 (58%) of isolates, Figure 43. The PI-2 pili was found among 94 (44%) isolates as has the presence of both PI-1 and PI-2 was also among 94 (44%) of isolates, Figures 44 and 45. Out the 215 isolates, 82 carried both *mef*(A) and *erm*(B) genes and the presence of PI-1, PI-2, and both PI-1 and PI-2 among these isolates was 78% (64/82), 95% (78/82), and 78% (64/82), respectively, Figures 35-37. One hundred and thirty three isolates out of the 215 carried *erm*(B) genotype (61), *mef*(A) genotype (65) and neither *erm*(B) or *mef*(A) genotype (7). The presence of PI-1, PI-2 and both PI-1 and PI-2 among these isolates was 45% (60/133), 35% (46/133) and 23% (30/133), Figures 43-45. Among the 61 *erm*(B) carrying isolates 24 (39%) carried PI-1, 17 (23%) carried PI-2 and 6 (4%) carried both. Among the 65 *mef(A)* carrying isolates 33 (51%) carried PI-1, 26 (40%) carried PI-2, and 24 (37%) carried both. Among the 7 isolates neither containing nether *mef*(A) nor *erm*(B). PI-1 and PI-2 was carried in 3 (43%) of isolates, and no isolates carried both pili (data not shown).

Figure 43. Presence of Pili-type 1 among all, *mef*(A) and *erm*(B) and non *mef*(A) and *erm*(B) 19A or 19F macrolide resistant *S. pneumoniae*. All is defined as isolates of various macrolide resistance genotypes [*mef*(A), *erm*(B), both *mef*(A) and *erm*(B) and those having neither *mef*(A) nor *erm*(B) having either a 19A or 19F serotype; *erm*(B) and *mef*(A) are those carrying both *erm*(B) and *mef*(A) macrolide resistance genes and having a 19A and 19F serotype; non-*erm*(B)/*mef*(A) are isolates other than those carrying both *mef*(A) and *erm*(B) (*mef*(A) only, *erm*(B) only or isolates having neither *erm*(B) nor *mef*(A) but also having a 19A or 19F serotype].

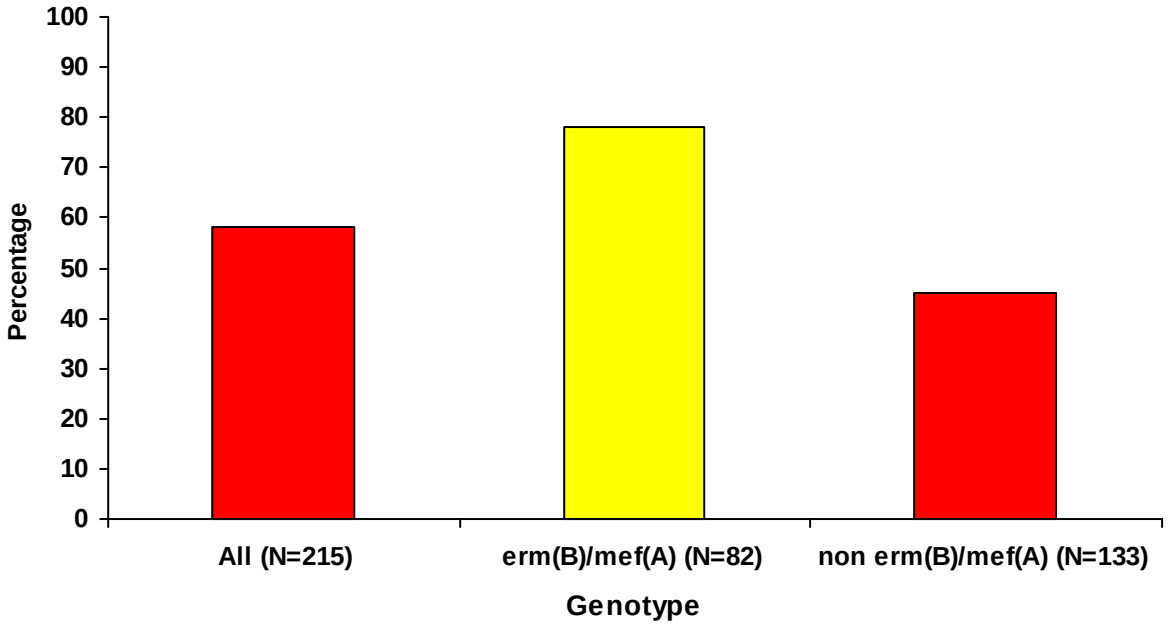


Figure 44. Presence of Pili-type 2 among all, *mef*(A) and *erm*(B) and non *mef*(A) and *erm*(B) 19A or 19F macrolide resistant *S. pneumoniae*. All is defined as isolates of various macrolide resistance genotypes [*mef*(A), *erm*(B), both *mef*(A) and *erm*(B) and those having neither *mef*(A) nor *erm*(B) having either a 19A or 19F serotype; *erm*(B) and *mef*(A) are those carrying both *erm*(B) and *mef*(A) macrolide resistance genes and having a 19A and 19F serotype; non-*erm*(B)/*mef*(A) are isolates other than those carrying both *mef*(A) and *erm*(B) (*mef*(A) only, *erm*(B) only or isolates having neither *erm*(B) nor *mef*(A) but also having a 19A or 19F serotype].

