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Serotype Emergence and Genotype Distribution among Macrolide-Resistant Invasive *Streptococcus Pneumoniae* Isolates in the Postconjugate Vaccine (PCV-7) Era

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We conducted population-based surveillance for pneumococcal bacteremia within a 5-county region surrounding Philadelphia from October 2001 through September 2008, the period following introduction of the seven-valent pneumococcal conjugate vaccine. Erythromycin resistance increased from 14.7% in 2001-2002 to 20.3% in 2007-2008, while the resistance rate to penicillin (MIC, ≥2 μg/ml) decreased from 7.2% to 4.2% during the same period. The most predominant serotypes associated with erythromycin resistance in 2007-2008 included 19A (29.7%), 15A (29.2%), 6C (10.1%), 3 (5.6%), and 6A (4.5%). The molecular mechanisms for the increasing erythromycin resistance were mainly due to the growing presence of *mef*(A) negative *erm*(B)$^+$ and *mef*(A)$^+$ *erm*(B)$^+$ genotypes, which increased from 20.0% to 46.1% and from 1.8% to 19.1%, respectively, from 2001-2002 to 2007-2008. However, *mef*(A)-mediated erythromycin resistance decreased from 72.7% in 2001-2002 to 34.8% in 2007-2008. Serotypes related to the *erm*(B) gene were 15A (45.6%), 19A (20.9%), 3 (10.1%), and 6B (6.3%); serotypes related to the *mef*(A) gene were 6A (18.6%), 19A (15.0%), 6C (9.3%), and 14(8.4%); serotypes associated with the presence of both *erm*(B) and *mef*(A) were 19A (81.5%), 15A (7.7%), and 19F (6.2%). Pulsed-field gel electrophoresis analysis demonstrated that erythromycin-resistant isolates within the 19A serotype were genetically diverse and related to several circulating international clones. In contrast, erythromycin-resistant isolates within the 15A serotype consisted of clonally identical or closely related isolates.

Streptococcus pneumoniae is a major pathogen that causes pneumonia, bacteremia, and meningitis in humans (9, 17). The surface capsular polysaccharide is one of the most important virulence factors and is the basis for all licensed pneumococcal vaccine strategies (21), with more than 90 immunologically distinct serotypes. *S. pneumoniae* is notable for its ability to switch serotypes and acquire antimicrobial drug resistance genes, reflecting an ability to incorporate foreign DNA (13). Antimicrobial resistance, which is often multidrug resistance, among clinical isolates of *S. pneumoniae* is common, limiting options for effective antimicrobial therapy.

In particular, macrolide resistance among *Streptococcus pneumoniae* isolates has risen in recent years worldwide (8, 10, 11, 14). Erythromycin resistance is mainly due to the presence of *mef*(A) and *erm*(B) genes (4, 25). Mef A is an efflux pump that removes most intracellular macrolides, resulting in low- to intermediate-level macrolide resistance, termed the M phenotype. Erythromycin resistance may also be mediated by the presence of an erythromycin-ribosomal methylase, which is encoded by the *erm*(B) gene. *erm*(B)-encoded methylation of adenine at position 2059 in the 23S rRNA blocks the binding of macrolides (e.g., erythromycin), lincosamides (e.g., clindamycin), and streptogramin B (e.g., dalfopristin) and results in high-level resistance to these antibiotics (MLS_B phenotype), with high erythromycin MICs (≥256 μg/ml). In rare cases, macrolide resistance may also be caused by mutations in 23S rRNA (A2059G) or ribosomal proteins L4 and L22 (6, 25). The *erm*(B) gene is the most prevalent genotype globally, accounting for the majority of clinical isolates in Europe (5). In the United States, *mef*(A) is a common genotype (66% in 2001 to 2004 to 54% in 2005-2006) (11), and the presence of the *erm*(B) genotype has remained relatively low. However, recent reports have noted an increase in the percentage of isolates carrying both *erm*(B) and *mef*(A) (12, 15).

The introduction of the seven-valent pneumococcal conjugate vaccine (PCV-7) in 2000 had a profound impact on the seroepidemiology of pneumococcal disease, with significant declines observed in pediatric and adult disease due to vaccine serotypes (16, 26). Initial reports indicated that the decline in vaccine serotypes was also associated with a decline in the frequency of drug resistance, due to the fact that the serotypes targeted by PCV-7 were among the more common drug-resistant types in the prevaccine era (16, 26). However, the emergence of nonvaccine serotypes in recent years has been driven, in part, by antimicrobial drug selection pressures (12, 15) and is changing the epidemiology of pneumococcal drug resistance.

We investigated the prevalence and molecular epidemiology of macrolide resistance among invasive pneumococcal isolates in the post-PCV-7 era, with the aim of understanding the antimicrobial susceptibility profile, serotype distributions, and the genetic relatedness among macrolide-resistant isolates. We were especially interested in examining the prevalence of non-PCV-7 serotypes among erythromycin-resistant isolates, including those that would and would not be covered by the newly introduced 13-valent pneumococcal conjugate vaccine (PCV-13).

