

**Antibiotic resistance in human periodontitis
and peri-implant microbiota**

Thomas Edwin Rams

Cover images

top: Photograph of human mandibular lateral incisor tooth, and radiograph of mandibular endosseous root form dental implant.

lower left: Photograph in 1928 of first *in vitro* antibiotic resistance testing on culture plate leading to initial discovery of first antibiotic, penicillin, by Sir Alexander Fleming. Note zone of staphylococcal growth inhibition between penicillin-producing mold colonies at top, and uninhibited staphylococcal colonies on bottom. Published in Fleming, A. (1929) On the antibacterial action of cultures of a penicillium with special reference to their use in the isolation of *B. influenzae*. *British Journal of Experimental Pathology* 10: 226-236.

lower right: Photograph 85 years later in 2013 of *in vitro* antibiotic resistance testing of a *Prevotella intermedia/nigrescens* strain isolated from the subgingival microbiota of a chronic periodontitis patient. Note antibiotic-resistant *P. intermedia/nigrescens* colonies exhibiting uninhibited growth on enriched Brucella blood agar in presence of clindamycin released from E-test strip (numbers indicate mg/L of clindamycin eluted from segment of E-test strip).

Back: Anaerobically-incubated culture colonies of polymicrobial subgingival microbiota characteristic of human periodontitis and peri-implantitis.

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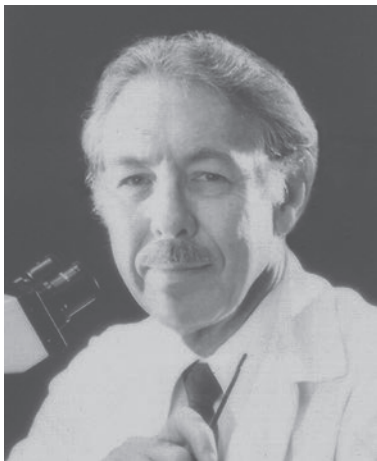
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To my first periodontal mentor:



Paul H. Keyes, D.D.S., M.S.
National Institute of Dental Research
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Bethesda, Maryland USA

Pioneered anti-infective non-surgical periodontal therapy starting in the mid-1970s.

Arrest of periodontal lesions will become more predictable as clinicians modulate therapeutic measures until periodontopathic microorganisms have been brought under control (1).

Successful management of periodontal diseases will depend upon recognition of the microbiological targets that need to be eliminated to arrest and prevent periodontitis, and on the proper [microbiological] monitoring and modulation of whatever type of therapy is selected to control crevicular infections (2).

The end point of our [periodontal] therapy is conversion from disease-associated bacterial complexes to health-associated populations. It is increasingly important that clinicians tell patients whether they are in the safety zone microbiologically and whether they are free of bacterial risk factors associated with destructive periodontitis (3).

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Paranimfen:

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Chapter 1

General introduction

The use of microbiological diagnostics to help guide treatment of human bacterial infections dates back to the second decade of the 20th century during World War I. According to Edberg (1), French surgeons used quantitative microbiological findings to assist in their management of traumatic war wounds in French troops as follows:

A wounded soldier is carried to an aid station from the trenches of France – the year is 1917. His uniform is cut away and, if the wound is greater than 15 hours old, a wide debridement is performed. Disinfectant is added to the wound at the time of surgery, and the soldier is evacuated to a rear military hospital. Accompanying the soldier is something new in wound treatment – a petri plate. In addition to the standard “incision and drainage”, this soldier has had his injury cultured and the petri plate will accompany him. When he arrives at the hospital the petri plate will be inspected and if there are no streptococci and fewer than five colonies of all other bacteria, the wound will be closed. If these criteria are not met, the wound will be left open to heal by secondary intention. Guidance by quantitative microbiology became quite common in the French army in World War I, the first time the counting of bacteria was used to dictate therapy. Soon after the war, however, the procedure was forgotten.

Edberg (1) also points out that it was not until 1955, nearly 40 years later, that there was a revival, in place to the present day, in the use of quantitative microbiology to guide treatment of traumatic injuries.

In addition to monitoring of medical infections for their microbiological composition, the discovery of antibiotics in 1928 by Sir Alexander Fleming marked the start of antimicrobial susceptibility testing in the field of clinical microbiology. As depicted on the cover of this thesis, Fleming (2) noted a zone of staphylococcal growth inhibition immediately adjacent to penicillin-producing mold colonies on a contaminated agar plate that had been left inadvertently exposed to the outside environment in his laboratory. This observation of the inhibitory effects of penicillin on staphylococcal growth represents the outcome of the first known *in vitro* antibiotic susceptibility test. Over the ensuing decades, *in vitro* antibiotic susceptibility testing has greatly evolved to its present-day widespread use in infectious disease management (3).

As recognized by Murray (4), even though antimicrobial susceptibility testing is generally limited to assessing interactions between cultivable microorganisms and test antimicrobials within an *in vitro* setting, and fails to capture the many *in vivo* host/bacterial pathogen interactions that influence the clinical status of an infectious process (5), clinical data for many bacterial infections demonstrate a good correlation between *in vitro* antimicrobial susceptibility test results and *in vivo* clinical patient responses. In general, it is recognized that patients with antibiotic drug-resistant bacterial pathogens in a clinical infection more frequently demonstrate a poorer microbiological response to antibiotic therapy than patients with bacterial pathogens shown to be sensitive *in vitro* to the antibiotic (4). As a result, the identification of antibiotic-resistant pathogens plays a key role in clinical decision-making relative to treatment of many medical infections. There is presently in infectious disease management an increased reliance upon *in vitro* antimicrobial susceptibility test results to help guide selection and administration of the most appropriate antimicrobial therapies in order to enhance patient outcomes and minimize clinical treatment failures, since use of antimicrobials which fail to exert activity against bacterial pathogens in an infection is considered the same as not using any antimicrobial agents at all (5).

It is within this historical background and context that this thesis examines the occurrence of antibiotic resistance among bacterial pathogens associated with human periodontitis and peri-implantitis infections.

Human periodontitis: an overview

Periodontitis is a destructive form of periodontal disease that adversely impacts tooth-supporting soft and hard tissues, and ultimately, may lead to loss of natural teeth in the human dentition. Periodontitis is considered to be multifactorial in its etiology, but nevertheless, is primarily driven in its progression by tooth surface growth of highly-organized, polymicrobial, biofilms of pathogenic bacteria that comprise what is commonly referred to as dental plaque.

In periodontal health, teeth are anchored to maxillary and mandibular alveolar bone by numerous Sharpey's fibers, composed of Type I collagen, that are the main constituent of the periodontal ligament covering tooth root surfaces from the cemento enamel junction to the apices of teeth. Sharpey's fibers are embedded, at one end, into cementum on tooth root surfaces, and on the other end, into alveolar bone proper that surrounds and houses tooth roots. The attachment apparatus of teeth is thus composed of periodontal ligament, tooth cementum, and alveolar bone (6) (Figure 1).

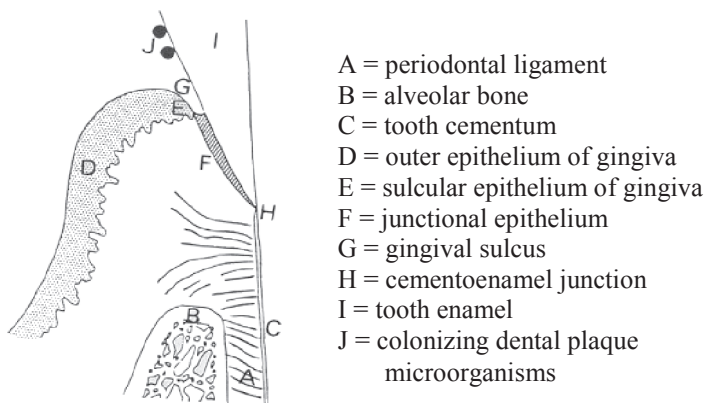


Figure 1. Cross-sectional depiction of tooth interface with gingival tissues and alveolar bone in periodontal health. Modified from Slots & Rams (6).

Gingival soft tissues surrounding teeth, and covering the periodontal attachment apparatus, are relatively devoid of inflammatory cells in periodontal health, and maintain an interfacing knife-edge marginal relationship with tooth surfaces. The gingival sulcus in periodontal health (Figure 1) is shallow (1-3 millimeters) in its coronal-apical dimension, and does not bleed upon probing with gentle insertion and apical advancement of a periodontal probe instrument (Figure 2).

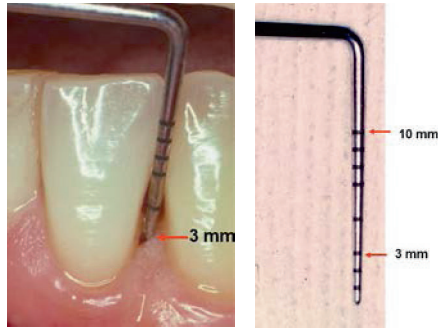


Figure 2. (left) Healthy gingival tissues around a natural tooth exhibiting no visible inflammation and a shallow gingival sulcus measured (right) with a periodontal probe.

In contrast, periodontitis induces a progressive loss from teeth of anchoring gingival connective tissue and periodontal ligament fibers, along with resorption of surrounding crestal alveolar bone. The junctional epithelium, which in periodontal health is normally located with its most coronal aspect at the cemento-enamel junction, migrates apically as progressive destruction of periodontal ligament fibers occurs subjacent to it (Figure 3).

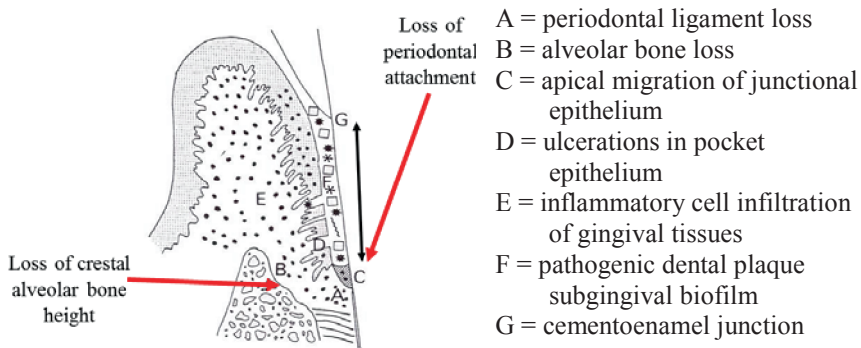


Figure 3. Cross-sectional depiction of tooth interface with gingival tissues and alveolar bone in periodontitis. Modified from Slots & Rams (6).

Clinically, periodontitis is characterized by the presence of gingival inflammation, with gingival tissues appearing red in color and edematous, and bleeding on probing is detected with a periodontal probe. Marked increases in periodontal probing depths are measured adjacent to periodontitis-affected tooth surfaces as the gingival sulcus is transformed into a periodontal pocket, with moderate (4-6 millimeters) and severe (≥ 7 millimeters) probing depths forming that may progress, in the absence of periodontal therapy, to the tooth root apex (Figure 4).

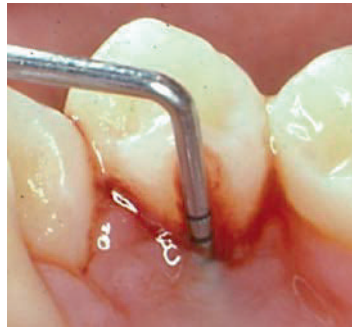


Figure 4. Inflamed gingival tissues on tooth affected by periodontitis, with bleeding on probing and an 8 millimeter probing depth.

With advanced periodontal tissue breakdown, increased tooth mobility may be detected upon gentle clinical luxation of teeth in a facial-lingual direction, and radiographic loss of crestal alveolar bone height and density may be noted.

Periodontitis is a relatively common affliction in humans around the world. In the United States, the most recent national epidemiologic prevalence survey of periodontitis involved the clinical examination of 3,742 dentate adults, aged 30 years and older, in the civilian non-institutionalized population. Clinical periodontal attachment loss (as measured from the cemento-enamel junction of teeth to the most apical periodontal probe penetration into periodontal pockets and gingival sulci) and periodontal probing depths (as measured from the free gingival margin to the most apical periodontal probe penetration into periodontal pockets and gingival sulci) were assessed in each of the examined adults at six sites per tooth on all teeth except third molars (7). It is estimated from this data that approximately 47.2% of the population sample, representing 64.7 million United States dentate adults ≥ 30 years old, exhibited some form of periodontitis, with severe and moderate periodontitis found in 8.5% and 30% of the study population, respectively (7). In addition, a particularly high prevalence of periodontitis was noted in elderly persons, with 64% of dentate adults aged ≥ 65 years presenting with either moderate or severe periodontitis (7). Moreover, periodontitis was found to be significantly higher or highest among certain population groups in the United States, including males, Mexican Americans (Hispanic) (followed closely by blacks, who were both significantly more affected by periodontitis than whites), less educated (with less than high school graduation), poor (below federal poverty levels in income), and current smokers (7). In addition to these potential risk factors, other research studies have associated a family history of periodontitis, specific genetic polymorphisms, certain systemic diseases (such as uncontrolled diabetes mellitus), obesity, poor oral hygiene, subgingival calculus, overhanging margins of dental restorations, and psychosocial stress, with an increased risk of periodontitis (8). Additional predisposing conditions to periodontitis have been proposed, and are under study (9).

Subgingival infection with specific pathogenic bacteria, methanogenic archaea, and possibly activation of certain herpesviruses in gingival tissues, have been related to the onset and progression of human periodontitis (6,8,10-12). Among the major bacterial species implicated as periodontal pathogens are a number of gram-negative species, including

Porphyromonas gingivalis, *Aggregatibacter actinomycetemcomitans*, *Treponema denticola* and other oral spirochetes, *Tannerella forsythia*, *Selenomonas noxia*, *Prevotella intermedia/nigrescens*, *Dialister pneumosintes*, *Fusobacterium nucleatum*, *Fusobacterium animalis*, and *Campylobacter rectus*. Several gram-positive species have been identified with periodontitis, including *Parvimonas micra*, *Filifactor alocis*, *Eubacterium nodatum* and other *Eubacterium* species, and each of the three anginosus streptococci group species, *Streptococcus constellatus*, *Streptococcus intermedius*, and *Streptococcus anginosus*.

In some periodontitis patients, atypical subgingival periodontal pathogens may be present in high numbers, such as the gram-positive facultative species *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and other staphylococci; the gram-negative facultative organisms classified as enteric rods and pseudomonads; and yeasts most frequently identified as *Candida albicans*. Microbial species presently classified as phylotypes, since they have yet to be successfully cultivated in laboratory settings, but can be identified via 16s ribosomal RNA bacterial gene analysis, have also been recovered from subgingival biofilms in periodontitis (13).

In addition to specific bacterial species, *Methanobrevibacter oralis*, a methanogenic *Archaea* species that has the capability to support anaerobic bacterial growth via interspecies hydrogen transfer, is increased in the subgingival pocket environment with periodontitis severity, decreased with successful periodontal therapy, and absent in periodontal health (11). Herpesviruses, such as cytomegalovirus and Epstein Barr virus, when present in an active lytic phase in gingival tissues, may be locally-immunosuppressive and promote overgrowth of periodontopathic bacteria in periodontal pockets (12).

As a result, the onset of periodontitis involves a series of complex interactions between a variety of local and systemic host susceptibility factors with the effects of pathogenic bacterial biofilm growth on tooth surfaces, and invasion into gingival tissues adjacent to tooth surfaces, leading to detrimental immunoinflammatory reactions which induce periodontal connective tissue loss and alveolar bone resorption around affected teeth (Figure 5).

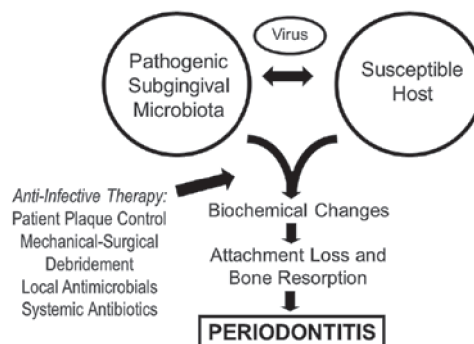


Figure 5. Model relating the interactions between host risk factors and pathogenic bacterial populations that result in inflammatory-mediated periodontal tissue breakdown characteristic of periodontitis.

Beyond the loss of teeth ultimately resulting in edentulism (a loss of all natural teeth), periodontitis, and periodontitis-associated microorganisms, have been implicated in the pathogenesis of a number of systemic medical conditions at non-oral body sites, including, but not limited to, cardiovascular disease, stroke, rheumatoid arthritis, premature/low birth weight pregnancy outcomes, tubal-ovarian abscess, infectious arthritis of knee, aspiration pneumonia and other types of lung infections, chronic conjunctivitis, and clenched-fist wounds (14,15). Periodontitis can thus not only adversely affect teeth in the oral cavity, and their normal functions in supporting mastication of food, stimulating maintenance of alveolar bone in the jaws, and contributing to dental esthetics, but it may also contribute to a decreased life-span for afflicted individuals by potentiating serious systemic medical disorders (14,15).

Interestingly, the exact etiologic mechanism underlying the onset or “triggering” of periodontitis disease-activity remains uncertain, and a recent systematic review even questions the role of pathogenic bacteria in the pathogenesis of periodontitis (16). While pathogenic bacterial biofilms on tooth surfaces appear necessary to initiate periodontal disease, it is noteworthy that most periodontal tissue damage from periodontitis is largely attributable to hyper-responsive host immunoinflammatory reactions to pathogenic bacterial biofilms (17).

Treatment of periodontitis

Because host-related risk factors for periodontitis are immutable or difficult to therapeutically alter, most periodontitis treatment strategies focus on suppression or elimination of pathogenic bacterial biofilms on supragingival and subgingival tooth surfaces. Mechanical debridement of pathogenic dental plaque biofilms (carried out with or without periodontal surgical flap access), and meticulous patient supragingival home plaque control, form the traditional basis for treatment of periodontitis. However, despite years of documented success with this type of treatment approach, a subset of periodontitis patients suffer further progressive periodontal breakdown (18). While a number of factors may be responsible for such treatment failures, such as inadequate root instrumentation, poor home oral hygiene compliance by patients with supragingival plaque control, presence of plaque-retentive dental restorations, it is also clear that many treatment failures in periodontitis are related to, despite extensive efforts of clinicians and patients, a persistence of a pathogenic subgingival dental plaque microbiota (19,20). In one such study by Rams et al. (19), 25 of 78 (32.1%) adults with severe periodontitis treated with root debridement, surgical pocket elimination, three-month maintenance care, and good supragingival plaque control experienced recurrent periodontitis disease activity within 12-months post-treatment. In multivariate analysis, the post-treatment persistence of elevated subgingival proportions of one or more of five major periodontal bacterial pathogens (i.e., *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia/nigrescens*, *P. micra*, *C. rectus*) was associated with a statistically significant 2.5 (150%) excess relative risk for periodontitis recurrence within 12-months post-treatment (19). Similarly, Mombelli et al. (21) reported 59% of 17 adults with chronic periodontitis remaining culture-positive for subgingival *P. gingivalis* after extensive non-surgical root debridement and supragingival dental plaque control, with a direct correlation found between residual deep periodontal probing depths, indicative of a poor clinical treatment outcome, and persistence of subgingival *P. gingivalis*. Using digital computer subtraction radiology to detect small changes in crestal alveolar bone mass, Chaves et al. (22) found post-treatment persistence of subgingival *P. gingivalis* after

mechanical root debridement of chronic periodontitis patients to be strongly associated with elevated risk of subsequent progressive periodontal bone loss (31.9 odds ratio; 84% positive predictive value).

To augment the antimicrobial effects of conventional mechanical-surgical periodontal therapy, and to better suppress or eliminate recalcitrant subgingival periodontal bacterial pathogens, systemic antibiotic therapy has been increasingly employed in the treatment of periodontitis. The use of systemic antibiotics in periodontal disease therapy represents a conceptual shift in the field of periodontology towards application of an anti-infective treatment strategy, where specific disease-associated bacterial pathogens, such as *P. gingivalis*, are primarily targeted for suppression or elimination from oral cavity of periodontitis patients (Figure 6).

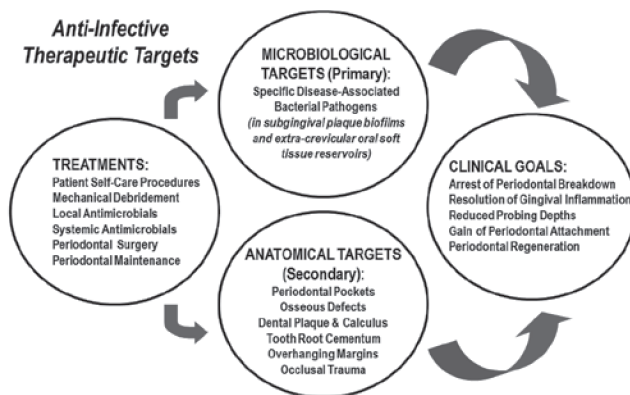


Figure 6. Specific periodontal bacterial pathogens are primary therapeutic targets in an anti-infective periodontal disease treatment model, with anatomical abnormalities in the periodontium being secondarily targeted for alteration. However, successful treatment interventions on both microbiological and anatomical targets help attain periodontal clinical goals and optimize patient outcomes.

Clinical trials, systematic reviews, and meta-analysis reported during the past decade have documented a beneficial clinical and microbiological effect of systemic periodontal antibiotic therapy on periodontitis patients (23-29). Antibiotics shown to have some efficacy in periodontitis therapy include single drug regimens with tetracycline-HCl, minocycline, doxycycline, amoxicillin, clindamycin, metronidazole, azithromycin, and moxifloxacin, as well as combination drug regimens involving amoxicillin plus metronidazole, and ciprofloxacin plus metronidazole (23-29).

Considerable questions remain to be resolved as to how to identify which periodontitis patient will benefit from systemic antibiotic therapy, and how to select the most appropriate antibiotic for the specific patient. An indication of a differential response to systemic antibiotic therapy among patients is illustrated by data showing that the administration of systemic amoxicillin plus metronidazole therapy on periodontitis patients leads to some patients with an enhanced clinical outcome relative to conventional non-antibiotic, mechanical-surgical treatment approaches, whereas other patient outcomes are found to be no better than a placebo-associated treatment (30-32).

Several explanations may account for an inadequate patient response to systemic periodontal antibiotic therapy. Because systemic antibiotics are generally prescribed to periodontitis patients for unsupervised oral consumption in home settings, inadequate compliance with taking the antibiotic regimen as instructed may occur. Loesche et al. (33) reported only 56% of 18 periodontitis patients were compliant with a prescribed systemic metronidazole drug regimen, with a significantly greater reduction of periodontal surgical needs found in compliant patients, as compared to non-compliant individuals.

Variability among patients in their ability to successfully absorb oral antibiotics may also impair systemic periodontal antibiotic therapies. Sakellari et al. (34) found serum and gingival crevicular fluid drug concentrations to vary widely among periodontitis subjects consuming tetracycline family antibiotics, and concluded that “*poor absorption of orally-administered tetracyclines in many individuals may account for much of the variability in clinical response to antibiotics observed in practice.*”

Antibiotic drug penetration into intact dental plaque biofilms is another problematic challenge in periodontics. Markedly higher minimal inhibitory concentration values, up to 250 times greater, of antibiotics are needed when periodontal bacterial pathogens are organized in dental plaque biofilms, as compared to being separated apart as planktonic cells (35).

In addition, targeted dental plaque microorganisms need to be susceptible to therapeutic concentrations of antibiotics in order to be inhibited by them. Limited data indicates that antibiotic-resistant periodontal bacterial pathogens have been increasing in their occurrence over time in the United States. Walker (36) observed an increased *in vitro* resistance to amoxicillin, tetracycline, and macrolide antibiotics among subgingival bacteria tested in the early 1990s as compared to the early 1980s. More recently, an increased occurrence of clindamycin resistance in subgingival *P. gingivalis* clinical isolates was reported in 2003 (37). An urgent need exists to better quantify the extent to which antibiotic resistance occurs among pathogenic subgingival bacterial species in human periodontitis patients.

Human peri-implantitis: an overview

Dental implants, which are employed as replacements for lost natural teeth in the oral cavity, may be affected by marginal bone loss that may lead to loss of the implants. Dental implants, in comparison to teeth, do not possess an attachment apparatus comprised of cementum, periodontal ligament and alveolar bone. Instead, successful titanium osseointegrated dental implants ankylose after a healing phase to adjacent alveolar bone after their surgical placement into osteotomy channels created where natural teeth were lost.

Controversy exists relative to the cause of marginal bone loss on dental implants, with three major theories currently being debated (38). First, is the “infection” theory, which regards peri-implantitis to be a destructive infectious complication of dental implants, analogous to periodontitis on natural teeth. The primary etiologic factor in peri-implantitis is considered to be pathogenic bacterial biofilm growth on dental implant surfaces, with possible contributions by methanogenic *Archaea* and herpesviruses (39-41). If allowed to persist without therapeutic intervention, immunoinflammatory host tissue reactions may occur that result in peri-implant mucosal redness, edema, and bleeding on probing, increased peri-

implant probing depths, and progressive peri-implant crestal alveolar bone loss. The marginal alveolar bone loss may ultimately encompass the apical extent of the dental implant, leading to dental implant mobility and loss (exfoliation). Peri-implantitis is estimated to occur in 10.7% to 47.2% of dental implant patients after 10 years of post-treatment observation (Figure 7) (42).

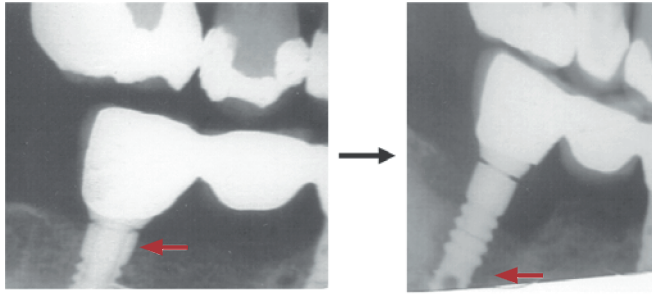


Figure 7. Radiograph appearance of peri-implantitis on a dental implant.
 (left) Dental implant status in 1994 with normal crestal alveolar bone height (arrow), as well as open margin between the dental implant crown and fixture.
 (right) Dental implant in 1998 - note marked loss of crestal alveolar bone height (arrow). Clinical bleeding on probing and deep peri-implant probing depths were detected, along with high submucosal proportions of *P. gingivalis*, *P. intermedia/nigrescens*, and *P. micra*.

The “adverse occlusal load” theory suggests that excessive overloading of dental implants by heavy occlusal forces may initiate progressive marginal bone loss leading to implant failure (38). Isidor (43), using a monkey model, found dental implants restored in supra-occlusal contact with an “antagonizing splint” (occlusal overload) exhibited significantly greater marginal bone loss over 18 months, and greater dental implant loss, as compared to dental implants subjected to a dental plaque-accumulating cotton cord placed around the peri-implant marginal soft tissues. Interestingly, when occlusal overloading is combined with ligature-induced, bacterial-mediated, peri-implant inflammation, peri-implant angular bone loss is significantly increased on buccal and lingual surfaces in beagle dogs than is observed with either risk factor alone (44).

The “compromised healing/adaptation” theory proposes that local and systemic host factors that compromise post-treatment healing after dental implant placement, such as poor bone quality, genetic factors, traumatic implant surgery, and improper prosthodontic treatment planning, are responsible for most, if not all, dental implant failures, with little contribution by submucosal bacterial populations (38).

