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Molecular Epidemiology of Multiresistant *Streptococcus pneumoniae* with Both *erm*(B)- and *mef*(A)-Mediated Macrolide Resistance

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Of a total of 1,043 macrolide-resistant *Streptococcus pneumoniae* isolates collected from 24 countries as part of PROTEKT 1999-2000, 71 isolates tested positive for both the *mef*(A) and *erm*(B) genes. Of 69 isolates subjected to further molecular investigations, all were resistant to tetracycline, 63 (91.3%) were resistant to penicillin, and 57 (82.6%) were resistant to trimethoprim-sulfamethoxazole. One isolate was also fluoroquinolone resistant, and another was resistant to quinupristin-dalfopristin. The ketolide telithromycin retained activity against all of the isolates. Of the 69 of these 71 isolates viable for further testing, 46 were from South Korea, 13 were from the United States, 8 came from Japan, and 1 each came from Mexico and Hungary. One major clonal complex (59 [85.5%] of 69 isolates) was identified by serotyping (with 85.5% of the isolates being 19A or 19F), pulsed-field gel electrophoresis, and multilocus sequence typing. The remaining isolates were less clonal in nature. Representative isolates were shown to carry the mobile genetic elements Tn*1545* and mega, were negative for Tn*1207.1*, had tetracycline resistance mediated by *tet*(M), and contained the *mef*(E) variant of *mef*(A). All isolates were positive for *mel*, a homologue of the *msr*(A) efflux gene. These clones are obviously very efficient at global dissemination, and hence it will be very important to monitor their progress through continued surveillance. Telithromycin demonstrated high levels of activity (MIC for 90% of the strains tested, 0.5 μg/ml; MIC range, 0.06 to 1 μg/ml) against all isolates.

Streptococcus pneumoniae is the major bacterial pathogen in community-acquired respiratory tract infections, both in prevalence and in its ability to cause systemic infection. The penicillin and macrolide resistance of this organism has increased rapidly worldwide, and considerable geographical variations in both genotypes and phenotypes have been observed (10, 12, 15, 25). Over the past decade, the application of serotyping combined with molecular epidemiological techniques, such as multilocus sequence type (MLST) determination and pulsed-field gel electrophoresis (PFGE), has revealed that several pneumococcal clones have spread successfully across the globe and contribute greatly to the burden of pneumococcal disease (4, 7, 8, 14, 17).

Macrolide resistance in *S. pneumoniae* occurs by two mechanisms: target modification and efflux. In the most common form of target modification, a specific adenine residue on the 23S rRNA (A2058 [*Escherichia coli* numbering]) is dimethylated by rRNA methylases (16). In *S. pneumoniae*, methylation is *erm*(B) mediated in almost all cases (31). Worldwide, the predominant mechanism responsible in *S. pneumoniae* is the Erm(B) methylase (10, 31). *erm*(B) is harbored, along with other resistance genes such as the tetracycline resistance gene *tet*(M), on the conjugative transposon Tn*1545* (29). Although rare at present, target modification by point mutations in the 23S rRNA and/or ribosomal L4 and L22 protein genes can also cause macrolide resistance and has been demonstrated in clinical isolates from widely distributed global sites (11).

In certain countries, such as the United States, efflux mediated by *mef*(A) is the predominant mechanism responsible (10). The *mef*(A) gene was originally isolated in *Streptococcus pyogenes*, while a similar gene, *mef*(E), was identified in *S. pneumoniae* (2, 27). As homology between these genes is >80%, the two genes have been amalgamated under the name *mef*(A) (23). Importantly, the two *mef*(A) variants exist on different genetic elements—*mef*(A) on Tn*1207.1* and *mef*(A) subclass *mef*(E) on mega (13, 26). A second efflux operon has been found on the mega element and has been designated *mel* (13). *mel* is a homologue of the ATP-binding cassette gene *msr*(A), which is known to be responsible for macrolide and streptogramin efflux in *Staphylococcus epidermidis* (24). A gene with 98% homology to *mel* has also been found on Tn*1207.1* (13).

