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Prevalence of tetracycline resistance genes in the oral cavity of Greek subjects

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Antibiotic resistance is a major public health issue, but limited data exist in the literature regarding its presence in the oral environment. The objective of the present cross-sectional study was to investigate the prevalence of the tetracycline resistance genes *tetM* and *tetQ* in the oral cavity of systemically healthy Greek subjects. After screening 649 subjects, the sample included 44 periodontally healthy, 36 gingivitis and 48 chronic periodontitis cases. Clinical parameters were assessed with an automated probe, samples were collected from tongue, first molars and pockets > 6 mm and analyzed with Polymerase Chain Reaction for *tetM* and *tetQ*. Findings have shown high prevalence in plaque and tongue samples (69.8-94.3% for *tetM* and 25.5-60.9% for *tetQ*). Deep pockets harbored these genes less frequently (50.0% for *tetM* and 16.7% for *tetQ*). Statistical analysis revealed that *tetM* was more frequently found in plaque samples from gingivitis cases and *tetQ* less frequently found in plaque samples from chronic periodontitis cases. The presence of these genes could not be correlated with previous intake of tetracyclines, suggesting other routes of transmission. The oral cavity of Greek subjects often harbor *tetM* and *tetQ*, even though tetracyclines were not frequently prescribed in this population.

Key words: tetracycline resistance, periodontal diseases, antimicrobial drug resistance, molecular biology, prokaryotic genes, PCR.

INTRODUCTION

The issue of antibiotic resistance is considered a major public health issue which has been recognized and widely investigated during the last decades (World Health Organization, 2012). Few relevant data exist in the literature regarding the presence of resistance in oral bacteria and the contribution of clinical dentistry in disseminating this phenomenon (Walker, 1996; Sweeney, 2004).

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It is well documented that several bacteria, including important pathogens, have developed resistance to various classes of antibiotics among which some are agents of choice to be administered for orofacial or periodontal infections. This property is frequently spread among bacteria by antibiotic resistance genes which can regulate several mechanisms, such as production of enzymes (which inactivate antibiotics), ribosomal protection proteins or the function of efflux pumps (which transport antimicrobial molecules outside the bacterial cell) (Chopra & Roberts, 2001).

It has also been shown that antibiotic resistance is directly correlated to their consumption and therefore to the policy of each country for antibiotic use (Van Winkelhoff *et al*., 2000). Antibiotic resistance rates in Greece are among the highest in Europe and a possible cause could be the widespread practice of consuming and dispensing antimicrobials, even without prescription (Plachouras *et al*., 2010; Miyakis *et al*., 2011).

Tetracyclines are among the antibiotics widely used in dentistry in the past and nowadays in periodontology in local delivery forms (in microspheres). They are bacteriostatic agents that block protein synthesis by connecting to the bacterial ribosomal subunit 30S. Both *tetM* and *tetQ* genes encode for ribosomal protection proteins, that bind to the bacterial ribosome and do not allow for the tetracycline molecule to connect and perform its blocking action. *tetM* is found mainly in Gram positive and *tetQ* in Gram-negative periopathogenic bacteria. Bacterial resistance to the tetracyclines is a very frequent phenomenon, limiting their effectiveness against possible pathogens. Until now, 38 different genes encoding for resistance mechanisms have been identified in several genera (Chopra & Roberts, 2001; Roberts, 2005). Despite this fact, the recognition of their anticollagenolytic properties, in addition to their antimicrobial ones, have reintroduced interest to this class of antibiotics by the medical and dental community, since they have been shown to be effective even at subantimicrobial doses for treatment of periodontal disease or arthritis (Stone *et al*., 2003; Kirkwood *et al*., 2007). The tetracyclines –especially doxycycline– have also been proven to be effective in malaria treatment and therefore are still an important and low-cost option for treatment of this widespread and life threatening disease (Dahl *et al*., 2006).

