

Original Article

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High Frequency of Macrolide-Resistant *Streptococcus pneumoniae* Colonization in Respiratory Tract of Healthy Children in Ardabil, Iran

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Background: *Streptococcus pneumoniae* (*S. pneumoniae*) is one of the most common causes of human diseases in young children. Macrolides are commonly antibiotics used for empirical treatment of community-acquired respiratory infections. The purpose of this study was to determine antibiotic resistance pattern as well as the relationship between macrolide resistance and the major mechanisms of resistance in pneumococci isolated from healthy children.

Materials and Methods: In this cross-sectional study, 43 isolates of *S. pneumoniae* were collected from healthy children in Ardabil. Resistance pattern against tested antibiotics was determined using the disk diffusion method. The Minimum Inhibitory Concentration (MIC) of erythromycin was determined using the E-test strips. The *mefA/E* and *ermB* gene were detected in erythromycin-resistant isolates using the specific primers and Polymerase Chain Reaction (PCR) technique.

Results: According to antimicrobial susceptibility testing, 74.4 % of the isolates were resistant to erythromycin, 95.3 % to penicillin, 81.3 % to co-trimoxazole, 72 % to azithromycin, 41.8 % to tetracycline, 27.9 % to clindamycin, and 16.2 % to chloramphenicol. All isolates were susceptible to levofloxacin and vancomycin. In the case of rifampin, 95.3% of the isolates were sensitive and 4.6% semi-sensitive. The MIC of erythromycin for resistant isolates was between 1.5 and ≥ 256 $\mu\text{g/ml}$. PCR results revealed that 100% of erythromycin-resistant isolates contained *mefA/E* gene and 81.25 % contained both the *ermB* and *mefA/E* genes.

Conclusion: The prevalence of antibiotic-resistant strains of *S. pneumoniae*, especially resistance to macrolides, was high among healthy children in Ardabil. According to the results of this study, we suggest using levofloxacin, rifampin and vancomycin antibiotics as an appropriate prophylactic regimen in pneumococcal infections.

Key words: *S. pneumoniae*, Healthy children, Macrolide, Antibiotic resistance, *ermB*, *mefA/E*

INTRODUCTION

Streptococcus pneumoniae (*S. pneumoniae*) remains as one of the most important pathogens of human in the world (1). The bacterium is cause of life-threatening diseases such

as sepsis, meningitis, pneumonia and otitis in children and immunocompromised elderly patients (2). Already, all isolates of *S. pneumoniae* were susceptible to penicillins; however, due to the emergence and spread of penicillin

resistance, these antibiotics were replaced by other types, such as macrolides, lincosamides, streptogramin, ceftriaxone, cefotaxime and vancomycin (3-5). Macrolide antibiotics are group of broad-spectrum antibiotics containing erythromycin, azithromycin and clarithromycin which are used in order to treat respiratory infections. Erythromycin was the first macrolide discovered in 1952 and originally considered as an excellent alternative against penicillin-resistant gram-positive bacterial infections (6). However, failures in the treatment of pneumococcal infections with macrolide antibiotics have been reported earlier. High macrolide use is correlated with the increase of macrolide-resistant *S. pneumoniae* (7). Globally, macrolide resistance among *S. pneumoniae* is geographically variable but ranges from <10% to >90% of isolates (8). Local studies from Iran showed macrolide resistance ranges from 8.2-57.2% (9, 10) and >70% among *S. pneumoniae* isolates collected from healthy and sick subjects, respectively (11).

Understanding the antibiotic resistance pattern of *S. pneumoniae* is necessary for appropriate antibiotic treatment of pneumococcal infections. Therefore, this study was conducted to determine the extent of macrolide resistance and elucidate the major underlying mechanisms in *S. pneumoniae* isolates collected from healthy children less than six years old in Ardabil, Iran.

MATERIALS AND METHODS

This cross-sectional study was conducted on 43 isolates of *S. pneumoniae* collected, using nasopharyngeal swab from 280 healthy children less than 6 years old attending kindergartens in Ardabil in 2015. The isolates were previously identified based on conventional methods and confirmed by presence of *lytA* gene using Polymerase Chain Reaction (PCR) method.

1. Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed by disk diffusion method using Mueller-Hinton agar containing sheep blood (5%). The antibiotic disks were as follows: levofloxacin (LEV, 5 µg), trimethoprim-Sulfamethoxazole

(SXT, 1.25/23.75 µg), clindamycin (DA, 2 µg), erythromycin (E, 15 µg), tetracycline (TE, 35 µg), chloramphenicol (C, 35 µg), azithromycin (AZM, 15 µg), penicillin (determined using oxacillin disk, 1 µg), vancomycin (VA, 35 µg), and rifampin (RA, 5 µg). The results were interpreted in accordance with the Clinical and Laboratory Standards Institute (CLSI) criteria (12).

The Minimum Inhibitory Concentrations (MICs) of erythromycin against isolates were determined using the E-test strips (Epsilon Test), with gradient concentrations ranging from 0.016 to 256 µg/ml. In this test, inhibition zone of growth was observed, pear shape, and the minimum concentration of antibiotic that inhibits the growth of bacteria is considered as the MIC value. According to the CLSI guideline, erythromycin susceptibility patterns are reported as follows: MIC ≤ 0.25 µg/ml as sensitive, 0.5 µg/ml as intermediate, and ≥ 1µg/ml as resistant.

2. Evaluation of inducible clindamycin resistance

Isolates that were sensitive to clindamycin and resistant to erythromycin tested for inducible resistance using the D-test. The test was performed by double-disk diffusion method. Erythromycin (15 µg) and clindamycin (2 µg) disks were placed close together within 20 mm apart from centre to centre on Mueller-Hinton agar plates. The plates were incubated overnight at 37 °C, D-shaped inhibition zone around the clindamycin disk adjacent to erythromycin disk indicated inducible clindamycin resistance (iMLSB). If an isolate was resistant to erythromycin but sensitive to clindamycin, without flattening of zone around clindamycin, was considered as MS phenotype. If the isolate was resistant to both erythromycin and clindamycin with circular shape of zone of inhibition was labeled as constitutive macrolide-lincosamide-streptogramin B resistant phenotype (cMLSB) (12).

3. PCR amplification of *mefA/E* and *ermB* genes

Chromosomal DNA was extracted from erythromycin-resistant *S. pneumoniae* isolates using the DNP™Kit (CinnaGen, Iran) according to the manufacturer's protocol.

Quality and quantity of extracted DNA was assayed by measuring OD₂₆₀ and OD₂₈₀ nm using Nanodrop (Termo Scientific, USA) and then stored at -20 °C for subsequent uses. Specific primers were used to amplify the *mefA/E* (forward: 5'- AGT ATC ATT AAT CAC TAG TGC-3' revers: 5'- TTC TTC TGG TAC TAA AAG TGG-3') and *ermB* (forward: 5'- GAA AAG GTA CTC AAC CAA ATA-3', revers: 5'- AGT AAC GGT ACT TAA ATT GTT TCA -3') genes (13). PCR was performed in a 20 × μL AccuPower™ PCR PreMix (Bioneer) with 10 pmol of each primer under the following conditions: initial denaturation at 95°C for 5 min, followed by 34 cycles of 95°C for 1 min, 55°C (*ermB*) and 50°C (*mefA/E*) for 1 min and 72°C for 1 min, and a final incubation at 72°C for 5 min. The amplified DNA fragments (PCR products: *mefA/E*, 348 bp, and *ermB*, 639 bp) were separated on 1% (w/v) agarose gel, stained with ethidium bromide and visualized under ultraviolet light.

Statistical analysis

Statistical analysis was carried out using SPSS software version 16.0. The associations of erythromycin resistance genotypes with resistance to other antibiotic classes were calculated using the chi-square test. Statistical significance was set at p< 0.05.

