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DETECTION OF MACROLIDE RESISTANCE GENES *ERMB* AND *MEFA/E* IN IRAQ

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ABSTRACT

The results of amplification of *ermB* gene by monoplex-PCR revealed that 83.8% of *S. pneumoniae* isolates gave positive result for detection of *ermB* gene, only 16.2% isolates of *S. pneumoniae* gave negative result for detection of *ermB* gene. The results showed that 100% of *S. pneumoniae* isolates had *mefA/E* gene. The presence of *mefA/E* gene was an absolute predictor of phenotypic. Multiplex PCR results showed that two amplification bands of 640 bp (*ermB*) and 346 bp (*mefA/E*) were observed in 83.8% of isolates showing a corresponding in the present of two bands, while only 16.2% isolates of *S. pneumoniae* appeared one band (*mefA/E* gene). All the *S. pneumonia* isolates revealed resistance to erythromycin antibiotic were tested for the ability to produce M phenotype by using the double-disk test. The result revealed that (18.9%) isolates showed an M phenotype, 81.1% isolates showed a constitutive MLS_B phenotype, and no isolates (0%) showed an inducible MLSB phenotype. **M phenotype isolates**: 85.7% of M phenotype isolates harbored the *mef(A/E)* gene, and 14.2% harbored the *mef(A/E)* and *erm(B)* genes. **MLS_B phenotype isolates**: 100% of MLS_B phenotype isolates had the *erm(B)* and *mef(A/E)*genes.

KEYWORDS: *Streptococcus pneumoniae*, monoplex-PCR, Resistant genes *ermB* and *mefA/E*.

INTRODUCTION

Streptococcus pneumoniae is responsible for high rates of morbidity and mortality worldwide (Rudan *et al.*, 2008). There are many mechanisms of resistance to antimicrobials and with macrolides, the *erm(B)* and *mef(A/E)* genes are responsible for the most-reported mechanisms

of resistance to this class. The *ermB* gene is known to confer resistance to other antimicrobial classes and a high level of resistance to the macrolides (Shortridge *et al.*, 1999). The resistance rate of *S. pneumoniae* to antibiotics which varies with the locality or region studied, is influenced by the frequency and intensity of utilization, and empirical use of the antimicrobial drugs is frequent (Borg *et al.*, 2009 ; Gossens, 2009).

The rise in drug resistance of *S. pneumoniae* underscores the need for clinical microbiology laboratories to accurately determine its antimicrobial susceptibility profile in a timely manner. In light of the medical importance and to demonstrate the molecular expression of several characterized *S. pneumoniae* virulence factors in Lower Respiratory Tract Infection (LRTI) patients. The present study was designed to detect phenotypically and genotypically of identifying of *mefA/E* and *ermB* genes in erythromycin resistant isolates of *S. pneumoniae.*

MATERIAL AND METHODS

Patients and Clinical Specimens

A total of 600 sputum samples were collected from out- and inpatients suffering from lower respiratory tract infection (LRTI) (pneumonia, COPD) attending to the Chest Unit in Al-Sadder Medical City, Al-Hakeem General Hospital and Clinic Consultive Center for Chest Disease and Al-Zahra'a Hospital for Childbirth and Children in Al-Najaf province during the period from February 2013-Aprile 2014. The patients included both sex (male and female) and the age range (1-80 years).

Isolation and Identification of *Streptococcus pneumoniae*

S. pneumoniae was isolated and identified according to traditional biochemical diagnostic to, by using the routine methods e.g. according to Macfaddin (2000) ; Collee *et al.*, (1996) ; Forbes *et al.*, (1998).

Diagnostic Kits: To confirm the diagnosis of pneumococcal isolates, the following kits were used: STREPTO-SYSTEM 9R Kit and Vitek-2 as recommended by Guido and Pascale (2005).

Extraction and Isolation of DNA

Genomic DNA Extraction Kit (Geneaid) was used for DNA extraction. Concentration of DNA was determined spectrophotometrically by measuring its optical density at 260 nm (Extinction coefficient of dsDNA is 50 µg/ml at 260 nm) the purity of DNA solution is

indicated by ratio of OD 260-280 which is in the range of 1.8±0.2 for pure DNA. PCR program that apply in the thermocycler. The PCR products and the ladder marker are resolved by electrophoresis on 1.2% agarose gel (Sambrook and Russell, 2001).

