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Molecular Epidemiology of Penicillin-Susceptible Non-β-Lactam-Resistant *Streptococcus pneumoniae* Isolates from Greek Children

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A total of 128 *Streptococcus pneumoniae* isolates that were susceptible to penicillin but resistant to non-β-lactam agents were isolated from young carriers in Greece and analyzed by antibiotic susceptibility testing, serotyping, restriction fragment end labeling (RFEL), and antibiotic resistance genotyping. The serotypes 6A/B (49%), 14 (14%), 19A/F (11%), 11A (9%), 23A/F (4%), 15B/C (2%), and 21 (2%) were most prevalent in this collection. Of the isolates, 65% were erythromycin resistant, while the remaining isolates were tetracycline and/or trimethoprim-sulfamethoxazole resistant. Fifty-nine distinct RFEL types were identified. Twenty different RFEL clusters, harboring 2 to 19 strains each, accounted for 76% of all strains. Confirmatory multilocus sequence typing analysis of the genetic clusters showed the presence of three international clones (Tennessee^{23F}-4, England^{14}-9, and Greece^{6B}-22) representing 30% of the isolates. The *erm*(B) gene was present in 70% of the erythromycin-resistant isolates, whereas 18 and 8% contained the *mef*(A) and *mef*(E) genes, respectively. The pneumococci representing *erm*(B), *erm*(A), and *mef* genes belonged to distinct genetic clusters. In total, 45% of all isolates were tetracycline resistant. Ninety-six percent of these isolates contained the *tet*(M) gene. In conclusion, penicillin-susceptible pneumococci resistant to non-β-lactams are a genetically heterogeneous group displaying a variety of genotypes, resistance markers, and serotypes. This suggests that multiple genetic events lead to non-β-lactam-resistant pneumococci in Greece. Importantly, most of these genotypes are capable of disseminating within the community.

Streptococcus pneumoniae is a common cause of invasive diseases, such as meningitis and bacteremia, and of respiratory tract infections (5). *S. pneumoniae* isolates that are resistant to penicillin and/or non-β-lactam agents have been frequently reported (6, 12, 35). Resistance of *S. pneumoniae* to erythromycin and the other macrolides is increasing in many parts of the world (15, 17). Strains resistant to erythromycin are also resistant to azithromycin, clarithromycin, and roxithromycin (37). This global increase in antibiotic-resistant and especially multidrug-resistant pneumococci is the result of the spread of various highly resistant pneumococcal clones (7, 25).

In Greece, the emergence of antibiotic resistance among pneumococcal isolates was recognized in the mid-1990s (30). Recently, pneumococcal isolates susceptible to penicillin and resistant to chloramphenicol, tetracycline, erythromycin, clindamycin, and trimethoprim-sulfamethoxazole (SXT) have been isolated from young Greek carriers. During the period from December 1995 to February 1996, 24% of the pneumococci isolated from healthy carriers attending day care centers were demonstrated to be penicillin-susceptible, non-β-lactam-resistant isolates (29). In a recent study in which 2,448 children younger than 2 years old living in various areas in Greece were screened during a 2-year period (1997 to 1999) for pneumococcal carriage, 15% of the pneumococci appeared to have reduced susceptibility to non-β-lactam agents (31).

The present study was undertaken to investigate the molecular epidemiological characteristics of the Greek pneumococci susceptible to penicillin but resistant to erythromycin and/or other non-β-lactam agents. Furthermore, penicillin, erythromycin, and tetracycline resistance determinants were studied at a molecular level.

MATERIALS AND METHODS

Bacterial isolates. We studied a collection of 128 *S. pneumoniae* isolates susceptible to penicillin but resistant to erythromycin and/or other non-β-lactam agents; the isolates were recovered from nasopharyngeal cultures obtained from children during two independent studies in Greece (3, 30, 31). The first study was performed in 338 children attending seven day care centers in the city of Patras in southwestern Greece during the 2-month period from December 1995 to February 1996. In this study, 30 penicillin-susceptible pneumococci resistant to one or more non-β-lactam agents were recovered from 132 carriers. Of these 30 *S. pneumoniae* isolates, 26 were available for further analysis in the present investigation. The second study, the Hellenic Antibiotic-Resistant Respiratory Pathogens (HARP) study, was conducted from February 1997 to February 1999. Nasopharyngeal cultures for *S. pneumoniae* were performed in 2,448 children younger than 2 years old living in central and southern Greece. Ninety-five (3.9%) of the 2,448 children attended day care centers. In the HARP study, screening of the children revealed 119 pneumococci which were penicillin susceptible but resistant to non-β-lactam agents. Of these 119 pneumococci, 102 were available for further analysis. Thirteen of these pneumococci were isolated from children attending day care. Only two of these children attended the same day care center.

