Comparative genomic analysis of multidrug-resistant Streptococcus pneumoniae isolates

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Introduction: Multidrug resistance in Streptococcus pneumoniae has emerged as a serious problem to public health. A further understanding of the genetic diversity in antibiotic-resistant S. pneumoniae isolates is needed.

Methods: We conducted whole-genome resequencing for 25 pneumococcal strains isolated from children with different antimicrobial resistance profiles. Comparative analysis focus on detection of single-nucleotide polymorphisms (SNPs) and insertions and deletions (indels) was conducted. Moreover, phylogenetic analysis was applied to investigate the genetic relationship among these strains.

Results: The genome size of the isolates was ~2.1 Mbp, covering >90% of the total estimated size of the reference genome. The overall G+C% content was ~39.5%, and there were 2,200–2,400 open reading frames. All isolates with different drug resistance profiles harbored many indels (range 131–171) and SNPs (range 16,103–28,128). Genetic diversity analysis showed that the variation of different genes were associated with specific antibiotic resistance. Known antibiotic resistance genes (pbps, murMN, ciaH, rplD, sulA, and dpr) were identified, and new genes (regR, argH, trkH, and PTS-EII) closely related with antibiotic resistance were found, although these genes were primarily annotated with functions in virulence as well as carbohydrate and amino acid transport and metabolism. Phylogenetic analysis unambiguously indicated that isolates with different antibiotic resistance profiles harbored similar genetic backgrounds. One isolate, 14-LC.ER102S, showed a much weaker phylogenetic relationship with the other isolates, possibly caused by genomic variation.

Conclusion: In this study, although pneumococcal isolates had similar genetic backgrounds, strains were diverse at the genomic level. These strains exhibited distinct variations in their indel and SNP compositions associated with drug resistance.

Keywords: Streptococcus pneumoniae, antimicrobial resistance, whole-genome sequencing, insertions/deletions, SNPs, phylogenetic analysis

Introduction

Streptococcus pneumoniae causes a series of severe infective disorders ranging from noninvasive infections (otitis media and pneumonia) to invasive infections (meningitis and sepsis), which could become life-threatening. Worldwide, S. pneumoniae is responsible for ~582,000–926,000 deaths annually in children aged 1–59 months. Antimicrobial agents are recommended as the first choice for treating these infections; however, the appearance of drug-resistant S. pneumoniae, especially multidrug-resistant (MDR) strains, decreases the effectiveness of antibiotics, and a number of such strains have been reported clinically. A study reported by the Asian
Network for Surveillance of Resistant Pathogens (ANSORP) illustrated that the overall rate of MDR in pneumococcal isolates was 59.3% (59.4% and 57.5% in nonmeningeal and meningeal isolates, respectively), with the highest MDR rate being 83.3% in China. Several factors contribute to this phenomenon. Some studies have shown that drug-resistant isolates can be created via the transmission of already resistant strains (primary resistance) or by selection of resistance-conferring mutations via inadequate therapy (secondary resistance). Especially, horizontal transmission through the transfer of genetic material between bacteria has the greatest effect on drug resistance transmission. As a transformable strain, the mechanisms of MDR S. pneumoniae involve different genes associated with different antibiotics. Pneumococcal resistance to β-lactams is mainly caused by mutations in penicillin-binding proteins (PBPs; pbp1a, pbp2a, and pbp2b) which have a decreased affinity for the antibiotic as well as other non-PBP mechanisms, including mutations in ciaH and cpoA as well as encoding histidine protein kinase and putative glycosyltransferase, respectively. Simultaneously, pneumococcal strains with resistance to macrolides are detected to be associated with two major mechanisms: target modification by ribosomal methylation encoded by the ermA gene and drug efflux encoded by the mefA gene. However, owing to the lack of comprehensive understanding, complicated resistance mechanisms of S. pneumoniae require investigation.

Over the past few decades, the methods in place to investigate the genetic characteristics of drug-resistant S. pneumoniae are restricted to PCR-related molecular approaches such as nest PCR, real time-PCR, and multilocus sequence typing (MLST). Although these methods have significantly enhanced our knowledge of S. pneumoniae, their discriminatory power for identifying potential drug resistance mechanisms is limited. However, in recent years, new genome-scale technologies have started to make an important contribution to our comprehension of the biology and molecular epidemiology of S. pneumoniae as an important bacterial pathogen.