Polymerase Chain Reaction (PCR) Technique

Selection of PCR Primers

In this study, monoplex and multiplex PCR was done to detect a number of genes that encode antibiotic resistance properities in *S. pneumoniae* isolates. Monoplex and multiplex PCR were used to detect *ermB* and *mef(A/E)*.

Monoplex PCR Mixture

monoplex The DNA extract of *S. pneumoniae* isolates were subjected to different genes by PCR. The protocols used depending on manufacturer's instruction. All PCR components were assembled in PCR tube and mixed on ice bag under sterile conditions as in Table 1.

Multiplex PCR Mixture

Isolates were subjected to two genes, by using premix multiplex PCR protocol. Single reaction (final reaction volume 20 µl) consisted of multiplex mix 2X 5 µl, *mef A/E* and *ermB* genes primers10µM (each one consist primer forward 2.5 µl and reverse 2.5 µl), DNA template 5 µl. All materials were mixed in same PCR tube on ice bag under sterile condition.

Table 1: The primers and their sequences used in conventional PCR for detection of *S. pneumoniae* virulence factors

Target Gene	DNA sequence(5'-3')	Product Size (bp)	References
ermB	F:GAA AAG GTA CTC AAC CAA ATA R:AGT AAC GGT ACT TAA ATT GTT TAC	640	Sutcliffe *et al.*, (1996)
mefA/E	F:AGT ATC ATT AAT CAC TAG TGC R:TTC TTC TGG TAC TAA AAG TGG	346	Sutcliffe *et al.*, (1996)

PCR Cycling Conditions

PCR mixture was set up in a total volume of 30 µl included 15µL of PCR premix, 2µl of each primer and 5µl of extracted DNA have been used. The rest volume was completed 6µl of sterile deionized distilled water, then vortexed. Negative control contained all material except template DNA, so instead that distilled water was added. PCR reaction tubes were centrifuged briefly to mix and bring the contents to the bottom of the tubes, and placed into thermocycler PCR instrument where DNA was amplified as indicating in below (table2):

Table2. Program used to amplify the genes by PCR

Gene	Stage	Temperature (time)	
ermA	Initial denaturation	93C° for 3min	
	Denaturation	93C° for 1min	35cycle
	Annealing	52C° for 1min	
	Extension	72C° for 1min	
	Final extension	72C° for 5min	
MefA/E	Initial denaturation	93C° for 3min	
	Denaturation	93C° for 1min	35cycle
	Annealing	52C° for 1min	
	Extension	72C° for 1min	
	Final extension	72C° for 5min	

Phenotypic Detection of Resistance Mechanisms

Double disc test using erythromycin (78 µg) and clindamycin (25 µg) were placed 15-20 mm apart on a Mueller-Hinton agar supplemented with 5% defibrinated horse blood on which a bacterial suspension equivalent to that of a 0.5 McFarland standard had been inoculated previously . Following overnight incubation at 37˚C under 5% CO_2, the precence inhibition zone around the two discs determined the inducible, constitutive or M-resistance phenotype of the isolate (Seppälä *et al.*, 1993).

RESULTS AND DISCUSSION

Detection of macrolide resistance genes *ermB* and *mefA/E* by monoplex and multiplex PCR.

The results of amplification of *ermB* gene by monoplex-PCR revealed that (83.8%) isolates of *S. pneumoniae* gave positive for detection of *ermB* gene, only 16.2% isolates of *S. pneumoniae* gave negative result for detection of *ermB* gene (Figure 1). The result of study showed that 100% of *S. pneumoniae* isolates had *mefA/E* gene. The presence of *mefA/E* gene was an absolute predictor of phenotypic (Figure 2).

Multiplex PCR results (figure 3) showed that two amplification bands of 640 bp (*ermB*) and 346 bp (*mefA/E*) observed in 83.8% of isolates showed a corresponding in the present of two bands, while only 16.2% isolates of *S. pneumoniae* appeared one band (*mefA/E* gene).

