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# Characterization of Antibiotic Resistance Determinants in Oral Biofilms

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**Oral biofilms contain numerous antibiotic resistance determinants that can be transferred within or outside of the oral cavity. The aim of this study was to evaluate the prevalence and the relative level of antibiotic resistance determinants from oral biofilms. Oral biofilm samples that were collected from healthy subjects and periodontitis patients were subjected to qualitative and quantitative analyses for selected antibiotic resistance determinants using PCR. The prevalence of *tet(Q)*, *tet(M)*, *cfxA*, and $bla_{TEM}$ was very high both in the patient and the healthy subject group, with a tendency toward higher values in the patient group, with the exception of *erm(F)*, which was more prevalent in the healthy group. The two extended spectrum β-lactam (ESBL) resistance determinants $bla_{SHV}$ and $bla_{TEM}$ showed a dramatic difference, as $bla_{TEM}$ was present in all of the samples and $bla_{SHV}$ was not found at all. The *aacA-aphD*, *vanA*, and *mecA* genes were rarely detected, suggesting that they are not common in oral bacteria. A quantitative PCR analysis showed that the relative amount of resistance determinants present in oral biofilms of the patient group was much greater than that of the healthy group, exhibiting 17-, 13-, 145-, and 3-fold increases for *tet(Q)*, *tet(M)*, *erm(F)*, and *cfxA*, respectively. The results of this study suggest that the oral antibiotic resistome is more diverse and abundant in periodontitis patients than in healthy subjects, suggesting that there is a difference in the diversity and distribution of antibiotic resistance in oral biofilms associated with health and disease.**

***Keywords*:** antibiotic resistance, resistance determinants, periodontitis, oral biofilm, polymerase chain reaction

Dental plaque, as a biofilm consisting of more than 700 different bacterial species (Socransky and Haffajee, 1994; Kroes *et al.*, 1999; Paster *et al.*, 2001; Aas *et al.*, 2005), is considered to act as a dynamic and complex microbial community, interacting with the host (Marsh, 2004). In general, the composition of the indigenous flora, along with host defense factors, is consistent with a state of periodontal health. Epidemiological data, both longitudinal and cross-sectional, strongly suggest that a population shift toward certain gram-negative anaerobic species in the dental plaque biofim is responsible for the initiation and progression of periodontal diseases (Moore and Moore, 1994; Socransky and Haffajee, 1994; Kroes *et al*., 1999). Therefore, understanding the microbial pathogenesis of periodontal disease requires a detailed understanding of the dynamic interactions between the host and Gram-negative pathogenic bacterial species in the periodontal environment.

Widespread use of antibiotics in humans, animals, and agriculture has resulted in a global problem of bacterial resistance due to the selection and dissemination of bacteria harboring antibiotic resistance (Levy, 1997). There are a number of resistant organisms in both hospitals and the community that defy conventional therapy, since they have become resistant to not one but a variety of antibiotics. To devise rational approaches to suppress the emergence and spread of antibiotic resistance, we need to better understand the mechanisms by which the resistance determinants develop, disseminate and persist among bacterial populations. Research on the diversity and distribution of the resistance determinants has been mainly focused on the cultivable pathogenic bacteria (Mazel and Davies, 1999), as almost all known antibiotic resistance genes have been identified and characterized from cultured microorganisms. Recent data, however, have demonstrated that numerous bacterial species can not be cultivated by standard techniques. In fact, it is estimated that more than 99% of the micro-organisms observed in nature may fall into this category (Amann *et al.*, 1995). Several known and novel antibiotic resistance determinants have been identified and discovered from oral microflora using molecular techniques adopting a metagenomic approach, instead of using conventional culture techniques. The genes encoding tetracycline resistance, including *tet*(*M*), *tet*(*O*), *tet*(*Q*), *tet*(*W*), *tet*37, and *tet*(*A*) and erythromycin resistance, including *erm*(*B*), *erm*(*V*), and *erm*(*E*), were identified from oral metagenomic libraries (Diaz-Torres *et al.*, 2003, 2006; Seville *et al.*, 2009). These results suggest that oral microflora are a reservoir for antibiotic resistance determinants and that uncultivable bacteria could play a major role in the acquisition and spread of antibiotic resistance (Sommer *et al.*, 2009).

