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Clonal Diversity and Resistance Mechanisms in Tetracycline-Nonsusceptible *Streptococcus pneumoniae* Isolates in Poland[▽]

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The frequency of tetracycline resistance in *Streptococcus pneumoniae* isolates in Poland is one of the highest in Europe. The aim of this study was to analyze the clonal diversity and resistance determinants of tetracycline-nonsusceptible *S. pneumoniae* isolates identified in Poland and to investigate the effect of tetracycline resistance on their susceptibilities to tigecycline, doxycycline, and minocycline. We have analyzed 866 pneumococcal isolates collected from 1998 to 2003 from patients with respiratory tract diseases, and 242 of these (27.9%) were found to be resistant to tetracycline. All of the resistant isolates were characterized by testing of their susceptibilities to other antimicrobials, serotyping, pulsed-field gel electrophoresis (PFGE), and identification of tetracycline resistance genes and transposons. Selected isolates representing the main PFGE types were analyzed by multilocus sequence typing. Among the isolates investigated, 27 serotypes and 146 various PFGE patterns, grouped into 90 types, were discerned. The most common PFGE type, corresponding to serotype 19F and sequence type 423, was represented by 22.3% of all of the tetracycline-resistant isolates. The *tet*(M) gene was the sole resistance gene in the group of isolates studied, and in over 96% of the isolates, the Tn*916* family of *tet*(M)-containing conjugative transposons was detected. Several isolates contained specific variants of the transposons, the Tn*1545*-like, Tn*3872*-like, or Tn*2009*-like element. The correlation between the MICs of tetracycline, doxycycline, and minocycline was revealed, whereas no cross-resistance to tetracycline and tigecycline was observed.

Streptococcus pneumoniae is a common cause of serious and often life-threatening infections such as pneumonia, bacteremia, and meningitis, as well as upper respiratory tract infections, like otitis media or sinusitis. During the past few years, pneumococcal populations have become increasingly resistant to various antimicrobials, including tetracyclines. In Poland the rates of resistance of *S. pneumoniae* and other gram-positive bacteria to tetracycline are among the highest in Europe (20, 22), but despite this, the level of tetracycline consumption in Poland remains high in comparison to the levels of consumption in other European countries (http://www.esac.ua.ac.be/main.aspx?c=*ESAC2&*n*=4666).

Three distinct mechanisms of resistance to tetracycline in bacteria have been described so far, including active efflux, ribosome protection by chaperone proteins, and enzymatic inactivation of the compound. In *S. pneumoniae*, this resistance results from the acquisition of one of two genes, *tet*(M) or, sporadically, *tet*(O), both of which encode ribosome protection proteins (50). The main source of the *tet*(M) gene is conjugative transposons of the Tn*916* family (41), the particular members of which, e.g., Tn*5251*, differ from Tn*916* mostly in their nucleotide sequence (40). Some of these elements also contain other resistance genes, e.g., Tn*1545* [*erm*(B), which encodes resistance to macrolides, and *aph3′-III*, which encodes resistance to kanamycin] (6, 7), Tn*3703* [*erm*(B)] (27, 28), Tn*3872* [*erm*(B) within Tn*917*] (32), and Tn*2009* [*mef*(E), which encodes resistance to macrolides and which exists within the mega element] (11). On the other hand, several Tn*916*-like transposons with *tet*(M) [and, sometimes, *erm*(B)] got inserted into other elements, thus forming larger structures, e.g., Tn*3701* with Tn*3703* inside (8, 28). Some of these structures also carry the chloramphenicol resistance gene *cat*, e.g., Tn*5253* (insertion of Tn*5251* into *cat*-containing Tn*5252*) (1) and Tn*3951* (21). As a result, clinical isolates of *S. pneumoniae* are often resistant to tetracycline and to other compounds. In the United States, more than 60% of erythromycin-resistant pneumococci are simultaneously resistant to tetracycline (13); and in some European countries, e.g., Spain and Italy, over 80% (31, 46) of erythromycin-resistant pneumococci are simultaneously resistant to tetracycline. In 95% of such isolates, the presence of the *int-Tn* gene, which encodes the transposase of the Tn*916* family, was detected (34).

Only limited data concerning tetracycline resistance in *S. pneumoniae*, as well as the susceptibilities of resistant strains to a novel antibiotic, tigecycline, are available. The main objective of this analysis was to reveal the molecular epidemiology of tetracycline-resistant *S. pneumoniae* isolates in Poland and to investigate the relationship between tetracycline resistance and susceptibilities to tigecycline, doxycycline, minocycline, and other antimicrobials.

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MATERIALS AND METHODS

Bacterial strains. Eight hundred sixty-six clinical isolates of *S. pneumoniae* from respiratory tract infections were collected by the National Medicines Insti-