Preliminary data from Greek subjects indicated an abundance of resistance genes in both tongue and subgingival samples for the tetracyclines and b-lactamic antibiotics but not for metronidazole (Ioannidis *et al*., 2009). Since we are interested in finding possible differences in resistance genes in patients with various periodontal conditions, we decided to detect the presence of *tetM* and *tetQ* genes, as they are present in different types of bacteria (Gram-positive *versus* Gram-negative, respectively).

The purpose of the present study was to investigate the prevalence of the tetracycline resistance genes *tetM* and *tetQ* in the oral cavity of Greek subjects with various periodontal conditions.

MATERIALS AND METHODS

Study population

649 subjects attending the Clinic of Periodontology at 251 Air Force Hospital, Athens, Greece and the Clinic of the Department of Preventive Dentistry, Periodontology and Implant Biology, Dental School, Aristotle University of Thessaloniki, Greece, were screened in order to be enrolled in the study from September 2011 to November 2012.

To be included in the study, patients should fulfill the following criteria:

- Age > 30 years
- Absence of systemic diseases or medications known to affect periodontal tissues, infectious conditions (hepatitis, HIV) or pregnancy and lactation
- No periodontal treatment or antibiotic intake during the last six months
- One of the following three periodontal conditions were met:
  1. Periodontal Health (no periodontal pockets or clinical attachment loss > 3 mm and bleeding on probing < 10%)
  2. Gingivitis (no periodontal pockets > 4 mm or attachment loss > 3 mm and bleeding on probing > 20%, without radiographic bone loss)
  3. Moderate or Advanced Periodontitis (at least 6 periodontal pockets with probing depth > 5 mm and clinical attachment loss > 3 mm and radiographic bone loss).

The study was conducted according to the protocol outlined by the Research Committee, Aristotle University of Thessaloniki, Greece and approved by the Ethical Committee of the School of Dentistry (#120), in compliance with the ethical principles of the World Medical Association Declaration of Helsinki. All patients read and signed an appropriate informed consent document prior to the participation in the study.

Study design

The present cross-sectional study, included three groups as follows: a) periodontal health (n = 44), b) gingivitis (n = 36), and c) chronic periodontitis (n = 48). From the 649 patients initially screened, most were excluded because of the strict periodontal criteria and two did not agree to enter the study.

All participants received the following assessments and analysis:

Clinical parameters of periodontal disease

a. Probing Depth.
b. Recession.
c. Clinical Attachment Loss.
d. Bleeding on Probing.

Clinical recordings were performed at six points of all teeth present at the dentition (mesio-buccal, buccal, disto-buccal, mesio-lingual, lingual, distolingual). Recordings were performed by a calibrated examiner (GK) using an automated probe (Florida probe, Florida Probe Corporation, USA).

Clinical sampling and analysis

Two clinical samples were collected from each patient: a pooled subgingival plaque sample from the mesiobuccal surface of the four first molars (or premolars when molars were missing) taken with sterile Gracey curettes, and a sample collected from the dorsal surface of the tongue with a sterile straight surgical bone curette (Sklar Instruments, PA, USA) after applying three consecutive strokes. All samples were immediately placed in 200 μl of TE buffer (Tris HCL 10 mM, EDTA 1 mM, pH 7.5) and stored at –20°C, until assayed. In addition chronic periodontitis patients contributed with one more sample from their deepest periodontal pocket.

Polymerase Chain Reaction (PCR)

Analysis of samples was performed blindly (coded samples). All experiments were performed in the Department of Microbiology, School of Biology, Aristotle University of Thessaloniki, Greece.

