Molecular Resistance Mechanisms of Macrolide-Resistant Invasive *Streptococcus pneumoniae* Isolates from Alaska, 1986 to 2010

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The rapid emergence of antibiotic-resistant pneumococcal strains has reduced treatment options. The aim of this study was to determine antimicrobial susceptibilities, serotype distributions, and molecular resistance mechanisms among macrolide-resistant invasive pneumococcal isolates in Alaska from 1986 to 2010. We identified cases of invasive pneumococcal disease in Alaska from 1986 to 2010 through statewide population-based laboratory surveillance. All invasive pneumococcal isolates submitted to the Arctic Investigations Program laboratory were confirmed by standard microbiological methods and serotyped by slide agglutination and the Quellung reaction. MICs were determined by the broth microdilution method, and macrolide-resistant genotypes were determined by multiplex PCR. Among 2,923 invasive pneumococcal isolates recovered from 1986 to 2010, 270 (9.2%) were nonsusceptible to erythromycin; 177 (66%) erythromycin-nonsusceptible isolates demonstrated coresistance to penicillin, and 167 (62%) were multidrug resistant. The most frequent serotypes among the macrolide-resistant isolates were serotypes 6B (23.3%), 14 (20.7%), 19A (16.7%), 9V (8.9%), 19F (6.3%), 6A (5.6%), and 23F (4.8%). *mef* and *erm*(B) genes were detected in 207 (77%) and 32 (12%) of the isolates, respectively. Nineteen (7%) of the erythromycin-nonsusceptible isolates contained both *mef* and *erm*(B) genotypes; 15 were of serotype 19A. There was significant year-to-year variation in the proportion of isolates that were nonsusceptible to erythromycin ($P < 0.001$). Macrolide resistance among pneumococcal isolates from Alaska is mediated predominantly by *mef* genes, and this has not changed significantly over time. However, there was a statistically significant increase in the proportion of isolates that possess both *erm*(B) and *mef*, primarily due to serotype 19A isolates.

Streptococcus pneumoniae continues to be an important cause of community-acquired pneumonia, meningitis, and acute otitis media worldwide. The rapid emergence of strains of pneumococci resistant to a number of well-known antibiotics, including macrolides, has reduced treatment options (1). Macrolides are commonly used for empirical treatment of community-acquired pneumococcal pneumonia, and pneumococcal isolates that are resistant to macrolides are often resistant to other first-line antibiotics. In the United States, the rate of resistance to macrolides steadily increased during the 1990s and then appeared to level off at approximately 30% (2, 3). In a recent study, however, the rate of macrolide resistance increased significantly in all regions of the United States and among all age groups (4).

Macrolide resistance in pneumococci is most often mediated by two mechanisms. Target-site modification encoded by the *erm*(B) gene usually confers high-level macrolide resistance ($\text{MIC}_{90} \geq 32 \mu\text{g/ml}$) and cross-resistance to lincosamides and streptogramin B drugs (MLS_B phenotype) (5). In pneumococci, *erm*(B) is carried on members of the Tn916 family of transposons, which also carry the *tet*(M) gene, conferring tetracycline resistance (6). The second macrolide resistance mechanism is active drug efflux mediated by a membrane efflux pump encoded by the *mef* class of genes, typically resulting in low- to mid-level resistance (MIC_{90} of 1 to $8 \mu\text{g/ml}$) and conferring resistance only to macrolides (M phenotype) (5, 7, 8). There are two variants of the *mef* gene, *mef*(A), which was originally found in *Streptococcus pyogenes*, and *mef*(E), which was originally described for *S. pneumoniae* (9, 10). While *mef*(A) and *mef*(E) are 90% identical at the nucleotide level, they are carried on different genetic elements. *mef*(A) is carried on the transposon Tn1207.1, whereas *mef*(E) is carried on the 5.4- to 5.5-kb macrolide efflux genetic assembly (mega) element (11). There have been other mechanisms of mac-

rolide resistance described for a limited number of clinical isolates. These mechanisms involve changes in a highly conserved region of domain V of the 23S rRNA and in ribosomal proteins L4 and L22 (5).