Isolates with both the *erm*(B) and *mef*(A) genes have been previously reported. Corso et al., in a 1996 to 1997 U.S. study, found a 7% prevalence (3). McGee et al. described a serotype 19F multiresistant clone with both genes in 30.5% of the isolates from five laboratories in South Africa (18).

PROTEKT (prospective resistant organism tracking and epidemiology for the ketolide telithromycin) is a longitudinal, global, multicenter surveillance study of respiratory tract pathogens. All macrolide-resistant *S. pneumoniae* isolates from PROTEKT 1999-2000 were screened for the common efflux and methylase genes associated with macrolide resistance to determine their global distribution (10). Of 1,043 isolates screened, 71 (6.8%) tested positive for both *erm*(B) and *mef*(A). Although the majority of these isolates came from South Korea, other isolates originated from geographically diverse locations (South Korea, 46 isolates; United States, 13; Japan, 8; Canada, 2; Mexico, 1; Hungary, 1). Most isolates were multiresistant, demonstrating high-level resistance to

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TABLE 1. Susceptibility and MIC range of 69 isolates of *S. pneumoniae* with combined *mef*(A) and *erm*(B) to various antimicrobials

Antimicrobial	% (*n*) Susceptible	% (*n*) Intermediate	% (*n*) Resistant	MIC range (μg/ml)	Comments
Penicillin G	2.9 (2)	5.8 (4)	91.3 (63)	0.06–8	Both penicillin-susceptible isolates and 2 of 4 intermediate isolates were from Japan
Erythromycin A	0 (0)	0 (0)	100 (69)	64–>64	
Telithromycin	100 (69)	0 (0)	0 (0)	0.06–1	MIC_{50} = 0.5 μg/ml, MIC_{90} = 0.5 μg/ml
Tetracycline	0 (0)	0 (0)	100 (69)	8–32	
Levofloxacin	98.6 (68)	0 (0)	1.4 (1)		Isolate P1071261: levofloxacin MIC = 8 μg/ml, penicillin G MIC = 2 μg/ml, tetracycline resistant, cotrimoxazole resistant, telithromycin MIC = 0.5 μg/ml
Trimethoprim-sulfamethoxazole	5.8 (4)	11.6 (8)	82.6 (57)	0.25–32	
Quinupristin-dalfopristin	87.0 (60)	11.6 (8)	1.4 (1)	0.25–4	1 resistant isolate[a], 8 intermediate isolates[b]
Linezolid	100 (69)	0 (0)	0 (0)	0.25–1	
Vancomycin	100 (69)	0 (0)	0 (0)	0.25–0.5	

[a] From South Korea.
[b] Five from South Korea, two from the United States, and one from Japan.

penicillin G, tetracycline, the macrolides, clindamycin, and trimethoprim-sulfamethoxazole. In addition, one of these isolates was also fluoroquinolone resistant and another was resistant to quinupristin-dalfopristin. Telithromycin demonstrated potent in vitro activity against all isolates (MIC for 90% of the strains tested [MIC_{90}], 0.5 μg/ml; MIC range, 0.06 to 1 μg/ml).

The rationale for the present study was to determine the genetic relatedness of these isolates and the mobile elements they carry to obtain a better understanding of their evolution and potential for further dissemination. Genetic relatedness was investigated by using serotyping, MLST determination, PFGE, and identification of the mobile genetic elements carrying resistance genes (Tn*1545*, Tn*1207.1*, and mega). In addition, the mechanisms of tetracycline and fluoroquinolone resistance were investigated.

MATERIALS AND METHODS

Bacterial isolates and control strains. From PROTEKT 1999-2000, a total of 1,043 macrolide-resistant *S. pneumoniae* isolates from 24 countries were tested for the presence of macrolide resistance mechanisms by using a previously published PCR- and probe-based method (9, 10). For initial MIC determination and before further testing of isolates, macrolide resistance was confirmed by using the National Committee for Clinical and Laboratory Standards (NCCLS) broth microdilution method (21). NCCLS breakpoints were used to determine susceptibility status (22). Breakpoints of ≤1 μg/ml (susceptible), 2 μg/ml (intermediate), and ≥4 μg/ml (resistant) were used for telithromycin (these breakpoints were approved by the NCCLS Subcommittee on Antimicrobial Susceptibility Testing in January 2003).

