Identification and molecular characterization of penicillin-nonsusceptible *Streptococcus pneumoniae* isolates recovered from invasive infections in a pre-pneumococcal vaccine era

Mehrdad Mosadegh¹ | Soheila Habibi Ghahfarokhi¹ | Ali Ahmadi² | Mohammad Reza Pourmand¹ | Yousef Erfani³ | Rahil Mashhadi⁴

¹Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
²Applied Microbiology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
³Department of Medical Laboratory Sciences, School of Allied Medical Sciences, Tehran University of Medical Sciences, Tehran, Iran
⁴Urology Research Center, Tehran University of Medical Sciences, Tehran, Iran

Correspondence
Mohammad Reza Pourmand, Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Poursina St., Tehran, Iran. Email: m.pourmand@tums.ac.ir
Yousef Erfani, Department of Medical Laboratory Sciences, School of Allied Medical Sciences, Tehran University of Medical Sciences, Shafiei St., Tehran, Iran. Email: yerfani@tums.ac.ir

Funding information
This work was supported by the [Tehran University of Medical Sciences] under Grant [number 39370].

Abstract

**Background:** Given the significant role of penicillin-nonsusceptible *Streptococcus pneumoniae* in inducing severe infectious diseases, identifying serotypes and genotypes that can mediate antimicrobial resistance has become a pillar of treatment strategies. This study aims to determine the correlation between the minimum inhibitory concentration of antimicrobial agents and amino acid mutations in penicillin-binding proteins. Moreover, molecular serotyping and multiple-locus variable number tandem repeat analysis typing were first-ever performed to characterize the invasive penicillin-nonsusceptible *S. pneumoniae* isolates in Iran.

**Methods:** Of 149 isolates, antimicrobial susceptibility tests were performed against penicillin, ceftriaxone, and cefotaxime by the MIC Test Strip, and sequence analysis of the *pbp* genes was performed through PCR-sequencing method. All penicillin-nonsusceptible *S. pneumoniae* isolates were serotyped and genotyped by sequential multiplex PCR and multiple-locus variable-number tandem repeat analysis, respectively.

**Results:** Among pneumococcal isolates, 53 isolates were classified as penicillin-nonsusceptible *S. pneumoniae*, of which 28 (71.7%) and 15 (28.3%) were resistant and intermediate to penicillin, respectively. Furthermore, ceftriaxone- and cefotaxime-nonsusceptible pneumococci constituted 33 (62.2%) and 29 cases (54.7%), respectively. Of note, there were 8 and 41 different serotypes and multiple-locus variable-number tandem repeat analysis types, respectively.

**Conclusions:** Due to the increasing resistance to antimicrobial agents, the most efficient approach to preventing pneumococcal infection mortality as vaccine-preventable diseases is focusing on wide-spectrum vaccination. Based on our findings, the 13-valent pneumococcal conjugate vaccine could considerably reduce the incidence of invasive pneumococcal diseases due to the high rate of serotype coverage.
1 | INTRODUCTION

The emergence of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has promoted the significance of identifying causative agents of respiratory infections. Although Streptococcus pneumoniae (S. pneumoniae) initially inhabits the mucosal membranes of the upper respiratory tract, they serve as a reservoir of the lower respiratory tract infection and bacteremia. Moreover, S. pneumoniae, the fourth most frequent cause of lethal infections, such as septicemia and meningitis, is the cause of the death of 341,029 children below the age of five, 494,340 deaths in the elderly adults (>70 years), and 1,189,937 deaths among all ages in 2016. Due to the high mortality rate, the emergence of β-lactam-resistant S. pneumoniae has turned into a public health concern to a degree that the World Health Organization (WHO) has listed these resistant bacteria as one of the priorities in managing infectious diseases in 2017. The cases of multi-drug resistant (MDR) S. pneumoniae are associated with high morbidity and mortality rates among children in Asia. To compensate for the toxic effects of antibiotics and attenuate the activity of β-lactams, S. pneumoniae induces genetic alterations in a protein called penicillin-binding protein (PBP), especially in pbp1a, pbp2b, and pbp2x. The recurrent alteration of DNA bases in the flanking positions of three conserved motifs, including SXXK, SXN, and K(T/S)G, located on the active site of PBPs, is clearly associated with low affinity with β-lactams. The global progressive increase in the proportion of penicillin-resistant isolates in many countries, for example, Iran, justifies the importance of studying β-lactam antibiotics to treat pneumococcal diseases. According to the findings of several studies, although the serotype of S. pneumoniae varied based on the study period, geographical region, and population age, it appears that only some serotypes can cause IPD around the world. Although numerous epidemiologic and molecular studies have studied the characterization of MDR S. pneumoniae isolates, still little is known about the precise mechanism. Given the high frequency of penicillin-resistant isolates in many countries, particularly Iran, it is reasonable to put the mechanism exhibiting β-lactam resistance in S. pneumoniae under scrutiny.