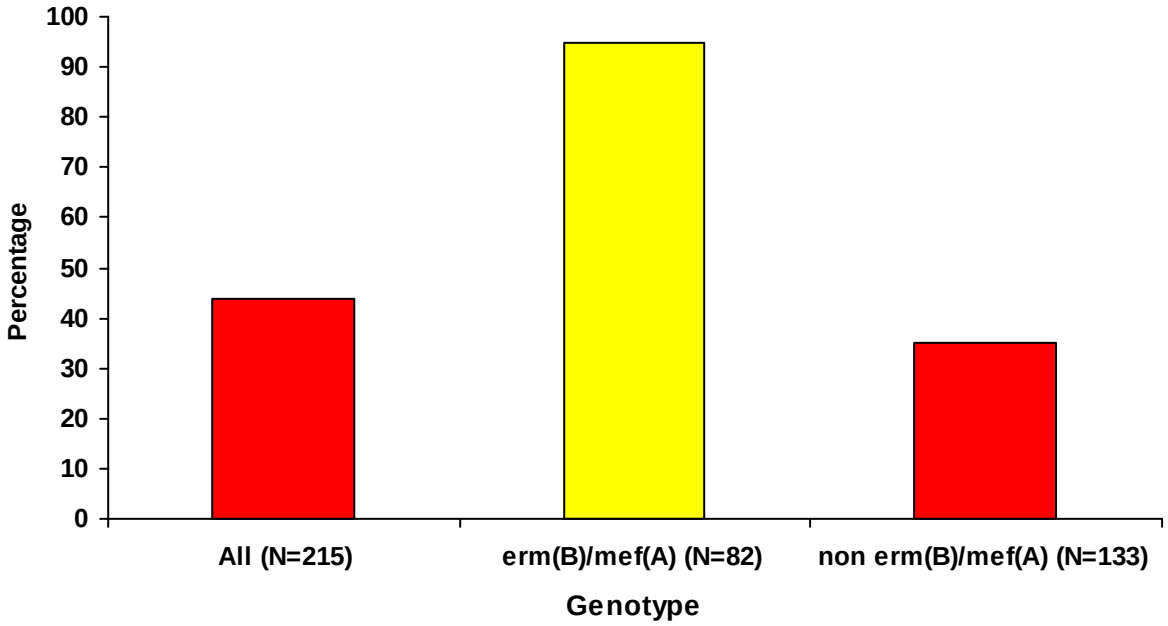
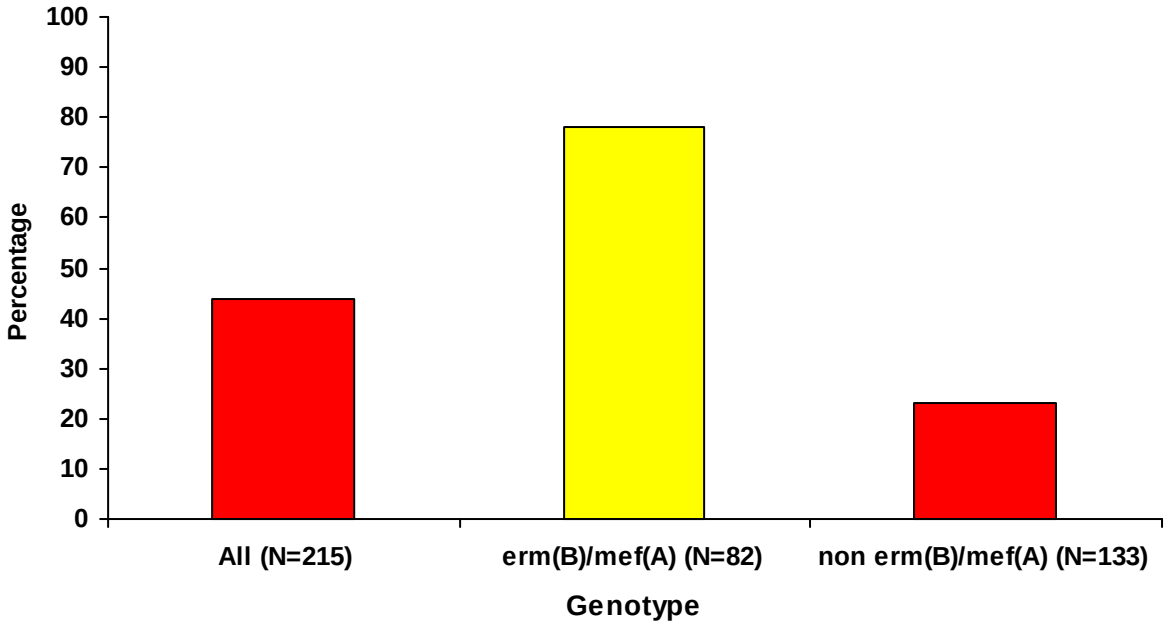


Figure 45. Presence of Pili-type 1 and Pili-type 2 among all, *mef*(A) and *erm*(B) and non *mef*(A) and *erm*(B) 19A or 19F macrolide resistant *S. pneumoniae*. All is defined as isolates of various macrolide resistance genotypes [*mef*(A), *erm*(B), both *mef*(A) and *erm*(B) and those having neither *mef*(A) nor *erm*(B) having either a 19A or 19F serotype; *erm*(B) and *mef*(A) are those carrying both *erm*(B) and *mef*(A) macrolide resistance genes and having a 19A and 19F serotype; non-*erm*(B)/*mef*(A) are isolates other than those carrying both *mef*(A) and *erm*(B) (*mef*(A) only, *erm*(B) only or isolates having neither *erm*(B) nor *mef*(A) but also having a 19A or 19F serotype].



G. DISCUSSION

Part I Characterization of Canadian Clinical Macrolide-Resistant *S. pneumoniae* Isolates

As macrolide antibiotics are among the recommended initial empiric treatment for community-acquired pneumonia most often caused by *S. pneumoniae*, it is imperative to monitor its extent of resistance in *S. pneumoniae*, characterize its resistance patterns and mechanisms of resistance and identify when new resistance patterns develop in order to determine the likelihood of their effectiveness against this bactrium. CROSS and CANWARD studies have greatly contributed to the knowledge of Canadian antimicrobial resistance development for over 10 years (92, 215). More importantly to this thesis, these studies monitored macrolide resistance, which allowed thorough characterization of macrolide-resistant *S. pneumoniae* in Canada. Characterization of clinical respiratory macrolide-resistant *S. pneumoniae* isolates between 1998 and 2008 has been the focus of this thesis. Five major findings were found. First, macrolide resistant *S. pneumoniae* increased significantly over time (208). Second, efflux was found as the predominant mechanism of macrolide resistance among clinical respiratory macrolide-resistant *S. pneumoniae* isolates (208). Third, the efflux mechanism of macrolide resistance was predominantly mediated by *mef*(A) class E gene variant of the *mef*(A) class gene (209). Fourth, higher *mef*(A) class E gene expression was found to be associated with higher macrolide MICs (204). Fifth, isolates containing both *mef*(A) and *erm(B)* gene (dual macrolide resistant) increased significantly over time (209).

During the 1998 and 2008 study period, macrolide resistance in Canadian *S. pneumoniae* isolates increased significantly ($p<0.0001$) from 8% to 21%. It remained stable at 8% during the 1998-2002 years of the study (208) and started to increase gradually thereafter, reaching 21% by the end of the study. The early low macrolide resistance found in CROSS was consistent with an 11.4% macrolide resistance reported by another group in Canada, the Canadian Bacterial Surveillance Network in 2000 (164). The low macrolide resistance rate in the late 1990s and early 2000s in Canada was in contrast to the US, where the rates of macrolide resistance being reported were 20% - 30% (105). Similarly, higher rates of macrolide resistance were noted in some countries in Europe, particularly France (53%), Italy (35%) and Spain (27%) as well as in Asia, particularly, in Japan (71%) and Hong Kong (80%) (100). In the United States, although higher than in Canada, macrolide resistance also remained stable at around 30% until 2005, however the PROTEKT study has documented its first significant increase to 35% since the study began in 2000 (106). Whether this represents a new upward trend will become evident as surveillance studies continue. It is imperative to monitor macrolide resistance, as any further rise in macrolide resistance would be a major cause for concern because macrolides remain in widespread use for treatment of community-acquired respiratory tract infections in the Untied States.

Many theories have been postulated to explain the increases in macrolide resistance. One of the theories is that increased macrolide resistance is due to increased macrolide use (137). Few studies have been published looking at this association and showed an increased macrolide resistance associated with increased macrolide consumption, more specifically, increased overall consumption of macrolides and use of longer acting macrolides such as clarithromycin and azithromycin. Other studies have showed a

decline in macrolide resistance following a decline in macrolide use as shown by a decline in prescriptions (25). Recently, a regression model was used to determine relationships between antimicrobial prescription rates and emergence of macrolide resistance in *S. pneumoniae* in Canada (112). This study demonstrated that changes in the antimicrobial prescription rates were significantly associated with changes in isolation of macrolide-resistant *S. pneumoniae*. It confirmed the findings of others that the increased use of clarithromycin and more so of azithromycin has an association with increased macrolide resistance. What was interesting and novel about this study was that it showed that the strongest association resides in the reduction in erythromycin prescriptions. The increased macrolide resistance in Canada observed in this thesis may be explained partially not only by the increased use of longer acting clarithromycin and azithromycin but also by decreased use of erythromycin.

