AMERICAN SOCIETY FOR MICROBIOLOGY | Clinical Microbiology Reviews

Biological and Epidemiological Features of Antibiotic-Resistant *Streptococcus pneumoniae* in Pre- and Post-Conjugate Vaccine Eras: a United States Perspective

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SUMMARY

Streptococcus pneumoniae inflicts a huge disease burden as the leading cause of community-acquired pneumonia and meningitis. Soon after mainstream antibiotic usage, multiresistant pneumococcal clones emerged and disseminated worldwide. Resistant clones are generated through adaptation to antibiotic pressures imposed while naturally residing within the human upper respiratory tract. Here, a huge array of related commensal streptococcal strains transfers core genomic and accessory resistance determinants to the highly transformable pneumococcus. β-Lactam resistance is the hallmark of pneumococcal adaptability, requiring multiple independent recombination events that are traceable to nonpneumococcal origins and stably perpetuated in multiresistant clonal complexes. Pneumococcal strains with elevated MICs of β-lactams are most often resistant to additional antibiotics.

Published 13 April 2016

Citation Kim L, McGee L, Tomczyk S, Beall B. 2016. Biological and epidemiological features of antibiotic-resistant *Streptococcus pneumoniae* in pre- and post-conjugate vaccine eras: a United States perspective. Clin Microbiol Rev 29:525–552. doi:10.1128/CMR.00058-15.

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Basic underlying mechanisms of most pneumococcal resistances have been identified, although new insights that increase our understanding are continually provided. Although all pneumococcal infections can be successfully treated with antibiotics, the available choices are limited for some strains. Invasive pneumococcal disease data compiled during 1998 to 2013 through the population-based Active Bacterial Core surveillance program (U.S. population base of 30,600,000) demonstrate that targeting prevalent capsular serotypes with conjugate vaccines (7-valent and 13-valent vaccines implemented in 2000 and 2010, respectively) is extremely effective in reducing resistant infections. Nonetheless, resistant non-vaccine-serotype clones continue to emerge and expand.

INTRODUCTION

Streptococcus pneumoniae (pneumococcus) remains the leading cause of community-acquired pneumonia, meningitis, and bacteremia in children and adults (1) and the most common cause of otitis media in infants and young children. Globally, pneumonia remains the most common cause of death in children younger than 5 years of age, causing 1.6 million deaths annually (2). Pneumococcal disease continues to cause the most deaths among vaccine-preventable diseases according to the World Health Organization (WHO) (3). Persons at higher risk for invasive pneumococcal disease (IPD) (i.e., pneumococcus recovered from a normally sterile site) include children <2 years of age, adults ≥65 years of age, those with underlying chronic conditions (e.g., cardiovascular or pulmonary diseases, etc.), and those with immunosuppression (e.g., congenital immunodeficiency, human immunodeficiency virus [HIV] infection, leukemia, or systemic corticosteroid use, etc.) (4–6). Pneumococcal conjugate vaccines are effective against IPD and have had a significant direct effect on infants and young children as well as an indirect effect on those not targeted to receive the vaccine (7, 8).

Antimicrobial-resistant pneumococcal infections were documented as early as 1912, when optochin resistance in experimental mice was described (9). Acquired optochin resistance was seen in humans 5 years later (10). In 1939, treatment-acquired sulfonamide resistance was reported in a human case of pneumococcal meningitis (11). Penicillin-resistant pneumococci were also selected in laboratories (12, 13); however, it was not until 1965 that the first clinical isolate with reduced penicillin susceptibility was reported (14). During the 1970s and 1980s, pneumococci resistant to penicillin (MIC of ≥0.1 μg/ml), erythromycin, and trimethoprim-sulfamethoxazole (TMP-SMX) spread rapidly globally, including to Australia, Papua New Guinea, Israel, Spain, Poland, South Africa, and the United States (15–19). Tetracycline and chloramphenicol resistances were also identified, with rates varying by region and population (20). Finally, fluoroquinolone resistance has been documented at relatively low levels compared to those for the above-mentioned antibiotics (21).

Multidrug-resistant pneumococci, defined as strains resistant to three or more classes of antimicrobials, were first identified in children (22) via nosocomial transmission and are predominantly associated with pediatric serotypes, or serotypes associated with carriage and disease among the pediatric population (i.e., serotypes 6A, 6B, 9V, 14, 19A, 19F, and 23F) (20, 23–25). Among 21 European Union and European Economic Activity countries, multidrug resistance was observed among isolates of serotypes 19A, 14, 1, 19F, and 23F (26). In the United States, residual multidrug resistance is much less common after 14 years of conjugate vaccine use and more frequently seen among isolates of serotypes 15A, 15B, 15C, 6C, 23A, and 35B (data from Active Bacterial Core surveillance [ABCs], 2013 to 2015). Multiresistant serotype 19A isolates still show the highest MICs for β-lactams, macrolides, lincosamides, tetracycline, and co-trimoxazole (17 of 772 total ABCs isolates according to partial 2015 ABCs data), although the present frequency of multiresistant 19A is low compared to its frequency during 2003 to 2010.

IMPACT OF ANTIMICROBIAL RESISTANCE

In 2013, the U.S. Centers for Disease Control and Prevention (CDC) released the first national report on antibiotic resistance threats in the United States, underscoring their increasing importance (27). The CDC estimated that at least 2 million people acquired serious infections from pathogens that were antimicrobial resistant and that at least 23,000 people died as a result of antimicrobial-resistant infections annually in the United States (27). Antimicrobial resistance complicates treatment and can result in additional antibiotic courses and outpatient visits, excess hospitalizations, and work loss (27).

Specific to antibiotic-resistant pneumococcal pneumonia, a study by Reynolds et al. found that resistance led to 32,398 additional outpatient visits and 19,336 additional hospitalizations, accounting for $91 million (4%) in direct medical costs and $233 million (5%) in total costs, including work and productivity losses (28). A Canadian study found that increased costs associated with penicillin-resistant IPD in children ≤18 years of age admitted to two hospitals was due mainly to antibiotic choice (29). In adults, increased costs due to penicillin-nonsusceptible pneumonia and bacteremia were due to prolonged hospitalization and the use of more expensive antibiotics (30, 31).

Other studies have also examined the association between antibiotic-resistant IPD and clinical outcomes, with differing results, although no recent studies have been reported. Moroney et al. compared persons with bacteremic pneumonia with MICs of cefotaxime of ≥0.25 μg/ml with persons with less resistant bacteremic pneumonia; they found that the proportions of those who died did not differ significantly between the two groups (32). This finding was also documented by Plouffe et al. among pneumococcal bacteremia cases in 10 adult care hospitals in Franklin County, OH, but those authors found that the duration of hospitalization was significantly longer for patients with penicillin-nonsusceptible *S. pneumoniae* (PNSP) than for those with penicillin-susceptible disease (15.8 days versus 12.1 days; $P = 0.05$) (33). In contrast to previous studies that found no difference in mortality, both Metlay et al. and Feiken et al. demonstrated that there was an increased risk of mortality among persons infected with nonsusceptible and resistant *S. pneumoniae* (34, 35). Metlay et al. found that in-hospital mortality was significantly increased among cases with PNSP bacteremic pneumonia compared to those with penicillin-susceptible *S. pneumoniae* (relative risk, 2.1; 95% confidence interval, 1.0 to 4.3) (34), while in the study by Feiken et al., mortality was significantly increased in U.S. persons infected with pneumococci with penicillin MICs of ≥4.0 μg/ml after the fourth day of hospitalization (35).

DETECTION OF ANTIBIOTIC RESISTANCE

Determination of antimicrobial susceptibility is essential not only for treatment of an individual patient but also for tracking anti-

microbial resistance patterns to inform antimicrobial guidance. Even though we can now identify pneumococci and many resistance patterns based upon genetic features, bacterial culture-based phenotypic susceptibility methods remain the gold-standard approach in clinical laboratories.

In the clinical setting, methods and interpretations used to assess antibiotic resistance in *S. pneumoniae* have been established by a number of professional bodies, such as the Clinical and Laboratory Standards Institute (CLSI), the British Society for Antimicrobial Chemotherapy (BSAC), and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (36). For some antibiotics, such as penicillin, defining resistance is a complex issue. Breakpoints are determined by a combination of the pharmacokinetic/pharmacodynamic properties of an antibiotic and patient outcome, and since antimicrobial resistance patterns continue to evolve over time, changes to breakpoints can also occur during the lifetime of an antibiotic. One example is the revised CLSI breakpoints for penicillin adopted in January 2008 (37, 38). This revision occurred when study data from patients with and without pneumococcal meningitis were reevaluated as a clinical response to penicillin was shown among nonmeningitis isolates despite reduced susceptibility *in vitro*. These changes in penicillin breakpoints for *S. pneumoniae* allow clinicians the choice of using penicillin instead of broad-spectrum antimicrobials to treat penicillin-susceptible nonmeningitis pneumococcal infections. These breakpoint differences should also be kept in mind when data from surveillance studies on pneumococcal penicillin resistance are compared. Clinically sensitive strains with 12- to >60-fold-higher MICs (0.12 to 2 μg/ml) than those for wild-type penicillin-sensitive strains (MIC = 0.01 μg/ml) could have profound advantages in the carriage reservoir.

Detection of *S. pneumoniae* typically relies on culture of clinical specimens and subsequent antibiotic susceptibility testing to guide treatment options. However, these methods are often slow, taking up to 48 h, and results are often negative due to prior antibiotic use before sampling or the tendency of *S. pneumoniae* to undergo autolysis. To improve detection from clinical specimens, PCR-based methods have been developed (39, 40). Knowledge of the molecular determinants of resistance to a number of antibiotics has also led to the development of a variety of molecular assays to detect the presence of resistance genes in pneumococcal isolates and also directly from clinical specimens (40–46). The majority of these assays are solely PCR based (40–43), although sequencing approaches and microarrays have also been used (45, 46). Several recent studies have also compared phenotypic drug susceptibility testing results with predictions based on whole-genome sequencing (WGS) data for a variety of bacterial pathogens (47), including *S. pneumoniae* (48, 49). Our CDC-based laboratory has developed a promising WGS-based "typing pipeline" for rapid and automated predictions of pneumococcal serotypes, MICs, genotypes, and additional features (50). Employing continually enhanced bioinformatic pipelines for querying WGS data will greatly expand the depth of laboratory-based strain surveillance efforts. For example, ARG-ANNOT (antibiotic resistance gene annotation) is a very useful bioinformatics tool that provides a periodically updated database (http://en.mediterranee-infection.com/article.php?laref=283&titer=arg-annot-) of known accessory resistance genes to screen bacterial whole-genome sequence data (51).

RISK FACTORS THAT CONTRIBUTE TO ACQUISITION OF RESISTANT INFECTIONS

Recent antibiotic use has been identified as the foremost risk factor for the development of resistance among IPD cases (52, 53), but other risk factors include age (particularly children under 5 years of age), female gender, hospitalization, living in an urban area, attending day care, pediatric serotypes (i.e., serotypes found commonly in children), HIV infection, and immunosuppression (53–55). Currently, >40% of isolates are penicillin resistant in several countries that lack significant conjugate vaccine coverage (56–62). Studies have found that previous use of β-lactam antibiotics (63), extremes of age (e.g., children <5 years of age and the elderly) (63–66), and child care attendance (in a carriage study using PNSP defined as an MIC of >0.06 μg/ml) (65) were associated with penicillin-nonsusceptible pneumococcal infections.

Fewer studies of the acquisition of multidrug resistance have been conducted; however, these studies have found that extremes of age (i.e., <5 years and >65 years of age), previous use of β-lactam antibiotics by patients with noninvasive disease, antibiotic use in the last month by patients with nasopharyngeal colonization, population density, geographic location, and pneumococcal seven-valent conjugate vaccine (PCV7) serotype are all independent risk factors (67–70). With the advent of HIV/AIDS in sub-Saharan Africa, risk factors for the acquisition of multidrug resistance in this immunocompromised group included extremes of age, PCV13 serotypes, pediatric serotypes, previous antibiotic use, previous hospital admission in the last 12 months, and tuberculosis treatment (63).

ANTIBIOTIC TREATMENT

Antibiotics have been a mainstay of IPD treatment and function by decreasing or eradicating the bacterial load (70, 71). Additionally, with severe pneumococcal disease, the inflammatory response needs to be controlled through antibiotics. For example, macrolides inhibit the production of pneumococcal virulence factors by macrolide-susceptible and macrolide-resistant pneumococci and have secondary anti-inflammatory properties to combat infection (72), including the control of neutrophil-mediated inflammation and inhibition of superoxide generation by neutrophils (73). Additionally, corticosteroids are also thought to be an adjunctive treatment for the early management of severe pneumococcal infections, including pneumococcal meningitis or sepsis (74). Unlike macrolides, corticosteroids are not as effective in controlling neutrophil-mediated inflammation (75) but might be best used in conjunction with β-lactams and macrolides to reduce morbidity and mortality associated with pneumococcal infection.