It is not presently known which of these three theories, or combinations of them, are applicable to the majority of dental implant failures. It is of interest that when systemic antibiotics are given as adjuncts to mechanical debridement and/or surgical procedures on peri-implantitis-affected dental implants heavily colonized by putative bacterial pathogens, marked clinical and/or radiographic improvements occur, leading to arrest of the peri-implantitis lesions (45-47). This suggests the applicability of the “infection” theory, and a

role for systemic antibiotics, for at least a subset of human dental implants experiencing marginal bone loss.

Purpose and goals of thesis

The hypothesis underlying this thesis is that antibiotic-resistant periodontal pathogens are present in the subgingival microbiota of United States periodontitis and peri-implantitis patients that have the ability to survive therapeutic concentrations of systemic antibiotics that are commonly prescribed in the treatment of these two oral diseases. At present, little or no data of recent origin is available on the occurrence of antibiotic-resistant bacterial pathogens in periodontitis and peri-implantitis patients in the United States.

The study of antibiotic-resistant periodontal and peri-implant bacterial pathogens may help account for clinical treatment failures in periodontitis and peri-implantitis therapies. If substantial antibiotic resistance is detected in affected patients, then new clinical strategies, which encompass assessments of subgingival and submucosal bacterial pathogen antibiotic susceptibility testing as part of treatment planning for periodontitis and peri-implantitis patients, may be more appropriate for clinical periodontal and oral implantology practice.

To help address the issue of how frequent are antibiotic-resistant periodontal pathogens found in United States periodontitis patients, an *in vitro* study was conducted on the antibiotic susceptibility of subgingival clinical isolates of *S. constellatus* and *S. intermedius* (**Chapter 2**), since little data is available in the scientific literature on the occurrence of antibiotic resistance among these species. A similar *in vitro* evaluation of antibiotic susceptibility testing was carried out on subgingival clinical isolates of *E. faecalis*, which may occur on occasion in refractory periodontitis patients, and for which little antibiotic resistance data is also available for strains recovered from periodontal lesions in the United States (**Chapter 3**).

Chapter 4 evaluated a wide range of organisms in the cultivable subgingival microbiota of chronic periodontitis subjects in the United States to further assess the occurrence of β -lactamase enzyme producing subgingival bacterial test species, which would potentially have the ability to degrade β -lactam antibiotics, leaving them pharmacologically inactive as antimicrobial agents, and compromising their efficacy in systemic periodontal antibiotic therapy.

Chronic periodontitis subjects in the United States were also evaluated in **Chapter 5** for the occurrence of subgingival periodontal pathogens that exhibited *in vitro* resistance to therapeutic breakpoint concentrations to several antibiotics used frequently in clinical periodontal practices, including clindamycin, doxycycline, amoxicillin, metronidazole, and the joint use of amoxicillin and metronidazole. A similar evaluation of United States chronic periodontitis subjects was carried in **Chapter 6** with a focus on the macrolide antibiotic spiramycin alone, and with metronidazole.

Chapter 7 aimed to assess the occurrence of *in vitro* antibiotic resistance among submucosal bacterial pathogens in human peri-implantitis lesions in the United States.

These studies were undertaken with the goal of providing better characterization of the occurrence of antibiotic-resistant pathogens in chronic periodontitis and peri-implantitis patients in the United States. An additional goal was to identify, among a selected panel of putative periodontal and peri-implant bacterial pathogens, those subgingival and sub-mucosal species which most frequently exhibit *in vitro* resistance to antibiotics pertinent to treatment of periodontitis and peri-implantitis.

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Chapter 2

Antibiotic susceptibility of periodontal *Streptococcus constellatus* and *Streptococcus intermedius* clinical isolates

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Abstract

Purpose: *Streptococcus constellatus* and *Streptococcus intermedius* in subgingival dental plaque biofilms may contribute to forms of periodontitis that resist treatment with conventional mechanical root debridement-surgical procedures, and may additionally participate in some extraoral infections. Since systemic antibiotics are often employed in these clinical situations, and little is known of the antibiotic susceptibility of subgingival isolates of these two bacterial species, this study determined the *in vitro* susceptibility to six antibiotics of fresh *S. constellatus* and *S. intermedius* clinical isolates from human periodontitis lesions.

Materials and methods: A total of 33 *S. constellatus* and 17 *S. intermedius* subgingival strains, each recovered from separate chronic periodontitis patients, were subjected to antibiotic gradient strip susceptibility testing with amoxicillin, azithromycin, clindamycin, ciprofloxacin and doxycycline on blood-supplemented Mueller-Hinton agar, and to the inhibitory effects of metronidazole at 16 mg/L in an enriched Brucella blood agar dilution assay. CLSI and EUCAST interpretative standards were used to assess the results.

Results: Clindamycin was the most active antibiotic against *S. constellatus* ($MIC_{90} = 0.25$ mg/L), with amoxicillin most active against *S. intermedius* ($MIC_{90} = 0.125$ mg/L). 30% of the *S. constellatus* and *S. intermedius* clinical isolates were resistant *in vitro* to doxycycline, 98% were only intermediate in susceptibility to ciprofloxacin, and 90% were resistant to metronidazole at 16 mg/L.

Conclusions: Since subgingival *S. constellatus* and *S. intermedius* exhibit variable antibiotic susceptibility profiles, potentially complicating selection of periodontitis antibiotic therapy, microbiological analysis that encompasses antimicrobial sensitivity testing may be particularly helpful in periodontal treatment planning of species-positive patients with refractory periodontitis.

Introduction

Streptococcus constellatus and *Streptococcus intermedius* are two phenotypically and genetically distinct members of the classic “*Streptococcus milleri* group” of bacteria now taxonomically classified as anginosus group streptococci (1). The two species are considered commensal organisms in the human upper respiratory, gastrointestinal, and female urogenital tracts, but may act as life-threatening opportunistic pathogens in deep-seated abscesses and purulent surgical infections when translocated into normally sterile body sites (1).

In the human oral cavity, *S. constellatus* and *S. intermedius* preferentially inhabit subgingival dental plaque biofilms on interproximal tooth surfaces in untreated periodontitis patients (2), where after periodontal root instrumentation, no decrease in their detection rate is reported to occur (3). The two species also show a predilection to colonize oral soft tissue surfaces, such as the hard palate, floor of the mouth, and dorsum of the tongue, to a degree similar or greater than in subgingival tooth sites (4), which provides a potential post-periodontal treatment reservoir source for re-seeding of periodontal pockets by the organisms. Increases in adrenaline and noradrenaline hormonal levels in response to psychosocial-emotional stress, a recognized risk factor in the development and progression of human periodontitis (5), significantly stimulates *in vitro* growth of *S. constellatus* and *S. intermedius* (6), which may further favor their oral selection and outgrowth in periodontitis-susceptible subjects. As a result, the oral colonization properties of *S. constellatus* and *S. intermedius*, along with their strong anti-phagocytic resistance to human polymorphonuclear leukocytes (7), likely contributes to their association with forms of destructive periodontal disease that do not respond to conventional mechanical root scaling-surgical treatment procedures (8-10). In addition, these species are associated with brain abscesses and other extraoral infections when they are disseminated outside of the oral cavity via bacteremia through inflamed gingival tissues (11,12).

Since antimicrobial chemotherapy is frequently employed to treat patients with refractory periodontitis and various extraoral infections (13), the antibiotic susceptibility of subgingival *S. constellatus* and *S. intermedius* can be a decisive factor in the selection and clinical success of prescribed anti-infective drug regimens. Because relatively little is presently known about the antibiotic sensitivity of periodontal strains of *S. constellatus* and *S. intermedius*, the purpose of this study was to determine the *in vitro* susceptibility to six antibiotics of fresh clinical isolates of these two species recovered from human periodontitis lesions.

Materials and methods

Bacterial strains

A total of 33 *S. constellatus* and 17 *S. intermedius* fresh clinical isolates were recovered on enriched Brucella blood agar primary isolation plates, as previously described (14), by the Oral Microbiology Testing Service Laboratory at Temple University School of Dentistry, Philadelphia. The species were isolated from subgingival plaque biofilm specimens removed from 50 systemically-healthy adults with untreated advanced chronic periodontitis (15) (22 male and 28 female; mean age = 55.2 ± 12.4 (SD) years; age range 31-76 years;

geographically living in eastern United States), as diagnosed by 50 periodontists in private dental practices, with each subject contributing one isolate of either test species. *S. constellatus* comprised a mean of 8.9 ± 0.2 (SE) % (range 0.1-46.8%), and *S. intermedius* averaged 4.1 ± 1.6 (SE) % (range 0.1-17.4%), in the cultivable subgingival microbiota of the species-positive patients.

S. constellatus was defined as gram-positive, lactose MUG-test negative (16), non-motile, facultative cocci demonstrating small ($< 0.5 \mu\text{m}$ in diameter), white, opaque, circular, β -hemolytic, surface colonies with irregular edges. The species were further positive for acetoin production (Voges-Proskauer test positive), negative for β -D-fucosidase, and positive for α -D-glucosidase enzyme activity (with or without concurrent β -D-glucosidase activity), as determined using a commercial test kit (Fluo-Card Milleri test kit, Key Scientific Products Co., Stamford, TX, USA) (17). *S. intermedius* was recognized as gram-positive, lactose MUG-test positive, non-motile, facultative cocci exhibiting small ($< 0.5 \mu\text{m}$ in diameter), dry, white, raised colonies with wrinkled edges, which were positive for acetoin, β -D-fucosidase, β -D-glucosidase, and α -D-glucosidase (17). These phenotypic identification criteria for *S. constellatus* and *S. intermedius* are reported to provide 96-100% agreement with 16S rRNA gene sequencing analysis of the species (17-19).

***In vitro* antimicrobial susceptibility testing**

Pure culture cell suspensions of each of the *S. constellatus* and *S. intermedius* clinical isolates were adjusted to a 0.5 McFarland turbidity standard, and streaked with sterile cotton-tipped swabs onto 150 mm diameter plates containing Mueller-Hinton agar with 5% sheep blood. After drying, predefined antibiotic gradient strips (E-test, bioMérieux, Durham, NC, USA) (20), containing amoxicillin, azithromycin, clindamycin, ciprofloxacin or doxycycline, were applied onto the inoculated media surfaces. After 24 hours of incubation at 35°C in ambient air-5% CO₂, the intersection between the border of test species growth and the antibiotic gradient strip drug scale for each antimicrobial was read to determine the *in vitro* minimum inhibitory concentration (MIC) value, following manufacturer's instructions. *Streptococcus pneumoniae* ATCC 49619 was employed as a quality control strain in the antibiotic gradient strip susceptibility testing.

For *in vitro* assessment of metronidazole susceptibility, a separate agar dilution assay with antibiotic-supplemented enriched Brucella blood agar was used, as previously described (14, 21) to evaluate the inhibitory effects of metronidazole at a concentration of 16 mg/L on each of the *S. constellatus* and *S. intermedius* test species. *Bacteroides thetaiotaomicron* ATCC 29741, *Clostridium perfringens* ATCC 13124, and a multi-antibiotic-resistant clinical periodontal isolate of *Fusobacterium nucleatum* were employed as positive and negative quality controls.

Data analysis

MIC₅₀ and MIC₉₀ values for each antibiotic were defined as the MIC level that completely inhibited 50% and 90%, respectively, of the tested subgingival clinical isolates. MIC interpretative standards developed by the Clinical and Laboratory Standards Institute (CLSI) for *Streptococcus viridans* group species, which includes *S. constellatus* and *S. intermedius*, were used to categorize the *in vitro* inhibitory activity of azithromycin and

clindamycin against the test isolates (22). Clinical breakpoint values developed by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for viridans group streptococci were used for amoxicillin susceptibility testing, and EUCAST breakpoints for *S. pneumoniae* were applied to doxycycline and ciprofloxacin test results (23), since no CLSI interpretative guidelines for these antibiotics against *S. constellatus* and *S. intermedius* are available. Clinical isolates with MIC values less than or equal to the antibiotic susceptibility breakpoint concentration were classified as “susceptible”, with those more than or equal to antibiotic resistance breakpoint concentrations identified as “resistant”. Test strains with MIC values in-between antibiotic susceptibility and resistance breakpoints were designated as “intermediate”.

Approval for this study was provided by the Temple University Human Subjects Protections Institutional Review Board.

Results

Quality control

The antibiotic gradient strip MIC values for the *S. pneumoniae* ATCC 49619 quality control strain for amoxicillin, azithromycin, clindamycin, ciprofloxacin and doxycycline, as well as the test results for the three quality control strains subjected to *in vitro* metronidazole resistance testing, were within the expected ranges and outcomes (data not shown).

In vitro antibiotic susceptibility testing

Table 1 presents the cumulative distribution of MIC antibiotic values against all tested periodontal *S. constellatus* and *S. intermedius* clinical isolates. Table 2 provides the MIC range, MIC₅₀, and MIC₉₀ for these antibiotics against periodontal *S. constellatus* and *S. intermedius* clinical isolates individually, as well as together, since only relatively small differences in antibiotic sensitivity were found between the two species. By possessing the lowest MIC₉₀ values, clindamycin was the most active antibiotic against *S. constellatus* (MIC₉₀ = 0.25 mg/L), whereas amoxicillin was the most active against *S. intermedius* (MIC₉₀ = 0.125 mg/L). Both drugs were 32 times more active against the test species than doxycycline, which exhibited MIC₉₀ values of 8 mg/L against *S. constellatus*, and 4 mg/L against *S. intermedius*. Amoxicillin, azithromycin, and ciprofloxacin demonstrated a similar magnitude of antimicrobial inhibition against periodontal *S. constellatus* (MIC₉₀ = 0.38 mg/L for each drug). In comparison, amoxicillin and azithromycin were more active against *S. intermedius* than ciprofloxacin.

Table 2 also presents the number and percentage of test strains resistant to antibiotic breakpoint concentrations. Periodontal *S. constellatus* and *S. intermedius* were overall most frequently resistant *in vitro* to doxycycline, with 30% of all test strains yielding MIC values above the doxycycline resistance breakpoint concentration. No periodontal *S. constellatus* and *S. intermedius* were resistant *in vitro* to amoxicillin, and only a low frequency of *in vitro* resistance was detected to azithromycin, clindamycin, and ciprofloxacin (2-6% of clinical isolates). However, 98% of all *S. constellatus* and *S. intermedius* periodontal isolates were only intermediate in their susceptibility *in vitro* to ciprofloxacin (Table 2), and

90% demonstrated *in vitro* resistance to metronidazole at a 16 mg/L breakpoint concentration.

Table 1. *In vitro* activity of selected antibiotics on 50 total *S. constellatus* and *S. intermedius* subgingival clinical isolates.

Antibiotic	Cumulative % of isolates inhibited at indicated concentration (mg/L)									
	≤0.03	0.06	0.12	0.25	0.5	1	2	4	≥ 8	
amoxicillin	2	4	32	84	100					
azithromycin	4	10	72	94	94	94	94	94	100	
clindamycin	8	16	64	92	96	96	96	96	100	
ciprofloxacin	0	0	0	48	92	96	98	100		
doxycycline	8	16	28	50	54	58	68	86	100	

Table 2. *In vitro* MIC (mg/L) of selected antibiotics on *S. constellatus* and *S. intermedius* subgingival clinical isolates.

Species (No. of isolates)	Antibiotic (susceptibility/resistance breakpoints, mg/L)	MIC range	MIC ₅₀ ^a	MIC ₉₀ ^a	No. (%) susceptible	No. (%) intermediate	No. (%) resistant
<i>S. constellatus</i> (N = 33)	amoxicillin ($\leq 0.5 / > 2$)	$\leq 0.016 - 0.5$	0.25	0.38	33 (100)	0 (0)	0 (0)
	azithromycin ($\leq 0.5 / \geq 2$)	$0.032 - > 256$	0.19	0.38	31 (93.9)	0 (0)	2 (6.1)
	clindamycin ($\leq 0.25 / \geq 1$)	$0.016 - > 256$	0.094	0.25	30 (90.9)	1 (3.0)	2 (6.1)
	ciprofloxacin ($\leq 0.12 / > 2$)	$0.125 - 2$	0.25	0.38	0 (0)	33 (100)	0 (0)
	doxycycline ($\leq 1.0 / > 2$)	$\leq 0.016 - 16$	0.38	8	19 (57.6)	6 (18.2)	8 (24.2)
<i>S. intermedius</i> (N = 17)	amoxicillin ($\leq 0.5 / > 2$)	$0.032 - 0.19$	0.094	0.125	17 (100)	0 (0)	0 (0)
	azithromycin ($\leq 0.5 / \geq 2$)	$0.047 - 12$	0.25	0.38	16 (94.1)	0 (0)	1 (5.9)
	clindamycin ($\leq 0.25 / \geq 1$)	$0.047 - 0.19$	0.094	0.19	17 (100)	0 (0)	0 (0)
	ciprofloxacin ($\leq 0.12 / > 2$)	$0.25 - 3$	0.38	0.5	0 (0)	16 (94.1)	1 (5.9)
	doxycycline ($\leq 1.0 / > 2$)	$0.023 - 8$	0.19	4	10 (58.8)	0 (0)	7 (41.2)
both species (N = 50)	amoxicillin ($\leq 0.5 / > 2$)	$\leq 0.016 - 0.5$	0.125	0.38	50 (100)	0 (0)	0 (0)
	azithromycin ($\leq 0.5 / \geq 2$)	$0.032 - > 256$	0.19	0.38	47 (94.0)	0 (0)	3 (6.0)
	clindamycin ($\leq 0.25 / \geq 1$)	$0.016 - > 256$	0.094	0.25	47 (94.0)	1 (2.0)	2 (4.0)
	ciprofloxacin ($\leq 0.12 / > 2$)	$0.125 - 3$	0.38	0.5	0 (0)	49 (98.0)	1 (2.0)
	doxycycline ($\leq 1.0 / > 2$)	$\leq 0.016 - 16$	0.19	6	29 (58.0)	6 (12.0)	15 (30.0)

^a MIC value at which 50% and 90% of isolates of species are inhibited, respectively.

Discussion

The present study provides *in vitro* antibiotic susceptibility test results on the largest number to date (n = 50 total) of *S. constellatus* and *S. intermedius* clinical isolates of subgingival origin recovered from patients in the United States with chronic periodontitis. In general, the antibiotic susceptibility profiles of subgingival *S. constellatus* and *S. intermedius* parallel those of strains of the species recovered at other body sites (24).

Several of the study findings may have clinically relevant therapeutic implications with regard to human periodontitis management. First, 30% of the subgingival *S. constellatus* and *S. intermedius* clinical isolates were resistant *in vitro* to doxycycline. Interestingly, this corresponds with a nearly identical level of doxycycline non-susceptibility recently reported in *S. constellatus* and other anginosus group streptococci isolated from human, non-periodontal, submucosal oromaxillofacial inflammatory infiltrates and odontogenic abscesses in Germany (25). Resistance to doxycycline, as well as other tetracycline family antibiotics, among subgingival *S. constellatus* and *S. intermedius* may compromise clinical periodontal treatment outcomes, and increase risk of progressive periodontitis, as a result of drug-resistant strains surviving in periodontal pockets and oral tissues during active doxycycline or tetracycline therapy. In this regard, high numbers of tetracycline-resistant *S. intermedius* and *Streptococcus anginosus* were found persisting, in the absence of other putative periodontal pathogens, in post-treatment subgingival dental plaque biofilms in a patient with refractory periodontitis experiencing a poor clinical response to systemic tetracycline-HCl therapy administered in conjunction with mechanical root instrumentation and periodontal flap surgery (26). Similarly, doxycycline-resistant subgingival *S. constellatus* and *S. intermedius* were detected at baseline and persisted post-treatment in over 50% of monitored periodontal sites in patients with chronic periodontitis where systemic doxycycline therapy failed to provide significant clinically beneficial effects (27). In another clinical study of refractory chronic periodontitis (9), baseline subgingival recovery of *S. constellatus* at $\geq 3.5\%$ of total DNA probe counts conferred an 8.6 odds ratio for marked progressive periodontal attachment loss within 12 months after the completion of a 28-day systemic tetracycline drug regimen and periodontal flap surgery. These data, along with the present *in vitro* susceptibility findings, suggest a need for clinical caution in the use of doxycycline and other tetracycline family antibiotics in periodontal therapy employed on patients with high numbers of subgingival *S. constellatus* and/or *S. intermedius*.

As expected, nearly all subgingival *S. constellatus* and *S. intermedius* clinical isolates were resistant to metronidazole at a 16 mg/L concentration, consistent with clinical studies demonstrating no statistically significant alterations in drug-resistant subgingival strains of the two species following systemic metronidazole administration in patients with periodontitis (28). This highlights a potential limitation of single antibiotic drug regimens involving metronidazole alone in periodontitis patients harboring *S. constellatus* and *S. intermedius* within polymicrobial subgingival dental plaque biofilms.

Since ciprofloxacin demonstrated only marginal *in vitro* activity against periodontal *S. constellatus* and *S. intermedius*, with nearly all clinical isolates only intermediate in their drug susceptibility to this first-generation fluoroquinolone, its potential clinical value is limited when prescribed alone in test species-positive patients with chronic periodontitis, in

likely contrast to moxifloxacin, which appears to exert greater antimicrobial activity against oral anginosus group streptococci (25). The clustering of ciprofloxacin MIC values for the test species near the drug's non-susceptible breakpoint concentration was also found by Asmah et al. (29) among anginosus group streptococci isolated from pyogenic abscesses at non-periodontal body sites.

No test isolates were resistant *in vitro* to amoxicillin. Consistent with this, clinical studies have shown systemic therapy with amoxicillin plus the β -lactamase inhibitor clavulanic acid led to markedly reduced progressive periodontal attachment loss in patients with refractory chronic periodontitis heavily colonized by subgingival *S. intermedius* in the relative absence of gram-negative periodontal pathogens (30). However, Feres et al. (28) detected large transient increases in amoxicillin-resistant subgingival *S. constellatus* populations secondary to systemic amoxicillin therapy in patients with chronic periodontitis, suggesting that there is likely some degree of non- β -lactamase enzyme-based heterogeneity among United States periodontal *S. constellatus* strains in their sensitivity to amoxicillin. Further research is needed to evaluate the extent and molecular basis to which periodontal *S. constellatus* strains from various areas of the United States, as well as other geographic areas in the world, have amoxicillin susceptibility profiles which differ from our present study collection from the eastern United States.

Clindamycin and azithromycin each displayed a relatively high level of *in vitro* antimicrobial activity against subgingival *S. constellatus* and *S. intermedius*, with only a low proportion of drug-resistant strains identified in the present study. Clindamycin, in addition to its antimicrobial activity against *S. intermedius*, is also capable at sub-MIC concentrations to markedly downregulate the organism's extracellular release of intermedilysin, a cytolytic toxin that may contribute as a virulence factor in *S. intermedius*-associated infections (31).

Conclusions

In conclusion, subgingival isolates of *S. constellatus* and *S. intermedius* *in vitro* were all or nearly all susceptible to amoxicillin, clindamycin, and azithromycin, only intermediate in susceptibility to ciprofloxacin, frequently resistant to doxycycline, and nearly all resistant to metronidazole. Since subgingival *S. constellatus* and *S. intermedius* exhibit variable antibiotic susceptibility profiles, potentially complicating selection of periodontitis antibiotic therapy, microbiological analysis that encompasses antimicrobial sensitivity testing may be particularly helpful in periodontal treatment planning of species-positive patients with refractory periodontitis.

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Chapter 3

Antibiotic susceptibility of periodontal *Enterococcus faecalis*

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Abstract

Purpose: *Enterococcus faecalis* may contribute to periodontal breakdown in heavily infected subgingival sites, particularly in patients responding poorly to mechanical forms of periodontal therapy. Because only limited data are available on the antimicrobial sensitivity of enterococci of subgingival origin, this study evaluated the *in vitro* antibiotic susceptibility of *E. faecalis* isolated from periodontitis patients in the United States.

Materials and methods: Pure cultures of 47 subgingival *E. faecalis* clinical isolates were each inoculated onto specially prepared broth microdilution susceptibility panels containing vancomycin, teicoplanin, and six oral antibiotics of potential use in periodontal therapy. After incubation in ambient air for 18 to 20 hours, minimal inhibitory drug concentrations were determined using applicable Clinical and Laboratory Standards Institute criteria and interpretative guidelines. The organisms were additionally evaluated for *in vitro* resistance to metronidazole at 4 mg/L.

Results: Periodontal *E. faecalis* exhibited substantial *in vitro* resistance to tetracycline (53.2% resistant), erythromycin (80.8% resistant or intermediate resistant), clindamycin (100% resistant to 2 mg/L), and metronidazole (100 % resistant to 4 mg/L). In comparison, the clinical isolates were generally sensitive to ciprofloxacin (89.4% susceptible; 10.6% intermediate resistant), and 100% susceptible *in vitro* to ampicillin, amoxicillin/clavulanate, vancomycin, and teicoplanin.

Conclusions: Tetracycline, erythromycin, clindamycin, and metronidazole revealed poor *in vitro* activity against human subgingival *E. faecalis* clinical isolates, and would likely be ineffective therapeutic agents against these species in periodontal pockets. Among orally-administered antibiotics, ampicillin, amoxicillin/clavulanate, and ciprofloxacin exhibited marked *in vitro* inhibitory activity against periodontal *E. faecalis*, and may be clinically useful in treatment of periodontal infections involving enterococci.

Introduction

Enterococcus faecalis is a gram-positive, facultative coccus frequently implicated as an opportunistic pathogen in various types of nosocomial infections in immunocompromised patients (1). The organisms normally inhabit the gastrointestinal tracts of humans, farm animals, dogs, cats, ducks, and many types of insects, as well as environments contaminated by human and animal feces (1,2). The alarming emergence in North America during the past 20 years of vancomycin-resistant *E. faecalis* and *Enterococcus faecium* strains (3), which are potentially multi-resistant to currently available antibiotics, has sparked increased clinical concerns about how best to treat and prevent human enterococcal infections (4).

Enterococci appear to be absent or infrequently found on supragingival and subgingival tooth surfaces and mucosa in the healthy human oral cavity (5). When present, *E. faecalis* is most closely identified with persistent endodontic infections (6), possibly introduced into the oral microbiome via dietary consumption of certain types of aged cheese and other organism-positive food products (7,8).

E. faecalis may opportunistically colonize periodontal pockets, and in some persons, may also contribute to periodontal breakdown in heavily infected sites (9-11). The organism has been recovered from the subgingival periodontitis microbiota (12-16), especially in patients responding poorly to mechanical forms of periodontal therapy (9,10). Persons positive for human immunodeficiency virus (HIV) infection (17), particularly those with necrotizing gingival lesions (11), also frequently yield periodontal *E. faecalis*. Because local and/or systemic antimicrobial therapy is increasingly employed on these types of periodontitis patients (18-20), and only limited data are available on the antimicrobial sensitivity of enterococci of subgingival origin (9,14,21,22), particularly from the United States (9), there is a need to further assess the antibiotic susceptibility of *E. faecalis* colonizing periodontal pockets to minimize clinical use and potential adverse effects of periodontal antimicrobial chemotherapy to which subgingival enterococci are resistant in organism-positive patients. As a result, this study evaluated the *in vitro* susceptibility of fresh subgingival *E. faecalis* clinical isolates, recovered from advanced chronic periodontitis patients in the United States, to various antibiotics of potential use in periodontal therapy.