During this study, macrolide resistance has been analysed by region. A significant decrease in the prevalence of macrolide resistant isolates has been observed in the BC/AB region, while a significant increase in macrolide resistant isolates has been noted for the ON region. It would be interesting to see whether a change in macrolide prescription rates had an effect on the change in macrolide resistance in these provinces. Analysis by age group showed that the majority of macrolide resistant isolates were from adult and elderly population and only 20% were from the pediatric age group, with only less than half from those under the age of 2 years old. As the study progressed there was a gradual decrease among the pediatric under the age of 2 years group to zero by the end of the study. Although the overall prevalence of isolates from the under the age of 2 years group was low its complete elimination by the end of the study might be reflective of the PCV7 vaccine action which specifically targets that population. The demographic

characteristics of these macrolide resistant *S. pneumoniae* are not different and are reflective of the characteristics of the CROSS and CANWARD studies (92, 215).

As macrolide resistance increased significantly over the course of the study, the mechanisms of resistance changed and evolved, however efflux *mef*(A) class E mediated resistance still remained the most common mechanism of resistance among macrolide resistant *S. pneumoniae* in Canada. Overall, efflux was responsible for macrolide resistance in 51% of macrolide resistant isolates. Most years of the study, efflux mediated macrolide resistance has been identified as the more common mechanism of macrolide resistance in Canada. However, over the 1998 and 2008 study period, the prevalence of *S. pneumoniae* strains expressing *mef*(A) gene alone had declined in Canada from 54% to 50% (p=0.037). The decline was not as pronounced as in the USA where it declined by over 10% in some studies. Isolates with efflux mediated macrolide resistance are considered low-level macrolide resistant isolates and can still be successfully treated with macrolides as their MICs are in the range that is still achievable at the site of infection by most macrolides following a standard dosage. Therefore, the decline of *S. pneumoniae* isolates with efflux mechanism of resistance can have therapeutic consequences, especially if it is replaced by isolates with higher level macrolide resistance or multi-drug resistance that are unlikely to be treated with macrolides as the MICs to inhibit the growth of these isolates greatly exceed the concentration achievable at the site of infection.

It became evident from the 10-year study that the efflux-mediated resistance is the more common type of macrolide resistance in *S. pneumoniae* in Canada. Observing the isolates with the *mef*(A) gene class genotype, it became evident that the majority of the isolates fell into the typical *mef*(A) phenotype, having a low level macrolide resistance as

expected, depicted by a low MIC_{90} value of 4µg/mL. However, it was evident from the very beginning that the range of MICs these isolates were displaying was expanding and some isolates were being detected with higher MICs (erythromycin MICs 16µg/mL, 32µg/mL) not typically found among isolates with a *mef*(A) genotype (204). Changes in expression of the *mef*(A) gene and/or underlying ribosomal mutations could account for the observed increase in MICs among *mef*(A) isolates. Isolates expressing *mef*(A) genotype and having higher than previously reported macrolide resistance (MIC_{90} of 16µg/ml) have been reported previously in the US studies (68). In these studies a clear rightward shift in the MICs for these isolates over time has been observed (68). The *mef*(A) isolates with higher than typical macrolide MICs in this study were considered sporadic and no clear rightward shift in the macrolide MICs has been noted over time (data not shown). Nevertheless, these isolates raise a concern as they have the potential to affect the therapeutic options. The effect of macrolide resistance on the ability of macrolides to eradicate S. *pneumoniae* infections has been explored using a pharmacodynamic model (212). These studies have shown that clarithromycin at clinically achievable epithelium lining fluid concentrations has been unable to eradicate *mef*(A) carrying *S. pneumonaue* isolates with MICs ≥16µg/mL. Similar findings have been noted using a murine model of pneumococcal pneumonia, where macrolides were able to eradicate infections caused by low level (MIC 0.5-1µg/mL) *mef*(A) carrying isolates only. Therefore, reliance on just the genotype determination, although in most cases accurate, might lead to challenges in macrolide therapy, suggesting that the MIC distribution should be carefully monitored for the isolates with low-level *mef*(A) mediated resistance.

Knowing the *mef*(A) class gene mediated resistance is the predominant type of macrolide resistance in Canada, additional studies have been performed to further contribute to the knowledge of *mef*(A) mediated macrolide resistance in Canada. First, knowing that two variants of *mef*(A) gene class exist, a study looking at the differentiation of the *mef*(A) gene class into the two variants *mef*(A) class A and *mef*(A) class E was performed (55). In Canada, *mef*(A) gene class E was the predominant variant, however *mef*(A) gene class A isolates were also found (209). The class E variant is also the predominant type in US (83) and it is becoming more common in some European countries, like Germany and Spain (12, 27, 145). Typically however, *mef*(A) class A is the predominant gene responsible for macrolide efflux among macrolide-resistant *S. pneumoniae* isolates in most countries in Europe (13, 50, 156). The existence of the two variants of the *mef*(A) gene in *S. pneumoniae* has only recently been recognized due to the work of Italian group, Del Grosso et al. (55). Until then the two genes, *mef*(A) and *mef*(E) (which are now considered sub classes of the common gene *mef*(A) as named by Roberts et al.(172)) were considered species specific and due to a high degree of similarity they were grouped into one gene, *mef*(A) as described by Roberts et al. (172). A debate exists whether the two variants should be differentiated (115, 117). Due to important differences, particularly in antibiotic susceptibility profiles, and serotype distribution as well as genetic relatedness between the isolates carrying the different variants many feel that the two should be differentiated (55, 115). The differentiation of the efflux mediated macrolide resistant *S. pneumoniae* isolates in this thesis confirmed the finding of others that the *mef*(A) class E variant is resistant to more antibiotic classes, such as penicillin, T/S, and doxycycline. It also confirmed that isolates with class A variant belong to one serotype and were genetically related. Although,

higher macrolide MICs were found among isolates carrying the *mef*(A) class A variant in one study (7), such observation was not made among *mef*(A) class A isolates in this thesis. Interestingly, isolates described above as falling out of the typical range for efflux mediated resistance were all *mef*(A) class E and not class A.

Since the initial description of the two variants, simple procedures have been described that can be used to determine which variant is present, therefore many researchers are going back looking retrospectively at their collections of *S. pneumoniae* to determine the prevalence and emergence of the two variants of efflux gene (144, 157). Studies, especially from Europe have been published showing great changes in the epidemiology of efflux genes over time, describing greater and greater prevalence of *mef*(A) class E among their previously mostly *mef*(A) class A *S. pneumoniae* population (12, 27, 50). These studies support the idea of differentiation of the two *mef*(A) gene variants. The current nomenclature is confusing and therefore will probably have to be addressed in the future, especially if the two genes will become more and wide spread among *S. pneumoniae* and different species of bacteria. The differentiation will provide information regarding the distribution and prevalence of the *mef*(A) gene class variants in *S. pneumoniae* which in turn may provide information regarding the spread of efflux-mediated resistance in Canada and worldwide.