The major etiology of oral infectious diseases, including dental caries and periodontal disease, is oral biofilms. There

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exist more than 700 different bacterial species in the oral cavity (Socransky and Haffajee, 1994; Kroes *et al.*, 1999; Paster *et al.*, 2006), and oral biofilms are considered to act as a dynamic and complex microbial community interacting with the host (Marsh, 2005). These interactions mediate tissue destruction, ultimately leading to dental caries and periodontal disease. Among oral biofilm microorganisms, most are not considered to be pathogenic; rather, only a small fraction of the whole population is responsible for initiating and maintaining disease processes. Numerous studies have strongly suggested that the emergence of one or more specific bacterial population groups, driven by environmental changes in the biofilm community, are responsible for causing oral infectious diseases (Marsh, 2003). It has also been established that only about half of over 700 oral bacterial species can be cultivated (Kroes *et al.*, 1999; Paster *et al.*, 2006). Furthermore, recent epidemiological studies have indicated that antibiotic misuse and overuse affect not only the pathogens but also the commensals, and commensal bacteria could serve as reservoirs of antibiotic resistance determinants for pathogens (Diaz-Torres *et al.*, 2006; Roberts and Mullany, 2006). The oral biofilm appears to provide an excellent breeding ground for the exchange of resistance genes, since its major colonizers are commensals. Experimental evidence has suggested that horizontal gene transfer of resistance determinants can occur in the oral biofilm (Roberts *et al.*, 1999, 2001; Mercer *et al.*, 2001; Wang *et al.*, 2002). This strongly suggests that exchange of mobile genetic elements between commensals and pathogenic bacteria can contribute to the emergence of drug resistance in the oral cavity. Oral microflora do, in fact, exhibit numerous resistance mechanisms, presumably due to the complex microbial interactions and the genetic fluidity in oral biofilms (Roberts, 1998).

It is therefore important to reveal the true potential for antibiotic resistance determinants present in the oral cavity–the oral resistome (D'Costa *et al.*, 2006)–associated with health and disease in order for us to better understand the mechanisms by which antimicrobial resistance develops, spreads and maintains in oral biofilms. The purpose of this study was to evaluate the prevalence and the relative amount of antibiotic resistance determinants from oral biofilms, adopting a metagenomic approach. This approach enables investigators to access antibiotic resistance determinants from a complex mixture of microorganisms extracted directly from their natural niches, including those that can not be cultivated in the laboratory (Handelsman, 2004). Genotypic profiles of the diversity and distribution of selected antibiotic resistance determinants present in oral biofilms of healthy and periodontitis subjects were assessed using PCR. In addition, several antibiotic resistance determinants, *tet*(Q), *tet*(M), *erm*(F), and *cfxA*, were also quantitatively analyzed.

## Materials and Methods

### Subjects and sample collection

Oral biofilm samples were obtained from two groups of study subjects, aged 18-65: the healthy group (N=20) and the periodontitis group (N=20). The study protocol was approved by the Columbia University Medical Center Institutional Review Board and written informed consent was obtained from study subjects. All subjects were selected from patients seeking treatments at the Postgraduate Clinic, Columbia University College of Dental Medicine, New York, New York. Subjects in the healthy group had not received antibiotics in the past 4 months and did not have periodontal disease. Eligible patients in the periodontitis group (i) had no past history of periodontal therapy other than prophylaxis provided by the referring general dentist, (ii) received no systemic antibiotics or anti-inflammatory drugs for at least 4 months, (iii) had a minimum of 4 teeth with radiographic bone loss, (iv) did not suffer from diabetes mellitus, and (v) did not suffer from any of the systemic conditions or genetic disorders affecting periodontal tissues. In the healthy group, dental plaque samples were collected from gingival pockets at mesial surfaces of the upper and lower first molars with a sterile Gracey curette using one stroke from the bottom of the pocket to the coronal portion of the pocket. In subjects with chronic periodontitis, samples were collected from periodontal pockets

