ORIGINAL ARTICLE

Molecular serotyping and genotyping of penicillin non-susceptible pneumococci: the introduction of new sequence types, Tehran, Iran

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Abstract

The emergence of penicillin non-susceptible *Streptococcus pneumoniae* (PNSP) isolates can pose significant challenges to today's health-care system. Resistant clonal isolates are disseminated in different regions and countries, and this study was focused on the description of the epidemiological spread of these strains. Clinical samples were collected from individuals admitted to hospitals affiliated to the Tehran University of Medical Sciences, Iran. To investigate the molecular characteristics of PNSP isolates, they were subjected to molecular typing using multi-locus sequence typing (MLST). Serotype distributions of *S. pneumoniae* isolates were also evaluated by multiplex PCR assay. The most prevalent serotypes in the PNSP isolates were 23F, 19F, 14, 3 and 9V. Two isolates were considered as a non-vaccine serotype. The MLST analysis showed that PNSP isolates belonged to five different clonal complexes (CC180, CC217, CC81, CC63 and CC320) and 42% (5/12) of the sequence types were novel (12936, 12937, 12938, 12939 and 12940). This study indicates the high level of heterogeneity that is present among PNSP isolates. Unexpected high genetic diversity in small populations indicates consecutive diversification of resistant strains.

Keywords: Capsular serotyping, microbial drug resistance, molecular typing, multilocus sequence typing, penicillin non-susceptible pneumococci

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Introduction

Streptococcus pneumoniae is the leading aetiology of invasive pneumococcal diseases (IPD) such as community-acquired pneumonia, septicaemia, and meningitis [1]. IPD causes numerous cases of severe mortality and morbidity worldwide mainly in developing countries [2]. The antibiotic resistance rate is increasing among strains of *S. pneumoniae*, which may cause complications in treatment [3]. Overuse oβ-lactam antibiotics for the initial treatment of pneumococcal infections may be leading to a considerable increase in penicillin non-susceptible *S. pneumoniae* (PNSP) worldwide [1].

Moreover, increases in resistance to macrolides and tetracyclines have been reported in several studies in the last decade [4], which also pose a significant challenge in IPD management [5]. Resistant clones of *S. pneumoniae* are disseminated in different regions and countries, and affect the spread of bacterial resistance. There is a need for appropriate assessment of antibiotic resistance levels among these pathogens to provide proper antimicrobial guidelines based on resistance patterns across countries [6].

Introduction of pneumococcal vaccines may cause serotype replacement, and consequently lead to the emergence of

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vaccine-escape strains [7,8]. Recombination rates in pneumococci, on the other hand, are high [9]. Determination of the genetic diversity and serotype relevance of PNSP strains can be useful in controlling infections caused by this bacterium. Multilocus sequence typing (MLST) is considered the standard method for standardized nomenclature and classification of resistant clones and epidemiological surveillance of *S. pneumoniae* (web1.sph.emory.edu/PMEN/) [10]. It can provide unambiguous results and presents good resolving and discriminatory power that can be used for local and global epidemiology. MLST was used to determine *S. pneumoniae* genotypes based on comparing sequences of seven housekeeping genes (*aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt* and *ddl*) [11]. As the studies on the molecular typing of *S. pneumoniae* in Iran are rare, our research focuses on assessing the phenotypic and genotypic characteristics of PNSP strains from various non-IPD cases. To achieve this goal, the isolates were subjected to serotyping and molecular genotyping using multiplex PCR and MLST methods respectively, and to phenotypic antimicrobial susceptibility testing using the Kirby–Bauer disc diffusion and MIC broth microdilution assay.

Materials and methods

Isolation and growth conditions

In this study, a total of 560 clinical samples were collected from individuals admitted to hospitals affiliated to Tehran University of Medical Sciences with suspected pneumococcal infections during February–September 2015. All specimens were obtained from different sources such as cerebrospinal fluid, ascites fluid, blood, sputum, pleural fluid, bronchoalveolar lavage, ear discharge, synovial fluid and eye discharge. These clinical samples were cultured on blood agar containing 5% sheep blood and incubated for 24 h at 37°C with 5% CO_2. Suspected colonies were identified by Gram staining, bile solubility and optochin (Mast Group, Bootle, UK) susceptibility test. Isolates were cultured in trypticase soy broth medium, which contained 10% glycerol, and were preserved at −70°C [12]. Molecular assays confirmed cultural and biochemical characterization of *S. pneumoniae* isolates. Isolates were examined for the presence of the *lytB* gene in DNA extracts by real-time PCR assay as previously described [13].