Materials and methods

Patients and bacterial strains

A total of 47 subgingival *E. faecalis* clinical isolates were recovered over a 12-month time period from 2,764 patient subgingival plaque specimens (1.7% *E. faecalis*-positive recovery rate) analyzed by the Oral Microbiology Testing Service (OMTS) Laboratory at Temple University School of Dentistry, Philadelphia, Pennsylvania. Each of the clinical isolates originated from one of 47 systemically healthy, non-hospitalized, adults (19 males and 28 females; aged 31 to 76 years; mean \pm SD age: 55.2 \pm 12.4 years) exhibiting advanced chronic periodontitis (23), as diagnosed by 27 periodontists in private dental practices in the United States, of which nearly all were geographically located in East Coast states.

The *E. faecalis* clinical isolates were recovered from subgingival plaque specimens, obtained using a standardized sampling protocol, from 29 patients before periodontal therapy, and from 18 patients three to six months after the completion of conventional mechanical periodontal therapy when an unsatisfactory clinical outcome relative to resolution of inflamed deep probing depths was reported by the treating periodontist. None of the patients received any systemic antibiotic therapy within 3 months before subgingival sampling. After isolation with cotton rolls and removal of saliva and supragingival deposits, one to two sterile, absorbent paper points (Johnson & Johnson, East Windsor, NJ, USA) were advanced for approximately 10 seconds into each of the three to five deepest periodontal pockets (> 6 mm) per patient who exhibited bleeding on probing. After removal, all paper points per patient were pooled into a glass vial containing six to eight small glass beads and 2.0 ml of anaerobically prepared and stored VMGA III transport medium (24). The subgingival samples were then transported within 24 hours to the OMTS Laboratory, which is licensed for high-complexity bacteriologic analysis by the Pennsylvania Department of Health. The OMTS Laboratory is also federally-certified by the United States Department of Health and Human Services to be in compliance with Clinical Laboratory Improvement Amendments-mandated proficiency testing, quality control, patient test management, personnel requirements, and quality-assurance standards required of clinical laboratories engaged in diagnostic testing of human specimens in the United States (25). All laboratory procedures were performed by personnel who were masked to the clinical status of the patients, and their inclusion in the present analysis. The study was approved by the Temple University Human Subjects Protections Institutional Review Board and conducted in accordance with the Helsinki Declaration of 1975, as revised in 2000.

At the OMTS Laboratory, the specimen vials were warmed to 35°C to liquefy the VMGA III transport medium, and sampled microorganisms were mechanically dispersed from the paper points with a Vortex mixer at the maximal setting for 45 seconds. Serial 10-fold dilutions of the dispersed bacteria were prepared in Möller's VMG I anaerobic dispersion solution (24), and appropriate 0.1 ml dilution aliquots were spread with a sterile bent glass rod onto non-selective enriched Brucella blood agar (EBBA) primary isolation plates (26), comprised of 4.3% Brucella agar supplemented with 0.3% bacto-agar, 5% defibrinated sheep blood, 0.2% hemolyzed sheep red blood cells, 0.0005% hemin, and 0.00005% menadione. Additional sample dilutions were inoculated onto EBBA primary isolation plates supplemented with metronidazole (Sigma-Aldrich, St. Louis, MO, USA) at 4 mg/L. All EBBA plates were incubated at 35°C for seven days in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI, USA) containing 85% N₂-10% H₂-5% CO₂.

Periodontal *E. faecalis* was presumptively identified as relatively large (3.0-4.0 mm in diameter), shiny, gray-white, non-adherent, catalase-negative, flat, circular surface colonies with slightly irregular edges and variable hemolysis on anaerobically incubated EBBA (9). A micromethod kit system (RapID STR, Innovative Diagnostic Systems, Atlanta, GA, USA) was used to confirm *E. faecalis* clinical isolate identification and biotype, with preparation and inoculation of kit panels performed as recommended by the manufacturer. A nitrocefin-based qualitative chromogenic disk assay (BBL Cefinase, BD Diagnostic Systems, Sparks, MD, USA), following manufacturer's instructions, was used to test the *E. faecalis* clinical isolates for β -lactamase enzyme activity. The proportional subgingival recovery of *E. faecalis* per organism-positive patient was determined by comparing their

colony forming units (CFU) among total viable anaerobic CFU counts on EBBA primary isolation plates.

The occurrence and proportions of *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Parvimonas micra*, *Prevotella intermedia/nigrescens*, *Campylobacter rectus*, *Fusobacterium nucleatum*, *Streptococcus constellatus*, gram-negative enteric rods/pseudomonads, *Staphylococcus aureus*, and *Candida* species were also examined for in each patient's pool of subgingival specimens to assess the distribution of co-infecting putative periodontal pathogens in *E. faecalis*-positive chronic periodontitis patients, using EBBA and selective culture media, incubation conditions, and presumptive phenotypic methods previously described (27-29).

***In vitro* antimicrobial susceptibility testing**

The 47 *E. faecalis* clinical isolates were each grown in pure culture on EBBA incubated overnight in air plus 5% CO₂, from which cell suspensions were prepared and adjusted to a 5 x 10⁵ organisms/ml density using a spectrophotometer with a 1.0 cm light path at 625 nm (0.08-0.10 absorbance range). The suspensions were then inoculated onto specially prepared broth microdilution susceptibility panels (Dade Microscan, West Sacramento, CA, USA), containing cation-adjusted Mueller-Hinton broth and the following antibiotics in two-fold concentration ranges: ampicillin (0.12 to 8 mg/L), amoxicillin/potassium clavulanate (0.5/0.25 to 16/8 mg/L), ciprofloxacin (0.25 to 2 mg/L), clindamycin (0.25 to 2 mg/L), erythromycin (0.12 to 4 mg/L), teicoplanin (0.25 to 16 mg/L), tetracycline-HCl (2 to 8 mg/L), and vancomycin (0.25 to 16 mg/L). High-level aminoglycoside resistance was evaluated in wells containing 2,000 mg/L of either gentamicin or streptomycin. *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 were used as quality-control organisms. Minimal inhibitory concentrations (MIC), defined as the lowest antibiotic concentration that completely inhibited visible growth, were determined following panel incubation in ambient air at 35°C for 18 to 20 hours.

In vitro resistance to metronidazole at 4 mg/L was recorded per patient when *E. faecalis* growth was noted on metronidazole-supplemented EBBA primary isolation plates (9,26,29,30). *Bacteroides thetaiotaomicron* ATCC 29741, *Clostridium perfringens* ATCC 13124, and a multi-antibiotic-resistant clinical periodontal isolate of *F. nucleatum* were used as positive and negative quality controls for antibiotic resistance testing on metronidazole-supplemented EBBA primary isolation plates.

Data analysis

MIC₅₀ and MIC₉₀ values for each antibiotic were determined as the MIC level that completely inhibited 50% and 90%, respectively, of the tested subgingival *E. faecalis* clinical isolates. MIC interpretative guidelines published in 2012 for *Enterococcus* species from the Clinical and Laboratory Standards Institute (CLSI) (31) were used to categorize test antibiotic *in vitro* inhibitory activity against the *E. faecalis* clinical isolates, except for clindamycin and metronidazole, where no applicable CLSI standards against enterococci are available. Ampicillin MIC interpretive criteria were applied to amoxicillin/clavulanate *in vitro* test values.

Results

45 (95.7%) of the *E. faecalis* clinical isolates belonged to the same biotype, which were negative for hemolysin; fermented mannitol and sorbitol; hydrolyzed arginine, tyrosine, hydroxyproline, lysine, pyrrolidone, phosphatase and esculin; and elaborated α -D-glucosidase, α -D-galactosidase and N-acetyl- β -D-glucosaminidase. Two other biotypes exhibited similar reactions, but one failed to hydrolyze hydroxyproline, and another was positive for hemolysin and did not hydrolyze tyrosine and phosphatase. No β -lactamase enzyme activity was detected among the 47 *E. faecalis* clinical isolates with nitrocefin-based chromogenic testing.

E. faecalis comprised a mean \pm SE of $49.7 \pm 4.0\%$ (median, 55.4%; interquartile range, 24.5 to 72.7%; total range, 1.1 to 95.2%) of total subgingival anaerobic viable counts in the 47 organism-positive patients with advanced chronic periodontitis. *E. faecalis* was $> 50\%$ of the subgingival cultivable microbiota in 25 patients, from 10 to 50% in 20 patients, and $< 10\%$ in two patients. In 43 (91.5%) patients, ≥ 1 of the evaluated putative periodontal pathogens were detected in subgingival co-infection with *E. faecalis*, with *P. gingivalis* and *P. micra* most frequently recovered in the majority of patients with *E. faecalis*-positive chronic periodontitis (Table 1).

Table 2 provides MIC₅₀ and MIC₉₀ values of test antimicrobial agents against subgingival *E. faecalis*. Teicoplanin and amoxicillin/clavulanate exhibited the lowest MIC₉₀ values (≤ 0.25 and $\leq 0.5/0.25$ mg/L, respectively), whereas tetracycline-HCl displayed MIC₉₀ values which exceeded 8.0 mg/L against subgingival *E. faecalis*.

Table 2 also lists the distribution of susceptible, intermediate, and resistant test results against *E. faecalis* clinical isolates for test antibiotics with available CLSI interpretive standards for enterococci. Substantial *in vitro* *E. faecalis* resistance was found to tetracycline-HCl (53.2% resistant) and erythromycin (80.8% resistant or intermediate resistant), with 19 (40.4%) of the clinical isolates jointly non-susceptible *in vitro* to both tetracycline-HCl and erythromycin. In comparison, subgingival *E. faecalis* was generally sensitive to ciprofloxacin, with 89.4% of the clinical isolates classified as susceptible and 10.6% intermediate resistant (Table 2). All *E. faecalis* clinical isolates were 100% susceptible *in vitro* to ampicillin, amoxicillin/clavulanate, vancomycin, and teicoplanin, according to CLSI MIC interpretative guidelines (Table 2).

In addition, all *E. faecalis* clinical isolates were resistant *in vitro* to clindamycin at 2 mg/L and metronidazole at 4 mg/L. High-level aminoglycoside resistance was rare among subgingival *E. faecalis*, with only three (6.0%) clinical isolates demonstrating *in vitro* resistance to streptomycin at 2,000 mg/L, and none to gentamicin at 2,000 mg/L. The single hemolysin-positive *E. faecalis* clinical isolate was resistant *in vitro* to tetracycline-HCl, erythromycin, clindamycin at 2 mg/L, and metronidazole at 4 mg/L.

Table 1. Co-infecting putative periodontal pathogens recovered in 47 patients with *E. faecalis*-positive chronic periodontitis.

Organism	No. (%) positive patients	Mean \pm SE recovery (%)	Median recovery (%)	Interquartile range (%)	Total range (%)
<i>P. gingivalis</i>	31 (66.0)	8.7 \pm 2.4	2.4	0.3 to 12.2	0.04 to 41.7
<i>P. micra</i>	28 (59.6)	4.1 \pm 0.9	3.0	0.9 to 5.1	0.01 to 22.0
<i>P. intermedia/nigrescens</i>	21 (44.7)	3.0 \pm 0.8	2.1	0.1 to 4.9	0.002 to 13.5
<i>C. rectus</i>	21 (44.7)	1.8 \pm 0.4	0.9	0.2 to 3.3	0.01 to 7.8
<i>S. constellatus</i>	13 (27.7)	1.3 \pm 0.4	0.7	0.4 to 2.7	0.02 to 3.8
<i>F. nucleatum</i>	10 (21.3)	1.6 \pm 0.3	1.6	1.0 to 2.4	0.3 to 3.0
enteric rods/pseudomonads	9 (19.2)	19.2 \pm 9.6	5.2	0.3 to 32.0	0.04 to 85.4
<i>A. actinomycetemcomitans</i>	5 (10.6)	0.1 \pm 0.04	0.01	0.01 to 0.03	0.01 to 0.2
<i>Candida</i> species	4 (8.5)	0.2 \pm 0.2	0.2	0.01 to 0.6	0.01 to 0.6
<i>S. aureus</i>	1 (2.1)	21.2 \pm 0.0	21.2	21.2	21.2

Table 2. *In vitro* susceptibility (mg/L) of 47 periodontal *E. faecalis* clinical isolates to antimicrobials with available CLSI MIC interpretative standards for enterococci.

Antimicrobial agent	MIC ₅₀	MIC ₉₀	Range	No. (%) isolates per CLSI interpretative category		
				susceptible	intermediate	resistant
ampicillin	0.5	1	0.5-1	47 (100)	0	0
amoxicillin/clavulanate	≤ 0.5/0.25*	≤ 0.5/0.25*	≤ 0.5/0.25*	47 (100)	0	0
ciprofloxacin	1	2	≤ 0.25* -2	42 (89.4)	5 (10.6)	0
erythromycin	4	> 4 [†]	≤ 0.12* - > 4 [†]	9 (19.2)	26 (55.3)	12 (25.5)
teicoplanin	≤ 0.25*	≤ 0.25*	≤ 0.25*	47 (100)	0	0
tetracycline-HCl	> 8 [†]	> 8 [†]	≤ 2* - > 8 [†]	22 (46.8)	0	25 (53.2)
vancomycin	1	2	0.5-2	47 (100)	0	0

* = Lowest concentration tested.

† = Highest concentration tested.

Discussion

Relatively sparse data are available on the antibiotic sensitivity profile of subgingival enterococci. Rams et al. (9) reported in 1992 on the *in vitro* antibiotic susceptibility of 12 subgingival *E. faecalis* clinical isolates from United States periodontitis patients, and more recently, 23 to 106 subgingival *E. faecalis* strains were assessed from periodontitis patients in Norway (14,21,22). However, because antibiotic resistance patterns of *E. faecalis*, and subgingival bacterial species in particular, potentially change over time (3,4,32), and exhibit geographic-based differences (30,33,34), these previous studies of subgingival *E. faecalis* conducted 20 years ago (9) or in Europe (14,21,22) may not necessarily be pertinent to contemporary clinical periodontal practice in the United States. This study tested the *in vitro* antibiotic susceptibility of the largest group of subgingival *E. faecalis* clinical isolates (n = 47) assembled to date from periodontitis patients of United States origin.

Importantly, vancomycin resistance was not detected among United States subgingival *E. faecalis* clinical isolates. Because vancomycin resistance was also not found in periodontal *E. faecalis* in Norway (14,21,22), and *E. faecalis* recovered from root canals (35-39) and oral rinse/saliva/intraoral surface samples (40-41), it appears that the human oral cavity in community-dwelling dental patients is not a significant reservoir for carriage and potential dissemination of vancomycin-resistant *E. faecalis*. The occurrence of vancomycin-resistant *E. faecalis*, as well as the resistance of the organism to other antimicrobial agents, such as ciprofloxacin, are markedly higher and concentrated in clinical infection isolates from hospitalized patients compared to non-hospitalized, community population groups (34).

Tetracycline-HCl, erythromycin, clindamycin, and metronidazole were found to exert relatively poor *in vitro* activity against subgingival *E. faecalis*, similar to previous evaluations of other periodontal and intraoral *E. faecalis* strains (14,21,22,35-42). Although clindamycin and metronidazole may be used in periodontal therapy (18-20), *in vitro* clindamycin and metronidazole resistance among facultative *E. faecalis* was expected since the organism is intrinsically resistant to clindamycin, and metronidazole possesses an antimicrobial spectrum limited to anaerobic bacteria and protozoa (43). As a result, these drugs, as well as tetracycline-HCl and erythromycin, would likely be ineffective therapeutic agents against *E. faecalis* in periodontal pockets, and may even be contraindicated in patients with heavy subgingival enterococcal colonization in order to reduce the risk of post-drug emergence of potentially-adverse *E. faecalis* superinfections by resistant strains. In contrast, all subgingival *E. faecalis* were negative for β -lactamase enzyme activity in the present study, similar to previous reports on oral *E. faecalis* strains (36,42), and all were susceptible *in vitro* to both ampicillin and amoxicillin/clavulanate at therapeutically attainable concentrations, also consistent with previous studies of periodontal and intraoral *E. faecalis* strains (14,21,22,35-42).

It is important to note that generally high subgingival proportions (mean: 49.7% of total subgingival viable count) of *E. faecalis* were recovered from the organism-positive patients, with most (91.5%) yielding *E. faecalis* as part of a mixed polymicrobial infection in deep periodontal pockets with various putative periodontal pathogens, most notably *P. gingivalis* and *P. micra* (Table 1). The occurrence and proportions of subgingival co-infecting putative periodontal pathogens in *E. faecalis*-positive chronic periodontitis has not

been previously reported and may help illuminate potential disease-associated subgingival microbial interactions, because the pathogenicity of *E. faecalis* is enhanced in experimental subcutaneous mice abscess formation via co-infection with *P. micra* (44).

Little attention has been given to potential therapeutic implications of heavy *E. faecalis* growth in periodontal pockets concurrent with large numbers of anaerobic periodontal pathogens, particularly in periodontitis patients refractory to mechanical forms of periodontal therapy (9,10). Colombo et al. (10) found subgingival *E. faecalis* and anaerobic periodontal pathogens in three of 14 (21.4%) periodontitis patients experiencing widespread progressive clinical periodontal attachment loss within 12 months after treatment with subgingival mechanical debridement, modified Widman flap surgery, systemic tetracycline-HCl therapy, and systematic three-month periodontal maintenance care. Refractory periodontitis patients also revealed high serum antibody titers to *E. faecalis* and several anaerobic periodontal pathogens, likely reflective of their oral colonization, more often as compared to successfully treated periodontitis patients and periodontally healthy subjects (45). High *E. faecalis* counts additionally have been detected with anaerobic periodontal pathogens in active necrotizing gingivitis/periodontitis lesions in HIV-positive individuals (11). *E. faecalis* can participate in biofilm formation on non-shedding surfaces (21,22), appears to be inadequately suppressed by subgingival mechanical debridement therapy (9,10), and may inactivate metronidazole drug regimens targeted against anaerobic bacterial pathogens (46,47). In turn, a number of anaerobic periodontal pathogens may elaborate β -lactamase enzymes (48,49), which have the potential to inactivate β -lactam antibiotics that otherwise would likely be active against subgingival *E. faecalis*. Thus, subgingival co-infection by high proportions of metronidazole-inactivating *E. faecalis*, along with β -lactamase producing anaerobic periodontal pathogens, may potentially resist amoxicillin plus metronidazole combination drug regimens frequently recommended for refractory periodontitis (18-20). In these situations, which appear to be infrequent given the low occurrence of subgingival enterococci in United States patients with periodontitis (9), metronidazole may be better paired with amoxicillin/clavulanate, which competitively inhibits β -lactamase enzymes (50), or alternatively with ciprofloxacin, if *in vitro* testing reveals the enterococci to be ciprofloxacin susceptible, and a review of the patient's medical history and current medications indicate that the antibiotic drugs can be safely administered. With all antimicrobial regimens, subgingival mechanical debridement immediately before or concurrent with antimicrobial chemotherapy appears essential with *E. faecalis* periodontal infections, because the organism in undisturbed surface biofilms compared to dispersed planktonic phase cells exhibits markedly enhanced antibiotic resistance (21), similar to biofilms formed by other subgingival microbial species (51,52). Because no consensus presently exists on optimal management of patients with refractory periodontitis presenting with mixed enterococci-anaerobic periodontal pathogen subgingival biofilm populations, additional clarifying clinical studies on these potentially problematic patient cases are urgently needed.

Conclusions

Tetracycline-HCl, erythromycin, clindamycin, and metronidazole revealed poor *in vitro* activity against human subgingival *E. faecalis* clinical isolates and, thus, would likely be ineffective therapeutic agents against these species in periodontal pockets. Among orally administered antibiotics, ampicillin, amoxicillin/clavulanate, and ciprofloxacin exhibited

marked *in vitro* inhibitory activity against all or most periodontal *E. faecalis* and may be clinically useful in treatment of periodontal infections involving enterococci.

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Chapter 4

Prevalence of β -lactamase-producing bacteria in human periodontitis

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Abstract

Purpose: β -lactam antibiotics prescribed in periodontal therapy are vulnerable to degradation by bacterial β -lactamase enzymes. This study evaluated the occurrence of β -lactamase enzyme-positive subgingival bacteria in chronic periodontitis subjects of USA origin, and assessed their *in vitro* resistance to metronidazole at a breakpoint concentration of 4 mg/L.

Materials and methods: Subgingival plaque specimens from deep periodontal pockets with bleeding on probing were removed from 564 adults with severe chronic periodontitis before treatment. The samples were transported in VMGA III, and then plated onto: (i) non-selective enriched Brucella blood agar (EBBA) and incubated anaerobically for seven days; and (ii) selective trypticase soy-bacitracin-vancomycin (TSBV) and incubated for three days in air + 5% CO₂. At the end of the incubation periods, the bacterial test species were identified and quantified. Specimen dilutions were also plated onto EBBA plates supplemented with 2 mg/L of amoxicillin, a combination of 2 mg/L of amoxicillin plus 2 mg/L of the β -lactamase inhibitor clavulanic acid, or 4 mg/L of metronidazole, followed by anaerobic incubation for seven days. Bacterial test species presumptively positive for β -lactamase production were identified by growth on EBBA primary isolation plates supplemented with amoxicillin alone and no growth on EBBA primary isolation plates containing both amoxicillin plus clavulanic acid. A subset of such isolates was subjected to nitrocefin-based chromogenic disk testing to confirm presence of β -lactamase activity. *In vitro* resistance to 4 mg/L of metronidazole was noted when growth of test species occurred on metronidazole-supplemented EBBA culture plates.

Results: 294 (52.1%) of the study subjects yielded β -lactamase-producing subgingival bacterial test species, with *Prevotella intermedia/nigrescens*, *Fusobacterium nucleatum* and other *Prevotella* species most frequently identified as β -lactamase-producing organisms. Of the β -lactamase-producing bacterial test species strains recovered, 98.9% were susceptible *in vitro* to metronidazole at 4 mg/L.

Conclusions: The occurrence of β -lactamase-positive subgingival bacterial species in more than half of the subjects with severe chronic periodontitis raises questions about the therapeutic potential of single-drug regimens with β -lactam antibiotics in periodontal therapy. The *in vitro* effectiveness of metronidazole against nearly all recovered β -lactamase-producing subgingival bacterial species further supports clinical periodontitis treatment strategies involving the combination of systemic amoxicillin plus metronidazole.

Introduction

β -lactamase production represents a major virulence factor by which pathogenic bacteria evade the broad-spectrum antimicrobial effects of β -lactam antibiotics and perpetuate human infections, including those in orofacial tissues (1). Bacterial β -lactamases rapidly hydrolyze amide bonds within the four-membered ring forming the foundational structure of β -lactam antibiotics, leaving them pharmacologically inactive as antimicrobial agents that disrupt bacterial cell wall peptidoglycan biosynthesis (1).

β -lactamase activity has been detected in subgingival sites of chronic periodontitis subjects at levels capable of inactivating β -lactam antibiotics passing into periodontal pockets through gingival crevicular fluid exudate (2). Subgingival β -lactamase has been significantly correlated with increasing periodontal probing depth measurements (2), recent treatment with systemic penicillin drugs (3), and carriage of β -lactamase-encoding genes by microbial species in subgingival plaque biofilms (4). Studies of patients with chronic periodontitis in the USA (3-5), The Netherlands (6,7), Spain (7), Norway (8), France (9,10), and the United Kingdom (11) have reported a 53.2% to 100% occurrence in subjects for subgingival β -lactamase-producing bacteria, with higher prevalence rates found in localities with greater over-the-counter access and consumption of systemic antimicrobial agents (7). However, these findings are limited by their inclusion of relatively few study subjects (12 to 47 patients with periodontitis per study), who were mostly dental school patients and/or from localized geographic regions that may not be representative of more diverse community populations. For example, data from the USA on the occurrence of subgingival β -lactamase-producing bacterial species in patients with chronic periodontitis is presently derived from a total of 42 dental school patients in Connecticut, and 25 in Florida (3,5).

As a result, there is a need to assess subgingival β -lactamase-producing bacteria in larger-sized subject groups that are geographically distributed beyond a single city or dental school patient population. The aim of the present study was to evaluate the occurrence of β -lactamase-positive subgingival bacteria in 564 geographically subjects in the USA with chronic periodontitis, and to assess their *in vitro* resistance to metronidazole at a breakpoint concentration of 4 mg/L.

Materials and methods

Subjects

A total of 564 adults (270 men and 294 women; age range, 33-91 years; mean age \pm standard deviation = 49.1 ± 11.7 years), diagnosed with severe chronic periodontitis (12) by periodontists in private dental practices in the USA, were included in the present study as their subgingival plaque samples were consecutively received for microbiological analysis by the Oral Microbiology Testing Service (OMTS) Laboratory at Temple University School of Dentistry, Philadelphia (PA, USA). 354 (62.8%) of the study subjects geographically originated from Maryland ($n = 174$), Pennsylvania ($n = 91$), New Jersey ($n = 51$), Delaware ($n = 25$), Virginia ($n = 7$), and the District of Columbia ($n = 6$) in the mid-Atlantic region of the USA, with all others from Connecticut ($n = 48$), Florida ($n = 35$), Illinois ($n = 20$), 11 other states in the eastern USA ($n = 47$) and Texas ($n = 60$). Persons identified with aggressive periodontitis, or antibiotic use in the past six months, were

excluded. Approval for the study was provided by the Temple University Human Subjects Protections Institutional Review Board.

Microbial sampling and transport

Subgingival plaque specimens were obtained by the diagnosing periodontists, who followed a standardized sampling protocol, before treatment from three to five deep (≥ 6 mm) periodontal pockets in each subject that exhibited bleeding on probing during the initial diagnostic evaluation. After isolation with cotton rolls, and removal of saliva and supragingival deposits, one to two sterile, absorbent paper points (Johnson & Johnson, East Windsor, NJ, USA) were advanced into each selected periodontal site for approximately 10 seconds. Upon removal, all paper points per study subject were pooled in a glass vial containing six to eight small glass beads and 2.0 ml of anaerobically prepared and stored VMGA III transport medium (13), which possesses a high preservation capability for oral microorganisms during post-sampling transit to the laboratory (13,14). The subgingival samples were then transported within 24 hours to the OMTS Laboratory, which is licensed for high-complexity bacteriological analysis by the Pennsylvania Department of Health. The OMTS Laboratory is also federally certified by the United States Department of Health and Human Services to be in compliance with Clinical Laboratory Improvement Amendments-mandated proficiency testing, quality control, patient test management, personnel requirements and quality assurance standards required of clinical laboratories engaged in diagnostic testing of human specimens in the USA (15). All laboratory procedures were performed by personnel who were blinded to the clinical status of the study subjects and their inclusion in the present analysis.

Microbial culture and incubation

At the OMTS Laboratory, the specimen vials were warmed to 35°C to liquefy the VMGA III transport medium, and sampled microorganisms were mechanically dispersed from the paper points with a vortex mixer, which was used at the maximal setting for 45 seconds. Serial, 10-fold dilutions of the dispersed bacteria were prepared in Möller's VMG I anaerobic dispersion solution, comprised of prerduced, anaerobically sterilized, 0.25% tryptose, 0.25% thiotone E peptone, and 0.5% NaCl (13). Then, 0.1 ml dilution aliquots were spread, with a sterile bent glass rod, onto nonselective enriched Brucella blood agar (EBBA) primary isolation plates (16), comprised of 4.3% Brucella agar supplemented with 0.3% bacto-agar, 5% defibrinated sheep blood, 0.2% hemolyzed sheep red blood cells, 0.0005% hemin and 0.00005% menadione, and onto selective trypticase soy-bacitracin-vancomycin (TSBV) agar (17). The inoculated EBBA plates were incubated at 35°C for seven days in a Coy anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI, USA) containing 85% N₂, 10% H₂ and 5% CO₂, and the TSBV plates were incubated at 35°C for three days in air + 5% CO₂.