Bacteriological procedures. Isolation, identification, and susceptibility testing of the Greek *S. pneumoniae* isolates were performed by applying standard methods as described previously (30, 31). Penicillin and erythromycin MICs for the

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Greek isolates were determined by the E-test method (AB Biodisk, Solna, Sweden). Susceptibility to clindamycin, chloramphenicol, tetracycline, and SXT was determined by the disk diffusion method according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) (21). Multidrug resistance was defined as resistance to three or more classes of antimicrobial agents. Pneumococci were serotyped by the capsular swelling method or the latex agglutination technique (30, 33).

Penicillin-binding protein (PBP) genotyping. Genetic polymorphism of the penicillin resistance genes *pbp1a*, *pbp2b*, and *pbp2x* of the pneumococcal isolates was investigated by restriction fragment length polymorphism (RFLP) analysis of the PCR-amplified genes, as described previously (13).

Detection and analysis of the *erm*(B) and *mef* genes. To detect the presence of *erm*(B) within the pneumococcal isolates, we used the protocol of Sutcliffe et al. (28). In summary, we amplified the genes by PCR and analyzed the amplified DNA products by agarose gel electrophoresis. The presence of the *mef* gene was also detected by PCR (28). In order to discriminate between *mef*(A) and *mef*(E), PCR-RFLP analysis was performed by the method of Del Grosso et al. (8). The amplicon was digested using *Bam*HI and *Dra*I. The *mef(*A) gene contains a single *Bam*HI site, which is absent in the *mef*(E) gene. Digestion of *mef*(A) and *mef*(E) with *Dra*I yields two and three fragments, respectively.

Detection of the *tet*(M) and *tet*(O) genes. In order to discriminate between *tet*(M) and *tet*(O), a PCR-RFLP analysis was performed as described previously (2). In summary, a PCR mix was made of 25-μl reaction buffer containing 0.5 U of thermostable DNA polymerase, diluted in the buffer supplied by the manufacturer (Integro, Leuvenheim, The Netherlands), 0.2 mM (each) deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 10 pmol of each primer, and 10 to 50 ng of pneumococcal DNA. Amplification cycling in a programmable thermal controller (PTC-100; MJ Research, Watertown, Mass.) consisted of the following steps: predenaturation for 1 min at 94°C, followed by 30 cycles, with 1 cycle consisting of 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C. Amplification was finished after 3 min at 72°C. A 1.5% agarose gel in 0.045 M Tris-borate buffer with 0.001 M EDTA (0.5× TBE buffer) containing 0.1 μg of ethidium bromide per ml was used to visualize the PCR products.

RFEL typing. Pneumococcal strain typing by restriction fragment end labeling (RFEL) was performed by the method of van Steenbergen et al. (36) as adapted by Hermans et al. (13). Briefly, purified pneumococcal DNA was digested by the restriction enzyme *Eco*RI. The DNA restriction fragments were end labeled at 72°C with [α-^{32}P]dATP using DNA polymerase (Goldstar; Eurogentec, Seraing, Belgium). After the radiolabeled fragments were denatured and separated electrophoretically on a 6% polyacrylamide sequencing gel containing 8 M urea, the gel was transferred onto filter paper, vacuum dried (HBI, Saddlebrook, N.Y.), and exposed for variable times at room temperature to ECL hyperfilm (Amersham Laboratories, Amersham, United Kingdom).

Computer-assisted analysis of DNA band patterns. RFEL autoradiographs were converted to images (Image Master DTS; Pharmacia Biotech, Uppsala, Sweden) and analyzed with a computer (Windows version Gelcompar software version 4; Applied Math, Kortrijk, Belgium). DNA fragments were analyzed as described previously (26). For evaluation of the genetic relatedness of the isolates, we used the following definitions: (i) isolates of a particular RFEL type are 100% identical by RFEL analysis; (ii) an RFEL cluster represents a group of RFEL types that differ in only one band (approximately >95% genetic relatedness); (iii) an RFEL lineage represents a group of RFEL types that differ in less than four bands (approximately >85% genetic relatedness).

