Genetic Analyses of Penicillin Binding Protein Determinants in Multidrug-Resistant *Streptococcus pneumoniae* Serogroup 19 CC320/271 Clone with High-Level Resistance to Third-Generation Cephalosporins

Margaret Ip, Irene Ang, Veranja Liyanapathirana, Helen Ma, Raymond Lai

Department of Microbiology, Faculty of Medicine, Chinese University of Hong Kong, Prince of Wales Hospital, Hong Kong SAR

We describe the dissemination of a multidrug-resistant (MDR) serogroup 19 pneumococcal clone of representative multilocus sequence type 271 (ST271) with high-level resistance to cefotaxime in Hong Kong and penicillin binding protein (*pbp*) genes and its relationships to Taiwan^{19F}-14 and the prevalent multidrug-resistant 19A clone (MDR19A-ST320). A total of 472 nonduplicate isolates from 2006 and 2011 were analyzed. Significant increases in the rates of nonsusceptibility to penicillin (PEN) (MIC $\geq$ 4.0 μ g/ml; 9.9 versus 23.3%; $P = 0.0005$), cefotaxime (CTX) (MIC $\geq$ 2.0 μ g/ml; 12.2 versus 30.3%; $P < 0.0001$ [meningitis MIC $\geq$ 1.0 μ g/ml; 30.2 versus 48.7%; $P = 0.0001$]), and erythromycin (ERY) (69.2 versus 84.0%; $P = 0.0003$) were noted when rates from 2006 and 2011 were compared. The CTX-resistant isolates with MICs of 8 μ g/ml in 2011 were of serotype 19F, belonging to ST271. Analyses of the penicillin binding protein 2x (PBP2x) amino acid sequences in relation to the corresponding sequences of the R6 strain revealed M339F, E378A, M400T, and Y595F substitutions found within the ST271 clone but not present in Taiwan^{19F}-14 or MDR19A. In addition, PBP2bs of ST271 strains and that of the Taiwan^{19F}-14 clone were characterized by a unique amino acid substitution, E369D, while ST320 possessed the unique amino acid substitution K366N, as does that of MDR19A in the United States. We hypothesize that ST271 originated from the Taiwan^{19F}-14 lineage, which had disseminated in Hong Kong in the early 2000s, and conferred higher-level β -lactam and cefotaxime resistance through acquisitions of 19 additional amino acid substitutions in PBP2b (amino acid [aa] positions 538 to 641) and altered PBP2x via recombination events. The serogroup 19 MDR CC320/271 clone warrants close monitoring to evaluate its effect after the switch to expanded conjugate vaccines.

Streptococcus pneumoniae serogroup 19 clonal complex 320/271 (CC320/271) includes important multidrug-resistant (MDR) clones, including sequence type 271 (ST271), responsible for both invasive pneumococcal disease (IPD) and noninvasive pneumococcal disease in most Asian countries (1, 2) and in parts of China (3). In the United States and other western countries, serotype 19A-ST320, a multidrug-resistant (MDR) clone, has been highly successful after the introduction of the seven-valent pneumococcal conjugate vaccine (PCV7) (4). ST320 is a double-locus variant (DLV) of the originally described Taiwan^{19F}-14 (Pneumococcal Molecular Epidemiology Network; PMEN14) clone, ST236, from isolates from a Taiwanese hospital in 1997 (5) and has undergone a capsular switch to nonvaccine serotype 19A, likely through recombination that altered the *wzy* polymerase gene. In mainland China, 43.8% of cases of IPD were reportedly due to serogroup 19 during 2005 to 2011, with 84.7% of isolates belonging to CC320/271, including ST271, ST320, and ST236 (3). In Hong Kong, we reported the introduction of the Taiwan^{19F}-14 (ST236) clone back in 1999 (6), prior to the introduction of PCV7. These strains were resistant to β -lactams, tetracyclines, and macrolides but susceptible to chloramphenicol and replaced the predominant MDR Spain^{23F}-1 and Spain^{6B}-2 clones in the late 1990s (7).