The prevalence of the respective macrolide resistance mechanisms varies by geographic region. Prior to the introduction of the 7-valent pneumococcal polysaccharide-protein conjugate vaccine (PCV7) in the United States in 2000, *mef*-mediated resistance was the most prevalent mechanism of macrolide resistance (12). Since then, the prevalence of *mef*-mediated resistance has decreased, and isolates harboring *erm*(B) and *mef* genes are becoming increasingly common not only in the United States but also worldwide (13–17). In addition, isolates carrying only the *mef* gene showed a higher level of resistance ($\text{MIC}_{90} \geq 16 \mu\text{g/ml}$) than observed previously (18). In most of the rest of the world, *erm*(B)-mediated resistance has been the predominant mechanism (19–23).

In Alaska, rates of invasive pneumococcal disease (IPD) have been among the highest reported in the world, particularly among children <2 years of age (24). PCV7 was introduced into the childhood vaccine schedule in Alaska in January 2001 using a 3 + 1 dosing schedule (primary series given at 2, 4, and 6 months of age followed by a booster dose at 12 to 15 months of age), resulting in

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a significant and rapid decline in rates of IPD caused by vaccine serotypes (25). The rates of IPD caused by nonvaccine serotypes subsequently increased, leading to the introduction of PCV13 in March 2010. Here we describe the prevalence and molecular epidemiology of macrolide resistance among invasive pneumococcal isolates collected in Alaska as part of the statewide invasive bacterial disease surveillance program prior to the introduction of PCV7 (1986 to 2000) and post-PCV7 introduction (2001 to 2010). The data presented here specifically describe the macrolide resistance genotypes, antimicrobial susceptibility profiles, and serotype distribution among the macrolide-resistant isolates. In addition, we were particularly interested in examining the prevalence of and genetic relatedness among non-PCV7 macrolide-resistant isolates by using multilocus sequence typing (MLST).

MATERIALS AND METHODS

Population studied. Alaska's population of 710,231 (2010 U.S. Census [50]) includes 142,000 (20%) Alaska Native (AN) and American Indian peoples, 7,100 of whom are younger than 2 years of age. Sixty-five percent of AN peoples live in rural communities, many of which are isolated villages with populations ranging from 50 to 1,000 persons.

Bacterial isolates. The Arctic Investigations Program (AIP) established a population-based statewide surveillance system to monitor IPD in 1986. Isolates of *S. pneumoniae* are received at the AIP laboratory, Anchorage, AK, from 23 regional hospital laboratories processing sterile-site specimens (blood and cerebrospinal, pleural, peritoneal, or joint fluid) in the state. From 1986 through 2010, 2,923 isolates of invasive *S. pneumoniae* were submitted to the AIP. Pneumococci were confirmed by colony morphology, susceptibility to optochin (Difco, Detroit, MI), and bile solubility. All isolates were serotyped by slide agglutination and confirmed by the Quellung reaction using group- and type-specific antisera (Statens Serum Institut, Copenhagen, Denmark).

Antimicrobial susceptibility testing. Susceptibility testing was performed by using the broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines for penicillin (Pen), erythromycin (Ery), trimethoprim-sulfamethoxazole (TMP-SMX), tetracycline (Tet), clindamycin, chloramphenicol, ceftriaxone, cefotaxime, vancomycin, and rifampin (26). The MIC was determined to be the lowest concentration of antibiotic that inhibited growth. The MIC results were interpreted according to 2007 CLSI criteria, which included the following penicillin breakpoints: susceptible at ≤ 0.06 $\mu\text{g/ml}$, intermediate at 0.12 to 1 $\mu\text{g/ml}$, and resistant at ≥ 2 $\mu\text{g/ml}$. Isolates were defined as nonsusceptible if they were intermediately resistant or resistant to an antibiotic and were defined as multidrug resistant if they were nonsusceptible to three or more different classes of antibiotics. Erythromycin-nonsusceptible (Ery^{NS}) isolates were identified as having the M phenotype if they were susceptible to clindamycin and as having the MLS_B phenotype if they were nonsusceptible to clindamycin.