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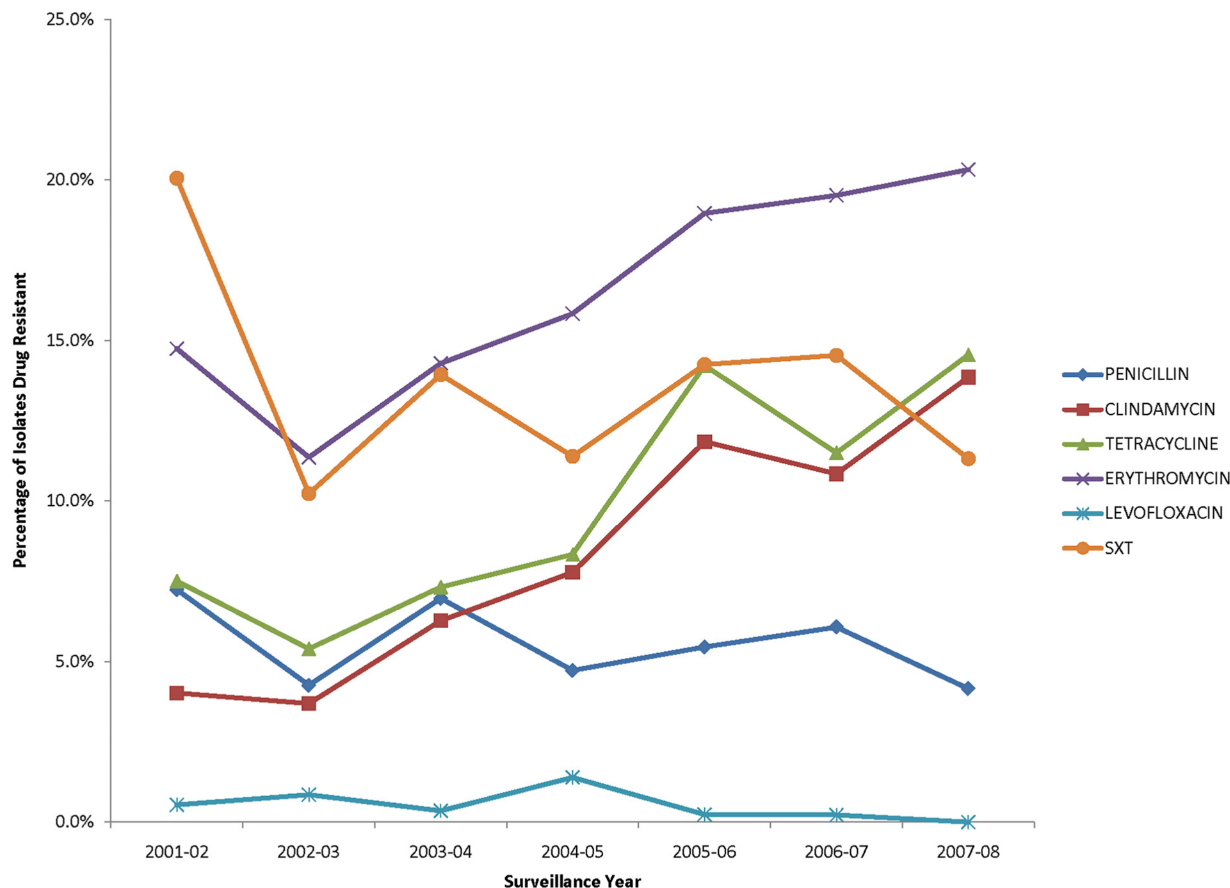

FIG 1 Antimicrobial resistance profiles of bacteremic *S. pneumoniae* isolates by year from 2001 to 2008. The percentages of clinical isolates in each year with resistance to each of the six antimicrobial drugs are plotted. Each year includes the period from October through September, to encompass a single respiratory season.

MATERIALS AND METHODS

Population-based surveillance for invasive pneumococcal disease. Data were collected as part of a population-based surveillance for bacteremic pneumococcal disease within the 5-county region surrounding Philadelphia (Bucks, Chester, Delaware, Montgomery, and Philadelphia Counties). Adult (age, ≥18 years) population surveillance was initiated in October 2001 as part of a larger study of risk factors for community-acquired bacteremic pneumococcal disease. The surveillance network currently encompasses 48 of the 49 acute care hospitals that serve the 3.7 million residents of the five counties. The one nonparticipating hospital is a small hospital that is closed to external studies and accounted for <2% of all cases in the region.

Subjects were identified through the microbiology laboratories at all hospitals. Hospital personnel were contacted by study personnel on a regular basis throughout the surveillance period in order to ensure complete capture of new cases. We confirmed the total number of eligible cases through contact with laboratory directors and review of their log books on an annual basis, as well as comparison with data from the City of Philadelphia Health Department, which mandates reporting of cases of pneumococcal bacteremia for sites within the city (19, 20).

Eligible patients were identified based on the parent study and included hospitalized adults residing in the five-county region with at least one set of blood cultures positive for *S. pneumoniae* drawn within 48 h of hospitalization and no prior hospitalization within 10 days of the episode of pneumococcal bacteremia, in order to exclude hospital-acquired infections.

Bacterial isolates and identification. All pneumococcal isolates were transported to the central laboratory at the Hospital of the University of Pennsylvania. Bacteria were cultured on blood agar medium in a 5% CO_2 atmosphere at a temperature of 35°C and identified using conventional methods, including bile solubility testing.