**Table 1.** Target resistance determinants and PCR primers used in the PCR assay

| Resistance determinants | Expected amplicon size (bp) | Primer sequence (5′→3′) | GenBank accession no. |
|---|---|---|---|
| *tet*(*Q*) | 656 | CTG TCC CTA ACG GTA AGG<br>TTA TAC TTC CTC CGG CAT CGG T | X58717, bp 1940-1957<br>X58717, bp 1301-1322 |
| *tet*(*M*) | 158 | AGT GGA GCG ATT ACA GAA<br>CAT ATG TCC TGG CGT GTC TA | X56353, bp 301-318<br>X56353, bp 459-440 |
| *ermF* | 465 | CGG GTC AGC ACT TTA CTA TTG<br>GGA CCT ACC TCA TAG ACA AG | AF203972, bp 4350-4370<br>AF203972, bp 3905-3924 |
| *aacA-aphD* | 227 | TAA TCC AAG AGC AAT AAG GGC<br>GCC ACA CTA TCA TAA CCA CTA' | M18086, bp 2144-2164<br>M18086, bp 2371-2350 |
| *cfxA* | 931 | GCA AGT GCA GTT TAA GAT T<br>TTA GTT TGC ATT TTC ATC | AM940017, bp 914-932<br>AM940017, bp 1-18 |
| $bla_{SHV}$ | 864 | GGT TAT GCG TTA TAT TCG CC<br>TTA GCG TTG CCA GTG CTC | AB374988, bp 5-24<br>AB374988, bp 852-869 |
| $bla_{TEM}$ | 867 | ATG AGT ATT CAA CAT TTT CG<br>CTG ACA GTT ACC AAT GCT TA | DQ916413.1, bp 7257-7271<br>DQ916413.1, bp 8105-8124 |
| *vanA* | 617 | GGG AAA ACG ACA ATT GC<br>AAA GGT CTG CGG GAA CG | EF206286, bp 110-126<br>EF206286, bp 741-727 |
| *mecA* | 532 | AAA ATC GAT GGT AAA GGT TGG C<br>AGT TCT GCA GTA CCG GAT TTG C | Y00688, bp 1282-1303<br>Y00688, bp 1814-1793 |

**Table 2.** PCR Primers used in the qPCR assay

| Resistance determinants | Primer sequence (5′→3′) | GenBank accession no. |
|---|---|---|
| *tet*(*Q*) | CAA GGT GAT ATC CGC TCT GA | X58717, bp 1665-1684 |
| | GGA AAA TCG TTC TTC CAG CA | X58717, bp 1812-1793 |
| *tet*(*M*) | ACA CGC CAG GAC ATA TGG AT | X56353, bp 443-462 |
| | GGG AAT CCC CAT TTT CCT AA | X56353, bp 588-569 |
| *ermF* | TAG ATA TTG GGG CAG GCA AG | AF203972, bp 4270-4289 |
| | CAC CTG TAA GAA GTT ACT AAT | AF203972, bp 4420-4402 |
| *cfxA* | TGA CAG TGA GAG ATT TGC TGC | AM940017, bp 356-376 |
| | GGT CAG CCG ACA TTT CCT CTT | AM940017, bp 526-506 |

(≥ 4 mm) at the mesial surfaces of the upper and lower first molars with a sterile Gracey curette as described above. All biofilm samples were stored at -20°C until use.

### Genomic DNA purification and preparation

Oral biofilm samples were washed three times with PBS at 8,000×g for 10 min at 4°C and bacterial pellets were acquired. Genomic DNA was extracted from the pellets using the Wizard Genomic DNA Purification kit (Promega, USA) according to the manufacturer's instruction. DNA concentration was measured with a UV spectrophotometer.

### Detection and characterization of antibiotic resistance determinants

Fifty nanograms of pooled genomic DNA was used for an end-point polymerase chain reaction (PCR) analysis for detection of selected antibiotic resistance determinants. PCR primers specific for *tet*(Q), *tet*(M), *erm*(F), *aacA-aphD*, *cfxA*, $bla_{SHV}$, $bla_{TEM}$, *vanA*, and *mecA* were designed based upon the DNA sequences available from GenBank (Table 1). These resistance determinants, encoding resistance to tetracycline [*tet*(*Q*), *tet*(*M*)], erythromycin (*ermF*), aminoglycoside (*aacA-aphD*), and β-lactam (*cfxA*, $bla_{SHV}$, $bla_{TEM}$), were selected since they are frequently detected from oral bacteria and are clinically relevant [*tet*(*Q*), *tet*(*M*), *cfxA*, $bla_{SHV}$, $bla_{TEM}$], as tetracycline and amoxicillin are used for treating certain types of periodontal disease (Van Winkelhoff *et al.*, 1996). PCR was carried out with *Taq* DNA polymerase (Promega) in an MJ Mini Personal Thermal Cycler (BioRad, USA) according to the methods described previously (Yoo *et al.*, 2007). Presence of antibiotic resistance determinants was ascertained by running 0.2 μg of PCR product on a 1% agarose gel and comparing the molecular size of the PCR products with the expected amplicon size (Table 1). When necessary, each DNA band was excised from the gel, eluted using the QIAquick Gel Extraction kit (QIAGEN, USA) and subjected to direct DNA sequencing at the Protein Core Facility, Columbia University Medical Center, New York, New York, using Applied Biosystems Genetic Analyzers with BigDye terminator (Sanger) chemistry. In the direct DNA sequencing procedure, the PCR primers used for end-point PCR (Table 1) were used as sequencing primers. Identified gene sequences were analyzed with Vector NTI Advance 10 software (Invitrogen, USA) and submitted to a comparative analysis against the publicly available DNA (NCBI server, http://www.ncbi.nlm.nih.gov/) and protein (ExPASy Molecular Biology server, http://expasy.cbr.nrc.ca/) databases to determine their identity and the degree of similarity to other known resistance determinants.