Following the application of pneumococcal conjugate vaccines (PCVs) and the replacement phenomenon, non-vaccine serotypes (NVT) were determined as the main cause of the remaining pneumococcal infections. Therefore, it is important to investigate the serotype distribution in IPD in the pre-vaccine era to select proper vaccine and evaluate its potential effectiveness. Multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) are effective methods for high-precision epidemiological typing; however, they are subject to some limitations, including time consumption and high expense. Multiple-locus variable-number tandem repeat analysis (MLVA) typing is characterized by various features, making it acceptable as the typing method. MLVA enjoys both high discriminatory power and simplicity of both techniques for epidemiological investigations. This method functions based on a variability observed in some tandemly repeated DNA sequences demonstrating genetic polymorphism in bacterial isolates. To date, few studies, especially in Asia, have addressed the genetic diversity of S. pneumoniae by the MLVA method. In the present study, the antimicrobial susceptibility of Penicillin-Non-susceptible Streptococcus pneumoniae (PNSP) isolates was investigated and the mutations in genes encoding PBPs were identified. Furthermore, invasive PNSP isolates were characterized by molecular serotyping and MLVA typing assays.

2 | MATERIALS AND METHODS

2.1 | Clinical isolate collection and growth conditions

A total of 149 nonduplicated invasive isolates were obtained from the patients admitted to the teaching hospitals affiliated to the Tehran University of Medical Sciences (TUMS) between 2017 and 2019 (74 isolates from 2017 to 2018 and 75 isolates from 2018 to 2019). All cases belonged to sporadic episodes and none of the patients had received any pneumococcal vaccines. The clinical samples were cultured on blood agar containing 5% sheep blood and incubated for 24 hours at 37°C in the presence of 5% CO₂. All isolates were confirmed as S. pneumoniae by conventional methods and PCR amplification of the species-specific lytA gene. The study’s inclusion criteria involved isolates recovered from patients with invasive pneumococcal diseases. All of them were included for AST analysis. Finally, PNSP isolates were characterized for epidemiological features. Fifty-three isolates for which penicillin MICs were ≥0.125 μg/mL were classified as PNSP. Ninety-six invasive penicillin-susceptible S. pneumoniae (PSSP) isolates (penicillin MICs, <0.125 μg/mL) were excluded for further analysis. The isolates recovered from patients aged 1 month to 88 years, with a mean age of 21 years.

2.2 | Susceptibility testing

All 149 pneumococcal isolates were screened for penicillin resistance with 1 μg oxacillin disk using Kirby–Bauer disk diffusion. The minimum inhibitory concentrations (MIC) of penicillin, ceftriaxone, and cefotaxime were determined for oxacillin-resistant isolates using MIC Test Strip (MTS; Liofilchem.). Interpretation was performed

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**KEYWORDS**

beta-lactam resistance, epidemiological monitoring, genotyping techniques, penicillin-binding proteins, pneumococcal infections, pneumococcal vaccines, drug resistance, respiratory tract infections, serotyping, vaccine-preventable diseases
using the recommended breakpoints in line with the guidelines set by Clinical Laboratory Standards Institute (CLSI-2015). The studied population was divided into meningeal and nonmeningeal isolates, which have different breakpoints to interpret antimicrobial susceptibility testing (AST) results. The oral penicillin breakpoint (MICs≥0.12 μg/mL) was considered to classify isolates as nonsusceptible to penicillin. S. pneumoniae ATCC 49619 was used as the control in this experiment.