PCR for macrolide resistance genes. Erythromycin-resistant isolates were analyzed for the presence of *erm*(B) and *mef* genes by using a duplex PCR assay. Bacterial cells were suspended in 0.1 ml of nuclease-free H₂O, heated at 100°C for 10 min, centrifuged at 13,000 rpm for 5 min, and stored at -30°C until use. The erythromycin resistance genes *erm*(B) and *mef* were screened by using primer sets designed previously by Sutcliffe et al. and Tait-Kamradt et al., respectively (10, 27). PCR was performed with a total volume of 25 μl consisting of 12.5 μl AmpliTaq Gold (Applied Biosystems, Foster City, CA), 3.0 μl of DNA lysate, 0.5 μM each *erm*(B) primer, and 2.0 μM each *mef* primer. Amplifications were performed with a Perkin-Elmer GeneAmp 9700 PCR system (Applied Biosystems) under the following conditions: 94°C for 3 min followed by 30 cycles of 94°C for 45 s, 53°C for 30 s, and 72°C for 2 min with a final extension step at 72°C for 5 min. Negative controls consisted of the PCR mixture without the DNA template; positive controls consisted of *S. pneumoniae* DNA from erythromycin-resistant strains known to carry either *mef* or *erm*(B). Am-

FIG 1 Proportion of invasive pneumococcal isolates nonsusceptible to erythromycin in Alaska by year, 1986 to 2010.

plification products were run through 3% ReadyAgarose gels (Bio-Rad, Hercules, CA) in 1× Tris-acetate-EDTA (TAE) (Invitrogen, Carlsbad, CA) at 100 V for 60 min. The PCR product sizes for the *erm*(B) and *mef* genes are 639 bp and 940 bp, respectively. The PCR products of the *mef* gene were digested with DraI (Promega, Madison, WI) in order to discriminate between the *mef*(A) and *mef*(B) subclasses (28).

MLST analysis. MLST was performed, as previously described (29), with modifications (30), on macrolide-resistant isolates recovered from 2000 to 2010. The sequence types (STs) were determined by comparing the sequences with alleles downloaded from the pneumococcal MLST database (<http://spneumoniae.mlst.net/>). Clonal complexes were assigned by using the eBURST algorithm with software available at the MLST website (<http://www.mlst.net/>).

Statistical analysis. Sixty MICs (2.1%) were tested to an indeterminate cutoff without an upper limit (e.g., >16 $\mu\text{g/ml}$). All were in the resistant range. These isolates were assigned the next highest MIC (>16 $\mu\text{g/ml}$ considered 32 $\mu\text{g/ml}$) for graphing and analysis. The proportions of isolates with macrolide resistance were compared across other factors by using a chi-squared test, Fisher's exact test, or a randomization test as appropriate. Average ages were compared with a *t* test. All *P* values are two sided, and a *P* value of <0.05 is considered statistically significant. Trends over time in the proportion of isolates that were antibiotic resistant were assessed by using logistic regression. Genetic diversity was assessed by applying Simpson's diversity index (*D*) to STs obtained by MLST (31).

RESULTS

Prevalence and trends in macrolide resistance. Of the 2,923 invasive pneumococcal isolates collected in Alaska from 1986 to 2010, 270 (9.2%) were nonsusceptible to erythromycin (MICs > 0.5 $\mu\text{g/ml}$). The prevalence of erythromycin-nonsusceptible (Ery^{NS}) isolates increased from a low of 0.8% in 1988 to 21.5% in 2000, and the proportion of isolates that were Ery^{NS} varied significantly over time (*P* < 0.001) (Fig. 1). For the period from 1986 to 1994, 3.5% (38/1,086) of isolates were Ery^{NS}. From 1995 to 2000, 16% (117/736) of isolates were Ery^{NS}, the majority (93%) of which were of serotypes contained in PCV7 (Fig. 2). Following the introduction of PCV7 in 2001, the number of Ery^{NS} isolates declined to 8.2% (37/452) for the period from 2001 to 2005. However, from 2006 to 2010, the proportion of Ery^{NS} isolates increased to 12% (78/649), the majority (58%; 45/78) of which were of serotypes 19A and 6A.

For this 25-year period, the average age of patients with Ery^{NS} isolates was 28.4 years (range, <1 year of age to 97 years of age). The proportion of Ery^{NS} isolates was significantly higher in chil-

FIG 2 Erythromycin-nonsusceptible invasive pneumococcal isolates from Alaska by serotype and time period, 1986 to 2010. *, serotypes included in PCV7; **, other serotypes included serotypes 1, 3, 4, 5, 6C, 8, 7F, 9A, 9N, 10A, 11A, 15A, 15B, 16F, 17F, 20, 23A, 23B, 31, 33F, 35B, and NT.

dren <5 years of age (13.5%; 136/1,004) than in individuals ≥ 5 years of age (7%; 134/1,917) ($P < 0.001$). Among the Ery^{NS} isolates, 44.4% (120/270) and 28% (75/270) were recovered from children <2 years of age and adults ≥ 55 years of age (49 adults were > 65 years of age), respectively. Overall, 5.7% (167/2,923) of isolates were recovered from cerebrospinal fluid (CSF) specimens. This included 79% (165/208) of the meningitis cases. Among the Ery^{NS} isolates, 6.7% (18/207) were recovered from CSF and included 78% (18/23) of the meningitis cases.