When an inappropriate antibiotic is selected and used for treatment, it can increase the risk of poor outcomes by leading to failed bacterial eradication, selection of resistant bacteria, and complications resulting from these resistant bacteria (72). Current guidelines recommend empirical, broad-spectrum antibiotic therapy for acute bacterial infections (76), with consideration of the common etiologic pathogens, probability of pneumococcus involvement, and antibiotic resistance trends in the local geographic area (71). For the treatment of bacteremic pneumococcal pneumonia in hospitalized children without underlying conditions, studies have shown that penicillin, ampicillin, or cefuroxime should be adequate treatment for those infections caused by isolates with penicillin MICs of ≤2 μg/ml (77). In children, oral monotherapy with amoxicillin, cefuroxime, or cefdinir should be effective after

TABLE 1 Molecular mechanisms responsible for most observed cases of pneumococcal antibiotic resistance

Antibiotic	Mechanism(s)
β-Lactams (penicillin and cephalosporins)	Mutations in penicillin-binding (transpeptidase) domains of *pbp* genes (primarily *pbp2x*, *pbp2b*, and *pbp1a*); mutations in aminoacyl-tRNA ligase gene (*murM*); mutations in other genes, including *pdgA*, *ciaH-ciaR*, and *stkP*
Macrolides	*erm* (23S rRNA methyltransferases) (*ermB* and rarely *ermTR*), *mef*-mediated efflux [*mef*(A) or *mef*(E)], mutations in 23S rRNA genes or L4 or L22 ribosomal protein genes (*rplD* and *rplV*, respectively)
Fluoroquinolones	Mutations in DNA gyrase (primarily *gyrA*) and/or topoisomerase IV genes (primarily *parC*), PmrA-mediated efflux
Tetracycline	Ribosomal protection proteins, primarily Tet(M) and rarely Tet(O)
Rifampin	Mutations in *rpoB* encoding the β-subunit of RNA polymerase
Chloramphenicol	Inactivation of chloramphenicol by *cat*-encoded chloramphenicol acetyltransferase
Trimethoprim-sulfamethoxazole	Mutations in the dihydrofolate reductase gene (*folA*) and dihydropteroate synthetase gene (*folP*)
Ketolides	Mutations in 23S rRNA or L4 or L22 ribosomal protein genes (*rplD* and *rplV*), *ermB* with deletion or mutation in leader sequence
Oxazolidinones	Mutations in 23S rRNA genes, deletions in L4 ribosomal protein gene *rplD*

initial parenteral therapy (77). Macrolides can also be used for outpatient management of pneumococcal pneumonia, although breakthrough and/or sepsis meningitis has occurred due to resistant pneumococci (78–81). For adults with bacteremic pneumococcal pneumonia, studies have shown lower mortality rates for patients treated with a cephalosporin and a macrolide (82, 83), although no pediatric studies have replicated this finding. If the pneumococcal infection is resistant to penicillin (MIC of up to 2 μg/ml), then a third-generation cephalosporin or clindamycin can provide adequate treatment (77). Finally, if a pneumococcal isolate has a penicillin MIC of ≥4.0 μg/ml, both clindamycin and vancomycin are recommended (77), although this is a recommendation that might be questioned since most present-day invasive strains with this level of penicillin resistance are also clindamycin resistant (50; ABCs, unpublished data). A newer quinolone or linezolid might also be considered (84). While there was an increasing trend of penicillin resistance within pneumococci prior to conjugate vaccine implementation, high-dose parenteral penicillin and other parenteral antibiotics continue to be effective for pneumonia and bacteremia (85). In summary, at present, all antibiotic-resistant pneumococcal infections can be treated with antibiotics (85). Table 1 lists most known pneumococcal resistance features and causal genetic determinants.

PNEUMOCOCCAL RESISTANCE TO β-LACTAM ANTIBIOTICS

Since the mass production of penicillin in the mid-1940s, treatment of pneumococcal infections has relied heavily upon penicillin and other β-lactam antibiotics, which are the most widely used and effective antibiotics against this species. Strains with reduced susceptibility to β-lactams were detected for the first time in 1967 (86), only about 20 years after penicillin was mass produced. Strains with higher penicillin MICs were observed during the late 1970s (22) and rapidly emerged and disseminated after this time (87, 88). In the United States, only 5% of 5,459 IPD isolates recovered during 1979 to 1987 were reported to be nonsusceptible (penicillin MIC of ≥0.1 μg/ml), with only 1 isolate being classified as resistant (MIC of ≥2 μg/ml) (89). This situation dramatically changed during the next few years in the United States. During 1993 to 1994, the percentage of nonsusceptible isolates was 14.1%, and 3.2% of these isolates were penicillin resistant with representation by a wider array of serotypes (90). Rates of IPD due to penicillin-nonsusceptible strains peaked in 1999, when such isolates accounted for 25.1% of all IPD isolates recovered in the United States. PCV7 serotypes accounted for ~80% of penicillin-nonsusceptible IPD cases (55, 91).

Peptidoglycan Synthesis and Penicillin-Binding Proteins

Peptidoglycan is a major cell wall component found only in bacteria, constituting more than half of the Gram-positive bacterium dry weight. Peptidoglycan serves essential roles in cell expansion, maintenance of cell integrity, cell division, surface anchoring, and cellular diffusion. It follows that this structure is the target for nearly all commonly used and prospective antibiotics that target cell wall synthesis (307). Pneumococcal peptidoglycan is composed of strands of alternating glucosamine and *N*-acetylmuramic acid residues, directly cross-linked by transpeptidases between two *N*-acetylmuramic acid residues via short stem peptides of up to 5 amino acids (L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala) between the L-Lys of one stem and the penultimate D-Ala of an adjacent stem. Facilitated by the structural similarity of the β-lactam ring to the D-Ala-D-Ala terminus of the peptidoglycan stem peptide, β-lactams irreversibly bind transpeptidases at their active site. Binding of β-lactams to the transpeptidase active site of these penicillin-binding proteins (PBPs) thus blocks cross-linking of muropeptide chains to prevent cell wall synthesis (92). A second peptidoglycan cross-linking activity relevant to pneumococcal β-lactam resistance involves an addition to the stem through the substitution of the lysyl ε-amino group with L-Ala-L-Ala or L-Ser-L-Ala. These branches serve as a PBP cross-linking substrate to an adjacent stem via the fourth-position D-Ala. This branching activity is carried out by the aminoacyl ligases MurM and MurN, which add the first and second amino acids to the stem lysine, respectively (93).

β-Lactam resistance in pneumococcal disease isolates is due to combinations of altered PBPs that have decreased affinities for these antibiotics (94, 95). These strains invariably reveal profound changes in corresponding key PBP genes, and a very wide range of "resistant" PBP gene alleles have been documented (96–99). β-Lactamases, whether introduced via mobile genetic elements or

expressed from the core genome, have never been observed within pneumococcal strains. It is remarkable that resistant PBP combinations expressed from the core genome somehow also serve their essential biosynthetic roles after structural alterations that prevent binding to analogs (β-lactams) of their normal substrates. Unlike the major beta-hemolytic streptococcal pathogens, the highly adaptable pneumococcus has rapidly reengineered these essential proteins required for cell growth and division in response to β-lactam selective pressure.

A seminal finding was that a combination of 3 cloned PBP gene alleles (*pbp2b*, *pbp2x*, and *pbp1a*) from a highly penicillin-resistant pneumococcal strain could be used in gene replacement experiments to transform a susceptible strain to the same high level of resistance (100). All three of these PBPs share a penicillin-sensitive N-terminal transpeptidase domain that contains three conserved motifs: SerXXLys, containing the active-site serine that is bound (acylated) by PBPs; SerXAsn; and LysSer(or Thr)Gly. In general, β-lactam-nonsusceptible pneumococci contain PBP gene substitutions that appear to affect the polarity, charge distribution, and flexibility of the region neighboring the active site to decrease PBP-binding affinities for penicillin and/or other β-lactam classes (101–103).

The involvement of these three PBPs in peptidoglycan expansion/cell shaping and cell division dictates that two (PBP2b and PBP2x) are also essential for cell viability (104), making it difficult to dissect specific steps in the acquisition of resistance. Although *pbp1a* can be inactivated under laboratory conditions, resulting in profound phenotypic defects (104), it undoubtedly serves irreplaceable roles in nature. The requirement of these PBPs for viability dictates that there are fitness costs associated with the development of pneumococcal β-lactam resistance. Nonetheless, the notion of reduced biological fitness within clinical isolates that are nonsusceptible to β-lactam antibiotics seems contradicted by the phenomenal disease burdens associated with individual resistant clonal complexes (CCs) that have emerged and thrived during the past 40 to 50 years (25, 105–112). It is likely that resistance-conferring mutations that profoundly affect cell fitness are usually rapidly lost in the population. In highly significant and rare instances, it also seems likely that these mutations are rapidly alleviated through compensatory chromosomal mutations. It is also clear that once stably established within a strain, successful resistance-conferring PBP alleles are readily disseminated among multiple pneumococcal clones.

A very large array of mutant transpeptidase combinations have been documented within the three PBP genes, resulting in various levels of reduced susceptibility to β-lactams. Since β-lactams each have differing affinities for individual PBPs (107, 113, 114), the variety of β-lactams consumed contributes to this great diversity. There are multiple chromosomal PBP gene alterations that are conserved within individual highly resistant, globally disseminated clones, and it is likely that β-lactam resistance has played a primary role in their global dissemination. The complex patterns of PBP gene changes observed, and the wide range of MICs of β-lactams represented, depict the amazing adaptability of this organism to various β-lactam selection intensities. Even though certain amino acid substitutions have been shown to be key to resistance, there is not a uniform and precise consensus of the specific contributing roles of the different substitutions within these three genes or of different allelic combinations. Resistance can have complex pathways involving additional PBP and non-PBP genes (115). In addition, bypass suppressor mutations can complicate assessments drawn from experiments with laboratory strains.

Nonetheless, certain key features of β-lactam resistance are consistent. A low level of β-lactam resistance results from alterations within the primary resistance PBPs PBP2x and PBP2b (114). PBP2x alterations effect low-level resistance to all or most β-lactams, while PBP2b mutations influence primarily penicillin resistance, consistent with its lack of binding to cephalosporins (113). An altered PBP1a is required for higher levels of β-lactam resistance, in combination with either or both of the primary PBPs. PBP2x mutations primarily affect expanded-spectrum cephalosporin resistance (107), while both PBP2x and PBP2b confer resistance to penicillins (116, 117). As one can surmise from large-scale surveillance data, nearly all highly β-lactam-resistant clinical isolates are resistant to both penicillin and cephalosporins (118). Clinical isolates of pneumococci with MICs of ≥0.5 μg/ml for penicillins or third-generation cephalosporins, such as ceftriaxone or cefotaxime, almost always contain profoundly altered *pbp2b*, *pbp2x*, and *pbp1a* genes. More subtly altered *pbp2a* genes have been reported for a smaller percentage of resistant clinical isolates, suggesting less typical involvement in the development of β-lactam resistance (119).

Involvement of Other Proteins in β-Lactam Resistance

Although the PBP genes *pbp1a*, *pbp2b*, and *pbp2x* have been clearly demonstrated to be required for high-level β-lactam resistance in naturally occurring clinical isolates, in some instances, low-level resistance is also dependent upon proteins that are not directly targeted by β-lactams. The scenario that different PBP allele combinations confer differing β-lactam resistance phenotypes is complicated by the finding that transformations using PBP genes from certain strains were not sufficient to transform wild-type strains to the same high level of resistance (120, 121). One study reported that strains exhibiting identical PBP transpeptidase domain sequences exhibited penicillin MICs ranging from 0.25 to 2.0 μg/ml (122).

Inactivation of the *murM* gene results in the lack of branching activity, resulting in the synthesis of peptidoglycan consisting of only linear muropeptides (93). The MurM aminoacyl ligase appears to be required for penicillin resistance since its inactivation within four different strains exhibiting penicillin MICs of 0.12 to 6 μg/ml resulted in a nearly complete loss of penicillin resistance (123). Besides this finding that suggested a direct role of aminoacyl ligase branching activity in penicillin resistance, highly modified *murM* genes have been revealed in some resistant pneumococcal strains, suggesting that *murM*-encoded branching activity evolves in a clone-specific manner to accommodate specific variant PBPs (124–127). Analysis of laboratory mutants with depleted levels of PBP2b revealed the increased incorporation of branched-stem peptides (128) theorized to mimic adaptation to PBP derivatives found in β-lactam-resistant strains that have low transpeptidase activity (126), and *murM*-null mutants required much higher PBP2b levels for continued growth than did wild-type *murM* strains (128). Despite these observations, most β-lactam-resistant strains appear to have unaltered *murM* genes (125). For example, the highly β-lactam-resistant emergent PMEN-14 clone and the closely related and highly successful serotype 19A switch variants of this clonal complex have a nonmosaic *murM* gene that is identical to that found in certain sensitive strains (49).