2.3 Amplification of pbp1a, pbp2b, and pbp2x genes and DNA sequencing

To investigate the structural alterations in pbp genes, PCR amplification of pbp1a, pbp2b, and pbp2x genes was assessed by sequencing based on the study conducted by Harimaya et al. with some modifications in three primer sets (Table 1). First, the genomic DNA of isolates was extracted using the high pure PCR template preparation kit (Roche). Each PCR assay contained 2 μl of the DNA template, 12.5 μl 2x HotstarTaq master mix (3 mM MgCl2, 0.4 mM dNTP, and 0.2 U/μl Taq DNA polymerase), 1 μl of 10 pmol/μl of each primer, and sterile distilled water to obtain a final volume of 25 μl with the following PCR program: initial denaturation at 94°C for 5 min, 35 cycles of 30 s at 94°C, 30 s at 57°C, and 60 s at 72°C for denaturation, annealing, and extension, respectively, and the final extension at 72°C for 10 min. After purification, bidirectional sequencing of PCR products was performed by Macrogen Inc. Ultimately, the results were aligned using BioEdit 7.2.5 and compared to DNA and amino acid sequences of S. pneumoniae R6 strain as the control.

2.4 Molecular serotyping

Serotypes of PNSP strains were determined through sequential multiplex PCR according to the instruction provided by Coskun-Ari et al. The present study focused on identifying the PCV13-covered serotypes. The primers in this study were previously described based on the recommendations of the Center for Disease Control and Prevention (CDC). The PCR conditions were as follows: initial denaturation at 94°C for 5 min, 30 cycles of 45 s at 94°C, 30 s at 58°C, 60 s at 72°C for denaturation, annealing, and extension, respectively, and final extension at 72°C for 10 min. Serotypes not belonging to the PCV13-covered serotypes were classified as NVT.

### Table 1 The primer sets for the amplification of pbp genes

| Gene | Primer Sequences (5′ → 3′) | Product Size (bps) | Ref. |
|------|--------------------------|-------------------|------|
| pbp1a | Forward: TGG GAT GGA TGT TTA CAC AAA TG<br>Reverse: GTC GTA CTA TTA TTT GTG CTT GG | 1197 | 21 |
| pbp2b | Forward: GGC TAT TCT CTA AAT GAC CGT<br>Reverse: AGC TTA GCA ATA GGT GTT GG | 1317 |
| pbp2x | Forward 1: TAT GAA AAG GAT CGT CTG GG<br>Forward 2: TAT GAA AAG GAC CGT GTA GC<br>Reverse: AGA GAG TCT TTC ATA GCT GAA GC | 1148 |
Thr$^{371}$ → Ala substitution in the STMK motif of PBP1a. 79.2% had Thr$^{328}$ → Ala substitution in the STMK motif of PBP2x (Figure 1). No amino acid substitutions were recognized in the three conserved motifs of PBP2b. In contrast, the substitution of Thr$^{445}$ → Ala was observed in all PNSP isolates in the amino acid position adjacent to the second conserved motif serine-serine-asparagine (SSN) of PBP2b. Only six PNSP isolates (Sp27, Sp33, Sp36, Sp43, Sp46, and Sp52) with very high levels of penicillin resistance (MIC ≥8 mg/L) exhibited the substitution of Ala$^{546}$ → Gly adjacent to the KTG motif of PBP2b (Figure 1). Approximately 26% of the PNSP isolates exhibited the substitution Leu$^{546}$ → Val adjacent to the KSG region of PBP2x, which showed 4 μg/mL MICs.