The Hong Kong Special Administrative Region (HKSAR) introduced PCV7 to the childhood immunization program in October 2009 and subsequently introduced the 10-valent vaccine in October 2010 and PCV13 in 2011 in keeping with the changes in the vaccine coverage of prevalent serotypes. Of the serotypes included in PCV7, serotype 19F is the least likely to evoke a protec-

tive immune response according to both vaccine efficacy trials and *in vivo* studies (8, 9). Therefore, despite the initial decrease in carriage and invasive disease due to serotype 19F, reemergence of the disease might be anticipated due to a waning of antibody responses over time. This is reflected by recent studies in Hong Kong comparing pre- and postvaccination serotype carriage, where a significant difference was not found for serotype 19F (10). In addition, during this period, an increasing level of resistance of *S. pneumoniae* to the third-generation cephalosporin cefotaxime was observed. Given the ability of pneumococci to adapt and transform, we sought to examine the levels of antimicrobial and β -lactam resistance of these isolates and study the polymorphisms of amino acid sequences of penicillin binding protein 1a (PBP1a), PBP2b, and PBP2x that might be related to different levels of

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Address correspondence to Margaret Ip, margaretip@cuhk.edu.hk.

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β -lactam resistance. In this paper, we describe the dissemination of a clone of pneumococci with high-level resistance to cefotaxime in Hong Kong, the penicillin binding protein translated amino acid sequences, and their relationships to that of the prevalent pneumococcal clones MDR19A-ST320 and Taiwan^{19F}-14. Specifically, we examined the genetic relatedness and the amino acid changes of PBP1a, -2b, and -2x of our serotype 19F isolates and compared them with those of the widespread prevalent clones reported in North America and Asia.

MATERIALS AND METHODS

Isolates and patients. A total of 472 nonduplicate *Streptococcus pneumoniae* isolates from clinical specimens of patients admitted to three hospitals, namely, Prince of Wales, Northern District, and Shatin Hospitals, Hong Kong, during 2006 and 2011 were included. *S. pneumoniae* isolates were isolated from blood cultures and body fluids ($n = 27$) and the lower respiratory tract (LRT) ($n = 445$), including bronchoalveolar lavage (BAL) fluid, tracheal aspirates, or sputa. *S. pneumoniae* isolates were identified by using conventional microbiological methods and stored at -70°C in 10% glycerol–brain heart infusion (BHI) broth.

Antimicrobial susceptibility testing. Antibiotic susceptibility was determined by the broth microdilution method described by the Clinical and Laboratory Standards Institute (CLSI) (11). The following antimicrobials were tested: penicillin (PEN), cefotaxime (CTX), chloramphenicol, lincomycin, erythromycin (ERY), tetracycline, linezolid, ciprofloxacin, levofloxacin, and vancomycin (Sigma-Aldrich, St. Louis, MO, USA).

Molecular typing of serotypes, PFGE, and MLST. Strains were serotyped by multiplex PCRs (28), and serotypes were determined, where indicated, by an agglutination method using serotype-specific antisera (Statens Serum Institut, Copenhagen, Denmark). Serogroup 19 isolates were further characterized by pulsed-field gel electrophoresis (PFGE) according to previously reported protocols, with modifications (12), and isolates from the predominant cluster were typed by multilocus sequencing typing (MLST) (<http://spneumoniae.mlst.net/>).