Antibiotic susceptibility testing. All the isolates were examined for antibiotic susceptibility against a panel of drugs, including oxacillin (for penicillin), erythromycin, tetracycline, clindamycin, penicillin, and trimethoprim-sulfamethoxazole (cotrimoxazole) by the disk-diffusion method (BD Diagnostics) and verified for resistant strains using the Etest (AB Biodisk, Solna, Sweden) for penicillin and erythromycin resistance. Testing for inducible *erm*(B) was conducted using apposed clindamycin and erythromycin disks placed ~10 mm apart. Interpretations of disk diffusion and Etest MICs were in accord with the Clinical and Laboratory Standards Institute (CLSI) 2007 criteria (3), with the exception of Etest erythromycin MICs, which were interpreted per AB Biodisk guidelines (1). An isolate with an erythromycin MIC of ≥2 μg/ml was considered resistant based on a 2-fold-higher MIC of erythromycin with the Etest and growth in 5 to 10% CO_2 versus results with ambient air agar dilution susceptibility testing. A penicillin MIC of ≥2 μg/ml was considered resistant based on the 2007 CLSI criteria and not the more recent 2010 criteria (MIC, ≥8 μg/ml). We used the earlier MIC breakpoint for penicillin in order to better compare results with those from previous studies during the same surveillance period. Erythromycin-nonsusceptible isolates were identified as the M phenotype if they were susceptible to clindamycin and the MLS_B phenotype if they were nonsusceptible to clindamycin.

Detection of erythromycin resistance genes. All erythromycin-resistant isolates were analyzed for the presence of the macrolide resistance genes *erm*(B) and *mef*(A) by using a SYBR green-based real-time

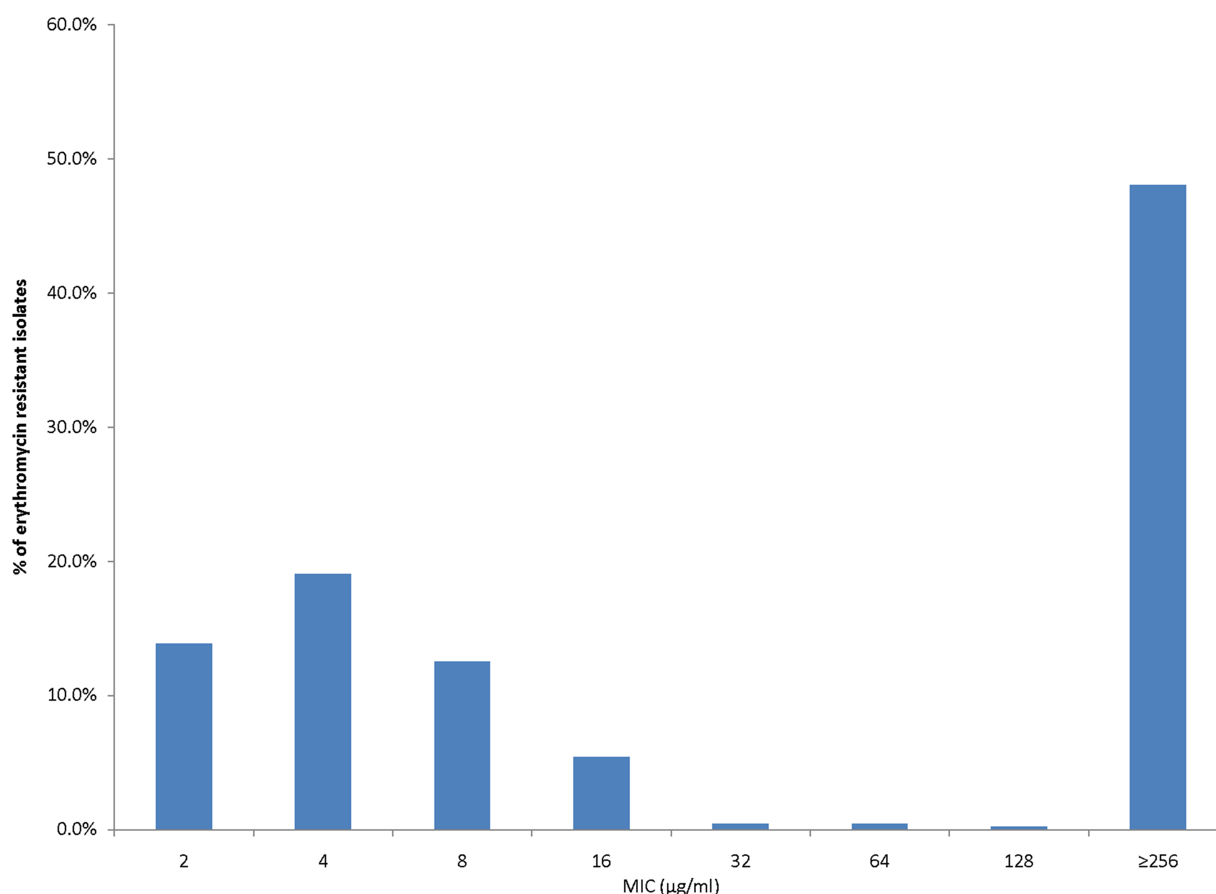

FIG 2 Distribution of erythromycin MICs among *S. pneumoniae* isolates collected from October 2001 to September 2009. Among a total of 2,688 isolates, 2,226 isolates were susceptible (MIC, ≤1 μg/ml). Among these, three isolates with MICs of 1 μg/ml were classified as susceptible according to the 2007 CLSI guideline. A total of 462 isolates were resistant (MIC, ≥2 μg/ml), and the MIC distribution is plotted.

PCR method. DNA was prepared by boiling bacterial cultures. The primers used to screen for the presence of *erm*(B) and *mef*(A) are available on request from the authors. PCR conditions followed the protocol described by J. Sutcliffe et al. (24). The PCR products for positive *mef*(A) and *erm*(B) isolates were confirmed by DNA sequencing for the first 10 positive isolates from each gene.

