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Molecular detection of genes responsible for macrolide resistance among *Streptococcus pneumoniae* isolated in North Lebanon

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ABSTRACT

In recent years, the increased use of macrolides was linked with the emergence of resistance *Streptococcus pneumoniae* worldwide. The main aim of this study was to determine the prevalence of *S. pneumoniae* resistant to macrolides and to identify the macrolide resistance genotypes among clinical isolates collected in North Lebanon. Disk diffusion susceptibility method was performed for 132 strains of *S. pneumoniae* isolated over a period of 5 years in North Lebanon. Polymerase Chain Reaction followed by pyrosequencing was carried out for confirmation of phenotypic diagnosis. The macrolide resistance genotypes were also identified by using PCR amplification of genes implicated in this resistance: *erm*(A), *erm*(B), *erm*(C), *msr*(A), *lin*(A) and *mef*(A/E). Macrolide resistance was found in 34.1% of *S. pneumoniae* isolates. We observed that the $cMLS_B$ phenotype (31/45, 68.9%) was the most common in these pneumococci and *erm*(B) was the most common resistance gene (32/45, 71.1%). This study shows that macrolide resistance in *S. pneumoniae* in North Lebanon is mainly related to target site modification with predominance of cMLSb phenotype but is also mediated by efflux pumps. *lin*(A) gene was reported for the first time in one *S. pneumoniae* strain in combination with *erm*(B) and *mef*(A/E) genes.

Introduction

Streptococcus pneumoniae (*S. pneumoniae*) is one of the major contributors to mortality and morbidity worldwide causing a variety of diseases ranging from simple respiratory infections such as otitis media and pneumococcal pneumonia to reach the threatening invasive infections such as meningitis and septicemia [1]. Even though proper antimicrobial treatments are available in the market, pneumococcal diseases kill 1.6 million people in developing countries each year, of which one million are children under the age of 5 years [2]. Beta-lactams, macrolides and fluoroquinolones are the three main classes of antibiotics used in the treatment of *S. pneumoniae*.

The worldwide increase in Beta-lactams resistance coincided with an increase in macrolide resistant pneumococci [3]. There are two phenotypic resistance profiles among *S. pneumoniae*, designated M and MLS_B, according to whether resistance to macrolides and/or lincosamides and/or streptogramin B agents is observed. Of these, MLS_B and M phenotypes account for the majority of drug infection and macrolide resistance mainly caused by target site modification and active drug efflux [4].

MLS_B phenotype is the end result of a methyltransferase, which is encoded by the *erm*(B) gene for erythromycin resistance methylase [5]. This gene confers high resistance to all macrolides (Minimum Inhibitory Concentration $\geq 64\ \mu g/mL$) by reduction in the binding affinity to the 23S rRNA (domain V). This mechanism depends on methylation of specific position Adenine 2058 (A2058) in 23S rRNA [4]. The expression of the MLS_B resistance may be constitutive ($cMLS_B$) or inducible ($iMLS_B$). Interestingly the constitutive resistance is characterized by *erm*(B) mRNA active methylase which is produced in the absence of an inducer [6,7]. However strains that carry an inducible *erm* gene are resistant to the inductor but remain susceptible to non-inductors macrolides. Macrolides C-14 and C-15 ring members are inducers as opposing to the 16-ring macrolides, lincosamides, and streptogramin, which are non-inducers [4,6].

The second mechanism of resistance in streptococci is the efflux mechanism that is encoded by *mef* (macrolide efflux) gene [8], which causes resistance to C-14 and C-15 membered macrolides compounds only, and the encoding phenotype is designed M [4].

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Table 1
Primers used in the study.

Gene	Primer sequence (5′-3′)	Size	Protocol cycle	Reference
erm(A)	F: 5′-AAGCGGTAAACCCCTCTGA-3′ R: 5′-TTCGCAAATCCCTTCTCAAC-3′	190	30 (30 at 94 °C; 30 s at 52 °C; 1 min at 72 °C)	[15]
erm(B)	F: 5′-CTATCTGATTGTTGAAGAAGGATT-3′ R: 5′-GTTTACTCTTGGTTTAGGATGAAA-3′	142	Same as erm(A)	[15]
erm(C)	F: 5′-AATCGTCAATTCCTGCATGT-3′ R: 5′-TAATCG TGGAATACGGGTTTG-3′	299	Same as *erm*(A)	[15]
lin(A)	F: 5′-GGTGGCTGGGGGGTAGATGTATTAACTGG-3′ R: 5′-GCTTCTTTTGAAATACATGGTATTTTTCGATC-3′	323	30 (30 s at 94 °C; 30 s at 57 °C; 1 min at 72 °C)	[16]
msr(A)	F: 5′-GGCACAATAAGAGTGTTTAAAGG-3′ R: 5′-AAGTTATATCATGAATAGATTGTCCTGTT-3′	940	25 (1 min at 94 °C; 1 min at 50 °C; 90sa at 72 °C)	[16]
mef(E)	F: 5′-ATGGAAAAATACAACAATTGGAAACGA-3′ R: 5′-TTATTTTAAATCTAATTTTCTAACCTC-3′	1218	35 (30 s at 94 °C; 30 s at 50 °C; 90 s at 72 °C)	[17]
mef(A)	F: 5′-AGTATCATTAATCACTAGTGC-3′ R: 5′-TTCTTCTGGTACTAAAAGTTG-3′	345	Same as *mef*(E)	[18]