Inactivation of another gene shown to be nonessential in the

laboratory setting, the peptidoglycan *O*-acetyltransferase encoded by the *adr* gene, also attenuates PBP variant-conferred penicillin resistance (129). A missense mutation within the *pdgA*-encoded peptidoglycan *N*-acetylglucosamine deacetylase (130) was involved in a late transformational step in achieving high-level penicillin resistance in PBP experiments employing donor chromosomal DNA from a highly penicillin-resistant pneumococcal strain.

StkP (Ser/Thr kinase) is involved in pneumococcal cell division (131) and has been shown to colocalize with PBP2x at the cell division site (132). Inactivation of *stkP* in a penicillin-resistant clinical isolate with altered PBPs abrogated penicillin resistance; however, a survey of penicillin-resistant isolates revealed no altered *stkP* alleles (133).

Mutations that can affect β-lactam resistance to a modest extent in the absence of PBP gene alterations are known. Alterations within the two-component sensor kinase CiaH have been found within β-lactam-resistant mutants selected in the laboratory and in nature (134). These mutations result in higher expression levels of genes upregulated by the transcriptional regulator CiaR, although the putative affected genes actually conferring β-lactam resistance are unknown. Mutations within *cpoA*, which encodes a glycosyltransferase required for the synthesis of diglycosyldiacylglycerols (135), conferred a modest level of piperacillin resistance (136) and were associated with reduced levels of PBP1a.

β-Lactams Influence the Nasopharyngeal Pneumococcal Strain Reservoir

Although current information indicates that high doses of parenteral β-lactams are currently effective against most penicillin-resistant pneumococci, carriage studies that reveal a high proportion of isolates with reduced β-lactam susceptibility suggest that β-lactam antibiotic use plays a significant role in the evolution of the species within its normal upper respiratory tract (URT) reservoir. This can be easily overlooked in the current clinical microbiology landscape, where typically a significant proportion of isolates have extensively remodeled PBP segments that allow for greatly increased penicillin MICs (≥0.12 to 2 μg/ml), which are now considered susceptible for nonmeningitis disease. This observation of stable genetic changes that have occurred in the post-penicillin era reflects extremely rapid and effective adaptation to the global selection pressure exerted by β-lactam antibiotics. Even in some relatively remote geographic areas, one can readily isolate from healthy individuals a high proportion of nasopharyngeal pneumococcal PBP gene mutants with elevated MICs of penicillin (137–139). Even in the absence of antibiotic usage data for a given region, these observations provide direct evidence of prior β-lactam selection pressure in the community or in individuals (140, 141) and suggest the fitness of these resultant emergent mutants in their natural reservoir. While pneumococcal isolates with penicillin MICs in the range of 0.12 to 2 μg/ml are considered resistant for meningitis, these values are not relevant for the treatment of other invasive infections (142). Nonetheless, treatment recommendations should take into account the need to prevent resistance from developing since there is little understanding of the evolution of these phenotypes in the carriage reservoir and how it shapes the evolution of the species as a whole. Patients with pneumococcal bacteremia who had been treated with either β-lactams or macrolides within the previous 6 months were highly likely to be infected with a penicillin-resistant strain, indicative of a prior causative selective effect (141).

The vast reservoir of related mitis group streptococci in the upper respiratory tract has repeatedly provided the genetic basis for the emergence of β-lactam-resistant pneumococcal strains. Although normally harmless, upper respiratory tract carriage of pneumococci, especially in the nasopharynx, is an obligatory step in the various types of infections caused by this major pathogen (143, 144). Pneumococcal carriage most frequently occurs in children, with very high carriage rates in certain undeveloped areas and lower rates in many other regions. The URT is highly colonized with multiple species of closely related members of the *Streptococcus mitis* group, with which pneumococci freely exchange core genetic loci that contribute to β-lactam resistance (145).

In this environment, microbial species are often subjected to various, and often nonlethal, concentrations of β-lactams. Pneumococci are naturally transformable by the uptake and chromosomal integration of DNA from other pneumococcal strains and other related nonpathogenic mitis group species. Compatible with the importance of recombination in the evolution of β-lactam-resistant pneumococci is the fact that even within neutral housekeeping genes, the majority of observed allelic changes have occurred through horizontal recombination events rather than through intrachromosomal mutation events (146). There is irrefutable observational evidence that β-lactam resistance in pneumococci has repeatedly occurred in nature through recombination events with highly related nonpathogenic fellow members of the *Streptococcus mitis* group. Since colonization with closely related nonpneumococcal species occurs in all humans, it is logical that β-lactam nonsusceptibility would arise first in such strains prior to its appearance in pneumococci. This prediction is supported by the remarkable observation that virtually all clinical isolates that display reduced penicillin susceptibility (MICs of >0.5 μg/ml) are characterized by one or more mosaic PBP1a, -2b, and -2x genes. Within these PBP genes, there are clearly discernible regions of sequence that clearly originated within nonpneumococcal mitis group species (96–100). In contrast, within basally penicillin-susceptible wild-type pneumococci, there is very little sequence variation between different alleles of the same PBP gene. *Streptococcus mitis* and *Streptococcus oralis* have been identified as PBP gene sequence donors for variant PBP2x, -2b, -1a, and -2a (108, 147, 148) found in certain PNSP strains. The observation of mosaic pneumococcal PBP genes is consistent with the finding that commensal mitis group nonpneumococcal species, which are also normally β-lactam susceptible, exhibit high β-lactam MICs in areas where rates of pneumococcal resistance are also high (149). It follows that optimal pneumococcal recipients for the development of β-lactam resistance would have optimal exposure to penicillin-nonsusceptible resistant DNA donors in the human URT. The pneumococcal recipient strain would be an efficient long-term colonizer and also highly transformable. These features, while obviously conducive for the development of the β-lactam nonsusceptible (NS) phenotype, are also conducive for the acquisition of other resistance determinants through horizontal transfer events.

There are relatively few highly resistant β-lactam-resistant pneumococcal lineages, although they constitute the vast majority of β-lactam-resistant (and multiple-drug-resistant [MDR]) pneumococci recovered from infections (25). Even incremental

changes that increase β-lactam resistance are relatively rare, as evidenced by the long-observed low-level β-lactam resistance phenotypes of certain well-known common clonal complexes, such as serotype 19A/sequence type 199 (ST199), serotype 23A/ST338, and serotype 15A/ST63 (PMEN-25) (50). The relatively small number of β-lactam-resistant lineages may be due to the requirement for mutation events within multiple PBP genes. These PBP genes are not situated closely on the chromosome, presumably making cotransfer of mutated alleles from related resistant streptococcal strains relatively infrequent. These resultant mutated enzymes with poor binding affinity for an analog of their natural cell wall component substrate must somehow still carry out essential roles for normal growth and division. For these reasons, β-lactam resistance is an excellent measure of the adaptability of this species.

Our experiences attempting to analyze oropharyngeal specimens for pneumococcal species- and serotype-specific DNA sequences highlight the extremely high abundance and diversity of closely related homologs of pneumococcal genes (150), consistent with the nonpneumococcal mitis group streptococci providing a huge reservoir for selectable markers transferrable to pneumococci through homologous recombination. The initial acquisition of penicillin resistance in mitis group streptococci is presumably also a low-frequency event that requires multiple changes to occur within unlinked essential genes, and the mutational combinations that occur must be tolerated in order for a given strain to emerge to detectable numbers. Since there is much more genetic substrate for PBP gene changes to occur within the more common commensal nonpneumococcal mitis group members of the URT, the inevitable appearance of nonpneumococcal gene segments within β-lactam-nonsusceptible pneumococcal PBP genes is easier to reconcile. Although it is intuitive that many of the key point mutations that confer pneumococcal β-lactam resistance have in fact occurred in nonpneumococcal species, it is very difficult to quantitate the accumulation of point mutations within specific pneumococcal strains after the initial resistance-conferring recombination events from nonpneumococcal donors. Certain important point mutations have been localized to specific hot spots mapped on PBP crystal structures that have been frequently found in clinical isolates (101–103). For example, the Q552E and T550A PBP2x substitutions (117, 151, 152) are widespread in several different clones of resistant pneumococci. In our laboratory, through genomic analysis, we have localized 27 transpeptidase positions within the 3 key PBPs, where an amino acid change relative to a wild-type sensitive pneumococcal strain occurs within each of the ~200 highly resistant (penicillin MIC of ≥4 μg/ml) pneumococcal clinical isolates that we have examined (Y. Li, B. J. Metcalf, S. Chochua, P. A. Hawkins, R. Gierke, T. Pilishvili, L. McGee, and B. W. Beall on behalf of the Active Bacterial Core surveillance team, unpublished data).

Dissection of a β-Lactam Resistance Pathway in the Laboratory

A recent study carefully employed genomic analysis of laboratory strains derived from stepwise transformations of a sensitive pneumococcal strain with genomic DNA from a naturally occurring highly penicillin-resistant *S. mitis* strain (124, 153). This study provided a glimpse of the genetic complexity of the transfer of β-lactam resistance from a highly resistant *S. mitis* strain to a pneumococcal donor within a controlled laboratory setting. In this study, a total of 78 different genes were affected by 36 different recombination events that occurred during the four consecutive transformation events that were required to confer high-level penicillin resistance (124). As expected, mosaic PBP genes were essential in the stepwise process. In particular, *pbp2b* and *murM* sequences from this *S. mitis* strain were essential for the final high level of penicillin resistance. Certain mosaic sequences were observed among a subset of pneumococcal clinical isolates, confirming the key role of *S. mitis* in the evolution of pneumococcal β-lactam resistance.

It is likely that next-generation genome sequencing (NGS) will be incorporated routinely for the analysis of pneumococcal disease isolates collected through national surveillance programs. This will lead to a more detailed understanding of the different pathways leading to pneumococcal β-lactam resistance. NGS will soon lead to bioinformatic approaches that will allow immediate deduction of the various MICs of different β-lactam antibiotics exhibited by this organism. From a practical clinical perspective, we have found that accurate predictions of MICs of 5 different β-lactam classes for the vast majority of invasive isolates can be simply predicted through an automated system that compiles 3 allele combinations of transpeptidase amino acid sequences of PBP1a, PBP2b, and PBP2x (50; our unpublished data).

Intrapneumococcal Exchange of β-Lactam Resistance Loci

Interspecies recombination events involving pneumococcal recipients result in the cotransformation of multiple unlinked genes, some of which are intrinsically required for resistance and some of which may or may not provide compensatory mutations that relieve deleterious effects on cell fitness (115). Although many different mosaic pneumococcal PBP genes have been associated with β-lactam resistance (48), existing multilocus sequence typing (MLST) data for genes that are under neutral selection indicate that most recombinant pneumococcal alleles reflect intraspecies recombination events. It is insightful that of the seven MLST targets used for typing of pneumococci, only one (*ddl*) is frequently associated with sequences of a nonpneumococcal origin and is due to its proximity to *pbp2b* causing it to be frequently cotransferred into pneumococci (154). Not coincidentally, nearly all such divergent *ddl* alleles are associated with a β-lactam NS phenotype.

Recombinational exchange events at the *cps* loci have been shown to have occurred often between pneumococcal strains (49, 98, 155, 156), providing a mechanism of immune escape from serotype-specific antibody. The first account of pneumococcal serotype switching was described in 1928 (157), leading to the discovery of the transforming substance in 1944 (158). Through transforming a sensitive strain with chromosomal DNA from a resistant strain and selecting for β-lactam resistance, another hitchhiking effect was observed: the cotransfer of the large *cps* locus and the closely flanking *pbp1a* and *pbp2x* genes (159). This experiment demonstrated the potential for the appearance of serotype-switching events through selection for β-lactam resistance. It logically follows that the reverse is also possible through immune selection of serotype-switching events. Soon after the implementation of PCV7, genotyping results from a set of invasive serotype 19A isolates suggested that a cotransformation event of this nature had occurred within a serotype 4 recipient strain, where an intermediately penicillin-resistant non-PCV7 strain served as a donor of the *cps19A cps* locus along with the flanking *pbp1a* and *pbp2x* alleles (160). This single cotransfer of both cap-

sular serotype and mosaic PBP alleles was verified through genome sequence analysis of multiple progeny as well as identified donor and recipient strains (161). Multiple serotype 19A/CC695 strains of this nature were subsequently detected within many sites, all of which appeared to have occurred within the post-PCV7/pre-PCV13 period (112, 162). Subsequent genomic analysis demonstrated that the simultaneous transfer of multiple unlinked, and often large, recombinational fragments (up to at least 44 kb) had occurred simultaneously with the vaccine escape recombination event (163). At least five independent serotype switch events involving type 4 recipients and type 19A donors were identified by a combination of MLST and genomic analyses. It was found that one of these variants disseminated west across the United States (163). During the late 2000s, this serotype 19A/ST695 variant became the third most prevalent serotype 19A strain (112). Through NGS-based typing in the post-PCV13 period, we detected two of these independent variants among the few remaining serotype 19A clones (50). Our current NGS typing pipeline verified the identification of the originally described donors (161, 163) through recognition of specific MLST and PBP alleles within the two progeny strains (50). We detected linked *cps19A* and cotransferred *pbp2x* from the donor and in addition detected that a second unlinked recombination event had occurred, resulting in the cotransfer of *pbp2b* and *ddl* alleles that were also highly associated with the specific serotype 19A donor strain.