Identification of the 23S rRNA A2059G point mutation. All macrolide-resistant isolates were screened for the presence of the 23S rRNA A2059G point mutation by using a TaqMan single-nucleotide polymorphism (SNP) genotyping assay. There are four copies of 23S rRNA in *S. pneumoniae*, and the level of resistance to macrolides depends on the copy number of the mutated gene. The probes and primers for detecting the 23S rRNA A2059G mutation were designed by Applied Biosystems using the TaqMan SNP genotyping assay. The assay reagent consisted of a 40× mix of unlabeled PCR primers (23SRNA_F, GACTCGGTGAAATT TTAGTATCTGTGAAGA; 23SRNA_R, TCAATATCAAACTGCAGTAA AGCTCCAT) and TaqMan MGB probes (labeled with the fluorochrome dyes 6-carboxyfluorescein [FAM] and VIC; FAM-AGGACGGAGA GACC and VIC-AGGACGGAAAGACC). The TaqMan real-time PCR was performed according to the method provided by the manufacturer. To evaluate the accuracy of the assay, we selected 20 clinical macrolide-resistant isolates and 2 additional strains with a known 23S rRNA A2059G mutation (provided generously by A. Tait-Kanradt). The accuracy and specificity of the TaqMan SNP method were 100% identical to those for DNA sequencing. The primers for amplifying each of the four alleles of the 23S rRNA are available upon request from the authors. All PCR-positive isolates for the A2059 mutation were then confirmed by sequencing.

TABLE 1 Genotype distributions among erythromycin-resistant *S. pneumoniae* isolates collected from 2001 to 2008

	% of isolates with indicated genotype during period							
Genotype[a]	2001-2002 (n = 55)	2002-2003 (n = 41)	2003-2004 (n = 42)	2004-2005 (n = 57)	2005-2006 (n = 84)	2006-2007 (n = 93)	2007-2008 (n = 89)	P value[b]
mef(A)$^+$ *erm*(B) negative	72.7	70.7	52.4	50.9	40.5	44.1	34.8	<0.0001
mef(A) negative *erm*(B)$^+$	20.0	26.8	26.2	36.8	40.5	31.2	46.1	0.01
erm(B)$^+$ *mef*(A)$^+$	1.8	0.0	9.5	10.5	17.9	23.7	19.1	<0.0001
23S rRNA (A2059G)	3.6	2.4	7.1	0.0	1.2	1.1	1.1	0.17

[a] Isolates with an unknown genotype constituted 1.8%, 0%, 4.8%, 1.8%, 0%, 1.1%, and 0% in each year, respectively.
[b] Determined using the Mantel-Haenszel chi-square test for trend.

TABLE 2 Serotype distributions, overall and by genotype, among erythromycin-resistant isolates

Serotype	% of isolates in genotype group with the indicated serotype			
	All resistant isolates ($n = 448$)	*mef*(A)$^+$ *erm*(B) negative	*mef*(A) negative *erm*(B)$^+$	*mef*(A)$^+$ *erm*(B)$^+$
19A[a]	27.57	15.0	20.9	81.5
15A	17.2	0.4	45.6	7.7
6A[a]	9.2	18.6	0.6	0.0
14	6.3	8.4	5.7	0.0
6C	4.7	9.3	0.6	0.0
19F[b]	3.8	5.8	0.6	6.2
3[a]	3.6	0.4	10.1	0.0
6B[b]	3.6	1.8	6.3	0.0
33F	3.6	5.8	1.3	1.5
23F[b]	3.4	4.9	1.3	0.0
9V[b]	2.9	4.9	0.6	0.0
20	2.7	5.3	0.0	0.0
7F[a]	1.8	3.5	0.0	0.0
4[b]	1.8	3.1	0.0	0.0
23A	1.6	1.8	1.9	0.0
23B	1.1	2.2	0.0	0.0
11A	0.9	1.3	0.6	0.0
35B	0.7	0.9	0.0	0.0
Others[c]	3.5	6.6	6.9	3.1

[a] Serotype that was included only in the PCV-13 vaccine.
[b] Serotype that was included in the PCV-7 and PCV-13 vaccines.
[c] Other serotypes identified included the following (number of isolates shown in parentheses): 1 (1), 7C (1), 9A (2), 9C (1), 10A (2), 12F (2), 15 (1), 15C (2), 15F (1), 22F (3), and 25A (1).

Serotyping. All pneumococcal isolates were serotyped by the Quellung reaction using antisera from the Staten Serum Institut (SSI; Copenhagen, Denmark) (2, 23).

PFGE. Chromosomal DNA for pulsed-field gel electrophoresis (PFGE) was prepared as described by M. C. McEllistrem et al. (18) and was then digested with the restriction enzyme SmaI. The DNA fragments were resolved in a CHEF-Mapper apparatus (Bio-Rad) at 6.0 V/cm for 20 h with pulse times of 1 to 30 s, followed by another 6 h with pulse times of 5 to 9 s. We completed PFGE analysis on erythromycin-resistant isolates collected from 2005 to 2008 because we received funding for this component during the second half of the surveillance period. Seven international clones identified in the Pneumococcal Molecular Epidemiology Network were purchased from American Type Culture Collection and used for comparison.

Statistical analyses. We calculated descriptive statistics for all cases, using means and medians as appropriate and geometric means for susceptibility results. We compared the frequency of macrolide resistance phenotypes and genotypes by using chi-square test statistics. We analyzed linear trends in proportions over time using the Mantel-Haenszel chi-square test for trend. We calculated population rates of disease for individual serotypes by using adult population estimates from the U.S. Census population intercensal estimates for 2001 to 2007. These county-level population estimates are based on the 2000 decennial Census, with annual population adjustments based on sampling and boundary adjustments (http://www.census.gov/popest/archives/2000s/vintage_2008/). Since our analyses focused on 1 October through 30 September analysis periods, we used the estimated population denominator of the year at the start of the observation period for each annual incidence rate calculation. We analyzed linear trends in the incidence of infection due to each genotype by using linear regression. The PFGE profiles were analyzed with the Fingerprinting II Informatix software (Bio-Rad).

