Reports of Biochemistry & Molecular Biology
Vol.12, No.2, Jul 2023

Original article

www.RBMB.net

Serotype Distribution and Multi Locus Sequence Type (MLST) of Erythromycin-Resistant *Streptococcus Pneumoniae* Isolates in Tehran, Iran

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Abstract

Background: The number of erythromycin-resistant *Streptococcus pneumoniae* has significantly increased around the world. The present study aimed to determine the serotype distribution and molecular epidemiology of the erythromycin-resistant *Streptococcus pneumoniae* (ERSP) isolated from patients with invasive disease.

Methods: A total of 44 *Streptococcus pneumoniae* isolates were tested for susceptibility to several antimicrobial agents. Additionally, the polymerase chain reaction (PCR) was applied to evaluate ERSP isolates in terms of the presence of erythromycin resistance genes (e.g., *ermB* and *mefA*). The isolates were serotyped using the sequential multiplex-PCR method, and molecular epidemiology was assessed through the multilocus sequence typing (MLST) analysis.

Results: The results represented multidrug resistance (MDR) in approximately half of the pneumococcal isolates. Among 22 ERSP isolates, 20 (90.9%) and 12 (56%) ones contained *erm*B and *mef*A, respectively. Further, 14 (31.8%), 3 (22.7%), and 19A (18.1%) were the common serotypes among the isolates. No significant correlation was observed between serotypes and erythromycin resistance genes. Furthermore, the MLST results *revealed* 18 different sequence types (STs), the top ones of which were ST3130 (3 isolates) and ST166 (3 isolates). Population genetic analysis disclosed that CC63 (32%), CC156 (18%), and CC320 (18%) were identified as the predominant clonal complexes.

Conclusions: The ERSP isolates exhibited high genetic diversity. The large frequency of MDR isolates suggests the emergence of high resistant strains, as well as the need to implement vaccination in the immunization schedule of Iran. These accumulating evidences indicate that 13-valent pneumococcal conjugate vaccines provided higher serotype coverage in the ERSP isolates.

Keywords: Erythromycin Resistance, Genotyping Techniques, Multilocus Sequence Typing, Serotyping, Pneumococcal Vaccines.

Introduction

Streptococcus pneumoniae is considered an opportunistic pathogen and a leading cause of invasive diseases such as sepsis and meningitis (1). The mortality rate from invasive pneumococcal disease (IPD) among young children is approximately one million cases per year, mostly in developing countries (2). In this regard, antimicrobial agents are the first choice for treating pneumococcal infections. However, the increasing incidence of antibiotic-resistant *S. pneumoniae* (e.g., β-lactams and macrolides) makes the treatment of pneumococcal infections challenging, especially in high-risk groups and areas like Iran (3). Macrolides are a well-established class of antibiotics used for a range of indications like community-acquired pneumonia (CAP), sinusitis, and otitis. The

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Received: 29 Apr, 2023; Accepted: 4 Jul, 2023

highest resistance rates have been estimated in many Asian countries, particularly China, South Korea, and Japan (4). The macrolide resistance of *S. pneumoniae* is based on two main mechanisms of the change in target site by methylases, encoded by the *erm*B gene (MLS_B phenotype), as well as efflux pump, encoded by the *mef* gene family (M phenotype) (5). Globally, the *erm*B gene is known as the most common cause of macrolide resistance in *S. pneumoniae* strains (6).

Despite the presence of various pneumococcal serotypes (more than 98), some have a significantly greater propensity to cause invasive infections in specific age groups or geographic regions (3, 7). In addition, some serotypes are associated with antibiotic resistance; for example, high antibiotic resistance has been reported in some clones such as 19A clonal complex (CC) 320 (8). Multilocus sequence typing (MLST) is considered as a gold standard approach for the molecular typing of *S. pneumoniae*. In this method, strains are located in a CC which is similar to at least six loci of seven housekeeping genes (9). Further, MLST plays an essential role in monitoring the clones acquiring antibiotic resistance and global distribution, called international multi-resistant clones (10). According to the molecular epidemiological monitoring network (http://spneumoniae.mlst.net/pmen/), 43 international clones of pneumococci are related to serotypes 6A, 6B, 14, 15A, 19F, 19A, 23F, and 35B, which are accompanied by antibiotic resistance (11).

The present study focused on determining capsular types and clonal diversity, as well as the presence of macrolide resistance genes in the erythromycin-resistant *S. pneumoniae* (ERSP) isolates associated with invasive pneumococcal infections.