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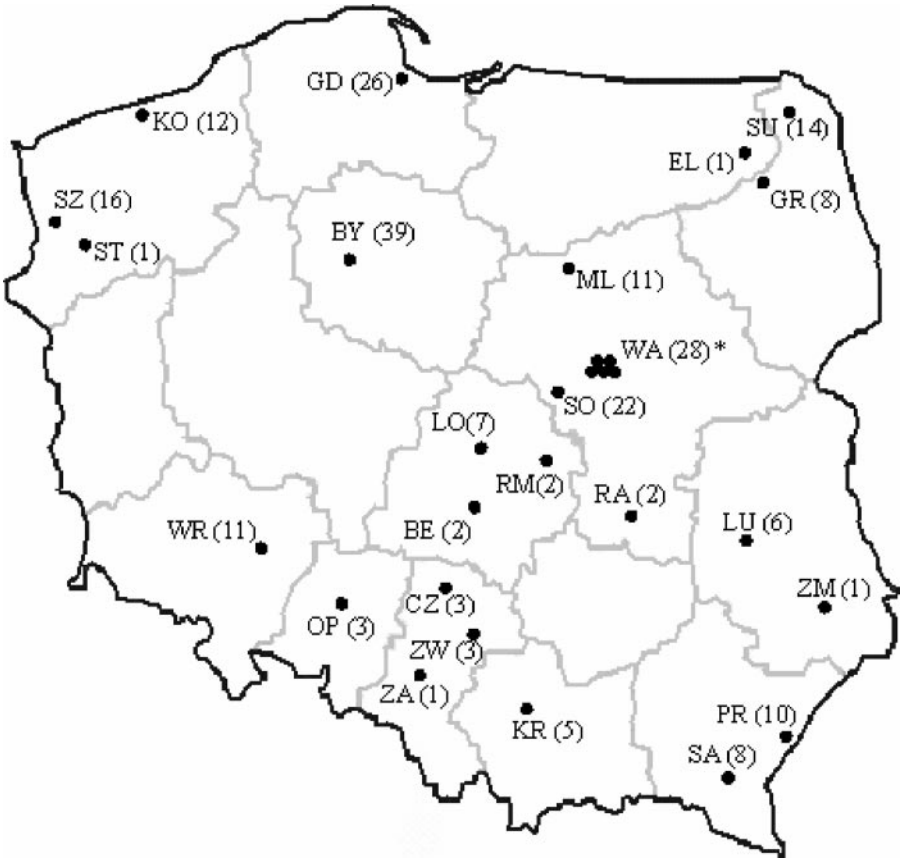

FIG. 1. Geographic distribution of tetracycline-resistant *S. pneumoniae* isolates in the study. The solid circles indicate the health care centers where the isolates were identified. Abbreviations of towns: BE, Be[strok]lchatów; BY, Bydgoszcz; CZ, Częstochowa; EL, E[strok]lk; GD, Gdańsk; GR, Grajewo; KO, Ko[strok]lobrzeg; KR, Kraków; LU, Lublin; LO, [strok]Lódź; ML, M[strok]lawa; OP, Opole; PR, Przemyśl; RA, Radom; RM, Rawa Mazowiecka; SA, Sanok; SO, Sochaczew; ST, Stargard; SU, Suwa[strok]lki; SZ, Szczecin; WA, Warszawa; WR, Wroc[strok]law; ZA, Zabrze; ZM, Zamość; ZW, Zawiercie. Numbers of isolates are shown in parentheses. *, 28 is the total number of isolates from five health care centers in Warsaw.

tute (NMI) in Warsaw, Poland, during an ongoing national surveillance program. They were recovered between 1998 and 2003 from various specimens (sputum specimens, bronchoalveolar lavage fluid, or tracheal aspirates) in 29 health care centers in 25 towns all over Poland (Fig. 1). The species identification was confirmed at NMI by conventional tests, such as susceptibility to optochin (bioMerieux, Marcy l'Etoile, France) and solubility in sodium deoxycholate (35). The collection of isolates was a part of that used in studies of the nonsusceptibility of Polish pneumococci to β-lactams and fluoroquinolones. Serotype, pulsed-field gel electrophoresis (PFGE), and multilocus sequence typing (MLST) data, as well as the β-lactam MICs for 52 isolates among those that were nonsusceptible to penicillin and tetracycline, are published separately (44). The data for four ciprofloxacin- and tetracycline-nonsusceptible isolates are also published separately (43). In this work, the PFGE typing of these isolates was repeated for the whole group of tetracycline-resistant organisms.

Susceptibility testing. The initial screening of tetracycline susceptibility was performed by the disk diffusion method, as recommended by the Clinical and Laboratory Standards Institute (CLSI) (5). For all tetracycline-resistant isolates, the MICs of a wider set of antimicrobials were determined by the broth microdilution method, as recommended by the CLSI (5). The following agents were tested: tetracycline, doxycycline, penicillin G, erythromycin, and chloramphenicol (Sigma-Aldrich, Steinheim, Germany); tigecycline and minocycline (Wyeth, Pearl River, NY); clindamycin and linezolid (Pharmacia Upjohn, Kalamazoo; MI); levofloxacin and telithromycin (Aventis Pharma, Romainville, France); teicoplanin and rifampin (Gruppo Lepetit, Lainate, Italy); cefotaxime (ICN Biomedicals, Aurora, OH); meropenem (Astra Zeneca, Cheshire, United Kingdom); vancomycin (Eli Lilly, Indianapolis, IN); and trimethoprim-sulfamethoxazole (Roche, Basel, Switzerland). *S. pneumoniae* strain ATCC 49619 was included for quality control purposes. The appropriate CLSI criteria were used for the interpretation of the results (5). For doxycycline, minocycline, and teicoplanin, the guidelines of the Societe Francaise de Microbiologie (http://www.sfm.asso.fr/nouv/general.php?pa=2) were adopted. For tigecycline the FDA breakpoints (http://www.fda.gov/cder/foi/label/2005/021821lbl.pdf) were applied.