Test species identification

Total anaerobic viable counts, and counts of the test species *Porphyromonas gingivalis*, *Prevotella intermedia/nigrescens*, other *Prevotella* species (including *Prevotella melaninogenica* and non-pigmented *Prevotella* species), *Fusobacterium nucleatum*, *Parvimonas micra*, *Capnocytophaga* species, *Streptococcus constellatus*, *Centipeda periodontii*, *Enterococcus*

coccus faecalis, and staphylococci, were made on non-selective EBBA primary isolation plates using a ring-light magnifying loupe, presumptive phenotypic methods previously described (18-21), and the RapID ANA II (Innovative Diagnostic Systems, Atlanta, GA, USA) micro-method kit system for selected isolates. *Aggregatibacter actinomycetemcomitans*, gram-negative enteric rods/pseudomonads, and *Candida* species were quantitated on selective TSBV agar, as previously described (16,17). The proportional recovery of each test species was ascertained in each subject by calculating as the percentage of each test species colony-forming units relative to total subgingival anaerobic viable counts, as determined on non-selective EBBA primary isolation plates.

Detection of β -lactamase-producing organisms

Additional 0.1 ml aliquots of subgingival sample dilutions were inoculated onto EBBA primary isolation plates supplemented with either 2 mg/L of amoxicillin, which was previously established as a susceptibility breakpoint for aminopenicillin antibiotics (22), or a combination of 2 mg/L of amoxicillin plus 2 mg/L of the β -lactamase-inhibitor, clavulanic acid (23), followed by anaerobic incubation. Direct colony suspensions (equivalent to a 0.5 McFarland standard) of pure *A. actinomycetemcomitans* isolates from selective TSBV were subcultured onto these media as their recognition is frequently obscured within mixed bacterial populations on non-selective EBBA primary isolation plates (17).

A surface-overlay technique was used to add clavulanic acid, at a concentration of 2 mg/L, to amoxicillin-containing EBBA primary isolation plates (24). Using a sterile glass rod, 0.1 ml of a fresh 400 mg/L solution of clavulanic acid, prepared by dissolving 4.2 mg of a 95.3% pure lithium clavulanate powder (provided by SmithKline Beecham, Collegeville, PA, USA) into 10 ml of sterile 0.1 M phosphate buffer (pH 6.0), was evenly spread over the surfaces of 20 ml EBBA plates supplemented with amoxicillin. The plates were held at room temperature for 30 minutes to allow surface drying and subsurface drug diffusion, and then inoculated with subgingival plaque specimens.

Test species positive for β -lactamase production were presumptively identified by growth on EBBA primary isolation plates supplemented with amoxicillin alone and by no growth on EBBA primary isolation plates containing both amoxicillin plus clavulanic acid (6,7). A subset of 50 such presumptively identified isolates was subjected to a nitrocefin-based qualitative chromogenic disk assay (BBL Cefinase; BD Diagnostic Systems, Sparks, MD, USA), following manufacturer's instructions, to confirm presence of β -lactamase activity.

In vitro antibiotic resistance testing

Aliquots (0.1 ml) of subgingival sample dilutions were also inoculated onto EBBA primary isolation plates supplemented with metronidazole at a susceptibility breakpoint concentration of 4 mg/L (22), and incubated anaerobically for seven days, in order to assess *in vitro* resistance to metronidazole among β -lactamase-producing clinical isolates. *In vitro* resistance to metronidazole was defined as test species growth on metronidazole-supplemented EBBA primary isolation plates (16,20,25,26). *Bacteroides thetaiotaomicron* ATCC 29741, *Clostridium perfringens* ATCC 13124 and a multi-antibiotic-resistant clinical periodontal isolate of *F. nucleatum* were employed as positive and negative quality

controls for the antibiotic resistance testing. All antimicrobials were obtained as pure powder from Sigma-Aldrich (St. Louis, MO, USA).

Data analysis

Descriptive analysis were used to calculate mean subject age and standard deviation values, the occurrence and proportional cultivable recovery of test species in subjects, the occurrence and proportional recovery of β -lactamase-producing organisms in subjects, and the occurrence in subjects of *in vitro* metronidazole drug resistance among the subgingival clinical isolates. Data analysis was performed using the SAS 9.2 for Windows (SAS Institute, Inc., Cary, NC, USA) statistical software package.

Results

A subset of 50 test species, presumptively identified as β -lactamase positive by their growth on amoxicillin-supplemented EBBA primary isolation plates and by no growth on amoxicillin plus clavulanic acid-supplemented EBBA primary isolation plates, were all confirmed as β -lactamase-producing organisms with the nitrocefin chromogenic disk assay (data not shown).

A total of 294 (52.1%) study subjects yielded β -lactamase-producing subgingival test species. *P. intermedia/nigrescens*, *F. nucleatum*, and other *Prevotella* species were most frequently identified as β -lactamase-positive species, with 51.0% of all study subjects with cultivable *P. intermedia/nigrescens*, 24.6% with *F. nucleatum* and 66.2% with other *Prevotella* species exhibiting β -lactamase-producing strains of these species (Table 1). β -lactamase production was also found among 0.8% of *P. micra* species, 2.0% of *Capnocytophaga* species and 5.0% of gram-negative enteric rods/pseudomonads in culture-positive subjects (Table 1).

A single β -lactamase-producing subgingival test species was recovered from 236 (80.3%) subjects, whereas 54 (18.4%) and four (1.3%) subjects each yielded two and three different β -lactamase-producing test species, respectively (Figure 1).

Subgingival proportions of β -lactamase-positive *P. intermedia/nigrescens*, *F. nucleatum* and *P. micra* averaged 6.7-9.9%, whereas mean subgingival recovery levels of < 1% were found for β -lactamase-positive isolates of other *Prevotella* and for *Capnocytophaga* species (Table 1). 353 (98.9%) of the 357 β -lactamase-producing subject test species recovered (Table 1) were susceptible *in vitro* to 4 mg/L of metronidazole, except for one *P. intermedia/nigrescens* strain, one *F. nucleatum* strain, and gram-negative enteric rods/pseudomonads in two subjects.

All recovered strains of *P. gingivalis*, *A. actinomycetemcomitans*, *S. constellatus*, *C. periodontii*, *E. faecalis*, and *Staphylococcus* species, failed to demonstrate presumptive β -lactamase activity (Table 1).

Table 1. Occurrence and proportional subgingival recovery of β -lactamase-positive test species in 564 adults with severe chronic periodontitis.

Species	No. (%) culture positive subjects ^b	No. (%) subjects with β -lactamase enzyme-positive isolates ^c	Mean % \pm SE recovery of β -lactamase enzyme-positive isolates	Range %
<i>P. intermedia/nigrescens</i>	449 (79.6)	229 (40.6 ^b /51.0 ^d)	9.6 \pm 0.8	0.001-62.1
<i>F. nucleatum</i>	293 (52.0)	72 (12.8/24.6)	6.7 \pm 0.9	0.001-29.4
other <i>Prevotella</i> species ^a	68 (12.1)	45 (8.0/66.2)	0.6 \pm 0.5	0.001-24.2
<i>Capnocytophaga</i> species	251 (44.5)	5 (0.9/2.0)	0.3 \pm 0.1	0.002-0.6
<i>P. micra</i>	495 (87.8)	4 (0.7/0.8)	9.9 \pm 3.9	1.5-19.7
enteric rods/pseudomonads	40 (7.1)	2 (0.4/5.0)	1.7 \pm 0.8	0.9-2.5
<i>P. gingivalis</i>	197 (34.9)	0	0	0
<i>A. actinomycetemcomitans</i>	85 (15.1)	0	0	0
<i>S. constellatus</i>	267 (47.3)	0	0	0
<i>C. periodontii</i>	4 (0.7)	0	0	0
<i>E. faecalis</i>	5 (0.9)	0	0	0
<i>Staphylococcus</i> species	4 (0.7)	0	0	0

^a Includes *Prevotella melaninogenica* and non-pigmented *Prevotella* species.

^b Among all study subjects.

^c Species growth on amoxicillin-supplemented enriched Brucella blood agar (EBBA) primary isolation plates, with no growth on similar plates with a clavulanic acid overlay.

^d Among species-positive study subjects.

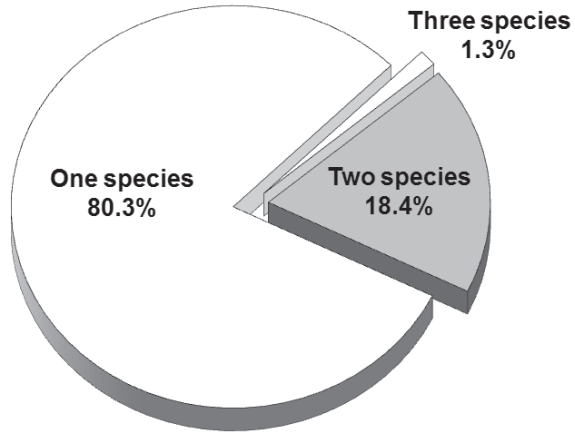


Figure 1. Occurrence of one or more periodontal bacterial pathogens producing β -lactamase among 294 β -lactamase-positive subjects with chronic periodontitis.

Discussion

These findings confirm and extend previous studies revealing subgingival β -lactamase-producing bacteria to be present in a majority of patients with chronic periodontitis (3-11). A strength of the present study is that it assessed the largest group, to date, of subjects with chronic periodontitis for subgingival β -lactamase-producing bacteria ($n = 564$); similar, previous, investigations have collectively examined a total of only 255 subjects with chronic periodontitis (3,5-11). The present study data also evaluated a private dental practice-based convenience sample of subjects with chronic periodontitis originating from a wider geographic region than in previous reports from the USA on subgingival β -lactamase activity (2,3,5), which may permit the findings to be more generalizable to a broader range of USA community population groups.

Owing to the instability of clavulanic acid in agar dilution plates stored for more than three days (24), a surface-overlay technique was used in this study to add clavulanic acid to amoxicillin-containing EBBA primary isolation plates immediately before *in vitro* testing. Since clavulanic acid binds irreversibly to β -lactamase enzymes and prevents inactivation of β -lactam antibiotics (23), microbial growth patterns on these plates were compared with those on EBBA plates supplemented with amoxicillin only as a presumptive method for detecting β -lactamase-positive subgingival bacterial species. This approach was validated with confirmatory nitrocefin chromogenic disk testing for β -lactamase activity from presumptively detected subgingival bacterial species in previous studies (6,7), as well as on a subset of such subject isolates in the present study.

In this study, 52.1% of subjects with untreated chronic periodontitis yielded β -lactamase producing subgingival bacteria, which is at the lower end of the prevalence rates of 53.2% to 100% reported in previous studies (3,5-11). This may be because of differences in periodontal disease severity, treatment status, and recent systemic antibiotic usage among the evaluated subjects with chronic periodontitis, and the microbiological methods employed. For example, some previous studies evaluated only subjects with “refractory” chronic periodontitis, responding poorly to conventional mechanical periodontal therapy (5,8). As systemic antimicrobial therapy is frequently employed in such patients (27), which is positively correlated with increased subgingival β -lactamase activity (3), it is not surprising that a greater occurrence of β -lactamase-producing subgingival bacteria would be reported for patients with “refractory” periodontitis compared with patients with untreated chronic periodontitis without antibiotic use within the past six months, as in the present study. In this regard, the 52.1% occurrence of subgingival β -lactamase-producing bacteria in the present study is similar to the 48% rate documented among 21 patients with chronic periodontitis not exposed to antibiotic therapy over the previous 12 months (3). Moreover, a higher occurrence of subgingival β -lactamase would also be expected among subjects with chronic periodontitis from countries, such as in southern Europe, with greater antibiotic over-the-counter access and consumption rates than found in the USA (28).

P. intermedia/nigrescens, *F. nucleatum*, and other *Prevotella* species were the most frequently identified β -lactamase-positive species in the present study, consistent with previous investigations (3,5-10). Subgingival recovery of cultivable enzyme-producing strains of *P. intermedia/nigrescens* and *F. nucleatum* averaged 9.6% and 6.7%, respectively, of total anaerobic viable counts in β -lactamase-positive subjects, whereas other *Prevotella*

species occurred in lower proportions, averaging $< 1\%$. *P. micra*, *Capnocytophaga* species, and gram-negative enteric rods/pseudomonads were also found to be β -lactamase positive in some study subjects. Interestingly, all 197 strains of *P. gingivalis* in subjects in the present study were β -lactamase negative, in agreement with most previous studies of subgingival isolates (6,9,29). In contrast, 7.7% of 26 subgingival *P. gingivalis* strains were reported β -lactamase-positive in one study (30), and 25.5% of 51 strains in another (31) were found *in vitro* to be resistant to amoxicillin, but susceptible to amoxicillin/clavulanic acid, indicative of β -lactamase production. Additional β -lactamase positive microbial species and subjects would likely be identified with more sensitive methods, such as molecular analysis of bacterial 16S ribosomal RNA gene sequences (5,8) or β -lactamase-encoding genes (4,10,32,33), than the culture-based procedures employed in the present study.

This study also did not classify β -lactamase phenotypes, test for β -lactamase groups resistant to clavulanic acid, or quantitate the strength of subgingival β -lactamase activity present in β -lactamase-positive subjects (1,2). Additionally, the private practice periodontists who diagnosed the study subjects were not calibrated in their assessments, although support for their diagnosis of severe chronic periodontitis was evidenced by their identification for microbiological sampling of three or more periodontal sites per subject with probing depths ≥ 6 mm, which strongly correlates (94.1% positive predictive value) with the presence of severe periodontal attachment loss in adults (34).

The presence of β -lactamase activity in periodontal pockets poses potentially important therapeutic implications relative to control of mixed populations of subgingival periodontal bacterial pathogens in dental plaque biofilms. Enzymatic degradation of β -lactam antibiotics may permit subgingival perpetuation of β -lactamase-producing microbial species and other organisms in the subgingival microbiota that otherwise would be suppressed by β -lactam antibiotics (1). An increasing prevalence of β -lactamase activity among oral bacterial isolates has been reported (35), which is largely thought to be the result of plasmid and transposon-mediated conjugal transfer and dissemination of β -lactamase-encoding genes among microorganisms surviving following selective pressure by β -lactam antibiotics (1). β -lactamases may predispose systemic penicillin monotherapy, unprotected by β -lactamase-inhibitors, to oral cavity treatment failures, such as recalcitrant orofacial infections (36,37) and periodontal surgical flap necrosis (38), as well as enhance periodontal abscess risk in untreated periodontitis subjects (39,40).

Penicillin and amoxicillin are among systemic antibiotics most frequently prescribed by periodontists (41). However, relatively little research attention has been given to their adjunctive use in periodontal therapy in the absence of β -lactamase inhibitors (42,43), with systemic phenoxymethyl penicillin failing to provide significant treatment benefits in a clinical trial of patients with aggressive periodontitis (44). It is noteworthy that subgingival bacterial isolates resistant to 2 mg/L of amoxicillin were found to show a transient increase from a pre-treatment baseline of 0.5% to 35% (a 70-fold increase), over the course of an adjunctive 14-day systemic amoxicillin drug regimen, in patients with chronic periodontitis (45). In that study, *P. intermedia/nigrescens* and *P. melaninogenica*, often identified as β -lactamase-producing species in the present and previous studies (3,5-10), were found to be among the most prevalent amoxicillin-resistant organisms (45). The extent to which bact-

erial β -lactamase activity contributed to these *in vivo* microbiological shifts was not addressed in the study, and remains to be delineated in future investigations.

Finally, the present study found 98.9% of recovered subgingival β -lactamase-producing bacteria to be susceptible *in vitro* to 4 mg/L of metronidazole, an observation consistent with previous reports (3,5,8). As a result, metronidazole suppression of β -lactamase-positive microbial species may, in part, help protect concurrently administered amoxicillin from *in vivo* degradation by bacterial β -lactamases, better enabling pharmacologically active amoxicillin to reach penicillin-binding proteins on bacterial cell membranes to exert antimicrobial effects (1), and contributing to the documented clinical and microbiological benefits of systemic amoxicillin plus metronidazole in periodontal therapy (46-49). However, this may not occur in patients from certain geographic regions, such as in Columbia, where marked *in vitro* metronidazole resistance may be found among β -lactamase-positive subgingival isolates of *P. gingivalis*, *P. intermedia/nigrescens*, *P. melaninogenica* and *F. nucleatum* (31).

Conclusions

In conclusion, the occurrence of β -lactamase-positive subgingival bacterial species in more than one-half of subjects with severe chronic periodontitis raises questions about the therapeutic potential of single-drug regimens with β -lactam antibiotics in periodontal therapy. The *in vitro* effectiveness of metronidazole against nearly all recovered β -lactamase-producing subgingival bacterial species further supports clinical periodontitis treatment strategies involving the combination of systemic amoxicillin plus metronidazole.

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Chapter 5

Antibiotic resistance in human chronic periodontitis microbiota

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Abstract

Purpose: Chronic periodontitis patients may yield multiple species of putative periodontal bacterial pathogens that vary in their antibiotic drug susceptibility. This study determined the occurrence of *in vitro* antibiotic resistance among selected subgingival periodontal pathogens in chronic periodontitis patients.

Materials and methods: Subgingival biofilm specimens from inflamed deep periodontal pockets were removed prior to treatment from 400 adults in the United States with chronic periodontitis. The samples were cultured, and selected periodontal pathogens tested *in vitro* for susceptibility to amoxicillin at 8 mg/L, clindamycin at 4 mg/L, doxycycline at 4 mg/L, and metronidazole at 16 mg/L, with a post-hoc combination of data for amoxicillin and metronidazole. Gram-negative enteric rods/pseudomonads were subjected to ciprofloxacin disk diffusion testing.

Results: Overall, 74.2% of the periodontitis patients revealed subgingival periodontal pathogens resistant to at least one of the test antibiotics. One or more test species, most often *Prevotella intermedia/nigrescens*, *Streptococcus constellatus* or *Aggregatibacter actinomycetemcomitans*, were resistant *in vitro* to doxycycline, amoxicillin, metronidazole, or clindamycin, in 55%, 43.3%, 30.3%, and 26.5% of the chronic periodontitis patients, respectively. 15% of patients harbored subgingival periodontal pathogens resistant to both amoxicillin and metronidazole, which were mostly either *S. constellatus* (45 persons) or ciprofloxacin-susceptible strains of gram-negative enteric rods/pseudomonads (nine persons).

Conclusions: Chronic periodontitis patients in the United States frequently yielded subgingival periodontal pathogens resistant *in vitro* to therapeutic concentrations of antibiotics commonly utilized in clinical periodontal practice. The wide variability found in periodontal pathogen antibiotic resistance patterns should concern clinicians empirically selecting antibiotic treatment regimens for chronic periodontitis patients.

Introduction

Over the last 35 years, the issue of systemic antibiotics in periodontal therapy has evolved from initial research and controversy (1,2) to a scientific consensus recognizing their beneficial impact in the treatment of both aggressive and chronic forms of human periodontitis (3-9).

However, many aspects related to the selection and administration of systemic periodontal antibiotic therapy remain unresolved (4). At present, most systemic periodontal antibiotic treatment regimens appear to be empirically prescribed by clinicians without guidance from a microbiological analysis of subgingival bacterial biofilm populations (10), even though patients with periodontitis frequently yield multiple species of periodontal pathogens that potentially vary in their antibiotic drug resistance (5). One of the risks of this approach is that an antibiotic drug may be selected to which the targeted periodontal pathogens are intrinsically resistant or poorly susceptible, compromising the efficacy of the antimicrobial therapy, and increasing the likelihood of a clinical treatment failure. A position paper for the American Academy of Periodontology (5) expressed concern about this potential adverse outcome, and advocated evaluation of antimicrobial susceptibility patterns of suspected periodontal pathogens prior to administration of systemic periodontal antibiotic therapy. In contrast, a European workshop on antimicrobial agents in periodontics concluded that “*since the antimicrobial profiles of most putative periodontal pathogens are quite predictable, antimicrobial susceptibility testing seems to have no benefit*” (11).

Relative to this, recent data documents antibiotic resistance to be rare among fresh subgingival clinical isolates of periodontal pathogens tested from periodontitis patients residing in northern European countries, where antibiotic usage is generally restricted and infrequent (12). However, markedly higher levels of periodontal pathogen antibiotic resistance has been reported in other geographic regions of the world, such as in Spain in southern Europe and Columbia in South America, where there is less controlled antibiotic access and greater non-supervised consumption of these drugs than in northern Europe (13,14).

In the United States, relatively little recent data exists on the extent to which antibiotic resistance occurs among subgingival periodontal pathogens in patients with periodontitis (15). As a result, it is not clear whether patients with periodontitis in the United States harbor subgingival periodontal pathogens with predictable antimicrobial susceptibility profiles, or instead, present with a more variable occurrence of sensitivity and resistance to antibiotics. To address this issue, the purpose of this study was to examine the occurrence of *in vitro* antibiotic resistance of selected periodontal pathogens in patients with chronic periodontitis in the United States to therapeutic antibiotic breakpoint concentrations of clindamycin, doxycycline, amoxicillin, and metronidazole, as well as to both amoxicillin and metronidazole.

Materials and methods

Patients

A total of 400 adults (193 males, 207 females; aged 35-78 years; mean 50.5 ± 9.9 (SD) years), diagnosed with severe chronic periodontitis (16) by periodontists in private dental practices in the United States, were included in the present study. Their subgingival plaque samples were consecutively received and processed for microbiological analysis by the Oral Microbiology Testing Service (OMTS) Laboratory at Temple University School of Dentistry, Philadelphia. More than one-half of the study patients originated from dental practices located in the mid-Atlantic region of the United States (Maryland, Pennsylvania, New Jersey, Delaware, New York, Virginia, West Virginia and the District of Columbia), with the rest from 11 other eastern states and six midwest and western states. Samples from patients identified with aggressive periodontitis, or antibiotic use six months prior to sampling, were excluded. Approval for the study was provided by the Temple University Human Subjects Protections Institutional Review Board.

Microbial sampling and transport

Subgingival plaque specimens were obtained by the diagnosing periodontists, following a standardized sampling protocol, before treatment from three to five deep (> 6 mm) periodontal pockets per patient that exhibited bleeding on probing. After isolation with cotton rolls, and removal of saliva and supragingival plaque, one to two sterile, absorbent paper points (Johnson & Johnson, East Windsor, NJ, USA) were advanced into each selected periodontal site for approximately 10 seconds. Upon removal, all paper points per study patient were pooled in a glass vial containing six to eight small glass beads and 2.0 ml of anaerobically prepared and stored VMGA III transport medium (17), which possesses a high preservation capability for oral microorganisms during post-sampling transit to the laboratory (17,18). The subgingival samples were then transported within 24 hours to the OMTS Laboratory, which is licensed for high-complexity bacteriological analysis by the Pennsylvania Department of Health. The OMTS Laboratory is also federally-certified by the United States Department of Health and Human Services to be in compliance with Clinical Laboratory Improvement Amendments-mandated proficiency testing, quality control, patient test management, personnel requirements, and quality assurance standards required of clinical laboratories engaged in diagnostic testing of human specimens in the United States (19). All laboratory procedures were performed by personnel who were blinded to the clinical status of the study subjects, and their inclusion in the present analysis.

Microbial culture and incubation

At the OMTS Laboratory, the specimen vials were warmed to 35°C to liquefy the VMGA III transport medium, and sampled microorganisms were mechanically dispersed from the paper points with a Vortex mixer, which was used at the maximal setting for 45 seconds. Serial, 10-fold dilutions of the bacterial suspensions were prepared in Möller's VMG I anaerobic dispersion solution, comprised of pre-reduced, anaerobically sterilized, 0.25% tryptose, 0.25% thiotone E peptone, and 0.5% NaCl (17). Then, 0.1 ml dilution aliquots were spread, with a sterile bent glass rod, onto non-selective enriched Brucella blood agar

(EBBA) primary isolation plates (20), comprised of 4.3% Brucella agar supplemented with 0.3% bacto-agar, 5% defibrinated sheep blood, 0.2% hemolyzed sheep red blood cells, 0.0005% hemin, and 0.00005% menadione, and onto selective trypticase soy-bacitracin-vancomycin (TSBV) agar (21). EBBA plates were incubated at 35°C for seven days in a Coy anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI, USA) containing 85% N₂, 10% H₂, and 5% CO₂, and TSBV plates were incubated at 35°C for three days in air + 5% CO₂.

Microbial identification

Putative periodontal pathogens examined for in this study were *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia/nigrescens*, *Parvimonas micra*, *Fusobacterium nucleatum*, *Streptococcus constellatus*, *Staphylococcus aureus*, *Enterococcus faecalis*, gram-negative enteric rods/pseudomonads, and *Candida* species.

Total anaerobic viable counts, and counts of *P. gingivalis*, *P. intermedia/nigrescens*, *P. micra*, *F. nucleatum*, *S. constellatus*, *S. aureus* and *E. faecalis* were made on EBBA primary isolation plates using a ring-light magnifying loupe, presumptive phenotypic methods previously described (15,22,23), and an enzyme-based, micro-method kit system (RapID ANA II, Innovative Diagnostic Systems, Atlanta, GA, USA) for selected isolates. *A. actinomycetemcomitans*, gram-negative enteric rods/pseudomonads, and *Candida* species were quantitated on TSBV agar, as previously described (20,21). The proportional recovery of each test species was ascertained in each patient by calculating as the percentage of test species colony-forming units relative to total subgingival anaerobic viable counts, as determined on non-selective EBBA primary isolation plates.

In vitro antibiotic resistance testing

Additional 0.1 ml aliquots of subgingival sample dilutions were inoculated onto EBBA primary isolation plates supplemented with either amoxicillin at 8 mg/L, clindamycin at 4 mg/L, doxycycline at 4 mg/L, or metronidazole at 16 mg/L (all antimicrobials obtained as pure powder from Sigma-Aldrich, St. Louis, MO, USA), and incubated anaerobically for seven days. These antimicrobial concentrations represent non-susceptible/resistant breakpoint concentrations against anaerobic bacteria for amoxicillin, clindamycin, and metronidazole as recommended by the Clinical and Laboratory Standards Institute (CLSI) (24), and for doxycycline as recommended by the French Society for Microbiology (25). Direct colony suspensions (equivalent to a 0.5 McFarland standard) of pure *A. actinomycetemcomitans* isolates from selective TSBV plates were subcultured onto these media as their recognition is frequently obscured within mixed bacterial populations (21). *In vitro* resistance to the antibiotic breakpoint concentrations was recorded when test species growth was noted on the respective antibiotic-supplemented EBBA plates (15,20,26,27). *Bacteroides thetaiotaomicron* ATCC 29741, *Clostridium perfringens* ATCC 13124, and a multi-antibiotic-resistant clinical periodontal isolate of *F. nucleatum* were used as positive and negative quality controls for all antibiotic resistance testing on drug-supplemented EBBA plates.

Gram-negative enteric rods/pseudomonads recovered on TSBV primary isolation plates were subjected to *in vitro* ciprofloxacin disk diffusion testing. Direct colony suspensions of

the organisms, equivalent to a 0.5 McFarland standard, were inoculated onto Mueller-Hinton agar, incubated in ambient air at 35°C for 16 to 18 hours, and assessed with CLSI interpretative guidelines (28).