The ramifications of these and similar independent findings (164) are profound: one recombinational event (e.g., one that confers a different serotype and/or resistance feature) can unpredictably result in conferring additional unselected and chromosomally unlinked traits (165). It is likely that genomic traits that contributed to the success of the major serotype 4 CC695 in the pre-PCV7 era (111, 166) contributed to the success of the more resistant serotype 19A ST695 variant during the 2000s (112).

RESISTANCE TO MACROLIDES AND LINCOSAMIDES

Macrolide antibiotics (e.g., erythromycin, clarithromycin, and azithromycin) have been widely used to treat community-acquired respiratory tract infections globally. In more recent years, resistance to macrolides in *S. pneumoniae* has increased substantially, and in many parts of the world today, macrolide-resistant pneumococci are now more common than penicillin-resistant *S. pneumoniae* (167). Erythromycin resistance has become more common in U.S. IPD isolates than penicillin resistance (MIC ≥2 μg/ml) since PCV13 implementation, due largely to the removal of penicillin-resistant strains (50). The most common IPD serotypes in children <5 years of age during 2013, in order of incidence, were serotypes 15B/15C (included together since they interconvert), 33F, 22F, and 35B and together comprised 48% of IPD cases (50). Nearly half (49%) of these isolates were erythromycin resistant (with serotype 35B isolates being predominantly penicillin nonsusceptible with an MIC of 2 μg/ml), predominantly due to the *mef* determinant (efflux mechanism described below). Both macrolide resistance proportions among isolates and resistance mechanisms vary considerably depending on the country and vaccine implementation. Reported proportions of erythromycin-resistant pneumococci are ~15% in Latin American isolates, 30.2% in U.S. isolates (http://www.cdc.gov/abcs/reports-findings/survreports/spneu14.html), and as high as 80% among isolates in Southeast Asian countries (168). Besides conjugate vaccination status, these differences may reflect the variability in antibiotic prescribing rates among various countries. Although macrolide resistance accounted for far more IPD cases in 2009 than in 2013 (169), the proportion of erythromycin-resistant cases was actually lower (24.9% relative to 28.2% [see http://www.cdc.gov/abcs/reports-findings/surv-reports.html for ABCs data from 1997 to 2013]). It is also important to note that ~28% of erythromycin-resistant cases during 2013 were also clindamycin resistant (almost all were constitutively resistant). Clindamycin resistance during 2013 was observed only among erythromycin-resistant strains. (Note that in the above-mentioned URLs, we refer to ABCs, the Centers for Disease Control and Prevention Active Bacterial Core surveillance program, a population-based laboratory and active surveillance system for bacterial pathogens at 10 U.S. sites.)

Macrolides are microbiostatic agents that inhibit bacterial protein synthesis through binding to the 23S rRNA component of the 50S ribosomal subunit. Macrolide resistance in *S. pneumoniae* is mediated by two major mechanisms: target modification and active efflux.

Target Modification

The most common form of target modification in macrolide-resistant pneumococci is ribosomal modification by 23S rRNA methylation, encoded primarily by the *erm*(B) gene. This methylation mostly confers constitutive high-level resistance to 14-, 15-, and 16-member macrolides as well as resistance to lincosamides and type B streptogramins (MLS_B phenotype) (170). In a small percentage of isolates, *erm*(B) confers clindamycin resistance that is inducible by low concentrations of a macrolide.

Although rare, a methylase encoded by the *erm*(A) subclass gene *erm*(TR) has also been shown to confer MLS_B resistance in pneumococci (171, 172). In pneumococci, Tn*916* family transposons comprise most *erm*(B)-carrying mobile genetic elements. A number of Tn*916* derivatives carrying *erm*(B) have been described (Tn*1545*, Tn*3872*, Tn*6002*, and Tn*6003*), and the *tet*(M) gene is typically also carried by these transposons (173). A large percentage of current macrolide-resistant *S. pneumoniae* isolates (31.5% from 2013 [unpublished ABCs data]) are therefore also resistant to tetracycline; however, some recent studies have shown Tn*916*-related elements where the *tet*(M) gene is present in a silent form (174). Other less commonly described target modifications are point mutations in domains II and V of 23S rRNA and in riboproteins L4 and L22 (175).

Efflux Mechanism

Lower-level erythromycin resistance affecting only 14- and 15-membered macrolides, but not lincosamides or streptogramins (M phenotype), is associated with efflux pumps. Active drug efflux is mediated by *mef* class genes, which include several variants: *mef*(A) and *mef*(E), which are the most common and share 90% sequence identity, and the rare variant *mef*(I), which has been described in only two clinical isolates from Italy (176). The genes for the three variants have been associated with different, but related, mobile genetic elements: the *mef*(A) gene on the defective transposon Tn*1207.1* or the closely related transposon Tn*1207.3* (177), *mef*(E) on the "macrolide efflux genetic assembly" (*mega*) element (178), and *mef*(I) on a nonmobile composite structure designated the 5216IQ complex (179).

Dual Mechanism for Macrolide Resistance

In the last decade, isolates with the dual resistance mechanism [both *erm*(B) and the *mef* gene] have been increasingly reported from the United States, Canada, South Africa, Mexico, and a number of countries in Asia and Europe (172, 180). The prevalence of isolates in the United States carrying both genes increased as a result of the diversification and expansion of lineages of Taiwan^{19F}-14 (PMEN-14 clone) following conjugate vaccine introduction. This was especially true of the major serotype 19A ST320 variant prior to PCV13 implementation (162), as described in more detail below. This strain complex and the dual mechanism accounted for ~47% of macrolide resistance among pediatric isolates during 2009 (50).

FLUOROQUINOLONE RESISTANCE

With increased rates of resistance to macrolide and β-lactam antibiotics among strains of *S. pneumoniae*, fluoroquinolones are now among the first choices for empirical treatment of respiratory tract infections and pneumonia in some countries. While the development of fluoroquinolone resistance has been linked with fluoroquinolone use (181), the rates of resistance in *S. pneumoniae* remain relatively low (<1% in the United States and <3% in Europe) (182), although higher rates have been reported in Asia (10.5%) (183, 184) and Canada (7.3%) (185). Resistance to fluoroquinolones can also develop during treatment, and there are several reports describing treatment failures in pneumococcal infections where fluoroquinolones were used (186, 187). These cases were primarily elderly patients with chronic lung disease, a patient population that is frequently exposed to fluoroquinolones and in which higher rates of resistance have been reported (188).

Fluoroquinolones target type II DNA topoisomerase enzymes (DNA gyrase and topoisomerase IV), which are vital for DNA supercoiling and chromosome segregation. Each of the enzymes consists of subunits that are structurally related to each other. The DNA gyrase subunits *gyrA* and *gyrB* are homologous to the *parC* and *parE* subunits of type IV topoisomerase. Fluoroquinolones inhibit DNA synthesis by binding to target sites within these proteins. Ciprofloxacin and levofloxacin target primarily topoisomerase IV (subunit ParC), while the principal target of moxifloxacin is DNA gyrase (subunit GyrA) (189, 190).

In pneumococci, two mechanisms that contribute to fluoroquinolone resistance have been identified, namely, target alteration and active efflux.

Target Alteration

Resistance mediated by target modification results from the alteration of the fluoroquinolone-binding site due to the stepwise accumulation of mutations in the quinolone resistance-determining region (QRDR) of the genes encoding the DNA gyrase (primarily *gyrA*) and DNA topoisomerase IV (primarily *parC*) subunits. Strains with mutations in only a single target enzyme often have susceptible phenotypes (first-step mutants) but present with an elevated risk of acquiring additional mutations during fluoroquinolone treatment, resulting in resistance (191). These so-called first-step mutants are considered precursors of resistant strains (191). Single mutations within the QRDRs of either *parC* or *gyrA* have also been frequently associated with clinically relevant fluoroquinolone resistance (192–194).

Studies focusing on the genetic basis of fluoroquinolone resistance in *S. pneumoniae* have reported that the most frequent mutations are in the *parC* S79 and *gyrA* S81 codons (192–194). Several other substitutions have been described in the QRDRs, but only a few have been reported to confer resistance through *in vitro* studies: *gyrA*-E85K, *gyrA*-Q118K, *gyrB*-E474K, *parC*-A63T, *parC*-D83N, *parE*-E474K, and *parE*-D435N or -H (193, 195, 196). Other frequently described substitutions are K137N in *parC* and I460V in *parE*, which are commonly found in susceptible strains and appear not to contribute to fluoroquinolone resistance (197). Our recent data for ABCs isolates from 2015 are in agreement with previous surveillance data that demonstrate the association of specific causative QRDR substitutions with a wide range of increased fluoroquinolone MICs (194), quite likely due to the added effects of separate mechanisms such as increased active efflux (198–202).

Efflux Pumps

A second mechanism contributing to nonsusceptibility to fluoroquinolones in some isolates is an increase in active efflux. Quinolones, like ciprofloxacin, that are small molecules seem to be more affected by active efflux than larger molecules such as moxifloxacin (199). In contrast to the *mefA* gene conferring macrolide resistance, the efflux mechanisms in fluoroquinolone resistance are not well characterized and have been reported mostly in isolates with low-level quinolone resistance. Overexpression of the ABC transporter genes *patA* and *patB*, which are also linked to stress responses, have been reported to confer efflux-mediated resistance to fluoroquinolones in pneumococci (199). Little is known about the expression regulation mechanism, but the efflux pump can be blocked by the plant alkaloid reserpine and, to a lesser degree, by verapamil (200). Efflux may not confer complete resistance but may be able to decrease the levels of intracellular fluoroquinolone to sublethal concentrations, fostering the occurrence of QRDR mutations (201).

Horizontal Gene Transfer and the Clonal Concept

In contrast to β-lactam resistance, the role of horizontal gene transfer and recombination in the evolution of fluoroquinolone resistance is uncertain. At least within the United States, where fluoroquinolone resistance has been rare for decades, it seems to have a modest role. Both intra- and interspecies transfers of fluoroquinolone resistance loci have been found to occur *in vivo*, but the impact of such events on fluoroquinolone resistance in the species might be small. *In vitro* models showed a higher frequency of recombination of QRDRs between viridans group streptococci and *S. pneumoniae* than of spontaneous mutations (202); however, this rate of recombination does not appear to be replicated in *vivo* (203). Reported studies addressing this question of recombination have estimated horizontal gene transfer in 0 to 11% of fluoroquinolone-resistant clinical isolates, and interestingly, this ratio appears to be higher for respiratory isolates than for invasive isolates (204–207).

Fluoroquinolone resistance has been documented in a number of international pneumococcal clones that have been associated with resistance to both penicillin and macrolides (208, 209). However, the role that clonal expansion plays in the increased frequency of fluoroquinolone resistance is controversial, with studies placing different significances on its importance. There has been little indication of clonal expansion of individual fluoroquinolone-resistant clones within the United States, where we screen for, but rarely recover, fluoroquinolone-resistant IPD isolates from an IPD surveillance population of >30 million individuals

(http://www.cdc.gov/abcs/reports-findings/survreports/spneu14.html). The increased prevalence of levofloxacin resistance documented in Hong Kong between 1995 and 2001 was suggested to be associated with the spread of strains related to the Spain^{23F}-1 clone. However, numerous studies have shown that clonal dissemination has not been a major contributor to the increase of fluoroquinolone resistance (209–211). Data on pneumococci resistant to levofloxacin from 25 countries, analyzed through the PROTEKT study (1999 to 2000), showed that while 34% were of the Spain^{23F}-1 lineage, the majority of isolates were genetically unrelated (211). These reports suggest that during this period, both the emergence of newly resistant strains and the clonal dissemination of strains contributed to the spread of fluoroquinolone resistance.

RESISTANCE TO OTHER ANTIBIOTICS

Tetracycline Resistance

Tetracyclines are broad-spectrum bacteriostatic drugs previously used in clinical practice and shown to be active against *S. pneumoniae*. Nonsusceptibility to tetracyclines remains the most frequently observed resistance phenotype in some countries, perhaps reflecting patterns of previous antibiotic usage (212). In pneumococci, resistance to tetracycline occurs by the protection of the bacterial 30S ribosome subunit against antibiotic binding by the acquisition of *tet* genes (213, 214). The acquisition of *tet*(M) is the most common mechanism, with the *tet*(O) gene rarely being reported in pneumococci. In streptococci, *tet*(M) is most often located on mobile conjugative transposons of the Tn*916*-Tn*1545* family and large composite structures like Tn*5253* and Tn*3872*. A recent study discovered the oldest known examples of two different Tn*916*-like *tet*(M)-containing elements identified among *S. pneumoniae* isolates from 1967 and 1968 (212). These transposons often contain genes for resistance to other classes of antibiotics, such as *erm*(B), and the selection of these transposons by macrolide antibiotics could possibly explain the continued persistence of tetracycline resistance. Comparison of *tet*(M) sequences among 8 multidrug-resistant isolates representing 5 diverse species revealed a high degree of variation indicative of mosaicism traced to two distinct alleles (215). In pneumococci, there is evidence of both the clonal distribution of a small number of divergent selected alleles and the horizontal movement of mobile elements carrying the *tet*(M) gene (216, 217).