Analysis of resistance determinants. Bacterial genomic DNAs were prepared with a Genomic Mini kit (A&A Biotechnology, Gdynia, Poland) and were used as templates for PCR. The isolates were tested for the presence of the *tet*(M) and *tet*(O) genes by PCR, as described elsewhere (14, 49). The PCR detection of the *erm*(B) and *mef*(E) genes was carried out with all isolates resistant to erythromycin [with primers ermBF and ermBR and primers MEF3 and MEF4 for the *erm*(B) and *mef*(E) genes, respectively], as reported previously (10, 48). The transposase gene *int-Tn*, specific for the Tn*916* family of transposons, was detected by PCR, as described by others (38). The identification of Tn*916*-like and Tn*1545*-like transposons was performed by PCR with primers O13 and O14 as reported by Poyart et al. (37). The distinction of Tn*1545*-like elements was carried out by amplification of the *aph3′-III* gene promoter (primers aphF [5′-GGAACAGTGAATTGGAGTTC-3′] and aphR [5′-GACATTCCTTCCGTATC-3′]), as well as of the *aph3′-III–erm*(B) region, also with primers aphF and ermBR. For the detection of Tn*5253*, the region of the right junction between Tn*5251* (Tn*916*-like) and Tn*5252* was analyzed by using primers 5252F (5′-CCTCCTGATTCCAGTGTC-3′) and 5251R (5′-GATTCTTCGCTGAACGAC-3′). Tn*5252* alone was detected by PCR of its transposase gene, *int-Tn*5252 (47). The isolates that were negative by PCR with primers O13 and O14 (37) were subjected to other analyses. Tn*3872* was detected by three PCRs with primers O13 and O20, O23 and O14, and O19 and O22, respectively (37). The presence of Tn*3951* was tested by PCR of the region between *erm*(B) and *tet*(M) with primers ermBF and tetMR. The isolates that were *mef*(E) positive were analyzed

for the presence of the Tn*2009* element (11) by PCR with primers MEF4 and O14 (10, 37).

In order to study the structural polymorphism of the *tet*(M) gene, *tet*(M) amplicons were digested with the SsiI restriction enzyme (an isoschizomer of AciI; Fermentas, Vilnius, Lithuania) and separated in 1% agarose gels (14).

Serotyping. Serotyping was performed by PCR with primers specific for genes responsible for the biosynthesis of types 1, 3, 4, 6A, 6B, 14, 18C, 19A, 19F, and 23F of the capsular polysaccharide (4, 26, 36). The serotypes of the remaining isolates and of randomly selected representatives of the pneumococci serotyped by PCR were determined by the capsular swelling method at the Statens Serum Institute, Copenhagen, Denmark.

PFGE and MLST analyses. Genomic DNA for PFGE was isolated as described by others (29), digested with the SmaI restriction enzyme (Fermentas), and separated in a CHEF-DR III system (Bio-Rad Laboratories, Hercules, CA). Two isolates were considered indistinguishable when they shared PFGE patterns and were considered related when they showed a difference of one to three bands from each other. The dendrogram was constructed with Molecular Analyst software (version 1.12; Bio-Rad) by using the unweighted pair-group method with arithmetic averages clustering method with a Dice coefficient and a position tolerance of 1.5%. MLST of selected isolates representing the main PFGE types was performed as described previously (17). Particular allele numbers and sequence types (STs) were identified by using the MLST database (http://www.mlst.net).

Statistical analysis. For statistical analysis, the χ^2 test, the Fisher exact test, and the Kolmogorov-Smirnov test were used.

RESULTS

Tetracycline-resistant isolates. Two hundred forty-two isolates, which represented 27.9% of all 866 isolates studied, were resistant to tetracycline. The percentages of tetracycline-resistant isolates were 25.4, 34.6, 28.5, 26.5, 26.3, and 21.6 during six consecutive years (1998 to 2003), respectively. Resistant isolates were recovered in all of the collaborating medical centers. The frequency of tetracycline resistance did not vary significantly over the 6 years studied, as shown by the χ^2 and Fisher exact tests. However, a constant downward trend in the prevalence of resistant isolates was observed from 1999 to 2003 ($P < 0.03$; Kolmogorov-Smirnov test).

Susceptibilities to antimicrobial agents. The tetracycline-resistant isolates showed various levels of susceptibility to the other antimicrobials studied (Table 1). A total of 80.1% and 75.6% of the isolates were nonsusceptible to doxycycline and minocycline, respectively, but all isolates were susceptible to tigecycline (MICs, ≤0.003 to 0.06 μg/ml). A total of 11.2% and 14.9% of the isolates were penicillin intermediate and penicillin resistant, respectively. The rates of resistance to erythromycin and chloramphenicol were 26.4% and 39.2%, respectively. A good correlation between the MICs of tetracycline, doxycycline, and minocycline was observed (Table 2), whereas no correlation existed between the tetracycline and the tigecycline MICs. Similarly, no link between the level of resistance to penicillin or erythromycin and the MICs of tigecycline was observed.

Serotyping, PFGE, and MLST analyses. Serotypes, PFGE types, and the MLST data are shown in Table 3. Altogether, 27 serotypes were present in the group of isolates investigated. The majority of isolates (78.1%) comprised five serotypes: 19F (69 isolates [28.5%]), 23F (60 isolates [24.8%]), 6B (27 isolates [11.2%]), 6A (17 isolates [7.0%]), and 14 (16 isolates [6.6%]).