Genetic analyses of penicillin binding protein genes. Selected isolates with CTX MICs of ≥ 1 $\mu\text{g/ml}$ (and covering the range of 1 to 8 $\mu\text{g/ml}$) were subjected to this analysis ($n = 17$). The penicillin binding protein genes *pbp1a*, *pbp2b*, and *pbp2x* were amplified and sequenced by the Sanger method. The PCR primer pair for *pbp1a* was 64F1 (5'-GTGCCTTCCCTCAAAATTC-3') and 2234R10 (5'-CTCAGGCTTTTGTTAAGCA C-3') (2,298 bp on the R6 genome), and sequencing was performed by using the same primers and two inner primers, 1357R7 (5'-TGGCGTTTGCATAATGCATG-3') and 1719R9 (5'-GCTACGTAGCCAGTGTTC-3'). For *pbp2b*, primers 872F3 (5'-GCAATATGGAAAGCGTGGAT-3') and 2012R2 (5'-TTGATAATGTCRCGCGCAAT-3') were used for both amplification (1,141 bp on the R6 genome) and sequencing. For *pbp2x*, primers 633F (5'-GAGYTTCTGGAACYTCTGG-3') and 2251R (5'-AATTCCAGCACTGATGGAAATAACATATTA-3') were used for amplification (1,649 bp on the R6 genome), and sequencing was performed by using the same internal primers and an additional inner primer, 1329R (5'-ATCMGCGWGAAGYTGAACAGC-3'). The partial amino acid sequences of PBP1a, -2b, and -2x were aligned to the corresponding sequences of *S. pneumoniae* R6 (GenBank accession no. [AAK99133](#) for PBP1a, [AAL00321](#) for PBP2b, and [AAK99108](#) for PBP2x). For analysis, the partial amino acid sequences of PBP1a (positions 264 to 654; 391 amino acids [aa]), PBP2b (positions 313 to 654; 342 aa), and PBP2x (positions 266 to 616; 351 aa) were compared with the corresponding amino acid sequences of Taiwan^{19F}-14 (GenBank accession no. [ACO23867](#), [ACO22394](#), and [ACO24263](#)) and MDR19A (GenBank accession no. [ZP06978248](#), [ZP06978009](#), and [ZP06978328](#)) and with PBP sequences from clinical isolates of *S. pneumoniae* with various penicillin and cefotaxime MICs reported previously (13). A neighbor-joining tree was constructed to illustrate the relatedness of these *pbp1a*, *pbp2b*, and *pbp2x* genes by using Molecular Evolutionary Genetic Analysis software (MEGA 5.05) (14).

Statistical analyses. The Fisher exact test or the chi-squared test was used to compare serotypes or antimicrobial susceptibility rates among different groups and time periods. A P value of <0.05 was considered statistically significant.

RESULTS

A total of 472 nonduplicate *S. pneumoniae* isolates from 2006 ($n = 172$) and 2011 ($n = 300$) were examined. Most strains were isolated from adults, and the mean ages of patients were 64.5 and 67.2 years for 2006 and 2011, respectively. A total of 79.0% (373/472) of *S. pneumoniae* isolates were obtained from male patients.

Antimicrobial susceptibility and serotypes. Significant increases in the rates of nonsusceptibility to PEN (MIC ≥ 4.0 $\mu\text{g/ml}$; 9.9 versus 23.3%; $P = 0.0005$), CTX (MIC ≥ 2.0 $\mu\text{g/ml}$; 12.2 versus 30.3%; $P < 0.0001$ [meningitis MIC ≥ 1.0 $\mu\text{g/ml}$; 30.2 versus 48.7%; $P = 0.0001$]), and ERY (69.2 versus 84.0%; $P = 0.0003$) were noted in 2011, compared to those in 2006. The rates of nonsusceptibility to PEN and CTX have nearly tripled in these two periods. All isolates remained susceptible to ≤ 0.25 $\mu\text{g/ml}$ vancomycin and ≤ 2 $\mu\text{g/ml}$ linezolid.

The significant increments in rates of nonsusceptibility to PEN, CTX, and ERY were further analyzed according to serotype. All CTX-resistant isolates (MIC ≥ 4 $\mu\text{g/ml}$) in 2011 were attributed to the single serotype 19F. Among all serotype 19F isolates (87/300) tested in 2011, 46% (40/87; 46.0%) were highly resistant to CTX, with MICs of 8 $\mu\text{g/ml}$. A total of 25.3% (22/87) of these serotype 19F isolates had PEN MICs of 4 $\mu\text{g/ml}$. For 2006, 8 isolates of serotype 19F with CTX MICs of ≥ 4 $\mu\text{g/ml}$ were obtained.

Serotypes 19F (29.1% versus 29.0%) and 6B (9.3% versus 9.3%) remained the most prevalent serotypes in the two periods, while the prevalence of serotype 23F (18.0% versus 5.3%) dropped significantly ($P < 0.001$) (data not shown). Serotype replacement with a rising prevalence of non-PCV7 serotypes, including serotypes 19A (2.3 versus 5.3%), 6C (1.2 versus 3.0%), and 34 (0 versus 1.7%), was observed in 2011, but the percent changes were not statistically significant.