First of all, PCR was performed for the detection of the 16S ribosomal RNA gene, in order to verify that the clinical samples contained identifiable bacterial DNA (Goncharoff *et al*., 1993). Samples were further analyzed by PCR for the presence of genes encoding resistance to tetracycline (*tetQ* and *tetM*). PCR conditions and primers are described in Table 1 (Goncharoff *et al*., 1993; Lacroix & Walker, 1995, 1996). The final volume of the reaction mixture for each PCR assay was 50 μl, containing 5 μl of DNA template, 200 μM of each deoxynucleotide triphosphate (dNTPs) (1 μl of 10 mM dNTPs mix solution), 5 μl of ×10 Standard Taq Reaction Buffer (10 mM Tris-HCl, 50 mM KClm 1.5 mM $MgCl_2$, pH 8.3), 0.5 μM of each primer (2.5 μl of 10 μM of each primer), 2.5 U (0.5 μl) Taq DNA polymerase (New England Biolabs Inc.) and distilled H_2O (33.5 μl). A Peltier Thermal Cycler (PTC-100, Peltier Thermal Cycler, MJ Research) was used for PCR.

For each set of samples analyzed by PCR a negative and a positive control were used. Sterile water for injection (Demo S.A. Pharmaceutical Industry), was used as negative control (replacing DNA template into the PCR reactions), and *tetM* positive clinical samples of *Staphylococcus aureus*, *tetQ* positive clinical samples of *Bacteroides fragilis* were used as positive controls (Ioannidis *et al*., 2009). The products of the DNA amplification were electrophoresed through a 1% agarose gel, stained with ethidium bromide, exposed under UV light and photographed. A 100 bp DNA ladder (New England Biolabs Inc.) was also loaded on agarose gel as a molecular weight standard. The amplified fragment sizes were 625 bp for 16S rRNA, 754 bp for *tetQ* and 397 bp for *tetM*. The elec-

TABLE 1. PCR conditions for 16S, *tetM* and *tetQ*

PCR conditions	*16S*	*tetM*	*tetQ*
Primers	*5′-CAG GAT TAG ATA CCC TGG TAG TCC ACG C-3′* and *5′- GAC GGG CGG TGT GTA CAA GGC CCG GGA ACG-3′* (Goncharoff *et al.*, 1993)	*5′-GAC ACG CCA GGA CAT ATG G-3′* and *5′-TGC TTT CCT CTT GTT CGA G-3′* (Lacroix & Walker, 1995)	*5′-GGC TTC TAC GAC ATC TAT TA- 3′* and *5-CAT CAA CAT TTA TCT CTC TG-3′* (Lacroix & Walker, 1996)
Initial denaturation	95°C for 5 min	95°C for 5 min	95°C for 5 min
Denaturation	94°C for 1 min	94°C for 30 sec	94°C for 30 sec
Annealing	55°C for 1 min	55°C for 1 min	50°C for 1 min
Extension	72°C for 34 sec	72°C for 90 sec	72°C for 160 sec
Step 2 to 4	34 cycles	37 cycles	37 cycles
Final extension	72°C for 3 min	72°C for 10 min	

trophoresis for each PCR product was carried out twice in order to test the reproducibility of the method.

Questionnaire

All subjects completed a questionnaire by an interview from one of the authors (DS) regarding the following: smoking, frequency of antibiotic intake for medical and dental reasons, class of antibiotics used 6-12 months before the interview and a period dating until 5 years ago, whether they have ever obtained antibiotics without prescription, whether they have antibiotics available at home and whether they are aware about the activity of antibiotics and the phenomenon of antibiotic resistance. In order to avoid wrong reporting by participants, the class of antibiotics that they have used was recorded based on their personal National Health Record.

Statistical analysis

The statistical analysis of the data was carried out with the statistical package SPSS, (SPSS 19.0 version, SPSS Inc., Chicago, IL, USA). The experiment was set to have at least 80% power to detect changes of 25% with a significance level (alpha) of 0.05 (two-tailed). A pilot study provided the expected values of the primary outcome variable (Ioannidis *et al*., 2009).

For clinical parameters, indicators of Descriptive Statistics were used, such as mean and standard deviation for each group, with the patient as the observational unit. Differences in clinical parameters, and age were sought by applying the Kruskal-Wallis test. Differences regarding the distribution of participants according to gender and smoking were sought by applying the z-test for proportions adjusted with Bonferroni corrections.