The PFGE analysis revealed 146 various DNA patterns and classified these into 90 types. Among the most prevalent isolates of serotype 19F, 13 PFGE types were present, with type 1 (55 isolates) being highly predominant. Together with PFGE

TABLE 1. Distribution of MICs of 17 antimicrobial agents for tetracycline-resistant isolates

Antimicrobial agent	No. of isolates for which the MIC (μg/ml) was:																	MIC (μg/ml)		Percent[a]		
	≤0.003	≤0.0075	0.0075	≤0.015	0.015	≤0.03	0.03	0.06	0.12	≤0.25	0.25	0.5	1	2	4	>4	8	16	>16	32	64	128	2	50%	90%	S	I	R
Tetracycline												1	7	40	133		14	48		109	69	2	32	64	19.9	54.9	100.0
Doxycycline													3	23	89		55	6					4	8	24.4	36.8	25.2
Minocycline																	71	23					8	0.015	100.0		38.8
Tigecycline	2		37		123	73	7				5	5	6	18	14		4						0.015	0.03			
Penicillin G		67			86	21	5	11					27	10	3		2						0.015	2	74.0	11.1	14.9
Cefotaxime		69			94	23	5	6			3		2										0.015	1	93.8	4.1	2.1
Meropenem				173	3	1	17	5	54		14	30											≤0.015	0.5	86.8	12.8	0.4
Vancomycin					26	5	12	54	1		140	28											0.25	0.5	100.0		
Teicoplanin		3				145	67	1											1		56		0.03	0.06	100.0		
Erythromycin										175			3	1	3				56				≤0.25	>16	72.4	1.2	26.4
Clindamycin						154					2				1	56							≤0.03	>4	76.9		23.1
Telithromycin				220			24	5			3	3	3	3	1								≤0.015	≤0.015	98.8	0.8	0.4
Chloramphenicol						3	1	5	2		1	7	23	59	55		21	69		5			4	16	70.7		39.2
Linezolid											5	79	147	11									1	1	100.0		
Rifampin							15	2			1			1	1	1							≤0.03	≤0.03	98.8	0.4	0.8
Levofloxacin				2		221					2	73	161	2	1		1						1	1	98.8	0.4	0.4
Trimethoprim-sulfamethoxazole							1	3	2		39	27	39	37	32		41	16	5				2	8	30.2	31.4	38.4

[a] S, susceptible; I, intermediate; R, resistant.

TABLE 2. Number of isolates and correlation between MICs of tetracycline and doxycycline, tetracycline and minocycline, and tetracycline and tigecycline

Drug and MIC (μg/ml)	No. of isolates for which the tetracycline MIC (μg/ml) was:					
	8	16	32	64	128	Total
Doxycycline						
0.5	1					1
1	3	3	1			7
2	7	22	11			40
4	3	23	67	39	1	133
8			29	25	1	55
16			1	5		6
Total	14	48	109	69	2	242
Minocycline						
0.5	2	1				3
1	9	11	3			23
2	1	12	16	4		33
4	2	19	51	17		89
8		5	27	37	2	71
16			12	11		23
Total	14	48	109	69	2	242
Tigecycline						
≤0.003		1	1			2
0.0075	4	8	13	12		37
0.015	7	30	50	34	2	123
0.03	3	8	42	20		73
0.06		1	3	3		7
Total	14	48	109	69	2	242

types 2, 10, and 12, type 1 corresponded to ST423 and its single-locus variant (SLV), ST1815. Consideration of three non-type 19F isolates of PFGE type 1 and ST423 as well shows that the ST423 clonal group consisted of 63 tetracycline-resistant isolates (26.0%) altogether. Isolates of serotype 23F showed higher levels of diversity, comprising 19 PFGE types. Almost half of these isolates (26 isolates) were represented by ST602 and its two SLVs, ST440 and ST1479. The isolates of serotype 6B represented four PFGE types. This group was dominated by ST315, which corresponds to international clone Poland^{6B}-20 (16 isolates).

Tetracycline resistance determinants. All of the tetracycline-resistant isolates possessed the *tet*(M) gene and were negative by the *tet*(O) PCR. For the further analysis of *tet*(M)-carrying transposons, the isolates were split into four phenotypic groups with respect to resistance to tetracycline (TET^r), erythromycin (ERY^r), and chloramphenicol (CHL^r), namely, TET^r; TET^r and ERY^r; TET^r and CHL^r; and TET^r, ERY^r, and CHL^r. Among the 96 TET^r isolates, the *int-Tn* gene, which codes for the transposase of the Tn*916* family of elements, was detected in 91 isolates; and the O13-O14 PCR, positive results of which indicate an intact structure of the Tn*916*-like transposons behind the *tet*(M) gene (37), was positive for 94 isolates (PCR product size, ~600 bp). Forty-eight of the 51 isolates in the TET^r and ERY^r group were positive by PCR of the *int-Tn* gene. Four of these most probably carried the Tn*1545*-like elements, as suggested by the positive O13-O14 PCR result (37) and amplification of the *aph3′-III* gene promoter (product size, ~100 bp) and the *aph3′-III–erm*(B) region (~3.5 kb). Five further isolates were found to contain the Tn*3872*-like transposons, being negative by the O13-O14 PCR but yielding products of the expected size in PCRs with primers O13 and O20 (~1.4 kb), O23 and O14 (~2.7 kb), and O19 and O22 (~2.4 kb) (37). The vast majority of the remaining isolates of the TET^r and ERY^r group were positive by the O13-O14 PCR and possessed the *erm*(B) gene; however, PCR of the *erm*(B)-*tet*(M) region excluded the possibility of the presence of a transposon associated with Tn*3951* (28). Two isolates were *erm*(B) negative but carried the *mef*(E) gene, which is responsible for erythromycin resistance; and these two isolates were found to contain the Tn*2009*-like elements, as suggested by the positive PCR with primers MEF4 and O14 (product size, ~3.3 kb). All of the 82 isolates in the TET^r and CHL^r group carried the *int-Tn* gene and were positive by the O13-O14 PCR (37). Only five of these isolates produced the amplicon of the transposase gene specific for the *cat* gene-containing Tn*5252* (product size, ~900 bp); however, the Tn*916*-like and Tn*5252*-like elements did not form a Tn*5253*-like structure in any of the isolates, as revealed by the lack of a product by PCR mapping of the junction region (1). All of the 13 isolates in the TET^r, ERY^r, and CHL^r group carried the *int-Tn* gene. Three of these contained the Tn*3872*-like element and one contained the Tn*1545*-like element. The presence of an isolate with Tn*3951* carrying *cat*, *erm*(B), and *tet*(M) (28) was excluded. One other isolate possessed *mef*(E) instead of *erm*(B); however, *tet*(M) and *mef*(E) were not located in the Tn*2009*-like structure (11). The *cat* gene of this isolate was present in the Tn*5252*-like transposon but, again, not in the Tn*5253*-like configuration (1).