Rifampin Resistance

The use of rifampin in combination with either β-lactam antibiotics or vancomycin has been recommended for the treatment of meningitis caused by multidrug-resistant *S. pneumoniae*. Rifampin has been used in combination therapy for the treatment of tuberculosis and infections due to resistant staphylococci. It has also been used increasingly for prophylaxis against *Neisseria meningitidis* and *Haemophilus influenzae* type b exposure. The rates of resistance to rifampin reported among pneumococcal isolates are relatively low at present and vary between 0.1% and 1.5% (218, 219). We observed 5 rifampin-resistant (MIC of >2 μg/ml) isolates among a total of 2,932 (0.17%) IPD isolates recovered from the ABCs program in 2013 (our unpublished data). Rifampin resistance occurs due to alterations in the *rpoB*-encoded β-subunit of RNA polymerase and has been described in several bacterial species. In pneumococci, resistance has been linked primarily to mutations in regions I and II of *rpoB* (220). *rpoB* sequence data indicated past horizontal transfer from related mitis group strains in rifampin-resistant pneumococci, which is a common theme for certain pneumococcal resistances, such as resistance to β-lactams, co-trimoxazole, and rifampin, that rely upon alterations within essential core genome determinants.

Chloramphenicol Resistance

In *S. pneumoniae*, resistance to chloramphenicol is due to enzymatic inactivation of the antibiotic by the production of a chloramphenicol acetyltransferase (CAT), encoded by the *cat* gene. This *cat* gene is carried on the conjugative transposon Tn*5253*, a composite structure made up of the tetracycline resistance transposon, Tn*5251*, and Tn*5252*, which carries the *cat* gene (221). Chloramphenicol-resistant strains have been reported to contain sequences homologous to cat_{pC194} and other flanking sequences from *S. aureus* plasmid pC194 (222).

Trimethoprim-Sulfamethoxazole (Co-Trimoxazole) Resistance

Trimethoprim and sulfamethoxazole antibiotics are broadly used in combination as the drug co-trimoxazole. Co-trimoxazole has been used as a treatment option for a range of pneumococcal diseases, particularly in children, because it is relatively inexpensive and generally effective. Resistance to co-trimoxazole has increased substantially worldwide, with recent studies showing rates ranging from 19% in Europe to 50% in Africa (in HIV-associated disease) to >60% in parts of Asia (22, 223, 224). Co-trimoxazole resistance is often accompanied by resistance to other antibiotics, especially penicillin. Trimethoprim resistance in pneumococci results from a single amino acid substitution (Ile100→Leu) in the dihydrofolate reductase (DHFR) protein (225) encoded by *folA* and is often associated with mosaic alleles. Additional mutations have been reported, which appear to modulate these alterations, affecting the affinity of DHFR for its natural substrates and thereby enhancing resistance (226). Resistance to sulfonamides is most often associated with localized 1- or 2-codon insertion mutations within the *folP* gene encoding dihydropteroate synthase (DHPS). Numerous studies have reported the occurrence of single- and/or multiple-amino-acid substitutions in DHPS of sulfonamide-resistant clinical pneumococcal isolates (227–229). In Africa, where sulfadoxine-pyrimethamine (Fansidar) therapy for malaria is common, studies have shown that its use contributes to increased co-trimoxazole resistance in pneumococci (230). Previous information revealed that both mutations (*folA*-I100L and *folP* insertion) are required for full co-trimoxazole (trimethoprim-sulfamethoxazole) resistance (MICs of >4/76 μg/ml) (231). We also find that most strains with intermediate co-trimoxazole resistance (MICs of 1/19 to 2/38 μg/ml) either contain a *folP* insertion or contain the *folA*-I100L mutation, with full resistance requiring both *folA* and *folP* mutant alleles (50).

Ketolide Resistance

Ketolides are a class of semisynthetic agents derived from erythromycin A and designed specifically to act against macrolide-resistant organisms. They bind to a secondary region on domain II of the 23S rRNA subunit and therefore have a stronger binding affinity for the ribosome, thereby maintaining activity against most erythromycin-resistant pneumococci. Telithromycin was the first ketolide drug approved for clinical use; however, safety

issues have limited the clinical utility of this drug (232). Both cethromycin (ABT-773) and solithromycin (CEM-101), a novel fluoroketolide, have shown improved activity against macrolide-resistant as well as telithromycin-intermediate and telithromycin-resistant organisms (233–235). This enhanced potency shows promise for future clinical use for these compounds, subject to pharmacokinetic/pharmacodynamic, toxicity, and animal infection model study findings.

High-level telithromycin resistance in *S. pneumoniae* has been experimentally generated by mutations in domain II or V of 23S rRNA and ribosomal proteins L4 and L22 (236), and telithromycin-resistant mutants are easily generated *in vitro* from *erm*(B)-positive strains that exhibit mutations within the region upstream of *erm*(B) (237). In contrast, clinical telithromycin resistance in *S. pneumoniae* remains rare. Farrell et al. reported that among a global collection of 13,874 *S. pneumoniae* isolates (1999 to 2003), 10 were telithromycin resistant, with MICs of ≥4 μg/ml, and they all contained the *erm*(B) gene (238). Mutations in 23S rRNA, L4, and L22 have also been reported in clinical telithromycin-resistant isolates (239, 240), and a combination of mutated genes can result in higher telithromycin resistance than a mutation in only a single gene (241, 242). Wolter and colleagues demonstrated that *erm*(B) with a deletion in the leader sequence was responsible for high-level telithromycin resistance in a strain isolated in Canada in 2007 (243).

Oxazolidinone Resistance

Linezolid is the first antibiotic in the oxazolidinone drug class that was approved for clinical use in 2000 for the treatment of nosocomial and community-acquired pneumonia. Linezolid blocks protein synthesis by binding to the 50S subunit of the bacterial ribosome via interactions with the central loop segment of domain V of the 23S rRNA. To date, linezolid-nonsusceptible pneumococcal strains are rare (238, 244). Recent data from the U.S. LEADER and global ZAAPS surveillance systems show no linezolid-nonsusceptible isolates among 2,150 *S. pneumoniae* isolates tested in 2011 (245, 246), and a review of ABCs data revealed that of 45,165 isolates tested during 1997 to 2014, only 11 (0.02%) are recorded as being nonsusceptible to linezolid (our unpublished data). Nonsusceptibility to linezolid has also been rarely reported among clinical isolates of staphylococci and enterococci, and resistance in these organisms has been found to be conferred by mutations in domain V of 23S rRNA (247). For pneumococci, Wolter et al. (248) described two clinical isolates with decreased susceptibility to linezolid (MICs of 4 μg/ml) that contained 6-bp deletions in the *rplD* gene encoding the riboprotein L4. The *rplD* deletion alleles were also found to confer a novel mechanism of simultaneous resistance to macrolides, oxazolidinones, and chloramphenicol. A more recent study identified 2 additional linezolid-nonsusceptible pneumococci with mutations and deletions within the *rplD* gene from the United States (249) in the ABCs program. Whole-genome sequencing of linezolid-resistant laboratory-generated mutants also revealed a role in resistance for a 23S rRNA methyltransferase (spr0333) and for the ABC proteins PatA and PatB (250). A proteomic and transcriptomic screen suggested increased energy requirement needs associated with the burden of resistance in these laboratory-derived mutants (251).

Expanded-spectrum oxazolidinones like tedizolid, which is a protein synthesis inhibitor, are in clinical development for the treatment of Gram-positive infections. Tedizolid has demonstrated potent *in vitro* activity against penicillin-resistant *S. pneumoniae*, including linezolid-resistant strains (252).

Streptogramin Resistance

Quinupristin-dalfopristin is a 30:70 combination of a type B and a type A streptogramin. The two components target the late and early stages of bacterial protein synthesis, respectively, and thus have a synergistic inhibitory effect. Resistance to quinupristin-dalfopristin among Gram-positive cocci is rare. Two clinical isolates among 8,837 (0.02%) pneumococcal isolates were identified in 2001 to 2002 with MICs of 4 μg/ml. They both had a 5-amino-acid tandem duplication (RTAHI) in the L22 ribosomal protein gene (*rplV*) preventing synergistic ribosomal binding of the antibiotic (253).

CORESISTANCE

It has long been recognized that pneumococcal strains with elevated β-lactam MICs are most often resistant to additional antibiotics (22, 70, 90). The factors that lead to multidrug resistance are complex; however, key observations have been made over the years since this was initially observed (22). Pneumococci were uniformly sensitive to antibiotics prior to their introduction, and it follows that antimicrobial resistance has been directly linked to their usage. There are numerous studies describing that previous recent usage of β-lactams increases the risk for β-lactam-nonsusceptible systemic and nonsystemic pneumococcal infections (142, 254, 255). Suboptimal dosing for long periods of time increases the likelihood of carriage of β-lactam-nonsusceptible strains (256), and the presence of recent β-lactam selective pressure allows an advantageous environment for survival, spread, and subsequent infections caused by such strains (257).

The genesis of the β-lactam-resistant parental strains of the major MDR complexes likely required multiple unlinked and rare recombination events. Certain strains have been identified as being particularly efficient in recombination. In one study, it was found that serotype 3 and 18C strains were much less transformable with a selective marker than were serotype 6B, 14, 19F, 9V, and 23F strains (258). Not coincidentally, these 5 serotypes accounted for most penicillin-nonsusceptible IPD strains prior to PCV7 implementation, and penicillin-nonsusceptible isolates of serotypes 3 and 18C have rarely been identified within the ABCs system (our unpublished data). Interspecies and intraspecies genetic transformations probably play a very large role in most pneumococcal antimicrobial resistance mechanisms. Recombination events have been shown to encompass very large chromosomal fragments. In extreme instances, recombinational fragments of >70,000 bp in length have been implicated (259). Besides β-lactam resistance, resistance gene mosaicism has been demonstrated for several different core genome determinants, including those that encode resistance to trimethoprim, sulfonamides, and rifampin (109, 220, 225, 228).

A recent observational study identified a hyperrecombining set of pneumococcal strains based upon their higher degree of mosaicism within housekeeping genes and found a striking correlation of this strain set with resistance to β-lactams and other antibiotics (260). Unexpectedly, there was no strong association of these strains with resistant conjugate vaccine serotypes, suggesting that despite strong associations of only certain serotypes with β-lactam resistance, reemergence of resistance or other adaptations obtained through this increased propensity to incorporate foreign

DNA are possible in the post-PCV13 era. Recently, an appendage on the surface of competent pneumococci was discovered and described as a type IV pilus required for genetic transformation (261). This structure, composed of the ComGC pilin, was shown to capture extracellular DNA with high efficiency in two divergent lineages and is likely to be expressed by most or all pneumococcal strains (261).

Efficient colonization allows for increased exposure to cocolonizing resistant bacterial strains, increasing the opportunity for interstrain homologous-recombination events that can lead to the incorporation of resistance determinants carried on the core genome (i.e., resistance to β-lactams, fluoroquinolones, and co-trimoxazole) and also selecting for integrative transposable elements (i.e., resistance to macrolides, lincosamides, tetracycline, and chloramphenicol). Subsequent continued colonization allows more exposure to sublethal concentrations of β-lactams, macrolides, and other antibiotics, selecting for the incremental accumulation of resistance-conferring genetic determinants. The ability to withstand a variety of selective agents allows still further opportunities as a recipient of additional accessory resistance genes from horizontal genetic transfer events. Besides selectively driving pneumococci to acquire specific resistance mechanisms, certain aminoglycosides, fluoroquinolones, and the DNA-damaging antibiotic mitomycin C actually trigger pneumococcal competence for genetic transformation through induction of the competence (*com*) regulon (262). The authors of that study proposed that pneumococcal competence is a stress response that seems analogous to the similarly induced SOS response that is lacking in pneumococci but very well characterized in *Escherichia coli*. Both responses result in increased levels of RecA, which is a key enzyme required for homologous recombination between host and recipient DNA. This finding gives further insight into the incompletely understood hazards of inappropriate antimicrobial treatments for all infectious diseases. For example, recent genomic evidence indicated that the use of tetracycline in the 1940s rapidly altered the normal population structure of the opportunistic pathogen *Streptococcus agalactiae* and "fixed" these strains within human hosts (263).