RESULTS

In the present study, antibiotic susceptibility test was performed by disk diffusion method and the MIC through the E-test strips for each 43 pneumococcal isolates. As shown in table 1, according to the disk diffusion test 100% of the isolates were susceptible to vancomycin and rifampin and 95.40% for levofloxacin. Forty-one of 43 isolates (95.34%) were resistant to penicillin and for erythromycin 30 of 43 isolates (69.76%) were resistant, 2 (4.6%) were intermediate and 11 (25.5%) were susceptible. Based on the E-test, 32 isolates were resistant to erythromycin. In the present study, the most resistance was obtained to penicillin (95.34%), trimethoprim (81.3%), erythromycin (74.4%), azithromycin (72%) and tetracycline (41.86 %), respectively. Overall, 28% of isolates were resistant to clindamycin. No inducible resistance to clindamycin was observed.

As shown in table 2 the majority of the isolates were resistant against multiple classes of antibiotics. Overall, 74.60 % of isolates were resistant to ≥ 3 antibiotics classes tested.

Table 1. Antibiotic resistance patterns of *Streptococcus pneumoniae* strains isolated from children in Ardabil, Iran, using agar diffusion method

Antibiotics	Total isolates (N = 43), n (%)			Genotypes						P
				<i>mef A/E</i> (N=32), n(%)			<i>erm B + mef A/E</i> (N= 26), n(%)			
	S	I	R	S	I	R	S	I	R	
Erythromycin	11(25.6)	-	32(74.4)	-	-	32 (100)	-	-	26 (100)	1
Azithromycin	11(25.6)	1(2.3)	31(72.1)	3 (9.38)	-	29(90.62)	3 (11.53)	-	23(88.46)	0.8
Clindamycin	31(72.1)	-	12(27.9)	20 (62.5)	-	12(37.5)	15(57.70)	-	11(42.3)	0.65
Tetracycline	25(58.4)	-	18(41.86)	16 (50)	-	16(50)	11(42.30)	-	15(57.70)	0.44
Levofloxacin	43(100)	-	-	-	-	-	-	-	-	-
Trimethoprim	7(16.3)	1(2.3)	35(81.4)	5 (15.62)	-	27(84.37)	4(15.39)	-	22(84.61)	0.9
Chloramphenicol	36(83.7)	-	7(16.3)	27(84.37)	-	5(15.62)	22(84.61)	-	4(15.38)	0.8
Penicillin ¹	2(4.65)	-	41(95.34)	-	-	32(100)	-	-	26 (100)	1
Vancomycin	43(100)	-	-	-	-	-	-	-	-	-
Rifampin	41(95.34)	2(4.6)	-	-	-	-	-	-	-	-

S; Susceptible, I; Intermediate, R, Resistant

¹ Determined using oxacillin disk, 1μg

Table 2. Antimicrobial susceptibility profile for *S. pneumoniae* isolates collected from children in Ardabil, Iran

Isolates N= 43 n (%)	Antibiotic resistance pattern	Antibiotic types	Antibiotic class	Total ^a n (%)
		n	n	
1(2.32)	-	0	0	1 (2.32)
1(2.32)	P	1	1	2 (4.65)
1(2.32)	SXT	1	1	
5(11.62)	SXT, P	2	2	8 (18.60)
1(2.32)	AZM, P	2	2	
2(4.65)	E, AZM, P	3	2	
1(2.32)	SXT, C, P	3	3	13 (30.20)
1(2.32)	DA, TE, P	3	3	
11(25.56)	SXT, E, AZM, P	4	3	
1(2.32)	SXT, E, C, P	4	4	7 (16.26)
1(2.32)	SXT, C, AZM, P	4	4	
1(2.32)	SXT, DA, TE, P	4	4	
4(9.30)	SXT, E, TE, AZM, P	5	4	
5 (11.61)	SXT, E, TE, C, AZM, P	6	5	12 (27.90)
3(6.97)	SXT, DA, E, TE, AZM, P	6	5	
4(9.30)	SXT, DA, E, TE, AZM, P	6	5	

^a Total number of isolates resistant to same number of antibiotic class

Levofloxacin (LEV, 5µg), trimethoprim (SXT, 25µg), clindamycin (DA, 2µg), erythromycin (E, 15µg), tetracycline (TE, 35µg), chloramphenicol (C, 35µg), azithromycin (AZM, 15µg), penicillin (determined using oxacillin disk, 1µg), vancomycin (VA, 35µg), rifampin (RA, 5µg)

The MIC range for erythromycin was between 256 to ≥ 0.032 µg/ml and the MIC50 value was determined as 12 µg/ml. According to the MIC test results, 32 isolates of *S. pneumoniae* (74.4%) were resistant to erythromycin. The erythromycin MIC results in resistant isolates were variable between 1.5 and ≥256 µg/ml. The MIC50 value for erythromycin resistant isolates was 32 µg/ml. PCR testing revealed the presence of *mefA/E* and *ermB* genes in resistant isolates (Figures 1 and 2).