High-throughput whole-genome sequencing (WGS) – a powerful tool based on next-generation sequencing – offers new opportunities for studying pathogenic bacteria and is now used widely to investigate pathogenesis and drug resistance mechanisms in a wide range of pathogens. Supplementary to WGS, is whole-genome resequencing (WGRS) – a technique whereby a genome is sequenced and compared with the genome of a previously sequenced reference strain to identify sequence polymorphisms between the two sequences, such as single-nucleotide polymorphisms (SNPs), gene acquisition, recombination, loss as well as insertions and deletions (indels), that are potentially important in epidemiological analyses, drug resistance detection, and phylogenetic investigations. Although there are several pneumococcal genomes in the public domain, WGRS is rarely used for investigation of drug resistance of S. pneumoniae isolated in China. In this study, we conducted WGRS to analyze 25 S. pneumoniae pneumococcal isolates and determine the genetic diversity including SNPs and indels among them.

**Methods**

**Microbiology methods**

Twenty-five pneumococcal strains were part of the routine hospital laboratory procedure and were selected according to their similar resistance patterns. They were isolated from children <14 years of age who were diagnosed with pneumonia and had received antibiotic therapy before the samples collected at the Shanghai Children’s Hospital, Shanghai, People’s Republic of China. Written informed consent was obtained from the patients’ guardians on behalf of the children enrolled in this study. All strains were identified on the basis of typical characteristics (ie, colony morphology, alpha hemolysis, and gram-positive diplococcus), after which the optochin sensitivity (Oxoid, Hampshire, UK) and bile solubility tests were used for final confirmation. The strains were stored at −80°C in 40% sterilized glycerin–bouillon medium until required.

The minimal inhibitory concentrations of penicillin (PEN), cefuroxime (CXM), ceftriaxone (CRO), erythromycin, azithromycin, levofloxacin (LEV), moxifloxacin (MXF), vancomycin (VAN), and sulfamethoxazole/trimethoprim (SXT) were determined by the broth microdilution method. The breakpoints used for interpretation were those recommended by the Clinical and Laboratory Standards Institute (CLSI) in 2016. In addition, all isolates were investigated by MLST analysis as described previously.

**DNA extraction and WGRS**

Chromosomal DNA was isolated from overnight cultures of the isolates previously grown on agar plates supplemented with 5% sheep blood using a TIANamp bacteria DNA kit (Tiangen, Beijing, People’s Republic of China) according to the manufacturer’s instructions. The extracted DNA was dissolved in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0), and
its concentration and purity were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The total DNA quantity in each sample was ≥10 μg.

Next, the genomic DNA was fragmented in a Covaris instrument (Woburn, MA, USA) to an average size of 250–300 nucleotides. Library preparation was done using TruSeq Nano DNA Sample Preparation Kit (Illumina, San Diego, CA, USA), and the libraries were then sequenced using an Illumina HiSeq 2000 instrument (125-bp paired-end reads), and the individual samples were barcoded using Illumina’s Multiplex Sample Preparation Oligonucleotide Kit. Sequence-read adapters were removed using AdapterRemoval (version 2.1.7). Low-quality bases were trimmed using a 5-bp sliding window, cutting once the average PHRED quality scores to below 20. After trimming, if either read from a pair of reads were <50 bp in length, then both reads were discarded. The sequence data files have been deposited in the National Center for Biotechnology Information’s Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra/).