Materials and Methods

Bacterial isolates

A total of 44 *S. pneumoniae* isolates were collected from the patients admitted to teaching hospitals in Tehran during 2018. Confirmatory biochemical tests were based on the Centers for Disease Control and Prevention (CDC) guidelines. Furthermore, the *S. pneumoniae*-specific *lytA* gene was amplified for the molecular confirmation of the isolates (12). The subjects did not receive any pneumococcal vaccine. This study was approved by the Ethics Committee of Tehran University of Medical Sciences (IR.TUMS.SPH.REC.1397.178) and conducted based on the principles of the Declaration of Helsinki. The written informed consent was obtained from participants or the legally authorized representative.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) was carried out following the Clinical Laboratory and Standards Institute (CLSI, 2017) guidelines (13). In this regard, the isolate susceptibility to erythromycin (15 µg), tetracycline (30 µg), clindamycin (2 µg), chloramphenicol (30 µg), trimethoprim/sulfamethoxazole (25 µg), and vancomycin (30 µg) was evaluated using the disk diffusion method, which were purchased from MAST Co., Merseyside, UK. Additionally, the minimum inhibitory concentrations (MICs) of penicillin and cefotaxime were determined by using a MIC test strip (MTS, *Liofilchem, Italy*) for oxacillin-resistant isolates. Multidrug resistance (MDR) was defined as acquired nonsusceptibility to at least one agent in three or more antimicrobial categories (14). Further, *S. pneumoniae* ATCC 49619 was utilized as the quality control strain.

Identification of ermB and mefA genes in the ERSP isolates

The macrolide resistance genes (*erm*B and *mef*A) were amplified through the polymerase chain reaction (PCR) using 2x hot start Taq master mix (containing 3 mM $MgCl_2$, 0.4 mM of each dNTP, 0.08 U/µl Taq DNA polymerase) (New England Biolabs Co.). The PCR was conducted in 25 µl volume with 2 µl of the target sample, 1 µl of each primer (10 pmol), and 11.5 µl of ddH_2O. In the process, an initial denaturation at 95 ºC for 30 min was

followed by 35 cycles of denaturation at 94 ºC for 60 s, and annealing at 58 ºC (*erm*B) and 60 ºC (*mef*A) for 35 s. Then, a 45-second extension at 72 ºC and a final 10-minute extension at 72 ºC were performed (15).

Molecular capsular typing through multiplex PCR assay

Capsular typing was carried out using a sequential multiplex PCR approach according to Ghahfarokhi et al. (16). Furthermore, the *cps*A gene-specific primers were used as an internal control in each reaction.

Multilocus sequence typing (MLST) analysis

The MLST analysis was employed to examine the genetic heterogeneity of the ERSP isolates. The seven specific housekeeping genes (*aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt*, and *ddl*) were amplified, and the PCR products were sent to Bioneer Company (Daejeon, South Korea) for purification and sequencing (17). In addition, the sequence types (STs) were determined by comparing the allelic profiles and identified STs on the MLST website (http://spneumoniae.mlst.net). The eBURST V3 program was utilized to separate bacterial populations into CCs (http://eburst.mlst.net).

Statistical analysis

Statistical analysis was performed in IBM SPSS Statistics 19.0 (IBM Crop, released 2010, IBM SPSS Statistics for Windows, version 19.0, Armonk, NY: IBM Corp). Further, the chi-square test or Fisher's exact one was applied for comparisons. A cut-off P-value of ≤ 0.05 (two-tailed) was considered statistically significant.

Results

Bacterial isolates

Among 44 *S. pneumoniae* isolates, 22 ones (50.0%) were classified as ERSP. The clinical sources of the isolates included blood (22.7%, 5), CSF (18.1%, n=4), pleural fluid (9.0%, 2), eye discharge (9.0%, 2), ear discharge (4.5%, 1), and respiratory specimens such as broncho-alveolar lavage (18.1%, 4), trachea aspirate (9.0%, 2), and sputum (9.0%, 2). Furthermore, approximately 52% of the isolates were obtained from the female subjects. The mean age of the patients was 38 years, and their minimum and maximum age was 2 months and 67 years, respectively. The results indicated no statistically significant relationship between IPD with age (P = 0.647) and sex (P = 0.749).

Antimicrobial susceptibility testing

The antibiotic susceptibility profile of 44 isolates is provided in Figure 1 and Table 1. As shown, about 54% of 44 *S. pneumoniae* strains, as well as 90.90% of 22 ERSP isolates are MDR. Additionally, ERSP isolates were significantly related to MDR isolates (P = 0.001), and both tetracycline- and penicillin-resistant ones (P< 0.05).

Table 1. Minimum Inhibitory Concentrations of *S. pneumoniae* isolates.