Data analysis

The recovered test periodontal pathogens were grouped for reporting purposes into subgingival bacterial clusters (i.e., red complex, orange complex, and other species), as previously described (29). Descriptive analysis was used to tabulate the occurrence and proportional cultivable recovery of test species in patients, as well as the occurrence and subgingival proportions of antibiotic-resistant test species. Based on previous studies demonstrating excellent agreement (98.5%) between periodontal pathogen antibiotic resistance patterns on EBBA plates jointly supplemented with both amoxicillin and metronidazole, and those determined from a post-hoc combination of findings from EBBA plates individually supplemented with amoxicillin or metronidazole (30), the *in vitro* antibiotic resistance data for the 400 study patients was combined and analyzed post-hoc for amoxicillin and metronidazole-supplemented EBBA primary isolation plates. Data analysis was performed using a statistical software package (SAS 9.2 for Windows, SAS Institute, Inc., Cary, NC, USA).

Results

Total cultivable counts and test species recovery

Total subgingival anaerobic viable counts on non-antibiotic-supplemented EBBA primary isolation plates averaged $4.0 \times 10^7 \pm 2.4 \times 10^6$ (SE) organisms/ml of sample (range = 9.0×10^5 to 4.2×10^8 organisms/ml).

Table 1 lists the occurrence and proportional cultivable recovery of subgingival test species recovered from the 400 study patients. *P. micra*, *P. intermedia/nigrescens* and *P. gingivalis* were isolated from most study patients (78% to 91%), with mean subgingival proportions of these species in positive patients ranging from 10.3% to 18.1% of cultivable anaerobic viable counts. *A. actinomycetemcomitans*, *S. constellatus*, and *F. nucleatum* were detected in 20.3% to 36.8% of the study patients. Gram-negative enteric rods/pseudomonads averaged 24.6% of the cultivable subgingival microbiota in nine (2.3%) positive patients, of which six had gram-negative enteric rods/pseudomonads >10% of total subgingival counts. *E. faecalis* and *S. aureus* were detected in four (1.0%) and one (0.3%) of the study patients, respectively. No subgingival *Candida* species were isolated in the study population with the employed microbiological methods.

In vitro antibiotic resistance testing

Table 2 lists the occurrence of *in vitro* antibiotic resistance among the test periodontal pathogens in the 400 study patients. *P. gingivalis* rarely showed *in vitro* resistance to any of the test antibiotics. In comparison, 76 of 81 (93.8%) patient strains of *A. actinomycetemcomitans* exhibited *in vitro* resistance to clindamycin at 4 mg/L, and 38 of 81 (46.9%) strains to doxycycline at 4 mg/L, whereas relatively few were resistant *in vitro* to either amoxicillin or metronidazole (3.7% to 6.1% of patient strains). Subgingival proportions of

antibiotic-resistant *A. actinomycetemcomitans* clinical isolates averaged from 2.1% to 22.7% of the subgingival cultivable microbiota.

P. intermedia/nigrescens was rarely resistant *in vitro* to metronidazole, and infrequently to clindamycin. However, 89 of the 320 (27.8%) *P. intermedia/nigrescens* patient strains were resistant to doxycycline, and 108 (33.8%) resistant to amoxicillin, with antibiotic-resistant strains averaging 13.7% of cultivable subgingival viable counts. Both *P. micra* and *F. nucleatum* displayed *in vitro* resistance to doxycycline in 67 and 11 persons, respectively, and less frequently to other test antibiotics. *S. constellatus* in most species-positive individuals (89.1%) was resistant *in vitro* to metronidazole. A total of 52 (35.4%) *S. constellatus* patient strains were resistant to amoxicillin, 35 (23.8%) resistant to doxycycline, and nine (6.1%) resistant to clindamycin. Antibiotic-resistant *S. constellatus* strains averaged 5% to 6% of cultivable subgingival organisms in culture-positive persons (Table 2).

All isolated subgingival gram-negative enteric rods/pseudomonads in nine positive patients were resistant *in vitro* to each of the four test antibiotics, but were susceptible to ciprofloxacin in disk diffusion testing. Each of these individuals was co-colonized by one or more metronidazole-susceptible periodontal pathogens, including *P. gingivalis*, *P. intermedia/nigrescens*, and *P. micra*, and one patient additionally had metronidazole-resistant *S. constellatus*.

Subgingival *S. aureus* in the one positive person was resistant *in vitro* to each of the four test antibiotics. Subgingival *E. faecalis* was resistant to metronidazole and doxycycline in all four positive patients, to clindamycin in three patients, and to amoxicillin in two patients (Table 2).

When data for *in vitro* resistance to amoxicillin at 8 mg/L and metronidazole at 16 mg/L were combined and analyzed post hoc (Table 2), resistance to both antibiotic concentrations occurred with 45 patient strains of *S. constellatus*, nine of gram-negative enteric rods/pseudomonads, two of *E. faecalis*, and one each of *S. aureus*, *P. intermedia/nigrescens*, and *A. actinomycetemcomitans*.

Overall, antibiotic-resistant subgingival periodontal pathogens were detected in 297 (74.2%) of the 400 study patients. One or more test periodontal pathogens resistant *in vitro* to doxycycline were found in 220 (55.0%) patients, to amoxicillin in 173 (43.3%) patients, to metronidazole in 121 (30.3%) patients, and to clindamycin in 106 (26.5%) patients. In addition, 60 (15.0%) of the study patients harbored subgingival test periodontal pathogens resistant *in vitro* to both amoxicillin and metronidazole.

Table 1. Occurrence and proportional subgingival recovery of test species in 400 adults with chronic periodontitis.

Test species	No. (%) culture positive patients	Mean % \pm SE recovery in positive patients	Range %
<u>Red complex species:</u>			
<i>P. gingivalis</i>	312 (78.0)	18.1 \pm 1.0	0.1-78.0
<u>Orange complex species:</u>			
<i>P. micra</i>	364 (91.0)	10.9 \pm 0.5	0.1-55.8
<i>P. intermedia/nigrescens</i>	320 (80.0)	10.3 \pm 0.6	0.01-62.2
<i>S. constellatus</i>	147 (36.8)	3.9 \pm 0.6	0.03-46.8
<i>F. nucleatum</i>	122 (30.5)	4.4 \pm 0.5	0.02-26.3
<u>Other species:</u>			
<i>A. actinomycetemcomitans</i>	81 (20.3)	2.7 \pm 1.1	0.001-71.1
enteric rods/pseudomonads	9 (2.3)	24.6 \pm 6.4	2.7-59.0
<i>E. faecalis</i>	4 (1.0)	15.8 \pm 4.5	7.7-26.2
<i>S. aureus</i>	1 (0.3)	58.7 \pm 0.0	58.7
<i>Candida</i> species	0	0	0

Table 2. Number (%) of study patients with periodontal pathogens resistant *in vitro* to antibiotic breakpoint concentrations.

Test species		clindamycin (4 mg/L) ^a	doxycycline (4 mg/L)	amoxicillin (8 mg/L)	metronidazole (16 mg/L)	amoxicillin plus metronidazole ^b
Red complex species:						
<i>P. gingivalis</i>	<i>n</i>	2 (0.6) ^c	2 (0.6)	1 (0.3)	0	0
	%	1.0 ± 0.6 ^d	29.5 ± 19.5	2.4	0	0
Orange complex species:						
<i>P. micra</i>	<i>n</i>	5 (1.4)	67 (18.4)	2 (0.6)	2 (0.6)	0
	%	14.8 ± 6.4	14.0 ± 1.3	8.9 ± 6.8	35.2 ± 1.3	0
<i>P. intermedia/nigrescens</i>	<i>n</i>	12 (3.8)	89 (27.8)	108 (33.8)	1 (0.3)	1 (0.3)
	%	4.6 ± 1.6	13.7 ± 1.3	13.1 ± 1.2	2.7	2.7
<i>S. constellatus</i>	<i>n</i>	9 (6.1)	35 (23.8)	52 (35.4)	131 (89.1)	45 (30.6)
	%	6.0 ± 2.8	5.0 ± 1.3	5.3 ± 1.2	5.2 ± 0.8	5.9 ± 1.3
<i>F. nucleatum</i>	<i>n</i>	0	11 (9.0)	8 (6.6)	1 (0.8)	0
	%	0	3.6 ± 1.8	2.3 ± 1.4	9.1	0
Other species:						
<i>A. actinomycetemcomitans</i>	<i>n</i>	76 (93.8)	38 (46.9)	5 (6.1)	3 (3.7)	1 (1.2)
	%	2.7 ± 1.1	3.5 ± 2.0	22.7 ± 13.6	2.1 ± 1.2	4.4
enteric rods/pseudomonads	<i>n</i>	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)
	%	24.6 ± 6.4	24.6 ± 6.4	24.6 ± 6.4	24.6 ± 6.4	24.6 ± 6.4
<i>E. faecalis</i>	<i>n</i>	3 (75.0)	4 (100)	2 (50.0)	4 (100)	2 (50.0)
	%	12.3 ± 4.1	15.8 ± 4.5	14.7 ± 5.9	15.8 ± 4.5	14.7 ± 5.9
<i>S. aureus</i>	<i>n</i>	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
	%	58.7	58.7	58.7	58.7	58.7

^a Non-susceptible antibiotic breakpoint concentrations incorporated into primary isolation plates.

^b Post hoc combination of *in vitro* resistance data for amoxicillin at 8 mg/L and metronidazole at 16 mg/L.

^c Percent of antibiotic-resistant strains among total number of patient isolates of species.

^d Mean % recovery ± SE of antibiotic-resistant strains in positive patients.

Discussion

This *in vitro* study assessed subgingival periodontal pathogen antibiotic resistance in the largest number of periodontitis patients in the United States (n = 400) since the report of Listgarten et al. (31) 20 years ago on 196 refractory periodontitis patients. The major finding was the frequent occurrence of antibiotic resistance among subgingival periodontal pathogens colonizing chronic periodontitis patients in the United States, which appears to be greater than is found in chronic periodontitis patients in northern Europe (12,13,27). No single evaluated antibiotic, or combination of antibiotics, demonstrated *in vitro* inhibition of all of the assessed periodontal pathogens across all of the study patients.

Resistance to doxycycline in the study patients was most striking, with 55% revealing one or more of the test periodontal pathogens resistant to doxycycline at a breakpoint concentration of 4 mg/L. *P. micra*, *P. intermedia/nigrescens*, and *A. actinomycetemcomitans* most frequently displayed doxycycline resistance among 18.4% to 46.9% of recovered patient strains. These frequencies of doxycycline resistance are relatively similar to tetracycline-HCl resistance detected in *P. micra* and *P. intermedia/nigrescens* subgingival strains in Germany (32), but markedly higher than reported for periodontal *A. actinomycetemcomitans* clinical isolates in France (33). Moreover, the doxycycline resistance detected in present-day periodontal *A. actinomycetemcomitans* clinical isolates in the United States (Table 2) appears to be considerably greater compared to the rare *A. actinomycetemcomitans* resistance to tetracycline family antibiotics that was reported more than 30 years ago (34). Consistent with these *in vitro* findings, no statistically significant reductions in doxycycline-resistant subgingival strains of *P. micra* and *P. intermedia/nigrescens* were found *in vivo* following systemic doxycycline administration in patients with periodontitis (35).

Amoxicillin resistance was present in 43.3% of the study patients, primarily among subgingival *P. intermedia/nigrescens*, which often elaborates β -lactamase enzymes capable of hydrolyzing β -lactam antibiotics such as amoxicillin (36). About one-third of 147 subgingival *S. constellatus* patient strains were resistant to amoxicillin, which is higher than reported in another recent *S. constellatus* study by our group involving a smaller number of clinical isolates (unpublished observations). Large transient increases in amoxicillin-resistant subgingival *S. constellatus* have been detected following systemic amoxicillin therapy in patients with chronic periodontitis (35).

Resistance to metronidazole occurred in subgingival biofilm specimens from 30.3% of the study patients, and as expected, was found almost exclusively among non-anaerobic test species, including *S. constellatus*, gram-negative enteric rods/pseudomonads, *E. faecalis*, and *S. aureus*. Metronidazole resistance among anaerobic periodontal pathogens was rarely noted in this study (Table 2), similar to anaerobic periodontal isolates in Europe (12,13,27), but in contrast to the approximately 20% to 25% metronidazole resistance rate reported in Columbia for subgingival *P. gingivalis*, *P. intermedia/nigrescens*, and *F. nucleatum* clinical isolates (14). Clinical studies have found no statistically significant reductions in metronidazole-resistant subgingival strains of *S. constellatus* after systemic metronidazole administration in patients with periodontitis (35).

About one-fourth of the study patients had periodontal pathogens resistant to clindamycin, most frequently among *A. actinomycetemcomitans* clinical isolates, consistent with other studies performed elsewhere in the world (12-14,33). Clindamycin resistance is absent or infrequent in anaerobic periodontal pathogens (Table 2) (12), consistent with clinical studies documenting suppression of a similar array of anaerobic periodontal pathogens, leading to marked clinical improvements, after systemic clindamycin therapy in patients with refractory periodontitis (37).

A total of 60 (15%) of the study patients harbored subgingival test periodontal pathogens resistant to both amoxicillin at 8 mg/L and metronidazole at 16 mg/L, which was the lowest level of patient resistance detected among the evaluated antibiotics. This confirms suggestions that the complementary antimicrobial spectrums of amoxicillin and metronidazole inhibit a wider array of periodontal pathogens than individual antibiotics, and pose a lower risk of encountering drug-resistant pathogenic species (5). Species resistant to both antibiotic concentrations included *S. constellatus* (30.6% of patient strains), all gram-negative enteric rods/pseudomonads and *S. aureus* clinical isolates, two of four *E. faecalis* subject strains, and one patient strain each of *A. actinomycetemcomitans* and *P. intermedia/nigrescens*. Despite a number of clinical studies finding marked improvements in patients with periodontitis prescribed systemic amoxicillin plus metronidazole (6-9), not all periodontitis patients treated with this drug combination appear to clinically respond in an equivalent fashion. A subset of periodontitis patient outcomes from systemic amoxicillin plus metronidazole therapy appear to be no better than placebo control groups (38-40), particularly in the absence of subgingival *P. gingivalis* (38). It is tempting to speculate that another potential determinant for this differential clinical effect of systemic amoxicillin plus metronidazole antibiotic therapy may be the presence of periodontal pathogens resistant to therapeutic concentrations of both drugs, similar to those detected in this study.

In this regard, nine study patients yielded subgingival gram-negative enteric rods/pseudomonads resistant to both amoxicillin and metronidazole, which suggests that potential administration of these two antibiotics as a part of periodontal therapy may be ineffective against these species, potentially compromising clinical outcomes (20,41). Doxycycline and clindamycin also appear unlikely to be viable alternatives in light of their negligible antimicrobial activity against gram-negative enteric rods/pseudomonads (Table 2). A combination of ciprofloxacin plus metronidazole (5) may be more appropriately considered for the six study patients with ciprofloxacin-sensitive gram-negative enteric rods/pseudomonads greater than 10% of the cultivable subgingival microbiota, and additionally harboring metronidazole-susceptible co-colonizing periodontal pathogens, because mechanical periodontal debridement does not reliably suppress large subgingival populations of gram-negative enteric rods/pseudomonads (41). In contrast, the three study patients with lower proportions of subgingival gram-negative enteric rods/pseudomonads may have adequate suppression of these species via conventional periodontal debridement procedures without a need for systemic antibiotic therapy (41).

The present *in vitro* study data has several limitations. The actual systemic periodontal antibiotic treatment needs, if any, of the study patients with chronic periodontitis were not determined. The study patients represent a private dental practice-based convenience sample that may not be statistically representative of chronic periodontitis patients in the

United States. Because no clinical or radiographic evaluations of the study subjects were conducted by calibrated examiners, independent of the private practice periodontists who submitted the subgingival biofilm specimens for microbiological testing, doubt may be raised about their diagnosis of chronic periodontitis in the study patients. However, their identification of three or more periodontal sites with deep probing depths and bleeding on probing in the study patients strongly correlates (94.1% positive predictive value) with a finding of severe periodontal attachment loss in adult patients (42). Only selected periodontal pathogens were evaluated in the subgingival biofilm specimens studied, without inclusion of additional cultivable periodontal bacterial species and *Archaea* implicated in the pathogenesis of periodontitis (43), which may possess even more diverse antibiotic susceptibility profiles. The occurrence and counts of *F. nucleatum* were likely underestimated on EBBA primary culture plates examined without a stereomicroscope in addition to a ring-light magnifying loupe (15). Exact minimal inhibitory concentration values of the test antibiotics against the detected periodontal pathogens were not determined with the clinical laboratory methods followed, and antibiotic resistance genes in the test periodontal pathogens were not studied. Moreover, the extent to which our *in vitro* laboratory findings may assist periodontal therapy *in vivo* and enhance clinical treatment outcomes remains to be fully established and validated.

In addition, the present study design differs from traditional investigations of bacterial antibiotic susceptibility, where evaluated species are tested individually after subculture, and minimal inhibitory concentrations reported without regard to the antimicrobial profile of other periodontal pathogens colonizing homologous persons (12-14). Instead, *in vitro* antibiotic resistance testing was performed on primary isolation plates, and tabulated across all test periodontal pathogens positive within each study patient (15), which more closely parallels what clinicians routinely confront in practice in determining potential periodontal antibiotic therapy needs of individual periodontitis patients. This direct plating method has been employed in previous periodontal microbiology studies (15,20,26,27,31,36), and correlates well ($r^2 = 0.99$) with the CLSI-approved agar dilution susceptibility assay in identifying antibiotic-resistant periodontal microorganisms (26). Similarly, a 94% agreement rate has been reported between primary (direct) versus secondary (subculture) antibiotic susceptibility plate testing on acute dentoalveolar abscess bacterial isolates (44).

It is important to emphasize that *in vitro* identification of antibiotic sensitivity in subgingival periodontal pathogens does not necessarily confer *in vivo* drug effectiveness against the organisms (45), since additional factors may impact passage of antibiotics into periodontal pockets, and alter their activity against mixed infections growing in biofilms (5). As a result, this study focused on periodontal pathogen antibiotic resistance, because it is recognized that patients presenting with drug-resistant pathogens generally demonstrate a poorer bacteriological response to antimicrobial therapy than patients infected with drug-susceptible organisms (46). However, documentation of antibiotic-resistant periodontal pathogens causing clinical periodontal treatment failure is limited in the periodontology scientific literature, and likely underrepresents its occurrence in clinical periodontal practice. Examples of such patient outcomes were described nearly 20 years ago by Fine (47). Tetracycline-resistant strains of subgingival *Aggregatibacter actinomycetemcomitans* were associated in one patient with progressive periodontitis occurring after long-term systemic tetracycline-HCl therapy and multiple periodontal flap surgeries (47). Another patient revealed high subgingival levels of erythromycin-resistant *Staphylococcus aureus* in

highly disease-active periodontitis lesions secondary to prolonged systemic erythromycin therapy and multiple periodontal surgeries (47). In both of these patients, alternative systemic antibiotic therapy, selected on the basis of microbiological analysis and *in vitro* antibiotic susceptibility testing, led to suppression of the pathogenic species and a sustained clinical resolution. Similarly, Colombo et al. (48) reported that elevated baseline proportions of subgingival *S. constellatus*, which is frequently resistant to tetracycline antibiotics (Table 2), and poorly removed by periodontal root instrumentation (49), conferred an 8.6 odds ratio for post-treatment progressive periodontal attachment loss on refractory chronic periodontitis patients treated with surgical debridement and a 28-day systemic tetracycline drug regimen. It is likely that antibiotic susceptibility testing in clinical situations like these may be clinically directive to dental professionals in their selection of periodontal antimicrobial therapies, and help reduce the risk of treatment failures attributable to the subgingival presence of antibiotic-resistant periodontal pathogens.

Conclusions

Chronic periodontitis subjects in this study frequently yielded subgingival periodontal pathogens resistant *in vitro* to therapeutic concentrations of antibiotics commonly utilized in clinical periodontal practice. The wide variability in periodontal pathogen antibiotic resistance patterns should concern clinicians empirically selecting antibiotic treatment regimens for chronic periodontitis patients, and suggests a role for microbiological analysis and antibiotic susceptibility testing as an aid in the selection of systemic periodontal antibiotic therapy.

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Chapter 6

Spiramycin resistance in human periodontitis microbiota

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Abstract

Purpose: The occurrence of *in vitro* resistance to therapeutic concentrations of spiramycin, amoxicillin, and metronidazole was determined for putative periodontal pathogens isolated in the United States.

Materials and methods: Subgingival plaque specimens from 37 consecutive adults with untreated severe periodontitis were anaerobically cultured, and isolated putative periodontal pathogens were identified to a species level. *In vitro* resistance to spiramycin at 4 mg/L, amoxicillin at 8 mg/L, and/or metronidazole at 16 mg/L was noted when putative periodontal pathogen growth was noted on the respective antibiotic-supplemented primary culture plates.

Results: A total of 18 (48.7%) subjects yielded antibiotic-resistant putative periodontal pathogens with spiramycin at 4 mg/L in drug-supplemented primary isolation plates, as compared to 23 (62.2%) subjects with amoxicillin at 8 mg/L, and 10 (27.0%) subjects with metronidazole at 16 mg/L. Spiramycin *in vitro* resistance occurred among species of *Fusobacterium nucleatum* (44.4% of organism-positive subjects), *Prevotella intermedia/nigrescens* (11.1%), *Parvimonas micra* (10.8%), *Streptococcus constellatus* (10%), *Streptococcus intermedius* (10%), *Porphyromonas gingivalis* (6.7%), and *Tannerella forsythia* (5.3%). Amoxicillin *in vitro* resistance was found in *P. intermedia/nigrescens* (55.5%), *T. forsythia* (15.8%), *S. constellatus* (10%), *F. nucleatum* (5.6%), and *P. micra* (2.7%). Only *S. constellatus* (70%) and *S. intermedius* (40%) exhibited *in vitro* resistance to metronidazole. When subject-based resistance data for spiramycin and metronidazole were jointly considered, all isolated putative periodontal pathogens were inhibited *in vitro* by one or the other of the antibiotic concentrations, except for one strain each of *S. constellatus* and *S. intermedius* from one study subject. Similarly, either amoxicillin or metronidazole at the drug concentrations tested inhibited *in vitro* all recovered putative periodontal pathogens, except *S. constellatus* in one subject.

Conclusions: *In vitro* spiramycin resistance among putative periodontal pathogens of United States origin occurred in approximately one-half of severe periodontitis patients evaluated, particularly among subgingival *F. nucleatum* species. *In vitro* resistance patterns also suggest that therapeutic concentrations of spiramycin plus metronidazole may have potential antimicrobial efficacy in non-*Aggregatibacter actinomycetemcomitans*-associated periodontitis similar to amoxicillin plus metronidazole, which may be beneficial, where spiramycin is clinically available, for patients hypersensitive to amoxicillin or other beta-lactam antibiotics.

Introduction

Spiramycin is a medium-spectrum, 16-membered, macrolide antibiotic widely used in the treatment of respiratory infections (1), except in the United States, where it is available by special permission for treatment of toxoplasmosis in women during the first trimester of pregnancy. Since the drug enters the oral cavity through gingival crevicular fluid and saliva, and persists for extended periods at potentially therapeutic concentrations (2), spiramycin is also prescribed in some countries for various odontogenic infections, including endodontic abscesses, pericoronitis, and destructive forms of periodontal disease (periodontitis) (3-6).

A number of studies have assessed the antimicrobial activity of spiramycin on putative periodontopathic bacteria in subgingival plaque biofilms (7-17). Except for ones completed over 25 years ago (7-9), these studies focused on clinical isolates from periodontitis patients located outside of the United States. Since geographic differences (18), and emergence of drug-resistant strains over time (19), may markedly alter antibiotic susceptibility patterns of subgingival bacterial species, the present-day antimicrobial effects of spiramycin on putative periodontal pathogens in the United States remain speculative.

The present study examined, using fresh subgingival isolates from periodontitis patients in the United States, the occurrence of *in vitro* resistance among putative periodontal pathogens to therapeutic concentrations of spiramycin, as well as amoxicillin and metronidazole.

Materials and methods

Subjects

A total of 37 adults (17 males, 20 females; aged 35-85 years; mean 57.5 ± 12.0 (SD) years), diagnosed with severe periodontitis (20) by periodontists in United States private dental practices, were consecutively included in the present study as their microbiological samples were received by the testing laboratory. Persons identified with aggressive periodontitis, or antibiotic use within the past six months, were excluded. Only four (10.8%) of the study subjects were reported as current smokers.

Microbial sampling and transport

Subgingival plaque specimens were obtained by each study subject's periodontist, following a standardized sampling protocol, prior to treatment from the three to five deepest periodontal pockets (mean 7.5 ± 0.3 (SE) mm) per subject which exhibited bleeding on probing. In brief, after isolation with cotton rolls, and removal of saliva and supragingival deposits, one to two sterile, absorbent paper points (Johnson & Johnson, East Windsor, NJ, USA) were advanced into each selected periodontal site for approximately 10 seconds. Upon removal, all paper points per study subject were pooled into a glass vial containing six to eight small glass beads and 2.0 ml of anaerobically prepared and stored VMGA III transport medium (21), which possesses a high preservation capability for oral microorganisms during post-sampling transit to the laboratory (21,22). The subgingival samples were then transported within 24 hours to the Oral Microbiology Testing Service

(OMTS) Laboratory at Temple University School of Dentistry, which is licensed for high complexity bacteriological analysis by the Pennsylvania Department of Health.

Microbial culture and incubation

At the OMTS Laboratory, the specimen vials were warmed to 35°C to liquefy the VMGA III transport medium, and sampled microorganisms were mechanically dispersed from the paper points with a vortex mixer at the maximal setting for 45 seconds. Serial 10-fold dilutions of dispersed bacteria were prepared in Möller's VMG I anaerobic dispersion solution (21). Appropriate dilution aliquots were plated onto non-selective enriched Brucella blood agar (EBBA) (23), Hammond's selective *Campylobacter* medium (24), and TSBV agar (25). Aliquots were also plated onto EBBA supplemented with either spiramycin at 4 mg/L, amoxicillin at 8 mg/L, or metronidazole at 16 mg/L (all antimicrobials were obtained as pure powder from Sigma-Aldrich, St. Louis, MO, USA). These antimicrobial concentrations represent non-susceptible/resistant breakpoint concentrations for anaerobic bacteria recommended by the French Society for Microbiology (26) for spiramycin, and the Clinical and Laboratory Standards Institute (CLSI) (27) for amoxicillin and metronidazole. All EBBA and Hammond's selective medium plates were incubated at 35°C for seven days in a Coy anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI, USA) containing 85% N₂-10% H₂-5% CO₂, and TSBV plates were incubated at 35°C for three days in 5% CO₂-95% air.