In *Streptococcus* spp., *mef* genes include a number of subclasses, of which *mef*(A) and *mef*(E) are the most significant [9]. *mef*(A) gene, originally found in *Strepococcus pyogenes* [10] and *mef*(E) gene discovered in *S. pneumoniae* [11] are very common in *S. pneumoniae*. In addition, the cotranscription of *mef*(E) and *msr*(D), an msr-class gene with homology to *msr*(A) found in staphylococci, in *S. pneumoniae* suggested that the products of the two genes may act as a dual efflux system [12]. *mef* genes provides a low-level of macrolide resistance (Minimum Inhibitory Concentration = 1–32 μg/mL) [13].

In order to better understand the epidemiology of macrolide resistance in *S. pneumoniae* in Lebanon, the main aim of this study was to determine the prevalence of resistance and to identify the genes responsible for this resistance among clinical *S. pneumoniae* strains isolated in North Lebanon.

Material and methods

Sample collection

This study was conducted in North Lebanon during the period 2010–2015. 132 non-duplicate *S. pneumoniae* isolates were collected from several clinical specimens including blood, sputum, bronchial wash, cerebrospinal fluid, deep tracheal aspirate and nasal and ear secretions. All isolates were identified by using gram staining and optochin susceptibility followed by latex agglutination testing (Pastorex™ Meningitis, Biorad, France).

Antimicrobial susceptibility test

The susceptibility of different strains to antibiotics was performed by the disk diffusion according to CA-SFM 2015 recommendations.

In order to highlight the macrolide resistance we tested the following antibiotics and its concentrations: Erythromycin (15 μg), Clindamycin (2 μg) and Pristinamycin (15 μg) (Biorad, France). We also determined the sensitivity of isolates to penicillin using the oxacillin disk diffusion method (oxacillin disk charged of 1 μg).

DNA extraction and molecular identification

All macrolide resistant *S. pneumoniae* isolates were used for molecular confirmation of the species identification. DNA was extracted using the QIAmp DNA Mini Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's recommended procedures. The DNA was eluted in 100 μL of elution buffer (Qiagen) and stored at −20 °C until use. 16S rRNA sequencing analysis targeting V3 region was performed using pyrosequencer according to the instructions of the manufacturer [14]. The sequences obtained were aligned using the BioEdit v7.0.1 package (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), then compared with sequences of *S. pneumoniae* published on the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/) using the basic local alignment search tool (BLAST) program.

Detection of macrolide resistance genes

In order to detect the genes known to be responsible for resistance to macrolide (*erm*(A), *erm*(B), *erm*(C), *msr*(A), *mef*(A/E) and *lin*(A) genes) in *S. pneumoniae*, PCR targeting these genes were performed using primers specific to each gene (Table 1).

Results

Molecular identification by pyrosequencing was accomplished and confirmed that all isolates were belonged to *S. pneumoniae*. Resistance to macrolide was found in 45 strains (34.1%) out of all 132 tested *S. pneumoniae*. Resistance phenotypes that were determined by disk diffusion susceptibility method with erythromycin, clindamycin and pristinamycin, showed that among 45 macrolide-resistant isolates, 32 (71.1%) exhibited the MLS_B phenotype: 31 (68.9%) belonged to the $cMLS_B$, and 1 (2.2%) to the $iMLS_B$ phenotype. The remaining 13 isolates (28.9%) were confirmed as M phenotype (Table 2).

PCR analysis of the 45 macrolide-resistant pneumococcal isolates showed that 37.8% (17/45) harbored the *erm*(B) gene and 28.9% (13/45) harbored the *mef*(A/E) gene. 14 isolates (31.1%) carried both *erm*(B) and *mef*(A/E) genes and only 1 strain (2.2%) possessed a combination of *erm*(B), *lin*(A) and *mef*(A/E). All the strains belonged to the MLS_B phenotype harbored the *erm*(B) gene, while all the strains with M phenotype had the *mef*(A/E) gene (Table 3).

In the other hand, *S. pneumoniae* with decreased susceptibility to penicillin (PNSP) is detected by oxacillin disk charged of 1 μg (OXA-1). Among the 45 strains of *S. pneumoniae* resistant to macrolide, 28 (62.2%) showed co-resistance to OXA-1 and 17 (37.8%) showed susceptibility to OXA-1.

Discussion

Nowadays, antibiotic resistance is one of the biggest threats to global health. It can affect anyone, of any age, in any country. This threat is influencing public health and patient safety by decreasing

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