Of these 1,043 isolates tested, 71 were positive for both *mef*(A) and *erm*(B). Of these 71 isolates, 69 were recoverable for further analysis. In South Korea (overall macrolide nonsusceptibility, 83.2%), isolates with both *mef*(A) and *erm*(B) represented 38.3% of the macrolide-resistant strains (similar distributions in both of the two centers), while in the United States (overall macrolide nonsusceptibility, 33.0%), this genotype represented 12.5% of the macrolide-resistant isolates (distribution varied greatly among the nine centers, ranging from 0.0 to 25.0%). All of the isolates were resistant to tetracycline, 63 (91.3%) were resistant to penicillin (MIC, ≥2.0 μg/ml), and 57 (82.6%) were resistant to trimethoprim-sulfamethoxazole (Table 1). One isolate from South Korea demonstrated resistance to quinupristin-dalfopristin (MIC = 4 μg/ml), and eight isolates showed intermediate susceptibility (MIC = 2 μg/ml). One isolate was resistant to levofloxacin (MIC = 8 μg/ml). All isolates were susceptible to telithromycin, linezolid, and vancomycin.

Reference strain *S. pneumoniae* R6 was analyzed in parallel to provide a control for all of the sequencing procedures.

Isolate preparation. All isolates were subcultured after storage (in a −70°C freezer or on plates kept at 4°C) on horse blood agar and incubated overnight at 36°C in 5 to 6% CO_2. An aliquot (100 μl) of RNase- and DNase-free H_2O (Sigma, Poole, United Kingdom) was added to each of the 96 wells of a MicroAmp plate (Applied Biosystems, Warrington, United Kingdom). For each isolate, a confluent area of growth was sampled by using a 1-μl plastic loop and transferred to a well in the MicroAmp plate. The plate was incubated at 95°C for 8 min in a PE 9700 thermocycler (Applied Biosystems) and then placed in a Jouan C4.12 centrifuge (Jouan Ltd., Ilkeston, United Kingdom) at 2,290 × *g* for 5 min. The resultant supernatant was used for further analysis.

Detection of other resistance mechanisms and mobile genetic elements. Primers TnIntS810F (5′GTGGCGAACGTCAAGTTCCT 3′) and TnIntS877R (5′ TCGATTCGCTAACACTCGCTTA3′) and probe TnIntS831T (VIC 5′TGGT TGAAGAAGCCTATC3′ MGB), which were used to detect the integrase gene (*int*) of Tn*1545*, were designed by using the *int* sequence (GenBank accession number X61025) and the Primer Express software package (Applied Biosystems). Detection of *int* was confirmed with a previously described assay (5). The allelic discrimination assay was performed on an ABI 7000 sequence detection system with the manufacturer's standard assay parameters and conditions (Applied Biosystems).

Detection of *tet*(M), *msr*(A), Tn*1207.1*, and mega was done as previously described (1). Discrimination between *mef*(A) and *mef*(A) subclass *mef*(E) was performed by amplifying a segment of the *mef*(A) gene and then comparing the sequence to the sequences with GenBank accession numbers U70055 [*mef*(A)] and U83667 [*mef*(A) subclass *mef*(E)]. Detection of type II topoisomerase genes *gyrA*, *gyrB*, *parC*, and *parE* was performed as described previously (19).

Sequencing of PCR products. PCR products were prepared for cycle sequencing by using shrimp alkaline phosphatase (SAP; Amersham Pharmacia, Little Chalfont, United Kingdom) and exonuclease I (Exo I; Amersham Pharmacia) treatment. Briefly, 5 μl of each PCR product was added to 5 μl of a reaction mixture containing 1 U of SAP and 1 U of Exo I in DNase- and RNase-free H_2O and incubated at 37°C for 60 min, followed by 75°C for 15 min to inactivate the enzymes. Each sample was then diluted 1 in 5 by adding 40 μl of DNase- and RNase-free H_2O. Each diluted SAP- and Exo I-treated product (5 μl) was added to 15 μl of a reaction mixture containing 1 μl of Ready Reaction Mix (Applied Biosystems), 4 μl of 5× sequencing buffer (Applied Biosystems), 9.5 μl of RNase- and DNase-free, sterile, distilled H_2O (Sigma), and 3.2 pmol of each target-specific forward and reverse primer. Cycling parameters were 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min.