During the course of this study, efflux mediated macrolide resistant *mef*(A) class E *S. pneumoniae* isolates which were phenotypically not characteristic of low-level macrolide resistant isolates were studied. These isolates were assessed in terms of the *mef*(A) class E gene copy number and insertion sites, possibility of dual efflux system and *mef*(A) class E gene expression in effort to explain the elevated MICs. Isolates with variety of low and high macrolide MICs (erythromycin MIC 1-32µg/mL) were studied and showed that

mef(A) class E gene is present in a single gene copy number that incorporates into the pneumococcal genome in more than 4 sites, a finding that was consistent with the study in the US. Results from these experiments have been published but are not shown in this thesis. (204). Correlation between the insertion site and the macrolide MIC was found for some isolates but for the majority no correlation was noted, making it difficult to show that mega insertion site has an effect on macrolide resistance (204). It is known that the mega element that carries the *mef*(A) class E gene also carries *mel*, a gene with homology to the efflux Msr(A) protein in *S. aureus* and that the two genes are co-transcribed (6, 50). Therefore, it has been speculated that the macrolide efflux system might be a dual-efflux system (6, 50). The *mef*(A) class E and *mel* intergenic region is normally 199bp long, and is designated class I insert. Sometimes, however this intergenic region is only 20bp long and is designated as Class II insert as described by Gay and Stevens et al. (83). In this thesis, unlike that found by Gay and Stevens et al., where the Class I predominated, the prevalence of Class I and Class II inserts was equal. No correlation between macrolide MICs and the different class of mega element were made, therefore linking a dual efflux system to higher MICs was not possible. Characterization of *mef*(A) class E gene expression showed a positive correlation between gene expression and increased MICs (204). The expression of the *mef*(A) class E genes was also shown to be inducible by all macrolide antibiotics (204). This study further contributes to the knowledge of efflux mediated macrolide resistance, however although increased expression was found associated with isolates with higher macrolide MICs, this study did not show that the increased mRNA levels translate into an increased amount of the *mef*(A) class E pump protein.

The most important finding during the characterization of macrolide resistant *S. pneumoniae* isolates over the last decade has been the emergence and increasing prevalence of isolates carrying both efflux *mef*(A) class E gene and ribosomal methylase, *erm*(B) gene (dual macrolide resistant isolates). Isolates carrying both macrolide resistance genes increased 16% while isolates carrying either *mef*(A) or *erm*(B) decreased, 5% and 14%, respectively throughout the course of this study. The emergence and increasing prevalence of dual macrolide resistant *S. pneumoniae* isolates might be due to the fact that majority of these isolates belong to a small group of clonal strains that exhibit multi-drug resistance, therefore these isolates might have an evolutionary advantage over strains with the single resistance determinants. These dual macrolide resistant isolates were first identified in 1999 in a single isolate (0.8%) and the prevalence of these isolates increased gradually each year of the study, reaching 19% of all macrolide resistant isolates at the end of the study (p=0.001). The dual *erm*(B) and *mef*(A) isolates are not only highly resistant to macrolide antibiotics and clindamycin, but also to other groups of antibiotics, making them multi-drug resistant as shown in this thesis by a non-susceptible rate of 97.3%, 81.4% and 35.7% for penicillin, T/S, and doxycycline, respectively. In addition to being multi-drug resistant, these isolates belong most commonly to 19F or 19A serotype; although other serotypes are have also been found. It is, however the 19F and 19A dual macrolide resistant *S. pneumoniae* isolates that are of greatest significance as these are the ones that are the most highly multi-drug resistant and genetically related. It has been observed worldwide that in recent years it is the emergence of these isolates that has contributed to the increases in macrolide resistance in *S. pneumoniae*, suggesting a global clonal spread of these highly multi-drug resistant isolates (106). Until recently, the dual *erm*(B) and *mef*(A) isolates were being reported

occasionally in the US; 3.3% of macrolide resistant strains in 1996-97, however by 1999-2000 its prevalence reached 12.4% and 25% by 2006 (106). Higher prevalence of these dual *erm*(B) and *mef*(A) isolates however was being noted in Asian countries and in South Africa during the same time, where the rates were 16% and 30% respectively (121, 142, 199). Susceptibilities to macrolide and clindamycin for the *erm*(B) and *mef*(A) strains are identical to those of strains carrying *erm*(B) alone, which suggests that the presence of *mef*(A) gene cannot be inferred from the phenotypic expression of MIC. The clinical impact for dual macrolide resistant isolates is likely to be similar to that of *erm*(B) alone, as high-level resistance is the same. However, other drugs such as penicillin, T/S and doxycycline might not be as effective. Genotyping is necessary to determine the presence of dual macrolide resistant *S. pneumoniae*.

Since the implementation of the PCV7 vaccine, the incidence of these dual macrolide resistant *S. pneumoniae* has not declined as one might have expected, owing to the fact that they were mostly 19F serotype, a serotype that was covered by the vaccine. In years post the vaccine introduction, the dual *erm*(B) and *mef*(A) isolates continued to increase, while escaping the vaccine pressure by 19F to 19A serotype switch (21, 30, 146, 163, 168, 175, 195). In the US the highest prevalence of dual macrolide resistant isolates was found among children younger than 2 year of age, while in Canada in this study, these isolates were least common among this age group (21). This could be due to earlier implementation and greater affect of the PCV7 vaccine in the United States which was designed to target this particular age group and hence might have contributed to the increase in the dual *mef*(A) and *erm*(B) carrying isolates which escaped the vaccine by capsular switch. During this study, 19A serotype started to emerge among the dual isolates in 2003, but it was not until 2007 that it was more common than 19F. Now most

of dual *erm*(B) and *mef*(A) macrolide resistant *S. pneumoniae* isolates are of 19A serotype. Ongoing surveillance is necessary to monitor the changing epidemiology of macrolide mechanisms of resistance especially since the introduction of vaccination programs.

Part II Serotype Distribution

As *S. pneumoniae* is the leading cause of community- acquired bacterial respiratory tract infections such as pneumonia and invasive infections such as bacteremia and meningitis, especially in early childhood, and because antibiotics commonly used to treat these infections are becoming less effective due to the increasing resistance, it is not surprising that prevention rather than treatment has become an interesting new area of research and pneumococcus has become known as a vaccine-preventable pathogen (162). Pneumococcal vaccine, PCV7 was developed based on the knowledge of the serotype distribution among the target group in hopes of reducing the burden of these serotype specific invasive pneumococcal infections (32). The seven serotypes included in the vaccine were chosen based on the seven most common serotypes causing invasive pneumococcal disease in the US. Coincidently, the majority of these serotypes were also antibiotic resistant. It has been estimated that PCV7 provided protection against 80-90% of isolates causing invasive pneumococcal disease in the US. In order to be effective, ongoing surveillance of serotype distribution is necessary to ensure good coverage and to monitor emergence of new serotypes, which in turn will facilitate the development of new vaccines. The second part of this thesis was devoted to studying the serotype distribution and PCV7 vaccine coverage among the macrolide resistant respiratory *S. pneumoniae*

isolates. Three main findings were observed during the course of this study. First, the PCV7 vaccine coverage decreased over time. Second, non-PCV7 vaccine serotypes emerged. Third, 19A serotype emerged among multi-drug resistant *S. pneumoniae*.