**Variant analysis**

High-quality sequence reads were mapped to the *S. pneumoniae* R6 strain reference genome (GenBank accession no. ASM704v1) using BWA (version 0.7.12-r1039). The alignments were improved using the Picard package (http://sourceforge.net/projects/picard/) with the following two commands: the “FixMateInformation” command was used to ensure that all mate-pair information was in sync between each read and its mate pair, and the “MarkDuplicates” command was used to mark potential PCR duplicates. Where multiple read pairs had identical external coordinates, only the pair with the best mapping quality was retained; the others were marked as PCR duplicates. We then undertook a local realignment of the mapped reads around indels using the GATK package in two steps: the “RealignerTargetCreator” command was used to determine suspicious intervals that probably need realigning, and the “IndelRealigner” command was used for realignment of such intervals. After alignment, we carried out variant calling using the Bayesian approach as implemented in the GATK package (https://software.broadinstitute.org/gatk/). The variants were further filtered according to the following criteria: RMS mapping quality of ≥25, site quality score of ≥30, variant confidence/quality by depth of ≥2, ≥16 reads covering each site with eight reads mapping to each strand, and the reads covering a major variant were at least five times greater than that of the minor variant. Sites that failed these criteria in any strain were removed from the analysis.

**Genome assembly and comparative analysis**

The high-quality reads were de novo assembled with Newbler 2.9 (version 20130529_1641) (http://www.454.com/products/analysis-software). Gaps inside the scaffold were closed with GapCloser (version 1.12) (http://soap.genomics.org.cn/soapdenovo.html). All newly sequenced genomes were ordered on the basis of the reference genome with Mauve (version 2.3.1). For each genome, regions that were absent in the reference genome were extracted with custom Perl script, and were then searched against the SwissProt database using blastx (version 2.2.26). Possible genes associated with drug resistance were identified by the following criteria: identity ≥45%, e-value <1e-6, and coverage ≥70%.

**Phylogenetic analysis**

A SNP supermatrix for each strain with reference alleles was constructed using custom Perl scripts. A neighbor-joining phylogeny was generated using the Kimura 2-parameter model of nucleotide substitution in MEGA 6.02. For each method, 1,000 bootstrap replicates provided support for nodes on the tree. The phylogenies derived from each method were congruent. We conducted principal component analysis on the SNPs using EIGENSOFT 5.02, and the eigenvectors were obtained from the covariance matrix using the R function.

**Results**

**Epidemiological data and bioinformatics analysis of the pneumococcal isolates**

The detailed epidemiological data from the selected *S. pneumoniae* clinical isolates are summarized in Table 1. Antibacterial susceptibility tests revealed that 21 strains were resistant to one or more antibiotics, whereas four isolates were susceptible to all the antibiotics we tested. None were resistant to LEV, MXF, and VAN. Most pneumococci (72%, 18/25) were defined as MDR *S. pneumoniae*. Additionally, MLST analysis identified 19 sequence types (STs), and different serotypes had different STs.

The WGS statistics for the 25 isolates are shown in Table S1. The sequencing read assembly revealed that the pneumococcal isolate sequences were ~2.1 Mbp in size, covering >90% of the total estimated size of the genome. The overall G+C% content was ~39.5% and the genomes contained 2,200–2,400 open reading frames. The scaffold range among the isolates was 46–86. The largest scaffold of each isolates...
Table 1 Epidemiological data from *S. pneumoniae* strains isolated from children