The *tet*(M) PCR-RFLP analysis showed seven polymorphs of the gene with three predominant profiles: profiles D (38.8%), A (27.7%), and G (19.0%). Three of these corresponded to restriction maps of *tet*(M) alleles previously identified in Tn*916* (GenBank accession number U09422; profile G), Tn*5251* (GenBank accession number X90939; profile A), and Tn*1545* (GenBank accession number X04388; profile B). There was no strict correlation between *tet*(M) polymorphs and particular transposon variants, as illustrated by the fact that profile B (2.5%) was found in only the Tn*916* family of elements, which had no Tn*1545*-like structure, whereas profile A was observed not only in Tn*916*-like transposons but also in Tn*1545*-like and Tn*3872*-like transposons. The majority of the *tet*(M) polymorphs were present in multiple *S. pneumoniae* clones; the only exception was polymorph B, which fully correlated with ST272 and ST2140 (SLVs of Poland^{23F}-16).

Characterization of tetracycline-susceptible isolates. Since the group of tetracycline-resistant *S. pneumoniae* isolates contained a highly prevalent fraction of closely related 19F/ST423 isolates, we decided to compare them with isolates of the same serotype from among the remaining 624 tetracycline-susceptible isolates. Among these, only three isolates (0.5%) of serotype 19F were found, and these represented ST423 (two isolates) and unrelated ST2142 (one isolate). One of the ST423 isolates, for which the tetracycline MIC was 2 μg/ml, possessed the *tet*(M) gene; and its PFGE profile was identical to that of PFGE subtype 1a, which was the most prevalent in the tetracycline-resistant serotype 19F group. The other ST423 and ST2142 isolates had tetracycline MICs of 0.12 μg/ml and lacked the *tet*(M) and *tet*(O) genes. The ST423 isolate had a unique PFGE pattern, although its PFGE pattern was related to that of the most frequent subtype, subtype 1a (pattern 1k).

TABLE 3. Distribution of serotypes, PFGE types and subtypes, STs, and resistance to antimicrobial agents among tetracycline-resistant isolates

