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Tetracycline Resistance in Group A Streptococci: Emergence on a Global Scale and Influence on Multiple-Drug Resistance▽†

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A global sample of group A streptococci (GAS) revealed ≥80 separate acquisitions of tetracycline resistance. Of 244 clones, 38 and 25% displayed resistance to tetracycline and erythromycin, respectively; a relatively high proportion (15%) were resistant to both classes of drugs. *tet*(M) displayed a highly significant association with *erm*(B).

Tetracycline in the environment can shape the evolution of many bacteria. In gram positive organisms, resistance to tetracycline is typically conferred by ribosome protection genes, such as *tet*(M) and *tet*(O). The number of acquisitions of resistance to tetracycline among group A streptococci (GAS) is unknown. Using a global collection of GAS isolates of defined genetic backgrounds, the number of independent acquisitions of tetracycline resistance is estimated. Since tetracycline resistance genes can reside on mobile genetic elements that carry macrolide resistance genes (2, 4, 8), the co-occurrence of resistance to both classes of drugs was also assessed.

emm types and multilocus sequence types (STs), which are based on seven housekeeping loci, were previously reported for most GAS isolates analyzed (5, 7). Twenty-eight new ST profiles were identified by previously described methods (5).

Susceptibility versus resistance to erythromycin and tetracycline was established by the Etest (7) for 188 GAS isolates recovered from >20 countries; antibiotic resistance profiles were unknown at the onset of the study. Antibiotic susceptibility was defined by MICs of <0.25 and 2 μg/ml for erythromycin and tetracycline, respectively. All isolates were distinct in *emm* type and ST. Only 2.1% of the isolates were resistant to erythromycin, whereas 60 (31.9%) were resistant to tetracycline. Tetracycline-resistant GAS (TRGAS) outnumbered macrolide-resistant GAS (MRGAS) 15-fold.

Tetracycline resistance is usually acquired by GAS via horizontal gene transfer. The number of distinct acquisitions can be estimated by identifying distant genetic backgrounds associated with resistance. A set of 291 isolates, which includes ~79% of known *emm* types (*Streptococcus pyogenes emm* sequence database, available at http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm) and additional MRGAS (7), was screened for tetracycline resistance by the Etest and/or by PCR targeting *tet*(M) and *tet*(O) at an annealing temperature of 50°C using primers tetMF1–tetMR1 and tetOF1–tetOR1 (Table 1). TRGAS isolates (n = 112), represented by 90 STs from 27 countries (see Table S1 in the supplemental material), were identified.

Genetic distances between the TRGAS isolates were defined by the eBURST clustering algorithm for analyzing relationships between STs (3). STs sharing five or six housekeeping alleles—double- or single-locus variants, respectively—were assigned to the same clonal complex (CC). Among the 90 STs, 61 were singletons that

TABLE 1. Oligonucleotide primers used for PCR and nucleotide sequence determination of amplicons

Forward primer		Reverse primer	
Name	Nucleotide sequence	Name	Nucleotide sequence
tetMF1	GCATAAGATTTCAGAATTGTTC(C/T)CTGTTCC	tetMR1	GTAATATCGTAGAAGCGGATCACTATCTGAG
tetMF2	CCTTTATAGTGGAGTACTACATTTACGAGATTCGG	tetMR2	CAACGGAAGCGGTGATACAGATAAACCAATGG
tetOF1	GAACAGGAAGAAAACAGGAGATTCCAAAACG	tetOR1	GGGTCGCCATCTGAAATTTCTGTAAGTGCCC
tetOF3	GTGCGTATATATAGCGGAACATTGCATTTGAGG	tetOR3	CTTCCAATAGGGAGCGGCTCTATGGACAACCCG