### 3.3 Serotype distribution in PNSP isolates

Among PNSP isolates, serotype 14 was the most frequent one (13/53, 24.5%), followed by serotype 19F (10/53, 18.9%) and serotype 3 (8/53, 15.1%). Only one isolate of serotype 19A was found in the studied collection. In addition, seven isolates (13.2%) were classified as NVT. Table 2 shows the frequency of serotypes. Serotypes 14 (10/28, 35.7%), 3 (7/28, 25%), and 23F (4/28, 14.2%) were the most common serotypes in children ≤5 years. In elderly patients (≥64 years), serotypes 19F (4/10, 40%) and 14 (3/10, 30%) exhibited the highest frequency. In other age groups, 19F (4/15, 26.6%), 9 V (4/15, 26.6%), and 23F (3/15, 20%) exhibited the highest portions. However, no significant difference was found between serotype and age. Between the isolates with penicillin MIC ≥8 μg/mL, eight serotypes were detected, with serotype 14 being the most predominant one (10/38, 26.3%). In the PNSP population, the predominant serotype in CSF (n = 6, 11.3%) and blood (n = 7, 13.2%) specimens was 14 (Figure 2). The serotype coverage rate of PCV-13 among PNSP isolates was 86.7%. There was, statistically, no significant relationship between the serotype and the source of infection.

### 3.4 Cephalosporin susceptibility of PNSP isolates

According to meningeal and nonmeningeal criteria, the rates of nonsusceptibility to ceftriaxone and cefotaxime in invasive pneumococcal
isolates were 62.2% and 54.7%, respectively (Table 2). Among PNSP isolates, serotypes 14 and 19F were the most prevalent ones with nonsusceptibility to ceftriaxone and cefotaxime, respectively (Table 2). Overall, the CSF isolates had higher resistance rates than blood isolates. A significant correlation between meningitis infection and penicillin resistance was seen ($p = 0.02$). The proportion of coresistance to ceftriaxone and cefotaxime was 24.5% ($n = 13$) in PNSP isolates, of which five cases (9.5%) and eight cases (15.0%) belonged to meningitis and nonmeningitis isolates, respectively. Of a total of 53 PNSP isolates, 15 isolates (28.3%) were susceptible to both ceftriaxone and cefotaxime.

### 3.5 | MLVA types of PNSP isolates

After comparing the MLVA profile with the MLVA databank, 41 different MTs were identified in 53 PNSP isolates, among which MT282 (13.2%) and MT279 (7.5%) were the most frequent genotypes (Figures 1 and 3). All isolates belonging to MT282 were associated with serotype 19F and resistant to penicillin ($p = 0.001$). The genetic relationship and antibiotic susceptibility status observed by MLVA among 53 PNSP isolates are presented in Figure 3. Calculation of Simpson’s Index of Diversity (SID) for all eight BOX loci indicated that BOX-03 was highly polymorphic and discriminatory for PNSP isolates (DI = 0.827), while BOX-11 had the least diversity index (DI = 0.449) (Table 3). The UPGMA dendrogram based on MLVA data using the Dice coefficient of similarity was drawn by BioNumerics software version 7.6.3 (Applied Maths) (Figure 1).

### 4 | DISCUSSION

With the emergence of SARS-CoV-2, accurate identification and characterization of other etiological agents of lower respiratory tract infections has become significantly vital to public health. The expanding prevalence of nonsusceptibility to β-lactam drugs has posed serious threats to public health worldwide. The results of Asian Network for Surveillance of Resistant Pathogens (ANSORP) studies in 11 Asian countries in 2012 (2184 isolates) revealed that the prevalence of PNSP isolates was 62.1%, of which 58.2% of the isolates were resistant to penicillin. The present study results revealed that the PNSP rate in IPD was 35.5%, of which 25.5% and 10% were PRSP (penicillin-resistant *S. pneumoniae*) and PISP (penicillin-intermediate *S. pneumoniae*), respectively. The findings of surveillance studies performed in Iran between the years 2014 and 2019 indicated that 20.8% and 58% of the studied isolates were PNSP. Therefore, the prevalence of nonsusceptibility to penicillin emphasizes the necessity of continued monitoring of the pneumococcal population in Iran. The highest genetic diversity of pbp genes was found in the genetic profile of pbp2x, followed by pbp2b and pbp1a genes. The results showed 16 and 42 amino acid substitutions in the STMK conserved motif of *PBPA* (Thr$^{371} \rightarrow$ Ala) and PBPA (Thr$^{338} \rightarrow$ Ala), respectively. Moreover, we found no substitution in the three conserved motifs of *PBP2b*, being in agreement with the results of Talebi et al., and in contrast with the results reported by Ling Goh et al. Ther efore, the prevalence of nonsusceptibility to penicillin emphasizes the necessity of continued monitoring of the pneumococcal population in Iran. The highest genetic diversity of *pbp* genes was found in the genetic profile of *pbp2x*, followed by *pbp2b* and *pbp1a* genes. The results showed 16 and 42 amino acid substitutions in the STMK conserved motif of *PBPA* (Thr$^{371} \rightarrow$ Ala) and PBPA (Thr$^{338} \rightarrow$ Ala), respectively. Moreover, we found no substitution in the three conserved motifs of *PBP2b*, being in agreement with the results of Talebi et al., and in contrast with the results reported by Ling Goh et al. In addition, the findings indicate that 20.7% of the PNSP isolates reveal no alterations in three conserved motifs of PBPs. All PRSP isolates and three PNSP isolates (Sp26, Sp38, and Sp41) harbored Pro$^{432}$ to Thr substitutions next to the *PBPA* conserved motifs SRN, which is consistent with previous findings. One of the significant findings of the