Microbial identification

On non-selective EBBA plates examined with a ring-light magnifying loupe and a dissecting stereomicroscope, the presence and levels of total anaerobic viable counts, *Porphyromonas gingivalis*, *Prevotella intermedia/nigrescens*, *Parvimonas micra*, staphylococci and enterococci were determined using presumptive phenotypic methods previously described (28-31); *Tannerella forsythia* was identified as gram-negative, non-motile, anaerobic rods exhibiting grey-pink speckled, convex, pinpoint colonies seen with a stereomicroscope, lack of long-wave ultraviolet light autofluorescence, and a positive CAAM test for trypsin-like activity (28); *Fusobacterium nucleatum* was identified as long-wave ultraviolet light autofluorescent chartreuse-positive (32), gray, iridescent colonies of gram-negative, filamentous, spindle-shaped, non-motile rods; *Streptococcus intermedius* was recognized as gram-positive, lactose MUG-test positive (33), non-motile, facultative cocci exhibiting small dry, white, raised colonies with wrinkled edges; and *Streptococcus constellatus* was defined as gram-positive, lactose MUG-test negative, non-motile, facultative cocci demonstrating small white, opaque, circular, beta-hemolytic, surface colonies with irregular edges, and positive for α -D-glucosidase enzyme activity, with or without β -D-fucosidase or β -D-glucosidase positive reactions, as determined with the Fluo-Card Milleri test kit (Key Scientific Products Co., Stamford, TX, USA) (34). *Campylobacter rectus* was quantitated on Hammond's medium, and *Aggregatibacter actinomycetemcomitans*, gram-negative enteric rods, pseudomonads, and *Candida* species on TSBV agar, as previously described (23,25,35). Proportional subject recovery of the various test putative periodontal pathogens was calculated as the percent recovery of the test species colony forming units (CFU) among the total subgingival anaerobic viable CFU count as determined on non-selective EBBA plates.

***In vitro* antibiotic resistance testing**

In vitro resistance to the antibiotic breakpoint concentrations of spiramycin (4 mg/L), amoxicillin (8 mg/L), or metronidazole (16 mg/L) was recorded per subject when test putative periodontal pathogen growth was noted on antibiotic-supplemented and non-supplemented primary isolation EBBA plates (23,36,37). *Bacteroides thetaiotaomicron* ATCC 29741, *Clostridium perfringens* ATCC 13124, and a multi-antibiotic-resistant clinical periodontal isolate of *F. nucleatum* were employed as positive and negative quality controls for all antibiotic resistance testing on drug-supplemented EBBA plates.

Data analysis

Recovered test putative periodontal pathogens were grouped for reporting purposes into subgingival bacterial clusters (i.e., red complex, orange complex, and other species) described by Socransky et al. (38). Descriptive analysis was used to calculate the subject occurrence and proportional cultivable recovery of test putative periodontal pathogens on non-selective EBBA, and the subject occurrence of *in vitro* test putative periodontal pathogen antibiotic resistance. *In vitro* data was also combined post-hoc for spiramycin and metronidazole, and for amoxicillin and metronidazole, to determine the number and proportion of organism-positive study subjects where test putative periodontal pathogens exhibited *in vitro* resistance to both antibiotics at the employed non-susceptible breakpoint concentrations.

Study parameters and approval

All microbiological procedures were performed on a standardized, blinded basis without knowledge of the clinical status of the study subjects, or their inclusion in the present analysis. Approval for the study was provided by the Temple University Human Subjects Institutional Review Board.

Results

Total cultivable counts and putative periodontal pathogen recovery

Total subgingival anaerobic viable counts on non-antibiotic-supplemented EBBA plates averaged $7.32 \times 10^7 \pm 1.6 \times 10^7$ (SE) organisms/ml of sample in the study subjects (range = 2×10^6 to 3.5×10^8 organisms/ml).

Table 1 lists the distribution of recovered subgingival test putative periodontal pathogens in the 37 study subjects. Among red complex bacterial species, *P. gingivalis* was isolated from 15 (40.5%) subjects, and *T. forsythia* from 19 (51.4%) subjects, at mean subgingival proportions of 14.4% and 1.4%, respectively, in culture-positive subjects. Among orange complex and other species, *P. micra* was isolated from all 37 subjects, *P. intermedia/nigrescens* and *F. nucleatum* were each found in 36 (97.3%) subjects, *C. rectus* was recovered from 20 (54.1%) subjects, and *S. constellatus* and *S. intermedius* were each present in 10 (27.0%) subjects (Table 1). Cultivable subgingival *A. actinomycetemcomitans*, gram-negative enteric rods, pseudomonads, staphylococci, enterococci and *Candida* species were not detected in any of the study subjects.

***In vitro* antibiotic resistance testing**

A total of 18 (48.7%) subjects yielded antibiotic-resistant putative periodontal pathogens with spiramycin at 4 mg/L in drug-supplemented primary isolation plates, as compared to 23 (62.2%) subjects with amoxicillin at 8 mg/L, and 10 (27.0%) subjects with metronidazole at 16 mg/L.

F. nucleatum most frequently exhibited *in vitro* spiramycin resistance, with 44.4% of subject strains resistant to 4 mg/L of spiramycin in primary isolation plates (Table 2). Between 10-11% of subject strains each of *P. intermedia/nigrescens*, *P. micra*, and *S. intermedius* demonstrated *in vitro* spiramycin resistance, and one subject strain each of *P. gingivalis* and *T. forsythia* were resistant *in vitro* to non-susceptible breakpoint concentrations of spiramycin (Table 2).

Amoxicillin at 8 mg/L in primary isolation plates inhibited all subject strains of *P. gingivalis*, *C. rectus*, and *S. intermedius*. In contrast, 55.5% of *P. intermedia/nigrescens* subject strains, and 2.7-15.8% of *T. forsythia*, *S. constellatus*, *F. nucleatum*, and *P. micra* subject strains displayed *in vitro* resistance to 8 mg/L of amoxicillin in primary isolation plates (Table 2).

All subject strains of *P. gingivalis*, *T. forsythia*, *P. intermedia/nigrescens*, *P. micra*, *C. rectus*, and *F. nucleatum* were inhibited *in vitro* by 16 mg/L of metronidazole in primary isolation plates. However, 70% of *S. constellatus*, and 40% of *S. intermedius* subject strains, revealed *in vitro* resistance to 16 mg/L of metronidazole (Table 2).

When subject-based *in vitro* resistance data for 4 mg/L of spiramycin and 16 mg/L of metronidazole were jointly considered post hoc, all isolated test periodontal pathogens were inhibited *in vitro* by one or the other of the antibiotic concentrations, except for one strain each of *S. constellatus* and *S. intermedius* in one (2.7%) study subject. Similarly, either 8 mg/L of amoxicillin or 16 mg/L of metronidazole inhibited *in vitro* all recovered test periodontal pathogens, except *S. constellatus* in one (2.7%) subject (Table 2).

Table 1. Occurrence and proportional subgingival recovery of selected putative periodontal bacterial pathogens in 37 adults with severe periodontitis.

Test species:	No. (%) of culture positive subjects	Mean % \pm SE recovery in positive subjects	Range %
<u>Red complex species:</u>			
<i>P. gingivalis</i>	15 (40.5)	14.4 \pm 4.4	0.1-59.1
<i>T. forsythia</i>	19 (51.4)	1.4 \pm 0.3	0.1-6.2
<u>Orange complex species:</u>			
<i>P. intermedia/nigrescens</i>	36 (97.3)	5.5 \pm 1.0	0.1-26.7
<i>F. nucleatum</i>	36 (97.3)	7.3 \pm 0.8	1.4-21.6
<i>P. micra</i>	37 (100)	10.8 \pm 1.4	2.5-38.5
<i>C. rectus</i>	20 (54.1)	2.1 \pm 0.6	0.1-9.1
<i>S. constellatus</i>	10 (27.0)	2.1 \pm 0.5	0.4-5.0
<u>Other species:</u>			
<i>S. intermedius</i>	10 (27.0)	4.7 \pm 1.8	0.2-16.3

Table 2. Occurrence of *in vitro* resistance among subgingival test putative periodontal pathogens to non-susceptible antibiotic breakpoint concentrations in primary isolation plates.

Test species:	Spiramycin (4 mg/L)	Amoxicillin (8 mg/L)	Metronidazole (16 mg/L)	Spiramycin (4 mg/L) plus Metronidazole (16 mg/L) ^b	Amoxicillin (8 mg/L) plus Metronidazole (16 mg/L) ^b
<u>Red complex species:</u>					
<i>P. gingivalis</i>	1 (6.7) ^a	0	0	0	0
<i>T. forsythia</i>	1 (5.3)	3 (15.8)	0	0	0
<u>Orange complex species:</u>					
<i>P. intermedia/nigrescens</i>	4 (11.1)	20 (55.5)	0	0	0
<i>F. nucleatum</i>	16 (44.4)	2 (5.6)	0	0	0
<i>P. micra</i>	4 (10.8)	1 (2.7)	0	0	0
<i>C. rectus</i>	0	0	0	0	0
<i>S. constellatus</i>	1 (10.0)	1 (10.0)	7 (70.0)	1 (10.0)	1 (10.0)
<u>Other species:</u>					
<i>S. intermedius</i>	1 (10.0)	0	4 (40.0)	1 (10.0)	0

^a No. (%) of species-positive subjects with strains resistant *in vitro* to non-susceptible antibiotic breakpoint concentrations in primary isolation plates.

^b Joint post hoc consideration of *in vitro* resistance data for each antibiotic.

Discussion

The present study findings represent the first data in over 25 years on *in vitro* spiramycin resistance in putative periodontal pathogens in the United States. *In vitro* resistance among recovered putative periodontal pathogens to spiramycin at 4 mg/L was found in 48.7% of severe periodontitis patients evaluated, which was less *in vitro* resistance than occurred with amoxicillin at 8 mg/L (62.2% of subjects), but more than was found with metronidazole at 16 mg/L (27.0%). These findings highlight the considerable subject variation seen across single antibiotic drugs in their antimicrobial effects against putative periodontal pathogens, which can markedly influence the selection and efficacy of periodontal antibiotic treatment regimens (39).

Among individual microbial species, most red and orange complex organisms, which are associated with increasingly more severe forms of periodontitis (38), were inhibited *in vitro* by spiramycin at 4 mg/L in primary isolation plates (Table 2). However, *F. nucleatum* was frequently resistant *in vitro* to spiramycin (44.4% of subject strains), consistent with previous reports on periodontitis stains from the United States (8,9) and other countries (5,12,13,16,17,40). Some subject strains of *P. gingivalis*, *T. forsythia*, *P. intermedia/nigrescens* and *P. micra* also exhibited *in vitro* resistance to spiramycin, but at lower proportions than homologous species strains previously isolated and tested in France (16). For reasons to be determined, all *C. rectus* in the present study were inhibited *in vitro* by spiramycin at 4 mg/L in primary isolation plates, as compared to 80% *C. rectus* resistance to spiramycin at the same *in vitro* concentration in France (17).

Since there is negligible spiramycin use in the general United States population, it is unlikely that the spiramycin resistance detected in the present study subjects is the result of prior spiramycin drug exposures promoting selection of resistant microbial species, in contrast to persons in countries where spiramycin is extensively prescribed. Instead, the increasing dissemination among gram-negative bacteria, via mobile plasmids and/or conjugative transposons, of various drug resistance genes active against all macrolide-class antibiotics may account for *in vitro* spiramycin resistance among periodontal microorganisms not previously exposed to spiramycin (41,42). However, the molecular basis for the *in vitro* spiramycin drug resistance detected in the present study remains to be determined.

In vitro resistance to amoxicillin included most *P. intermedia/nigrescens* subject strains, and a smaller subset of *T. forsythia*, *S. constellatus*, *F. nucleatum*, and *P. micra*, with metronidazole resistance found only among most *S. constellatus* and some *S. intermedius* clinical isolates, similar to previous reports (13,18,37,40).

The direct plating method used in this study to detect antibiotic resistance on primary culture plates has been previously used in a number of periodontal microbiology studies (23,36,37), and shown to highly correlate ($r^2 = 0.99$) with the CLSI-approved multiple agar dilution assay in successfully identifying antibiotic-resistant periodontal microorganisms (36). Similarly, Lewis et al. (43) reported a 94% agreement between primary versus secondary (subculture) antibiotic susceptibility plate testing of acute dentoalveolar abscess bacterial isolates. The relatively high viable anaerobic counts (on average in excess of 10^7 organisms/ml) recovered on non-selective EBBA media in the present study suggests that

an adequate subgingival sample inoculum was spread on antibiotic-supplemented primary isolation plates examined for resistant species growth. However, the inability to determine exact minimal inhibitory concentration (MIC) values of antimicrobials tested against microbial species is a shortcoming of this approach, since only non-susceptible/resistant breakpoint concentrations, as recommended by the French Society for Microbiology (26) for spiramycin, and the Clinical and Laboratory Standards Institute (27) for amoxicillin and metronidazole, were employed per antibiotic in the *in vitro* resistance testing. Nevertheless, identification of *in vitro* periodontal pathogen resistance to therapeutic concentrations of antimicrobials contemplated for patient care is highly relevant and clinically practical to dentists striving to avoid prescribing antibiotics ineffective against drug-resistant pathogens.

Since increasing interest has developed on use of combination systemic antibiotic regimens in periodontitis treatment, where two antibiotics with complementary antibacterial activity are administered to broaden the spectrum of antimicrobial effects against subgingival and soft tissue-invading bacterial pathogens (39,44), the present study also jointly considered subject-based resistance data post hoc for spiramycin at 4 mg/L plus metronidazole at 16 mg/L. Interestingly, all isolated putative periodontal pathogens were inhibited *in vitro* by one or the other of the antibiotic concentrations in primary isolation plates, except for one strain each of *S. constellatus* and *S. intermedius* from one (2.7%) study subject. Since the two antibiotic concentrations were not incorporated together into antibiotic-supplemented primary isolation plates, no conclusions can be drawn about synergistic drug effects of spiramycin plus metronidazole against oral bacterial species, which have been noted in some previous studies (5,10,11,40). However, these favorable microbiological effects are consistent with the results of a double-blind clinical trial finding systemic spiramycin plus metronidazole to significantly enhance gains of clinical periodontal attachment on initially deep periodontal pockets at six months post-treatment on adults with severe periodontitis (45).

A similar post hoc joint analysis also found either amoxicillin at 8 mg/L or metronidazole at 16 mg/L in primary isolation plates inhibited all recovered putative periodontal pathogens, except *S. constellatus* in one (2.7%) subject, which is also consistent with clinical trials demonstrating enhanced therapeutic benefits of systemic amoxicillin plus metronidazole in aggressive and chronic periodontitis therapy (44).

While the periodontists who performed clinical examinations in the present study were not formally calibrated, support for their severe periodontitis diagnoses was evidenced by their identification in the study subjects of three or more periodontal sites with deep probing depths with bleeding on probing (mean 7.5 mm at microbiologically-sampled periodontal sites), which strongly correlates (94.1% positive predictive value) with the presence of severe periodontal attachment loss in adult patients (46).

Overall, these findings suggest that the *in vitro* effectiveness of spiramycin against putative periodontal pathogens can be enhanced by broadening its antimicrobial spectrum with metronidazole, and that the joint *in vitro* antimicrobial effects of spiramycin plus metronidazole against a wide range of putative periodontal pathogens appears to be similar to that seen with amoxicillin plus metronidazole. Thus, a clinical periodontitis treatment strategy involving systemic spiramycin plus metronidazole, but not spiramycin alone, may

have potential therapeutic efficacy similar to the combination of amoxicillin plus metronidazole, and may be particularly beneficial as an alternative for periodontitis patients hypersensitive to amoxicillin or other beta-lactam antibiotics, and not colonized by *A. actinomycetemcomitans* strains resistant to spiramycin and metronidazole (47).

Conclusions

In vitro spiramycin resistance among putative periodontal pathogens of United States origin was found in approximately one-half of severe periodontitis patients evaluated, particularly among *F. nucleatum* species. *In vitro* resistance patterns also suggest that therapeutic concentrations of spiramycin plus metronidazole may have potential antimicrobial efficacy in non-*A. actinomycetemcomitans*-associated periodontitis similar to amoxicillin plus metronidazole, which may be beneficial, where spiramycin is clinically available, for patients hypersensitive to amoxicillin or other beta-lactam antibiotics. Further clinical and microbiological studies of spiramycin, particularly in combination with metronidazole, are indicated to further clarify its potential value in treatment of human periodontitis.

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Chapter 7

Antibiotic resistance in human peri-implantitis microbiota

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Abstract

Purpose: Because antimicrobial therapy is often employed in the treatment of infectious dental implant complications, this study determined the occurrence of *in vitro* antibiotic resistance among putative peri-implantitis bacterial pathogens.

Materials and methods: Submucosal biofilm specimens were cultured from 160 dental implants with peri-implantitis in 120 adults, with isolated putative pathogens identified to species level, and tested *in vitro* for susceptibility to 4 mg/L of doxycycline, 8 mg/L of amoxicillin, 16 mg/L of metronidazole, and 4 mg/L of clindamycin. Findings for amoxicillin and metronidazole were combined post-hoc to identify peri-implantitis species resistant to both antibiotics. Gram-negative enteric rods/pseudomonads were subjected to ciprofloxacin disk diffusion testing.

Results: One or more cultivable submucosal bacterial pathogens, most often *Prevotella intermedia/nigrescens* or *Streptococcus constellatus*, were resistant *in vitro* to clindamycin, amoxicillin, doxycycline or metronidazole in 46.7%, 39.2%, 25%, and 21.7% of the peri-implantitis subjects, respectively. Only 6.7% subjects revealed submucosal test species resistant *in vitro* to both amoxicillin and metronidazole, which were either *S. constellatus* (one subject) or ciprofloxacin-susceptible strains of gram-negative enteric rods/pseudomonads (seven subjects). Overall, 71.7% of the 120 peri-implantitis subjects exhibited submucosal bacterial pathogens resistant *in vitro* to one or more of the tested antibiotics.

Conclusions: Peri-implantitis patients frequently yielded submucosal bacterial pathogens resistant *in vitro* to individual therapeutic concentrations of clindamycin, amoxicillin, doxycycline or metronidazole, but only rarely to both amoxicillin and metronidazole. Due to the wide variation in observed drug resistance patterns, antibiotic susceptibility testing of cultivable submucosal bacterial pathogens may aid in the selection of antimicrobial therapy for peri-implantitis patients.

Introduction

Peri-implantitis is a destructive biological complication affecting dental implants after successful intraoral placement and prosthetic restoration (1). Peri-implantitis presents as an inflammatory lesion of peri-implant soft and hard tissues, characterized by increased peri-implant probing depths, bleeding on probing and/or suppuration, progressive peri-implant marginal bone loss, and ultimately, dental implant mobility and loss (2,3). Peri-implantitis is estimated to occur in 10.7-47.2% of dental implant patients after 10 years of post-treatment observation (4). A multitude of risk factors have been associated with the onset and progression of peri-implantitis, including submucosal presence of various bacterial species, *Archaea*, yeasts, and herpesviruses (5-7); inadequate oral hygiene (2,8); smoking (2); excessive occlusal forces (9,10); contamination, corrosion, and residual dental cement on submucosal implant surfaces (11,12); history of periodontitis on adjacent natural teeth (13); poorly controlled diabetes mellitus (2); and host carriage of IL-1RN gene polymorphisms (14).

Because the etiopathogenesis of peri-implantitis is not well delineated, it is not surprising that the most effective treatment for peri-implantitis has yet to be conclusively identified (15). However, studies and case reports in both animal models and humans have reported arrest of peri-implantitis lesions, leading to marked clinical and/or radiographic improvements, when systemic antibiotics were given as adjuncts to mechanical debridement and/or surgical procedures on affected dental implants heavily colonized by putative bacterial pathogens (16-18). As a result, systemic antibiotic therapy is often advised as a part of peri-implantitis treatment protocols (19-21), similar to use of systemic antibiotics in periodontitis treatment (22), despite an absence to date of strong supporting scientific data (23).

Testing subgingival bacterial species for their antibiotic susceptibility is recommended to help optimize periodontitis-directed antibiotic drug regimens in order to avoid administration of antimicrobial agents to which targeted pathogens are resistant (22). In contrast, little attention has been given to this issue in peri-implantitis disease management, with most dentists empirically employing antibiotics against peri-implantitis lesions without any prior microbiological testing (24). Because relatively few studies to date have examined the *in vitro* antibiotic susceptibility profiles of peri-implantitis-associated microorganisms (25-29), the extent to which antibiotic resistance occurs in submucosal microbial populations associated with peri-implantitis remains largely unknown. To address this need, the present study assessed the occurrence of *in vitro* antibiotic resistance among putative bacterial pathogens isolated from human peri-implantitis lesions.

Materials and methods

Subjects

A retrospective examination of archival records was performed at the Oral Microbiology Testing Service (OMTS) Laboratory at Temple University School of Dentistry, Philadelphia. A total of 120 partially dentate adults were consecutively identified and included in the present study as their peri-implant submucosal plaque samples were submitted for microbiological analysis to the OMTS Laboratory between 2006 and 2012.

Each of these adults were diagnosed, in the professional judgment of periodontists in private dental practices in the USA, with peri-implantitis (1) on one to five prosthetically restored, root-form dental implants, for a total of 160 dental implants, which were microbiologically sampled for culture analysis and *in vitro* antibiotic resistance testing. Laboratory records were used to ascertain, as reported by the treating periodontist, the geographic location of their private dental practice, and the patient gender, age, current smoking habit, peri-implant probing depths, and presence of peri-implant bleeding on probing, suppuration, and progressive radiographic marginal bone loss. Independent clinical and radiographic evaluations were not performed on the study subjects, and data were not available on their ethnicity, systemic medical status, past antibiotic use, oral hygiene performance, parafunctional occlusal habits, periodontitis history and treatment, and length of time since dental implant prosthetic loading. The study was approved by the Temple University Human Subjects Protections Institutional Review Board, and conducted in accordance with ethical principles detailed in the World Medical Association's Declaration of Helsinki, as revised in 2008.

Microbial sampling and transport

Submucosal plaque biofilm specimens were obtained by the diagnosing periodontists, who followed a standardized sampling protocol, before treatment from two to five deep peri-implant probing depths per subject that exhibited bleeding on probing with or without suppuration. After isolation with cotton rolls, and removal of saliva and supramucosal deposits, one to two sterile, absorbent paper points (Johnson & Johnson, East Windsor, NJ, USA) were advanced into each selected peri-implant site for approximately 10 seconds. Upon removal, all paper points per study subject were pooled in a glass vial containing six to eight small glass beads and 2.0 ml of anaerobically prepared and stored VMGA III transport medium (30), which possesses a high preservation capability for oral microorganisms during post-sampling transit to the laboratory (30,31). The submucosal samples were then transported within 24 hours to the OMTS Laboratory, which is licensed for high complexity bacteriological analysis by the Pennsylvania Department of Health. The OMTS Laboratory is also federally certified by the USA Department of Health and Human Services to be in compliance with Clinical Laboratory Improvement Amendments (CLIA)-mandated proficiency testing, quality control, patient test management, personnel requirements, and quality assurance standards required of clinical laboratories engaged in diagnostic testing of human specimens in the USA (32). All laboratory procedures were performed following a protocol standardized during the 2006-2012 study time period, and by laboratory personnel who were blinded to the clinical status of the study subjects, and their inclusion in the present analysis.

Microbial culture and incubation

At the OMTS Laboratory, the specimen vials were warmed to 35°C to liquefy the VMGA III transport medium, and sampled microorganisms were mechanically dispersed from the paper points with a vortex mixer, which was used at the maximal setting for 45 seconds. Serial, 10-fold dilutions of the dispersed bacteria were prepared in Möller's VMG I anaerobic dispersion solution, comprised of pre-reduced, anaerobically sterilized, 0.25% tryptose, 0.25% thiotone E peptone, and 0.5% NaCl (30). Then, 0.1 ml dilution aliquots were spread, with a sterile bent glass rod, onto non-selective enriched Brucella blood agar

(EBBA) primary isolation plates (33), comprised of 4.3% Brucella agar supplemented with 0.3% bacto-agar, 5% defibrinated sheep blood, 0.2% hemolyzed sheep red blood cells, 0.0005% hemin, and 0.00005% menadione, onto Hammond's selective *Campylobacter* medium (34), and onto selective trypticase soy-bacitracin-vancomycin (TSBV) agar (35). EBBA and Hammond's selective medium plates were incubated at 35°C for seven days in a Coy anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI, USA) containing 85% N₂, 10% H₂, and 5% CO₂, and TSBV plates were incubated at 35°C for three days in air + 5% CO₂.

Microbial identification

Submucosal test species examined for in this study were *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Prevotella intermedia/nigrescens*, *Parvimonas micra*, *Fusobacterium nucleatum*, *Campylobacter rectus*, *Streptococcus constellatus*, *Centipeda periodontii*, *Staphylococcus aureus*, *Enterococcus faecalis*, gram-negative enteric rods/pseudomonads, and *Candida* species.

Total anaerobic viable counts and counts of *P. gingivalis*, *T. forsythia*, *P. intermedia/nigrescens*, *P. micra*, *F. nucleatum*, *S. constellatus*, *C. periodontii*, *S. aureus*, and *E. faecalis* were made on EBBA primary isolation plates using a ring-light magnifying loupe, a dissecting stereomicroscope, and presumptive phenotypic methods previously described (36-41). *C. rectus* was quantitated on Hammond's medium, and *A. actinomycetemcomitans*, gram-negative enteric rods/pseudomonads, and *Candida* species on TSBV agar, using methods and criteria previously described (33,35,42). The proportional recovery of each test species was ascertained in each subject by calculating the percentage of test species colony-forming units relative to total submucosal anaerobic viable counts as determined on non-selective EBBA primary isolation plates.

In vitro antibiotic resistance testing

Additional 0.1 ml aliquots of submucosal sample dilutions were inoculated onto EBBA primary isolation plates supplemented with either 4 mg/L of doxycycline, 8 mg/L of amoxicillin, 16 mg/L of metronidazole, or 4 mg/L of clindamycin (all antimicrobials obtained as pure powder from Sigma-Aldrich, St. Louis, MO, USA), and incubated anaerobically for seven days. These antimicrobial concentrations represent non-susceptible/resistant breakpoint concentrations against anaerobic bacteria for amoxicillin, metronidazole and clindamycin as recommended by the Clinical and Laboratory Standards Institute (CLSI) (43), and for doxycycline disk diffusion testing as recommended by the French Society for Microbiology (44). Direct colony suspensions (equivalent to a 0.5 McFarland standard) of pure *A. actinomycetemcomitans* isolates from selective TSBV plates were subcultured onto these media as their recognition is frequently obscured within mixed bacterial populations (35). *In vitro* resistance to the antibiotic breakpoint concentrations of doxycycline, amoxicillin, metronidazole, or clindamycin was recorded when test species growth was noted on the respective antibiotic-supplemented EBBA plates (33,40,41,45,46). *Bacteroides thetaiotaomicron* ATCC 29741, *Clostridium perfringens* ATCC 13124, and a multi-antibiotic-resistant clinical periodontal isolate of *F. nucleatum* were employed as positive and negative quality controls for all antibiotic resistance testing on drug-supplemented EBBA plates.

In vitro data were combined post-hoc for amoxicillin and metronidazole to identify test species resistant to both antibiotics (40). A pilot study evaluated the validity of this approach using subgingival plaque biofilm samples from 52 subjects with severe chronic periodontitis who were not part of the present study. The microbial specimens were processed as described above, with inoculation onto EBBA primary isolation plates without antibiotics, or supplemented with either 8 mg/L of amoxicillin, 16 mg/L of metronidazole, or a combination of both 8 mg/L of amoxicillin and 16 mg/L of metronidazole, followed by anaerobic incubation for seven days.

Gram negative enteric rods/pseudomonads recovered on TSBV primary isolation plates were subjected to *in vitro* ciprofloxacin disk diffusion testing, with direct colony suspensions of the organisms equivalent to a 0.5 McFarland standard inoculated onto Mueller-Hinton agar incubated in ambient air at 35°C for 16-18 hours, and assessed with CLSI interpretative guidelines (47).

