ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 2000, p. 2979–2984 Vol. 44, No. 11
0066-4804/00/$04.00+0
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Genetic Diversity of the *tet*(M) Gene in Tetracycline-Resistant Clonal Lineages of *Streptococcus pneumoniae*

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Received 5 May 2000/Returned for modification 8 July 2000/Accepted 31 July 2000

The aim of the present study was to examine the stability and evolution of *tet*(M)-mediated resistance to tetracyclines among members of different clonal lineages of *Streptococcus pneumoniae*. Thirty-two tetracycline-resistant isolates representing three national (Spanish serotype 14, Spanish serotype 15, and Polish serotype 23F) and one international (Spanish serotype 23F) multidrug-resistant epidemic clones were all found to be *tet*(M) positive and *tet*(O), *tet*(K), and *tet*(L) negative. These isolates all carried the integrase gene, *int*, which is associated with the Tn*1545*-Tn*916* family of conjugative transposons. High-resolution restriction analysis of *tet*(M) products identified six alleles, *tet*(M)1 to *tet*(M)6: *tet*(M)1 to *tet*(M)3 and *tet*(M)5 in isolates of the Spanish serotype 14 clone, *tet*(M)4 in both the Spanish serotype 15 and 23F clones, and *tet*(M)6, the most divergent allele, in the Polish 23F clone. This indicates that *tet*(M) variation can occur at the inter- and intraclone levels in pneumococci. Two alleles of *int* were identified, with *int1* being found in all isolates apart from members of the international Spanish 23F clone, which carried *int2*. Susceptibility to tetracycline, doxycycline, and minocycline was evaluated for all isolates with or without preincubation in the presence of subinhibitory concentrations of tetracyclines. Resistance to tetracyclines was found to be inducible in isolates of all clones; however, the strongest induction was observed in the Spanish serotype 15 and 23F clones carrying *tet*(M)4. Tetracycline was found to be the strongest inducer of resistance, and minocycline was found to be the weakest inducer of resistance.

The gram-positive pathogen *Streptococcus pneumoniae* (the pneumococcus) is a major cause of pneumonia, otitis media, and meningitis (12). The evolution and broad global distribution of multiple antibiotic resistance determinants in bacteria have resulted in a situation in which pneumococci are commonly resistant to penicillin, the broad-spectrum cephalosporins, macrolides, lincosamides, co-trimoxazole, chloramphenicol, and tetracyclines, as well as rifampin (11), sulphonamides (42), and fluoroquinolones (13, 24, 30), making the treatment of serious pneumococcal disease increasingly difficult (17, 22). The transformable nature of *S. pneumoniae* (which has played an important role, along with point mutations) in the evolution of resistance [1, 4, 11, 13, 24, 30] has in no small part also led to a population structure characterized by free genetic exchange, punctuated by clonal expansion of successful variants. The best studied of these are the Spanish 23F, Spanish 6B, and French/Spanish 9V14 multidrug-resistant clones that have now spread intercontinentally (see reference 10 for a recent review).

One class of antimicrobial agents found most often in clinical use is the tetracyclines, broad-spectrum bacteriostatic drugs shown to be active against pneumococci (33). In some European (9, 16, 23), Asian (35, 36, 41, 47), and African (31, 52), countries lack of susceptibility to tetracyclines is the most frequently observed resistance phenotype in pneumococci. This situation has also been seen in Poland (45) and the United Kingdom (6). In the mid-1990s tetracyclines were the second most commonly prescribed antimicrobial drugs after the penicillins in both countries (14, 33). The only known mechanism of tetracycline-resistance in *S. pneumoniae* is the protection of the bacterial 30S ribosome subunit against antibiotic binding by the TetM (2) or TetO (50) proteins, with the *tet*(M) gene being more common than the *tet*(O)gene in pneumococci (7, 18, 19, 40, 43). Analysis of the nucleotide sequences of *tet*(M) genes from a diverse range of bacteria clearly reveals that *tet*(M) has evolved by recombination (28); however, it is unclear what is responsible for driving this recombination.