International comparison. The Greek genotypes were compared with an international collection of pneumococcal isolates representing 751 distinct RFEL types originating from 17 different countries in America, Europe, Africa, and Asia (2, 12;M. Sluijter, unpublished observations). The international collection includes the first 16 international pandemic clones described by the Pneumococcal Molecular Epidemiological Network in 2000 (http://www.pneumo.com/physician/pmen_clone_collection.asp) (18).

MLST. The genotypes of all clusters were verified by multilocus sequence typing (MLST) analysis. For this purpose, a fully automated method for MLST was used as described previously (14), and one or two isolates per cluster were analyzed. The MLST types were compared with the global database at www.mlst.net.

RESULTS

The serotypes 6A/B (49%), 14 (14%), 19A/F (11%), 11A (9%), 23A/F (4%), 15B/C (2%), and 21 (2%) were most prevalent in the collection of 128 Greek pneumococcal isolates. All isolates were invariably susceptible to penicillin, while 84 (65%), 78 (60%), 69 (54%), 63 (49%), and 62 (48%) isolates were resistant to erythromycin, SXT, tetracycline, chloramphenicol, and clindamycin, respectively.

The isolates were classified as penicillin-susceptible erythromycin-resistant (65%) and penicillin-susceptible erythromycin-susceptible (35%) isolates. The latter group was represented by tetracycline- and/or SXT-resistant pneumococci. The 84 penicillin-susceptible erythromycin-resistant pneumococci displayed capsular types 6B (56%), 14 (18%), 19F (13%), 11A (11%), 10A (1.2%), and 15C (1.2%). Of these erythromycin-resistant isolates, 59 (70%), 15 (18%), and 7 (8.3%) isolates carried the *erm*(B), *mef*(A), and *mef*(E) erythromycin resistance determinants, respectively. In four erythromycin-resistant isolates belonging to a serotype 11A clone, an *erm*(A) gene was previously detected (32). Furthermore, 62 (74%), 57 (68%), 46 (55%), and 42 (50%) of the 84 penicillin-susceptible erythromycin-resistant pneumococci, were also resistant to clindamycin, tetracycline, SXT, and chloramphenicol, respectively. Clindamycin resistance was always identified in combination with erythromycin resistance. All 62 clindamycin- and erythromycin-resistant isolates carried an *erm* resistance gene. Of the 54 erythromycin-resistant isolates that were resistant to tetracycline, 51 carried the *tet*(M) resistance gene. The three remaining isolates were negative for both the *tet*(M) and *tet*(O) genes.

The 44 penicillin-susceptible erythromycin-susceptible isolates belonged to serotypes 6B (29%), 23F (11%), 14 (6.7%), 21 (6.7%), 1 (4.4%), 6A (4.4%), 15B (4.4%), 18C (4.4%), 19A (4.4%), 8 (2.2%), 10A (2.2%), 11A (2.2%), 16F (2.2%), 19F (2.2%), 20 (2.2%), and 24F (2.2%) and nontypeable serotypes (4.4%).

Sulfamethoxazole resistance was identified in 34 of the 44 penicillin-susceptible erythromycin-susceptible isolates (78%), whereas tetracycline resistance was identified in 12 (27%) isolates. All but one carried the *tet*(M) resistance gene. All isolates were resistant to a single agent except for one isolate that was resistant to tetracycline and chloramphenicol and two isolates that were resistant to tetracycline and SXT.

Fourteen distinct PBP genotypes were observed. Of the 128 isolates, 76 (59%), 24 (19%), and 4 (3.1%) displayed a known penicillin-susceptible PBP 1A-2B-2X genotype, being 2-2-71, 2-2-3, and 2-2-2, respectively. The remaining isolates displayed

FIG. 1. Dendrogram of the 59 RFEL types observed among the 128 Greek *S. pneumoniae* nasopharyngeal isolates. The molecular sizes of reference bands (in bases [b]), serotypes, PBP types, RFEL types, MLST types, number of isolates per RFEL type, clusters, and cluster codes are depicted. One or two strains per RFEL clusters were analyzed by MLST. Day care center isolates from the 1995 to 1996 study (asterisks) and day care center isolates from the 1997 to 1999 study (degree symbols) are indicated. If more than one day care center isolate belongs to a specific genotype, the total number is displayed in parentheses. Two new strains with new MLST types (new^1 and new^2) are indicated. These two strains have been submitted to the MLST database. nt, nontypeable.