Antibiotic susceptibility testing

The isolates were screened for penicillin G resistance with a 1-μg oxacillin disc using the Kirby–Bauer disc diffusion method (with inhibition zone size ≥20 mm). The MICs of penicillin G and cefotaxime were determined for all oxacillin-resistant isolates using the MIC broth microdilution method, and interpretation of results was according to the CLSI guidelines [14]. According to the CLSI guidelines, penicillin susceptibility breakpoints for meningeal *S. pneumoniae* are ≤0.06 mg/L and ≥0.12 mg/L defining susceptibility and resistance, respectively (no intermediate category for meningeal strains). The recommended penicillin MIC susceptibility breakpoints for non-meningeal infections are ≤0.06 mg/L, 0.12–1 mg/L and ≥2 mg/L for susceptibility, intermediate susceptibility and resistance, respectively. Moreover, cefotaxime MIC breakpoints for meningeal infections defining susceptibility, intermediate susceptibility and resistance are ≤0.5 mg/L, 1.0 mg/L and ≥2 mg/L, respectively, and for non-meningeal infections are ≤1.0 mg/L, 2.0 mg/L and ≥4 mg/L.

Serotyping

Serotypes of the PNSP isolates were determined by the molecular method using multiplex PCR, as described by available procedures on the CDC Streptococcus Laboratory website. Capsular types identified by this method steadily produced concordant results with the conventional capsular serotyping assay [15].

Molecular genotyping

The MLST was conducted according to the method of Enright and Spratt [11]. Briefly, the internal fragments of seven housekeeping genes (*aroE*, *spi*, *gdh*, *xpt*, *gki*, *recP* and *ddl*) were amplified by PCR and the products were sequenced. Sequence types (STs) were determined by submitting the sequences to the MLST database (http://spneumoniae.mlst.net).

Results

From 560 clinical samples recovered from suspected pneumococcal infections, 46 *S. pneumoniae* were isolated (Fig. 1). Overall, ten non-meningitis isolates (10/42) were PNSP, based on CLSI revised breakpoints. In addition, penicillin resistance was detected in two of four meningitis isolates (MICs 0.12 mg/L and 0.5 mg/L) according to the meningeal breakpoint.

The serotypes of all PNSP isolates belonged to five different capsular serotypes. The serotypes of two (16.6%) isolates could not be determined that classified as non-vaccine serotypes. Serotype 23F was the most frequently identified serotype (3/12; 25.0%), followed by serotypes 19F, 14 and 3 (2/12; 16.6% each), and 9V (1/12; 8.3%).

Twelve different STs among *S. pneumoniae* isolates were revealed by MLST. Forty-two per cent (5/12) of the STs were novel (12936, 12937, 12938, 12939 and 12940) and results showed that they belonged to four different clonal complexes: ST12936 (CC180), ST12937 (CC63), ST12938 (CC81),