All sequencing was performed on an ABI PRISM 3100 genetic analyzer (Applied Biosystems). Sequence analysis was performed with the DNASTAR analysis program (DNASTAR, Madison, Wis.).

MLST determination. MLST determination was carried out as described previously (7). Briefly, genomic DNA was prepared from each isolate and internal fragments of around 450 bp were amplified from seven housekeeping genes: *aroE* (shikimate dehydrogenase), *gdh* (glucose-6-phosphate dehydrogenase), *gki* (glucose kinase), *recP* (transketolase), *spi* (signal peptidase I), *xpt* (xanthine phosphoribosyltransferase), and *ddl* (D-alanine–D-alanine ligase). These fragments were then sequenced (as described above) on both strands, and the sequences were compared with those included in the MLST database (www.mlst.net). Each isolate was ascribed to a known or unknown sequence type. If one of the seven loci differed from a known sequence type, the strain was designated a single-locus

variant (SLV) of that sequence type; if two loci differed, the isolated was designated a double-locus variant (DLV).

PFGE. PFGE analysis was carried out by *Sma*I digestion as described previously (4). Data analysis and dendrogram construction were performed with Phoretix software. PFGE categories were determined by the method of Tenover et al. (28). Briefly, isolates are classified as indistinguishable if they had identical banding patterns, closely related if two or three bands differed, possibly related if four to six bands differed, and different if seven or more bands differed.

Serotyping. Isolates were serotyped with antisera from the Statens Serum Institute (Copenhagen, Denmark).

Clonal complex designation. Strains were designated in a particular clonal complex if they were highly related to each other on the basis of serotyping, MLST, and PFGE pattern. To be included in a clonal complex, strains must have had at least closely related PFGE patterns and MLST patterns that were identical in at least five out of the seven alleles. Ideally, strains in the clonal complex should have been in the same serogroup but because of capsular replacement, a change in serogroup was allowed if the other two requirements were met.

RESULTS

The male-to-female ratio was approximately 2:1, with the age distribution spread evenly by age category. Approximately one-half of the isolates from known sources came from sputum, 13 came from ear swabs, and 5 were obtained from blood cultures. The most common diagnosis was community-acquired pneumonia, followed by acute otitis media. Demographics were similar for the total population of isolates from the study (n = 3,435), apart from a higher representation of ear swabs and otitis media in the subpopulation discussed here (13 of 69 ear swabs compared to 203 of 3,435; 16 of 69 otitis media cases compared to 265 of 3,435). The susceptibilities and MIC ranges of the 69 isolates of *S. pneumoniae* with both *erm*(B)- and *mef*(A)-mediated macrolide resistance to various antibacterials are presented in Table 1. All isolates were susceptible to telithromycin (MIC_{90}, 0.5 μg/ml; MIC range, 0.06 to 1 μg/ml).

Serotype distribution of isolates was limited, with most isolates being 19A or 19F (Table 2). Isolates from Japan were more diverse in serotype distribution. The range of MLSTs was also limited, with most MLSTs being SLVs or DLVs of each other, suggesting that several clones predominated. These results were confirmed by PFGE analysis. The epidemiological data demonstrate that clonal complex I was predominant in both of the countries, with the highest prevalence (South Korea and the United States), but not in Japan, where only one isolate belonged to this complex. The history of these strains obtained from the MLST database shows that the majority of known MLSTs have been previously isolated in the Far East, in particular Taiwan. ST 320 (22 isolates) was the exception, being previously isolated in Norway and Australia. None of the MLSTs have been previously reported in the United States, suggesting that these clones migrated from the Far East to the United States at some point in the recent past. However, it is important not to exclude the presence of these clones in the United States on the basis of their not being present in the MLST database, as it could be that they have simply not been investigated, reported, and hence added to the database.