### Table 2: Association between serotypes and antimicrobial nonsusceptibility rates of PNSP isolates with respect to the CLSI meningeal and nonmeningeal breakpoints

| Serotype | Penicillin | Ceftriaxone | Cefotaxime |
|----------|------------|-------------|------------|
|          | R | I | Total | R | I | Total | R | I | Total |
| 14       | 10 | 3 | 13 | 2 | 7 | 9 | 3 | 3 | 6 |
| 19F      | 7 | 3 | 10 | 3 | 5 | 8 | 4 | 3 | 7 |
| 3        | 5 | 3 | 8 | 3 | 0 | 3 | 3 | 1 | 4 |
| 23F      | 6 | 1 | 7 | 2 | 3 | 5 | 2 | 2 | 4 |
| 9 V      | 3 | 1 | 4 | 2 | 1 | 3 | 2 | 2 | 4 |
| 6A/6B    | 2 | 1 | 3 | 0 | 1 | 1 | 0 | 0 | 0 |
| 19A      | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 |
| NVT§     | 4 | 3 | 7 | 1 | 2 | 3 | 1 | 2 | 3 |

*Resistant.

†Intermediate.

§Nonvaccine serotypes.

FIGURE 2 The frequency of serotypes in CSF and blood sources
The current study is the higher discriminatory power of MLVA than MLST. Although we have found strains with a high level of penicillin resistance (MIC = 8 μg/mL) belonging to 12 different MLVA types, in another study, the eight strains with MIC > 8 μg/mL were highly related and they belonged to only two STs (321 and 2615).

Serotype-based pneumococcal vaccines have significant restrictions on serotype coverage, including nonvaccine serotype replacement or the emergence of nontypeable pneumococcal strains, known as the serotype replacement phenomenon and induce invasive pneumococcal disease. Based on the findings, serotype 14 was the most prevalent, followed by 19F, 3, and 23F. A similar distribution of some serotypes has been reported in Europe, indicating that the major serotypes causing IPD were 14, 6B, 19F, and 23F in the pre-pneumococcal vaccine era. In Iran, the findings of Talebi et al. suggested that the major PNSP serotypes found in 2019 were 14, 23F, and 19F. It should be noted that our results differed from previous studies, which indicated a significant increase in the proportion of serotype 19A in the pre-PCV7 era. In many parts of the world, the distribution of serotype 19A has been promoted in the area without PCV7 and PCV10 implementation, while the present study results demonstrated that the prevalence of this serotype in Iran was still low. In the current study, the vaccine coverage rate of PCV13 was 86.7%, while some recent surveillance studies conducted in Iran, China, and Japan pointed out that the PCV13 coverage rates were 95%, 85.8%, and 80.4%, respectively. Hence, based on the high serotype coverage rate of PCV13, it appears that this vaccine is the most preferred one for the Iranian vaccination program.

MLVA analysis revealed that the predominant MT was 282, which belonged to serotype 19F. All isolates belonging to the MT282 were resistant to penicillin, among which two and three isolates were ceftriaxone- and cefotaxime-resistant, respectively. In the present study, serotype 14, the most prevalent serotype, was further divided into nine MTs. The results reported by Ohkusu et al. demonstrated that MLVA could divide serotype 12F into five different MTs.