Among macrolide resistant *S. pneumoniae* studied in the thesis, the PCV7 vaccine covered 57% and 36% were not covered. Seven percent were possibly covered, assuming cross protection of similar serotypes within the serogroup occurred. The overall coverage of the respiratory *S. pneumoniae* isolates in this study was very good, considering PCV7 efficacy against pneumonia estimated at 5-25%. An assumption is made that pneumonia is caused by respiratory rather than invasive *S. pneumoniae*. However, although PCV7 coverage was very good at the beginning of the study it declined by the end of the study, indicating that the PCV7 vaccine has an effect on respiratory tract isolates as well as on invasive isolates although not to the same extent. The PCV7 vaccine coverage ranged from a high of 74% in 2000 and 2001 to a low of 18% in 2007. Overall, PCV7 vaccine coverage decreased 36% from 67% to 31% from 1998 to 2008 ($p = 0.0072$). Individual serotypes decreased from 5 to 16%, with the highest decrease of 16% observed for serotype 19F during the course of the study. Although serotype 19F decreased the most, it was still present in significant numbers in the last year of the study, probably due to the fact that it was the most prevalent serotype and probably also due to the fact that 19F serotype is the least immunogenic, therefore one would expect that it would be not fully subjected to the vaccine pressure and therefore affected by the vaccine. The decreases in individual serotypes over time in this study were statistically significant ($p<0.05$); however they were lower than those reported in the literature. Majority of the reports describing the vaccine effect indicate much higher reductions in the PCV7 serotypes following the introduction of the vaccine. The higher declines than those observed in this

study might be due to the fact that this study involved respiratory and not invasive *S. pneumoniae* isolates based on which most of the studies in the literature are published. The other probably more important factor is that the PCV7 vaccine which was introduced in the US in 2000 was not introduced in Canada until 2001 to 2004 depending on the province, therefore the study period might be too short to truly notice the effect of the vaccine. The last two years of the study show the most significant decrease in PCV7 vaccine coverage and probably reflect the true effect of the vaccine and until then it was the lag period.

Analysis of the PCV7 serotypes coverage by age group revealed that during the course of the study, the prevalence of the PCV7 serotypes decreased the most among the age group that the vaccine was intended for, the <2 age group. Concurrent with the literature the PCV7 serotypes decreased not only among the target group, those under the age of 2 but also among the elderly (>65 years old) and pediatric (2-4 year old) age group; results showing that the PCV7 vaccination not only reduced the prevalence of PCV7 serotypes in the group for which the vaccine was intended for (<2 years old) but also in the unvaccinated population, showing the presence of herd immunity. Among the pediatric age group <2 and 2-4, the greatest decreases were for serotype 6B, 14 and 19F and 19F, respectively and among the elderly age group for serotype 9V, 19F, and 6B.

Comparing the presence of PCV7 serotypes pre (1998-2000), during (2001-2004) and post (2005-2008) PCV7 vaccine introduction, 6 out of 7 PCV7 serotypes decreased by 1 to 11% (some did not reach statistical significance). Again the declines among the individual PCV7 vaccine serotypes were lower than the 80-90% decreases reported in the literature, possibly due to small and focused nature of this study and also due to later implementation of the PCV7 vaccine in Canada.

As the prevalence of vaccine serotypes decreased, an increase in the non-vaccine serotypes has been observed. Overall, 36% of macrolide resistant isolates during the study were not covered by the PCV7 vaccine. The percentage of isolates with non-vaccine serotypes ranged from a low of 18% in 1999 to a high of 65% in 2007 (p= 0.0152). The non-PCV7 serotypes increased on average 3.2% per year. The prevalence of isolates not covered by the PCV7 vaccine increased 24% from 33% in 1998 to 57% in 2008. The most common serotypes not covered by the vaccine were: 6A (6.3%), 19A (4.1%), 15A (3.8%), 12F (3.6%), 15B (2.8%), 11A (2.8%), 15C (2%), and 33F (1.9%).

Comparing the first and the last year of the study, isolates with serotype 33F, although the least prevalent among the top 8 emerging serotypes, increased the most by 17.1%. Majority of these isolates were highly resistant to macrolide antibiotics and clindamycin, mostly mediated by *erm*(B) gene, however, remained non-resistant to other classes of antimicrobials, such as penicillin and T/S. They were found to be genetically related; therefore their emergence might be facilitated by clonal spread. Serogroup 15 emerged as well, with the greatest increases noted for serotype 15A. Slightly different rates were noted when the comparisons were made by looking at pre (1998-2000), during (2001-2004) and post (2005-2008) PCV7 introduction periods. Comparing the pre (1998-2000) to post (2005-2008) PCV7 introduction periods, serotype 15B increased 5%, serotype 6A and 15C increased 4% and serotypes 11A, 15A, and 33F increased 3%. Serotype 12F is interesting; it was the 4th most common non-PVC7 serotype, it has emerged sporadically throughout the study, however overall it showed no change in its prevalence when comparing the pre (1998-2001) and post (2005-2008) PCV7 vaccination periods and a decline of 3% when comparing the first and the last year of the study. Interestingly, the few sporadic 12F isolates were localized to western provinces and the adult population.

These observations are consistent with a recent report from the Public Health Agency of Canada, Health Canada, which indicated the prevalence of this serotype across Canada in 2010 at 5% and its prevalence in western provinces, particularly Manitoba at 22% (personal communication, Irene Martin). The high prevalence of this serotype in Manitoba has been implicated in an outbreak causing pneumonia among select group of patients. In the current study, isolates with serotype 12F were found exclusively among low-level, *mef*(A)-mediated macrolide-resistant *S. pneumoniae* isolates; leaving macrolides as an effective treatment option. However, they were genetically related, and their emergence especially in a setting like the outbreak in Manitoba among the homeless might be due to clonal spread. Future surveillance will be very important to monitoring changes in the prevalence of the 12F serotype in Canada and also to see if it will become more common in other provinces and other target groups and also to determine changes in the macrolide resistance if it occurs.