### Quantitative real-time PCR (qPCR)

The level of antibiotic resistance determinants was analyzed by quantitative real-time PCR analysis (qPCR). Several antibiotic resistance determinants, *tet*(*Q*), *tet*(*M*), *cfxA*, and *erm*F, were selected after confirming their presence from the results of an end-point PCR. The amplified PCR products of these genes were directly isolated from the gel slice after electrophoresis by using the QIAquicl Gel Extraction kit (QIAGEN). The optical density (260 and 280 nm absorbance) was measured to estimate the amount of the purified PCR products. The purified PCR products were standardized as described previously (Maeda *et al.*, 2003) and used as controls in the qPCR assay. The real-time PCR reaction was carried out using a QuantiTect SYBR Green PCR kit (QIAGEN) in quadruplicate with Mx3000P (Stratagene, USA). The primers used in the reaction are listed in Table 2. The amplification parameters used were: 1 cycle of 95°C for 15 min; 40 cycles of 95°C for 30 sec, 57°C for 30 sec, 72°C for 25 sec; and 79°C for 5 min. Data analysis was carried out by MxPro QPCR Software (Stratagene), and the relative amount of antibiotic resistance determinants present in biofilm samples was calculated by comparing the threshold cycle number differences to a standard curve, as described previously (Livak and Schmittgen, 2001).

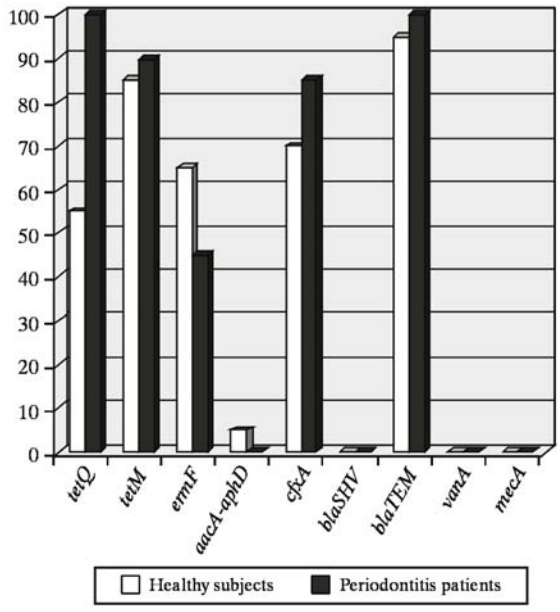


**Fig. 1.** Prevalence (%) of antibiotic resistance determinants in oral biofilms from periodontitis patients (N=20) and healthy subjects (N=20). Molecular detection of the antibiotic resistance genes was performed using an end-point PCR. The subjects harboring these genes are shown as percentage. A comparison is made between the patient and healthy groups.