The *tet*(M)-mediated resistance to tetracyclines and *erm*(B)-mediated resistance to macrolides, lincosamides, and streptogramins in pneumococci is due to the acquisition of highly mobile conjugative transposons of the Tn*916*-Tn*1545* type and large composite structures like Tn*5253* and Tn*3872* which carry these and other resistance determinants (8, 21). A core element in the biology of these transposons is the integrase. Allelic variation within the integrase gene, *int*, can therefore be used to help track the movement and population biology of these conjugative transposons.

To progress from a descriptive analysis of antibiotic consumption and the evolution of resistance to a more quantitative understanding of the dynamics of resistance and the implications for changing practices in antimicrobial chemotherapy, an important factor is an understanding of the stability or plasticity of the resistance genotype and phenotype. The aim of this study was to examine the stability and evolution of transposon-associated *tet*(M)-encoded tetracycline resistance among members of four different multiply drug-resistant clonal lineages of *S. pneumoniae*. This was undertaken by high-resolution restriction analysis (HRRA) of allelic variation within

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tet(M) and the transposon integrase gene (*int*). The inducibility of tetracycline resistance in these isolates was also investigated.

MATERIALS AND METHODS

Bacterial strains. Thirty-two *S. pneumoniae* isolates were included in this study, and 29 of these have already been described. They represented three different national (Spanish serotype 14, Spanish serotype 15 and Polish serotype 23F) or international (Spanish 23F) multidrug-resistant epidemic clones identified in previous studies (see Table 1 for details). All isolates were confirmed to be *S. pneumoniae* by optochin susceptibility and bile solubility tests in previous studies and were stored at −80°C, in 15% glycerol. All cultures subsequently grown from stored stocks were streaked to single colonies prior to use. Strains were grown overnight at 37°C in 5% CO_2 on brain heart infusion (Becton Dickinson Europe, Meylan, France) supplemented with 1.5% Bacto Agar (Becton Dickinson) and 5% (vol/vol) defibrinated sheep's blood (Oxoid, Basingstoke, United Kingdom) (BHI agar). The capsular types of three isolates for which no published data were available were determined with Danish pneumococcal typing antisera (Statens Seruminstitut, Copenhagen, Denmark) by the Quellung reaction.

Susceptibility testing. Antibiotic resistance was determined by the agar dilution and the disk diffusion methods. For medium preparation, plate inoculation, and interpretation of results, the methodology recommended by the National Committee for Clinical Laboratory Standards was applied (25, 26), with the exception that MICs were determined by the agar dilution method rather than the broth dilution method. Antibiotic dilutions were prepared in Mueller-Hinton agar (Oxoid) supplemented with 5% defibrinated sheep blood. After inoculation the plates were incubated aerobically for 18 to 20 h at 37°C and were supplemented with 5% CO_2. Susceptibility to the following antimicrobial agents was tested: penicillin, cefotaxime, erythromycin, chloramphenicol, rifampin, ofloxacin, doxycycline, and minocycline (all supplied by Sigma-Aldrich, Poole, United Kingdom) and tetracycline (NBL Gene Sciences, Cramlington, United Kingdom).

For all isolates, tetracycline, doxycycline, and minocycline MICs were evaluated both with and without induction of resistance. For each strain a few colonies from an overnight growth on BHI agar were used to inoculate three BHI agar plates: unsupplemented medium (lack of induction) and BHI agar supplemented with tetracycline at concentrations of 0.5 and 5 mg/liter, doxycycline at 0.5, 2, and 5 mg/liter, and minocycline at 0.5, 1, and 5 mg/liter (for induction of resistance to tetracyclines). Cells were harvested after not longer than 20 h of growth and were used for MIC evaluation.

Resistance to tetracycline, doxycycline, and minocycline was also determined by the disk diffusion method (26) with disks containing 30 μg of each antimicrobial agent (Oxoid). Macrolide and lincosamide resistance phenotypes were determined on the basis of the erythromycin MIC evaluation by the agar dilution method and the double-disk test method (15) with erythromycin (15-μg), lincomycin (15-μg), and clindamycin (2-μg) disks (all from Oxoid).