Another interesting emerging serotype was serotype 6A. It was the most prevalent serotype among the emerging serotypes and 6th most common serotype overall. Its prevalence remained unchanged when the first year (1998) was compared to the last year (2008) and increased 4% between the pre (1998-2000) and post (2005-2008) PCV7 vaccine introduction periods. Serotype 6A is a vaccine related serotype as antibodies against vaccine serotype 6B are protective against future challenge with serotype 6A *S. pneumoniae* as shown by many studies (99). Therefore, PCV7 vaccine was expected to have reduced its prevalence. In some studies, including this one, 6A serotype has not been shown to decrease as expected (152). The lack of reduction or an increase in serotype 6A post vaccination even though the vaccine was supposed to have an effect on this serotype can be explained by the emergence of a novel serotype 6C which was

undistinguishable from serotype 6A serotype (152, 159, 160). In the literature, there are some studies describing a decrease of serotype 6A, but the effect was cancelled out by the increased number of 6C serotypes (101). Prior to conjugate vaccine use the prevalence of 6C serotype was low and increased significantly following the introduction of the vaccine (101). A CDC study from 1999 to 2006 showed that 16.7% of previously identified 6A isolates were actually 6C serotype in 1999 and by 2006 this number increased to 61% (99). During this thesis, a retrospective study looking at the prevalence of 6C serotype within the previously identified 6A has been done. Among the previously identified 6A, 9.5% (7/74) turned out to be 6C. In contrast to the CDC study, isolates with 6C serotype emerged in 2003 and none were found prior to the vaccine implementation. From 2003 to 2008 isolates with serotype 6C remained low making up only 2% of macrolide resistant *S. pneumoniae* in 2008. The low rate of serotype 6C in this study is probably due to the nature of this study, which focused only on macrolide-resistant *S. pneumoniae*. 6C serotype isolates have been found more commonly among susceptible isolates than among resistant (152) so that probably contributed to the low prevalence rate in this study. In addition, the majority of studies describing the increasing prevalence of 6C serotype in *S. pneumoniae* are invasive studies, so the fact that this is a respiratory study might have also contributed to low rates of 6C serotype. Even though at low rates, this study shows that 6C serotype is emerging among respiratory *S. pneumoniae* isolates and in fact data from a different study in our laboratory whose purpose was to assess the baseline circulating serotypes of *S. pneumoniae* from all age groups in Canada from 2007 to 2009, before the introduction of the new PCV-13 vaccine, showed that 6C serotype was more common among respiratory than blood isolates (personal communication, Heather Adam). The discrepancy might be due to differences in the isolate selection

process. In the US, the increase in serotype 6C was also evident in the increase in 6C serotype invasive diseases, predominantly among children younger than 2 years old and among elderly >65 years old. Results from thesis show some demographic differences between 6A and 6C serotypes; 6C was mostly isolated from elderly (>65 year old) age group, mostly from maritime region, solely among *mef*(A) genotype and male gender. Consistent with the literature, isolates with 6C serotype were less antibiotic resistant than isolates with 6A serotype.

Among the non-vaccine serotypes, 19A is the most concerning. Although it was the 8th most common serotype overall, present at 4.1% of all macrolide resistant *S. pneumoniae,* its emergence and isolation in increasingly higher numbers as the study progressed and also its multidrug resistance profile and genetic relatedness makes it the top non-vaccine serotype in this study. Since it emerged in 1999, making up 1.4% of all macrolide resistant *S. pneumoniae,* it increased 14.4%, gradually every year to 15.5% by the end of the study in 2008 (p=0.0145). Comparing its prevalence pre and post the PCV7 vaccine introduction periods, it increased by 5%. It increased across all age groups with the highest increase (60%) noticed for the adult (18-64) unvaccinated age group. The emergence of the 19A among adult population is especially concerning as this age group was generally not considered at risk for pneumococcal infections. Majority of isolates with 19A serotype were the least susceptible to penicillin, T/S, doxycycline, clindamycin among all non-vaccine serotypes. They were also multidrug resistant. Genetic analysis showed that 50% of these isolates were related by PFGE.

Introduction of the PCV7 vaccine has been extremely successful at reducing vaccine serotypes and therefore reducing the burden of infections caused by these serotypes. There are many studies looking into the rates of invasive diseases pre and post vaccine

introduction and the results show remarkable decreases not only in the target group but also in non-vaccinated populations. The success of the vaccine has however been hampered by the emergence of isolates with serotype 19A. Initially this serotype was considered vaccine related serotype as it was thought that antibodies raised towards serotype 19F in the vaccine will be protective against serotype 19A, however, this turned out not to be the case, therefore this serotype is considered non-PCV7 in this thesis. In the literature sometimes it is still referred to as vaccine - related serotype. The increases in the prevalence of 19A serotype described in the literature from US studies were shown to be greater (as high as 400%) than those seen in this study (101). The reasons might be due to the nature of the studies as most of the US studies were performed on all *S. pneumoniae* received during a specified period of time, while this study characterized only macrolide-resistant *S. pneumoniae*, therefore decreasing the number of isolates and possibly affecting the rates of the emerging serotypes. In addition, the current study involved respiratory isolates and 19A serotype increased most predominantly among invasive isolates. Nevertheless it is evident that serotype 19A is becoming more prevalent, possibly replacing serotype 19F among macrolide-resistant *S. pneumoniae* and possibly among all *S. pneumoniae* as the last two years of the study depict the number and percentage of 19A serotype higher than 19F serotype. In 2007, 5 (5%) and 10 (10%) isolates were serotype 19F and serotype 19A, respectively and in 2008, 8 (9%) and 13 (15%) isolates were serotype 19F and serotype 19A. The emergence of 19A serotype in Canada was also shown in the study, performed by members of our group, the goal of which was to assess the baseline circulating serotypes of respiratory and invasive *S. pneumoniae* from all age groups in Canada from 2007 to 2009 before the introduction of the new PCV-13 vaccine (personal communication, Heather Adam). During this period

the ratio of 19A to 19F was roughly 2:1 (69 19A isolates and 34 19F isolates). Interestingly, the majority of these 19A isolates were isolates from the eastern region of Canada, which was defined as Ontario and Quebec, similarly to the 19A isolates found in this study. In contrast to the findings in this study 19F was still slightly more common than 19A among respiratory isolates in that study. This discrepancy might be due to the different selection process for the two studies. Since the introduction of PCV7 vaccine , isolates with seroype 19A have not only been found to be the cause of invasive pneumococcal disease but have been found to cause new and more complicated disease processes, especially in children. These include pediatric pneumococcal empyema (PPE), which is a rare complication of pneumonia, hemolytic uremic syndrome (HUS), which is an uncommon complication of invasive pneumococcal disease, mastoiditis, which refers to complications of acute otitis media in children (24, 33, 44, 155). Isolates with 19A serotype have also been increasing found to cause pneumonia. An implication of isolates with serotype 19A with more complicated diseases together with the multi-drug resistance profile of these isolates emphasizes the need to monitor its trend.

Among the non-vaccine serotypes, serotypes 1, 3, 7F, and 22F have been described in the literature as emerging since the implementation of PCV7 vaccine (2, 101). During this thesis 10 isolates with serotypes 3 and 22F have been detected, which contributed 0.9% each to macrolide resistant *S. pneumoniae*. One isolate with serotype 7F was found, and no serotype 1 was detected at all. Low numbers of these serotypes in this study might be due to the fact that these are considered mostly invasive serotypes and this study focused on respiratory isolates. Future studies will determine whether these will become more common among respiratory *S. pneumoniae* in Canada.

PCV13 is a next generation PCV7 vaccine, which provides coverage against 6 additional serotypes, including 1, 3, 4, 5, 6A, 7F, and 19A. Since this vaccine was implemented into the use in Canada in 2010, the serotype distribution in this study is not reflected by its use. However, retrospective analysis allows using the data as a base point based on which changes can be noticed. The retrospective analysis showed that PCV13 would have provided coverage for 68% of isolates. The coverage ranged from a high of 80% in 1999 to a low of 50% in 2007. The PCV13 vaccine coverage decreased 11% since the beginning of the study. Future studies will determine the effectiveness of the vaccine and are imperative in determining the evolution of serotypes among *S. pneumoniae*.