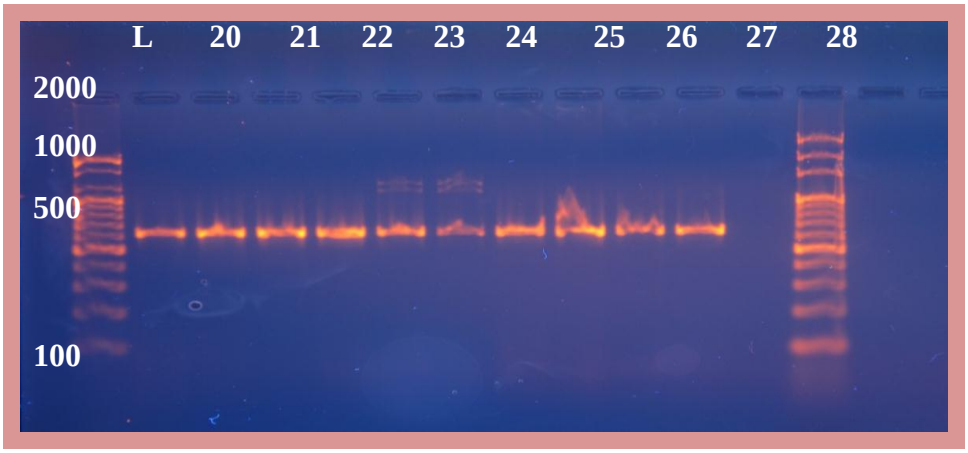

Figure (1): Gel electrophoresis of PCR product of *ermB* gene primer with product 640 bp. Lane (L), DNA molecular size marker (2000-bp ladder), Lanes (20-29) show positive results with *ermB* gene and lane 30 show negative results with *ermB* gene.

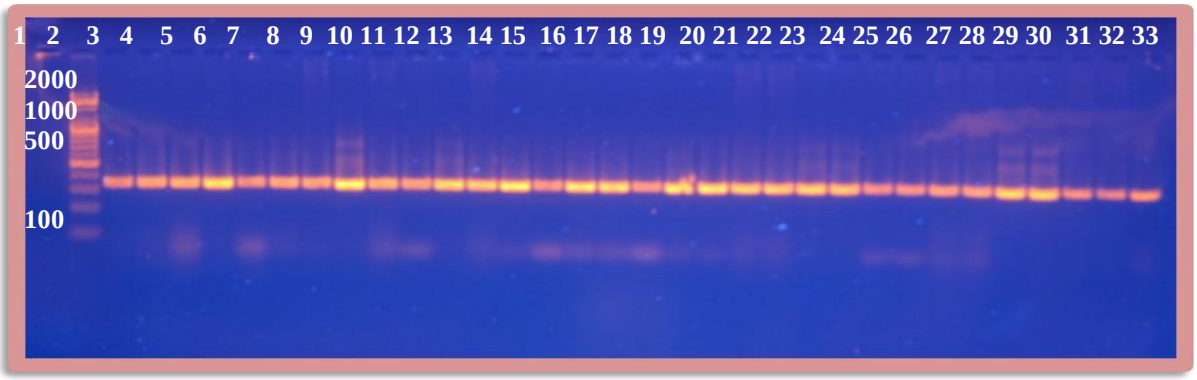

Figure (2): Gel electrophoresis of PCR product of *mefA/E* gene primers with product 346 bp. Lane (L), DNA molecular size marker (2000-bp ladder), Lanes (1-32) show ositive results with *mefA/E* gene.

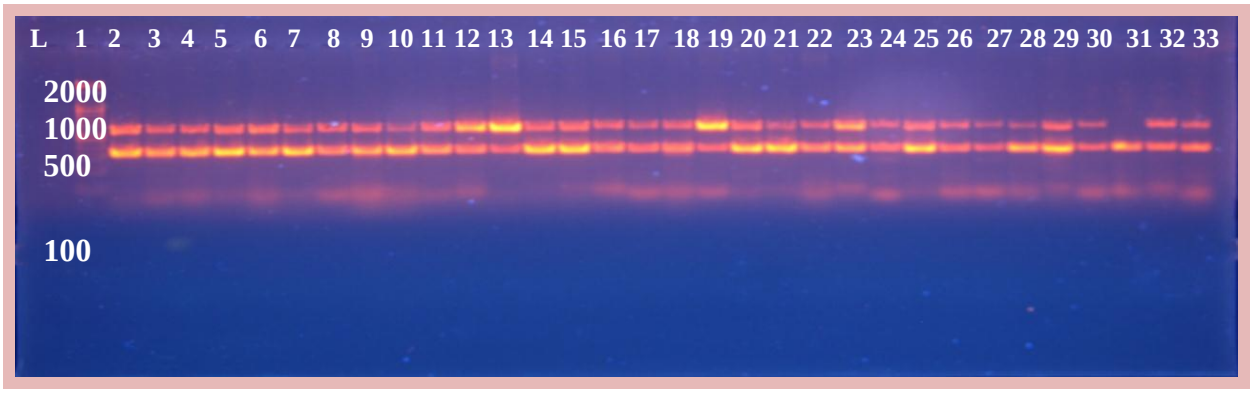

Figure (3): Gel electrophoresis of PCR amplified products of *ermB* and *mefA/E* genes, by Multiplex PCR for *S. pneumoniae* isolates that amplified with *ermB* gene primers with product 640 bp and *mefA/E* gene primers with product 346 bp. Lane (L), DNA