| Sample ID | Age (y) | Gender | Year of isolation | MIC value of isolates (μg/mL) | Drug resistance profiles | Serotype | Sequence types | Sequence depth |
|-----------|---------|--------|-------------------|-----------------------------|-------------------------|----------|---------------|---------------|
| 14LC.E1023 | 10m | Male | 2012 | 8/8/4 ≥16 ≥16 8/152 | PEN-CXM-CRO-MAC-SXT | 19F | 271 | 127.89 |
| 14LC.E1024 | 3m | Female | 2012 | 8/8/8 ≥16 ≥16 8/152 | PEN-CXM-CRO-MAC-SXT | 19F | 1,464 | 119.24 |
| 14LC.E1025 | 2y | Male | 2012 | 2/8/4 ≥16 ≥16 8/152 | CXM-CRO-MAC-SXT | 6A | 2,971 | 103.09 |
| 14LC.E1026 | 2y | Male | 2012 | 2/8/4 ≥16 ≥16 8/152 | CXM-CRO-MAC-SXT | 19F | 271 | 117.43 |
| 14LC.E1027 | 3y | Female | 2012 | 2/8/4 ≥16 ≥16 8/152 | CXM-CRO-MAC-SXT | 6B | 744 | 118.7 |
| 14LC.E1028 | 6y | Male | 2012 | 2/16/4 ≥16 ≥16 8/152 | CXM-CRO-MAC-SXT | 19F | 271 | 125.03 |
| 14LC.E1029 | 1y | Male | 2012 | 2/8/4 ≥16 ≥16 0.25/4.75 | CXM-CRO-MAC | 14 | 876 | 128.97 |
| 14LC.E1030 | 1y | Male | 2012 | 2/8/4 ≥16 ≥16 0.25/4.75 | CXM-CRO-MAC | 19A | 3,111 | 114.82 |
| 14LC.E1031 | 5m | Male | 2012 | 0.25/16/4 ≥16 ≥16 0.125/0.25 | CXM-MAC-MAC | 19A | 876 | 130.6 |
| 14LC.E1032 | 10m | Male | 2012 | 1/4/1 ≥16 ≥16 8/152 | CXM-MAC-MAC | 19F | 271 | 119.63 |
| 14LC.E1033 | 5m | Female | 2012 | 1/4/1 ≥16 ≥16 8/152 | CXM-MAC-MAC | 6A | 9,789 | 116.8 |
| 14LC.E1034 | 6m | Male | 2013 | 1/4/1 ≥16 ≥16 8/152 | CXM-MAC-MAC | 19A | 320 | 113.15 |
| 14LC.E1035 | 1y | Female | 2012 | 1/8/0.5 ≥16 ≥16 0.25/4.75 | CXM-MAC | 19F | 236 | 115.43 |
| 14LC.E1036 | 3y | Male | 2012 | 1/8/0.5 ≥16 ≥16 0.25/4.75 | CXM-MAC | 14 | 876 | 123.41 |
| 14LC.E1037 | 2y | Male | 2013 | 1/4/0.5 ≥16 ≥16 0.25/4.75 | CXM-MAC | 14 | 876 | 111.1 |
| 14LC.E1038 | 4y | Male | 2012 | 0.5/1/0.125 ≥16 ≥16 8/152 | MAC-SXT | 19F | 2,754 | 78.74 |
| 14LC.E1039 | 6y | Male | 2013 | 1/1/1 ≥16 ≥16 4/76 | MAC-SXT | 19F | 8,250 | 120.77 |
| 14LC.E1040 | 2y | Male | 2012 | 1/1/1 ≥16 ≥16 4/76 | MAC-SXT | 23F | 6,325 | 119.97 |
| 14LC.E1041 | 7m | Male | 2012 | 0.125/0.125/0.125 ≥16 ≥16 0.25/4.75 | MAC | 19F | 7,751 | 130.99 |
| 14LC.E1042 | 4y | Male | 2012 | 0.125/0.125/0.125 ≥16 ≥16 0.25/4.75 | MAC | 6A | 855 | 116.59 |
| 14LC.E1043 | 1y | Male | 2013 | 0.125/0.125/0.125 ≥16 ≥16 0.25/4.75 | MAC | 7F | 3,545 | 127.01 |
| 14LC.E1044 | 4y | Male | 2012 | 0.125/0.125/0.125 ≥16 ≥16 0.25/4.75 | MAC | 19A | 416 | 121.17 |
| 14LC.E1045 | 1m | Male | 2012 | 0.5/0.5/0.5 0.125 0.125 0.25/4.75 | MAC | 19F | 4,662 | 109.32 |
| 14LC.E1046 | 4y | Female | 2012 | 0.5/0.5/0.5 0.125 0.125 0.25/4.75 | MAC | 6B | 3,173 | 106.37 |
| 14LC.E1047 | 19m | Female | 2013 | 0.125/0.125/0.125 ≥16 ≥16 0.125/2.38 | None | 6A | 180 | 136.59 |