Identification of tetracycline resistance determinants by PCR. Chromosomal DNA was purified as described previously (48). PCR were carried out with primers at a concentration of 4 ng/μl of the final reaction volume. Primer sequences were as follows: *tet*(M) forward, 5′-AGT TTT AGC TCA TGT TGA TG-3′; *tet*(M) reverse, 5′-TCC GAC TAT TTG GAC GAC GG-3′; *int* forward, 5′-GCG TGA TTG TAT CTC ACT-3′; and *int* reverse, 5′-GAC GCT CCT GTT GCT TCT-3′. Both primer sets were designed on the basis of the available database sequences of *tet*(M) and *int* (GenBank accession numbers X90939 and L29324, respectively). Cycling parameters were denaturation at 95°C for 1 min, annealing at 55 or 50°C for 1 min [for *tet*(M) and *int* primers, respectively], and extension at 72°C for 1.5 or 1 min [for *tet*(M) and *int* primers, respectively], followed by a final extension step of 72°C for 10 min. The PCR amplicons were visualized by agarose gel electrophoresis as described by Sambrook et al. (39). Detection of the *tet*(O), *tet*(K), and *tet*(L) determinants was performed by PCR by previously described protocols (44). A tetracycline-susceptible isolate of *S. pneumoniae* (isolate R6) was used as a negative control for each of the *tet*- and *int*-specific PCRs *S. pneumoniae* isolates from a previous study (28) were used as positive controls for *tet*(M) and *int*, and *Staphylococcus aureus* isolates (44) were used for *tet*(K) and *tet*(L). *Streptococcus mutans* strain DL5 was used as a positive control for *tet*(O). Products from positive controls were sequenced by using the PCR primers to confirm the identities of the amplicons.

HRRA. HRRA was undertaken essentially as described previously (45, 49). Specifically, 10 μl of each PCR product was digested with the following restriction enzymes: *Aci*I, *Alu*I, *Dde*I, *Mse*I, and *Rsa*I. Restriction fragments were separated by polyacrylamide gel electrophoresis in 4 and 8% gels and were visualized by staining with ethidium bromide. The restriction footprint of the enzymes used covered 8.03% of the *tet*(M) PCR product on the basis of the published sequence of the gene (34). Each restriction pattern given by each digest was assigned a pattern number. Alleles were determined on the basis of the combined restriction patterns for all five digests. The percent divergence between the alleles described was calculated by using the band matching algorithm of Nei and Li (27). The same methodology was applied for HRRA of the *int* PCR product.

AP PCR and BOX PCR. PCR-based typing methods were used to establish the degrees of similarity among the strains analyzed. Arbitrarily primed (AP) PCR was carried out with AP4 (51) and AP7 (46) primers. Each reaction was carried out in a final volume of 50 μl with 20 mM Tris-HCl (pH 8.4) and 50 mM KCl, 5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 1 μg of primer, 5 U of *Taq* DNA polymerase (Gibco BRL, Paisley, United Kingdom), and approximately 20 ng of DNA. Cycling parameters were 95°C for 2 min, followed by 10 cycles of 30 s at 95°C, 30 s at 35°C, and 1 min at 72°C and then 30 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C and a final extension step of 72°C for 4 min. BOX PCR was performed as described above, with the following exceptions: 0.2 μg of each primer was used per 50-μl reaction mixture. Primers were designed on the basis of available published BOX sequences (20) and sequences derived by other workers in our laboratory (S. King, unpublished results): BOX A forward, 5′-CCA CGT CAG CKT CRC CTT RCC GT-3′; BOX A reverse, 5′-CAA GGC GAM GCT GAC RTK GTT TGA-3′. Cycling parameters were 35 cycles of 30 s at 95°C, 30 s at 50°C, and 4 min at 72°C, with a final extension step of 72°C for 10 min. Different AP PCR and BOX PCR types were identified the basis of a single band difference between the electrophoretic patterns of PCR products detected after separation through 1% agarose visualized with ethidium bromide.