	MIC break point (µg/mL)				**Number of Isolates**			
	Penicillin		**Cefotaxime**		**Penicillin**		**Cefotaxime**	
	I*	R**	I	R	I (%)	R (%)	I (%)	R (%)
Meningitis (N=4)	---	≥ 0.12	1	≥ 2	0(0)	1(25)	0(0)	2(50)
Non-meningitis (N=40)	4	≥ 8	2	≥ 4	2(5)	8(20)	0(0)	5(12.5)

* Intermediate, ** Resistance.

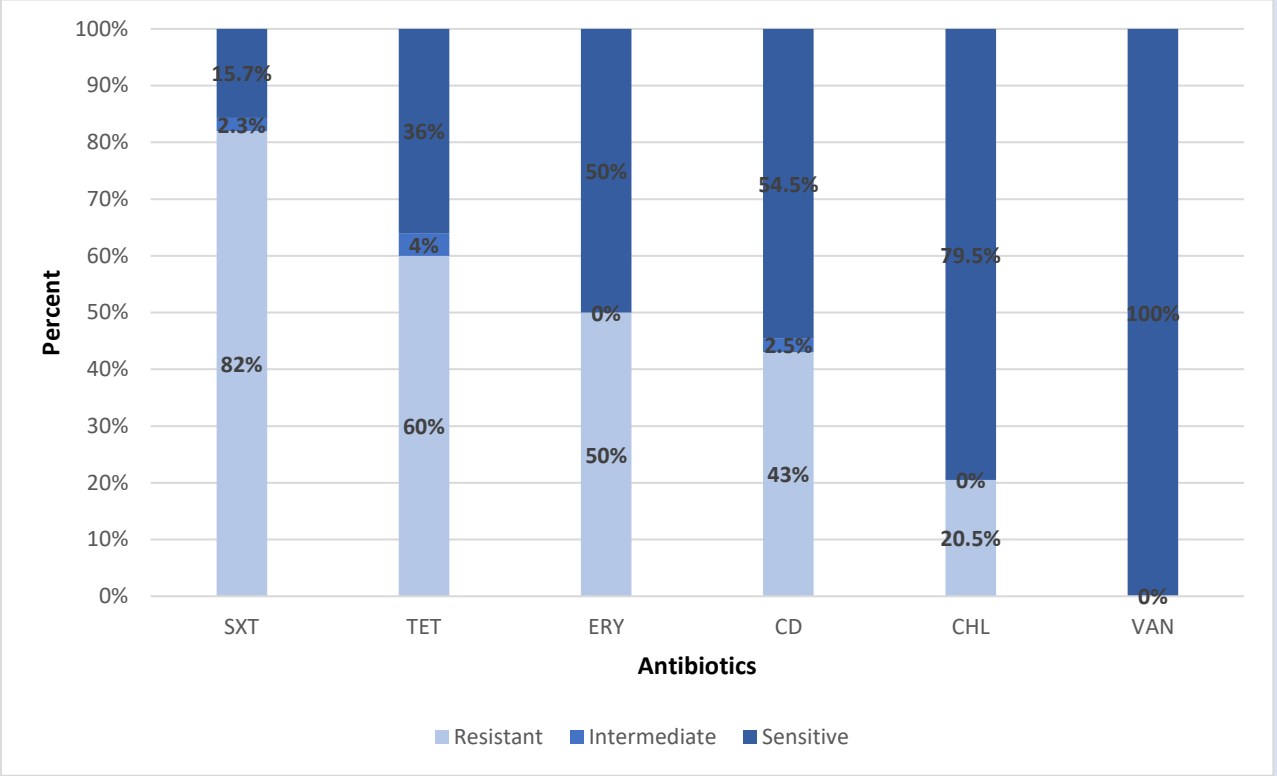

Fig. 1. Prevalence of antimicrobial susceptibility among pneumococcal isolates. SXT: trimethoprim/sulfamethoxazole; TET: tetracycline; ERY: erythromycin; CD: clindamycin; CHL: chloramphenicol; VAN: vancomycin.

Identification of ermB and mefA genes in the ERSP isolates

Almost 10 (45.4%) ERSP isolates expressed both the *erm*B and *mef*A genes, while 10 (45.4%) and 2 (9.2%) ones carried only *erm*B (cMLS phenotype) and *mef*A genes (M phenotype), respectively. Further, no statistically significant correlation was found between erythromycin resistance genotype and IPD (P = 0.414).

Molecular capsular typing through the multiplex PCR assay

Among the studied population, 14 (31.8%) was determined as the most prevalent serotype, followed by 3 (22.7%), 19A (18.1%), 23F (13.6%), and 19F (13.6%). As for the ERSP isolates, the serotype coverage rates of 7-, 10-, and 13-valent pneumococcal conjugate vaccines (PCV7, PCV10, and PCV13) were 59.0, 59.0, and 100%, respectively.

