Genetic diversity of the *tet(M)* gene in tetracycline-resistant oral streptococci

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Tetracycline resistance occurs at a high frequency among clinical isolates of both gram-positive and gram-negative bacteria. The mechanism and genetics of tetracycline resistance have not been extensively studied in streptococci, although the overwhelming majority of clinical isolates are tetracycline resistant. *tet(M)* is the most common tetracycline-resistance gene in streptococci. The aim of this study was to examine the genetic diversity of *tet(M)* genes in tetracycline-resistant oral streptococci from dental plaque. Streptococci were isolated from supragingival plaque samples of healthy persons. The isolates were then identified at the species level, and the minimum inhibitory concentration (MIC) of tetracycline was determined. Genomic DNA was extracted from tetracycline-resistant isolates and *tet(M)* was amplified using polymerase chain reaction with *tet(M)*-specific primers. The polymerase chain reaction products were cloned, DNA sequencing was performed, and the sequences were compared using an alignment program. The estimated nucleotide divergence between different *tet(M)* alleles ranged from 0.00% to 6.07% among oral streptococci. The *tet(M)* genes from oral streptococci consisted of regions similar in sequence, interspersed with regions that differed at some nucleotide sites, revealing a mosaic structure. The percent nucleotide divergence of *tet(M)* was unrelated to the MIC values of tetracycline for oral streptococci, and bacterial strains in the same streptococcal species showed different heterogeneity in *tet(M)*. The divergences of *tet(M)* nucleotide sequences among oral streptococci were comparable with those of other bacterial genera. Our findings may provide basic information about the transposition processes associated with *tet(M)* in oral streptococci.

Key Words: Diversity; Resistance; Sequence; Streptococci; Tetracycline

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Introduction

Tetracyclines are broad-spectrum antibiotics that exhibit activity against a wide range of bacteria [1]. They are a family of antibiotics that inhibit protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor site. Tetracyclines also have a number of non-antibacterial effects, such as inhibition of collagenase,

which was found to be therapeutically useful in periodontitis [2]. However, the extensive use of these agents has led to the emergence of antibiotic resistance.

Resistance to tetracycline occurs at high frequency among clinical isolates of both gram-positive and gram-negative bacteria [3]. The first tetracycline-resistant bacterium was isolated in 1953 [4]. Tetracycline-resistance genes mediate resistance mainly by two different mechanisms:

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active efflux or ribosomal protection. The tetracycline-resistance genes *tet(K)* or *tet(L)* confer tetracycline resistance due to active efflux, which exports tetracycline from the cell, while the *tet(M)*, *tet(O)*, *tet(S)*, or *tet(W)* genes express proteins that protect ribosomes from the action of tetracycline [1,5].

The *tet(M)* gene is widely distributed among both gram-positive and gram-negative bacteria and has been found in 59 genera [5]. Over the past two decades, many oral bacterial strains have been reported to be tetracycline resistant [6]. *tet(M)* is the most tetracycline-resistance gene in streptococci [1,4]. Particularly in gram-positive streptococci and enterococci, *tet(M)* has been associated with Tn916/Tn1545-like conjugative transposons that form the basis of a family of conjugative transposons having an extremely broad host range [7,8]. The mobile units of conjugative transposons have enabled the *tet(M)* gene to move between species and into a wide range of genera by conjugative transposition [1]. In previous studies, analysis of the nucleotide sequences of *tet(M)* genes from a diverse range of bacteria revealed that *tet(M)* has evolved by recombination following transformation of DNA from a different strain carrying a different *tet(M)* allele [9,10]. Recombination between evolutionarily divergent *tet(M)* genes exhibiting nucleotide variations [11] and genetic diversities of *tet(M)* in *Staphylococcus aureus* [7] and *Streptococcus pneumoniae* [9] have been reported.

Dental plaque is comprised of more than 700 species of bacteria, including bacteria related to periodontal diseases [12]. Because oral streptococci constitute the major bacterial species found in human dental plaque [13], tetracycline-resistance genes present in oral streptococci may be a source for transferring tetracycline resistance to other bacteria present in the oral cavity. Although many clinical oral streptococcal isolates are tetracycline resistant, the genetics of tetracycline-resistance genes have not been extensively studied in oral streptococci [14,15]. Here, we examined the genetic diversity of *tet(M)* genes in tetracycline-resistant oral streptococci from dental plaque.