RESULTS

Trends in antimicrobial resistance among invasive pneumococcal isolates. A total of 2,688 clinical isolates of *S. pneumoniae* were collected from October 2001 to September 2008 from blood cultures from adult patients admitted to any of the 46 hospitals within the surveillance region. The number of isolates per year was as follows: 373 (2001-2002), 352 (2002-2003), 287 (2003-2004), 360 (2004-2005), 422 (2005-2006), 461 (2006-2007), and 433 (2007-2008). Overall, the percentage of isolates resistant to each antimicrobial drug was as follows: penicillin, 5.5% ($n = 148$), erythromycin, 17.2% ($n = 462$), clindamycin, 8.7% ($n = 234$), tetracycline, 10.2% ($n = 274$), cotrimoxazole, 13.7% ($n = 368$), and levofloxacin, 0.5% ($n = 13$). Figure 1 displays the trends in antimicrobial resistance patterns over the study period for each of the antimicrobial drugs. An increasing trend in antimicrobial resistance was observed from the 2001-2002 season to the 2007-2008 season for erythromycin (14.7% to 20.3%; $P = 0.0004$), clindamycin (4.0% to 13.9%; $P < 0.0001$), and tetracycline (7.5% to 14.5%; $P < 0.0001$). In contrast, resistance to penicillin decreased over the years (7.2% to 4.2%; $P = 0.23$), and resistance to cotrimoxazole also decreased (20.1% to 11.3%; $P = 0.06$). Applying the newer 2010 CLSI criteria for penicillin susceptibility, the percentage of resistant isolates decreased from 3.7% to 2.8% ($P = 0.44$).

Mechanisms of resistance among macrolide-resistant isolates. Among 462 erythromycin-resistant isolates, the penicillin, tetracycline, and cotrimoxazole resistance rates were 26.0%, 53.7%, and 49.6%, respectively. Approximately half ($n = 226$) of the erythromycin-resistant isolates displayed the MLS_B phenotype (i.e., clindamycin resistant), and of these, 98.2% ($n = 222$) had high levels of erythromycin resistance (MIC, $\geq$256 μg/ml) (Fig. 2).

The genotype distribution of macrolide-resistant isolates was as follows: *mef*(A)$^+$ *erm*(B) negative, 48.9%; *mef*(A) negative *erm*(B)$^+$, 34.2%; *mef*(A)$^+$ *erm*(B)$^+$, 14.1%. Among those isolates with the *mef*(A)$^+$ *erm*(B)-negative genotype, 98.7% displayed the M phenotype. In contrast, among the isolates with the *mef*(A)-negative *erm*(B)$^+$ genotype, 97.5% displayed the MLS_B phenotype. No isolates with an inducible *erm*(B) phenotype were detected. In addition, a higher proportion of the *mef*(A)$^+$ *erm*(B)$^+$ isolates demonstrated resistance to penicillin (76.9%) than did the *mef*(A)-negative *erm*(B)$^+$ isolates (9.5% penicillin resistant) or the *mef*(A)$^+$ *erm*(B)-negative isolates (23.5% penicillin resistant).

In terms of secular trends, erythromycin resistance mediated by *mef*(A) was the most common genotype (72.7%) in 2001-2002, and this percentage fell to 34.8% in 2007-2008 (Table 1). In contrast, erythromycin-resistant isolates expressing the *erm*(B) gene alone increased from 20.0% to 46.1%, and *mef*(A)$^+$ *erm*(B)$^+$ isolates increased significantly from 1.8% to 19.1% over the same time period. From a population perspective, the annual rate of infection due to isolates with the *mef*(A)$^+$ *erm*(B)$^+$genotype increased from 0.03 cases per 100,000 adults to 0.6 cases per 100,000 adults over the study period ($P = 0.003$ for trend). The rate of infection due to isolates with the *mef*(A)-negative *erm*(B)$^+$ genotype increased from 0.4 to 1.4 cases per 100,000 adults ($P = 0.002$ for trend), and the rate of infection due to isolates with the *mef*(A)$^+$ *erm*(B)-negative genotype declined from 1.4 cases to 1.0 cases per 100,00 adults ($P = 0.97$ for trend).

There were a total of nine strains (1.8%) with the 23S rRNA point mutation. Two *mef*(A)$^+$ *erm*(B)-negative strains were de-