MLST of the ERSP isolates

Based on the MLST data, the eBURST algorithm resolved 18 STs into seven CCs and one singleton (Table 2). The most frequent STs were ST3130 (3 isolates) and ST166 (3 isolates), while each of other isolates (n=16) contained 16 distinct STs. Considering the single-locus variant (SLV), the STs were classified into three clonal groups and 12 singletons (Table 3). However, they were categorized into three clonal groups and six singletons according to the double-locus variant (DLV). Compared with the MLST database (http://mlst.net), 21 STs in the present study belonged to seven CCs, and ST708 was a singleton. Furthermore, CC63 was the most common CC, which included 32% of the STs (Fig. 2). The CC was not significantly related to age group (P = 0.629) and sex (P = 0.493). Finally, four meningeal isolates belonged to different CCs (CC63, CC156, CC180, and CC217) and contained the *erm*B gene, while the *mef*A gene was detected in three ones.

Table 2. Clonal complexes and the relationship among the molecular types and resistance genes of erythromycin-resistant *S. pneumoniae* isolates.

Clonal complex	Sequence Type	Number of Isolates	Serotype	Resistance genes	
				ermB	ermB + *mefA*
CC63	ST11483	1	14		1
	ST3130	3	19F		1
			14		1
			3	-	-
	ST2678	1	14		1
	ST12937	1	3	1	
	ST12940	1	23F	1	
CC156	ST166	3	14 (2)		2
			19A	1	
	ST92	1	19F	1	
CC320	ST320	1	19A		1
	ST9386	1	3	1	
	ST2393	1	19F		1
	ST3971	1	3	1	
CC15	ST3981	1	23F	1	
CC81	ST81	1	19A	1	
	ST9695	1	3	1	
	ST12938	1	23F	1	
CC180	ST12936	1	19A		1
CC217	ST12939	1	14	-	-
Singletons	ST708	1	14		1

Table 3. Grouping based on the difference in one allele (SLV) with the eBURST V3 software.

Number of isolates: 22 | Number of sequence types: 22 | Number of re-samplings for bootstrapping: 1000 Number of loci per isolate: 7 | Number of identical loci for group def.: 6 | Number of groups: 3						
Group 1: Number of isolates: 2 | Number of sequence types: 2 | Predicted Founder: None						
ST[a]	FREQ[b]	SLV[c]	DLV[d]	TLV[e]	SAT[f]	Distance
12937	1	1	0	0	0	1.0
2678	1	1	0	0	0	1.0
Group 2: Number of isolates: 2 | Number of sequence types: 2 | Predicted Founder: None						
ST	FREQ	SLV	DLV	TLV	SAT	Distance
9695	1	1	0	0	0	1.0
81	1	1	0	0	0	1.0
Group 3: Number of isolates: 2 | Number of sequence types: 2 | Predicted Founder: None						
ST	FREQ	SLV	DLV	TLV	SAT	Distance
2393	1	1	0	0	0	1.0
320	1	1	0	0	0	1.0
Singletons | Number of sequence types: 12						
11483, 166, 12940, 12939, 12938, 12936, 3130, 708, 9386, 3981, 3971, 92						

[a]ST: sequence type, [b]FREQ: frequency, [c]SLV: Single Locus Variants, [d]DLV: Double Locus Variants, [e]TLV: Triple Locus Variants, [f]SAT: Satellites.