Eighteen strains were chosen for further testing, and as each serotype, MLST, and PFGE pattern was represented, it is valid to assume that this group was representative of the total population of 69 isolates. All 18 were shown to carry the mobile genetic elements Tn*1545* and mega, and all were negative for Tn*1207.1*. Tetracycline resistance was mediated by *tet*(M) in all

TABLE 2. Epidemiological parameters and geographical distribution of the isolates described in this study

Clonal complex	No. of isolates	Serotype(s) (no. of isolates)	MLST	PFGE category	Country(ies) (no. of isolates)	Center(s) (no. of isolates)[a]
I	22	19A (15), 19F (6), 14 (1)	320	C	South Korea (20), United States (2)	SK70 (11), SK71 (9), USA7 (2)
I	17	19F (16), 19A (1)	271	C	South Korea (10), United States (7)	SK71 (9), SK70 (1), USA1 (3), USA4 (1), USA7 (2), USA8 (1)
I	10	19F	236	C	South Korea (6), United States (2), Mexico (1), Hungary (1)	SK71 (5), SK 70 (1), USA1 (1), USA8 (1), M33 (1), H650 (1)
I	7	19F (4), 19A (2), 14 (1)	SLV or DLV of 236, 271, 320, and 283	C	South Korea (5), United States (2)	SK71 (4), SK70 (1), USA5 (1), USA7 (1)
I	2	19F	283	C	South Korea (2)	SK71 (2)
II	1	Nontypeable	81	E	South Korea (1)	SK71
II	1	Nontypeable	DLV of 81	E	South Korea (1)	SK71
	1	Nontypeable	Unknown	H	South Korea (1)	SK71
	1	23F	Unknown	A	Japan	J81 (1), J82 (3), J83 (2), J85 (2)
	1	19F	257	B	Japan	
III	1	23F	DLV of 242	D	Japan	
III	1	23F	242	D	Japan	
I	1	19F	236	F	Japan	
	1	6B	Unknown	G	Japan	
	1	19A	Unknown	Not done	Japan	
	1	23F	Unknown	Not done	Japan	

[a] SK, South Korea, USA, United States, J, Japan, M, Mexico, H, Hungary (e.g., SK70 is center number 70 from South Korea). As noted, the eight isolates from Japan were from four different centers, but the particular center from which each isolate came is not identified here.

cases. Sequence analysis of *mef*(A) revealed that *mef*(A) subclass *mef*(E) was present in all of the isolates (GR Micro Limited data on file). All isolates were positive for the *msr*(A) gene and sequence analysis showed this gene to be the homologue *mel* (GR Micro Limited data on file). The mechanism(s) of fluoroquinolone resistance in isolate P1071261 remains unknown, as sequence analysis did not detect whether any of the type II DNA topoisomerase gene mutations commonly associated with resistance were present. Perhaps up-regulation of the *pmrA* efflux gene is responsible for this resistance; this requires further investigation. The ciprofloxacin (MIC, 16 μg/ml) and moxifloxacin (MIC, 2 μg/ml) MICs were also raised for this strain.

DISCUSSION

These data suggest that a multiresistant clonal complex of *S. pneumoniae*, possibly originating in the Far East, is prevalent in South Korea and the United States and also present in Mexico, Hungary, and Japan. The other strains described in this study were less clonal in nature. All of the strains possessed at least three mechanisms of macrolide resistance on at least two mobile genetic elements. The Erm(B) methylase gene *erm*(B) is most likely located on Tn*1545*, along with the tetracycline efflux gene *tet*(M). The *mef*(E) variant of *mef*(A) and the ATP-binding cassette gene *msr*(A) homologue *mel* were found to be present. The genes *mef*(E) and *mel* are known to coexist on the mobile genetic element mega; hence, it was not surprising that all of the isolates were negative for *mef*(A)-carrying genetic element Tn*1207.1* (1, 13).