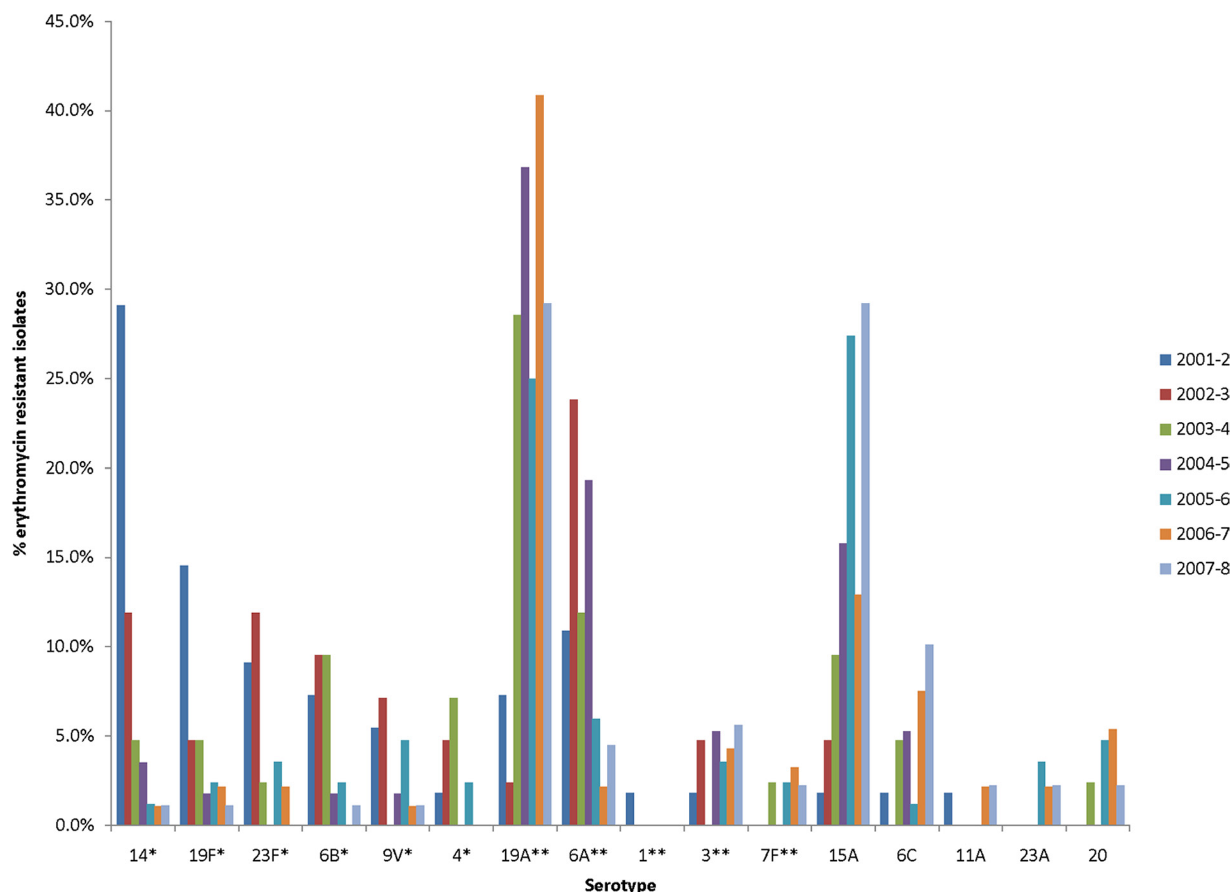

FIG 3 Serotype distribution of erythromycin-resistant *S. pneumoniae* isolates from October 2001 to September 2008. There were a total of 29 serotypes distributed among the 462 erythromycin-resistant isolates. The percentage of resistant isolates in each year of each serotype is depicted. Serotypes marked with an asterisk are PCV-7 serotypes; serotypes marked with double asterisks are PCV-13 serotypes. The following isolates were not included in this figure: 33F (3.5%; $n = 16$), 23B (1.1%; $n = 5$), 35B (0.6%; $n = 3$), 22F (0.6%; $n = 3$), 15C (0.4%; $n = 2$), 12F (0.4%; $n = 2$), 10A (0.4%; $n = 2$), 8 (0.4%; $n = 2$), 9L (0.2%; $n = 1$), 7C (0.2%; $n = 1$), 25A (0.2%; $n = 1$), 15F (0.2%; $n = 1$), 15B (0.2%; $n = 1$).

tected with the A2059G mutation in all four copies of the 23S rRNA. The other seven isolates with the A2059G mutation were *mef*(A) negative *erm*(B) negative. Among these, five had the A2059 mutation in all four gene copies and two had the mutation in two copies. Strains with the mutation in four copies of the 23S rRNA displayed an MLS_B phenotype and high-level resistance to erythromycin (MIC, $\geq$256 μg/ml). Two isolates, each with two copies of the A2059G mutation, exhibited the M phenotype and low-level erythromycin resistance. In addition, there were five erythromycin-resistant isolates harboring no detectable *erm*(B) or *mef*(A) genes or A2059G mutations. Further DNA sequence analyses of the 23S rRNA and ribosomal protein L4 and L22 for these five isolates did not identify any mutations in these genes.

Serotype patterns among erythromycin-resistant isolates. Of the 462 erythromycin-resistant isolates, 448 isolates were typeable and comprised 30 different serotypes. The overall serotype distribution among these erythromycin-resistant isolates was as follows: serotype 19A (27.5%), 15A (17.2%), 6A (9.2%), and 14 (6.3%) (Table 2). PCV-7 serotypes 14, 19F, 6B, and 23F were the most frequent serotypes associated with erythromycin resistance in 2001-2002. These serotypes decreased significantly over the study period (Fig. 3). By the end of the study period, the most common non-PCV-7 serotypes, 19A, 15A, 6C, 3, and 6A, accounted for 29.7%, 29.2%,10.1%, 5.6%, and 4.5% of the erythromycin-resistant isolates, respectively, compared with 7.3%, 1.8%,1.8%, 1.8%, and 10.9% in 2001-2002. Among all of the serotypes represented in Fig. 3, the proportion of isolates with macrolide resistance demonstrated a statistically significant increase over the surveillance period only for serotypes 9V (from 11.5% to 100%; $P = 0.02$) and 19A (from 13.8% to 27.4%; $P = 0.02$). The proportion of isolates with macrolide resistance within each of the remaining serotypes did not change significantly over the surveillance period. For example, 100% of 15A isolates were macrolide resistant at the start and end of the surveillance period, and 25% of 6C isolates were macrolide resistant at both time points.