Abbreviations: *PEN*, penicillin; *CXM*, cefuroxime; *CRO*, ceftriaxone; *MAC*, macrolides (erythromycin and azithromycin) and clindamycin; *SXT*, sulfamethoxazole–trimethoprim; *AZM*, azithromycin; *ERY*, erythromycin; *MIC*, minimal inhibitory concentrations; *m*, months; *y*, years.
WGS of MDR S. pneumoniae was 91,339–310,534 bp, and the N50 scaffold length was 41,305–82,460 bp. Furthermore, we observed that similar numbers of indels (range 131–171) were present in isolates exhibiting different levels and profiles of drug resistance. The number of SNPs representing nonsynonymous/synonymous mutations in coding regions and nonsynonymous/synonymous mutations in noncoding regions ranged from 16,103 to 28,128 among these isolates. The WGRS for 25 clinical isolates (14LC.ER1023–14LC.ER1047) have been deposited in GenBank under accession numbers: MCGY00000000, NAOM00000000, NAOL00000000, NAOK00000000, NAOJ00000000, NAOI00000000, NAOH00000000, NAOG00000000, NAOF00000000, NAOE00000000, NAOD00000000, NAOC00000000, NAOB00000000, NAOA00000000, NANZ00000000, NANY00000000, NANX00000000, NanW00000000, NanV00000000, NanU00000000, NanT00000000, NANS00000000, NANR00000000, NANQ00000000, and NAP00000000, respectively.

### Correlation analysis of pneumococcal isolates at the genome level

Sequence alignments of the pneumococcal isolates against *S. pneumoniae* R6 revealed that the mapping rates were 70.71–87.93% and the read depth range was 703–1,930. To further assess the relationships of the isolates, we conducted a phylogenetic analysis based on the SNPs identified in their whole-genome alignments. The phylogenetic tree revealed unambiguously that isolates with different antibiotic resistance profiles harbored similar genetic backgrounds (Figures 1 and 2); furthermore, the STs of the isolates (Table 1) were consistent with this finding. However, one isolate (14-LC.ER1025) showed a dissimilar genetic relationship to the other isolates, possibly as a result of genomic variation.

Compared with the *S. pneumoniae* R6 reference strain, the strain-specific regions in the isolates varied from 146,809 to 303,600 bp in size, which is proportionately 7.3–13.8% of the reference strain. Next, we conducted a functional analysis of these specific regions using the SwissProt database and

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**Figure 1** Phylogenetic relationships of *S. pneumoniae* isolates based on single-nucleotide polymorphisms from whole DNA sequences.

**Notes:** A tree representing the isolates. The branch lengths show the evolutionary distances among the isolates. The bootstrap values of the nodes represent the reliability of a branch being formed by all isolates in this branch, and values >70% are considered to be reliable. Resistant profiles: 14LC.ER1023–25, PEN-CXM-CRO-MAC–SXT; 14LC.ER1026-28, CXM-CRO-MAC–SXT; 14LC.ER1029-31, CXM-CRO-MAC; 14LC.ER1032-34, CXM–MAC–SXT; 14LC.ER1035-37, CXM-MAC; 14LC.ER1038-40, MAC–SXT; 14LC.ER1041–43, MAC; and 14LC.ER1044-47, NONE.

**Abbreviations:** PEN, penicillin; CXM, cefuroxime; CRO, ceftriaxone; MAC, macrolides (erythromycin and azithromycin) and clindamycin; SXT, sulfamethoxazole–trimethoprim.
found that they were located in 76 genes involved in functions related to virulence, protein metabolism, and antimicrobial resistance (Table S2). This suggests that the sequences share similarities and that horizontal genetic transfer may have occurred during their evolution.

Genetic variations potentially associated with drug resistance

Analysis of genetic variation across the whole genomes of the isolates revealed that indels and SNPs occurred in both antimicrobial-susceptible and antimicrobial-resistant strains. To find variations potentially associated with drug resistance, indels and SNPs were compared between antimicrobial-susceptible and antimicrobial-resistant strains.