RESULTS

On the basis of serotype, AP PCR and BOX PCR genomic patterns, *tet*(M)::*int* allele pattern, and resistance profile, all *S. pneumoniae* isolates analyzed in this study fell into one of four clusters of related isolates (Table 1). AP PCR and BOX PCR were found to discriminate between isolates of different clusters, whereas at least two of the four AP PCR or BOX PCR amplification patterns generated for each isolate were identical to the patterns for other isolates defined as being clonally related in previous studies (3–5, 11, 29, 49; http://mlst.zoo.ox.ac.uk).

Tetracycline resistance was determined by the agar dilution method for all 32 isolates studied (MIC range, 8 to 64 mg/liter). PCR screening revealed that all isolates were *tet*(M) positive and *tet*(K), *tet*(L), and *tet*(O) negative. Detection of the 1,862-bp fragment from positions 21 to 1882 of the published sequence of the *tet*(M) gene was indicative of the presence of the *tet*(M) determinant (34). HRRA of *tetM* gene PCR amplicons revealed that the *tet*(M) loci from all of the strains analyzed fell into six distinct restriction types [alleles *tet*(M)1 to *tet*(M)6]. The data generated from the *tet*(M) allele assignments (data not shown) were used to calculate the degree of genetic diversity among the alleles. The results of this analysis are shown in Table 2. The estimated nucleotide divergence between different *tet*(M) alleles ranged from 0.44% [*tet*(M)1 and *tet*(M)2] to 8% [*tet*(M)2 and *tet*(M)6]. HRRA did not reveal any variation in *tet*(M) within the Polish serotype 23F clone, Spanish serotype 15 clone, or Spanish serotype 23F pandemic clone. The Spanish serotype 14 clone was found to differ in that the isolates examined carried four alleles, *tet*(M)1 to *tet*(M)3 and *tet*(M)5, that were divergent by up to 3.18%. Isolates of the Spanish serotype 15 clone and the Spanish serotype 23F pandemic clone both carried *tet*(M)4.

All isolates were *int* positive, in that they produce the expected 1,046-bp fragment. After testing with a range of enzymes, two alleles of *int* that could be discriminated on the basis of a single *Dde*I digest were identified. Isolates of all national clones possessed a common *int* allele type 1, and all members of the Spanish 23F clone possessed the *int*2 allele.

According to the geometric mean MICs, the highest level of resistance to the tetracyclines tested was observed among isolates of the Polish 23F clone (32 mg/liter for tetracycline, 10.96 mg/liter for minocycline, and 7.05 mg/liter for doxycycline). The lowest MICs were observed for isolates of the Spanish serotype 15 clone and were equal to 12.13, 3.03, and 4 mg/liter for the same drugs, respectively. These isolates were all shown to possess *tet*(M)4.

Induction of resistance to the tetracyclines by subinhibitory concentrations of tetracycline was observed in nearly all isolates of each of the epidemic clones. The highest induction

TABLE 1. Combined data on origin, typing results, *tet*(M) gene variant analysis results, and tetracycline resistance patterns for 32 penicillin-nonsusceptible *S. pneumoniae* strains