The most common serotypes among *mef*(A)-negative *erm*(B)$^+$ strains included serotypes 15A, 19A, 3, and 6B, which comprised 45.6%, 20.9%, 10.1%, and 6.3% of the *mef*(A)-negative *erm*(B)$^+$ strains (Table 2). Serotype 19A was the most common serotype among the *mef*(A)$^+$ *erm*(B)$^+$ isolates (81.5%).

Genetic relatedness analysis by PFGE. We performed PFGE analysis on the 293 erythromycin-resistant strains collected from 2005 to 2008. There were a total of 12 major clusters (Table 3, A to L). Serotype 19A was the largest group of the macrolide-resistant isolates, which was distributed into six major clusters: isolates car-

TABLE 3 PFGE analysis of erythromycin-resistant *S. pneumoniae* isolates collected from 2005 to 2008[c]

Cluster	Related clone(s) [genotype][a]	Serotype	No. of strains	Susceptibility pattern[b]	Genotype
A	NA	6A	10	Clin^s Pen^i Tet^s Sxt^s	*mef*(A)$^+$ *erm*(B) negative
B	NA	33F	8	Clin^s Pen^s Tet^s Sxt^r	*mef*(A)$^+$ *erm*(B) negative
C	N. Carolina^{6A}-23 [*mef*(A)$^+$ *erm*(B) negative]	15A	60	Clin^r Pen^i Tet^r Sxt^s	*mef*(A) negative *erm*(B)$^+$ (55), *mef*(A)$^+$ *erm*(B)$^+$ (5)
		19A	7	Clin^r Pen^i Tet^r Sxt^s	*mef*(A) negative *erm*(B)$^+$
D	NA	6C	15	Clin^s Pen^i Tet^s Sxt^r	*mef*(A)$^+$ *erm*(B) negative
		3	10	Clin^r Pen^s Tet^r Sxt^s	*mef*(A) negative *erm*(B)$^+$
E	Tennessee^{23F}-4 [*mef*(A)$^+$ *erm*(B) negative]	23B	3	Clin^s Pen^r Tet^s Sxt^s	*mef*(A)$^+$ *erm*(B) negative
		23A	4	Clin^s Pen^s Tet^s Sxt^s	*mef*(A)$^+$ *erm*(B) negative
		19A	15	Clin^s Pen^s Tet^s Sxt^s	*mef*(A)$^+$ *erm*(B) negative
F	England^{14}-9 [*mef*(A)$^+$ *erm*(B) negative], Spain ^{23F}-1 [*mef*(A) negative *erm*(B) negative]	14	3	Clin^s Pen^s Tet^s Sxt^s	*mef*(A)$^+$ *erm*(B) negative
		19A	5	Clin^s Pen^i Tet^s Sxt^r	*mef*(A)$^+$ *erm*(B) negative
		23F	4	Clin^s Pen^s Tet^r Sxt^r	*mef*(A)$^+$ *erm*(B) negative
G	NA	11A	3	Clin^s Pen^s Tet^s Sxt^s	*mef*(A)$^+$ *erm*(B) negative
		15A	2	Clin^r Pen^s Tet^r Sxt^s	*mef*(A) negative *erm*(B)$^+$
		19A	10	Clin^r Pen^i Tet^r Sxt^r	*mef*(A) negative *erm*(B)$^+$
H	NA	20	11	Clin^s Pen^s Tet^s Sxt^r	*mef*(A)$^+$ *erm*(B) negative
I	Spain ^{9V}-3 [*mef*(A) negative *erm*(B) negative]	9V	6	Clin^s Pen^r Tet^s Sxt^r	*mef*(A)$^+$ *erm*(B) negative
J	NA	7F	7	Clin^s Pen^s Tet^s Sxt^s	*mef*(A)$^+$ *erm*(B) negative
K	S. Africa ^{19A}-13 [*mef*(A) negative *erm*(B)$^+$]	23A	3	Clin^r Pen^s Tet^r Sxt^s	*mef*(A) negative *erm*(B)$^+$
		19A	2	Clin^r Pen^i Tet^r Sxt^s	*mef*(A) negative *erm*(B)$^+$
L	Taiwan ^{19F}-14 [*mef*(A)$^+$ *erm*(B) negative]	19A	44	Clin^r Pen^r Tet^r Sxt^s	*mef*(A)$^+$ *erm*(B)$^+$
		19F	4	Clin^r Pen^r Tet^r Sxt^r	*mef*(A)$^+$ *erm*(B)$^+$

[a] Referenced from the Pneumococcal Molecular Epidemiology Network (http://www.sph.emory.edu/PMEN/). NA, not available.
[b] Clin, clindamycin; Pen, penicillin; Tet, tetracycline; Sxt, trimethoprim-sulfamethoxazole.
[c] Isolates (and counts) not listed in the above clusters: cluster A [23B (1), 23F (1), 19A (1), untypeable (1)]; cluster C [6C (1), 23F (1), 15C (2), 22F (1)]; cluster D [6A (1), 6B (1), 22F (1), 19A (1)]; cluster E [15A (1), 4 (2), 3 (1), 14 (1)]; cluster F [10A (1), 19F (1)]; cluster H [7C (1)]; cluster I [19A (1), 15F (1)]; cluster J [6B (1), 35B (2)]; cluster K [6B (1), 12F (1)].

rying both the *erm*(B) and *mef*(A) genes were related to international clone Taiwan 19F; isolates harboring the *mef*(A) gene alone were related to international clones Tennessee-23F and England14-9; isolates expressing the *erm*(B) gene alone were related to international clones Spain 23F-1 and North Carolina 6A-23. Serotype15A was the second largest group among macrolide-resistant isolates, and it appeared highly clonal. The majority of serotype15A isolates displayed close relatedness to the North Carolina 6A-23 clone.