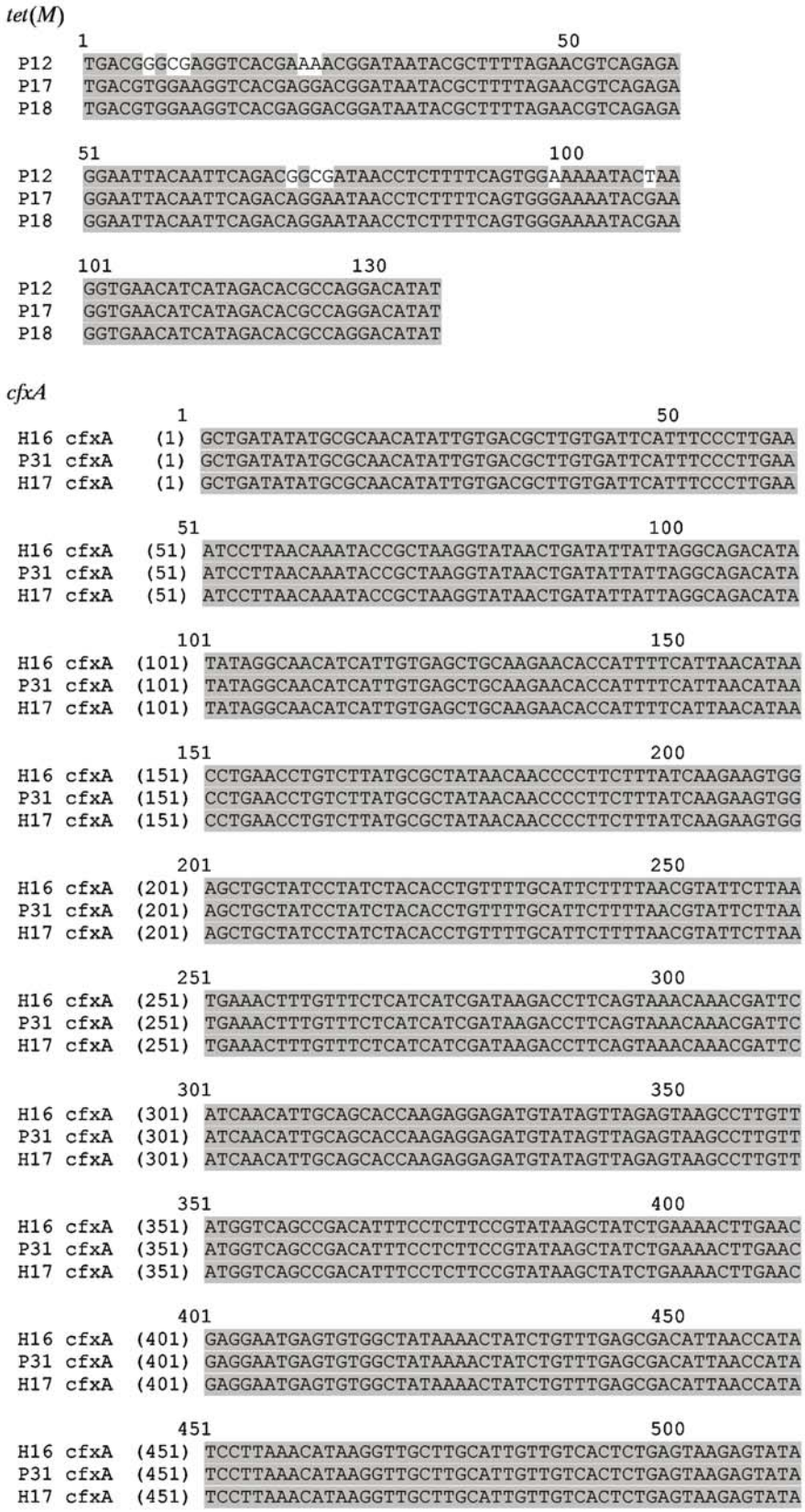


**Fig. 2.** Comparison of DNA sequences of selected resistance determinants amplified by PCR. DNA sequences from the resistance determinants ere obtained after direct sequencing of the PCR products obtained from selected subjects.

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                501                                             550
H16 cfxA  (501) ACGCAGCAAATCTCTCACTGTCAATGATATGACTGGCCCTGAATAATCTT
P31 cfxA  (501) ACGCAGCAAATCTCTCACTGTCAATGATATGACTGGCCCTGAATAATCTT
H17 cfxA  (501) ACGCAGCAAATCTCTCACTGTCAATGATATGACTGGCCCTGAATAATCTT

                551                                             600
H16 cfxA  (551) TCAGCATAGGACTCCAAGTCTTTGGGTCAAGTTTATCCCTATTTATATTT
P31 cfxA  (551) TCAGCATAGGACTCCAAGTCTTTGGGTCAAGTTTATCCCTATTTATATTT
H17 cfxA  (551) TCAGCATAGGACTCCAAGTCTTTGGGTCAAGTTTATCCCTATTTATATTT