Clonal relationship of serotypes 19F and 19A determined by PFGE and MLST. Serogroup 19 isolates (87 and 16 isolates of serotypes 19F and 19A, respectively) were subjected to PFGE analysis (Fig. 1). A major cluster was detected at a cutoff of 85% similarity, as calculated by the Dice coefficient with 1.5% position tolerance and 1.5% band optimization. This cluster consisted of 82% (71 of 87) serotype 19F isolates, belonging to ST271, its single-locus variants (SLVs), or ST320, which had high CTX MICs ranging from 2 to 8 $\mu\text{g/ml}$ and PEN MICs of 1 to 4 $\mu\text{g/ml}$ and was multidrug resistant, with high rates of nonsusceptibility to lincomycin (92.5%), erythromycin (98.8%), and tetracycline (100%). Thirty-six isolates had high CTX MICs of 8 $\mu\text{g/ml}$. In contrast, serotype 19A strains predominantly belonged to ST320, with CTX MICs of 1.0 $\mu\text{g/ml}$, PEN MICs of 1 to 4 $\mu\text{g/ml}$, and variable resistance to lincomycin, erythromycin, and tetracycline.

Comparison of amino acid sequences of PBPs of serogroup 19 isolates. The relatedness of amino acid sequences of PBP1a, -2b, and -2x of high-level CTX-resistant serogroup 19 isolates was examined (Fig. 2A to D). Seventeen clinical isolates of ST271 and ST320 covering the range of CTX MICs of 1 to 8 $\mu\text{g/ml}$ were selected for further examination of these genes. The polymorphisms in amino acid sequences of these PBPs reflecting different levels of β -lactam resistance among clinical isolates of *S. pneumoniae* and their reported profiles of PBP1a (strain no. A1 to C3),

FIG 1 PFGE patterns of SmaI-restricted chromosomal DNA of serotype 19F and 19A isolates. The dendrogram was obtained by using the unweighted-group method using average linkages. The cluster was classified by using a cutoff of 85% similarity, as calculated by the Dice coefficient (1.5% position tolerance and 1.5% band optimization). The MIC values of 8 antimicrobials are listed. Cip, ciprofloxacin; Lev, levofloxacin; Lin, linezolid; CTX, cefotaxime; Pen, penicillin; Chl, chloramphenicol; Ery, erythromycin; Tet, tetracycline. The MIC values of vancomycin and linezolid for all the isolates were ≤ 0.25 $\mu\text{g/ml}$ and ≤ 1 $\mu\text{g/ml}$, respectively. *SLV indicates a strain with new allelic profiles (4-16-19-15-6-20-new), which are single-locus variants of ST271/320; #Other indicates a strain with a non-ST271/320 sequence type.

FIG 2 Genetic relatedness of PBP amino acid sequences. (A to C) Neighbor-joining trees showing the relatedness of the PBP1a (A), PBP2b (B), and PBP2x (C) proteins among R6, ST271, Taiwan^{19F}-14, and MDR19A and previously reported PBP amino acid sequences (strain no. A1 to C3 for PBP1a, A1 to F1 for PBP2b, and A1 to C3 for PBP2x) with different levels of β -lactams resistance (16). * indicates that the sequences were obtained from GenBank under the accession number indicated. The bar represents a genetic distance of 0.5%. (D) Amino acid substitution sites of PBP2b and PBP2x. The sequences were aligned according to the corresponding amino acid sequences of R6. The amino acid positions are numbered in vertical format. PEN, penicillin; CTX, cefotaxime.

PBP2b (strain no. A1 to F1), and PBP2x (strain no. A1 to C3) were included in this analysis (13). Also, the sequences of the prevalent MDR19A (ST320) (GenBank accession no. [ZP06978248](#), [ZP06978009](#), and [ZP06978328](#), respectively) from North America and Taiwan^{19F}-14 (ST236) (accession no. [ACO23867](#), [ACO22394](#), and [ACO24263](#), respectively) clones, which are SLVs of ST271, were also included in the comparison. Figure 2D lists the strains analyzed, their ST types, their corresponding PEN and CTX MICs, and the amino acid substitutions of the major domains of PBP2b and -2x.