Excluding the variations in indels from the susceptible isolates, we analyzed the relationships between indel variations and antibiotic resistance (Table S3). The antibiotic-related genes with high mutation rates (≥0.38) are depicted in Table 2. The numbers of indel changes associated with PEN, CXM, and CRO β-lactams were 77, 237, and 139, respectively. Furthermore, 27 mutations in 21 genes (eg, dpr, dagA, zmpB, dfs, axe1, dctA, pspC, sulA, and other genes encoding hypothetical protein) showed high mutation rates and shared close relationships with CXM resistance. At the same time, 465 mutations were identified that may be related to macrolide and lincosamide resistance, and 16 mutations in five genes (lacE, ugd, pspC, pepB, and a gene encoding a hypothetical protein) were associated with this resistance. There were 253 mutations in the SXT-resistant isolates, and 14 associated with six genes (dpr, pspC, dfs, dctA, sulA, and a gene encoding a hypothetical protein) had high mutation rates.

Further analysis of the SNPs associated with drug resistance is shown in Table S4, and the genes closely associated with antibiotic resistance are depicted in Table 3. The number of SNPs associated with β-lactam resistance in PEN (3480), CXM (7707), and CRO (5214) involved 1,524, 1,494, and 1,189 genes, respectively. Specifically, 35 mutations in seven genes (sulA, gdhA, hom, xpt, bglA, regR, and a gene encoding a hypothetical protein) showed high mutation rates, and these were closely related to CXM resistance. Moreover, 14,380 mutations were identified that may be related to macrolide and lincosamide resistance, and trkH and PTS-EII genes were associated with this resistance mechanism. The number of mutations related to SXT resistance was 8,012, involving 1,524 genes, and six of them (udg, dfs, sulB, dpr, ...
dctA, and pspC) were closely associated with SXT resistance. An overview of the genetic diversity among the isolates is shown in Figure 3.

### Antibiotic resistance-associated regions previously reported in S. pneumoniae

We also detected known antibiotic resistance-associated genes (Figure 4A–C). Mutations in pbp2a, pbp2b, pbpX, murMN, and ciaH genes were mainly confined to PEN resistance, whereas mutations in pbp2a, pbp2b, pbp1b, murMN, and ciaH genes were associated with CXM and CRO resistance. Additionally, macrolide-resistant isolates were found to carry ermA and mefA genes, which encode ribosomal methylase and drug-efflux proteins, respectively. Most macrolide-resistant S. pneumoniae were also resistant to tetracycline and harbor the tetM gene. We found that only mutations in one gene (rplD) conferred macrolide resistance, whereas mutations in dfr and sulA genes were closely related to SXT resistance. These characteristics were not detected among the antibiotic-susceptible isolates.

### Discussion

The emergence of WGS as a powerful replacement for traditional molecular technologies has enabled highly detailed molecular epidemiology studies to be conducted on the genomic diversity of bacterial pathogens. As a comparative tool for the bacterial genome, WGRS can confirm a suspected disease outbreak of clinical drug-resistant isolates (eg, MRSA, MDR-tuberculosis).20,21 In addition to tracking drug-resistant bacteria, WGRS allows the discovery of specific
drug resistance mechanisms, such as motifs in mobile genetic elements such as transposons, and the mechanisms underlying gene transfer. The major challenge for WGRS is predicting the patterns of evolution involving genetic diversity in SNPs and indels. In this study, we selected 25 strains with differing antibiotic resistance patterns from different clinical isolates obtained from pediatric samples for WGRS analysis.

Phylogenetic analysis of the 25 isolates found that strains with the same antibiotic resistance profiles harbored different genetic backgrounds and vice versa. That is to say, the genetic changes we observed may not be independent of the drug resistance phenotype. Composite effects of selection, mutation, and recombination can force organisms toward bacterial evolution. Spontaneously, horizontal acquisition of transposons conferring drug resistance may promote the adaptation of organism to an environment with high drug resistance. The dynamics of these mobile elements might be attributed to a clinically important drug resistance shift in *S. pneumoniae*. We found that macrolide resistance strains have acquired *ermB* and *mefA* genes, which are carried on transposons such as Tn916 and Tn5251 which have been previously reported in other articles.