Materials and Methods

Bacterial strains and media

Streptococci were isolated from the supragingival plaque samples of healthy persons. All volunteers willing to donate their plaques for this study were informed about the procedure and gave written consent for inclusion in the study. This study was approved by the Institutional Review Board of Gangneung-Wonju National University Dental Hospital (IRB 2011-2). Isolates were identified to the species level using the Rapid ID 32 Strep system and a mini API reader (bioMérieux, Marcy-l'Etoile, France). Seven specific viridans-group streptococcal species (*Streptococcus anginosus*, *Streptococcus gordonii*, *Streptococcus mitis*, *Streptococcus mutans*, *Streptococcus oralis*, *Streptococcus salivarius*, and *Streptococcus sanguinis*) were identified.

Susceptibility testing

To determine the minimal inhibitory concentration (MIC) of the antibiotic, stock antibiotic solution of tetracycline (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) was prepared. The MICs were determined according to Clinical and Laboratory Standards Institute (CLSI) guidelines using a microdilution method in cation-adjusted Mueller-Hinton broth supplemented with lysed horse blood. Using streptococcal colonies taken directly from sheep-blood agar plates (KOMED, Seongnam, Korea), which were incubated at 37°C for 18 hours in aerobic conditions, a suspension equivalent to that of the 0.5 McFarland standard ($\sim 1 \times 10^8$ CFU/mL) in cation-adjusted Mueller-Hinton broth was prepared. The bacteria were inoculated into serially diluted antibiotic solutions in 96-well microtitration plates at final concentrations of 5×10^5 CFU/mL. The microtitration plates were incubated in an ambient-air incubator at 37°C for 24 hours. The microtitration plates were read visually and the minimum concentration of the antibiotics that produced no turbidity was recorded as the MIC. Antibiotic resistance was determined by interpretive standard concentrations from CLSI guidelines [16] and tests were repeated at least twice. The range of concentrations tested for each antibiotic was from 0.001–1,024 µg/mL.

Identification of tetracycline-resistance determinants by polymerase chain reaction (PCR)

Genomic DNA was extracted from the tetracycline-resistant strains using an Accuprep Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea) and stored at -70°C . PCR analysis was performed using Bioneer HotStart Taq polymerase (Bioneer). Gene 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' [9]. PCR amplification was performed in a DNA Thermal Cycler (GeneAMP PCR System 9700; Perkin Elmer, Waltham, MA, USA) under the following conditions: an initial incubation at 95°C for 5 minutes, 35 cycles of 95°C for 1 minute, 50°C for 1 minute, and 72°C for 30 seconds, followed by a final incubation at 72°C for 5 minutes. PCR products were electrophoretically separated on a 1% agarose gel, stained with

ethidium bromide, and visualized by ultraviolet transilluminator. The size of the *tet(M)* gene following DNA amplification was 1,862 bp.

Table 1. Minimal inhibitory concentration (MIC) of tetracycline

Strains	Tetracycline MIC ($\mu\text{g/mL}$)
<i>Streptococcus anginosus</i> KN154	64
<i>S. anginosus</i> KN157	128
<i>Streptococcus gordonii</i> KN164	16
<i>S. gordonii</i> KN469	64
<i>Streptococcus mitis</i> KN82	64
<i>S. mitis</i> KN172	64
<i>Streptococcus mutans</i> KN533	16
<i>Streptococcus oralis</i> KN64	128
<i>S. oralis</i> KN152	4
<i>Streptococcus salivarius</i> KN460	128
<i>Streptococcus sanguinis</i> KN16	32
<i>S. sanguinis</i> KN158	32

Fig. 1. Illustration of *tet(M)* diversity highlighting only the polymorphic sites in both nucleotide sequence alignments. Numbering begins at the first residue of the forward primer site.