Part III Prevalence and characterization of virulence factor Pili-1(PI-1) and Pili-2 (PI-2)

The emergence of multidrug resistant 19A serotype isolates has tempered the success of PCV7 vaccine (101, 102). Among many non-vaccine serotypes, 19A has become the most prevalent, filling the gap left by the effectiveness of the vaccine (23, 45, 47, 48, 71, 84, 128, 161, 162, 190). Many hypotheses have been suggested to explain the success of the emergence and spread of these isolates. One of these hypotheses focuses on capsule switch, suggesting that MDR 19A is a result of 19F capsule switch (163). By switching the capsule an already MDR isolate has a fitness advantage over other vaccine serotypes by not being subjected to immune selective pressure, while retaining the characteristics of an already successful clone. Recently multi-locus sequence typing (MLST) project through the Canadian Bacterial Surveillance Network (CBSN) has shown that the

majority of MDR 19A isolates post PCV7 vaccine introduction were sequence type 320 and prior to PCV7 introduction ST 320 was mostly associated with serotype 19F in Canada(163). In the same study analysis of the antibiotic alleles for key resistance conferring residues in penicillin binding proteins by DNA sequencing has showed conservation between MDR 19A and MDR 19F. Similarly, MDR 19A and MDR 19F had complete conservation at residues of *erm*(B), *mef*(A) and *tet*(M) associated with resistance to macrolide and tetracycline (163).

Whole genome sequence of MDR 19A ST320 post PCV7 vaccine introduction was compared with whole genome sequence of MDR 19F ST320 from the pre-PCV7 vaccine period and revealed 99.7% identity between the two genomes proving that the MDR 19A ST320 emerged from pre-existing MDR 19F ST320 in Canada as a result of capsular switch (163).

Pili were recently recognized in *S. pneumoniae* and implicated in the virulence of these bacteria (3, 18, 149, 167, 177, 210). The two pili, pilus type 1 and pilus type 2 have been shown to mediate host-bacterial interactions as an adhesin, and a proinflammatory stimulus. These pili were present in approximately 25% of isolates in various populations prior to the widespread use of PCV7 (3, 149). More specifically, prior to PCV7 introduction these pili were shown to be associated with strains belonging to capsular types included in the PCV7 vaccine. Consequently, the initial decline in vaccine types following the PCV7 introduction produced a decline in the prevalence of these piliated *S. pneumoniae*. However, now there seems to be a re-emergence of these pili among *S. pneumoniae* with non-vaccine serotypes (167). It appears that the distribution of the pili among *S. pneumoniae* is highly clonal (149). Knowing that the MDR 19A were highly clonal in this study and that they probably emerged from highly clonal MDR 19F

following the PCV7 vaccination, the possibility of these isolates having an advantage by carrying the pili was explored by comparing the MDR 19A and 19F to other non MDR 19A and 19F *S. pneumoniae* isolates. First, all dual *erm*(B) and *mef*(A) isolates which are mostly MDR and mostly 19F and 19A serotype were compared. As all dual isolates are of vaccine serotypes, and knowing that the presence of pili is strongly associated with vaccine types, high proportion of these isolates was expected to have the pili. The pili-type 1 was present in 62% of isolates, pili-type 2 was present tin 70% of isolates, and both pili- type 1 and pili-type 2 were present in 57% of isolates.

Following this analysis, the focus was on 19A and 19F isolates. In total, there were 215 isolates with either 19A or 19F throughout the study. Among these, 82 were MDR, having both *erm*(B) and *mef*(A) gene, and being genetically related, while 133 were not MDR, not dual *mef*(A) and *erm*(B) carrying isolates, and not genetically related. The hypothesis was that the presence of pili-type 1 and pili-type 2 would be more strongly associated with 19A and 19F isolates that were MDR and genetically related than with the other 19A and 19F isolates. The presence of pili among the MDR 19A and 19F isolates would indicate that its initial presence among 19F MDR prior to PCV7 vaccine introduction provided an additional advantage that further supported the capsular switch and allowed the strains to escape the immune system while maintaining the virulence of the 19F MDR strain. The results showed strong association between MDR, clonal 19A and 19F strains and the presence of pili-type 1 and pili-type 2. The association was strongest for pili-type 2.

The presence of virulence factor among these MDR isolates suggests that an organism does not have to become less resistant as it becomes more virulent, or less virulent as it becomes more resistant. It has been generally accepted that an organism that is more

resistant is so at a cost of virulence (35). Indeed that can be observed by looking at antibiotic susceptibility profiles for invasive versus non-invasive isolates (123). Invasive *S. pneumoniae* are less antibiotic and less multidrug resistant than non-invasive but they cause more virulent disease (123). Studies have demonstrated that erythromycin-resistant mutants have reduced adhesion and invasion characteristics to intestinal epithelial cells, murine macrophage and short time intracellular survivability within macrophage compared to the susceptible strain. Co-inoculation of the two strains in the mice resulted in low colonization level of the resistant strain compared to the susceptible strain. Competition experiments resulted in mutant that grew significantly slower than the susceptible parent strain and the mutation imposed a fitness cost in Ery-resistant mutant (123). Based on this, MDR *S. pneumoniae* would be expected to be less virulent and maybe it is. However for the MDR 19F/19A serotype *S. pneumoniae* the presence of pili might be the compensation for the loss of virulence that would normally be expected in MDR strains. As these isolates are non-invasive, suggesting that they are not as virulent, the presence of pili might provide an additional advantage that may allow these clones to spread and cause more severe diseases targeting non-typical population. The relationship between antibiotic resistance, serotype distribution and virulence is very complex and more studies looking at these associations are needed to fully understand the spread and emergence of some and not other bacterial clones. Isolates with 19F serotype prior to vaccine introduction were MDR and carried pili virulence factor plus they are known to be least immunogenic and therefore not affected by the vaccine to the same extent as the other vaccine type may have contributed to its survival despite the vaccine pressure and allowed for the capsular switch to 19A to occur.

In conclusion, the data in this thesis provides a great deal of knowledge and contributes to the further understanding of macrolide resistance in *S. pneumoniae* in Canada. Macrolide resistance although still below the level of resistance that would restrict its use, has been shown to be increasing. Low level efflux *mef*(A) gene class E mediated resistance remains the more common; however multi-drug resistant, genetically related dual *mef*(A) and *erm*(B), 19A, pili positive are on the raise. PCV7 vaccine coverage decreased revealing new and emerging serotypes. PCV13 vaccine coverage remains high for now. Future surveillance of macrolide resistance, serotype distribution and virulence factor (pili) detection is necessary to monitor the trends in light of the new PCV13 vaccine implementation.