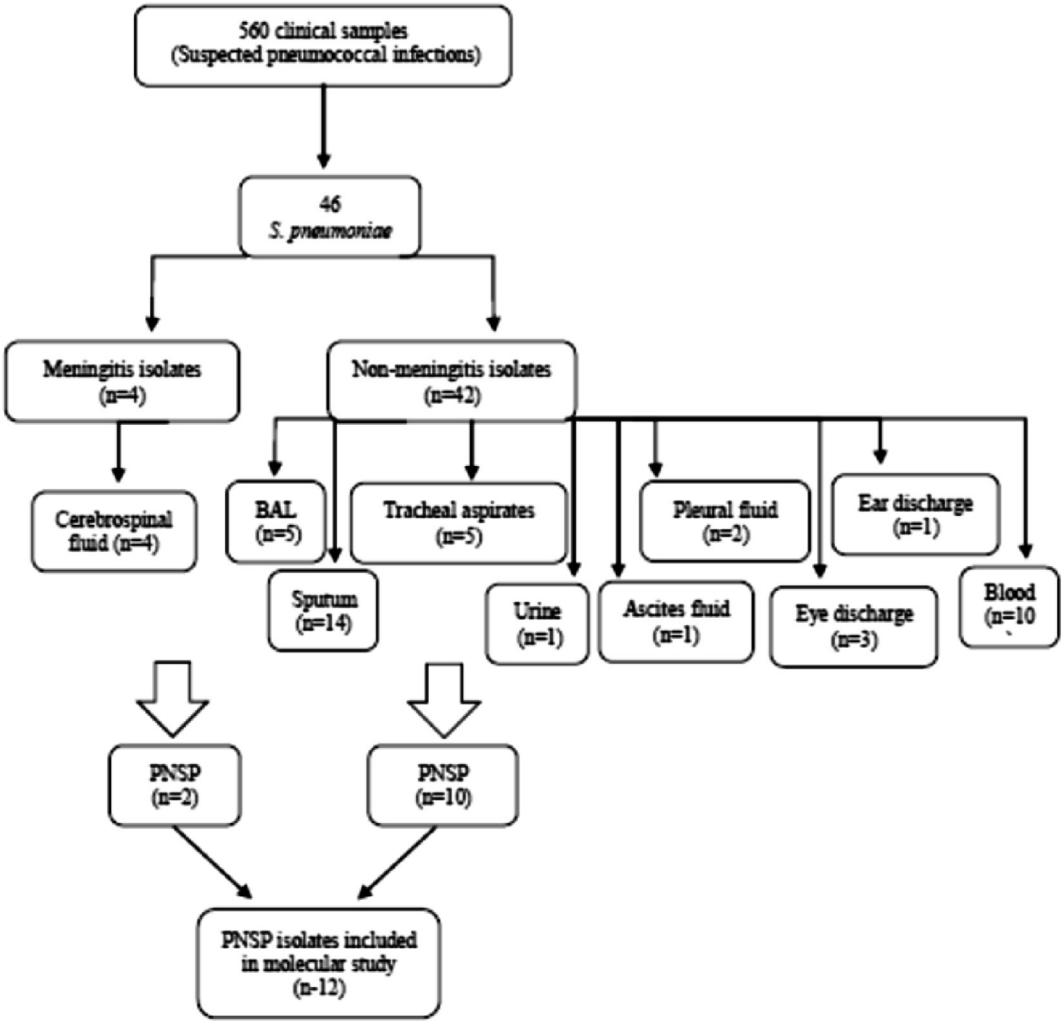

FIG. 1. Flow of clinical samples and penicillin non-susceptible pneumococci isolates included in the multilocus sequence typing study.

ST12939 (CC217) and ST12940 (CC63). The results are summarized in Table 1.

Forty-six PNSP were included in analyses. Examining the relationship between the type of infection and age, Pearson's chi-squared test and contingency table were used. Based on the results, there were two independent variables, and age was not related to the type of clinical infection (Fig. 1). Prevalences of PNSP among children and adults were 35% (n = 16) and 65% (n = 30), respectively (p 0.749). Resistance to penicillin was not associated with gender (18 (39%) men versus 28 (61%) women; p 1.000). There was a substantial relationship between resistance to penicillin and resistance to erythromycin (p 0.001). Among the 12 PNSP isolates, ten isolates were also resistant to erythromycin. According to our study, resistance to penicillin among strains with CC63 was more, but this was not statistically significant (p 0.306) (Table 1). There was no significant relationship between the type of clinical infection and resistance to penicillin (p 0.330).

Discussion

Increasing incidence of PNSP has become a worldwide problem since the 1980s [16,17] and has created severe problems regarding the management of IPD [1]. The prevalence of PNSP in Asian countries varies widely [5,18,19]. The rapid growth in incidence may result from the intercontinental spread of a few PNSP clones [20,21]. Very low PNSP frequencies were reported from Sweden (2%–3%) for a long period [22]. However, in Skåne, the prevalence of PNSP increased from 8% to 10% [23,24]. Non-susceptible isolates were reported from Turkey (19.1%), North America (9%–24%) and Europe (0%–43%) [18,25]. In recent studies from the USA (2011) and Asian countries (2012) prevalences were 14.8% and 4.8%, respectively [23]. Our data indicate that 23.8% of non-meningitis isolates were PNSP. The incidence of PNSP strains in the current study was relatively low compared with the previous study

TABLE 1. Molecular characteristics of penicillin non-susceptible pneumococci isolates