The amino acid sequences of PBP1a of the current serogroup 19 strains MDR19A (ST320) and Taiwan^{19F}-14 (ST236) were 100% identical and clustered together (Fig. 2A). For PBP2b (Fig. 2B), the sequence of MDR19A (ST320) was the closest reference sequence to those of our current serogroup 19 strains. MDR19A (ST320) possessed the amino acid substitution K366N, while both the serotype 19F (ST271) and Taiwan^{19F}-14 strains possessed the amino acid substitution E369D. However, the latter two differed by 19 aa at positions 538 to 641 (Fig. 2D). These 19 amino acid changes were present in all ST271, ST320, and strains reported

previously (5268D1' and 5204D1; PEN, 3 to 6 μ g/ml; CTX, 4 to 12 μ g/ml) and suggest their importance in conferring a higher level of resistance to β -lactams. These 19 amino acid alterations are likely to have arisen by a recombination event in the *pbp2b* gene.

For PBP2x (Fig. 2C), the amino acid sequences of MDR19F-ST271 clustered together with strain C3, with a known high PEN MIC of 6 μ g/ml and a CTX MIC of 12 μ g/ml. MDR19A clustered together with other ST320 strains. PBP2x of ST271 strains possessed the unique amino acid substitutions M339F, E378A, M400T, and Y595F, distinct from those of MDR19A-ST320 strains and Taiwan^{19F}-14.

DISCUSSION

Our data clearly demonstrated substantial increases in the prevalence of pneumococcal isolates with nonsusceptibility to penicillin, cefotaxime, and erythromycin during the 5-year period. Although the analysis was based on two single years, 2006 and 2011, these changes were not due to temporal fluctuations of the strains isolated, as continuing increases of rates of nonsusceptibility to β -lactams and erythromycin from 2009 were documented (our

unpublished data). This finding corroborated data from a recent surveillance report from 11 Asian countries; the prevalence rate of penicillin nonsusceptibility has been increasing from 1996 and reached 13.4% (nonmeningitis breakpoint according to CLSI guidelines) during 2008 to 2009 in China (1). The corresponding figures reported in this study were 9.9% in 2006 and 23.3% in 2011 for Hong Kong. A recent study from China reported that the rates of nonsusceptibility to PEN and CTX during 2009 to 2010 were >50% and >35%, respectively (15). The increasing rates of nonsusceptibility to β -lactams over the past years in our regions warrant concern.

Our results showed that high rates of β -lactam nonsusceptibility in 2011 were due mainly to the clonal expansion of serotype 19F ST271 and, to a lesser extent, of its SLVs. Similarly to recent surveillance data in European countries, serotype 19F was the predominant serotype that has persisted after introduction of PCV7 vaccine in pneumococcal carriage and disease (16). Successful expansion of MDR19A of ST320 in the post-PCV7 era has already posed a serious global public health risk (17). Regionally, in South East Asia, the prevalent serotype 19F isolates were recently reported to be of both ST236 in Malaysia and ST271 in Beijing, China (18, 19).

Recombination resulting in amino acid substitutions in the PBP1a, -2b, and -2x sequences at or surrounding the active site of transpeptidase domains has been associated with resistance to β -lactams. In our study, all the PBP2b enzymes of ST271 strains and the Taiwan^{19F}-14 (PMEN14) clone were characterized by a unique amino acid substitution, E369D, while ST320 possessed the unique amino acid substitution K366N, as does U.S. MDR19A. We hypothesize that ST271 originated from the Taiwan^{19F}-14 lineage that disseminated in Hong Kong in the early 2000s and developed higher β -lactam and cefotaxime resistance through the acquisition of an additional 19 amino acid substitutions in PBP2b (aa positions 561 to 641) (Fig. 2D) and in PBP2x via recombination events. This clone is characterized by a high cefotaxime MIC of 8 μ g/ml and accounted for the increased level of third-generation cephalosporin resistance in *S. pneumoniae*.