Antimicrobial susceptibility. Sixty-six percent (177/270) of Ery^{NS} isolates were nonsusceptible to penicillin, and 82% (222/270) were nonsusceptible to TMP-SMX, compared to 7.5% (199/2652) and 12% (314/2653) of erythromycin-susceptible isolates, respectively. The proportions of Ery^{NS} isolates that were nonsusceptible to cefotaxime, ceftriaxone, and tetracycline were much higher, at 32% (85/270), 26% (60/227), and 23% (63/270), compared to 0.7% (18/2652), 0.7% (18/2377), and 1% (36/2651) of erythromycin-susceptible isolates, respectively. Sixty-two percent (167/270) of Ery^{NS} isolates were nonsusceptible to at least two additional classes of antibiotics, compared to only 5% (128/2653) of erythromycin-susceptible isolates. The proportion of Ery^{NS} isolates that were nonsusceptible to other antibiotics changed over time. The proportion of Ery^{NS} isolates that were nonsusceptible to penicillin increased from 60% (93/155) during 1986 to 2000 to 73% (84/115) during 2001 to 2010 ($P = 0.03$); tetracycline resistance increased from 17% (27/155) to 31% (36/115) ($P = 0.007$). In contrast, the proportion of Ery^{NS} isolates that were nonsusceptible to TMP-SMX decreased significantly, from 90% (140/155) to 71% (82/115) ($P < 0.001$).

Routine susceptibility testing for clindamycin began in 2001. Among the 115 Ery^{NS} isolates recovered from 2001 to 2010, 32 (28%) displayed the MLS_B phenotype (clindamycin resistant).

The proportion of isolates with the MLS_B phenotype increased significantly from the 2001-2005 time period to the 2006-2010 time period (13% versus 87%; $P < 0.001$).

Macrolide resistance determinants. The majority (77%; 207/268) of invasive Ery^{NS} isolates carried the *mef* genes [*mef*(E), $n = 204$; *mef*(A), $n = 3$], 12% ($n = 31$) carried the *erm*(B) gene, and 7% ($n = 19$) carried both genes (dual resistance) (Table 1). Eleven (4.1%) isolates were negative for both macrolide resistance genes (Table 1). Among those isolates with the *mef*⁺ genotype for which clindamycin susceptibility testing results were available ($n = 77$), all displayed the M phenotype. Fourteen (93%) of Ery^{NS} isolates with the *erm*(B) genotype and all of the Ery^{NS} isolates ($n = 17$) that were positive for both the *mef* and *erm*(B) genes displayed the MLS_B genotype. The proportion of isolates with various genotypes differed significantly by time period ($P < 0.001$). In particular, the proportion of isolates that carried both the *mef* and *erm*(B) genes was significantly greater in the later time period (2006 to 2010) than in the previous time periods. This was due primarily to the presence of 15 serotype 19A isolates recovered from 2006 to 2010.

The proportion of Ery^{NS} isolates carrying the *erm*(B) gene and carrying the *erm*(B) and *mef* genes was significantly higher among individuals ≥ 5 years of age than among those <5 years of age (17.2% [23/134] versus 6.6% [9/136] [$P = 0.003$] and 9.0% [12/134] versus 5.2% [7/136] [$P = 0.003$], respectively). The proportion of persons with Ery^{NS} isolates carrying the *mef* genes did not differ significantly by age. Ery^{NS} isolates recovered from non-Native Alaskans were more likely to carry the *erm*(B) gene or both genes than Ery^{NS} isolates recovered from Alaska Native peoples (14.9% [21/141] versus 8.5% [11/129] and 10.6% [15/141] versus 3.1% [4/129], respectively).