To gain insight into the genomic basis for antibiotic resistance traits, we analyzed the genomes of pneumococcal isolates with MDR resistance. As reported previously, MDR isolates display a series of resistance mechanisms including modification of drug targets, inactivation of therapeutic agents, and overexpression of efflux pumps. Efflux pumps

### Table 3 SNP changes associated with antibiotic resistance in *S. pneumoniae*

| Gene symbol | Gene description | Mutations | Consequence | Potential antibiotics |
|-------------|------------------|-----------|-------------|-----------------------|
| pbpX        | Penicillin-binding protein 2X | 4         | NS/MNP      | PEN/CXM/MAC           |
| murF        | UDP-N-acetylmuramoylalane-D-glutamyl-lysine-D-alanyl-D-alanine ligase | 1         | NS          | PEN                   |
| ddl         | D-alanine-D-alanine ligase | 3         | NS          | PEN                   |
| rpmG        | 50S Ribosomal protein L33 | 1         | NS          | PEN                   |
| ppb2a       | Penicillin-binding protein 2a | 29        | NS/MNP      | PEN                   |
| sulA        | Dihydroproteate synthase | 4         | NS          | CXM/MAC               |
| gdhA        | NADP-specific glutamate dehydrogenase | 1         | NS          | CXM                   |
| hom         | Homoserine dehydrogenase | 1         | NS          | CXM                   |
| xpt         | Xanthine phosphoribosyltransferase | 1         | NS          | CXM                   |
| bgA         | 6-phospho-beta-glucosidase | 1         | NS          | CXM                   |
| regR        | Transcription regulator, member of GaIR family | 10        | NS/MNP      | CXM                   |
| ppbA        | Penicillin-binding protein 1A | 6         | NS/MNP      | CXM                   |
| zmpB        | Zinc metalloprotease | 1         | NS          | CXM                   |
| pepT        | Aminotripeptidase; tripeptidase | 3         | NS          | CXM                   |
| gcp         | Secreted metalloendopeptidase Gcp | 2         | NS/MNP      | CXM                   |
| kdgK        | 2-keto-3-deoxyglucurate kinase | 11        | NS/MNP      | CXM                   |
| gno         | 5-keto-D-glucurate 5-reductase | 2         | NS/MNP      | CXM                   |
| ugl         | Unsaturated glucuronyl hydrolase | 2         | NS/MNP      | CXM                   |
| recU        | Recombination protein U | 2         | NS/MNP      | CXM                   |
| dagA        | D-alanine glycine permease | 1         | NS          | CXM                   |
| galT        | Galactose-1-phosphate uridylyltransferase | 1         | NS          | CXM                   |
| trkH        | Trk transporter membrane-spanning protein-K+ transport | 1         | NS          | MAC                   |
| ppb2b       | Penicillin-binding protein 2B | 5         | NS          | MAC                   |
| PTS-EII     | Phosphotransferase system sugar-specific EII component | 4         | NS          | CXM/MAC               |
| adhP        | Alcohol dehydrogenase, propanol-prefering | 1         | NS          | MAC                   |
| ropA        | Trigger factor | 1         | NS          | MAC                   |
| bgIH        | 6-phospho-beta-glucosidase | 1         | NS          | MAC                   |
| miaA        | tRNA Isopentenylpyrophosphate transferase | 3         | NS/MNP      | MAC                   |
| divIB       | Cell division protein DivIB | 1         | NS          | MAC                   |
| truA        | tRNA pseudouridine synthase A | 1         | NS          | MAC                   |
| pspC        | Choline-binding protein A | 3         | NS          | MAC/SXT               |
| ugd         | UDP-glucose dehydrogenase | 1         | NS          | SXT                   |
| dfr         | Dihydrofolate reductase | 7         | NS/MNP      | SXT                   |
| suB         | Dihydrofolate synthetase | 2         | NS/MNP      | SXT                   |
| dpr         | DNA-binding protein starved cells-like peroxide resistance protein | 1         | NS          | SXT                   |
| dctA        | Dicarboxylate/amine acidication (Na+ or H+) symporter | 1         | NS          | SXT                   |

**Abbreviations:** NS, nonsynonymous; MNP, more than or equal to two bases mutations; PEN, penicillin; CXM, cefuroxime; MAC, macrolides (erythromycin and azithromycin) and clindamycin; SXT, sulfamethoxazole–trimethoprim SNP, single-nucleotide polymorphism.
that contribute to the MDR phenomenon are divided into the ATP-binding cassette (ABC) family of transporters, major facilitator superfamily proteins, small multidrug resistance family proteins, resistance-nodulation-division family proteins and, multidrug and toxic compound extrusion family proteins. In this study, we found that alterations in the ABC-membrane spanning domain efflux pump were observed in the MDR isolates, and this may have led to the MDR strains.