Differences in prevalence of investigated genes and answers regarding the questionnaire were also sought among periodontally healthy, gingivitis and periodontitis subjects and within each group both for tongue and plaque samples, by applying the *z*-test for proportions adjusted with Bonferroni corrections.

Possible correlations between the presence of *tetM* and *tetQ*, antibiotic intake for medical or dental reasons during the last five years, and intake of tetracyclines during the last 6-12 months were sought by estimating the non-parametric Spearman correlation coefficient ($a = 0.05$).

RESULTS

Demographic data for participants and clinical parameters of the subjects are presented in Table 2. All groups were age matched and clinical parameters of periodontal disease were statistically significantly different among groups (Kruskal-Wallis test, $p < 0.05$). Chronic periodontitis cases were more frequently in male and smokers (z-test for proportions adjusted with Bonferroni corrections, $p < 0.05$).

The distribution of resistance genes among groups with different periodontal conditions is presented in Table 3. In plaque samples *tetM* genes were more frequently found in gingivitis cases and *tetQ* genes less frequently found in periodontitis cases compared to the other groups (z-test for proportions adjusted with Bonferroni corrections, $p < 0.05$). Within group analysis revealed that *tetM* genes were found more frequently in plaque samples from gingivitis cases. Within chronic periodontitis patients, tongue samples harbored statistically significantly more frequently *tetQ* genes compared to plaque or deep pockets and less frequently *tetM* genes in deep pockets (z-test for propor-

TABLE 2. Demographic data and clinical parameters

Diagnosis	Total	Age (mean ± SD)	Male (%)	Smokers (%)	Probing Depth (mm) (mean ± SD)	Recession (mean ± SD)	Clinical Attachment Loss (mm) (mean ± SD)	Bleeding on Probing (%)
Periodontal Health	44	46.4 ± 7.9	52 (a)	16 (a)	1.51 ± 0.2 (a)	0.03 ± 0.06 (a)	1.54 ± 0.22 (a)	6 ± 5 (a,b)
Gingivitis	36	49 ± 9.2	53 (b)	13 (b)	1.80 ± 0.27 (b)	0.03 ± 0.07 (b)	1.86 ± 0.30 (b)	33 ± 16 (a)
Chronic Periodontitis	48	49.5 ± 9.5	79 (a,b)	42 (a,b)	3.00 ± 0.55 (a,b)	0.32 ± 0.43 (a,b)	3.36 ± 0.69 (a,b)	31 ± 22 (b)

Values in the same column that share the same letter display statistically significant differences (Kruskal-Wallis test and z-test for proportions adjusted with Bonferroni corrections, $p < 0.05$)

TABLE 3. Prevalence of *tetM* and *tetQ* in plaque and tongue samples according to periodontal conditions

	tetM			*tetQ*		
	Plaque % (positive/all)	Tongue % (positive/all)	Deep pockets % (positive/all)	Plaque % (positive/all)	Tongue % (positive/all)	Deep pockets % (positive/all)
Periodontal Health	71.4 (30/42)	73.8 (31/42)		41.9 (18/43)	59.1 (26/44)	
Gingivitis	**94.3** (33/35) (a)	79.4 (27/34) (a)		42.9 (15/35)	54.3 (19/35)	
Chronic Periodontitis	69.8 (30/43) (a)	76.2 (32/42) (b)	50 (11/22) (a,b)	**25.5** (12/47) (a)	60.9 (28/46) (a,b)	16.7 (5/30) (b)

Statistically significant differences among groups are indicated by bold lettering
Statistically significant differences within the same group are indicated by the same letter (z-test for proportions with Bonferroni corrections, $p < 0.05$)

tions adjusted with Bonferroni corrections, $p < 0.05$), In total, *tetM* genes were present in 77.5% (93/120) of plaque samples and in 76.3% (90/118) of tongue samples, whereas *tetQ* genes were present in 36% (45/125) of plaque samples and in 58.4% (73/125) of tongue samples.