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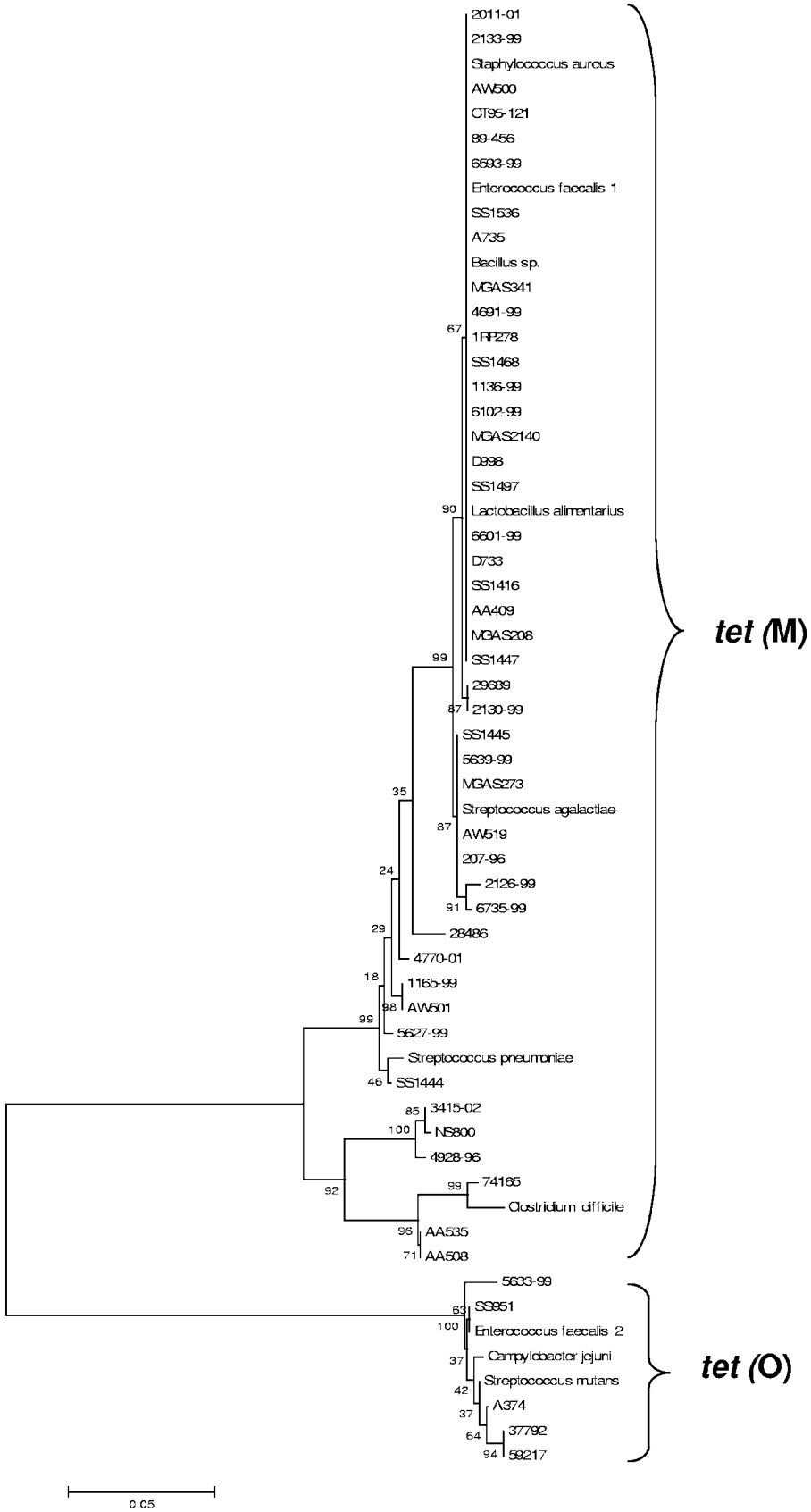