molecular size marker (2000-bp ladder), Lanes (1-29 , 31 and 32) show positive results with *ermB* and *mefA/E* genes, Lane 30 show negative results with *ermB* and positive result with *mefA/E*.

Bean and Klena, (2002) pointed out that PCR assay for macrolide resistance determinants detected *mef(A)* in 83 (66.9%) isolates and *erm(B)* in 118 (95.2%) isolates. Both *mef(A)* and *erm(B)* were detected in 77 (62.1%) isolates.

Resistance to macrolide antibiotics such as clindamycin is mediated by two major mechanisms: methylation of ribosomal macrolide target sites, encoded by the gene *erm(B),* and drug efflux, encoded by the *mef(A)* gene (Farrell *et al*., 2005; Klugman, 2002). *Erm(B)*- or *erm(B)*+*mef(A)*-positive strains have high resistance levels and are resistant to clindamycin (MLS phenotype), whereas *mef(A)*-positive strains generally have lower resistance levels and are susceptible to this antibiotic (M phenotype). A frequent association of erythromycin and tetracycline resistance is often related to insertion of *erm(B)* into a conjugative transposon of the Tn916 family that harbours the *tet(M)* gene and carries integrase (int) and excisase (xis) geneS (Brenciani *et al*., 2007). The two main subclasses of *mef* in *S. pneumoniae*, *mef(E)* and mef(A), are carried on different but related elements: *mef(A)* on *Tn1207.1* or *Tn1207.3*, and *mef(E)* on an element called ‘macrolide efflux genetic assembly’ (mega).

There are many mechanisms of resistance to antimicrobials and with macrolides, the *erm(B)* and *mef(A/E)* genes are responsible for the most-reported mechanisms of resistance to this class. The *erm(B)* gene is known to confer resistance to other antimicrobial classes and a high level of resistance to the macrolides (Shortridge *et al.,* 1999). Among 18 penicillin-resistant strains, 7 were resistant to at least two other antimicrobial drugs. All erythromycin-resistant strains, except one, contained the *erm*(*B*) and/or *mef*(*A*/*E*) genes, with a predominance of the former. The resistance rate to penicillin and erythromycin in Porto Alegre remained stable.

Bean and Klena, (2002) showed that 124 erythromycin-resistant pneumococcal isolates were examined for the presence of macrolide resistance genes. The *erm (B)* gene was detected in 118 (95.2%) isolates and the *mef (A)* gene in 83 (66.9%) isolates. Both the *mef (A)* and *erm (B)* genes were detected in 77 (62.1%) isolates. DNA macrorestriction analysis of these isolates identified them as belonging to a single multi-resistant clone.

Weber *et al.*, (2010) studied the relationship between the observed resistance and the presence of the *erm(B)* and *mef(A/E)* genes in *S. pneumoniae*. Six of ten resistance strains had *erm(B)*, two had *mef(A/E)*, one had both genes, and one did not have either gene. With these results, the relationship of these genes to the erythromycin resistance was confirmed. It has been shown that the strains had *erm(B)* showed MICs between 2 and >8µg/mL, and those that had *mef(A/E)* showed MICs between 1 and 2µg/mL. Strains that had both genes showed MICs >8µg/mL, as did the strain that lacked these genes. In spite of the small number of erythromycin-resistant strains tested, almost all of the strains that had *erm(B)* showed elevated MICs, compared to those having *mef(A/E).*

The presence of an rRNA methylase in pneumococci was recognized early as being responsible for erythromycin resistance (Leclercq and Courvalin, 1991). The *ermB* (*ermAM*) gene in pneumococci is often part of the Tn1545 transposon that carries determinants that confer resistance to tetracycline and streptomycin (Trieu-Cuot *et al.*, 1990).