Results regarding the questionnaire are presented in Table 4. According to statistical analysis of data, the following differences were observed among groups: chronic periodontitis cases were less aware of antibiotic resistance and also have been administered tetracyclines less frequently compared to periodontally healthy individuals (z-test for proportions adjusted with Bonferroni corrections, $p < 0.05$). No differences were observed among groups regarding the frequence of antibiotic intake 6-12 months before the interview, and b-lactamic antibiotics was the only class of antibiotics that they had been administered. During the last five years, according to their medical records, participants in all three groups have been administered b-

TABLE 4. Consumption, attitude and knowledge about antibiotics of the subject sample

		Periodontal Health	Gingivitis	Chronic Periodontitis
Antibiotic use for medical reasons during the last 5 years (%)	*1-2 times*	37	47.2	43.8
	3-4 times	28.3	27.8	29.2
	> 5 times	34.8	22.2	16.7
Antibiotic use for dental reasons during the last 5 years (%)	*1-2 times*	52.2	38.9	39.6
	3-4 times	8.7	8.3	8.3
	> 5 times		5.6	6.3
Available at home (%)	*Yes*	28.3	33.3	16.7
Without Prescription (%)	*Yes*	2.2	0	0
	By a pharmacist	13	30.6	16.7
Antibiotic intake last 6-12 months (%)	*Yes (b-lactams)*	30.4	14.3	29.2
– b-lactams (%)	*Yes*	78.2 (a,b)	72.2 (a,b)	70.8 (a,b)
– tetracyclines (%)	*Yes*	**27.5** (a)	17.1 (a)	**6.8** (a)
– imidazoles (%)	*Yes*	15.4 (b)	22.9 (b)	13.7 (b)
Knowledge about antibiotic activity (%)	*Effective against bacteria*	47.8	48.6	25.5
	Effective against viruses	6.5	20	10.4
	Both	41.3	22.9	50
Knowledge about antimicrobial resistance (%)	*Yes*	**47.8**	22.9	**20.8**

Statistically significant differences among groups are indicated by bold lettering. Statistically significant differences within each group are indicated by the same letter (z-test for proportions adjusted with Bonferroni corrections, $p < 0.05$)

lactams statistically significantly more frequently than other classes of antibiotics (z-test for proportions adjusted with Bonferroni corrections, $p < 0.05$).

When seeking for correlations between the presence of *tetM* and *tetQ* and previous antibiotic intake, no correlations were observed between the presence of these genes and any antibiotic use for medical or dental reasons during the last 5 years, or intake of tetracyclines during the last 6-12 months (Spearman's correlation coefficient greater than 0.05).

DISCUSSION

The present study investigated the presence of the bacterial genes *tetM* and *tetQ* which encode resistance to the tetracyclines in the oral cavity of Greek subjects with various periodontal conditions. Results have shown high rate of resistance to the tetracyclines in the oral microbiota, not connected to the use of tetracyclines for medical or dental reasons, as well as with the periodontal status of participants. The tongue appears to be an important niche of *tetM* and *tetQ* and therefore it seems reasonable to suggest that daily brushing of the tongue could assist in constraining the dissemination of these genes in other habitats of these subjects.

In the current study, tetracycline was selected mainly because this family of antibiotics has been widely used to treat periodontal diseases especially after the observation that doxycycline has been used to effectively treat localized aggressive (formerly localised juvenile) periodontitis and data have shown that during the 1980s tetracyclines were the favorite class of antibiotics of periodontists (Slots & Rosling, 1983; Slots & Rams, 1990). It is also known that tetracyclines (e.g. minocycline) are currently frequently incorporated in local delivery systems for treating periodontal disease.

Resistance to the tetracyclines has been observed as early as 1953, a few years after their introduction for therapeutic use and has been directly correlated to their extensive applications (Roberts, 2005). The wide spectrum of activity combined to the low cost of these antibiotics have established their use not only as antimicrobials for human and veterinary use, but as well as animal growth promoters, in agriculture, aquaculture and treatment of honeybees (World Health Organization, 2012).