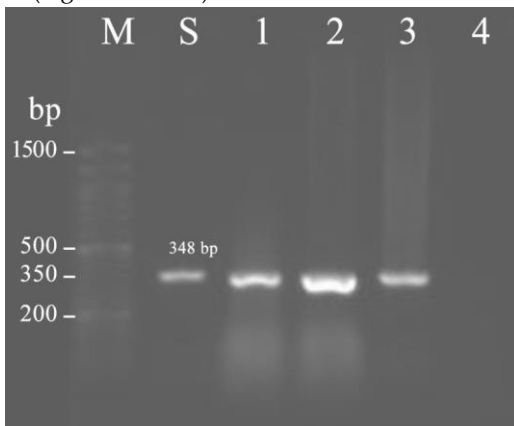


Figure 1. PCR detection of *mefA/E* gene in *Streptococcus pneumoniae* isolates. M: molecular weight markers, Lanes 1, 2 and 3 *mefA/E* positive isolates, Lane 4: Negative control, Lane S: Positive control.

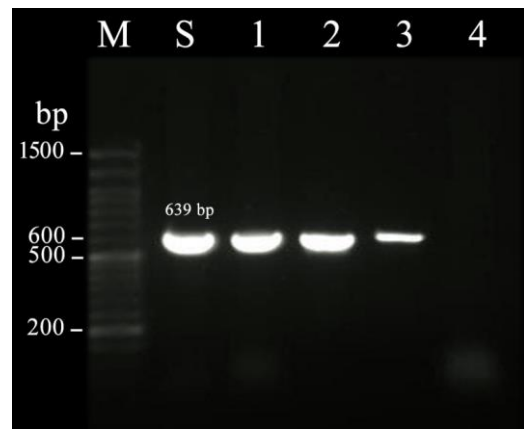


Figure 2. PCR detection of *ermB* gene in *Streptococcus pneumoniae* isolates. M: molecular weight markers, Lanes 1, 2 and 3 *ermB* positive isolates, Lane 4: Negative control, Lane S: Positive control strain PTCC 1240.

The *mefA/E* gene was detected in all of the erythromycin-resistant isolates and 26 (81.25%) of isolates had both the *mefA/E* and *ermB* genes. The erythromycin resistance level for isolates with both *mefA/E* and *ermB* genes was higher (MIC₅₀= 48 µg/ml) in comparison with that of with *mefA/E* gene alone (MIC₅₀= 32 µg/ml). There was no significant relationship between the erythromycin

resistance genotypes and resistance to the antibiotics tested ($p > 0.5$) (Table 1).

DISCUSSION

Macrolides are increasingly used in the treatment of diseases caused by *S. pneumoniae* (8). In this study, we evaluated the prevalence of macrolide resistance in *S. pneumoniae* isolates collected from healthy children in Ardabil. For erythromycin resistance, which is the most widely used macrolide drug, the results of the disk diffusion and E-test methods were not identical [30 (69.76%) vs. 32 (74.41%)]. This suggests that the E-test method is more accurate than disk diffusion method. Thirty-one (72.09%) isolates were resistant to azithromycin, a new semi-synthetic generation of macrolides, which is approximately identical to erythromycin. As compared to studies conducted in other cities of Iran, the prevalence of macrolide-resistant *S. pneumoniae* in healthy subjects in Ardabil was higher than in Kashan (8.2%), Zahedan (18.4%), Hamadan (25.5%), Mashhad (48.3%) and Tehran (57.2%) (9, 10, 14-16), as well as higher than the isolates collected from healthy children in Jordan, Hong Kong, Peru, Ghana, Uganda, Korea, Sri Lanka, Vietnam, Singapore, Thailand, China, India, Philippines and Russia (17-23). Previous studies showed a positive correlation between utilization of macrolides with the level of macrolide resistance in *S. pneumoniae* (24). Higher macrolide resistance in this study may be connected to the expanded utilization of macrolides in the study region. In a cross sectional study in 2016, it has been shown that antibiotics were contained within 54.9% of the prescriptions by general practitioners in Ardabil and macrolides were included in 18.3% of prescriptions (25).