The MLVA protocol in this study proposes a collection of VNTR markers with different diversity indices (0.449–0.827). VNTR markers with a moderate DI (<0.5) might reflect the slow rate of genetic evolution. Conversely, VNTRs with higher DI designate further diversity and greater rapid evolution. The findings showed the highest diversity indices in several VNTR markers, including BOX-02, BOX-03, and BOX-11. To the best of our knowledge, this is the first report of the genotype tracking data conducted by the MLVA method in Asia, which could be used as the baseline data for further studies on pneumococcal population genomics in IPD.

**TABLE 3** Calculation of Simpson's Index of Diversity and determination of nonamplified loci for MLVA markers

| Loci    | Simpson's Index of Diversity (SID) | Nonamplified loci (%) |
|---------|-----------------------------------|-----------------------|
| BOX-01  | 0.636                             | 0%                    |
| BOX-02  | 0.728                             | 4%                    |
| BOX-03  | 0.827                             | 0%                    |
| BOX-04  | 0.816                             | 4%                    |
| BOX-06  | 0.576                             | 2%                    |
| BOX-11  | 0.449                             | 4%                    |
| BOX-12  | 0.669                             | 9%                    |
| BOX-13  | 0.809                             | 30%                   |

**FIGURE 3** The minimum spanning tree of the PNSP isolates for MLVA typing was constructed with a categorical coefficient. Each circle represents a different MLVA type. The color of a circle indicates the antibiotic susceptibility status of the tested isolates. The size of the circle reflects the number of isolates while the distance between circles reflects the degree of genetic divergence.

Abbreviations: CR-RSP: ceftriaxone-resistant *S. pneumoniae;* CR-SSP: ceftriaxone-susceptible *S. pneumoniae;* CR-ISP: ceftriaxone-intermediate *S. pneumoniae;* CA-RSP: cefotaxime-resistant *S. pneumoniae;* CA-SSP: cefotaxime-susceptible *S. pneumoniae;* CA-ISP: cefotaxime-intermediate *S. pneumoniae*
5 | CONCLUSION

Based on the high distribution of β-lactam-resistant pneumococci, PCV-13 could play a vital role in reducing the incidence of IPD. In addition, the heterogeneous distribution of resistant isolates suggests the need for constant monitoring of the genetic diversity. The MLVA results proved its high efficiency in genetic characterization of the invasive pneumococcal population, especially in local and short-term studies. Furthermore, it is an appropriate method for detecting the emergence and outspread of a virulent clonal variant in local outbreaks.

AUTHOR CONTRIBUTIONS
Mosadegh M did the material preparation, data collection, analysis, and wrote the manuscript; Pourmand MR, Ghahfarokhi SH, and Ahmadi A were involved in analyzing and interpretation of results; Erfani Y and Mashhadi R edited the first draft and prepared the graphs; Pourmand MR and Erfani Y did the study design, supervised, and approved the final version of the manuscript.

ACKNOWLEDGMENT
This research was supported by the School of Public Health, Tehran University of Medical Sciences. Authors expressed their gratitude to the hospital’s staff.

CONFLICT OF INTEREST
Authors claimed no conflict of interest.

DATA AVAILABILITY STATEMENT
All the data and materials are described in the appropriate sections and provide citations to the external databases/publications. The data that support the findings of this study are available upon request from the corresponding author [Prof. Mohammad Reza Pourmand]. The data are not publicly available due to restrictions, for example, their containing information that could compromise the privacy of research participants.

PATIENT CONSENT STATEMENT
The requirement for written informed consent was waived because de-identified data were used.

ORCID
Mohammad Reza Pourmand https://orcid.org/0000-0003-1280-5765

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How to cite this article: Mosadegh M, Habibi Ghahfarokhi S, Ahmadi A, Pourmand MR, Erfani Y, Mashhadi R. Identification and molecular characterisation of penicillin-nonsusceptible Streptococcus pneumoniae isolates recovered from invasive infections in a pre-pneumococcal vaccine era. J Clin Lab Anal. 2022;36:e24566. doi: 10.1002/jcla.24566