CC type (*n*)	ST	MIC (mg/L)	Capsular Serotype	Source of isolate	Erythromycin
CC320 (3)	2393	16	19F	blood	R
	9386	4	3	pleural fluid	R
	3971	16	NVT	ear discharge	R
CC63 (4)	11483	4	14	sputum	R
	3130	8	NVT	tracheal aspirate	R
	12937	8	3	eye discharge	R
	12940	8	23F	sputum	R
CC81 (3)	9695	8	23F	blood	S
	12938	4	23F	BAL	R
	81	8	19F	BAL	R
CC180 (1)	12936	0.12	9V	CSF	R
CC217 (1)	12939	0.5	14	CSF	S

Abbreviations: BAL, bronchoalveolar lavage; CC, clonal complex; CSF, cerebrospinal fluid; NVT, non-vaccine serotype; R, resistant; S, susceptible; ST, sequence type.

in Iran (28%) [19], which is similar to results from other countries such as China (20%) [5,26].

There was a significant association between resistance to penicillin and erythromycin ($p < 0.05$). Additionally, a high rate of resistance to erythromycin, tetracycline, trimethoprim-sulfamethoxazole and clindamycin, and an increasing rate of resistance to penicillin and cefotaxime, in Iran have been noted in several studies [19,25,27,28].

In the Middle East, the most common serotypes associated with IPD are 14, 6B, 5, 6A and 19A [29]. 23F was the dominant serotype in the class of isolates with novel STs. These findings are contrary to those described in the Middle East. In our research, vaccine coverage among PNSP isolates for pneumococcal conjugate vaccines PCV-7, PCV-10 and PCV-13 were 58.3%, 58.3% and 83.3%, respectively. We found five new STs associated with four different serotypes that showed the high distribution of these novel STs in the pneumococcal population. The ability of *S. pneumoniae* to switch its polysaccharide capsule with other pneumococci causes replacement of non-vaccine serotypes with those represented in pneumococcal conjugate vaccines through serotype replacement. The possibility exists that these broad distributions in genotypes and serotypes affect serotype replacement; however it might be attributable to various circumstances [30].

Results from MLST revealed 12 different STs for *S. pneumoniae* isolates. The MLST analyses showed the PNSP strains belonged to five major clonal complexes, CC320, CC63, CC81, CC180 and CC217. In the current study, CC63 was shown as the predominant clonal complex, which is in accordance with another study in Iran [19]. Non-meningitidis PNSP isolates belonged to three clonal complexes: CC320, CC63 and CC81. ST320, the predicted founder of CC320, is the most common ST in various countries including USA, Canada, Colombia, Spain, China, and ten other Asian countries [31,32]. This ST is extensively drug-resistant and distributed worldwide. ST320 was not found in the present study, nor in the previous study in Iran. However, the STs 9386, 2393 and 3971 located in our study and ST320 belong to the same clonal complex (CC320) [25].

Among the invasive isolates of *S. pneumoniae* in Iran, the serotypes 14 (33.3%), 3 (21.6%), 19F (15%), 23F (15%), 19A (6.6%), 6A (3. 3%), 9V (3.3%) and 6B (1.6%) are prevalent. In order of frequency, the clones found in serotype 14 are related to ST3130, ST63, ST2678, ST166, ST557, ST6354, ST2253 and ST3772, respectively. Most of these STs belong to the CC63, which is one of the most common clonal complexes in Iran. Strains with serotype 3 belong to CC180 but were not found in this study. 19F and 23F serotypes belong to ST3130 and ST81, which are international multidrug-resistant clones. Based on the Asian Network for Surveillance of Resistant Pathogens studies the prevalence of serotype F19 in Iran is similar to that in other Asian countries, but serotype 14 in Iran is more prevalent than in other Asian countries [19].

Conclusion

This study presents data about the molecular epidemiology of PNSP in Iran and indicates the high level of heterogeneity that is present among PNSP. Epidemiological information is essential to better understand the population dynamics of *S. pneumoniae* before the introduction of pneumococcal vaccines. Unexpectedly high genetic diversity in these small populations is indicating consecutive diversification of resistant strains.

The small sample size is the most important limitation of this study. However, our findings provide basic knowledge of molecular epidemiology, and antimicrobial resistance of PNSP. Further studies should be carried out to obtain more information about molecular serotyping/genotyping of these strains in Iran.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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