Erythromycin MICs for isolates carrying the *mef* genes ranged from 0.5 $\mu\text{g/ml}$ to ≥ 64 $\mu\text{g/ml}$, including seven isolates with MICs

TABLE 1 Prevalence of erythromycin resistance determinants among invasive pneumococcal isolates in Alaska by time period

Erythromycin resistance determinant(s)	% of isolates with resistance determinant (no. of positive isolates/total no. of isolates tested)				Total ($n = 2,923$)	<i>P</i> value for trend
	1986–1994 ($n = 1086$)	1995–2000 ($n = 736$)	2001–2005 ($n = 452$)	2006–2010 ($n = 649$)		
<i>mef</i> ⁺	82 (31/38)	85 (99/117)	81 (30/37)	60 (47/78)	77 (207/268)	<0.001
<i>erm</i> (B) ⁺	8 (3/38)	11 (13/117)	11 (4/37)	15 (12/78)	12 (31/268)	0.274
<i>mef</i> ⁺ and <i>erm</i> (B) ⁺	0 (0/38)	2 (2/117)	3 (1/37)	21 (16/78)	7 (19/268)	<0.001
Neither	5 (2/38)	3 (3/117)	3 (1/37)	6 (5/78)	4 (11/268)	0.342

FIG 3 Distribution of erythromycin MICs among *mef*⁺, *erm*(B)⁺, and *mef*⁺ *erm*(B)⁺ pneumococcal isolates in Alaska, 1986 to 2010. *, MICs <4 μg/ml include MICs of 0.5 μg/ml (*n* = 1), 1 μg/ml (*n* = 5), and 2 μg/ml (*n* = 7).

of ≥64 μg/ml (Fig. 3). The majority (69%) of *mef*-positive isolates had MICs of 8 to 16 μg/ml. *mef*-positive isolates with MICs of ≥32 μg/ml were not seen until 2000 and accounted for 21% (16/77) of Ery^{NS} isolates recovered from 2001 to 2010. Erythromycin MICs of isolates harboring the *erm*(B) gene ranged from 4 μg/ml to ≥64 μg/ml (Fig. 3). Prior to the introduction of PCV7, only 6% (1/16) of isolates carrying the *erm*(B) gene had MICs of ≥32 μg/ml. After the introduction of PCV7, 100% (16/16) of isolates carrying the *erm*(B) gene had MICs of ≥32 μg/ml. Isolates carrying both the *erm*(B) gene and a *mef* gene had high MICs (32 μg/ml to ≥64 μg/ml). Among Ery^{NS} isolates that were negative for both genes,

MICs ranged from 0.5 μg/ml to 32 μg/ml, the majority (64%) of which had MICs of ≤8 μg/ml.

Serotype distribution. The distribution of pneumococcal serotypes among Ery^{NS} isolates is shown in Table 2. The most frequent serotypes among Ery^{NS} isolates were serotypes 6A, 6B, 9V, 14, 19A, 19F, and 23F, accounting for 86% (233/270) of the isolates. Among isolates carrying the *erm*(B) gene, 12 serotypes were represented; serotypes 6B, 15A, 19F, and 23F were the most frequently identified (66%; 21/32). Among isolates carrying the *mef* genes, 19 serotypes were represented. The majority of these isolates were of serotypes 6B, 9V, 14, and 19A (77%; 160/207). Iso-

TABLE 2 Distribution of pneumococcal serotypes and sequence types among erythromycin-nonsusceptible isolates according to resistance genotype

Serotype	No. (%) of Ery ^{NS} isolates	No. (%) of Ery ^{NS} isolates with resistance gene				Sequence type(s) ^a (no. of isolates)
		<i>mef</i> ⁺ only	<i>erm</i> (B) ⁺ only	<i>mef</i> ⁺ and <i>erm</i> (B) ⁺	Neither	
6B ^b	63 (23.3) ^c	56 (89)	6 (10)	0	0	90 (1), 146 (1), 1165 (1), 1518 (1), 1536 (6), 4590 (1), 4591 (1)
14 ^b	56 (20.7)	53 ^d (95)	2 (4)	1 (2)	0	9 (2), 13 (14), 343 (1), 671 (2), 782 (3)
19A	45 (16.7)	28 (62)	1 (2.2)	15 (33)	1 (2.2)	156 (3), 172 (2), 199 (15), 320 (13), 1451 (1), 1756 (1), 1936 (1), 3976 (6)
9V ^b	24 (8.9)	23 (96)	0	0	1 (4)	156 (4), 557 (3), 669 (1), 4589 (1)
19F ^b	17 (6.3)	11 (65)	3 (18)	3 (18)	0	81 (2), 271 (2), 2346 (1), 4588 (1)
6A	16 (5.6)	13 (81)	1 (6)	0	2 (12.5)	376 (2), 473 (9), 1339 (1), 1379 (1), 4097 (1)
23F ^b	13 (4.8) ^c	6 (46)	6 (46)	0	0	37 (1), 81 (1), 439 (1)
15A	6 (2.2)	1 (17)	5 (83)	0	0	63 (5), 817 (1)
6C	5 (1.9)	5 (100)	0	0	0	1292 (3), 1379 (1)
9N	3 (1.1)	1 (33)	1 (33)	0	1 (33)	66 (2), 405 (1)
3	2 (0.7)	0	2 (100)	0	0	180 (1)
33F	2 (0.7)	2 (100)	0	0	0	2705 (1), 7507 (1)
7F	2 (0.7)	1 (50)	0	0	1 (50)	191 (2)
23A	2 (0.7)	1 (50)	1 (50)	0	0	338 (2)
Other ^e	14 (5.2)	6 (43)	3 (21)	0	5 (36)	62 (1), 63 (1), 383 (1), 439 (1), 558 (1), 816 (1), 1030 (1), 4592 (1), 4593 (1) ^f
Total	270 (9.2)	207 (77)	31 (11.5)	19 (7)	11 (4)	