Clone and strain/year of isolation/ country of origin[a]	Reference(s)	Typing results[b]		*tet*(M)::*int* allele	Resistance to tetracyclines												
					Tetracycline				Doxycycline				Minocycline				
		BOX A rev.:: BOX A for.:: AP7::AP4	Resistance pattern		MIC (mg/liter)	Induction[c] 0.5	2	5	MIC (mg/liter)	Induction 0.5	2	5	MIC (mg/liter)	Induction 0.5	1	2	5
Spanish serotype 14 national clone																	
B12/1992	3; website[d]	A::A::B::A	PXTELC	1::1	32			++	8				16				+, ◆
GM32/1990	3; website[d]	A::A::A::C	PTELC	2::1	32			+	8				16				
VH33/1990	3; website[d]	A::A::A::A	PTELC	2::1	32			+	8				16				
VH40/1994	3; website[d]	A::A::A::A	PTELC	2::1	32			+	8				16				
T13/1990	3; website[d]	A::A::A::A	PTC	3::1	16	+		+	4	+		+	8		●	◆	+, ●
T9/1991	3; website[d]	A::A::A::A	PTEC	3::1	32				4	+		+	8				
GM23/1993	3; website[d]	A::A::C::A	PTC	3::1	16	+		+	4	+		+	8				+, ●
C30/1994	3; website[d]	A::A::A::A	PTC	3::1	16	+		+	4				8				+
C38/1994	3; website[d]	A::A::A::B	PTELC	5::1	32				8				8	+			+
Spanish serotype 15 national clone																	
GM49/1989	3	D::D::D::D	PTEC	4::1	16		◆		4				2	++			+
BS50/1998	3	D::D::D::D	PTC	4::1	16				4				4	+			+
C60/1993	3	D::D::D::D	PTC	4::1	16				4			++	4	+			+
B62/1991	3	D::D::D::D	PTELC	4::1	8	+	◆	+++	4		◆	++	2	++			+++
C69/1994	3	D::F::D::D	PTEC	4::1	8			++	4			++	4	+			+
Spanish serotype 23F international clone																	
SpI/1998/Spain	This study	G::G::G::G	PTC	4::2	16	+	◆	++	4	+		+	2	+	●	◆◆	++
89NB/—United States[e]	This study	G::G::G::H	PTEC	4::2	32				8			+	4	+			++◆
DN87/1987/United Kingdom	4	G::G::G::G	PTC	4::2	16	+		+	4			+	2	++			+
DN88/1988/United Kingdom	4	G::G::G::G	PTC	4::2	32			◆	8				4	++, ◆	●	◆	
264/1984/Spain	4,5; website[d]	G::G::G::G	PTC	4::2	64				8		◆	+	4	++			◆
267/1984/Spain	4, 5	G::G::G::G	PTC	4::2	16	+		+	4	+		++	2	++			+++
38G/1992/Mexico	This study	G::G::G::G	PTC	4::2	16				4			+	2	++			+
Polish serotype 23F national clone																	
233/1994	11, 49	I::I::L::L	PXTECR	6::1	32			+	8				16				
234/1994	11, 49	I::I::M::I	PXTECR	6::1	32			+	8				16				
256/1994	Website[d]	I::I::N::I	PXTELC	6::1	32			+	8				16	+			
22/1995	29	I::K::K::I	PXTELC	6::1	32			+	8				16				
26/1995	29	I::I::I::I	PXTELC	6::1	32				4			+	8				
9/1995	29	I::I::I::I	PXTELC	6::1	32				8				8				
29/1995	29	I::I::I::I	PXTELC	6::1	32				8				8	+			+, ◆
32/1995	29	I::I::I::K	PXTELC	6::1	32				8				8	+			
39/1995	29	I::I::I::K	PXTELC	6::1	32				8		◆		8	+			
40/1995	29	I::I::I::K	PXTELC	6::1	32				4				8				
6/1995	29	I::L::I::I	PXTELC	6::1	32				8				16	+			

[a] Country of origin for isolates of the Spanish 23F international clone.

[b] A to I represent different banding patterns obtained by typing by BOX PCR and AP PCR. Abbreviations for resistance pattern: P, penicillin; X, cefotaxime; T, tetracycline; E, erythromycin; L, lincosamide (lincoymcin and clindamycin); C, chloramphenicol; R, rifampicin. An underlined symbol indicates intermediate susceptibility.

[c] MICs determined after induction of tetracycline resistance by cells preincubation on BHI agar supplemented with tetracycline at concentrations of 0.5 or 5 mg/liter, doxycycline at concentrations of 0.5, 2, or 5 mg/liter, and minocycline at concentrations 0.5, 1, 2, or 5 mg/liter. The increases in the MICs after the induction of resistance with particular concentration or tetracycline (+), minocycline (●), and doxycycline (◆) are indicated: +, ●, and ◆, twofold (1-dilution) increases; ++ and ◆◆, fourfold (2-dilution) increases; +++, eightfold (3-dilution) increase.

[d] http://www.mlst.zoo.ox.ac.uk.

[e] —, data not available.