The *erm(B)* gene confers resistance to other classes of antimicrobials (streptogramines and lincosamines), affecting the therapeutic choice. The majority of strains that had *erm(B)* were penicillin-resistant. Similar observations were reported by other workers (Shortridge *et al.*, 1999).

Wolter *et al.*, (2007) pointed out that a rare clinical isolate of *S. pneumoniae*, highly resistant to telithromycin, contained *erm(B)* with a truncated leader peptide and a mutant ribosomal protein L4. By transformation of susceptible strains, this study shows that high-level telithromycin resistance is conferred by *erm(B)*, wild type or mutant, in combination with a $_{69}$GTG$_{71}$-to-TPS mutation in ribosomal protein L4.

Tait-Kamradt *et al*., (2001) described a highly resistant clinical isolate of *S. pneumoniae*, BSF11524 was serotype 19A and was highly resistant to erythromycin (MIC, >256 µg/ml), clindamycin (MIC, >256 µg/ml), and telithromycin (MIC, >256µg/ml). It is resistant to tetracycline (MIC, 12 µg/ml) and penicillin (MIC, 16 µg/ml) but susceptible to chloramphenicol (MIC, 2 µg/ml). It was confirmed, as described previously (Tait-Kamradt *et al.*, 2001), to be *erm(B)* positive and *mef(A)* negative. The *erm(B)* gene contained an adenine base insertion in the control peptide creating a stop codon and resulting in the truncation of the control peptide to 10 amino acids.

These previous studies incompatible with the results of the present study in which all isolates of *S. pneumoniae* showed 100% of *mefA/E* and less than of *ermB* 97.3%.

M phenotype

All the *S. pneumoniae* isolates resistant to erythromycin antibiotic were tested for the ability to produce M phenotype by using the double-disk test. The result in table (3) indicated that (18.9%) isolates showed an M phenotype, (81.1%) isolates showed a constitutive MLS_B phenotype, and (0%) showed an inducible MLS_B phenotype.

M phenotype isolates

(85.7%) of 7 M phenotype isolates harbored the *mef(A/E)* gene, and (14.2%) harbored the *mef(A/E)* and *erm(B)* genes. This result was in agreement with the result of Calatayud *et al.*, (2007) found fourteen (87.5%) of 16 M phenotype isolates harbored the *mef(E)* gene, and (12.5%) harbored the *mef(A)* gene. One *mef(E)* isolate was also resistant to tetracycline and harbored the *tet(M)*, *xis*, and *int* genes. No *tet(M)*, *xis*, and *int* genes were detected by PCR in the remaining 15 tetracycline-susceptible isolates.

MLS_B phenotype isolates

Therteen (100%) of MLS_B phenotype isolates had the *erm(B)* and *mef(A/E)*genes. This result was disagreement with the result of Calatayud *et al.*, (2007) found five of nine tetracycline-susceptible isolates had the *erm(B)*, int, and xis genes. Three of the four remaining isolates had the *erm(B)* gene alone. The last isolate had *int*, *xis*, *tnpA*, *tnpR*, *erm(B)*, *mef(E)*, and *tet(M)* genes and was of serotype 19A. After induction with subinhibitory concentrations of tetracycline (5), no variations in tetracycline MIC were found for this tetracycline-susceptibletet (M)-positive isolate, suggesting the presence of a silent form of the *tet(M)* gene.

Hsueh *et al.*, (2003) showed 33% of the erythromycin-resistant isolates had the M phenotype. The erythromycin resistant M phenotype was more common among PRSP isolates (46%) than among PSSP isolates (20%).

Table (3): Distribution of *S. pneumoniae* isolates according to the present of M and MLS_B phenotype.

Isolate	*ermB*	*mefA/E*	Mphenotype	MLS_B
S_1	+	+	-	+
S_2	+	+	-	+
S_3	+	+	-	+
S_4	+	+	-	+
S_5	+	+	-	+
S_6	+	+	-	+
S_7	+	+	-	+
S_8	+	+	-	+
S_9	+	+	-	+
S_{10}	+	+	-	+
S_{11}	+	+	-	+
S_{12}	+	+	-	+
S_{13}	+	+	-	+
S_{14}	+	+	-	+
S_{15}	+	+	-	+
S_{16}	+	+	-	+
S_{17}	+	+	-	+
S_{18}	+	+	-	+
S_{19}	+	+	-	+
S_{20}	+	+	-	+
S_{21}	+	+	-	+
S_{22}	+	+	-	+
S_{23}	+	+	+	-
S_{24}	+	+	-	+
S_{25}	+	+	-	+
S_{26}	+	+	-	+
S_{27}	+	+	-	+
S_{28}	+	+	-	+
S_{29}	+	+	-	+
S_{30}	-	+	+	-
S_{31}	+	+	-	+
S_{32}	+	+	-	+
S_{33}	-	+	+	-
S_{34}	-	+	+	-
S_{35}	-	+	+	-
S_{36}	-	+	+	-
S_{37}	-	+	+	-