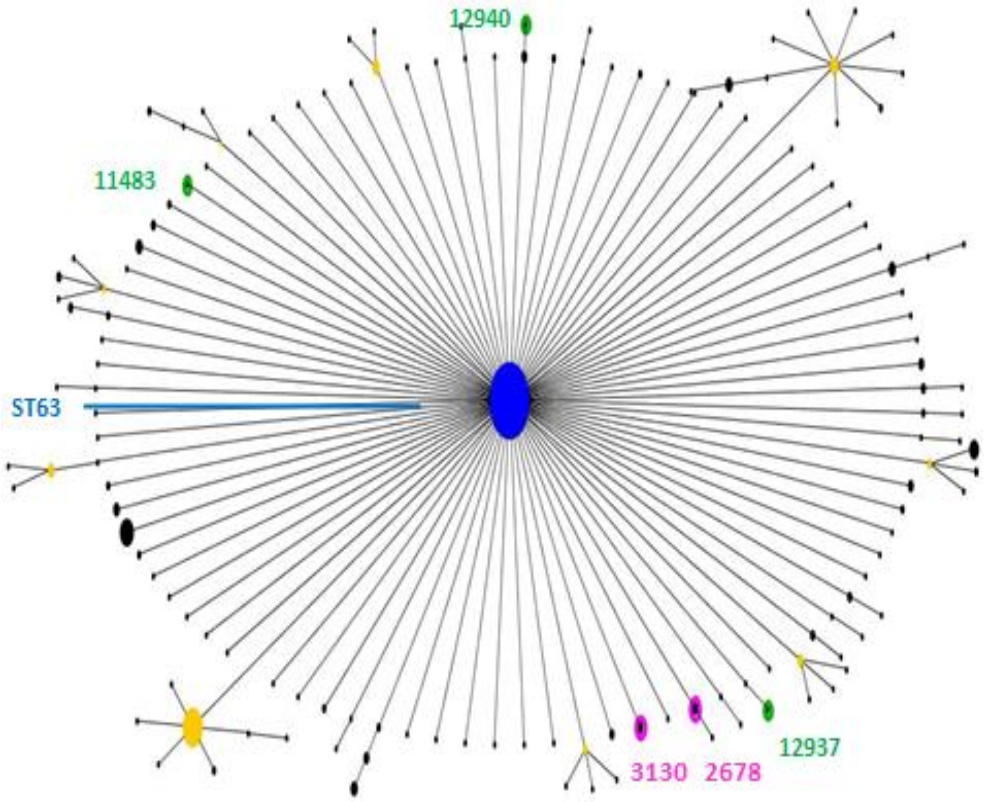

Fig. 2. Comparison of the sequence types in the present study with the available sequence types at PubMLST databases using the eBURST V3 software. Seven isolates (5 sequence types) are located in the CC63.

Discussion

According to the Asian Network for Surveillance of Resistant Pathogen (ANSORP) study, Asian regions have the highest levels of ERSP around the world (72.7%) (18). In the present study, 50% of the isolates were erythromycin resistant, which is inconsistent with the results of another study in Iran which estimated 71.4% as the rate of ERSP isolates (19). Approximately 91% of the ERSP isolates exhibited MDR phenotype, reflecting a significant correlation between erythromycin resistance and MDR. In addition, no statistically significant relationship was observed between erythromycin resistance and age group ($P> 0.05$). The result is not in line with that of Talebi et al. which revealed more prevalence of ERSP among the patients aged less than 15 years, as well as a significant difference in the erythromycin resistance rates of those under and over the age of 15 years ($P< 0.05$) (20). Despite the effectiveness of PCVs in decreasing the burden of the pneumococcal infection caused by vaccine serotypes, the vaccines are only given to a limited number of high-risk individuals in Iran (20). Similarly, the results of another study in Nepal indicated that the coverage rate of PCV7 and PCV10 was 59%, while that of the serotypes included in PCV13 was 100% (21).

Regarding the *S. pneumoniae*, macrolide resistance occurs mainly through two primary mechanisms of *erm*B and *mef*A genes. The *mef* genes (phenotype M) have been suggested as the primary mechanism of the resistance in North America, England, and Germany (9). However, the MLS_B phenotype is the most common mechanism in Iran (20), which is confirmed by the results of the present study. The *erm*B was found in five different pneumococcal serotypes (mostly in serotypes 3 and 14), and the *mef*A was associated with four different serotypes. There is a global increase in the number of MDR strains with both mechanisms of macrolide resistance. This type of resistance is attributed to the transposon Tn2010 carrying both macrolide

resistance genes (*erm*B and *mef*) (22). In the present study, 45.4% of the ERSP isolates simultaneously contained both resistance genes, which is relatively high compared with the other studies in the world. However, a high percentage of this type has been reported in some countries such as China (62.9%) (23).

Based on the results of the present study, 14, 3, 19A, and 23F were the predominant serotypes among the ERSP isolates, which are consistent with those of Talebi et al. introduced 14, 3, 19F, and 23F as the most prevalent serotypes in the isolates, respectively (20). Further, 14, 19F, and 23F are among the global top seven disease-causing serotypes before vaccine introduction (24). The serotypes 14 and 19F were the most common serotypes causing IPD, which is in agreement with results of Balaji et al. (25). Furthermore, serotype 1 is considered one of the five most prevalent serotypes in numerous developing countries such as South Africa, Kenya, and Philippines (18, 26, 27). However, this serotype was not detected in the present study and recent surveillance research in Iran (16, 20), which may represent that the circulation pattern of *S. pneumoniae* serotypes is geographically diverse (28). In the present study, serotype 14 belonged to ST3130, ST166, ST11483, ST2678, ST12939, and ST708, respectively, which is not in line with the results of Talebi et al. which demonstrated the association of the serotype with ST3130, ST63, ST2678, ST166, ST557, ST6354, ST2253, and ST3772, respectively (20). Most of the STs belonged to the CC63, which was the most common CC in this study (20). Additionally, the most frequent STs among ERSP isolates were ST3130 (serotypes 19F, 14, 3) and ST166 (serotypes 14 and 19A). According to Talebi et al., the isolates were of ST3130 (serotypes 19F and 14). Interestingly, ST166, one of the most prevalent STs (3 isolates) in the present study, was not found in the other similar studies in Iran (20, 29). Further, the results of this study failed to report the presence of ST180/CC180, which is not in concordance with those of Talebi et al. and Azarian et al. which introduced this ST as the dominant genotype among isolates with serotype 3 (20, 30). The isolates accompanied by this clone are more related to the invasive diseases in adults, which have been observed since the pre-vaccination period until the recent years. This clone is associated with IPD and leads to many deaths in Europe and North America (31).