TABLE 2. Percent nucleotide divergence between *S. pneumoniae tet*(M) alleles

Allele	% Nucleotide divergence for allele:				
	2	3	4	5	6
1	0.44	1.74	1.34	3.03	7.41
2		1.25	1.54	2.87	8.00
3			3.18	2.13	6.93
4				3.33	5.34
5					5.28

ratio (calculated as the increase in the geometric mean MICs evaluated for a particular drug and cluster of isolates with and without induction of resistance) was observed for isolates of Spanish serotype 15 and serotype 23F clones, all of which carried *tet*(M)4. Induction was not noted for two members of the Polish 23F clone (strains 9 and 40). The subinhibitory concentrations of minocycline tested did not induce resistance to tetracycline or doxycycline. However, minocycline did induce resistance to itself in four isolates, two members of the Spanish serotype 14 clone (T13 and GM23; both with the *tet*(M)3 allele) and two of the Spanish serotype 23F clone (SpI and DN88). Subinhibitory concentrations of doxycycline induced resistance in 10 isolates: tetracycline resistance in 4 isolates, to minocycline resistance in 7 isolates, and doxycycline resistance in 3 isolates.

According to National Committee for Clinical Laboratory Standards (26)-recommended breakpoints for the disk diffusion method, four isolates of the Spanish serotype 15 clone and one isolate of the Spanish serotype 14 clone fit into the category of intermediate susceptibility. One member of the serotype 15 clone (isolate B62) would be categorized as susceptible. All other isolates were identified as resistant to tetracycline by the disk diffusion method.

All isolates were found to be penicillin-nonsusceptible *S. pneumoniae*. The MICs of erythromycin for all Polish isolates were ≥256 mg/liter, and except for two isolates (isolates 233 and 234), all isolates were resistant to the lincosamides tested. A high level of resistance to erythromycin indicates the presence of the MLS_B phenotype coded by *erm*(B). In four isolates (isolates GM49, C69, 89NB and T9) low-level resistance to erythromycin (MIC range, 0.5 to 8 mg/liter) with susceptibility to lincosamides was observed, indicative of the presence of a macrolide efflux system (15, 38). All other isolates were susceptible to the macrolides and lincosamides tested. No single isolate was susceptible to chloramphenicol, and no single isolate was resistant to ofloxacin. All isolates were identified as multidrug resistant, being resistant to drugs of at least three different groups of antimicrobial agents.

DISCUSSION

The genes that encode tetracycline resistance in bacteria are numerous and are often evolutionarily distinct. By far the most widely distributed tetracycline resistance determinant in gram-positive bacteria is *tet*(M) (37). In this study isolates of different epidemic clones of multidrug-resistant pneumococci have been checked for the presence of four different tetracycline resistance determinants, *tet*(K), *tet*(L), *tet*(M), and *tet*(O), and all have been found to be positive only for *tet*(M). The identification of *tet*(O)-positive pneumococci has, however, been reported in a limited number of South African (50) and North American (18) isolates.

Six *tet*(M) alleles were identified by HRRA in *S. pneumoniae* isolates belonging to four epidemic clones. *tet*(M)6, identified in Polish isolates, was the most divergent of all alleles detected, differing from *tet*(M)2 by 8% at the nucleotide level. This is similar to the divergence found previously between progenitor alleles of *tet*(M) carried by Tn*1545* and *S. aureus*, and recombination between these alleles resulted in the mosaic structure of the *tet*(M) alleles found in a diverse range of organisms (28). The potential origin of *tet*(M)6 is under investigation. The most common allele was *tet*(M)4, identified in all isolates of the Spanish serotype 15 and 23F clones. In this study all of the members of the Spanish 23F clone carried the same *int2* allele, which was unique among the isolates examined.

The susceptibilities of the isolates to selected tetracyclines were analyzed to establish if there are any differences in tetracycline induction or resistance profiles among *S. pneumoniae* isolates of different allelic variants of *tet*(M). Interestingly, *S. pneumoniae* strains that belong to two different epidemic clones (Spanish serotypes 15 and 23F), both of which carry *tet*(M)4, shared similar profiles of inducible tetracycline resistance. For isolates of both clones, the MICs of minocycline were relatively low, ranging from 2 to 4 mg/liter for strains examined without induction of resistance. After induction of resistance with tetracycline, increases in the minocycline MICs to 8 and 16 mg/liter were observed for all these isolates. Of the three tetracyclines tested, minocycline was the weakest inducer of resistance and tetracycline was the strongest inducer of resistance. Only minocycline did not induce resistance to the other tetracyclines tested. However, the induction of resistance to minocycline by subinhibitory concentrations of the drug observed in four isolates indicates that minocycline should not be underestimated as an inducer of resistance.