^a MLST was performed on all Ery^{NS} isolates recovered from 2000 to 2010 (*n* = 137 [51%]).

^b Serotypes found in the 7-valent pneumococcal conjugate vaccine (PCV7).

^c Erythromycin resistance determinants could not be determined for one isolate each of serotype 6B and serotype 23F due to the lack of growth upon subculture from the freezer.

^d Three serotype 14 isolates carried the *mef*(A) gene. All other *mef*⁺ isolates carried the *mef*(E) gene.

^e Other serotypes include serotypes 1, 4, 5, 8, 9A, 10A, 11A, 15B, 16F, 17F, 20, 23B, 31, and 35B.

^f These STs represent the following serotypes in the order presented in the table: 11A, 8, 16F, 23B, 35B, 10A, 20, 17F, and 15B.

FIG 4 Distribution of serotypes among erythromycin-nonsusceptible pneumococcal isolates in Alaska by time period, 1986 to 2010. *, $P < 0.01$ over time.

lates carrying both genetic determinants included those of serotypes 14, 19A, and 19F, 79% (15/19) of which were of serotype 19A. Ten serotypes were represented among isolates negative for both *mef* and *erm*(B) genes (Table 2).

Overall, serotypes among macrolide-resistant isolates have varied significantly by time period (Fig. 4). From 1986 to 2000, the majority (94%; 145/155) of Ery^{NS} isolates were of serotypes contained in PCV7. The frequency of these serotypes had decreased significantly by the 2006–2010 time period, and these serotypes were replaced by non-PCV7 types 19A (47.4%; 37/78), 6A (10.3%; 8/78), 6C (6.4%; 5/78), and 15A (6.4%; 5/78). The proportion of isolates resistant to macrolides increased significantly over the surveillance time periods for serotypes 6A, 6B, 9V, 14, 19A ($P < 0.001$), 19F ($P = 0.009$), and 23F ($P = 0.018$) (Fig. 2). The proportion of isolates that were erythromycin nonsusceptible among the remaining serotypes did not change significantly over the surveillance time periods.

MLST. MLST of 137 macrolide-resistant isolates recovered during surveillance from 2000 through 2010 yielded 51 sequence types (STs), including eight new STs (ST4097, ST4588, ST4589, ST4590, ST4591, ST4592, ST4593, and ST7507) (Table 2). Among *mef*⁺ isolates, 35 STs were represented; 57% fell into 6 STs and included ST199 (15%; serotype 19A), ST13 (14%; serotype 14), ST473 (9%; serotype 6A), ST156 (7%; serotypes 9V and 19A), ST3976 (6%; serotype 19A), and ST1536 (6%; serotype 6B). The two serotype 14 *mef*(A)⁺ isolates were of ST9, indicating clonal relatedness to the England¹⁴⁻⁹ international clone. Ten STs were represented among the 15 isolates harboring the *erm*(B) gene; 40% were of ST63 and included isolates of serotypes 8 and 15A. Ery^{NS} isolates harboring both the *erm*(B) and *mef* genes were clustered into a single clonal complex (CC), CC271, which corresponds to the widely distributed antibiotic-resistant Taiwan^{19F-14} international strain. This CC was represented by 3 STs (ST271, ST320, and ST1415), the majority (88%; 14/16) of which were of ST320 and included serotype 19A isolates. The other STs included isolates of serotypes 19F and 19A. Using Simpson's diversity index (D), isolates harboring both the *mef* and *erm*(B) genes were significantly less diverse ($D = 0.34$) than isolates carrying the *mef* gene or the *erm*(B) gene ($D = 0.94$ and 0.86 , respectively) ($P < 0.01$). When we applied Simpson's diversity index to specific serotypes, we found that isolates of serotypes 6A and 14 had a lower probability of diversity ($D = 0.59$ and 0.58 , respectively) than