Serotype	PFGE (sub)type(s) (no. of isolates)	ST[a]	Drug(s) to which isolate(s) was resistant[b]	International clone
3	55a (2)	ND		
3	55a (1)	ND	DOX	
3	1a (1)	423	CHL	
4	56a (1)	ND		
6A	31a (1)	72	MIN	
6A	32a (1)	440	MIN	
6A	24a (1)	490	DOX, ERY, CLI	
6A	24b (1), 29a (1)	490	SXT	
6A	24c (1)	490	MIN, SXT	
6A	25a (1), 27a (1)	490		
6A	28a (1)	490	CHL	
6A	26a (1)	490	MIN, ERY, CLI, SXT	
6A	26a (1)	1049	MIN, DOX, ERY, CLI, SXT	
6A	30a (2)	**2185**	DOX	
6A	30a (1)	**2185**	DOX, MIN	
6A	30a (1)	**2185**	DOX, MIN, SXT	
6A	30a (1)	**2185**	DOX, MIN, ERY, CLI	
6A	30b (1)	**2184**	DOX, MIN	
6B	35a (1)	90	MIN, PEN, CHL, SXT	Spain^{6B}-2
6B	34b (1)	273	DOX, MIN, ERY, CLI	Greece^{6B}-22
6B	34c (1)	273	MIN, ERY, CLI	Greece^{6B}-22
6B	34a (1)	273	MIN, ERY, CLI, CHL	Greece^{6B}-22
6B	33a (5), 33e (1)	315	DOX, MIN, ERY, CLI, SXT	Poland^{6B}-20
6B	33f (1)	315	MIN, ERY, CLI, SXT	Poland^{6B}-20
6B	33a (1), 33i (1)	315	MIN, ERY, CLI	Poland^{6B}-20
6B	33a (2), 33f (1), 33h (1)	315	DOX, MIN, ERY, CLI	Poland^{6B}-20
6B	33d (1)	315	DOX, ERY, CLI	Poland^{6B}-20
6B	33a (1)	315	DOX, MIN, CLI, CHL	Poland^{6B}-20
6B	33g (1)	315	DOX, MIN, PEN, ERY, CLI, SXT	Poland^{6B}-20
6B	33b (1)	1032	DOX, MIN, MEM, ERY, CLI	SLV Poland^{6B}-20
6B	33b (1)	1032	DOX, MIN, ERY, CLI	SLV Poland^{6B}-20
6B	33c (1)	1053	DOX, ERY, CLI, SXT	SLV Poland^{6B}-20
6B	34a (1)	1490	MIN, ERY, CLI, CHL	SLV Greece^{6B}-22
6B	34a (1)	1490	ERY, CLI, CHL	SLV Greece^{6B}-22
6B	33a (1)	1505	ERY, CLI, SXT	SLV Poland^{6B}-20
6B	16b (1)	**2138**		
7F	57a (1)	ND	MIN	
8	58a (1)	ND		
9N	59a (2)	ND		
9N	60a (2)	ND		
10A	61a (1)	ND		
11A	63a (1)	62	DOX, MIN, ERY, SXT	
11A	62a (1)	1010	DOX, MIN	
11A	64a (1)	ND	DOX, MIN, CHL	
11A	65a (1)	ND	CHL	
12F	66a (1)	ND	CHL	
14	18a (1)	124	CHL	
14	19a (1)	124	DOX, MIN, ERY, CHL	
14	20a (1), 21a (1)	124		
14	14a (3)	143	DOX, MIN, PEN, ERY, CLI, SXT	
14	14a (1)	143	DOX, MIN, PEN, ERY, CLI, TEL	
14	14a (1)	143	DOX, MIN, PEN, CTX, ERY, CLI	
14	14b (1)	143	DOX, MIN, PEN, ERY, CLI	
14	15a (1)	143	PEN, ERY, CLI	
14	22a(1)	156	PEN, ERY, CLI, SXT	Spain^{9V}-3
14	23a (1)	378		
14	14a (1)	1477	DOX, MIN, PEN, ERY, CLI	
14	16a (1)	**2138**		
14	17a (1)	**2139**		
15A	67a (1)	63	DOX, MIN, ERY, CLI	Sweden^{15A}-25
15A	70a (1)	124	DOX, MIN, SXT	
15A	68a (1)	410	DOX, CHL	
15A	69a (1)	410	CHL, SXT	
15A	16b (1)	**2138**		
15B/C	71a (1)	ND	ERY, CLI, SXT	
15B/C	72a (3)	ND		
15B/C	73a (1)	ND	MIN, CHL	

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TABLE 3—*Continued*

Serotype	PFGE (sub)type(s) (no. of isolates)	ST[a]	Drug(s) to which isolate(s) was resistant[b]	International clone
15F	74a (1)	ND	MIN	
18B	76a (1)	ND		
18C	1a (1)	423	DOX, CHL	
18C	75a (1)	1016	CHL, SXT	
18C	75b (1), 75c (1)	1016		
19A	77a (1)	276	ERY, SXT, RIF	
19A	77b (1)	276	ERY, CLI	
19A	77b (1)	ND	MIN, PEN, ERY, CLI, SXT	
19A	77b (1)	ND	PEN, ERY, CLI, SXT	
19A	78a (2)	1625	SXT	
19F	13a (1)	81	PEN, CHL, SXT	Spain^{23F}-1
19F	7a (1)	179	DOX, ERY, CLI	SLV Portugal^{19F}-21
19F	6a (1)	236	DOX, MIN, PEN, CTX, ERY, SXT	Taiwan^{19F}-14
19F	9a (1)	236	SXT	Taiwan^{19F}-14
19F	11a (1)	236	MIN	Taiwan^{19F}-14
19F	8a (1)	257	ERY, CLI, SXT	
19F	5a (1)	410	DOX, CHL	
19F	1a (20)	423[c]	CHL	
19F	10a (1)	423	CHL	
19F	1a (2)	ND	DOX, MIN, CHL	
19F	1a (1)	ND	DOX, CHL	
19F	1a (1)	ND	MIN, CHL	
19F	2b(1)	423	MIN, CHL	
19F	1a (7)	ND	CHL, SXT	
19F	12a (1)	423	CHL, SXT	
19F	1a (1)	ND	DOX, MIN, CHL, SXT	
19F	1a (3)	423[c]	MIN, CHL, SXT	
19F	2a (1)	423	MIN, CHL, SXT	
19F	1a (2)	423[c]	SXT	
19F	1a (2)	ND	MIN, SXT	
19F	1a (1)	423	ERY	
19F	1a (1)	ND	CHL, RIF	
19F	1a (3)	ND		
19F	1e (1), 2c (1)	423		
19F	1b (1)	423	DOX, MIN, SXT	
19F	1c (2)	423[c]	SXT	
19F	1c (1)	ND	CHL, SXT	
19F	1g (1)	423	CHL, SXT	
19F	1f (1), 1g (1), 1i (1)	423	CHL	
19F	1h (1)	423		
19F	3a (1)	1489	DOX, ERY, CLI	
19F	1d (1)	1815		
19F	4a (1)	**2136**	DOX, MIN, SXT	
20	79a (1)	ND		
22F	80a (1)	ND	CHL	
22F	81a (1)	ND		
23F	52a (1)	72	DOX, MIN, ERY, CLI	
23F	37a (3), 37d (1), 37e (2), 37g (1)	81	PEN, CHL, SXT	Spain^{23F}-1
23F	37d (1)	81	DOX, PEN, CHL, SXT	Spain^{23F}-1
23F	37a (2), 37h (1)	81	CHL, SXT	Spain^{23F}-1
23F	37a (1), 37f (1)	81	PEN, ERY, CHL, SXT	Spain^{23F}-1
23F	37b (1)	81	PEN, SXT	Spain^{23F}-1
23F	37c (1)	81	PEN, CHL, SXT, LVX	Spain^{23F}-1
23F	38a (1)	81	PEN, ERY, CLI, CHL, SXT	Spain^{23F}-1
23F	54a (1)	242	DOX, MIN, PEN, ERY, CLI	Taiwan^{23F}-15
23F	47b (2)	272	DOX, MIN, PEN, ERY, CLI, CHL, SXT	SLV Poland^{23F}-16
23F	47c (2)	272	DOX, MIN, PEN, CTX, ERY, CLI, CHL, SXT	SLV Poland^{23F}-16
23F	47c (1)	272	MIN, PEN, CTX, ERY, CLI, CHL, SXT	SLV Poland^{23F}-16
23F	48a (1)	410	CHL, SXT	
23F	1a (1)	423	SXT	
23F	39a (3), 39f (2), 42a (1), 43a (1), 45a (1)	440	MIN	
23F	39a (2), 42b (1), 44a (1)	440		
23F	39a (1)	440	DOX, MIN, CHL	
23F	39a (1)	440	MIN, SXT	
23F	46a (1)	440	MIN, ERY, CLI	
23F	39a (1), 39c (1), 39e (1), 41a (1)	602		
23F	39a (1), 39b (1), 40a (1)	602	MIN	
23F	39a (3)	602	MIN, SXT	