Data analysis

Descriptive analysis was used to characterize the number of dental implants, their arch location (maxilla, mandible, or both), and intraoral region (anterior, posterior, or both), per each study subject. Submucosal proportions of the test microbial pathogens, and for strains exhibiting *in vitro* antibiotic resistance, were determined for each study subject. Then, the occurrence of test species, as well as their mean and median submucosal proportions, was tabulated across all subjects, which served as the unit of analysis in the results. Semi-quantification cut-off values were also applied to microbial proportional recovery data to identify peri-implantitis test species and subjects exhibiting moderately heavy (1-10% of total cultivable counts) and heavy (> 10%) microbial growth in submucosal plaque biofilm specimens, as previously described (48). Kappa analysis, with kappa values > 0.75 indicative of excellent agreement (49), quantified agreement in the pilot study data between test species growth on EBBA plates jointly supplemented with both amoxicillin and metronidazole, as compared to a post-hoc combination of data from EBBA plates individually supplemented with amoxicillin or metronidazole. Data analysis was performed using the SAS 9.2 for Windows (SAS Institute, Inc., Cary, NC, USA) statistical software package.

Results

The 120 peri-implantitis subjects included 66 men and 54 women, aged 28-90 years (mean 60.9 ± 10.3 (SD) years), and 11.7% current smokers. A total of 80 (66.7%) of the study subjects geographically originated from dental practices in Maryland ($n = 16$), Pennsylvania ($n = 30$), New Jersey ($n = 28$), Virginia ($n = 2$), and New York ($n = 4$) in the mid-Atlantic region of the USA, with all others from six other states in the eastern USA ($n = 32$), and four western states ($n = 8$).

Table 1 provides the intraoral subject distribution of the 160 microbiologically sampled dental implants, which were made by either Biomet *3i* (23.1%), Nobel Biocare (22.5%), BioHorizons (18.8%), Bicon 11.9%, or an undisclosed manufacturer (23.7%). In most study subjects (60.8%), a single dental implant with peri-implantitis was sampled, with

most located in posterior areas of the oral cavity, and similarly distributed between the maxilla and mandible (Table 1).

The sampled dental implants were reported by the diagnosing periodontists to have deep (> 6 mm) probing depths (mean 7.7 ± 0.2 (SE) mm), bleeding on probing, and progressive radiographic marginal bone loss. For 16 (13.3%) of the study subjects, suppuration was noted at the sampled dental implants.

Submucosal biofilm specimens from the study subjects averaged total anaerobic viable counts of $6.9 \times 10^7 \pm 8.4 \times 10^6$ (SE) organisms/ml of sample (range = 5.0×10^6 to 3.5×10^8 organisms/ml) on non-selective EBBA primary isolation plates. All study subjects yielded either heavy (75% subjects) or moderately heavy (25% subjects) submucosal growth by one or more of the bacterial test species.

Table 2 lists the occurrence and proportional cultivable recovery of submucosal test species from the study subjects. *P. micra*, *F. nucleatum*, and *P. intermedia/nigrescens* were the most frequent test species isolated, with heavy or moderately heavy submucosal growth of these organisms found in 83-100% of culture-positive subjects. Gram-negative enteric rods/pseudomonads, identified to a species level in one subject as *Escherichia coli*, exhibited heavy or moderately heavy submucosal growth in seven subjects. *Candida* species were recovered from four subjects in relatively low submucosal proportions (mean 0.2%). No submucosal *S. aureus* or *E. faecalis* were detected with the non-selective culture media used in the study.

For subgingival microbial samples processed in the pilot study (Table 3), 195 of 198 (98.5%) test species demonstrated excellent agreement (kappa value = 0.79 ± 0.11 (SE)) between their *in vitro* growth on EBBA plates jointly supplemented with both amoxicillin and metronidazole, and their growth patterns as determined from a post-hoc combination of *in vitro* resistance data from EBBA plates individually supplemented with amoxicillin or metronidazole. Based on this, *in vitro* antibiotic resistance data from amoxicillin and metronidazole-supplemented EBBA primary isolation plates were combined post-hoc for subjects with peri-implantitis.

Table 4 lists the occurrence of *in vitro* antibiotic resistance among peri-implantitis bacterial test species. *P. micra* and *F. nucleatum* rarely exhibited *in vitro* resistance to any of the test antibiotics. *C. rectus* and *C. periodontii* were sensitive to all of the test antibiotic concentrations. *P. gingivalis* was similarly susceptible, except for four subject strains resistant *in vitro* to clindamycin. *P. intermedia/nigrescens* revealed little or no *in vitro* resistance to doxycycline or metronidazole, but was resistant to amoxicillin among 38 subject strains, and to clindamycin in 35 subject strains, with antibiotic-resistant strains averaging 6.6% and 4.3%, respectively, of submucosal viable counts. *T. forsythia* similarly showed *in vitro* resistance to amoxicillin in six subject strains, and to clindamycin among seven subject strains. Most *S. constellatus* subject strains (18 out of 24) were resistant *in vitro* to metronidazole, with nine resistant to doxycycline, two resistant to amoxicillin, and 18 resistant to clindamycin. Antibiotic-resistant *S. constellatus* strains averaged 4-18.6% of cultivable submucosal organisms in culture-positive subjects. All submucosal gram-negative enteric rods/pseudomonads were resistant *in vitro* to doxycycline, amoxicillin, metronidazole, and clindamycin, but were all susceptible *in vitro* to ciprofloxacin in disk

diffusion testing. All six *A. actinomycetemcomitans* subject strains exhibited *in vitro* resistance to clindamycin, and five to doxycycline, whereas none were resistant *in vitro* to either amoxicillin or metronidazole (Table 4).

When *in vitro* data for amoxicillin and metronidazole were combined post hoc (Table 4), one *S. constellatus* subject stain (also resistant to doxycycline), and all gram-negative enteric rods/pseudomonads, were resistant to both antibiotic concentrations.

Among the seven study subjects with ciprofloxacin-susceptible, submucosal strains of gram-negative enteric rods/pseudomonads, two subjects yielded only gram-negative enteric rods/pseudomonads from their submucosal biofilm specimens, and three subjects additionally had moderate to heavy growth of co-colonizing metronidazole-susceptible test species (including *P. gingivalis*, *P. intermedia/nigrescens*, *P. micra*, *C. rectus* and *F. nucleatum*). In two other study subjects, one additionally had moderately heavy *P. micra* growth resistant *in vitro* to both metronidazole and doxycycline, whereas the other study subject additionally revealed moderate growth of metronidazole-susceptible test species (including *P. intermedia/nigrescens*, *P. micra*, *C. rectus* and *T. forsythia*), as well as moderate growth of metronidazole-resistant *S. constellatus* and *F. nucleatum* (which was also resistant *in vitro* to doxycycline). In the latter study subject, all of the test species accompanying the gram-negative enteric rods/pseudomonads were susceptible *in vitro* to clindamycin.

Table 5 presents the occurrence of one or more antibiotic-resistant peri-implantitis bacterial pathogens in the study subjects. A total of 46.7% of subjects with peri-implantitis yielded clindamycin-resistant bacterial test species. In comparison, 39.2%, 25% and 21.7% of subjects had submucosal bacterial test species resistant *in vitro* to amoxicillin, doxycycline, and metronidazole, respectively. In addition, 6.7% subjects with peri-implantitis harbored submucosal bacterial test species resistant *in vitro* to both amoxicillin at 8 mg/L and metronidazole at 16 mg/L (Table 4). Overall, 86 (71.7%) of the 120 subjects with peri-implantitis exhibited submucosal bacterial pathogens resistant *in vitro* to one or more of the test antibiotics, whereas 34 (28.3%) study subjects did not reveal any peri-implantitis bacterial test species resistant *in vitro* to any of the evaluated antibiotics.

In the four study subjects with sparse submucosal growth of *Candida* species, which were resistant *in vitro* to all of the test antibiotics, none of their recovered bacterial test species exhibited *in vitro* resistance to any of the test antibiotics.

Table 1. Intraoral distribution of peri-implantitis-affected dental implants in 120 adults.

Item	No. (%) of study subjects
<u>Implants/subject</u>	
one	73 (60.8)
2-3	32 (26.7)
4-5	15 (12.5)
<u>Arch location</u>	
maxilla	56 (46.7)
mandible	52 (43.3)
both	12 (10.0)
<u>Intraoral region</u>	
anterior	25 (20.8)
posterior	76 (63.3)
both	19 (15.8)

Table 2. Occurrence and proportional submucosal culture recovery of putative peri-implantitis microbial pathogens in 120 adults.

Test species:	No. (%) of culture positive subjects	Mean % ± standard error (median %) recovery in positive subjects	Range %	No. (%) of culture positive subjects	
				Moderately heavy growth ^a	Heavy growth ^a
<i>P. micra</i>	111 (92.5)	12.4 ± 0.8 (10.0)	1.4-40.0	53 (47.7)	58 (52.3)
<i>F. nucleatum</i>	98 (81.7)	10.6 ± 1.0 (7.5)	1.1-52.6	62 (63.3)	36 (36.7)
<i>P. intermedia/nigrescens</i>	94 (78.3)	6.4 ± 0.8 (3.4)	0.1-39.0	58 (61.7)	20 (21.3)
<i>C. rectus</i>	65 (54.2)	1.5 ± 0.3 (0.7)	0.1-15.5	28 (43.1)	1 (1.5)
<i>T. forsythia</i>	33 (27.5)	2.1 ± 0.4 (1.2)	0.1-10.2	20 (60.6)	1 (3.0)
<i>P. gingivalis</i>	32 (26.7)	6.4 ± 1.3 (2.9)	0.1-29.2	21 (65.6)	7 (21.9)
<i>S. constellatus</i>	24 (20.0)	5.6 ± 1.3 (4.1)	0.2-19.3	12 (50.0)	5 (20.8)
enteric rods/pseudomonads	7 (5.8)	29.5 ± 10.6 (18.5)	1.5-77.8	2 (28.6)	5 (71.4)
<i>A. actinomycetemcomitans</i>	6 (5.0)	4.4 ± 2.6 (2.2)	0.2-17.0	4 (80.0)	1 (16.7)
<i>Candida</i> species	4 (3.3)	0.2 ± 0.1 (0.1)	0.1-0.4	0	0
<i>C. periodontii</i>	2 (1.7)	0.2 ± 0.1 (0.1)	0.1-0.3	0	0
<i>S. aureus</i>	0	0	0	0	0
<i>E. faecalis</i>	0	0	0	0	0

^a Moderately heavy submucosal growth = 1-10% of total viable counts; heavy submucosal growth = > 10% of total viable counts

Table 3. Occurrence of test species growth on EBBA primary isolation plates where amoxicillin and metronidazole were individually incorporated and growth data combined post-hoc, as compared to plates jointly supplemented with both antibiotics, among 198 test species^a recovered from 52 severe chronic periodontitis subjects.

	Test species growth <i>present</i> on EBBA primary isolation plates supplemented with both amoxicillin plus metronidazole ^b	Test species growth <i>absent</i> on EBBA primary isolation plates supplemented with both amoxicillin plus metronidazole ^b
Test species growth <i>present</i> on both EBBA primary isolation plates individually supplemented with amoxicillin or metronidazole ^b	6 ^{c,d}	0
Test species growth <i>absent</i> on one or both EBBA primary isolation plates individually supplemented with amoxicillin or metronidazole ^b	3 ^e	189

^a Includes *A. actinomycetemcomitans* (4 subject strains), *P. gingivalis* (16), *P. intermedia/nigrescens* (47),

F. nucleatum (50), *P. micra* (50), *S. constellatus* (30), and gram-negative enteric rods/pseudomonads (1).

^b *In vitro* concentration of amoxicillin at 8 mg/L and metronidazole at 16 mg/L.

^c No. of test species.

^d Includes five subject strains of *S. constellatus* and one subject strain of gram-negative enteric rods/pseudomonads.

^e *S. constellatus* subject strains absent on amoxicillin, but present on metronidazole, antibiotic-supplemented plates.

Table 4. Number of peri-implantitis subjects with test bacterial pathogens resistant *in vitro* to antibiotic breakpoint concentrations.

Test species:	<i>n</i>	clindamycin (4 mg/L) ^a	doxycycline (4 mg/L)	amoxicillin (8 mg/L)	metronidazole (16 mg/L)	amoxicillin plus metronidazole ^b
<i>P. micro</i>	<i>n</i> %	4 ^c 4.8 ± 2.0 (5.9) ^d	1 4.0	2 3.4 ± 2.3 (3.4)	3 4.5 ± 0.3 (4.5)	0
<i>F. nucleatum</i>	<i>n</i> %	1 2.0	1 4.6	1 4.1	1 4.6	0
<i>P. intermedia/nigrescens</i>	<i>n</i> %	35 4.3 ± 1.2 (2.7)	7 13.0 ± 6.9 (3.3)	38 6.6 ± 1.5 (3.4)	0	0
<i>C. rectus</i>	<i>n</i> %	0	0	0	0	0
<i>T. forsythia</i>	<i>n</i> %	7 2.8 ± 1.3 (2.2)	1 0.2	6 1.9 ± 0.5 (2.3)	0	0
<i>P. gingivalis</i>	<i>n</i> %	4 1.5 ± 0.6 (1.7)	0	0	0	0
<i>S. constellatus</i>	<i>n</i> %	3 4.0 ± 1.8 (5.6)	9 7.0 ± 2.4 (5.3)	2 9.7 ± 7.2 (9.7)	18 5.8 ± 1.7 (4.2)	1
enteric rods/psuedomonads	<i>n</i> %	7 29.5 ± 10.6 (18.5)	7 29.5 ± 10.6 (18.5)	7 29.5 ± 10.6 (18.5)	7 29.5 ± 10.6 (18.5)	7 29.5 ± 10.6 (18.5)
<i>A. actinomycetemcomitans</i>	<i>n</i> %	6 4.4 ± 2.6 (2.2)	5 4.8 ± 3.1 (2.6)	0	0	0
<i>C. periodontii</i>	<i>n</i> %	0	0	0	0	0

^a Non-susceptible antibiotic breakpoint concentrations incorporated into primary isolation plates.

^b Joint post hoc consideration of *in vitro* resistance data for amoxicillin at 8 mg/L and metronidazole at 16 mg/L.

^c Number of subjects with antibiotic-resistant strains of species.

^d Mean % ± standard error (median %) recovery of antibiotic-resistant strains in positive subjects.

Table 5. Occurrence of antibiotic-resistant submucosal pathogens in 120 subjects with peri-implantitis.

Antibiotic (breakpoint concentration)	No. (%) of subjects with ≥ 1 putative peri-implantitis bacterial pathogens resistant <i>in vitro</i> to antibiotic breakpoint concentrations
clindamycin (4 mg/L)	56 (46.7)
amoxicillin (8 mg/L)	47 (39.2)
doxycycline (4 mg/L)	30 (25.0)
metronidazole (16 mg/L)	26 (21.7)
amoxicillin (8 mg/L) + metronidazole (16 mg/L) ^a	8 (6.7)

^a Post-hoc combination of *in vitro* resistance data for amoxicillin at 8 mg/L and metronidazole at 16 mg/L.

Discussion

All of the subjects with peri-implantitis in this study yielded heavy or moderately heavy submucosal growth by one or more of the bacterial test species. This suggests a potential contributing role for these microbial species in the pathogenesis of the evaluated peri-implantitis lesions, and supports a possible role for antimicrobial agents in their therapeutic management. As a result, assessment of their antibiotic susceptibility profile *in vitro* is clinically relevant.

The frequent submucosal occurrence in this study of *P. micra*, *F. nucleatum*, and *P. intermedia/nigrescens*, as well as the recovery of *C. rectus*, *T. forsythia*, *P. gingivalis*, *S. constellatus*, *A. actinomycetemcomitans*, and gram-negative enteric rods/pseudomonads, is consistent with previous culture-based investigations of human peri-implantitis (5). These species have also been implicated as putative pathogens in the pathogenesis of periodontitis (33,37,42,50). Additional bacterial species and novel phylotypes may be identified in peri-implantitis lesions with other types of selective culture media (37,38), and with various nucleic acid-based molecular methods, such as checkerboard DNA-DNA hybridization (51), end-point and real-time PCR (52,53), denaturing gradient gel electrophoresis (54,55), and 16S ribosomal RNA sequencing (56,57). It is not known from the present retrospective study design if these organisms initiated peri-implantitis similar to periodontitis, or merely colonized deep peri-implant probing depths secondarily after other etiologic factors triggered the onset of peri-implant marginal tissue breakdown.

Nevertheless, the purpose of the present study was to assess the occurrence of *in vitro* antibiotic resistance among cultivable bacterial pathogens in human peri-implantitis lesions. In this regard, over 70% of the peri-implantitis study subjects yielded submucosal bacterial pathogens resistant *in vitro* to therapeutic concentrations of one or more of clindamycin, doxycycline, amoxicillin, or metronidazole alone, or to both amoxicillin and metronidazole. This suggests that patients with peri-implantitis in the USA frequently harbor submucosal bacterial pathogens resistant to several antibiotics commonly employed in clinical dental and periodontal practice.

The highest subject frequency (46.7%) of *in vitro* antibiotic resistance among submucosal bacterial pathogens was to clindamycin, particularly in strains of *P. intermedia/nigrescens*, *T. forsythia*, and *A. actinomycetemcomitans*. Amoxicillin submucosal bacterial pathogen resistance was detected in 39.2% of subjects with peri-implantitis, most notably in *P. intermedia/nigrescens*, which is often positive for amoxicillin-degrading β -lactamase enzyme production (41,58). Doxycycline and metronidazole bacterial pathogen resistance was observed in 25% and 21.7% of subjects with peri-implantitis, respectively, most often among *S. constellatus* submucosal isolates. Interestingly, since metronidazole in the presence of titanium shows enhanced antimicrobial activity *in vitro* against *P. gingivalis* and *P. intermedia* biofilms (59), it is possible that similar improved drug effects may occur *in vivo* and potentially lessen metronidazole resistance in bacterial biofilms colonizing titanium dental implant surfaces.

The relatively low subject frequency (6.7%) in subjects of peri-implantitis bacterial pathogen resistance to both amoxicillin and metronidazole was similar to that found among subgingival bacterial pathogens in chronic periodontitis (40), and is consistent with reports

of improved peri-implantitis clinical and radiographic parameters following systemic administration of the combination of amoxicillin and metronidazole (18). Sbordone et al. (25) also found amoxicillin plus metronidazole to be highly active *in vitro* against peri-implantitis isolates of *P. gingivalis*, *P. intermedia*, and *F. nucleatum*. These findings suggest that the effects of adjunctive amoxicillin plus metronidazole in peri-implantitis treatment may mirror the drug combination's efficacy in enhancing periodontitis therapy (60,61).

It is noteworthy that submucosal *S. constellatus* in one subject, and gram-negative enteric rods/pseudomonads in seven subjects, were resistant *in vitro* to both amoxicillin at 8 mg/L and metronidazole at 16 mg/L. The relatively high submucosal levels of these multidrug-resistant organisms in species-positive subjects (Table 3) argue against administration of amoxicillin plus metronidazole in colonized patients with peri-implantitis. In considering alternative antimicrobial regimens, it is important to take into account the antimicrobial susceptibility of other co-colonizing bacterial pathogens within peri-implantitis lesions. Submucosal gram-negative enteric rods/pseudomonads in all seven species-positive study subjects were sensitive *in vitro* to ciprofloxacin, which exerts only weak activity against oral anaerobic bacteria (62). Three of these subjects also yielded species of metronidazole-susceptible anaerobic bacterial pathogens, whereas two other subjects revealed clindamycin susceptible bacterial pathogens, which were otherwise resistant *in vitro* to the other test antibiotics. Thus, for these latter two types of submucosal biofilm profiles, the use of ciprofloxacin plus metronidazole, or ciprofloxacin plus clindamycin, for adjunctive drug therapy may be more appropriate to consider than ciprofloxacin alone (22). This illustrates the variability of antibiotic susceptibility patterns that may occur in mixed facultative-anaerobic bacterial pathogen populations on ailing dental implants, and the potential complexities faced by clinicians contemplating selection and administration of peri-implantitis antimicrobial therapies.

While the present study evaluated, to date, the largest number of human subjects with peri-implantitis ($n = 120$) for *in vitro* antibiotic resistance among cultivable submucosal putative bacterial pathogens, it is important to appreciate several limitations in its retrospective study design. First, the study subjects embodied a private dental practice-based convenience sample that may not be statistically representative of peri-implantitis patients in the USA or other countries. This limits the generalizability of the present study findings to other peri-implantitis patient populations, which may possess geographic-based differences in antibiotic susceptibility patterns among oral bacterial species (46,63). Because no clinical or radiographic evaluations were conducted by calibrated examiners independent of the private practice periodontists who submitted submucosal biofilm specimens for microbiological testing, the diagnosis of peri-implantitis in the study subjects may be questioned. While the private practice periodontists were not formally calibrated in their classification of peri-implantitis, their identification of deep peri-implant probing depths (mean 7.7 mm) exhibiting bleeding on probing and/or suppuration and progressive marginal bone loss is consistent with recognized criteria for peri-implantitis (1), and correlates with data demonstrating a statistically significant odds ratio of 4.6 between peri-implant probing depths ≥ 6 mm and a history of marked progressive marginal bone loss on prosthetically-restored dental implants (64). Because information was also not accessible for the study subjects on their ethnicity, systemic medical status, past antibiotic use, oral hygiene performance, parafunctional occlusal habits, periodontitis history and treatment,

and length of time since dental implant prosthetic loading, the potential influence of these variables on the observed *in vitro* antibiotic resistance patterns could not be evaluated, and remains to be delineated.

In addition, exact minimal inhibitory concentration values of the test antimicrobials against the peri-implantitis bacterial pathogens were not determined with the clinical laboratory methods followed, and the molecular genetic basis for the detected peri-implant bacterial drug resistance was not elucidated. However, the direct plating method used in this study to detect antibiotic resistance on primary culture plates has been employed in previous periodontal microbiology studies (33,40,41,45,46), and correlates well ($r^2 = 0.99$) with the CLSI-approved agar dilution susceptibility assay in identifying antibiotic-resistant periodontal microorganisms (45). Similarly, a 94% agreement rate has been reported between primary (direct) versus secondary (subculture) antibiotic susceptibility plate testing on acute dentoalveolar abscess bacterial isolates (65). Indeed, identification of submucosal bacterial pathogens resistant to internationally-recognized therapeutic breakpoint concentrations of antibiotics being considered in peri-implantitis therapy, irrespective of the exact minimal inhibitory drug concentration values against the organisms, is highly relevant and clinically practical to treating dentists seeking to enhance their antimicrobial drug selections for patients.

However, because antibiotic susceptibility findings *in vitro* do not necessarily by themselves confer drug effectiveness *in vivo* against targeted bacterial pathogens (66), the extent to which the reported laboratory findings may potentially impact the treatment of human peri-implantitis lesions is not known. Additional variables influencing the success of antimicrobial therapy in clinical settings need to be considered, such as the ability of patients to safely take medications (i.e., no contraindicating allergies, drug interactions or side effects), differences among patients in oral antibiotic absorption (67), drug passage through peri-implant tissues, antibiotic penetration into antimicrobial-resistant submucosal bacterial biofilms (68), and patient compliance with prescribed drug dosing schedules. However, it is generally recognized that drugs lacking antimicrobial efficacy against targeted microorganisms under *in vitro* laboratory testing conditions are unlikely to be effective therapeutic agents *in vivo* against the organisms (69). Additional research is needed to address these issues specific to patients with dental implants, and to further elucidate the etiology of peri-implantitis.

Conclusions

A relatively wide range of *in vitro* antibiotic resistance was found among putative bacterial pathogens recovered from submucosal surfaces of 160 dental implants in 120 patients suffering from peri-implantitis. The highest frequency of *in vitro* antibiotic submucosal bacterial pathogen resistance in subjects with peri-implantitis was to clindamycin, whereas joint resistance to amoxicillin plus metronidazole was rare. No single antibiotic or antibiotic combination tested *in vitro* showed complete inhibition of peri-implantitis bacterial pathogens across all of the study subjects. These findings suggest that microbiological analysis and *in vitro* antibiotic susceptibility testing of cultivable isolates may aid in the selection of appropriate antimicrobial treatment regimens for peri-implantitis.

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Chapter 8

General discussion and future perspectives

The research findings presented in this thesis add to the scientific database that underlies the clinical application of systemic antibiotics in periodontal disease and peri-implantitis management. It is clear from the various studies described in Chapters 2-7 that there is considerable variability in the antibiotic susceptibility of the evaluated putative bacterial pathogens in human chronic periodontitis and peri-implantitis patients in the United States. From available data, it appears that the occurrence of antibiotic-resistant periodontal and peri-implant bacterial pathogens is greater than levels reported in northern European countries, but likely less than in South America. It is also clear that assertions about the predictability of antimicrobial profiles of putative periodontal pathogens, which led to conclusions about a perceived lack of benefit for antimicrobial susceptibility testing (1), do not appear to be applicable to periodontitis and peri-implantitis patients in the United States, since considerable resistance was found to several antibiotics among subgingival and submucosal clinical isolates. However, the present thesis research findings confirm that the combination of amoxicillin plus metronidazole, which have complementary and sometimes synergistic antimicrobial spectrums, encounter the lowest occurrence of antibiotic-resistant bacterial pathogens among the tested antibiotics. Further research is needed to gain more insight into the use of this antibiotic drug combination in dentistry, and why some periodontitis patients fail to experience clinical benefits with it.

Because of the lack of geographic-based data, there is a pressing need for the development of national and international surveillance systems to track antibiotic resistance in bacterial species important in periodontitis and peri-implantitis, with the occurrence of new outbreaks of drug resistance, and trends in antibiotic resistance patterns, used to help clinical patient care and influence policy on antibiotic use. No such organized surveillance system exists in the world today relative to dental-based infections, despite the increasing world-wide use of antibiotics in periodontal disease, peri-implantitis, and other oral disease treatments.

The antibiotic resistance variability observed in the present thesis research has important treatment implications for clinicians attempting to successfully treat patients with antibiotic-resistant pathogens, since an increased risk of clinical treatment failure is expected if an antibiotic to which the patient's pathogenic microbiota is resistant is empirically selected and administered. Thus, a more appropriate treatment planning strategy for clinical use is to base systemic periodontal and peri-implantitis antibiotic therapy selection in part on the results of *in vitro* antibiotic susceptibility testing of cultivable pathogens that are associated with the patient's subgingival or submucosal dental plaque biofilm. This information would augment the more conventional risk-benefit considerations involving the patient's medical status, concurrent medication use, and potential antibiotic drug side-effects. Access to *in vitro* antibiotic resistance test results would enable clinicians to earlier consider alternative antibiotic choices when confronted with antibiotic resistance before the patient suffers a clinical treatment failure.

Since antibiotic susceptibility testing presently requires viable microorganisms to perform, it is necessary to rely upon cultivation procedures in carrying out microbiological analysis of subgingival and submucosal bacterial populations. Because of the specialized nature of the subgingival and submucosal bacterial populations, only a limited number of laboratories are available in the world today to provide such culture-based microbiological testing, and

this resource may even become scarcer as the present laboratory directors approach retirement without a new generation in waiting.

In the future, detection of antibiotic-resistant bacterial pathogens may be potentially identified without cultivation by use of molecular methods seeking the presence of functional antibiotic resistance genes, but such approaches remain at present, only theoretical.