Serotypes such as serotypes 1, 5, and 7F are considered highly invasive since they are well represented in global IPD cases but are less commonly encountered in URT carriage in children (264). The possibility that isolates of these serotypes are not efficient colonizers is consistent with the observation that they are also represented primarily by strains that are uniformly sensitive to β-lactams and are also usually sensitive to other antibiotics. Not surprisingly, the majority of β-lactam-nonsusceptible strains in the pre-PCV7 era in the United States were of serotypes commonly carried by healthy children (serotypes 6B, 9V, 14, 19F, and 23F) and are still commonly carried in many unvaccinated or undervaccinated populations (264–267).

An increased selective advantage for dual macrolide and penicillin resistance was indicated by the much more rapid increase in the prevalences of invasive strains that were resistant to both antibiotics than of singly resistant strains revealed from U.S. surveillance data during the period from 1996 to 1999 (268). Coincidentally, the prevalence of strains resistant to erythromycin was increasing more rapidly among penicillin-resistant pneumococci, and resistance to penicillin was increasing only among erythromycin-resistant strains. During this period in the United States, much of this coresistance to erythromycin and penicillin may have originated from the singly erythromycin-resistant serotype 14 PMEN-9 strain (England^{14}-9), since closely related penicillin- and erythromycin-resistant isolates within this clonal complex caused a large percentage of IPD cases during the pre-PCV7 implementation period (111, 166) (see the PMEN-9 complex in Fig. 1). Thus, it appears likely that emergent strains that are singly resistant to erythromycin or penicillin have a significant survival advantage in carriage and a better opportunity to survive and acquire additional resistance mechanisms. Notably, over a 20-year period (1994 to 2013), 5,807 (75.2%) of 7,724 ABCs isolates with penicillin MICs of ≥2 μg/ml were also resistant to erythromycin (unpublished ABCs data). Of 7,882 ABCs isolates with intermediate penicillin resistance (MICs of 0.12 to 1.0 μg/ml), 3,681 (46.7%) were erythromycin resistant. In contrast, only 3,760 (7.5%) of 49,940 penicillin-susceptible isolates were erythromycin resistant. While ~24% (15,607/65,646) of the cumulative ABCs isolates were penicillin nonsusceptible, 22% (13,248/65,646) were erythromycin resistant. Of these 13,248 erythromycin-resistant isolates, 9,488 (71.6%) were penicillin nonsusceptible, which accounts for 60.8% of the total number of penicillin-nonsusceptible isolates.

β-Lactam-resistant pneumococci are also highly likely to be resistant to additional antibiotics such as lincosamides [most often coexpressed with macrolide resistance from *erm*(B)], tetracycline (*tetM*), and co-trimoxazole (core genome *folA* and *folB* mutations conferring resistance to trimethoprim and sulfonamides, respectively). This is also true of isolates with intermediate resistance levels. For example, nearly 80% of the ABCs penicillin-intermediate (MICs of 0.12 to 1.0 μg/ml) pediatric isolates from 2012 were resistant to at least one other antibiotic, with 52% being resistant to macrolides (unpublished ABCs data). Table 2 gives a general description of the genetic lineages that have comprised the majority of penicillin-resistant (MIC of ≥2 μg/ml) strains in the ABCs program since 1998 (Fig. 1). It is highly significant that almost all of these strains are coresistant to macrolides by virtue of containing the *mefA-mefE* and/or *erm*(B) determinant.

FITNESS COSTS

The emergence and stability of antibiotic resistance are complex biological processes driven by various factors, including antibiotic use, the rate of resistant mutant formation, the fitness cost imposed, and the compensatory mechanisms to decrease this cost (269, 270). Compensatory mutations can occur in the same altered gene or in others contributing to the developed resistance mechanism, thereby retaining the same resistance level (271). This compensatory phenomenon can occur with or without antibiotic exposure. However, under antibiotic pressure, only those mutations that provide a competitive advantage will be selected, thereby allowing the survival and persistence of resistant strains (269).

In *S. pneumoniae*, fluoroquinolone-resistant strains have a low frequency due to the high fitness cost imposed by point mutations in the genes that encode type II topoisomerases (topoisomerase IV and DNA gyrase) (272, 273). However, the E85K change in *gyrA* identified as a high-cost mutation can be compensated for by the presence of a recombinant topoisomerase IV enzyme (273) in some laboratory mutants. These specific alterations acquired by interspecific recombination may be selected as they reduce the fitness cost associated with fluoroquinolone resistance mutations. Similarly, an increase in fitness cost and a loss of virulence have

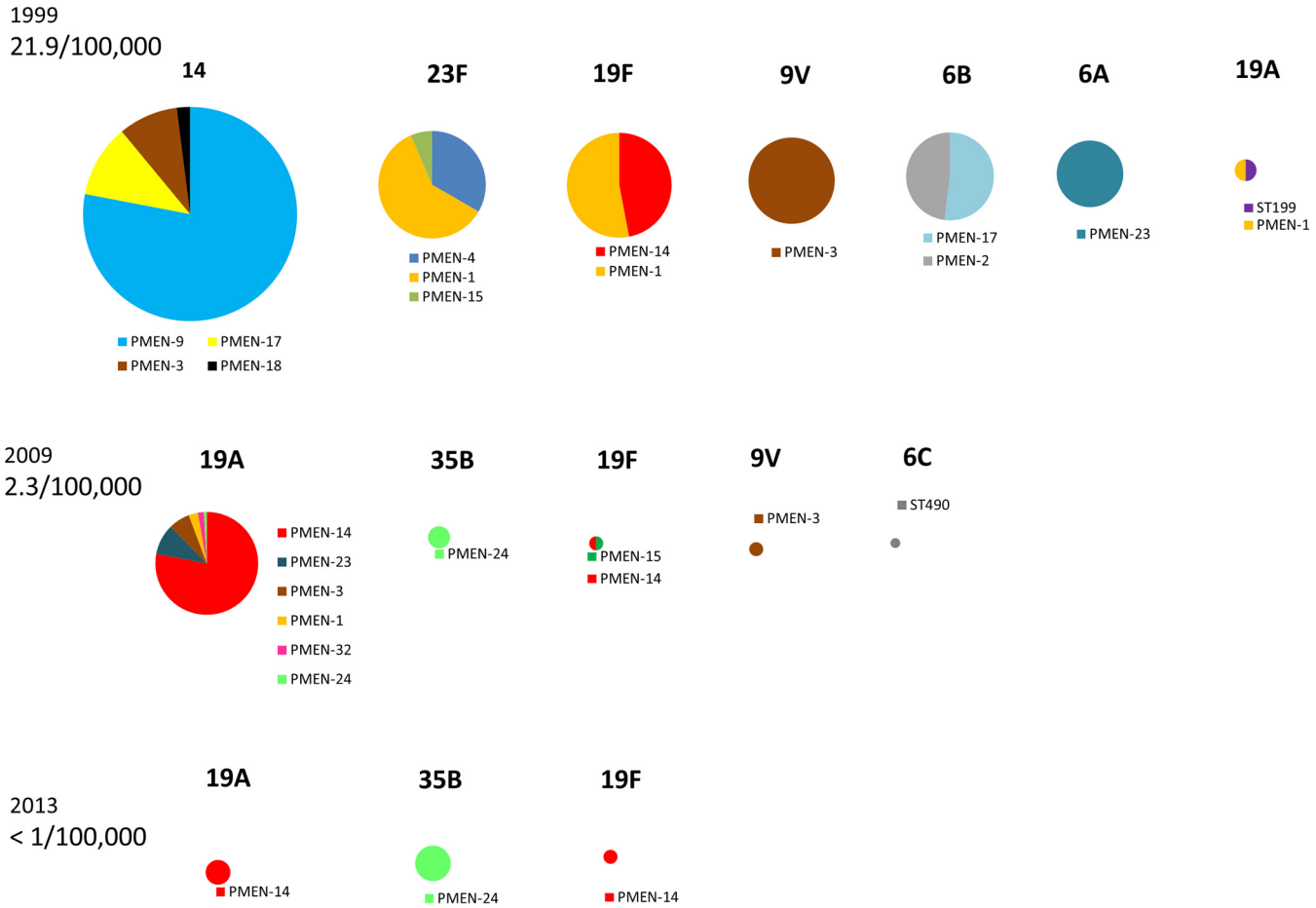

FIG 1 Approximate numbers of cases per 100,000 individuals caused by penicillin resistance for each year (left). These data encompass all clonal complexes and serotypes associated with penicillin resistance (MIC of ≥2 μg/ml) in cases of pediatric IPD during 1999, 2009, and 2013. The circle diameters reflect relative IPD incidences.

been described in laboratory strains where β-lactam resistance was conferred by mutant PBP gene alleles through transformation (274, 275). However, some clinical strains can also compensate for this fitness cost imposed by this resistance, producing a selective advantage for these strains with an increased potential to spread β-lactam resistance. In a transformation-based study employing resistant clinical strains as PBP gene sources, Albarracín Orio et al. (276) found that the fitness cost and cell division defects conferred by the given mutant *pbp2b* alleles were fully compensated for by the acquisition of the corresponding *pbp2x* and *pbp1a* alleles from the same strain. This suggests that *pbp1a* and *pbp2x* mutant alleles not only contribute to the development of higher β-lactam resistance levels but also are acquired for their compensatory effect on fitness, and this compensatory process may occur even without antibiotic pressure.

ANTIBIOTIC TOLERANCE

Antibiotic tolerance is described as the ability of bacteria to survive but not show any apparent growth in the presence of an antibacterial agent. Bacterial tolerance is not detected by routine *in vitro* susceptibility testing and has been proposed as a phenotype that could be a precursor to the phenotype of resistance (277). Tolerance was first described for β-lactam antibiotics, and the penicillin-tolerant phenotype was recognized in 1985 in eight clinical isolates of pneumococci (278). Tolerance to vancomycin has also been described in clinical isolates of *S. pneumoniae* (279–282) and has been linked with treatment failure (279). While the mechanism of tolerance has not been fully elucidated, it has been suggested that tolerance is due to a faulty system of regulation of autolysins or changes in the composition of the cell wall (283). Several studies have suggested that the molecular mechanism of tolerance to penicillin and vancomycin involves a defect in the activation of LytA, a murein hydrolase that mediates an endogenous process of death leading to cellular lysis (281, 284, 285). Sung et al. (285) also reported defective LytA autolysin and antimicrobial tolerance in clinical strains. Pep27, a 27-amino-acid secreted peptide that has an important role in controlling bacterial death, has also been suggested to play a role in the tolerance phenotype (281, 286).

VACCINES AND RESISTANCE

PCV-Driven Decrease of Antibiotic Resistance Has Targeted a Limited Array of Pneumococcal Clones

β-Lactam-resistant MDR clonal complexes originated within a surprisingly small number of strains, the most important of which

TABLE 2 Representative serotypes, common MLST types, and common resistance determinant patterns found within penicillin-resistant strains recovered through the ABCs program from individuals of all ages during 1998 to 2015

Clonal complex reference strain (serotype, MLST type)	Associated serotype(s)	Common MLST type(s)	Predominant resistance-associated PBP transpeptidase profile(s)[a]	Common non-beta-lactam resistance determinant profile(s)
PMEN-1 (23F, ST81)	23F, 19F, 19A[a]	ST81, ST2346[b]	15/12/18	*mef*, *cat*, *tetM*, *folA*-I100L, *folP* insertion; *ermB*, *cat*, *tetM*, *folA*-I100L, *folP* insertion
PMEN-2 (6B, ST90)	6B	ST90	34/57/56	*ermB*, *cat*, *tetM*, *folA*-I100L, *folP* insertion; *ermB*, *tetM*, *folA*-I100L, *folP* insertion
PMEN-3 (9V, ST156)	9V, 14, 19A,[b] 35B,[b] 11A,[c] 31[c]	ST156,ST166	15/12/18	*mef*, *folA*-I100L, *folP* insertion; *ermB*, *tetM*, *folA*-I100L, *folP* insertion; *folA*-I100L, *folP* insertion
PMEN-4 (23F, ST37)	23F	ST37	27/38/52	*mef*, *folA*-I100L, *folP* insertion
PMEN-5 (14, ST18)	14	ST18	Not available	*ermB*, *cat*, *tetM*, *folA*-I100L, *folP* insertion
PMEN-9 (14, ST9)	14	ST13	27/36/8	*mef*, *folA*-I100L, *folP* insertion
PMEN-14 (19F, ST236)	19F, 19A,[b] 3[c]	ST236, ST271, ST320,[b] ST1451[b]	13/54/33, 13/11/16,[b] 13/11/33,[b] 13/14/26[b]	*ermB*, *mef*, *tetM*, *folA*-I100L, *folP* insertion
PMEN-15 (23F, ST242)	23F, 6A[c]	ST242	13/31/73, 13/31/146[c]	*mef*, *tetM*, *folA*-I100L, *folP* insertion; *ermB*, *tetM*, *folP* insertion[b]
PMEN-17 (6B, ST384)	6B, 6A	ST384, ST147	41/36/8	*mef*, *tetM*, *folA*-I100L, *folP* insertion
PMEN-18 (14, ST67)	14		38/16/8, 38/16/36	*ermB*, *tetM*, *folA*-I100L, *folP* insertion
PMEN-23 (6A, ST376)	6A, 19A[b]	ST376, ST1339[b]	42/42/8, 27/30/8[b]	*mef*, *folA*-I100L, *folP* insertion
PMEN-24 (35B, ST377)	35B, 19A[b]	ST558	4/7/7	*mef*
PMEN-32 (14, ST230)	19A[a]	ST230, ST276	17/15/18, 17/7/8	*tetM*, *folA*-I100L, *folP* insertion

[a] PBP profile data and matching transpeptidase sequence databases are available in reference 50.
[b] First observed during the post-PCV7 period from 2002 to 2009.
[c] First observed during the post-PCV13 period from 2012 to 2015.

are linked to historical, phenotypic, and genotypic data at the PMEN website (http://www.pneumogen.net/pmen/). These PMEN MDR CCs represented PCV7 serotypes that thrived and disseminated throughout the world during the 1980s and 1990s (25). Nonvaccinated or poorly vaccinated populations in countries with unrestricted antibiotic availability continue to have high proportions of highly resistant disease isolates within these clonal complexes (62, 287). As shown in Fig. 1 and Table 2, these clonal complexes have been responsible for the vast majority of penicillin-resistant pneumococcal strains recovered through the ABCs program since before PCV7 was introduced.