Comparative analysis of all strains showed that mutations in the form of SNPs and indels occurred frequently among different strains of S. pneumoniae. Specially, in our search for genome regions with high mutation rates in the isolates, our analysis linked diversity in indels and SNPs in genes (eg, dpr, dagA, zmpB, pspC, adhP, truA, bglH) with resistance against several antibiotics, although the gene annotation indicated that these genes are mainly involved in virulence as well as carbohydrate and amino acid transport and metabolism. Furthermore, other genetic changes involved in lone antibiotic resistance were always detected. A large number of mutations in PBPs (eg, pbp1b, pbp2a, pbp2b, and pbpX) have

Figure 3  Indel and SNP variation in the complete genome of S. pneumoniae R6 compared with 25 S. pneumoniae clinical isolates.

Notes: The distribution of nonsynonymous mutations (SNPs and indels) in the genome sequences of 25 isolates compared with the S. pneumoniae R6 reference strain is shown. Protein-coding genes on the sense and anti-sense strands are shown in rings II and III (from inside to outside). The arrow directions indicate the translational directions. The distribution of nonsynonymous mutations in the genomes of 25 isolates (from isolate 14LC-ER1023 to 14LC-ER1047) are shown from ring IV to ring XXVII. Green bars represent nonsynonymous SNPs; red bars represent nonsynonymous indels.

Abbreviation: SNP, single-nucleotide polymorphisms.
been identified, and these are reported to reduce the binding affinities for β-lactam antibiotics, resulting in resistance. Moreover, other resistance determinants such as MurMN and CiaRH affecting peptidoglycan structure are known to influence β-lactam resistance. Among the indels observed in the resistant isolates in this study, *sulA* is characterized by a 6-bp insertion, resulting in the insertion of two amino acids (Arg and Pro) in dihydropteroate synthase, thereby conferring resistance to SXT. Concurrently, other studies have reported nonsynonymous SNPs in the dihydrofolate reductase encoding *dfr* gene in SXT isolates. Furthermore, it was found that mutations in the ion transporter membrane-spanning protein encoding *trkH* gene were responsible for increased antibiotic susceptibility in the aminoglycoside-resistant population. Moreover, the same study found mutations in a gene close to *trkH*, namely *PTS-EII*, which suggests that an operon in this

**Figure 4** (A) SNPs changes in PBPs associated with penicillin resistance in *S. pneumoniae*. (B) SNPs changes in PBPs associated with cephalosporins resistance in *S. pneumoniae*. (C) SNPs changes associated with sulfamethoxazole–trimethoprim resistance in *S. pneumoniae*.

**Abbreviations:** SNPs, single-nucleotide polymorphisms; PBPs, penicillin-binding proteins; PEN, penicillin; CXM, cefuroxime; CRO, ceftriaxone; SXT, sulfamethoxazole–trimethoprim.
region regulating the expression of the structural genes exists. However, whether these changes are necessarily related to drug resistance needs further analysis.

This study has several limitations. First, the sample size was fairly small: only 25 clinical isolates were used, and this may have affected the genetic divergence analysis. Second, the strains were isolated over 2 years, and were from different patients with different genetic backgrounds. Last, we did not conduct any experiments to confirm that the genetic diversity in indels and SNPs were associated with antibiotic resistance. In all, further investigation of the function of some of the genes identified in this study is essential.

In conclusion, by undertaking WGRS and comparative genomic analysis of 25 pneumococcal isolates, we have shown that although pneumococcal isolates were similar on genetic background, strains were diverse at the genomic level. These strains exhibited distinct variations in their indel and SNP compositions, which are potentially correlated with drug resistance. Here, we have simply reported the genomic variation present in the isolates. Further in-depth investigations of these variations and their associations with antibiotic resistance mechanisms are required.

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Disclosure

All authors report no potential conflicts of interest.

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