It has been reported that a significant number of erythromycin-resistant *S. pneumoniae* and *S. pyogenes* strains contain a determinant that mediates resistance via a putative efflux pump (Tait-Kamradt *et al.*, 1997). The gene encoding the erythromycin-resistant determinant was cloned and sequenced from three strains of *S. pneumoniae* bearing the M phenotype

(macrolide resistant but clindamycin and streptogramin B susceptible). The DNA sequences of *mefE* were nearly identical, with only 2-nucleotide differences between genes from any two strains.

Mendonça-Souza *et al.,* (2004) pointed out that investigated the occurrence and phenotypic and genotypic characteristics of erythromycin-resistant *S. pneumoniae* strains isolated in Brazil (1990 to 1999). Of the 931 pneumococcal strains evaluated, 40 (4.3%) were erythromycin-resistant (Ery-R). Most 37 (92.5%) of the 40 Ery-R isolates presented the MLSB phenotype and 3 (7.5%) strains showed the M phenotype. PCR testing indicated that all MLSB phenotype isolates harbored the *erm(B)* gene only, whereas the *mef(A/E)* gene was present in all isolates presenting the M phenotype. The *tet(M)* gene was the most frequent (86.1%) among Ery-R isolates that were also resistant to tetracycline.

Montanari *et al.,* (2001) pointed out that laboratory differentiation of erythromycin resistance phenotypes is poorly standardized for pneumococci. Montanari *et al.,* (2001) were tested 85 clinical isolates of erythromycin-resistant *S. pneumoniae* for the resistance phenotype by the erythromycin-clindamycin DDT and by MIC induction test. In DDT, 65 strains, all carrying the *erm(AM)* determinant, were assigned to the constitutive macrolide, lincosamide, and streptogramin B resistance (cMLS) phenotype, and the remaining 20, all carrying the *mef(E)* gene, were assigned to the recently described M phenotype; an inducible MLS resistance (iMLS) phenotype was not found.

A triple-disk test, set up by adding a rokitamycin disk to the erythromycin and clindamycin disks of the double-disk test, allowed the easy differentiation not only of pneumococci with the M phenotype from those with MLS resistance but also, among the latter, of those of the true cMLS phenotype from those of the iMcLS phenotype. While distinguishing MLS from M resistance in pneumococci is easily and reliably achieved, the differentiation of constitutive from inducible MLS resistance is far more uncertain and is strongly affected by the antibiotic used to test inducibility (Montanari *et al.*, 2001).

Carsenti-*Dellamonica et al.,* (2005) showed out of 15 erythromycin-resistant mutants, 10 were resistant to clindamycin and spiramycin without any blunting of the zone (phenotype MLSB constitutive) and remained susceptible to linezolid. The MIC of linezolid was increased 2- to 3-fold.

Bean and Klena, (2002) showed that all 77 isolates containing both genes (*ermB* and *mefA/E*) were multi-resistant; the most frequently associated combination of resistances was penicillin, erythromycin, co-trimoxazole and tetracycline, which was noted in 74 (96%) of these isolates. This isolate may contain a deleted or otherwise defective *erm(B)* gene, and the low level of resistance resulted from the product of a functional *mef(A)* gene, imparting the M phenotype. Typically, erythromycin-resistant pneumococci from any given geographical location possess only one of the two most commonly described resistance mechanisms. In the USA, the *mef(A)* gene is more dominant, being identified in 61% of 114 macrolide-resistant isolates examined (Shortridge *et al.*, 1999). In contrast, in Europe *erm(B)* has been found in >80% of erythromycin resistant isolates (Schmitz *et al.*, 2001). In Christchurch, the predominant macrolide resistance genotype is both *erm(B)* and *mef(A).* This genotype was identified in 62.1% of the Christchurch isolates examined in this study. Although an uncommon genotype, a recent report from South Africa found 36 of 118 (30.5%) erythromycin-resistant isolates tested contained both *erm(B)* and *mef(A)* genes, using PCR (McGee *et al.*, 2001).