The results of the present study suggested CC63 as the most common CC among the ERSP isolates, which is consistent with those of Talebi et al. As for the present study, *erm*B and *mef*A genes were identified in 7 (100%), and 4 (57%) isolates of CC63 in order. However, Talebi et al. reported that 4% of isolates in this CC carried both *ermB* and *mef* genes, while 57 and 39% contained *ermB* and *mef* genes, respectively (20). According to the Pneumococcal Molecular Epidemiology Network, the ST63 (serotype 15A), the predicted founder of CC63, is considered one of the international drug-resistant clones, which is detected in many parts of the world such as Asia, Europe, Africa, and Australia (32, 33). Despite the lack of a statistically significant relationship between the type of invasive disease and CC of MDR isolates, the strains from the individuals with respiratory diseases belonged to three CCs (CC156, CC81, and CC63). Furthermore, the strains obtained from the blood of the patients with septicemia were related to two different CCs (CC81 and CC320). Based on the results of the present study, CC81 was the most frequent CC (14%). Spain23F-1 ST81, associated with CC81, is one of the international MDR clones, as well as prevailing among penicillin-non-susceptible isolates. The ST is further related to resistance to macrolides and fluoroquinolones. Following the PCV7 introduction, the prevalence of this clone significantly reduced in various regions of the world (34). The international distribution of this clone can be ascribed to the adaptation to the colonization of human nasopharynx (35). In addition, nasopharyngeal colonization by pneumococci facilitates genetic exchanges among *S. pneumoniae* strains, even with closely related species (32, 34).

The results of the present study determined CC320 (serotypes 3, 19A, and 19F) as another

common CC, which constituted 18% of the isolates. However, none of the strains isolated from respiratory infections belonged to this CC. Further, ST320 is a predicted founder for CC320. The clone is known as one of the MDR clones in the world, which has been found in different countries (36). After introducing the PCV7, ST320 (serotype 19A) is still considered as one of the most frequent MDR clones in many Asian countries such as China and Taiwan (37). Furthermore, 18% of the isolates belonged to CC156 (serotypes 14, 19A, and 19F), another prevalent CC in this study. The predicted founder of the CC, ST156, is common in countries like the United States, Norway, France, and Spain (1), the strains of which spread globally and are accompanied by antibiotic resistance (32). The ST was first detected in 2001 and exhibits a high MIC to penicillin and cefotaxime. The clone is associated with different serotypes such as 11A, 9A, 6B, 14, 15B, 15C, 19A, 19F, 23F, and 24F (38).

The results of the present study indicated the large frequency of MDR and ERSP among the invasive pneumococcal isolates, as well as the high heterogeneity of the ERSP isolates. The serotypes 14, 3, and 19F constituted the majority (77.2%) of the ERSP isolates, which belonged to CC63, CC320, CC156, CC81, and CC217. Ultimately, the inclusion of PCV13 in immunization policies can be effective in decreasing the pneumococcal clinical burden.

Acknowledgment

We thank the scientists and personnel of hospitals affiliated to Tehran University of Medical Sciences, for their collaboration.

Funding

This study was funded and supported by Tehran University of Medical Sciences (TUMS) (Grant Number: 39112).

Conflict of Interest

The authors declare no potential conflict of interest.

References

1. Mosadegh M, Asadian R, Emamie AD, Rajabpour M, Najafinasab E, Pourmand MR, et al. Impact of Laboratory Methods and Gene Targets on Detection of *Streptococcus pneumoniae* in Isolates and Clinical Specimens. Rep Biochem Mol Biol. 2020;9(2):216.

2. O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Deloria-Knoll M, McCall N, et al. Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. Lancet. 2009;374(9693):893-902.

3. Mosadegh M, Habibi Ghahfarokhi S, Ahmadi A, Pourmand MR, Erfani Y, Mashhadi R. Identification and molecular characterization of penicillin-nonsusceptible Streptococcus pneumoniae isolates recovered from invasive infections in a pre-pneumococcal vaccine era. J Clin Lab Anal. 2022;36(8):e24566.