Both ST271 and ST320 possess the 19 additional amino acid substitutions detected beyond aa position 538 of PBP2b and were absent in their ancestor Taiwan^{19F}-14 (Fig. 2D). Among these 19 amino acid substitutions, A619G is close to the catalytic motif KTG spanning positions 615 to 617 and is next to residue Thr618, which is involved in stabilizing unhindered access to the active site. A619G and Q628E have been reported to close the active site in crystallization studies (20). Although the remaining 17 aa are not actively involved in enzyme activity, they are likely related to the protein structure that hinders the antibiotic binding site. A computational analysis of wild-type and mutant PBP2bs showed that the interaction between β -lactams and the catalytic cleft of PBP2b could be prevented by mutations toward the end of the C terminus (20), while those with amino acid alterations before position 538 show only intermediate resistance. This would explain the higher MICs of MDR19A (PEN, ≥ 4 μ g/ml; CTX, 2 μ g/ml) and ST271 (PEN, 4 μ g/ml; CTX, 8 μ g/ml) than those of the ancestor clone Taiwan^{19F}-14 (PEN, 2 μ g/ml; CTX, 1 μ g/ml).

A recent study utilized whole-genome association analysis of two pneumococcal populations, many with known phenotypes for β -lactam susceptibility, from Thailand and Massachusetts to search for single nucleotide polymorphisms (SNPs) and insertions and deletions (indels) that might infer β -lactam nonsuscep-

tibility and antimicrobial resistance (21). That study identified 71 nonsynonymous SNPs that potentially contribute to β -lactam nonsusceptibility. However, that whole-genome association study did not describe strains of high cefotaxime MICs of 8 μ g/ml or higher, whereas in our study, we have demonstrated changes in PBP2x associated with high-level cefotaxime resistance. The role of some of these substitutions, such as M339F and M400T, in the loss of affinity of PBP2x for cephalosporins was previously studied (22, 23), and the combination of changes at these key residues of ST271 may explain the high MIC values of cefotaxime. The four amino acid substitutions unique to ST271 were M339F, E378A, M400T, and Y595F. According to the crystal structure of PBP2x (24), M339 is next to the active-site lysine residue Lys340 in the catalytic motif SXXK (where X is an unspecified amino acid residue) spanning positions 337 to 340, while E378A is close to Trp374, which is involved in hydrophobic interactions with the β -lactam molecule. M400T is close to Ser395, which is involved in hydrogen bonding with the β -lactam. Y595F is next to Ser596, which does not directly interact with the antibiotic molecule and is suggested to have an effect on the protein's overall structure. Interestingly, the ST320 strains from this study have the same *pbp2x* mosaic gene as that of MDR19A and were phenotypically characterized by high PEN MICs (2 to 4 μ g/ml) while maintaining lower CTX MICs (1 to 2 μ g/ml). These strains are likely to have emerged independently of ST271 under such circumstances. Croucher et al. (25) analyzed 173 "vaccine escape" PMEN14 isolates of ST236 and ST320 and of serotypes 19F, 19A, and 23F and commented that PMEN14 is a rapidly recombining lineage, leading to multiple emergences of resistance through transformation.

The MDR CC320/271 clone remains a predominant clone in many Asian countries (1). The HKSAR was the first region in Asia Pacific to introduce PCV7 in the childhood immunization schedule in September 2009 and has subsequently switched to the extended-spectrum vaccines. As with other countries with childhood conjugate vaccination, capsular replacement with the new serotypes 6C and 6D (10) and new clones of serogroup 15A, 15B, and 15C have been reported to have a role in carriage and disease in children (26). In Hong Kong, the clonal expansion of ST271 serotype 19F, described here, was identified mainly for isolates from the adult group. Most of these isolates were also highly resistant to erythromycin (MIC ≥ 64 μ g/ml), and dual possession of the *mefE* and *ermB* genes carried by a new transposon, Tn2010, has been associated with high-level macrolide resistance in serotype 19A and 19F isolates of CC320/271 (27). The widespread nature and persistence of this MDR clone suggests survival fitness of this clone in our locality.

In conclusion, we have shown the clonal expansion of MDR ST271 serotype 19F with high-level resistance to β -lactams, including third-generation cephalosporins. ST271 strains possess characteristic and unique amino acid residues at the PBP determinants indicating high-level β -lactam resistance, compared to MDR19A-ST320 isolates. The serogroup 19 MDR CC320/271 clone in Hong Kong and in the Asian region warrants close monitoring to evaluate its effect after the switch to the expanded-spectrum conjugate vaccines.

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