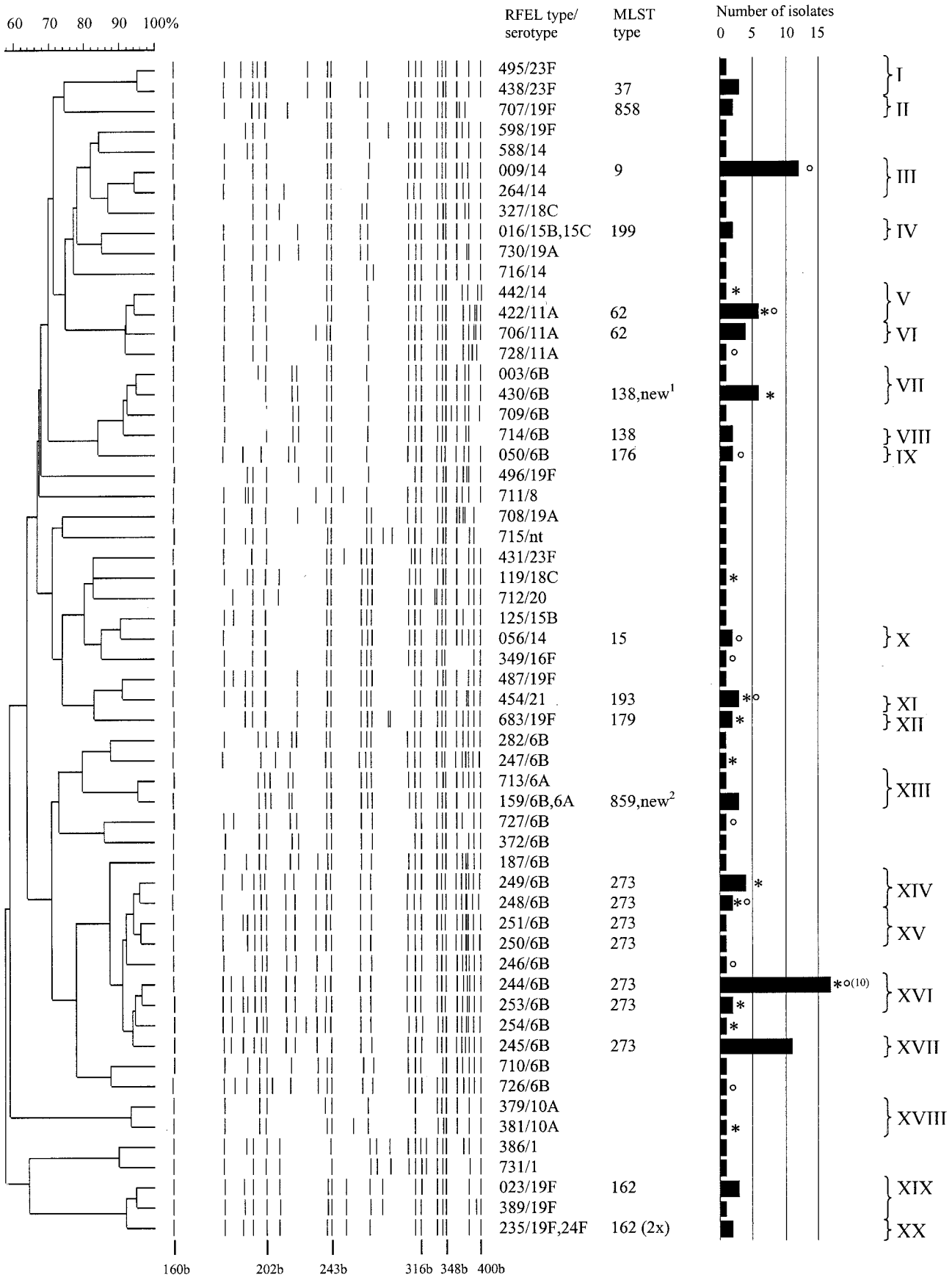
60 70 80 90 100%
RFEL type/ serotype
MLST type
Number of isolates
0 5 10 15
495/23F
438/23F 37
707/19F 858
598/19F
588/14
009/14 9
264/14
327/18C
016/15B,15C 199
730/19A
716/14
442/14
422/11A 62
706/11A 62
728/11A
003/6B
430/6B 138,new1
709/6B
714/6B 138
050/6B 176
496/19F
711/8
708/19A
715/nt
431/23F
119/18C
712/20
125/15B
056/14 15
349/16F
487/19F
454/21 193
683/19F 179
282/6B
247/6B
713/6A
159/6B,6A 859,new2
727/6B
372/6B
187/6B
249/6B 273
248/6B 273
251/6B 273
250/6B 273
246/6B
244/6B 273
253/6B 273
254/6B
245/6B 273
710/6B
726/6B
379/10A
381/10A
386/1
731/1
023/19F 162
389/19F
235/19F,24F 162 (2x)
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
XIII
XIV
XV
XVI
XVII
XVIII
XIX
XX
*(10)
160b 202b 243b 316b 348b 400b