4. Cheng AC, Jenney AW. Macrolide resistance in pneumococci- is it relevant? Pneumonia 2016;8(1):1-3.

5. Azadegan A, Ahmadi A, Lari AR, Talebi M. Detection of the efflux-mediated erythromycin resistance transposon in *Streptococcus pneumoniae*. Ann Lab Med. 2015;35(1):57-61.

6. Schroeder MR, Stephens DS. Macrolide resistance in *Streptococcus pneumoniae*. Front Cell Infect Microbiol. 2016;6:98.

7. Jauneikaite E, Tocheva AS, Jefferies JM, Gladstone RA, Faust SN, Christodoulides M, et al. Current methods for capsular typing of *Streptococcus pneumoniae*. J Microbiol Methods. 2015;113:41-49.

8. Setchanova L, Alexandrova A, Pencheva D, Sirakov I, Mihova K, Kaneva R, et al. Rise of multidrug-resistant *Streptococcus pneumoniae* clones expressing non-vaccine serotypes among children following introduction of the 10-valent pneumococcal conjugate vaccine in Bulgaria. J Glob Antimicrob Resist. 2018;15:6-11.

9. Harrison OB, Brueggemann AB, Caugant DA, Van Der Ende A, Frosch M, Gray S, et al. Molecular typing methods for outbreak detection and surveillance of invasive disease caused by *Neisseria meningitidis*, *Haemophilus influenzae* and *Streptococcus pneumoniae*, a review. Microbiology. 2011;157(08):2181.
10. Sadowy E, Skoczyñska A, Fiett J, Gniadkowski M, Hryniewicz W. Multilocus sequence types, serotypes, and variants of the surface antigen PspA in *Streptococcus pneumoniae* isolates from meningitis patients in Poland. Clin Vaccine Immunol. 2006;13(1):139-144.
11. Setchanova L, Alexandrova A, Pencheva D, Sirakov I, Mihova K, Kaneva R, et al. Rise of multidrug-resistant *Streptococcus pneumoniae* clones expressing non-vaccine serotypes among children following introduction of the 10-valent pneumococcal conjugate vaccine in Bulgaria. J Glob Antimicrob Resist. 2018;15:6-11.
12. Azarsa M, Salami SA, Pourmand MR, Forushani AR, Kazemian H. Evaluation of lytB gene for detection of *Streptococcus pneumoniae* in isolates and clinical specimens by real-time PCR. Jundishapur J Microbiol. 2017;10(6).
13. Clinical, Institute LS. Performance standards for antimicrobial susceptibility testing. Clinical and Laboratory Standards Institute Wayne, PA; 2017.
14. Basak S, Singh P, Rajurkar M. Multidrug resistant and extensively drug resistant bacteria: A study. J Pathog. 2016;2016.
15. Mosleh MN, Gharibi M, Alikhani MY, Saidijam M, Vakhshiteh F. Antimicrobial susceptibility and analysis of macrolide resistance genes in *Streptococcus pneumoniae* isolated in Hamadan. Iran J Basic Med Sci. 2014;17(8):595.
16. Ghahfarokhi SH, Mosadegh M, Ahmadi A, Pourmand MR, Azarsa M, Rahbar M, et al. Serotype Distribution and Antibiotic Susceptibility of *Streptococcus pneumoniae* Isolates in Tehran, Iran: A Surveillance Study. Infect Drug Resist. 2020;13:333.
17. Adamiak P, Vanderkooi OG, Kellner JD, Schryvers AB, Bettinger JA, Alcantara J. Effectiveness of the standard and an alternative set of *Streptococcus pneumoniae* multi locus sequence typing primers. BMC Microbiol. 2014;14(1):1-8.
18. Kim SH, Song J-H, Chung DR, Thamlikitkul V, Yang Y, Wang H, et al. Changing trends in antimicrobial resistance and serotypes of *Streptococcus pneumoniae* isolates in Asian countries: an Asian Network for Surveillance of Resistant Pathogens (ANSORP) study. Antimicrob Agents Chemother. 2012;56(3):1418-1426.
19. Houri H, Tabatabaei SR, Saee Y, Fallah F, Rahbar M, Karimi A. Distribution of capsular types and drug resistance patterns of invasive pediatric *Streptococcus pneumoniae* isolates in Teheran, Iran. Int J Infect Dis. 2017;57:21-26.
20. Talebi M, Azadegan A, Sadeghi J, Ahmadi A, Ghanei M, Katouli M, et al. Determination of characteristics of erythromycin resistant *Streptococcus pneumoniae* with preferred PCV usage in Iran. PloS one. 2016;11(12):e0167803.
21. Shah AS, Deloria Knoll M, Sharma P, Moisi J, Kulkarni P, Lalitha MK, et al. Invasive pneumococcal disease in Kanti Children's Hospital, Nepal, as observed by the South Asian Pneumococcal Alliance network. Clin Infect Dis. 2009;48(Supplement_2):S123-S128.
22. Chancey ST, Agrawal S, Schroeder MR, Farley MM, Tettelin H, Stephens DS. Composite mobile genetic elements disseminating macrolide resistance in *Streptococcus pneumoniae*. Front Microbiol. 2015;6:26.
23. Korona-Glowniak I, Siwiec R, Malm A. Resistance determinants and their association with different transposons in the antibiotic-resistant *Streptococcus pneumoniae*. Biomed Res Int. 2015;2015.
24. Hocknell RE, Cleary DW, Srifeungfung S, Clarke SC. Serotype distribution of disease-