Susceptibility to tetracycline has apparently been observed in *tet*(M)-positive isolates of the largest of national epidemic penicillin-nonsusceptible *S. pneumoniae* clones identified in the United States in 1996 and 1997 (7) and Romania (32), and intermediate susceptibility (MICs, 2 to 4 mg/liter) has been observed in some Italian isolates of the Spanish 23F clone (19). However, by routine susceptibility testing, resistance to tetracycline is evaluated without any induction of resistance, and it is therefore possible that some tetracycline-resistant *S. pneumoniae* strains may have been misidentified as susceptible. This possibility was confirmed in this study, when the disk diffusion method was used to evaluate resistance to tetracycline in isolates of the Spanish serotype 15 clone, for which tetracycline MICs were the lowest among the clones examined. The observed inducibility of resistance in these and the other isolates examined suggests that all *tet*(M)-positive *S. pneumoniae* strains should be considered resistant to all tetracyclines, and the lack of susceptibility to one of them should be interpreted as resistance to drugs of the whole group. The importance of inducible resistance in the proper identification of resistance to tetracyclines in *tet*(M)-positive strains of another gram-positive pathogen, *Staphylococcus aureus,* has already been documented (44).

It has previously been shown that members of the Tn*916* family of transposons can be found in the host chromosome in multiple copies (35). From the HRRA experiments performed in this work, it is apparent that there was no superposition of different *tet*(M) restriction types, indicating a single allele per isolate (multiple copies of the same allele could be present). Acquisition of different *tet*(M) alleles within a clonal lineage, as found for the Spanish serotype 14 clone, may have been mediated by loss of the original transposon and acquisition of a new transposon carrying a different allele of *tet*(M) or by recombination following transformation by DNA from a different strain, again carrying a different allele of *tet*(M).

The MLS_B phenotype of strains carrying *tet*(M)1, *tet*(M)2, *tet*(M)5, and *tet*(M)6 may indicate that the tetracycline resistance determinant is in the context of a *tet*(M)Tn*1545*-like transposon. The isolates of the pandemic 23F clone and the Spanish serotype 15 clone all possess *tet*(M)4 and lack macrolide resistance. One notable exception among the serotype 15 isolates is strain B62, which displayed the MLS_B phenotype. One explanation is that a Tn*917*-like element carrying *erm*(B) has been inserted into a Tn*916*-like element in a composite transposon-like structure, as observed previously for some other pneumococci resistant to macrolides, lincosamides, and streptogramin B (MLS_B) (21), or that the strain has acquired a Tn*1545*-like element.

The data presented here indicate that *tet*(M) gene variation in *S. pneumoniae* can occur at the inter- and intraclone levels. Identical genomic backgrounds with different allelic variants of *tet*(M) or the same *tet*(M) and *int* alleles observed in unrelated *S. pneumoniae* strains indicate active movement and evolution of these genes. It is also apparent that the relative stability or instability of transposon-encoded determinants differs between different clones of *S. pneumoniae.* The differences in resistance to tetracyclines among isolates and the inducibility of resistance indicate the importance of proper identification of this mechanism of resistance in pneumococci, the need to standardize tetracycline susceptibility tests, and the revision of current guidelines. It remains to be confirmed whether the different tetracycline resistance phenotypes observed in this study are due to differences within *tet*(M) alone.

ACKNOWLEDGMENTS

This work was supported by a grant from the Warwick University Research and Teaching Development Fund to Neil Doherty and by a European Respiratory Society Fellowship to Krzysztof Trzcinski. Both N.D. and K.T. contributed equally to this study.

We thank S. King for use of the BOX sequence data.

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