TABLE 1. Molecular and phenotypic characteristics of the 129 Greek pneumococcal isolates

RFEL cluster	No. of isolates/no. of RFEL types	Serotype (no. of strains)	Resistance pattern[a] (no. of strains)	PBP type (no. of strains)	Genotype (no. of strains)
I	4/2	23F (4)	S	2-2-3	
II	2/1	19F (2)	TECLS	2-2-3	*erm*(B), *tet*(M)
			ECLS		*erm*(B)
III	13/2	14 (13)	E (13)	2-2-71	*mef*(A)
IV	2/1	15B	S	2-2-44	
		15C	ECL	2-2-3	*erm*(B)
V	7/2	11A (6)	TECL (4)	2-2-3 (6)	*erm*(B) (4), *tet*(M) (4)
		14 (1)	S (2)	2-2-71 (1)	*mef*(E) (1)
			E (1)		
VI	4/1	11A (4)	TECL	2-2-3	*erm*(A), *tet*(M)
VII	7/2	6B (7)	S (6)	2-2-71	*erm*(B) (1), *tet*(M)(1)
			E (1)		*mef*(E)
VIII	2/1	6B (2)	S (2)	2-2-71	
IX	2/1	6B (2)	CTECLS	2-2-71	Unknown
			S		
X	2/1	14 (2)	S (2)	2-2-71	
XI	3/1	21	S (3)	2-2-7	
				2-2-98	
XII	2/1	19F (2)	TECL	2-2-98	*erm*(B) (2)
					tet(M) (2)
XIII	4/2	6B (2)	T (3), TS (1)	2-5-22 (2)	*tet*(M) (4)
		6A (2)		2-5-99 (1)	
				2-2-71 (1)	
XIV	6/2	6B (6)	CTECLS (5)	2-2-71	*erm*(B) (6)
			CTECL		*tet*(M) (6)
XV	2/2	6B (2)	CTECLS	2-2-71	*erm*(B) (2)
			CTECL		*tet*(M) (2)
XVI	19/2	6B (19)	CTECLS (17)	2-2-71 (18)	*erm*(B) (19)
			CTECL (1)	0-0-0 (1)	*tet*(M) (18)
			TECLS (1)		
XVII	11/1	6B (11)	CTECLS (11)	2-2-71 (10)	*erm*(B) (11)
				0-0-0 (1)	*tet*(M) (11)
XVIII	2/2	10A	TECL (1)	2-2-71	*erm*(B) (1), *tet*(M) (1)
			S	2-2-2	
XIX	4/2	19F (4)	E	2-2-2 (4)	*met*(A) (1)
					met(E) (3)
XX	2/1	19F (1)	S	2-2-2 (1)	
		24F (1)		2-2-3 (1)	

[a] Abbreviations: C, chloramphenicol; T, tetracycline; E, erythromycin; CL, clindamycin; S, trimethoprim-sulfamethoxazole.

alterations in *pbp2x* (11 distinct types), *pbp2b* (2 types), and *pbp1a* (1 type).