isolates of serotypes 6B, 9V, 19A, and 19F ($D = 0.77, 0.75, 0.77$ and 0.87 , respectively) ($P = 0.014$). Five STs were also represented among the 14 serotype 6A isolates, including ST376, ST473, ST1339, ST1379, and ST4097. Among the 22 serotype 14 isolates, 5 STs were represented, including ST9, ST13, ST343, ST671, and ST782.

DISCUSSION

The rate of macrolide resistance among invasive *S. pneumoniae* isolates from Alaska increased steadily from 1989 to 2000 but then declined significantly following the introduction of PCV7 in 2001. This was due mainly to a decline in the incidence of disease caused by serotypes included in the vaccine, namely, serotypes 6B, 9V, 14, 19F, and 23F. However, in more recent years, we have seen a steady increase in the number of isolates with reduced susceptibility to erythromycin, particularly among isolates of serotypes 19A, 6A, and 6C. Erythromycin nonsusceptibility was most prominent among isolates recovered from children <5 years of age, with almost one-half being recovered from children <2 years of age. In our population, the majority of erythromycin-nonsusceptible isolates were found to carry *mef* genes; however, we have seen an increase in the number of isolates carrying both the *mef* and *erm*(B) genes. Erythromycin-nonsusceptible isolates were more likely to have reduced susceptibility to penicillin and were frequently resistant to three or more classes of antibiotics.

We previously described the epidemiology of IPD in Alaska and the impact of PCV7 on IPD among Alaskan children in the post-PCV7 era (2001 to 2007) (25, 32, 33). In these reports, we described a rapid and significant decrease in the prevalence of disease caused by vaccine types coincident with a significant increase in the prevalence of IPD caused by nonvaccine types. The findings of the present study show that the majority of erythromycin-nonsusceptible isolates, prior to the introduction of PCV7, were serotypes contained in the vaccine. It was therefore not surprising that within a few years of the introduction of PCV7, the proportion of erythromycin-nonsusceptible isolates declined to levels not seen since the late 1980s. However, this decline was short-lived, as the proportion of erythromycin-nonsusceptible isolates increased to levels seen in the years just prior to PCV7 introduction. More than 50% of these isolates were of serotypes 19A and 6A.

In Alaska, macrolide resistance in *S. pneumoniae* is due mainly

to *mef* genes, in particular *mef(E)*. The *mef(E)* gene was found in strains of a variety of different serotypes, while the *mef(A)* gene was found only in serotype 14 isolates. These isolates were of the same sequence type (ST9) as the England¹⁴-9 international clone, which has been a significant contributor to the worldwide dissemination of M-phenotype erythromycin resistance (11). While we observed a slight decrease in the prevalence of *mef*-positive isolates in the later time period (2005 to 2010), this observation coincided with a significant increase in the prevalence of isolates expressing both the *erm(B)* and *mef(E)* genes. This is consistent with data reported by Bley et al., who found that isolates harboring both resistance mechanisms carried the *erm(B)* gene and only the *mef(E)* gene but not *mef(A)* (34). There have been a number of reports of pneumococci harboring both the *erm(B)* and *mef* genes (13, 15–17, 21, 23, 35, 36). In a study by Farrell et al., this dual resistance was found to be related to the clonal spread of the Taiwan^{19F}-14 clone (13). In contrast, Calatayud et al. found no clonal relationship among isolates harboring both resistance genes (22). In our study, we found that the majority (71%) of isolates expressing both resistance genes were of serotype 19A and were also found to be multidrug resistant. These findings are in agreement with the work of others who have reported an increase in the prevalence of IPD caused by multidrug-resistant serotype 19A isolates following the introduction of PCV7 (30, 37–42). These studies suggest that the increased prevalence worldwide of isolates harboring both *erm(B)* and *mef* genes is most likely due to the clonal dissemination of the Taiwan^{19F}-14 (ST236) clone, which is now expressing the serotype 19A capsule. None of the dual-resistance serotype 19A isolates in our study belonged to ST236, but one single-locus variant, ST320, represented 93% of the isolates.