DISCUSSION

Population-based surveillance for bacteremic pneumococcal disease among adults in the Philadelphia region between 2001 and 2008 demonstrated increasing antimicrobial resistance to erythromycin, tetracycline, and clindamycin, while resistance to penicillin declined over the same period. In particular, erythromycin resistance increased steadily from 14.7% in 2001-2002 to 20.3% in 2007-2008. Among all erythromycin-resistant isolates, the proportion of isolates from serotypes included in PCV-7 fell between 2001-2002 and 2007-2008, from 67.3% to 4.5%, and the proportion from serotypes included in PCV-13 fell from 89.1% to 46.1%. In 2001-2002, of the serotypes found in PCV-7, types 14, 19F, 23F, and 6B were the most prevalent erythromycin-resistant serotypes. In 2007-2008, types19A, 15A, and 6C emerged as the most common serotypes among erythromycin-resistant isolates. The increase in macrolide resistance over the surveillance period represented both an expansion of serotypes, with high macrolide resistance at the start of the period (e.g., 15A), and acquisition of macrolide resistance within specific serotypes (e.g., 19A), either through capsular switching or introduction of novel clones into the region.

We observed that the genotype distribution patterns of erythromycin resistance shifted during the study period. An efflux pump mediated by the *mef*(A) gene was the predominant mechanism responsible for macrolide resistance at the beginning of the study period. *mef*(A)-mediated macrolide resistance gradually dropped from its peak of 72.7% in 2001-2002, to 40.5% in 2004-2005, and to 34.8% in 2007-2008. In parallel, *erm*(B)-positive strains increased from 20.0% at the start of surveillance to 46.1%, becoming the most prevalent macrolide resistance genotype in

2007-2008. The most notable shift was the increase of *mef*(A)$^+$ *erm*(B)$^+$ isolates from 1.8% in 2001-2002 to 19.1% in 2007-2008. The higher prevalence of the *erm*(B) genotype was primarily related to the higher frequency of *erm*(B)$^+$ serotype 15A, a serotype not covered by the recently introduced PCV-13 vaccine. Our data are consistent with those of PROTEKT US nationwide *S. pneumoniae* surveillance, which showed the prevalence of the *mef*(A)$^+$ *erm*(B)$^+$ genotype increased from 9.7% in 2000-2001 to 24.1% in 2005-2006 among clinical isolates and that *erm*(B)$^+$ genotype prevalence increased slightly from 16.5% to 18.8%, while *mef*(A)$^+$ genotype prevalence decreased from 65.7% to 53.8% over the same time period (12).

Serotype19A has been widely recognized as the key emerging serotype in the post-PCV-7 era, notably for its multidrug-resistant phenotype (7). Of note, 19A is a component of PCV-13, which was introduced in 2010, and so future surveillance will need to determine the impact on this serotype. Since serotypes 15A, 23A, and 6C are not included in the PCV-13 vaccine, a future increase in cases caused by these serotypes may be expected, particularly in response to ongoing drug selection for antimicrobial-resistant isolates. It is very likely that these serotypes could play important roles in the expansion of macrolide resistance in the post-PCV-13 era, especially type 15A. The majority (96.3%) of 15A isolates displayed high-level macrolide resistance [*erm*(B) encoded] and also displayed multidrug resistance. In addition, a small percentage of 15A isolates expressed both the *erm*(B) and *mef*(A) genes and were genetically related, as demonstrated by PFGE. This suggests that serotype 15A acquired *erm*(B) and *mef*(A) genes through a horizontal gene transfer event within serogroup 15 rather than capsular switching with other serogroup strains positive for the *erm*(B) and *mef*(A) genes.

We did not detect a high frequency of the 23S rRNA A2059G point mutation. Among all serotypes, 23F isolates displayed the highest frequency of the A2509G mutation (14% of 23F macrolide-resistant isolates). This could have resulted from clonal spread or could have arisen from independent mutation events. Joloba et al. demonstrated that serotype 23F cannot be naturally transformed *in vitro* under inducing conditions (13). This may explain the relatively high rate of the 23S rRNA A2059G mutation as a mechanism for resistance among serotype 23F isolates.

Our work builds on prior studies demonstrating an increase in macrolide resistance among pneumococcal isolates, adding additional data on the serotypes and molecular types responsible for this increase. Taken together, surveillance of invasive *S. pneumoniae* in the Philadelphia region highlights the upward trend of macrolide resistance, especially of high-level macrolide resistance mediated by *erm*(B) alone or both the *erm*(B) and *mef*(A) genes. This study has identified several emerging serotypes associated with macrolide resistance, specifically, serotypes 15A, 6C, and 23A, which are not covered by the PCV-13 vaccine. It has been speculated that serotype 6A in PCV-13 may provide some cross-protection against 6C (22). Future surveillance studies will help assess the degree of cross-protection observed in practice. Moreover, it is imperative to monitor whether the existing non-PCV-13 serotypes will spread further or whether additional replacement serotypes will emerge following the introduction of PCV-13.

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