REFERENCE

1. Bean, D.C. and Klena, J.D. Prevalence of *erm(A)* and *mef(B)* erythromycin resistance determinants in isolates of *Streptococcus pneumoniae* from New Zealand. Journal of Antimicrobial Chemotherapy, 2002; 50: 597–599.
2. Borg, M.A.; Tiemersma, E.; Scicluna, E.; van de Sande-Bruinsma, N.; de Kraker, M.; Monen, J. and Grundmann, H. Prevalence of penicillin and erythromycin resistance among invasive *Streptococcus pneumoniae* isolates reported by laboratories in the southern and eastern Mediterranean region. Clin. Microbiol. Infec, 2009: 15(3): 232-237.
3. Brenciani, A.; Bacciaglia, A. Vecchi, M.; Vitali, L.A.; Varaldo, P.E. and Giovanetti, E. Genetic elements carrying *erm(B)* in *Streptococcus pyogenes* and association with tet(M) tetracycline resistance gene. Antimicrob Agents Chemother, 2007; 51: 1209–1216.
4. Calatayud, L.; Ardanuy, C.; Cercenado, E.; Fenoll, A.; Bouza, E.; Pallares, R.; Martin, R. and Linares, J. Serotypes, Clones, and Mechanisms of Resistance of Erythromycin-Resistant *Streptococcus pneumoniae* Isolates Collected in Spain. Antimicrob Agents Chemother, 2007; 51: 3240-6.
5. Carsenti-Dellamonica, H.; Galimand, M.; Vandenbos, F.; Pradier, C.; Roger, P.M.; Dunais, B.; Sabah, M.; Mancini, G. and Dellamonica, P. (2005). *In vitro* selection of mutants of *Streptococcus pneumoniae* resistant to macrolides and linezolid: relationship

with susceptibility to penicillin G or macrolides. Journal of Antimicrobial Chemotherapy, 2005; 56: 633-642.

6. Collee, J.G.; Fraser, A.G.; Marmison, B.P. and Simpson, S.A. (1996). Mackie and McCrtenty. Practical medical microbiology. 14th ed. Churchill Livingstone inc., USA, 1996.
7. Farrell, D.J.; Jenkins, S.G.; Brown, S.D.; Patel, M.; Lavin, B.S. and Klugman, K.P. Emergence and spread of *Streptococcus pneumoniae* with *erm*(B) and *mef*(A) resistance. Emerg Infect Dis, 2005; 11: 851–858.
8. Forbes, B.A.; Sahm, D.F.; Weissfeld, A.S.; Bailey and Scott's Diagnostic microbiology. 10th ed. Elsevier company, USA, 1998.
9. Gossens, H. Antibiotic consumption and link to resistance. Clin. Microbiol. Infec, 2009; 15(suppl.3): 12-15.
10. Guido, F. and Pascale, F. Performance of the New VITEK 2 GP Card for Identification of Medically Relevant Gram-Positive Cocci in a Routine Clinical Laboratory. J Clin Microbiol, 2005; 43(1): 84-88.
11. Hausdorff, W.P.; Feikin, D.R. and Klugman, K.P. Epidemiological differences among pneumococcal serotypes. Lancet Infect Dis, 2005; 5: 83-93.
12. Hsueh, P.R.; Teng, L.J.; Wu, T.L.; Yang, D. ; Huang, W.K.; Shyr, J.M.; Chuang, Y.C.; Wan, J.H ; Yan, J.J.; Lu, J.J. Wu, J.J.; Ko, W.C.; Chang, F.Y.; Yang, Y.C.; Lau, Y.J.; Liu, Y.C.; Lee, C.M.; Leu, H.S.; Liu, C.Y.L. and Luh, K.T. Telithromycin and Fluoroquinolone Resistant *Streptococcus pneumoniae* in Taiwan with High Prevalence of Resistance to Macrolides and β-Lactams: SMART Program 2001 Data. Antimicrobial Agents and Chemotherapy, 2003; 47(7): 2145–2151.
13. Klugman, K.P. The successful clone: the vector of dissemination of resistance in *Streptococcus pneumoniae*. J Antimicrob Chemother, 2002; 50 (Suppl. S2): 1-6.
14. Leclercq, R. and Courvalin, P. Bacterial resistance to macrolide, lincosamide, and streptogramin antibiotics by target modification. Antimicrob. Agents Chemother. 1991; 35: 1267–1272. (Erratum, 35:2165.)
15. Macfaddin, J.F. Biochemical tests for identification of medical Bacteria. 3rd-ed, williium and Wilkins, U. S. A, 2000.
16. McGee, L.; Klugman, K.P.; Wasas, A.; Capper, T. and Brink, A. Serotype 19F multiresistant pneumococcal clone harboring two erythromycin resistance determinants *erm (B)* and *mef (A)* in South Africa. Antimicrobial Agents and Chemotherapy, 2001; 45: 1595-8.