Only higher penicillin MICs (≥4 μg/ml) have been associated with treatment failures of nonmeningitis IPD cases (35). This level of resistance was associated with ~8% of IPD cases in the United States immediately prior to PCV7 introduction (data not shown). Nearly half (49%) of these cases associated with clinically relevant penicillin resistance during 1999 were due to PCV7 serotype 14, while the remainder were accounted for primarily by PCV7 serotypes 23F (21%), 9V (10%), 19F (8%), 6B (6%), and 6A (3%) (data not shown). Previously recognized globally disseminated clones (25) were the apparent sources of major penicillin-resistant (MICs of ≥2 μg/liter) clonal complexes causing IPD within children in the United States immediately prior to PCV7 introduction (108, 109, 111, 166). Individual penicillin-resistant (MICs of ≥2 μg/ml) PCV7 serotypes were comprised primarily of 1 to 3 major genetic complexes (97, 105–111, 288, 289) represented by the PMEN reference strains shown in Table 2. The vaccine-related serotype 6A also exhibited significant penicillin resistance, due to strains highly related to PMEN-23 (110). It is likely that the dramatic emergence, proliferation, and spread of these multiresistant strains were due in part to antibiotic selective pressure. Their equally impressive decline in the United States was due to conjugate vaccines specifically targeting the predominant IPD-causing serotypes, which were largely comprised of these MDR clones.

Serotype 19A IPD

Figure 2 depicts the breakdown of pediatric IPD cases in the United States during 1999, 2009, and 2013 according to serotype class and penicillin susceptibility (2007 guidelines are used since they are consistent with the fact that the lower MIC values of 0.12 to 1 μg/ml are reflective of adaptation to penicillin and are relevant for meningitis cases). Lower MICs to indicate high resistance (≥2 μg/ml) are used since these values differ from the clinically significant MICs for nonmeningitis disease (≥4 μg/ml) by only a single dilution. An error margin of a single dilution factor is sometimes encountered in the clinical or surveillance laboratory, depending upon the precise MIC of individual strains. As discussed above, prior to PCV7 introduction, virtually all high-level penicillin resistance was due to serotypes 14, 23F, 19F, 9V, 6B, and 6A, with this level of resistance within serotype 19A isolates being rare (Fig. 1 and 2). In contrast, during the post-PCV7 years, high-level penicillin resistance was greatly reduced and was due primarily to serotype 19A. At the same time, the relative proportion of intermediately penicillin-resistant infections also increased, due primarily to the continued expansion of the serotype 19A/CC199 complex and the serotype 19A/CC695 switch variant (162). Although the emergence of highly resistant serotype 19A had a high impact, the rate of pediatric serotype 19A IPD plateaued during the mid- to late 2000s (112). In addition, despite a relatively small eroding effect of nonvaccine serotypes, the overall disease rate also stabilized, indicative of the durable protective effect of PCV7 (8).

It is noteworthy that serotype 19A isolates accounted for only a small proportion of penicillin resistance before PCV7 introduction (Fig. 1 and 2), although at the time, it was the most frequent

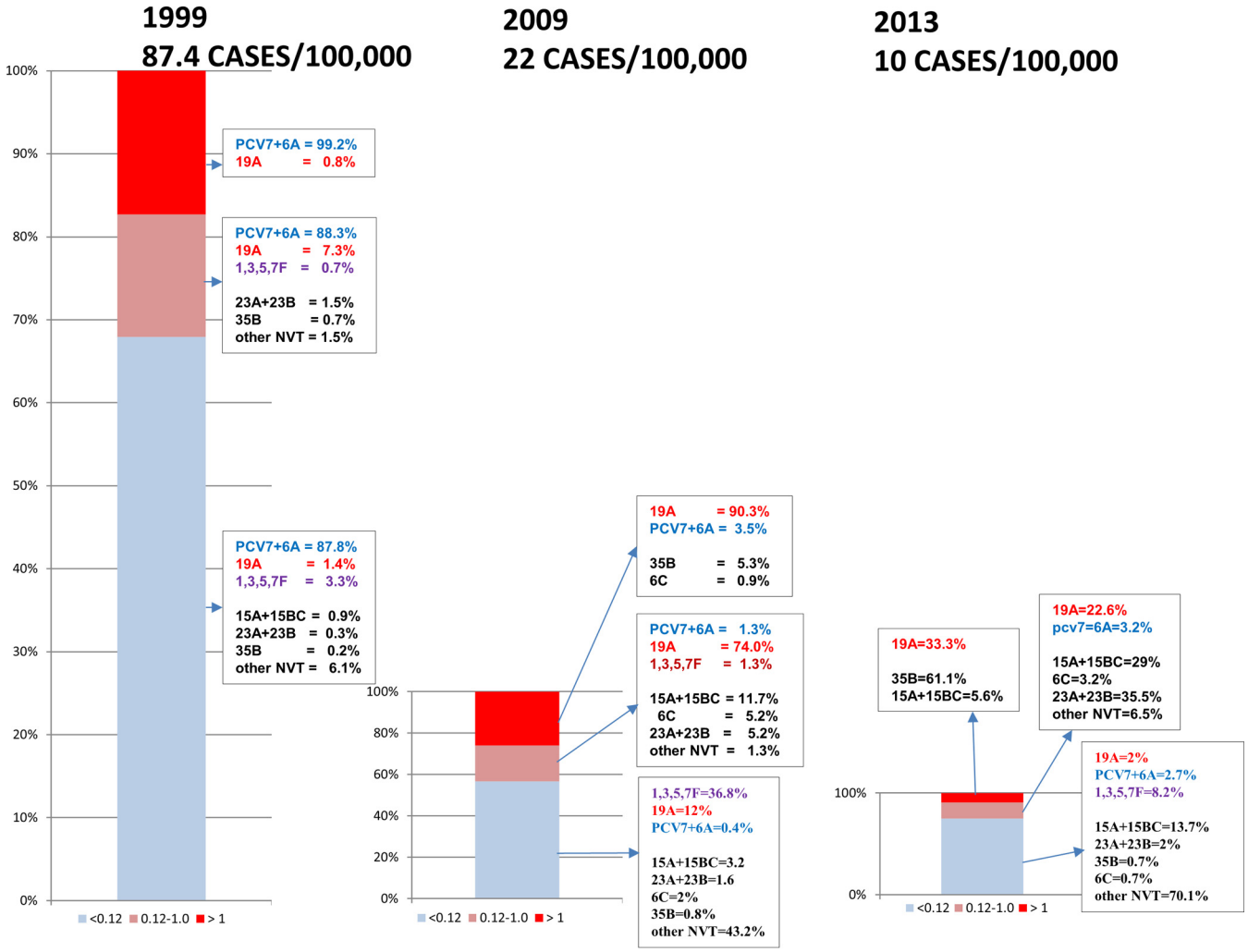

FIG 2 ABCs IPD rates in the population of individuals <5 years of age in 1999 (before PCV7 introduction), 2009 (9 years after PCV7 introduction), and 2013 (3 years after PCV13 introduction). The bright red portions indicate penicillin resistance (MICs of ≥2 μg/ml), lighter red indicates intermediate resistance (0.12 to 1.0 μg/ml), and gray indicates sensitivity (≤0.06 μg/ml). PCV13 types, besides those targeted by PCV7, are indicated in red and purple. Only nonvaccine types (NVT) that are found associated with intermediate or high penicillin resistance are specifically indicated in black boldface type.

non-PCV7-targeted serotype associated with high penicillin MICs. Besides its incidence in post-PCV7 IPD, serotype 19A was exceptional in its genetic diversity and resistance features (112, 162). Historical MLST data combined with data from genomic analysis indicated that multiple serotype 19A strains arose independently through serotype-switching events within various highly successful PCV7 serotype strains (49, 111, 155, 161) (Fig. 2). The most striking single change in post-PCV7 penicillin-resistant IPD in the United States was the total disappearance of the major resistant PMEN-9 clonal complex (Fig. 1). Of note, serotype 19A variants of this clonal complex did not appear post-PCV7, while several other serotype 19A variants of other PCV7 type clones emerged and proliferated (including the less penicillin-resistant ST695 serotype 19A variant of the nonresistant major PCV7 type 4 strain) (112, 162). All but one of these MDR serotype 19A variants disappeared as a cause of pediatric IPD in the post-PCV13 period, with the frequency of the remaining highly resistant serotype 19A variant of the PMEN-14 clonal complex being greatly reduced (50). These early results are reassuring in the sense that there are no indications of a structural serotype 19A serological variant that is not targeted by PCV13 in the same manner in which serotype 6C was not targeted by PCV7 and for many years was misidentified as serotype 6A (290, 291).

While individual PCV7 serotypes disappeared or drastically decreased soon after PCV7 introduction, much PCV7 clonal diversity was preserved within the emergent serotype 19A (Fig. 1). Throughout the post-PCV7 period, penicillin-resistant serotype 19A variants persisted, with multilocus sequence types being identical to or closely similar to (in order of abundance) the highly resistant clones PMEN-14, PMEN-23, PMEN-3, and PMEN-1 (Fig. 1). In addition, the intermediately resistant serotype 19A switch variant of a predominant antibiotic-sensitive serotype 4 clone (ST695) was first detected soon after PCV7 introduction and became one of the three most common serotype 19A invasive clonal complexes (described in more detail below). Additional minor penicillin-resistant "PCV7 genotypes" that were detected as serotype 19A variants during the post-PCV7 period

included PMEN-26 (Colombia^{23F}-26), PMEN-32 (Denmark^{14}-32), PMEN-24, and PMEN-11 (CSR^{19A}-11) (112, 162).

The most successful single clonal complex throughout the United States during the late 2000s was established by highly resistant serotype 19A ST320 strains within the PMEN-14 lineage (112); the emergence was first detected within the ABCs program among samples from pediatric IPD cases recovered in 2002 (160). Although tracking of this lineage throughout the 2000s documented its steady increase in frequency as a cause of IPD, this clonal complex was absent in pediatric IPD cases within the ABCs program during 1999 despite intensive strain surveillance (111, 166). The major fraction of the initial increase of the prevalence of serotype 19A IPD during the early 2000s was actually due to the moderately resistant CC199 (162), which was the main serotype 19A genotype in the pre-PCV7 period and rarely exhibited penicillin MICs of ≥0.5 μg/ml (111, 160, 166). CC199 rapidly emerged to become the single most abundant invasive clonal complex afflicting children during the early to mid-2000s (162). Although the ST199 genotype was the most common serotype 19A genotype prior to PCV7 implementation, the serotype 19A/CC199 incidence increased after PCV7 implementation, suggesting profound positive selection due to the removal of PCV7 serotypes from the carriage reservoir. From 2006 to 2010, there was also a pronounced shift in the serotype 19A genetic structure, where the prevalence of highly resistant PMEN-14-related serotype 19A strains (CC320) continued to increase with a concurrent decrease in the prevalence of CC199, suggesting that besides the expression of a nonvaccine serotype, high levels of antimicrobial resistance also strongly impacted serotype 19A emergence (112). The main CC320 genotype that became prevalent in serotype 19A strains appears to have originally originated within serotype 19F as a double-locus variant of PMEN-14 (ST236), although the most common serotype 19F genotypes (ST236 and ST271) were also documented within post-PCV7 resistant serotype 19A isolates by the ABCs program (49, 112, 162). These ST236 and ST271 serotype 19A variants originated through independent serotype switch events occurring within serotype 19F recipient strains (49). The serotype 19A/CC320 complex consisted of a high number of ST320 isolates and a plethora of less abundant single-locus variants, consistent with the notion of a single serotype 19A/ST320 strain becoming very abundant and gradually diversifying. Remarkably, throughout the post-PCV7 decade, it was still evident that PCV7 still offered sustained protection against most IPD (8, 292) (Fig. 1 and 2). Nonetheless, although the incidence of resistant infections decreased markedly, the proportion of IPD cases still associated with high penicillin MICs (≥4 μg/ml) actually increased from 8.2% to 9.1% during 1999 to 2009 due to the disproportionate relative incidence of serotype 19A/CC320 strains (50, 112, 162; unpublished ABCs data) (Fig. 1 and 2).