causing *Streptococcus pneumoniae* in Thailand: A systematic review. Vaccine. 2019;37(24):3159-3166.
25. Balaji V, Jayaraman R, Verghese VP, Baliga P, Kurien T. Pneumococcal serotypes associated with invasive disease in under five children in India & implications for vaccine policy. Indian J Med Res. 2015;142(3):286.
26. du Plessis M, Allam M, Tempia S, Wolter N, de Gouveia L, von Mollendorf C, et al. Phylogenetic analysis of invasive serotype 1 pneumococcus in South Africa, 1989 to 2013. J Clin Microbiol. 2016;54(5):1326-1334.
27. Brueggemann AB, Peto TE, Crook DW, Butler JC, Kristinsson KG, Spratt BG. Temporal and geographic stability of the serogroup-specific invasive disease potential of *Streptococcus pneumoniae* in children. J Infect Dis. 2004;190(7):1203-1211.
28. Wang L, Fu J, Liang Z, Chen J. Prevalence and serotype distribution of nasopharyngeal carriage of *Streptococcus pneumoniae* in China: a meta-analysis. BMC Infect Dis. 2017;17(1):765.
29. Azarsa M, Moghadam SO, Rahbar M, Baseri Z, Pourmand M. Molecular serotyping and genotyping of penicillin non-susceptible pneumococci: the introduction of new sequence types, Tehran, Iran. New Microbes New Infect. 2019;32:100597.
30. Azarian T, Mitchell PK, Georgieva M, Thompson CM, Ghouila A, Pollard AJ, et al. Global emergence and population dynamics of divergent serotype 3 CC180 pneumococci. PLoS Pathog. 2018;14(11):e1007438.
31. Nurse-Lucas M, McGee L, Hawkins PA, Swanston WH, Akpaka PE. Serotypes and genotypes of *Streptococcus pneumoniae* isolates from Trinidad and Tobago. Int J Infect Dis. 2016;46:100-106.
32. Calatayud L, Ardanuy C, Tubau F, Rolo D, Grau I, Pallarés R, et al. Serotype and genotype replacement among macrolide-resistant invasive pneumococci in adults: mechanisms of resistance and association with different transposons. J Clin Microbiol. 2010;48(4):1310-1316.
33. Miernyk KM, Bulkow LR, Case SL, Zulz T, Bruce MG, Harker-Jones M, et al. Population structure of invasive *Streptococcus pneumoniae* isolates among Alaskan children in the conjugate vaccine era, 2001 to 2013. Diagn Microbiol Infect Dis. 2016;86(2):224-230.
34. Korona-Glowniak I, Maj M, Siwiec R, Niedzielski A, Malm A. Molecular epidemiology of *Streptococcus pneumoniae* isolates from children with recurrent upper respiratory tract infections. PloS one. 2016;11(7):e0158909.
35. Croucher NJ, Walker D, Romero P, Lennard N, Paterson GK, Bason NC, et al. Role of conjugative elements in the evolution of the multidrug-resistant pandemic clone *Streptococcus pneumoniae* Spain23F ST81. J Bacteriol. 2009;191(5):1480-1489.
36. Shin J, Baek JY, Kim SH, Song J-H, Ko KS. Predominance of ST320 among *Streptococcus pneumoniae* serotype 19A isolates from 10 Asian countries. J Antimicrob Chemother. 2011;66(5):1001-1004.
37. Pan F, Han L, Huang W, Tang J, Xiao S, Wang C, et al. Serotype distribution, antimicrobial susceptibility, and molecular epidemiology of *Streptococcus pneumoniae* isolated from children in Shanghai, China. PloS one. 2015;10(11):e0142892.
38. Sjöström K, Blomberg C, Fernebro J, Dagerhamn J, Morfeldt E, Barocchi MA, et al. Clonal success of piliated penicillin nonsusceptible pneumococci. Proc Natl Acad Sci. 2007;104(31):12907-12912.