FIG. 1. Phylogenetic tree of partial *tet*(M) and *tet*(O) gene sequences. Partial *tet*(M) and *tet*(O) nucleotide sequences are shown for 49 TRGAS isolates (GenBank accession numbers EF363154 to EF363202) and isolates from 10 additional bacterial species. The phylogenetic tree was

TABLE 2. Conservative estimate for the number of independent acquisitions of tetracycline resistance among TRGAS isolates

Description of tetracycline-resistant isolates	No. of representatives	No. of isolates	No. of distinct STs	No. of independent acquisitions of tetracycline resistance
Singleton isolates, sharing <5 housekeeping alleles with all other strains and/or having different Tet resistance genotypes	61	61	61	62[a]
CCs, in which all isolates share at least 5 housekeeping alleles with another member of the complex	13[b]	51	29	13
Double-locus variant pairs having different *emm* types	5	NA[c]	NA	5
Total	NA	112	90	80

[a] Two isolates share the same *emm* type and ST (*emm12* ST36) but have different Tet resistance genes and therefore are scored as separate acquisitions of tetracycline resistance.
[b] All isolates within each of these CCs have *tet*(M), except for one isolate that lacks both *tet*(M) and *tet*(O) (*emm58* ST19).
[c] NA, not applicable.

TABLE 3. Distribution of resistance genes among 244 genetically distinct GAS isolates[a]

Macrolide susceptibility or resistance	No. of isolates				
	Susceptible to tetracycline	*tet*(M)	*tet*(O)	Tetracycline resistant, gene unknown	Total
Susceptible to erythromycin	128	51	3	2	184
erm(A)	*12*	**9**	**1**	**1**	23
erm(B)	*4*	**12**	**1**	0	17
mef(A)	*8*	**7**	**2**	0	17
erm(A) + *erm*(B)	0	**1**	0	0	1
Macrolide resistant, gene unknown	0	**1**	**1**	0	2
Total	152	81	8	3	244

[a] Thirty-six isolates are doubly resistant to both drugs (boldfaced), 56 are resistant to tetracycline but susceptible to erythromycin (underlined), 24 are resistant to erythromycin but susceptible to tetracycline (italicized), and 128 are susceptible to both ($P < 0.001$ by Fisher's exact test).

differed from all other STs by ≥3 housekeeping alleles (Table 2); each singleton ST represents an independent acquisition of tetracycline resistance. Two *emm12* ST36 isolates had distinct *tet* genes—*tet*(M) and *tet*(O)—bringing the subtotal to 62 acquisitions.

Thirteen CCs account for the remaining 29 STs (Table 2). If each CC arose from a single TRGAS progenitor, then acquisition of resistance by organisms belonging to the same CC should be scored as a single genetic event. Accordingly, ≥75 independent acquisitions of tetracycline resistance are estimated. Closer inspection of the 13 CCs reveals five sets of double-locus variants in which STs of each pair also differ in the *emm* type, signifying that ≥3 sequential genetic steps were required to evolve these CCs. If differences in two housekeeping alleles, plus a different *emm* type, represent at least two independent acquisitions of resistance, then the minimum estimate for separate acquisitions of tetracycline resistance among GAS rises to ≥80 events.

For representative TRGAS isolates corresponding to the 80 independent acquisitions, the vast majority ($n = 72$; 90%) have *tet*(M). Distinctions between the *tet*(M) and *tet*(O) amplicons were confirmed by nucleotide sequencing using the primers listed in Table 1. The findings for 49 TRGAS are summarized in a phylogenetic tree (Fig. 1). Two major sequence clusters distinguish the *tet*(M) and *tet*(O) alleles. The high levels of sequence similarity observed with *tet*(M) and *tet*(O) genes from other bacterial species provide additional support for the acquisition of tetracycline resistance genes by GAS via horizontal transfer.