It is remarkable that virtually all serotype 19A IPD isolates surveyed during the past 15 years reflect genotypes that were likely to have originated within strains expressing other serotypes. Besides the variants that share genotypes that originated within PCV7 serotypes, this extends to the serotype 19A/CC199 complex and variants of other genotypes associated primarily with nonconjugate vaccine serotypes (112). The majority of nonconjugate vaccine serotype 15B and 15C isolates recovered in the pre-PCV7 period were of ST199, although these strains lack the intermediate penicillin resistance exhibited by the serotype 19A/CC199 complex. A serotype 19A variant complex of penicillin-intermediate and macrolide- and lincosamide-resistant PMEN-25 (Sweden^{15A}-25) was also observed during the 2000s (112). Since 2006, multiple serotype 19A switch variants of resistant serotype 35B/ST558 (PMEN-24 complex) have been recovered through the ABCs program (50, 112). The detection of such variants is consistent with naturally occurring immune pressure exerted against resistant serotype 35B and 15A strains that became more abundant in the PCV7 implementation period. Besides involving putative vaccine escape events, the serotype switch events in the post-PCV7 implementation period also involved non-PCV7-type parental strains. These events seem similar in nature to those that occurred before PCV7 introduction (156). During both the pre- and post-PCV7 periods, strains that were not targeted by pediatric vaccines and were also abundant in carriage served as donor and recipient partners in capsular locus switch events.

Current Moderately Successful Antibiotic-Resistant Nonvaccine Types

A pressing question that currently exists is whether any of the most predominant non-PCV-type resistant clonal complexes will emerge as major pathogens. Through the ABCs program, we observed statistically significant, but small, clonal shifts within strains of nonvaccine serotypes 35B, 15A, and 23A during the post-PCV7 era, where single intermediately penicillin-nonsusceptible clonal complexes within these serotypes appeared and modestly emerged (293, 294). Similar increases in prevalence within nonsusceptible serotype 15B/15C (ST3280) and 23B (ST1373) complexes have continued in post-PCV13 IPD (Fig. 2) (see reference 50 for detailed MLST-based data for all serotypes). In the post-PCV7 period, resistant serotype 35B made a moderate emergence, and resistant serotype 6C appeared for the first time to be associated with pediatric IPD (290, 291). Penicillin-nonsusceptible serotype 6C was more genetically complex, with 4 different CCs associated with penicillin nonsusceptibility (291). Interestingly, 3 of the 4 penicillin-nonsusceptible CCs have been commonly associated with the closely related serotype 6A (291), suggesting that much of the increase in the prevalence of serotype 6C IPD (mostly in adults, where it caused a significant disease burden) and the increased resistance originated from independent serotype switch events involving individual type 6A capsular locus recipients. Increasing proportions of clinical isolates within serotypes 35B and 6C in the United States with decreased susceptibility to β-lactams have been reported (295). Resistant serotype 35B (serotype 35B/ST558) IPD was virtually absent among children in the preconjugate vaccine era, but during the past decade, serotypes 35B, 6C, and additional serotypes have increased in both carriage (296, 297) and IPD cases representing all ages (50, 294, 295), potentially as a consequence of the removal of competing conjugate vaccine serotypes from the pediatric carriage reservoir. The PMEN-24 serotype 35B clonal complex has emerged to become the predominant pediatric β-lactam-resistant IPD serotype (50). Although the impact of serotype 35B on resistant IPD is currently small, the frequency of penicillin-resistant (MIC of ≥2 μg/ml) IPD in children due to this clonal complex is higher than what was attributed to serotype 19A in the pre-PCV7 era (Fig. 1). Despite the detected emergence of minor clonal complexes with various levels of β-lactam resistance, the continued strategy for targeting prevalent serotypes with conjugate vaccines is a proven success for sustained decreases in rates of IPD associated with all levels of antimicrobial resistance. These observations provide an ob-

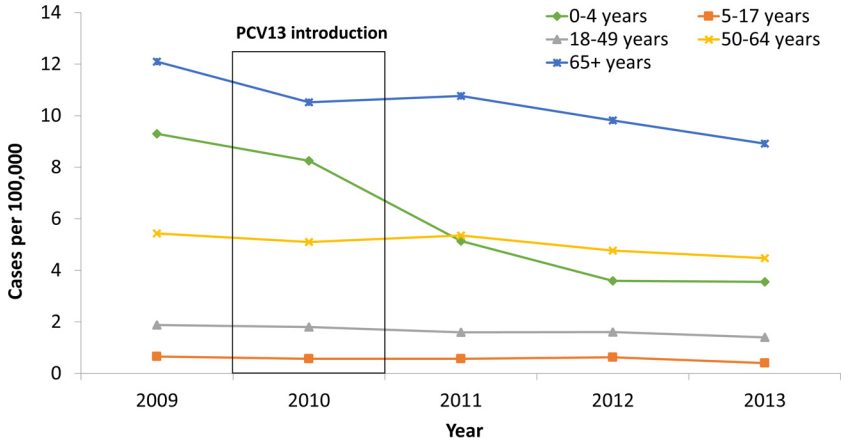

FIG 3 Incidences (number of cases per 100,000 individuals) of antimicrobial-nonsusceptible (nonsusceptible to one or more classes of antimicrobials, including macrolides, cephalosporins, tetracyclines, penicillins, fluoroquinolones, and glycopeptides) IPD. (Data from Active Bacterial Core surveillance, 2009 to 2013.)

vious rationale for the continued monitoring of serotypes and resistance features of remaining IPD isolates in the post-PCV13 era. While PCV13 appears likely to provide sustained protection against resistant IPD (292), we are currently in a relatively early phase of monitoring, and there is a continued need to closely monitor serotype-specific IPD, especially cases associated with resistance.

Pneumococcal Conjugate Vaccines and Antibiotic Resistance

To summarize, pneumococcal conjugate vaccines serve as a powerful tool against antibiotic resistance. PCV7-type MDR clones peaked immediately before PCV7 implementation, causing the vast majority of IPD cases. By 2004, 4 years after PCV7 introduction in the United States, there were well-documented decreases in the rates of invasive disease caused by penicillin-nonsusceptible strains (6.3 to 2.7 cases per 100,000 individuals) and multidrug-nonsusceptible strains (4.1 to 1.7 cases per 100,000) (91). Vaccine-type resistant disease decreased by 87%, but a significant increase was seen in disease caused by serotype 19A, not included in PCV7 (91). After PCV7 introduction in 2000, the MDR PCV7-type clones virtually disappeared as causes of IPD cases associated with the vaccinated population (7, 8); however, certain non-PCV7 serotype variants of these strains emerged (50, 160, 166, 294). An updated analysis using ABCs data and the 2008 CLSI penicillin breakpoints also found similar declines (298); however, by 2007 to 2008, serotypes in PCV13, but not in PCV7, caused 78% to 97% of penicillin-nonsusceptible IPD, depending on age, with serotype 19A alone accounting for 82% of these cases (298). This increase was hypothesized to be multifactorial: (i) selective pressure from antibiotic use among children that led to subsequent transmission to adults (299–302), (ii) the possible emergence of clones that have advantageous traits for expansion, (iii) capsular switching (161), and (iv) the ability of serotype 19A to cause both colonization and invasive disease (162).

Similar to the peak of antimicrobial-nonsusceptible IPD seen prior to PCV7 introduction, the incidence of nonsusceptible IPD (particularly serotype 19A) increased prior to PCV13 introduction (8, 169) (Fig. 3). Over 100 countries have introduced PCV13 into their childhood immunization schedules. Data documenting

TABLE 3 Incidences of antimicrobial-nonsusceptible IPD caused by all serotypes, serotypes included in PCV13 but not in PCV7, and multidrug-nonsusceptible IPD in the United States[d]

Age group (yr)	Antimicrobial-nonsusceptible IPD caused by all serotypes[a]			Antimicrobial-nonsusceptible IPD caused by serotypes in PCV13 but not in PCV7[a,b]			Multidrug-nonsusceptible IPD[a,c]		
	Rate (no. of cases per 100,000 individuals)			Rate (no. of cases per 100,000 individuals)			Rate (no. of cases per 100,000 individuals)		
	2009	2013	% difference	2009	2013	% difference	2009	2013	% difference
<5	9.3	3.6	−61.8	6.5	0.5	−92.7	5.2	0.8	−84.6
5–17	0.7	0.4	−39.0	0.2	0.1	−67.2	0.2	0.1	−53.2
18–49	1.9	1.4	−25.4	0.9	0.3	−60.0	0.8	0.3	−60.0
50–64	5.4	4.5	−17.6	2.4	0.9	−63.4	2.0	0.9	−55.4
≥65	12.1	8.9	−26.3	4.4	1.4	−67.4	4.2	1.8	−56.9

[a] Nonsusceptible to any of the following antimicrobials: macrolides, cephalosporins, tetracyclines, penicillins, fluoroquinolones, and glycopeptides.
[b] Serotypes in PCV13 but not in PCV7 include serotypes 1, 3, 5, 7F, and 19A. Serotype 6A was excluded due to cross-protection from serotype 6B, which was included in PCV7.
[c] Nonsusceptible to ≥3 antimicrobial classes.
[d] Data from Active Bacterial Core surveillance, 2009 to 2013.

the impact of PCV13 on antimicrobial-nonsusceptible pneumococci are of high interest. ABCs data were used to estimate trends of antimicrobial-nonsusceptible pneumococci 3 years after PCV13 introduction in the United States. During 2009 to 2013, the incidence of antimicrobial-nonsusceptible IPD (i.e., nonsusceptible to macrolides, cephalosporins, tetracyclines, penicillins, fluoroquinolones, and glycopeptides), antimicrobial-nonsusceptible IPD caused by all serotypes included in PCV13 but not in PCV7 (i.e., serotypes 1, 3, 5, 7F, and 19A), and multidrug-nonsusceptible IPD (i.e., nonsusceptibility to ≥3 antimicrobial classes) decreased in all age groups (Table 3) (169).

Other countries have also reported similar reductions in antimicrobial-resistant IPD after pneumococcal conjugate vaccine introductions. In a South African clinical trial for a 9-valent conjugate vaccine, rates of penicillin-resistant IPD and trimethoprim-sulfamethoxazole-resistant IPD decreased by 67% and 56%, respectively (303). dos Santos et al. also found that penicillin and ceftriaxone nonsusceptibility decreased after PCV10 introduction in Brazil, although no trend was found for clindamycin, chloramphenicol, erythromycin, rifampin, tetracycline, or levofloxacin (304). In Colombia, penicillin resistance among invasive isolates decreased after PCV7 introduction (41.1% versus 14.2%; $P = 0.02$) (305). Data from Israel also showed a significant decrease in the proportion of isolates with penicillin MICs of ≥0.125 μg/ml (26.2% to 16.4%), although there was an increase in non-vaccine-type strains with penicillin MICs of ≥2 μg/ml, particularly among serotype 19A isolates, similarly to the United States (306). Overall, continued surveillance is needed to document the effects of PCV13 on serotype distribution, antimicrobial resistance, and replacement.

CONCLUSION

In conclusion, we have attempted a broad review of pneumococcal antimicrobial resistance, including the public health impact and risk factors for resistance; mechanisms of resistance, tolerance, and fitness; and the role of conjugate vaccines in decreasing antimicrobial resistance. With the advent of more advanced laboratory techniques, including whole-genome sequencing, and continued, high-quality surveillance of antimicrobial resistance, we can continue to further expand our understanding of this area. Conjugate vaccines have been demonstrated to be a powerful tool and should continue to be introduced in countries to decrease not only the burden of disease but also antimicrobial-resistant pneumococci.

ACKNOWLEDGMENTS

We are indebted to all of the hospitals and laboratories, the RDB *Streptococcus* Laboratory, and the RDB Epidemiology section that participate in the CDC Emerging Infections Program Network and the Active Bacterial Core surveillance program.

We declare no conflict of interest.

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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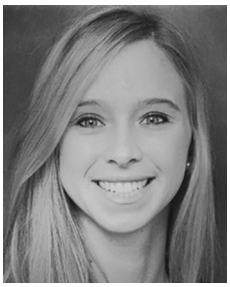

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