17. Mendonça-Souza, C.R.V.; Carvalho, M.G.S.; Barros, R.R.; Dias, A.D.; Sampaio, J.L.M.; Castro, A.C.D.; Facklam, R.R.; Teixeira, L.M. Occurrence and characteristics of erythromycin-resistant *Streptococcus pneumoniae* strains isolated in three major Brazilian States. Microb. Drug Resist. 2004; 10(4): 313-320.
18. Montanari, M.P.; Mingoia, M.; Giovanetti, M. and Varaldo, P.E. Differentiation of Resistance Phenotypes among Erythromycin-Resistant. Journal of clinical microbiology, 2001; 39(4): 1311-1315.
19. Rudan, I.; Boschi-Pinto, C.; Mulholland, K. and Campbell, H. pidemiology and ethiology of childhood pneumonia. Bull World Health Organ, 2008; 86(5): 408–416.
20. Sambrook, J. and Russell, R.W. Molecular cloning: A laboratory manual, 3rd ed. Cold spring harbor laboratory press, cold spring harbor, N.Y, 2001.
21. Schmitz, F.J.; Perdikouli, M.; Beeck, A.; Verhoef, J. and Fluit, A.C. Molecular surveillance of macrolide, tetracycline and quinolone resistance mechanisms in 1191 clinical European *Streptococcus pneumoniae* isolates. International Journal of Antimicrobial Agents, 2001; 18: 433–6.
22. Seppälä, H.; Nissinen, A.; Yu, Q. and Huovinen, P Three different phenotypes of erythromycin-resistant *Streptococcus pyogenes* in Finland. J. Antimicrob. Chemother. 1993; 32: 885–891.
23. Shortridge, V.D.; Doern, G.B.; Brueggemann, A.B.; Beyer, J.M. and Flamm, R.K. Prevalence of macrolide resistance mechanisms in *Streptococcus pneumoniae* isolates from a multicenter antibiotic resistance surveillance study conducted in the United States in 1994–1995. Clinical Infectious Diseases, 1999; 29: 1186-1188.
24. Sutcliffe, J.; Grebe, T.; Tait-kamradt, A. and Wondrack, L. Detection of erythromycin-resistant determinants by PCR. Antimicrob. Agents and Chemo, 1996; 40(11): 2562-2566.
25. Tait-Kamradt, A.; Clancy, J.; Cronan, M.; Wondrack, F.; Dib-Hajj, L.; Yuan, W. and Sutcliffe, J. *mefE* Is Necessary for the Erythromycin-Resistant M Phenotype in *Streptococcus pneumoniae*. Antimicrobial agents and chemotherapy, 1997; 41(10): 2251–2255.
26. Tait-Kamradt, A.; Reinert, R. R.; Al-Lahham, A.; Low, D.E. and Sutcliffe, J. High-level ketolide-resistant streptococci, p. 101. Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother. American Society for Microbiology, Washington, DC, 2001.

27. Trieu-Cuot, P.; Poyart-Salmeron, C.; Carlier, C. and Courvalin, P. Nucleotide sequence of the erythromycin resistance gene of the conjugative transposon Tn1545. Nucleic Acids Res, 1990; 18: 3660.
28. Weber, F.T.; Dias, C. and da Costa, M. Antimicrobial susceptibility of *Streptococcus pneumoniae* and genotypic characterization of erythromycin-resistant strains in Porto Alegre, Brazil. Brazilian Journal of Microbiology, 2010; 41: 1-5. ISSN 1517-8382.
29. Wolter, N.; Smith, A.M.; Low, D.E. and Klugman, K.P. (2007) High-Level Telithromycin Resistance in a Clinical Isolate of *Streptococcus pneumoniae*. Antimicrobial agents and chemotherapy, 2007; 51(3): 1092-1095.