                601                                             650
H16 cfxA  (601) ACTAAGGTATCAAGTGAAATTCCTTTATTGTCAAAGTCATTACAAAGAGC
P31 cfxA  (601) ACTAAGGTATCAAGTGAAATTCCTTTATTGTCAAAGTCATTACAAAGAGC
H17 cfxA  (601) ACTAAGGTATCAAGTGAAATTCCTTTATTGTCAAAGTCATTACAAAGAGC

                651                                             700
H16 cfxA  (651) TAATGCCTGATGAACCTTAAACACACTCATCATAGGATAAACACTCTTAT
P31 cfxA  (651) TAATGCCTGATGAACCTTAAACACACTCATCATAGGATAAACACTCTTAT
H17 cfxA  (651) TAATGCCTGATGAACCTTAAACACACTCATCATAGGATAAACACTCTTAT

                701                                             750
H16 cfxA  (701) TATTGACCTTAACCGTATCTCTGTTATTAACAATA
P31 cfxA  (701) TATTGACCTTAACCGTATCTCTGTTATTAACAATA
H17 cfxA  (701) TATTGACCTTAACCGTATCTCTGTTATTAACAATA
```

**Fig. 2.** Continued

### Statistical analysis

A non-parametric statistical method, Mann-Whitney U test, was used for all statistical analyses, as the assumptions of the t-test were not met. The significance level of all statistical analyses was set at 0.05 and all analyses were performed with SPSS statistical software, version 16 (SPSS Inc, USA).

## Results

### Molecular detection of antibiotic resistance determinants

The prevalence of *tet*(*Q*), *tet*(*M*), *cfxA*, and $bla_{TEM}$ was higher than 50% in both the patient and the healthy subject groups, with a tendency toward higher values in the patient group (Fig. 1). The *tet(Q)* gene was present in all patient samples, while its prevalence in the healthy group was just over 50%. For *tet(M)*, both groups exhibited almost identical prevalence. The *cfxA* gene was also more prevalent in the patient group. It is interesting to observe that *ermF* was found in the healthy group, suggesting that *ermF* is found in health-associated oral biofilms. The two extended spectrum β-lactam (ESBL) resistance determinants, $bla_{SHV}$ and $bla_{TEM}$, showed a dramatic difference, as $bla_{TEM}$ was present in all of the samples and $bla_{SHV}$ was not found at all. The *aacA-aphD*, *vanA*, and *mecA* genes were rarely detected, suggesting that they are not found in oral bacteria.

### Characterization of identified antibiotic resistance determinants

After resistance determinants were detected by PCR, the PCR products were randomly selected and isolated, and their DNA sequences were determined to check if the PCR amplicons were indeed targeted resistance genes. The results showed that the sequences of amplified PCR products were virtually identical to those of target genes (Fig. 2). When *cfxA* was sequenced and compared among two healthy subjects (H16 and H17) and one patient (P21), it was found that these *cfxA* genes were identical and the sequences matched perfectly to those of the *P. intermedia* class A β-lactamase precursor gene

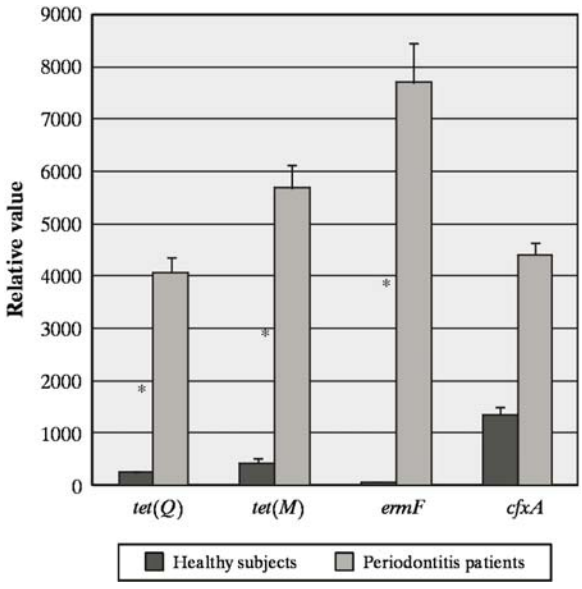


**Fig. 3.** Determination of relative values of antibiotic resistance determinants present in biofilms from healthy subjects and periodontitis patients. Relative amounts of *tet*(*Q*), *tet*(*M*), *ermF*, and *cfxA* were calculated (see text for detail) using the average from a quadruplicate run of qPCR analysis. The results indicated that there were significant statistical differences between healthy subjects and periodontitis patients in the levels of *tet*(*Q*), *tet*(*M*), and *ermF* ($p \leq 0.001$, *denoted as**), suggesting that these antibiotic resistance genes are more abundant in periodontitis patients, as compared to healthy subjects.

(*cfxA2*) [GenBank accession no. AY753592.1]. The sequences of *tet(M)* were also virtually identical among three patients, with a discrepancy of a few base pairs. In addition, it was found that the sequences of *ermF* and *aacA-aphD* were identical to those in the databases (data not shown).

### Quantitative analysis of antibiotic resistance determinants

The results of the quantitative analysis are shown in Fig. 3. Our findings indicate that there were significant differences in *tet*(*Q*), *tet*(*M*), and *ermF* levels between healthy subjects and patients ($p \leq 0.001$). It was found that relative amounts of *tet*(*Q*) and *tet*(*M*) in patients were about 17- and 13-fold greater than in healthy individuals. Interestingly, the amount of *ermF* present in samples from the periodontitis group was 145-fold higher than in the healthy group, although *ermF* was less prevalent in the periodontitis group than the healthy group. However, we have insufficient evidence to conclude that the patient group had higher *cfxA* levels than healthy subjects ($p = 0.095$), as the relative difference in the two groups was only about 3-fold, which was smaller than other antibiotic resistance genes. These results suggest that there is a difference in the level of resistance determinants between oral biofilms associated with health and disease.