A small proportion (4%) of our erythromycin-nonsusceptible isolates were found to be negative for both *erm(B)* and *mef*, which suggests that resistance in these isolates may be the result of changes in a highly conserved region of domain V of the 23S rRNA or in ribosomal proteins L4 and L22, requiring further investigation.

Isolates carrying *mef* genes have typically shown low- to mid-level resistance (MIC₉₀ of 1 to 8 µg/ml). Since the introduction of PCV7, we have seen a significant increase in the proportion of *mef*-positive isolates that have MICs of >8 µg/ml. This is consistent with other studies which have reported MIC₉₀ values of 16 µg/ml associated with *mef*-positive isolates (18). Such increases may result in increased treatment failures, as macrolides remain the drug of choice for empirical treatment of community-acquired respiratory tract infections in the outpatient setting. Also, because many isolates that carry both resistance mechanisms are also highly resistant to multiple antibiotics, treatment options become increasingly limited.

Macrolide resistance is reported to be frequently associated with resistance to other antibiotics (23, 43–46). In our study, macrolide-resistant isolates were more likely to have reduced susceptibility to other antibiotics, namely, penicillin (66%) and TMP-SMX (82%). Although the rate of coresistance to TMP-SMX remained high, it did decline significantly ($P = 0.001$) during the latter part of this 25-year surveillance period. Prior to the introduction of PCV7, the majority of erythromycin-nonsusceptible isolates that were multidrug resistant belonged to serotypes contained in the vaccine. It is important to note that we did not see a significant decline in the proportion of erythromycin-nonsus-

ceptible isolates that were resistant to multiple antibiotics after the introduction of PCV7, and in fact, we have seen an increase in multidrug resistance among isolates of serotypes not contained in this vaccine or the 13-valent conjugate vaccine, namely, serotypes 6C, 15A, and 23A.

In the early years of IPD surveillance in Alaska (1986 to 1994), the majority (65%) of erythromycin-nonsusceptible isolates were of serotype 6B (24). By the mid- to late 1990s, isolates of serotypes 6B, 9V, and 14 made up the majority (75%) of erythromycin-nonsusceptible isolates (47). With the introduction of PCV7 in 2001, we observed another shift in the serotype distribution among erythromycin-nonsusceptible isolates, with the near elimination of PCV7 types, replaced by nonvaccine types that included serotypes 19A, 6A, and 6C. In addition, we observed an increase in the rate of macrolide resistance among isolates of serotypes 15A and 23A and an increase in the variety of serotypes that were macrolide resistant, particularly in the most recent time period. Similar findings have been reported by others (48, 49). In a study by Calatayud et al., they reported that the incidence of IPD due to macrolide-resistant pneumococci among adults remained stable throughout the 1999–2007 period as a result of a significant decrease in the incidence of IPD due to macrolide-resistant serotypes included in PCV7 coincident with a significant increase in the incidence of IPD caused by isolates of macrolide-resistant nonvaccine types such as serotypes 15A, 16F, 19A, 23A, 24F, and 33F (48). These findings suggest that serotypes which were susceptible to macrolides prior to the introduction of PCV7 have now acquired macrolide resistance determinants as a result of macrolide selective pressure in the late-PCV7 era.

In conclusion, macrolide resistance among pneumococcal isolates from Alaska is mediated predominantly by *mef* genes. However, there was a statistically significant increase in the proportion of isolates that possessed both the *erm(B)* and *mef* genes. Moreover, isolates with this dual-resistance mechanism were more likely to be resistant to a number of other antibiotics, which may limit treatment options. The decline in the prevalence of macrolide resistance after the introduction of PCV7 was a result of a decrease in the incidence of IPD due to vaccine serotypes. Since much of the increase in the prevalence of macrolide resistance in the later time period (2006 to 2010) was due to serotype 19A, which is a component of PCV13, we expect to see a decline in the prevalence of Ery^{NS} pneumococci in the coming years. However, because we have seen an increase in the variety of serotypes that were macrolide resistant and not contained in the 13-valent vaccine, the decline in the prevalence of macrolide resistance among pneumococci may be short-lived. Continued surveillance of macrolide resistance among invasive pneumococci isolates, particularly highly resistant *erm(B)*- and *mef*-positive isolates, is therefore warranted.

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