Greece, has one of the highest antibiotic consumption among the European countries (39.4 Defined Daily Doses or DDDs per 1000 inhabitants), although tetracyclines according to the 2013 European Centre for Disease Prevention and Control (ECDC) relevant report, do not appear to be as frequently prescribed (2.3 DDDs) as other classes of antimicrobials such as b-lactams (12.9 DDDs) (ECDC, 2011). Findings of the present study are in agreement with this report since participants in the present study reported lower use of tetracyclines and imidazoles compared to b-lactamic antibiotics. In addition, 6-12 months before the interview, subjects had been administered antibiotics belonging only to the b-lactams.

A previous study concerning British children who have never been administered tetracyclines before, has also shown high frequencies of *tetM* and *tetW* in the oral cavity and other sources of these genes, unrelated to antibiotic intake, are therefore suggested, in agreement with findings from the present study (Lancaster *et al*., 2003). According to the European Medicines Agency 2012 report regarding sales of veterinary antimicrobial agents, tetracyclines are the most widely used antibiotics for livestock and poultry, for treatment of infections or prophylaxis (European Medicines Agency, 2012). In the European Community, tetracyclines are banned for non-antimicrobial use in animals, although according to the World Health Organization, legislative restrictions are unfortunately not always applied (World Health Organization, 2012). The United States Federal Drug Administration also recommends the judicious use of medically important antimicrobial drugs in food producing animals, although the agency provides guidance and does not establish legally enforceable responsibilities (World Health Organization, 2010).

These resistance genes can arrive to humans through for example direct contact, inadequately cooked meat or dairy and meat products. Most alarmingly, it has been shown that tetracycline resistance genes can be disseminated in the soil environment and groundwater from animal farms and therefore potentially through agriculture to the food chain, while their presence has also been shown in all aspects of salmon fish farming including marine sediments (Chee-Sanford *et al*., 2001; Miranda *et al*., 2003; Peak *et al*., 2007).

The prevalence of two such genes, *tetM* and *tetQ* in the periodontal environment has been investigated, and the literature indicates that 81-84% of bacteria carrying *tetM* are not periodontal pathogens, while *tetQ* is carried by periodontal pathogens, mainly *Prevotella* and *Bacteroides* spp (Olsvik *et al*., 1995; Lacroix & Walker, 1996; Manch-Citron *et al*., 2000; Chung *et al*., 2002).

According to the design of the current study, the bacterial genera carrying these genes were not identified, since total DNA was processed for determination of *tetM* and *tetQ* and cultural techniques for identification of bacterial species carrying these genes were not applied. However, it has been previously shown that these genetic determinants of resistance, since they are encoded in mobile elements can be readily transferred among bacterial species in the oral environment and certainly the biofilm structure of supragingival and subgingival plaque is known to favor this spread (Hausner & Wuertz, 1999; Roberts *et al*., 1999; Warburton *et al*., 2007). Therefore, the high prevalence of *tetM* and *tetQ* as reported in the present study, is indicative of high propability for transfer of resistance to the tetracyclines to several unrelated members of the oral microbiota, including periodontal pathogens. Nowadays tetracyclines are not being used very frequently for human therapeutic use, but since they are being used so extensively for veterinarian therapeutic and non therapeutic uses (e.g. growth promoters), in agriculture (to treat bacterial diseases that affect apples, peaches, pears) and aquaculture, it is interesting to observe how bacteria that colonise and affect humans respond to the immense challenge they are being subjected to in the enviroment (Martínez, 2008). This information could prove vital in decision making of how we should use other classes of antibiotics in anything else than human treatment, although we should be aware that the presence of a given resistance gene does not guarantee that it will be expressed. For this reason, function expression analyses such as proteomic or transcriptomic methodology should be applied. Findings of the present study cannot challenge the effectiveness of these antibiotics in treating periodontal disease, since the bacterial genera –sources of these genes have not been identified, but show the possible impact of high-scale use of antibiotics to antimicrobial resistance in the oral environment.

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