## Discussion

Oral biofilms provide a readily available model system for research on bacterial antimicrobial resistance determinants, since there exist more than 700 different, mostly commensal, bacterial species in the oral cavity. In this study, we tried to detect resistance determinants in oral bacteria and characterize their occurrence and distribution by a metagenomic approach in order to help understand how antibiotic resistance is maintained within oral biofilms. Major advantages of a nucleic acid-based molecular detection system for antimicrobial resistance include a rapid and sensitive detection of the presence of resistance genes and accessibility to uncultivable microflora (Fluit *et al.*, 2001). Therefore, to circumvent limitations of phenotypic characterization of antibiotic resistance profiles in oral bacteria, including uncultivable bacteria, genotypic profiles of resistant determinants were assessed. Several resistance determinants encoding resistance to tetracycline [*tet*(*Q*), *tet*(*M*)], erythromycin (*ermF*), aminoglycoside (*aacA-aphD*), and β-lactam (*cfxA*, $bla_{SHV}$, $bla_{TEM}$) were selected, since they are frequently detected from oral bacteria and are clinically relevant [*tet*(*Q*), *tet*(*M*), *cfxA*, $bla_{SHV}$, $bla_{TEM}$], as tetracycline and amoxicillin are used for treating certain types of periodontal disease (Van Winkelhoff *et al.*, 1996). Although not directly relevant to oral bacteria, two resistance determinants, *vanA* and *mecA*, encoding resistance to vancomycin and methicillin, respectively, were included in the assay to see if these determinants were present in oral biofilms.

As expected, *tet*(*Q*) was more prevalent in oral biofilms of periodontitis patients than in those of healthy subjects, since *tet*(*Q*) is common in gram-negative bacteria (Okamoto *et al.*, 2001). It has been well established that major bacterial populations associated with periodontitis are anaerobic gram-negative bacteria, while oral biofilms associated with health subjects contain mostly gram-positive species (Roberts, 1998; Chung *et al.*, 2002). For *tet*(*M*), both groups exhibited almost identical prevalence, which was corroborated from the previous findings indicating that *tet*(*M*) is also found in *streptococci* carrying transposons, along with gram-negative bacteria (Roberts, 1998). The *cfxA* gene was also more prevalent in periodontitis-associated biofilms and it is well established that *cfxA* in dental plaque mainly originates from *P. intermedia* species (Iwahara *et al.*, 2006). Interestingly, $bla_{SHV}$, a gene encoding one of the most common β-lactamases found in *Enterobacteriaceae* (Medeiros, 1997), was not detected in oral biofilms, although $bla_{TEM}$ was widely distributed among plaque samples, regardless of periodontal disease state. Multi-resistant plasmids containing TEM-1 β-lactamase have been isolated from oral commensal *Neisseria* spp. and *Eikenella corrodens* (Pintado *et al.*, 1985; Roberts, 1998). On the other hand, it has been observed that $bla_{\mathrm{SHV}}$ is found in *Klebsiella, Escherichia, Salmonella* and, in some cases, *Pseudomonas* spp. (Bradford, 2001). Taken together, it appears that these resistance determinants do not exist in oral biofilms. It is not entirely clear why *ermF* was more prevalent in biofilm samples from the healthy group than those from the periodontitis group. This result suggests that *ermF* is more closely associated with gram-positive bacteria, a major component of oral biofilms related to a healthy status. It was not expected that we would find a major presence of *aacA-aphD*, *mecA*, and *vanA*, since these determinants are not considered to be found in oral bacteria.