In streptococci, mobile genetic elements carrying *tet*(M) or *tet*(O) sometimes harbor genes encoding macrolide resistance (1, 4). Since selection for resistance to one antibiotic can influence the evolution of resistance to another drug in multiply resistant bacteria, it was of interest to determine the relationship between resistance to tetracycline and macrolides in GAS. The resistance genotype of MRGAS was determined by PCR for *erm*(A), *erm*(B), and *mef*(A) and has been reported previously for most isolates (7). The degree of growth inhibition by erythromycin was determined for other isolates.

Findings on susceptible phenotypes and resistant genotypes were combined with data on the *emm* type and ST. A set of 244 distinct GAS isolates was identified in which each isolate had a unique profile comprising the erythromycin susceptibility or macrolide resistance genotype, tetracycline susceptibility or tetracycline resistance genotype, and *emm* type–ST combination. Furthermore, all 244 isolates sharing the same antibiotic resistance profile were distant by ≥2 genetic steps; they are represented by 182 singleton STs and 21 CCs. Of the 244 distant clones, 92 (38%) were resistant to tetracycline and 60 (25%) were resistant to macrolides (Table 3). Thirty-six (15%) displayed resistance to both classes of drugs; 128 were susceptible to both. According to a two-by-two test for independence, the number of organisms resistant to both antibiotics exceeded the number of organisms expected to be doubly resistant simply by chance ($P < 0.001$ by a two-tailed Fisher exact test). Findings were similar for the 239 isolates differing by ≥3 genetic steps.

Of the 92 TRGAS clones, 81 (88%) harbor *tet*(M) (Table 3).

constructed by the neighbor joining method using the Kimura 2-parameter model for nucleotide substitution; branch support was assessed by bootstrap analysis (1,000 replicates), and confidence intervals are indicated at the nodes. The tree is based on 646 sites. Bar, 0.05 nucleotide substitution per site. A tree based on predicted amino acid sequences yields a similar topology. GenBank accession numbers for resistance genes from other species are as follows: *Staphylococcus aureus tet*(M), M21136; *Streptococcus pneumoniae tet*(M), AY466395; *Streptococcus agalactiae tet*(M), AE014233; *Enterococcus faecalis tet*(M), X92947; *Clostridium difficile tet*(M), AF333235; *Lactobacillus alimentarius tet*(M), AY149586; *Bacillus* sp. *tet*(M), AF491293; *E. faecalis tet*(O), M20925; *Streptococcus mutans tet*(O), M20925; *Campylobacter jejuni tet*(O), AY190525.

The *tet*(M) gene is found in association with *erm*(B) in 12 isolates, representing 71% of the total *erm*(B)-positive isolates. A two-by-two test for independence was used to compare the number of isolates observed to have both *tet*(M) and *erm*(B) ($n = 12$), *tet*(M) only ($n = 69$), *erm*(B) only ($n = 5$), or neither ($n = 158$), to the number of isolates expected if *tet*(M) and *erm*(B) were randomly associated. The frequency of co-occurrence of *tet*(M) and *erm*(B) is significantly greater than that expected by chance ($P = 0.002$). In contrast, the *erm*(A) gene co-occurs with *tet*(M) at a frequency that can be explained by chance, as does *mef*(A) (*P*, not significant).

The data support the hypothesis that *tet*(M) and *erm*(B) are often linked, and they are consistent with the idea that *tet*(M) and *erm*(B) may be coinherited. In streptococci, *tet*(M) is often carried by the conjugative transposon Tn*916* or the related Tn*1545*, which together have a broad bacterial host cell range (2, 6). Although mobile genetic elements carrying *tet*(M) were not identified in the TRGAS, members of the Tn*916*–Tn*1545* family of elements are strong candidates. In addition to *tet*(M), the Tn*1545* element harbors *erm*(B) (8). Since *tet*(M) is more widely distributed than *erm*(B), and >70% of *erm*(B) occurrences are found in *tet*(M)-positive GAS, it seems plausible that strong selection forces act to drive the spread of *tet*(M) among many different GAS strains, whereby *erm*(B) is the frequent hitchhiker.

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