Although, great deal of research on *S. pneumoniae* and macrolide resistance has been done worldwide, the data in this thesis contributes great Canadian data to the medial literature. This was the first study in Canada that studied and tied together macrolide resistance mechanisms, genetic relatedness and serotyping data along with PCV7 vaccine coverage over 10 year period. It analysed in detail the serotype evolution before, during and after the PCV7 vaccine introduction and provided a baseline serotype data before the introduction of new PCV13 vaccine. The baseline data can be used to monitor the changes that might occur as the new vaccine becomes widely used. The greatest contribution of this work to the medical literature was the identification of pili-virulence factors among the successful, multidrug resistant, genetically and serotypically related clone that emerged since the introduction of the PCV7 vaccine. This work might lead to new research in the area of bacterial virulence and resistance, which might help in further understanding of why some clones become successful despite vaccine and antibiotic pressure while other do not.

This study was not without limitations. The major limitation of this study was in the fact that 2 different surveillance studies were used as a source of *S. pneumoniae* isolates. This may have contributed to noticeable changes if different centres joined or left the study and if those centres had unusual resistance patterns, such as high level of macrolide resistance or high presence of 19A serotype, or predominant mechanism of resistance. These types of situations could have affected the resistance and its mechanims and serotype prevalence. This was not observed, all major centres participating in both studies remained unchanged throughout this thesis and the distribution of macrolide-resistant isolates was consistent and wide spread throughout all the centres and Canada. Although the major centres were the same between the two studies, the number of *S. pneumoniae* they contributed was different. Even in CROSS the number of *S. pneumoniae* collected decreased over time. Ideally, in a surveillance study, all centres, and amount of *S. pneumoniae* they contribute should be constant. However, that is not always the case as centres leave, others join and sometimes the numbers of collected organisms change due to workload or budget. The contribution of CANWARD would have more impact if greater number of *S. pneumoniae* were collected, however I feel it would not change the overall conclusion of the study, but make the statement of increased macrolide resistance and increased prevalence of 19A, dual *mef*(A) and *erm*(B) *S. pneumoniae* more pronounced.

H. Future Directions

This thesis has contributed to the knowledge and understanding of Canadian clinical macrolide-resistant respiratory tract *S. pneumoniae* isolates. It specifically monitored the changes in macrolide resistance over the last 10 years, characterized genetic determinants of this resistance, monitored the yearly changes, and looked for new and emerging trends. Work in this thesis also provided an excellent overview of serotype distribution among macrolide resistant *S. pneumoniae*, provided vaccine coverage, and observed the emergence of new serotypes. The data in this thesis provides a platform to potential future studies. Some of these studies are listed below:

1. The first and most important future study is the continuation of molecular macrolide resistance characterization. It is very important to continue to detect the mechanisms of resistance in order to be able to notice changes, and to observe potential new mechanisms over time.
2. Once mechanisms of resistance are detected, a second study can focus on low-level efflux mediated resistance. It will be important to monitor the prevalence of low-level efflux mediated resistance to make sure that it remains the predominant mechanism of resistance in Canada. Monitoring of the mechanisms of resistance is of particular importance in countries where low-level macrolide resistance predominates, as any decreases in such resistance can have profound effects on therapy even if rate of macrolide resistance remains the same. At the same time it will be important to monitor changes in the MIC distribution among these efflux strains to make sure that increases in the MICs of these low level macrolide resistant isolates are being noticed. Increases in the MICs in efflux-

mediated resistance can also have an effect on therapy as the previously low and treatable isolates may fail therapy.

3. A third study should focus on discrimination between the two variants of efflux mediated macrolide resistance genes, the *mef*(A) gene class A and *mef*(A) gene class E. Such discrimination should be ongoing, as changes in the prevalence of the two variants are likely to influence the pattern of susceptibility to other antibiotic groups, serotype distribution, as well as genetic relatedness, thereby providing information regarding the spread of efflux mediated resistance in Canada. Epidemiology of these two variants is changing in Europe, so it would be interesting to see if it changes in North America as well.
4. Another study should focus on dual *mef*(A) and *erm*(B) macrolide resistant *S. pneumoniae* isolates. As these isolates were shown to increase over the last 10 years, it will be important to monitor this trend and to characterize these isolates. In addition to characterizing the antibiotic resistance patterns, serotype distribution and genetic relatedness, the characterization should include the determination of the *mef*(A) gene variant in order to detect changes from the *mef*(A) gene class E if they occur in these dual macrolide resistant isolates.
5. A study looking into serotype distribution should be ongoing. The data in this thesis provides an excellent platform based on which changes in serotype distribution can be observed. Knowledge of the most common serotypes allows the determination of the vaccine efficacy/coverage and provides the necessary changes that need to be made in order to increase the vaccine coverage. Although this study involved respiratory isolates, and the data might not be directly applicable to vaccines specifically developed against invasive disease, it

still provides important information. A study looking into emerging serotypes is also very important. Serotypes which emerged in the post PCV7 introduction period in this thesis should be monitored. In light of the introduction of the new PCV13, which is suppose to provide protection against 19A, it will be important to monitor the 19A trend and also other emerging serotypes to see which one will increase over time as the 19A will most likely be shown to decrease. Ongoing study looking at serotype 6C should be done as well as the new, not described in this thesis, serotype 6D. The new PCV13 vaccine will most likely lead to decreases in 6C serotype, as PCV13 will include both 6A and 6B antigens and it is the 6A antigen that is supposed to be cross-reactive to 6C, but no prediction regarding 6D can be made at this point. Among emerging serotypes in this thesis was serotype 11A. Recently, serotype 11F has been discovered, therefore a future serotyping study should include the detection of the newest serotype 11F as well.

6. Detection of the pili type-1 and pili-type 2 among the isolates with the other emerging serotypes as descried in this thesis should be performed. This could lead to better prediction regarding the next new clone that is likely to take over once the new PCV13 is implemented. In addition, pili detection should extend to the non-typeable isolates, as recently these were shown to carry these virulence genes.
7. Due to the detection of inducible clindamycin resistance ($iMLS_B$ phenotype) among macrolide resistant but clindamycin susceptible *erm*(B) positive *S. pneumoniae* isolates, it is important to continue to screen for this type of resistance pattern. From an epidemiological point of view it is important to

provide information regarding how common this previously thought of as not very common type of resistance is. From a clinical point of view it is important to realize that a clindamycin susceptible isolate might fail therapy if clindamycin inducible resistance is not confirmed by D-test, as it may be assumed to be *mef(A)* (low-level) mediated macrolide resistant isolate especially if macrolides MIC are on the lower range. From a research point of view, an *erm*(B) positive *S. pneumoniae* that is susceptible to clindamycin should be characterized further.

8. Significant number of non-typeable *S. pneumoniae* isolates were detected in this thesis. Biochemical tests such as otichin and bile-solubility confirmed it as *S. pneumoniae.* So from a clinical microbiology laboratory point of view these isolates were *S. pneumoniae* and therefore included in this thesis. These isolates were subjected to molecular serotyping in order to confirm the results. Majority of these isolates failed to amplify a *cps*A gene, which encodes a capsule, and is included in the PCR reaction as a positive control, therefore suggesting that there is no capsule. These isolates were sequenced to determine their identification and the majority of them turned out to be *S. mitis*, however some were also *S. pneumoniae*. It is important to point out that some isolates were non-typeable but were positive for *cpsA* gene and were confirmed by sequencing as *S. pneumoniae*. A further study looking into the non-typable isolates is recommended.

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