Cloning and sequence analysis

Among the strains containing *tet(M)*, 12 were selected for further analysis of *tet(M)* genetic diversity. We included at least one strain from each of the seven streptococcal species and all the strains were randomly selected from within the same species. The PCR products were purified using an *Accuprep* Gel Purification Kit (Bioneer) and subsequently cloned into a PCR 4-TOPO vector from a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer instructions. The cloned *tet(M)* PCR products were transformed into One Shot TOP 10 *Escherichia coli* DH5 α -T1^R competent cells, and the plasmids were harvested from cultured *E. coli* with an *Accuprep* Nano-Plus Plasmid Mini Extraction Kit (Bioneer). DNA sequencing was performed by Macrogen (Seoul, Korea) using the following primers: T3, 5'-ATT AAC CCT CAC TAA AGG GA-3'; T7, 5'-TAA TAC GAC TCA CTA TAG GG-3'; OM139, 5'-TCG AGG TCC GTC TGA AC-3' [7], OM140, 5'-AAC AGA AGG TAG AAC TG T-3'. The OM139 and OM140 primers were designed with the GenScript DNA sequencing primer-design tool (http://www.genscript.com/cgi-bin/tools/sequencing_primer_design). The DNA sequences were compared using an alignment program [17].

Results

Tetracycline-susceptibility tests were performed for 635 streptococcal isolates, with 148 isolates being determined to be tetracycline resistant. Among the 148 tetracycline-resistant oral streptococcal strains, 68 (46%) contained *tet(M)*. Twelve strains from the 68 *tet(M)*-positive isolates were selected for *tet(M)* sequence analysis. The MICs of these isolates for tetracycline are presented in Table 1.

Comparison of only polymorphic sites among the sequences is shown in Fig. 1. It was revealed that genetic variation in *tet(M)* is not randomly distributed. The mosaic distribution of polymorphic sites suggested that localized recombination events may have occurred. The results of sequence analysis are shown in Table 2. The number of different nucleotides between different *tet(M)* alleles varied from 0 to 113, corresponding to the estimated nucleotide divergence ranging from 0% (*S. anginosus* KN154 and *S.*

Table 2. Percent nucleotide divergence of *tet(M)* sequences among oral streptococcal isolates

	<i>S. anginosus</i> KN157	<i>S. gordonii</i> KN164	<i>S. gordonii</i> KN469	<i>S. mitis</i> KN172	<i>S. mitis</i> KN82	<i>S. mutans</i> KN533	<i>S. oralis</i> KN64	<i>S. oralis</i> KN152	<i>S. salivarius</i> KN460	<i>S. sanguinis</i> KN16	<i>S. sanguinis</i> KN158
<i>S. anginosus</i> KN154	3.33	2.74	3.28	0.27	3.33	3.28	0.00	2.74	3.11	3.28	3.06
<i>S. anginosus</i> KN157		6.07	0.16	3.54	0.21	0.27	3.33	6.07	0.64	0.16	0.59
<i>S. gordonii</i> KN164			6.02	3.01	6.07	6.02	2.74	0.32	5.59	6.02	5.59
<i>S. gordonii</i> KN469				3.49	0.16	0.21	3.28	6.02	0.59	0.11	0.54
<i>S. mitis</i> KN172					3.54	3.49	0.27	3.01	3.33	3.49	3.28
<i>S. mitis</i> KN82						0.27	3.33	6.07	0.64	0.16	0.59
<i>S. mutans</i> KN533							3.28	6.02	0.70	0.21	0.64
<i>S. oralis</i> KN64								2.74	3.11	3.28	3.06
<i>S. oralis</i> KN152									5.59	6.02	5.59
<i>S. salivarius</i> KN460										0.59	0.05
<i>S. sanguinis</i> KN16											0.54

Table 3. The comparison of nucleotide sequences between *Streptococcus gordonii* KN164 and *tet(M)* from bacteria reported in the National Center for Biotechnology Information database

Bacterial species	Number of different nucleotides	Difference (%)	Reference
<i>Clostridium septicum</i>	5	0.27	Sasaki et al. [21]
<i>Enterococcus faecalis</i>	110	5.91	Perreten et al. [22]
<i>Escherichia coli</i>	105	5.64	Jones et al. [23]
<i>Lactococcus lactis</i>	110	5.91	Florez et al. [24]
<i>Lactobacillus sakei</i>	151	8.11	Ammor et al. [25]
<i>Staphylococcus aureus</i>	153	8.22	Nesin et al. [26]
<i>Streptococcus oralis</i>	6	0.32	Ciric et al. [27]
<i>Streptococcus pneumoniae</i>	71	3.81	Del Grosso et al. [28]
<i>Streptococcus pneumoniae</i>	43	2.31	Provvedi et al. [29]
<i>Streptococcus suis</i>	89	4.78	Ye et al. [30]

oralis KN64) to 6.07% (*S. gordonii* KN164 and *S. anginosus* KN157; *S. gordonii* KN164 and *S. mitis* KN82; *S. oralis* KN152 and *S. anginosus* KN157; *S. oralis* KN152 and *S. mitis* KN82). Genetic consistencies were not found within the same species.