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TABLE 3—*Continued*

Serotype	PFGE (sub)type(s) (no. of isolates)	ST[a]	Drug(s) to which isolate(s) was resistant[b]	International clone
23F	37d (1)	1051	PEN, CHL, SXT	SLV Spain^{23F}-1
23F	51a (1)	1116		
23F	53a (1)	1364	MIN, CHL	
23F	37e (1)	1476	CHL, SXT	SLV Spain^{23F}-1
23F	39d (1)	1479	MIN, ERY, SXT	
23F	39d (1)	1479	MIN, SXT	
23F	50a (1)	**2137**	CHL, SXT	
23F	47a (1)	**2140**	DOX, MIN, PEN, ERY, CLI, CHL, SXT	SLV Poland^{23F}-16
23F	49a (1)	**2141**	DOX	
23F	39a (1)	ND		
28	82a (1), 83a (1)	ND		
28F	84a (1)	ND	MIN	
28F	85a (1)	ND		
33F	66b (1)	ND	CHL	
35C	86a (1)	ND		
35C	87a (1)	ND	MIN	
37	88a (1)	ND	MIN	
37	89a (1)	ND	CHL, SXT	
Rough	90a (1)	317	MIN, ERY, CLI, SXT	
Rough	36a (1)	410	DOX, CHL	

[a] New STs are shown in boldface; allelic profiles are as follows: ST2136, 13-16-19-5-17-20-26; ST2137, 15-17-4-12-6-1-6; ST2138, 2-12-1-18-17-4-14; ST2139, 16-44-1-16-9-11-8; ST2140, 7-13-8-1-10-6-171; ST2141, 10-25-12-1-6-20-28; ST2184, 1-10-9-18-5-1-6; ST2185, 1-10-9-43-5-1-6. ND, not determined.

[b] CTX, cefotaxime; CHL, chloramphenicol; CLI, clindamycin; DOX, doxycycline; ERY, erythromycin; LVX, levofloxacin; MEM, meropenem; MIN, minocycline; PEN, penicillin; RIF, rifampin; TEL, telithromycin; SXT, trimethoprim-sulfamethoxazole.

[c] ST was determined for selective representative only.

DISCUSSION

Tetracyclines are low-cost antibiotics, and because of that they are attractive for use in countries with limited health care budgets. This could be one of explanations for their high level of consumption in Poland (http://www.esac.ua.ac.be/main.aspx?c=*ESAC2&*n*=4666), despite the significant prevalence of resistance to these drugs among clinically important pathogens (20, 22). Although tetracycline-resistant *S. pneumoniae* isolates have been reported in all parts of the world, only a limited number of studies have addressed the question of the genotypic and phenotypic diversities of such isolates. In particular, only fragmentary data of this kind have been available in Poland and other countries of Central and Eastern Europe. In accordance with the findings of previous studies (15, 20, 22), we found a relatively high frequency of resistance to this compound (27.9%; standard deviation, 4.2%), with a slight downward trend over the period from 1999 to 2003. This tendency may be associated with the fact that tetracycline consumption has been kept at a relatively constant level in recent years (4.6 defined daily doses per 1,000 inhabitants per day [DID] in 1997 and 4.5 DID in 2001), whereas the total level of antibiotic use in Poland has been systematically increasing (19.3 DID in 1997 to 27.2 DID in 2001) (http://www.esac.ua.ac.be/main.aspx?c=*ESAC2&*n*=4666).