McGee et al. have described a serotype 19F multiresistant clone with combined *erm*(B) and *mef*(A) genes in 30.5% of 118 erythromycin-resistant *S. pneumoniae* isolates from five laboratories in South Africa (18). Molecular epidemiological investigations showed that 83% of the isolates belonged to a single multiresistant serotype 19F clone closely related to the international Taiwanese clone. The ST 271 and ST 236 Taiwanese clones described here comprise 28 of the 29 strains isolated. The ST 320 clone is indistinguishable from these clones by PFGE and has only two variant alleles by MLST determination and hence is very closely related. Together with the other closely related strains in clonal complex I, 59 (85.5%) of the 69 isolates described in this study were very closely related to the international Taiwanese clone.

It is interesting that all but one of the isolates from Japan were less related to clonal complex I and each other yet had the same phenotype, the same mechanisms of resistance, and the same genetic elements carrying these genes. It is also interesting that each of the MLSTs was more prevalent at the local (i.e., center) level yet were also present in smaller numbers in other centers and countries (Table 2). This suggests that these multiresistant isolates have evolved from several ancestral strains and have been effective in both local and international dissemination. On the other hand, this could also suggest that the divergence seen in Japan is present because these strains have been established there longer than elsewhere.

It is of concern that quinupristin-dalfopristin and fluoroquinolone resistance was found in two separate isolates. These strains are obviously very efficient at global dissemination, and hence it is very important to monitor their progress through continued surveillance. As evidence of this, we recently described the successful expansion of a fluoroquinolone-resistant Spain^{23F}-1-14 variant clone (MLST 81) in Hong Kong (19). We are currently in the process of defining all of the isolates testing positive for both *erm*(B) and *mef*(A) from PROTEKT global years 2 (2000 to 2001) and 3 (2001 to 2002) to monitor further resistance development and changes in distribution and prevalence. Fortunately, telithromycin was shown to be highly active against all of the isolates described in this study, demonstrating this compound's potential for reducing the spread of these multiresistant clones. In addition, the ketolides are less likely to become resistant by mutation because of their strong binding to the dual ribosomal targets in domains II and V of the 23S rRNA (6).

The high level of resistance to cotrimoxazole (82.6%) in these isolates is of concern, particularly if these clones are able to, or indeed have already, spread to developing countries. The World Health Organization protocol for case management in children more than 2 months old directs that in patients with nonsevere pneumonia, cotrimoxazole is the preferred drug in most settings because of its broad-spectrum efficacy, low cost, ease of administration, antimalarial activity, and relatively low rate of adverse effects (33).

The seven-valent (7-V) formulation of the pneumococcal conjugate vaccine was licensed by the Food and Drug Administration and introduced in the United States in February 2000. The serotypes represented in 7-V are 4, 6B, 9V, 14, 18C, 19F, and 23F. Cross-coverage is thought to extend to other serotypes within the same serogroup. Several studies have shown that significant cross-reactivity of serotypes 6B and 19F with serotypes 6A and 19A, respectively, occurs (20, 30, 34). However, different amounts of cross-reacting opsonizing antibodies have been noted between different vaccine formulations, and different methods used to determine antibodies may be unreliable for assessment of cross-reaction (34). In a recent population-based survey carried out by the Active Bacterial Core Surveillance of the Centers for Disease Control and Prevention to examine the changes in invasive pneumococcal disease (IPD) postintroduction of 7-V, although a statistically significant reduction in IPD caused by vaccine serotypes was found in young children, a statistically significant reduction was not found for IPD caused by serotype 19A (32). As 19 of the multiresistant isolates described here are serotype 19A (including 4 from the United States), the lack of conclusive evidence that serotype 19A is adequately covered by 7-V is of concern and encourages careful monitoring of this multiresistant clone.

In summary, we have described the emergence and intercontinental spread of several multiresistant clones of *S. pneumoniae*. We also report evidence of resistance to the fluoroquinolones and quinupristin-dalfopristin, but not to linezolid or telithromycin, in these strains. We are monitoring the progress of these clones in the subsequent years of PROTEKT.

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