RFEL analysis divided the 128 penicillin-susceptible isolates, which were resistant to one or more non-β-lactam agents into 59 distinct RFEL genotypes (Fig. 1). Ninety-nine isolates belonged to 20 genetic clusters, representing 77% of the isolates and varying in size from 2 to 19 isolates (Table 1). The average cluster size was 5.0 isolates. Five of the 20 clusters contained two serotypes, while the remaining clusters contained only one serotype each. The 20 clusters belonged to 11 lineages. The three largest clusters were cluster III, XVI, and XVII. Cluster III consisted of 13 (10%) isolates and was identical to the pandemic clone England^{14}-9 which was confirmed by MLST analysis (Fig. 1 and Table 1). This cluster belonged to a single predominant lineage of 14 genetically related isolates, representing three RFEL genotypes and harboring the serotypes 11A, 14, and 18C. The serotype 14 pneumococci carried the *mef*(A) erythromycin resistance determinant and had a low to moderate level of resistance to erythromycin. The remaining pneumococci were resistant to SXT.

Clusters XVI (19 isolates) and XVII (11 isolates) belonged, together with cluster XIV (6 isolates) and XV (2 isolates), to one predominant lineage, representing 10 RFEL types and all harboring the serotype 6B. Most isolates were resistant to erythromycin, clindamycin, tetracycline, chloramphenicol, and SXT. These pneumococci carried the *erm*(B) erythromycin resistance determinant and had a high level of resistance to erythromycin. The latter clusters were identical to the pandemic clone Greece^{6B}-22 and closely related to the penicillin-resistant MDR clone Spain^{6B}-2 that has spread from Spain to Iceland in the late 1980s (29).

Clusters I (three isolates) and XIX (three isolates) represented genotypes identical to the pandemic clone Tennessee^{23F}-4 and closely related to the pandemic clone Spain^{9V}-3 (six of seven alleles) as described by the Pneumococcal Molecular Epidemiology Network. The genetic relatedness of the observed MLST profiles of the 20 RFEL clusters is depicted in Fig. 2.

The 39 isolates recovered from children attending day care centers displayed 21 different genotypes; 10 isolates displayed unique genotypes, whereas the remaining 29 isolates displayed 13 genotypes belonging to 11 clusters. Cluster XVI contained nine isolates from one day care center, whereas cluster XVII contained three isolates from a second single day care center.

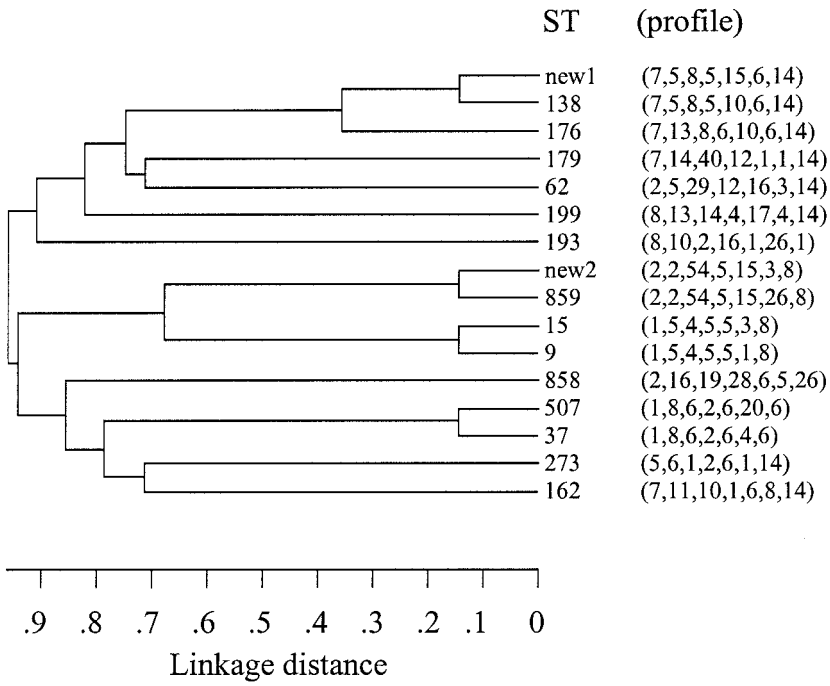

FIG. 2. Genetic relatedness of the 15 MLST sequence types (ST) observed within the 20 RFEL clusters.

The remaining clustering day care center isolates originated from different day care centers (Fig. 1).