The sequences of targeted resistance determinants were confirmed by direct sequencing. For example, the sequences of *tet*(*Q*) genes amplified from two patients and one healthy subject sample were found to be identical to that of *P. intermedia tet*(*Q*), *B. fragilis tetA*(*Q*)3, and *P. ruminicola tet*(*Q*). This result suggests that the predominant class of *tet*(*Q*) present in oral biofilms originates from Gram-negative anaerobic bacteria, such as *P. intermedia* and other *Bacteroides* spp. When *tet*(*M*) was sequenced and compared among three patient subjects, it was found that these genes were highly homologous (>97%) to each other and to *tet*(*M*) found in the streptococcal conjugative transposon Tn916 or Tn1545. These results confirm that a DNA-based amplification system not only can detect known determinants but also may identify closely related classes of resistance determinants whose DNA sequences are not identical to known determinants.

Molecular detection of resistance determinants by end-point PCR merely indicates the presence of these genes, not providing quantitative information that can be valuable in assessing true resistance potential in oral biofilms. One of the major objectives of this study was to compare antibiotic resistance profiles between oral biofilms associated with health and disease. For this purpose, the relative levels of resistance determinants present in oral biofilms of healthy subjects and periodontitis patients were compared, rather than estimating absolute amounts of those determinants. In this study, the quantitative analysis of resistance determinants was performed to determine a relative ratio between the two groups using a relative quantification method. The exact copy numbers of each resistance determinant present in biofilm samples can be determined in future studies using an absolute quantification scheme, as described previously (Maeda *et al.*, 2003). The results of a quantitative analysis of *tet*(*Q*), *tet*(*M*), *ermF*, and *cfxA* showed that the levels of resistance determinants were

higher in oral biofilms of periodontitis patients compared to those of healthy subjects, as expected, since oral biofilms isolated from periodontitis lesions contain an increased amount of Gram-negative species, in which these genes are known to be more prevalent (Socransky *et al.*, 1998; Socransky and Haffajee, 2002). It is not clear why the relative level of *ermF* in periodontitis biofilms increased as high as 145-fold, although the prevalence was higher in healthy biofilms. A further analysis using more samples will be required to elucidate a relationship between the prevalence and the level of *ermF*. So far, there have been very few reports regarding a quantitative measurement of resistance determinants in oral bacteria. Maeda *et al*. (2003) reported that higher copy numbers of *tet*(*Q*) were detectable after antibiotic therapy for periodontal disease. Taken together, it suggests that a qPCR analysis is a powerful tool for measuring the level of resistance genes present in oral biofilms and the quantitative information will allow a better understanding of the resistance profiles of oral biofilms.

The results of this study suggest that there is a difference in the diversity and distribution of antibiotic resistance determinants between periodontitis patients and healthy subjects and that, in general, the resistance determinants are more prevalent and abundant in periodontitis patients than in healthy subjects. Thus, it is plausible that patients with severe periodontitis may show increased antibiotic resistance to administered antimicrobial agents, reducing the efficacy of antibiotic therapy. For example, higher *tet*(*Q*) levels may hinder successful antibiotic treatment by tetracycline due to increased resistance to tetracycline. Therefore, surveying the genetic diversity and relative amounts of resistance determinants in periodontitis patients will be critical in designing antibiotic therapy for these patients. Compared to a conventional culture-based susceptibility test, DNA-based amplification systems, both qualitative and quantitative, can provide results much faster and also reveal information on uncultivable bacteria. The information on overall resistance profiles may be utilized to formulate guidelines for using systemic antibiotics in treating periodontal disease. This may lead to a more rational and prudent use of systemic antibiotics in periodontal therapy, which will ultimately reduce the spread of antibiotic resistance.

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