Interestingly, the prevalence of erythromycin-resistant *S. pneumoniae* in healthy children in Ardabil was higher than children with pneumococcal infection in America (29%), Italy (3.4%), Finland (21.5%), Russia (19%), Greece (24%), Morocco (16.7%), and Japan (4.69%) (26-31). While, it was lower than Vietnam (88.3%), Taiwan (87.2%), Korea (85.1%), Hong Kong (76.5%), and China (75.6%) (27). This

finding is in accordance with previous reports showing that colonizer pneumococci isolates are more resistant as compared with invasive isolates (32). However, reports from Iran showed higher erythromycin resistance in clinical isolates. Macrolide resistance in clinical isolates of *S. pneumoniae* has been increasing steadily in Iran. In 2001, 2011, 2016 and 2017, those erythromycin resistance rates were 25%, 65%, 75%, and 71.4%, respectively (11,33,34).

Macrolide resistance in *S. pneumoniae* is mediated by three main mechanisms, including; (1) mutations in ribosomal proteins, (2) discharge of antibiotics due to efflux pumps and (3) changes in the structure of the target molecule through methylation of 23s *rRNA* gene. The genes encoding efflux pumps (*mef A/E*) and methyltransferases enzymes (*erm B*) are carried on transposons, so spreading of resistance genes among the strains is possible (35).

In this study, we investigated erythromycin resistance genes by PCR method. Our results showed that the prevalence of *mefA/E* gene was higher than the *ermB* gene and 32 (100%) of the erythromycin resistant isolates had *mefA/E* gene and 26 (81.25%) isolates had both *ermB* and *mefA/E* genes. Similar results were demonstrated in the agreement with this study in other countries. In Malaysia (64.7%), Hong Kong (66.7%) as well as Germany (50%) and Greece (*mefA* 5.3% and *mefE* 41.8%), the prevalence of *mefA* gene was more than *ermB* gene (30-39). However, frequency of *ermB* gene in some countries including Taiwan (70.7%), Sri Lanka (75%), China (76.9%) and Turkey (95%) were higher than *mefA* gene prevalence (30, 40). The study from other Iranian city has reported the similar finding as 42 and 50% for *ermB* and *mefA*, respectively (41). It has been documented that resistance mediated by the *ermB* gene, is usually associated with high-level macrolide MICs and efflux, encoded by the *mefA/E* gene, shows low-level macrolide MICs (30). Similar findings were observed in this study. MIC₅₀ for isolates carrying both *ermB* gene was 48 µg/ml, whereas it was 32 µg/ml for isolates containing just *mefA/E* gene. However,

in this study all isolates contained *mefA/E* gene and higher MICs in the presence of *ermB* gene could be at least partially attributed to the coexistence of *mefA/E* gene.

The results obtained from other antibiotics studied showed that 74.36 % of isolates were resistant to ≥ 3 antibiotic classes and had multiple drug resistance phenotypes. These results are inconsistent with recent reports from other regions (41). Most of the isolates were susceptible to levofloxacin, vancomycin, rifampin and chloramphenicol. These results were similar to the findings of other study conducted on clinical isolates collected in Iran (34).

In conclusion, because of the high resistance rate to macrolides, erythromycin and azithromycin, using these antibiotics is not recommended for empiric treatment of suspected pneumococcal infections in the study region. However, levofloxacin, rifampin and vancomycin can be used against infections caused by *S. pneumoniae* in Ardabil. Our study further demonstrated that *erm B* and *mef A/E* genes are dominantly present in macrolide resistant isolates. Due to the carrying of these genes by transposons, the isolates could act as reservoir for persistence and dissemination of macrolide resistant pneumococcal isolates in the community.

Authors' Disclosure of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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