To examine the sequence divergence of *tet(M)* from oral streptococci used in this study with other bacterial species, we compared the nucleotide sequence of *S. gordonii* KN164, which showed the most divergent *tet(M)* sequence among the oral streptococcal strains used in this study, with the *tet(M)* sequences of other bacteria from the National Center for Biotechnology Information database. The divergence data is shown in Table 3. The sequence differences ranged from 0.27% with *Clostridium septicum* to 8.22% with *S. aureus*.

Discussion

Several studies reported the prevalence of tetracycline resistance in oral streptococci, with the severity of the prevalence varying among studies conducted in different countries. In London, Stapleton et al. [11] found *tet(M)* in 14 out of 22 strains (63.6%), while a study conducted in Spain by Rodriguez-Avial et al. [18] reported that 35% of oral streptococcal strains were resistant to tetracycline, and that among these, 77.8% contained *tet(M)*. In Belgium, 114 out of 157 strains (72.6%) exhibited tetracycline resistance and among these, 105 (92.1%) were *tet(M)* positive [19].

The *tet(M)* determinant is mostly non-plasmid associated and mediates resistance to minocycline, as well as tetracycline [3].

One of the reasons for the success of this gene is that it is commonly contained within conjugative transposons, which have an extraordinarily broad host range [1,8]. According to de Vries et al. [7], conjugative transposition plays an important role in the evolution and horizontal spread of *tet(M)* in *S. aureus*, with *S. aureus* of human origin containing diverse *tet(M)* variants located on Tn916- and Tn5801-like (Tn6014) transposons.

Previous studies demonstrated the heterogeneity of the *tet(M)* genes. Rizzotti et al. [20] reported divergence of the enterococcus *tet(M)* gene in up to 10.9% of the *tet(M)* sequence. Doherty et al. [9] examined the stability and evolution of *tet(M)*-mediated resistance to tetracycline among members of different clonal lineages of *S. pneumoniae* and reported that the estimated nucleotide divergence between different *tet(M)* alleles ranged from 0.44% to 8%.

In our data, the *tet(M)* genes of oral streptococci consisted of regions similar in sequence and interspersed with regions that differed at nucleotide sites, revealing a mosaic structure. The mosaic structure of *tet(M)* genes has also been observed in other bacterial species. Oggioni et al. [10] analyzed the nucleotide sequences of *tet(M)* genes from a diverse range of bacteria. They compared the sequences of eight different *tet(M)* genes and detected a mosaic structure that could be traced to two distinct alleles. It appeared that the mosaic structure within *tet(M)* had evolved after acquisition of the gene by mobile genetic elements. The block structure of these genes provides evidence for the contribution of homologous recombination to the evolution and the heterogeneity of the *tet(M)* locus. The effect of these

mosaic structures on tetracycline resistance has not been formally addressed. It is known that streptococci carrying different *tet(M)* gene subtypes can confer different levels of tetracycline resistance [9]; however, this phenomenon was not observed in the oral streptococci used in our study.

Streptococci make up a large contingent of oral bacteria. It is assumed that they are potential sources of tetracycline-resistance genes for other bacteria, including the bacteria that cause periodontal disease. When the transposon containing a tetracycline-resistance gene moves between the chromosomes of bacteria in the oral cavity, transfer of tetracycline resistance may occur. However, further studies are necessary to reveal the possibility of transferring genes associated with tetracycline resistance between oral bacterial species.

The data presented in our study provide basic information about the transposition process associated with *tet(M)* in oral streptococci. The mosaic distribution of polymorphic sites suggests that highly localized recombination events may have occurred during the transposition process. However, further studies on larger samples of accumulated data over time are required to clarify the transfer mechanism associated with this gene. This will aid the acquisition of a more detailed understanding of the diversity of the *tet(M)* gene and the course of its spread.

Conflicts of Interest

The authors declare that they have no competing interests.

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