DISCUSSION

We evaluated 128 *S. pneumoniae* isolates that were susceptible to penicillin but resistant to non-β-lactam agents from young carriers in Greece by antibiotic susceptibility testing, serotyping, RFEL, and antibiotic resistance genotyping. In general, the isolates could be divided into two groups: one group consisting of 84 erythromycin-resistant isolates, while the second group of 44 isolates were erythromycin susceptible. Multidrug resistance, i.e., resistance to three or more different classes of antibiotics, was seen predominantly within the first group of isolates, whereas the erythromycin-susceptible isolates predominantly displayed monodrug resistance to tetracycline or sulfamethoxazole. Furthermore, clustering of isolates, which is the result of horizontal spread of the isolates within the community, was higher among multidrug-resistant genotypes. This is in line with previous findings where multidrug-resistant clones have shown to spread from country to country (3, 4, 7, 20, 22, 25, 27, 35). This has led to the classification of pandemic clones by the Pneumococcal Molecular Epidemiological Network (18). Although the majority of these pandemic clones are penicillin resistant, our study as well as previous studies have shown that this is not a prerequisite for clonal spread.

With emerging non-β-lactam resistance among pneumococci, the spread of penicillin-susceptible non-β-lactam-resistant pneumococci has become apparent (1, 24, 29, 34). This is underlined by our observation that, in addition to one large lineage of serotype 6B isolates that were mostly susceptible to penicillin and resistant to erythromycin, tetracycline, chloramphenicol, and sulfamethoxazole, 16 smaller clusters were found. In total, 30% of the isolates displayed a genotype identical to those of the pandemic clones Tennessee^{23F}-4, England^{14}-9, and Greece^{6B}-22 as reported by the Pneumococcal Molecular Epidemiological Network. These findings clearly indicate heterogeneity among the penicillin-susceptible, non-β-lactam-resistant isolates. Furthermore, these clusters represent not only the conjugate vaccine serotypes 6B, 14, 19F, and 23F, but also non-vaccine serotypes, such as 11A, 15B/C, and 21. This observation implicates that non-vaccine serotypes are also able to spread among children; hence, vaccination with pneumococcal conjugate vaccines is not a solution for the emergence of multidrug resistance among pneumococcal isolates.

In this study, 70% of the erythromycin-resistant isolates harbored the *erm*(B) gene, while 18 and 8% of the isolates contained *mef*(A) and *mef*(E) genes, respectively. These data differ from previous findings made by Reinert and coworkers, who observed an almost equal distribution of *erm*(B) (43%) and *mef*(E) (56%) genes among erythromycin-resistant isolates in Germany (23). However, our data are in line with a recent study performed in Italy and Vietnam where the majority of the strains also displayed the *erm*(B) gene (2, 19). In the four low-level erythromycin-resistant isolates displaying an identical RFEL genotype and serotype 11A, the *erm*(A) gene was previously identified (32).

Genetic analysis of tetracycline resistance genes *tet*(M) and *tet*(O) revealed that the *tet*(M) gene was exclusively observed in 91% of the Greek isolates. This is in line with the Vietnamese study where the *tet*(M) gene was also exclusively observed (2). In contrast to other studies, the remaining tetracycline-resistant isolates did not harbor the *tet*(O) gene (16). So far, no other tetracycline resistance determinants have been

described; therefore, the underlying mechanism remains unclear. Also in line with the Vietnamese study is the isolation of *tet*(M)-containing but tetracycline-susceptible strains in Greece, suggesting the presence of a nonfunctional or unexpressed tetracycline resistance gene (2, 9). A similar phenomenon was seen for the PBP genes. Though the majority of the isolates displayed (known) susceptible genotypes, 10% of the isolates displayed alterations in one or two of the three major PBP genes, *pbp1a*, *pbp2b*, and *pbp2x*, which often implicates intermediate penicillin resistance (10). In our study, the observed alterations in *pbp1a*, *pbp2b*, and four of the *pbp2x* alterations were identified previously in non-penicillin-susceptible pneumococci isolated in Thailand, the United States, and The Netherlands (11). Although these alterations have shown to be related to intermediate susceptibility, in all cases an additional alteration in one or two of the PBP genes was present (Sluijter, unpublished). These findings support the hypothesis that not all genetic alterations lead to amino acid substitutions or to substitutions which are relevant for penicillin resistance.

We hypothesize that the ongoing antibiotic pressure will continue the process of alteration and spread of resistance genes among pneumococci. Our study underlines that antibiotic resistance in any form and irrespective of its serotype is of benefit for *S. pneumoniae* with respect to survival and spread in the community. This implies that despite the introduction of new and effective pneumococcal conjugate vaccines, restrictive use of antibiotics will be of major importance.

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