A positive correlation between tetracycline MICs and those of doxycycline and minocycline was observed in the analysis, which was in congruence with the findings presented in earlier reports (12, 25). All of the tetracycline-resistant isolates retained clear susceptibility to tigecycline, similar to the findings of previous reports from Europe, North America, and Asia (3, 12, 19, 52; T. S. Bouchillon, T. S., B. Johnson, J. Johnson, D. Hoban, M. Hackel, M. Person, M. Dowzicky, 15th Eur. Congr. Clin. Microbiol. Infect. Dis., abstr. P805, 2005; T. S. Bouchillon, T. S., B. Johnson, J. Johnson, D. Hoban, M. Hackel, M. Person, M. Dowzicky, 15th Eur. Congr. Clin. Microbiol. Infect. Dis., abstr. P806, 2005; S. B. Johnson, T. Stevens, J. Johnson, D. Hoban, A. Hsiung, and M. Dowzicky, 15th Eur. Congr. Clin. Microbiol. Infect. Dis., abstr. P807, 2005). So far no cross-resistance to tetracycline and tigecycline has been observed in pneumococci, and it is possible that the risk of selection of tigecycline-resistant organisms with tetracyclines may be low by definition. These findings, together with those presented in reports on the excellent activity of tigecycline against other agents that cause community-acquired respiratory tract infections (2, 16, 24, 42), position it to be among the promising therapeutic options under certain circumstances for these types of infections (18).

The *tet*(M) gene was the only determinant of resistance identified in the isolates studied, which was in accordance with the seldom occurrence of the *tet*(O) gene (30, 51). To our knowledge, in Europe only the *tet*(M) gene has been detected so far in pneumococci (15, 45). The analysis of the *tet*(M)-carrying mobile elements revealed their remarkable diversity in the population studied. Two hundred thirty-four isolates (96.7%) carried the *int-Tn* gene, which codes for the transposase of the Tn*916* family of transposons, which has confirmed the role of these conjugative elements as major determinants of tetracycline resistance in *S. pneumoniae* (34, 39). The sporadic isolates that were negative by the *int-Tn* PCR could either possess specific structural variants of the Tn*916*-like elements or, eventually, other transposon types. Interestingly, among the isolates that, apart from their resistance to the tetracyclines, were resistant to erythromycin and/or chloramphenicol, the presence of elements that determine such coresistance that have been described so far seems to be rare, as exemplified by the fact that only eight isolates had a Tn*3872*-like transposon; five isolates had a Tn*1545*-like transposon; two isolates had a Tn*2009*-like transposon; and no isolates had a

Tn*5253*, Tn*3951*, or Tn*2010* transposon (9). It is possible that in most of these isolates, *tet*(M) resided in Tn*916*-like elements, whereas the *erm*(B) and *cat* genes were located in separate transposons. This could, for example, be the case for several chloramphenicol-resistant isolates, in which *cat*-containing transposon Tn*5252* was detected, but not in association with *tet*(M), as is the case in Tn*5253*. However, the possibility could not be excluded that a significant fraction of isolates resistant to tetracycline and erythromycin possessed *tet*(M) and *erm*(B) located in other than Tn*1545*-like or Tn*3872*-like structures of the Tn*916* family, such as Tn*3701* (28).

A single *S. pneumoniae* serotype may include genetically diverse clones that can be distinguished by PFGE and MLST. In this study, a remarkable diversity of PFGE patterns and STs was observed in all major serotypes (serotypes 6A, 6B, 14, 19F, and 23F). Each of these serotypes was dominated by specific clones, namely, ST423 in serotype 19F; ST81, ST440, and ST602 in serotype 23F; ST315 in serotype 6B; ST490 in serotype 6A; and ST143 in serotype 14. This observation suggests that the high prevalence of resistance to tetracycline in *S. pneumoniae* in Poland is mainly the result of the spread of a relatively few clones, especially ST423 of serotype 19F. The particular role of serotype 19F is underlined by the fact that only 3 of 624 tetracycline-susceptible isolates in the study represented this common serotype, which means that almost each Polish pneumococcus of serotype 19F is resistant to tetracycline. Of the 26 international multiresistant clones of pneumococci recognized so far (33), 17 clones are resistant to tetracycline. In our analysis, representatives of eight such clones (with two SLVs) were detected, and these constituted 24.0% of the isolates. These results demonstrated that, apart from ST423, the international *S. pneumoniae* clones contribute significantly to the spread of tetracycline resistance as well. The currently available pneumococcal vaccines, PCV7 and PPV23, cover 75.2% and 86.8% of the serotypes of the isolates studied, respectively. However, clones of vaccine serotypes may be gradually replaced by those of nonvaccine serotypes because of, among other reasons, capsule switching (23). It probably occurred among the study isolates, although mostly to serotypes covered by the vaccines; e.g., three ST423 isolates represented serotype 3, 18C, or 23F instead of serotype 19F.

In summary, this work has provided useful comparative data for future studies, presenting the findings of a complex molecular analysis of Polish tetracycline-resistant *S. pneumoniae* isolates. The observed high frequency of resistance to tetracycline among the *S. pneumoniae* organisms isolated in Poland appears to arise from the spread of a relatively few epidemic clones that harbor the *tet*(M) gene, located in transposons of the Tn*916* family, as the sole resistance determinant. The data obtained confirmed the observations on the cross-resistance between tetracycline, doxycycline, and minocycline and showed that tigecycline has very good activity against the tetracycline-resistant isolates. Moreover, the results of the study create a strong experimental basis for elaboration of therapeutic recommendations aimed at the prevention of the further dissemination of tetracycline resistance among *S. pneumoniae* isolates in Poland.

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