Prime candidates for systemic periodontal antibiotic therapy are periodontitis patients not adequately responding to local mechanical-surgical periodontal therapy, or who have a history of recurrent or progressive periodontitis disease-activity despite the provision of thorough root instrumentation, meticulous patient supragingival plaque control, and regular periodontal maintenance care. The diversity of the subgingival microbiota in these types of periodontitis patients is often highlighted by multiple species with varying antibiotic susceptibilities, which pose problems for clinicians attempting to empirically select antibiotic therapies. For patients with dental implant complications of apparent infectious origin, systemic antibiotics may be prescribed as an adjunct to surgical procedures aimed at mechanical disruption of submucosal bacterial biofilms, and disinfection of dental implant surfaces exposed to the oral environment. At present, no systemic antibiotic therapy is indicated for patients with gingivitis, mild forms of periodontitis, and peri-implant mucositis.

Future studies are urgently needed to better determine the optimum doses and durations for systemic antibiotic therapies in dentistry. At present, a wide variety of antibiotic drug schedules are prescribed for patients with periodontitis and peri-implantitis, and concern exists that suboptimal antibiotic concentrations may compromise patient outcomes, and potentially contribute to increased antibiotic resistance in the bacteria in the oral cavity and other body sites.

The molecular basis for the antibiotic resistance detected in this thesis also needs to be assessed in future studies to help better understand the process by which antibiotic resistance genes are transferred among bacterial species, and disseminated in human population groups.

Despite the occurrence of antibiotic resistance among subgingival and submucosal bacterial pathogens, periodontal and peri-implantitis antibiotic-based treatments nevertheless have the potential to be more effective, predictable, and safe for patients if more emphasis is placed on the increased use of microbiological analysis, and antibiotic susceptibility testing, as a routine part of dental treatment planning.

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Chapter 9

Summary

Summary

Antibiotics play a key role in anti-infective treatment strategies directed against destructive periodontal diseases and infectious dental implant complications. As disease processes driven by the growth of specific pathogenic bacterial populations on subgingival tooth roots and submucosal dental implant surfaces, periodontitis and peri-implantitis exhibit clinical and radiographic improvements characteristic of a health-associated status when marked suppression or eradication of periodontal and peri-implant bacterial pathogens occurs with appropriate systemic antibiotic therapy administered in conjunction with mechanical debridement and plaque control procedures. However, an important determinant in the potential success of systemic periodontal and peri-implantitis antibiotic therapy is the occurrence of antibiotic resistance among targeted periodontal and peri-implant bacterial pathogens, which may survive antibiotic drug regimens and contribute to clinical treatment failure. Consequently, this thesis focused on assessing the extent of *in vitro* resistance within selected putative periodontal and peri-implant bacterial species, and among groups of chronic periodontitis and peri-implantitis patients, to several antibiotics frequently used in periodontal and oral implantology dental practice.

Chapter 1 provides an overview of the pathogenesis of chronic periodontitis and peri-implantitis, the key role systemic drug administration may play in the arrest of the diseases, and unresolved issues pertaining to the optimization of systemic periodontal and peri-implant antibiotic therapy.

Chapter 2 examines the *in vitro* antibiotic susceptibility profiles of *Streptococcus constellatus* and *Streptococcus intermedius*, often overlooked gram-positive periodontal pathogens that have been associated with refractory periodontitis patients. The *in vitro* inhibition of amoxicillin, azithromycin, clindamycin, ciprofloxacin, doxycycline, and metronidazole was evaluated on the largest number to date (n = 50 total) of *S. constellatus* and *S. intermedius* fresh clinical isolates of subgingival origin recovered from chronic periodontitis subjects. Using E-test susceptibility methodology to determine minimal inhibitory concentration (MIC) values, and Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) antibiotic susceptibility breakpoint concentrations and interpretative standards, clindamycin was identified as the most active test antibiotic against *S. constellatus* (MIC₉₀ = 0.25 mg/L), with amoxicillin most active against *S. intermedius* (MIC₉₀ = 0.125 mg/L). 30% of the *S. constellatus* and *S. intermedius* clinical isolates were resistant *in vitro* to doxycycline, 98% were intermediate in susceptibility to ciprofloxacin, and 90% were resistant to metronidazole at 16 mg/L. It was concluded that the variable antibiotic susceptibility profiles of subgingival *S. constellatus* and *S. intermedius* may complicate selection of periodontitis antibiotic therapy in species-positive patients, and that microbiological analysis encompassing antimicrobial sensitivity testing may be particularly helpful in periodontal treatment planning for refractory periodontitis patients.

Chapter 3 assesses the *in vitro* antibiotic susceptibility profiles of periodontal isolates of *Enterococcus faecalis*, an opportunistic gram-positive pathogen that may be recovered in periodontitis patients responding poorly to mechanical forms of periodontal therapy. Broth microdilution susceptibility panels, and CLSI criteria and interpretative guidelines, were employed to evaluate selected antibiotics against the largest group of subgingival *E.*

faecalis clinical isolates (n = 47) tested to date from chronic periodontitis patients in the United States. Periodontal *E. faecalis* clinical isolates exhibited substantial *in vitro* resistance to tetracycline (53.2% resistant), erythromycin (80.8% resistant or intermediate resistant), clindamycin (100% resistant to 2 mg/L), and metronidazole (100% resistant to 4 mg/L). In contrast, subgingival *E. faecalis* was generally sensitive to ciprofloxacin (89.4% susceptible; 10.6% intermediate resistant), and 100% susceptible *in vitro* to ampicillin, amoxicillin/clavulanate, vancomycin, and teicoplanin. These findings suggest that, among the evaluated antibiotics with oral administration routes, heavy *E. faecalis* growth in periodontal pockets of periodontitis patients may best respond to systemic antibiotic therapy involving ampicillin, amoxicillin/clavulanate, or ciprofloxacin, whereas tetracycline, erythromycin, clindamycin, and metronidazole would likely be ineffective therapeutic agents against this species.

Chapter 4 addresses the extent to which bacterial β -lactamase enzyme production, which has the capability to hydrolyze amide bonds of β -lactam antibiotics and render them pharmacologically inactive, occurs within the subgingival microbiota of chronic periodontitis patients. The largest group of chronic periodontitis subjects to date were evaluated for subgingival β -lactamase-producing bacteria (n = 564), which was detected test species growth on enriched Brucella blood agar primary isolation plates supplemented with amoxicillin alone, and their absence on similar primary isolation plates containing both amoxicillin plus clavulanic acid, a potent β -lactamase enzyme inhibitor. Approximately one-half (52.1%) of the study subjects yielded β -lactamase enzyme producing subgingival bacterial test species, with *Prevotella intermedia/nigrescens*, *Fusobacterium nucleatum*, and other *Prevotella* species most frequently identified as β -lactamase enzyme producing organisms. In addition, nearly all (98.9%) of the β -lactamase enzyme producing bacterial test species strains recovered were susceptible *in vitro* to metronidazole at 4 mg/L. These findings raise questions about the therapeutic potential of single-drug regimens with β -lactam antibiotics in periodontal therapy. In addition, the *in vitro* effectiveness of metronidazole against nearly all recovered β -lactamase enzyme producing subgingival bacterial species suggests its value in protecting against amoxicillin degradation by β -lactamase enzymes in systemic periodontal antibiotic therapy involving the combination of amoxicillin plus metronidazole.

Chapter 5 focuses on the occurrence of *in vitro* antibiotic resistance among selected subgingival periodontal pathogens in chronic periodontitis subjects, and assessed the largest number of chronic periodontitis subjects in the United States (n = 400) for *in vitro* antibiotic testing of subgingival periodontal pathogens in 20 years. Utilizing subgingival biofilm specimens from untreated inflamed and deep periodontal pockets, selected cultivable periodontal pathogens were tested on antibiotic-supplemented primary isolation plates *in vitro* for susceptibility to breakpoint concentrations of amoxicillin at 8 mg/L, clindamycin at 4 mg/L, doxycycline at 4 mg/L, and metronidazole at 16 mg/L, with gram-negative enteric rods/pseudomonads additionally subjected to ciprofloxacin disk diffusion testing. It was revealed that 74.2% of the chronic periodontitis subjects had subgingival periodontal pathogens resistant to at least one of the test antibiotics. Test species, most often *P. intermedia/nigrescens*, *S. constellatus* or *Aggregatibacter actinomycetemcomitans*, were resistant *in vitro* to doxycycline, amoxicillin, metronidazole, or clindamycin, in 55%, 43.3%, 30.3%, and 26.5% of the chronic periodontitis subjects, respectively. 15% of subjects harbored subgingival periodontal pathogens resistant to both amoxicillin and

metronidazole, which were mostly either *S. constellatus* or ciprofloxacin-susceptible strains of gram-negative enteric rods/pseudomonads. These findings indicate that chronic periodontitis subjects in the United States frequently yielded subgingival periodontal pathogens resistant *in vitro* to therapeutic concentrations of antibiotics, and demonstrated a wide variability in periodontal pathogen antibiotic resistance patterns. This frequent occurrence of, and considerable variability in, periodontal pathogen antibiotic resistance should concern clinicians empirically selecting antibiotic treatment regimens for chronic periodontitis patients, and suggests an important role for microbiological analysis and antibiotic susceptibility testing as an aid in the selection of systemic periodontal antibiotic therapy.

Chapter 6 determined the occurrence of *in vitro* resistance to therapeutic concentrations of spiramycin, a macrolide antibiotic often overlooked in periodontal chemotherapeutics, as well as amoxicillin and metronidazole, among subgingival periodontal pathogens in chronic periodontitis patients in the United States. Selected cultivable periodontal pathogens from 37 chronic periodontitis subjects were tested on antibiotic-supplemented primary isolation plates *in vitro* for susceptibility to breakpoint concentrations of spiramycin at 4 mg/L, amoxicillin at 8 mg/L, and metronidazole at 16 mg/L, with test results combined post-hoc for spiramycin and metronidazole, and for amoxicillin and metronidazole, to determine the number and proportion of organism-positive study subjects where test putative periodontal pathogens exhibited *in vitro* resistance to both antibiotics. Test periodontal pathogens were resistant *in vitro* to spiramycin, amoxicillin, or metronidazole in 48.7%, 62.2%, and 27% of the chronic periodontitis subjects, respectively. Spiramycin *in vitro* resistance occurred mostly among species of *F. nucleatum* (44.4% of organism-positive subjects), with less frequent resistance, approximating 10% or less of subject strains, found among clinical isolates of *P. intermedia/nigrescens*, *Parvimonas micra*, *S. constellatus*, *S. intermedius*, *Porphyromonas gingivalis*, and *Tannerella forsythia*. Only one subject harbored subgingival periodontal pathogens resistant to both spiramycin and metronidazole, which were *S. constellatus* and *S. intermedius* strains. Similarly, only one subject yielded any test species resistant *in vitro* to both amoxicillin and metronidazole, which also were strains of *S. constellatus*. These findings suggest that with one-half of chronic periodontitis subjects demonstrating *in vitro* spiramycin resistance among subgingival periodontal pathogens, particularly *F. nucleatum* species, spiramycin by itself appears to have only a limited potential as a systemic chemo-therapeutic agent in human periodontitis therapy. However, *in vitro* resistance patterns also suggest that therapeutic concentrations of spiramycin plus metronidazole may have potential antimicrobial efficacy in non-*A. actinomycetemcomitans*-associated periodontitis patients similar to amoxicillin plus metronidazole, and may be beneficial for patients hypersensitive to amoxicillin or other beta-lactam antibiotics.

Chapter 7 evaluates the occurrence of *in vitro* antibiotic resistance among putative peri-implantitis bacterial pathogens in the largest number of human peri-implantitis subjects studied to date (n = 120) on this issue. Submucosal biofilm specimens were cultured from 160 dental implants with peri-implantitis in 120 adults, with isolated putative pathogens tested on antibiotic-supplemented primary isolation plates for susceptibility to doxycycline at 4 mg/L, amoxicillin at 8 mg/L, metronidazole at 16 mg/L, and clindamycin at 4 mg/L, with gram-negative enteric rods/pseudomonads additionally subjected to ciprofloxacin disk diffusion testing. Test results for amoxicillin and metronidazole were combined post-hoc to identify peri-implantitis species resistant to both antibiotic concentrations. 71.7% of the

peri-implantitis subjects yielded submucosal bacterial pathogens resistant *in vitro* to one or more of the test antibiotics. Cultivable submucosal bacterial pathogens, most often *P. intermedia/nigrescens* or *S. constellatus*, were resistant *in vitro* to clindamycin, amoxicillin, doxycycline or metronidazole in 46.7%, 39.2%, 25%, and 21.7% of the peri-implantitis subjects, respectively. Only 6.7% subjects revealed submucosal test species resistant *in vitro* to both amoxicillin and metronidazole, which were either *S. constellatus* or ciprofloxacin-susceptible strains of gram-negative enteric rods/pseudomonads. These findings indicate that peri-implantitis patients frequently harbor submucosal bacterial pathogens resistant *in vitro* to individual therapeutic concentrations of clindamycin, amoxicillin, doxycycline or metronidazole, but only rarely to both amoxicillin and metronidazole. Due to this wide variation in antibiotic resistance patterns, antibiotic susceptibility testing of cultivable submucosal bacterial pathogens may aid in the selection of antimicrobial therapy for peri-implantitis patients.

Chapter 8 offers additional perspective on the above research findings relative to their potential clinical diagnostic implications and therapeutic applications in clinical periodontal and oral implantology practice. It is concluded that microbiological analysis encompassing evaluation of the antibiotic susceptibility of targeted pathogens offers practicing clinicians an enhanced ability to optimize selection and administration of systemic periodontal and peri-implantitis antibiotic therapies, and to reduce the risk of clinical failures due to the presence of antibiotic-resistant pathogenic bacterial species. Specific clinical recommendations are offered as to how to implement this approach into modern oral health care practice. Directions for future research on the use of systemic antibiotics in the treatment of destructive periodontal diseases and infectious dental implant complications are also proposed and discussed.

Chapter 10

Samenvatting

Samenvatting

Antibiotica spelen een belangrijke rol bij de behandeling van infectieuze, destructieve vormen van parodontale aandoeningen en infecties rond tandvervangende implantaten. Bij deze aandoeningen spelen subgingivale bacteriële populaties op het worteloppervlak en op het oppervlak van het submucosale implantaat een belangrijke rol. Reductie van de bacteriële biofilm op deze oppervlakken is essentieel voor klinische en radiologische verbeteringen van de parodontale en peri-implantaire status. Naast de mechanische reductie van de bacteriële biofilm kan een systemische antibioticum behandeling of het toepassen van een combinatie van antibiotica een belangrijke bijdrage leveren aan de reductie van de subgingivale en de submucosale pathogene bacteriën. Een goed mondhygiëneniveau is hierbij van groot belang. Echter, een belangrijke determinant voor het succes van een systemische antibioticum therapie bij parodontale en peri-implantaire infecties is de aanwezigheid van antibioticum resistentie bij parodontale en peri-implantaire bacteriële pathogenen die niet onderdrukt worden en zo een bijdrage kunnen leveren aan een verminderde therapieresponse. Dit proefschrift beschrijft onderzoek naar het voorkomen van *in vitro* resistentie bij geselecteerde parodontale en peri-implantaire bacteriesoorten in patiënten met chronische parodontitis en patiënten met peri-implantitis. In dit onderzoek zijn antibiotica onderzocht die regelmatig worden toegepast bij de behandeling van parodontitis en peri-implantaire infecties.

In **Hoofdstuk 1** wordt de pathogenese van parodontitis en peri-implantitis besproken. Er wordt stilgestaan bij de sleutelrol die het systemische gebruik van antibiotica kan spelen bij de behandeling van deze ziekten en bij vragen die betrekking hebben op de optimalisatie van het gebruik van systemische antibiotica bij de behandeling van genoemde aandoeningen.

In **Hoofdstuk 2** wordt de *in vitro* antibioticum gevoeligheid van *Streptococcus constellatus* en *Streptococcus intermedius* onderzocht. Deze gram-positieve parodontale bacteriële ziekteverwekkers zijn geassocieerd met refractaire parodontitis. De *in vitro* gevoeligheid voor amoxicilline, azitromycine, clindamycine, ciprofloxacin, doxycycline en metronidazol werd onderzocht in klinische subgingivale isolaten afkomstig van patiënten met chronische parodontitis. Met behulp van de E-test techniek werd de minimaal remmende concentratie (MIC) vastgesteld. Breekpunt concentraties werden berekend aan de hand van de gegevens van de Clinical and Laboratory Standards Institute (CLSI) en de European Committee on Antimicrobial Susceptibility Testing (EUCAST). Clindamycine bleek het meest werkzame antibioticum tegen *S. constellatus* ($MIC_{90} = 0,25$ mg/L), amoxicilline bleek het meest werkzame antibioticum tegen *S. intermedius* ($MIC_{90} = 0,125$ mg/L). 30% van de *S. constellatus* en *S. intermedius* klinische isolaten bleken resistent te zijn voor doxycycline, 98% was matig gevoelig voor ciprofloxacin en 90% was resistent voor metronidazol bij een concentratie van 16 mg/L. De conclusie van dit onderzoek is dat de variabele antibioticumgevoeligheid spectra van subgingivale *S. constellatus* en *S. intermedius* de keuze van een effectieve antibioticum behandeling in patiënten die positief zijn voor deze pathogenen kan bemoeilijken. Daarom kan microbiologische analyse en het vaststellen van de antibioticumgevoeligheid bijzonder nuttig zijn bij de behandeling van refractaire parodontitis in patiënten met subgingivale *S. constellatus* en *S. intermedia*.

In **Hoofdstuk 3** is de *in vitro* antibioticumgevoeligheid van parodontale isolaten van

Enterococcus faecalis (n=47, USA patiënten) onderzocht. Deze opportunistische, gram-positieve ziekteverwekker kan worden gevonden bij parodontitis patiënten die onvoldoende reageren op mechanische parodontale behandeling. De microdilutie methode werd gebruikt om de gevoeligheid voor ampicilline, amoxicilline, ciprofloxacine, clindamycine, erytromycine, teicoplanine, tetracycline-HCl, vancomycine, gentamicine en streptomycine vast te stellen. Daarnaast werd de resistentie voor metronidazol bij een concentratie van 4 mg/L gemeten met behulp van de agar-dilutie methode. CLSI criteria werden gebruikt voor het vast stellen van breekpuntconcentraties. De klinische subgingivale *E. faecalis* isolaten lieten een aanzienlijke *in vitro* resistentie zien tegen tetracycline (53,2% resistent), erytromycine (80,8% resistent of verminderd gevoelig), clindamycine (100% voor 2 mg/L) en metronidazol (100% voor 4 mg/L). Subgingivale *E. faecalis* bleek veelal gevoelig voor ciprofloxacine (89,4% gevoelig; 10,6% matig gevoelig) en 100% gevoelig voor ampicilline, amoxicilline/clavulaanaat, vancomycine en teicoplanine. Op basis van deze bevindingen kan worden geconcludeerd dat patiënten met hoge subgingivale aantallen *E. faecalis* mogelijk baat hebben bij een systemische behandeling met ampicilline, amoxicilline/clavulaanzuur, of ciprofloxacine, en dat systemische tetracycline, erytromycine, clindamycine en metronidazol waarschijnlijk ineffektieve middelen zijn bij de behandeling van een subgingivale infectie met deze pathogeen.

In **hoofdstuk 4** is de bacteriële β -lactamase productie in de subgingivale microflora van patiënten met chronische parodontitis onderzocht. Dit enzym is in staat om de amide verbinding in β -lactam antibiotica te hydrolyseren waardoor het antibioticum farmacologisch niet langer werkzaam is. Deze β -lactamase productie werd onderzocht door subgingivale plaquemonsters van een grote groep patiënten (n=564) te inoculeren op verrijkte Brucella agar platen waaraan amoxicilline, en op platen waaraan amoxicilline en clavulaanzuur (een β -lactamase remmer) werd toegevoegd. β -lactamase-producerende bacteriën groeien op het medium waaraan amoxicilline is toegevoegd maar worden geremd op het medium met amoxicilline en clavulaanzuur. In 52,1% van de patiënten werden β -lactamase-producerende subgingivale bacteriën gevonden. Het meest frequent werd deze eigenschap aangetroffen bij *Prevotella intermedia/nigrescens*, andere *Prevotella* species en bij *Fusobacterium nucleatum*. Tevens werd vastgesteld dat 98,9% van de gevonden β -lactamase-producerende bacteriesoorten gevoelig was voor 4 mg/L metronidazol. Deze bevindingen suggereren dat de effectiviteit van een monotherapie met een β -lactam antibioticum als ondersteuning van een parodontale behandeling beperkt is. Echter, de grote gevoeligheid van de β -lactamase-producerende bacteriesoorten voor metronidazol lijkt een belangrijke beschermende factor die wellicht afbraak van amoxicilline door β -lactamase-producerende bacteriesoorten voorkomt wanneer parodontitis wordt behandeld met de combinatie van metronidazol en amoxicilline.

In **Hoofdstuk 5** is het voorkomen van *in vitro* antibioticum resistentie van geselecteerde subgingivale parodontale pathogenen in chronische parodontitis patiënten onderzocht in 400 Amerikaanse patiënten. Subgingivale plaquemonsters uit diepe, ontstoken parodontale pockets werden geënt op primaire isolatie media waaraan breekpunt concentraties van amoxicilline (8 mg/L), clindamycine (4 mg/L), doxycycline (4 mg/L), en metronidazol (16 mg/L) werden toegevoegd. Op deze wijze werd de resistentie van een geselecteerd aantal paropathogene bacteriën vastgesteld. Daarnaast werd de gevoeligheid van geïsoleerde gram-negatieve staaftvormige enterobacteriën en *Pseudomonas* species voor ciprofloxacine onderzocht met behulp van de disc diffusie methode. In 74,2% van de chronische

parodontitis patiënten werd tenminste één paropathogene species gevonden die resistent was voor tenminste één van de onderzochte antibiotica. *P. intermedia/nigrescens*, *S. constellatus* of *Aggregatibacter actinomycetemcomitans* bleken resistent voor doxycycline, amoxicilline, metronidazol of clindamycine in resp. 55%, 43,3%, 30,3% en 26,5% van de chronische parodontitis patiënten. In 15% van de onderzochte patiënten werden pathogenen gevonden die resistent waren voor zowel amoxicilline als metronidazol. Dit betrof veelal *S. constellatus* of ciprofloxacin-gevoelige enterobacteriën of *Pseudomonas* species. Deze bevindingen laten zien dat chronische parodontitis patiënten in de Verenigde Staten subgingivaal frequent gekoloniseerd zijn met paropathogenen die resistent zijn voor therapeutische antibioticumconcentraties. Tevens werd aangetoond dat er een grote variatie bestaat in antibioticumresistentie bij paropathogene bacteriesoorten. Deze nieuwe informatie is van belang voor klinici die empirisch een antibioticum therapie kiezen bij de behandeling van chronische parodontitis. Er lijkt hier een belangrijke rol weggelegd voor microbiologische analyse van de subgingivale plaque en antibioticumgevoeligheid onderzoek bij het maken van een keuze voor een systemische parodontale antibioticum therapie.

In **Hoofdstuk 6** is de resistentie van een aantal geselecteerde paropathogene bacteriën onderzocht voor therapeutische concentraties van spiramycine (een macrolide antibioticum), amoxicilline en metronidazol. Subgingivale plaquemonsters van chronische parodontitis patiënten (n=37) werden geënt op primaire isolatie media waaraan breekpunt concentraties spiramycine (4 mg/L), amoxicilline (8 mg/L) en metronidazol (16 mg/L) werden toegevoegd. Testresultaten werden post-hoc gecombineerd voor spiramycine en metronidazol en voor metronidazol en amoxicilline om zo het aantal en het percentage paropathogenen bacteriesoorten vast te stellen dat resistent is voor een of beide antibioticum combinaties. Geselecteerde paropathogenen waren resistent voor spiramycine, amoxicilline of metronidazol in respectievelijk 48,7%, 62,2% en 27% van de chronische parodontitis patiënten. Spiramycine resistentie werd het meest frequent gevonden in *Fusobacterium nucleatum* (44,4% van de positieve patiënten) maar ook in lage frequentie ($\leq 10\%$ van de patiënten) in *Prevotella intermedia/nigrescens*, *Parvimonas micra*, *Streptococcus constellatus*, *Streptococcus intermedius*, *Porphyromonas gingivalis* en *Tannerella forsythia*. In één patiënt werd een *S. constellatus* en een *S. intermedius* gevonden die resistent waren voor zowel spiramycine als metronidazol. In een andere patiënt werd een *S. constellatus* stam gevonden die resistent was voor metronidazol en amoxicilline. In ongeveer 50% van de onderzochte chronische parodontitis patiënten werd een spiramycine resistente paropathogeen gevonden, in veel gevallen betrof het *F. nucleatum*. Deze bevindingen laten zien dat spiramycine als systemische monotherapie van beperkte betekenis is voor de behandeling van parodontitis. Echter, de *in vitro* gevoeligheid van paropathogenen, met uitzondering van *A. actinomycetemcomitans*, duiden op een mogelijke toepassing van de combinatie van spiramycine en metronidazol in patiënten met een overgevoeligheid voor amoxicilline of andere β -lactam antibiotica.

In **Hoofdstuk 7** is het voorkomen van antibioticum resistentie bestudeerd van bacteriën die betrokken zijn bij peri-implantitis. Submucosale monsters van 160 implantaten met peri-implantitis afkomstig uit 120 patiënten werden gekweekt op primaire isolatie media waaraan doxycycline (4 mg/L), amoxicilline (8 mg/L), metronidazol (16 mg/L) en clindamycine (4 mg/L) was toegevoegd. Gram-negatieve staafvormige enterobacteriën en *Pseudomonas* species werden apart getest op gevoeligheid voor ciprofloxacin met behulp

van de disc diffusie test methode. Test resultaten voor metronidazol en amoxicilline werden post-hoc gecombineerd om bacteriesoorten die resistent waren voor beide antibiotica te identificeren. In 71,7% van de patiënten werden pathogenen gevonden die resistent waren voor één of meerdere van de geteste antibiotica. Kweekbare submucosale pathogenen, veelal *Prevotella intermedia/nigrescens* of *Streptococcus constellatus*, bleken *in vitro* resistent voor clindamycine, amoxicilline, doxycycline of metronidazol in respectievelijk 46,7%, 39,2%, 25% en 21,7% van de peri-implantitis patiënten. In 6,7% van de patiënten werden pathogenen gevonden die resistent waren voor zowel metronidazol als amoxicilline. Dit betrof *Streptococcus constellatus* of ciprofloxacine-gevoelige gram negatieve staafvormige enterobacteriën of *Pseudomonas* species. De resultaten laten zien dat patiënten met peri-implantitis frequent submucosale pathogenen hebben die resistent zijn voor therapeutische concentraties van clindamycine, amoxicilline, doxycycline of metronidazol maar slechts zelden voor de combinatie van metronidazol en amoxicilline. Antibioticum gevoeligheid bepaling van submucosale pathogenen kan behulpzaam zijn bij de keuze van een antimicrobiële therapie bij de behandeling van patiënten met peri-implantitis.

In **Hoofdstuk 8** worden de gevonden onderzoeksresultaten besproken in het licht van parodontale diagnostiek en toepassing in de parodontale en de implantologische praktijk. Op basis van de bevindingen in dit proefschrift wordt geconcludeerd dat microbiologisch onderzoek, in casu de evaluatie van de antibioticumgevoeligheid van specifieke pathogenen, een extra mogelijkheid biedt aan clinici bij hun keuze voor de selectie van een systemische antimicrobiële therapie bij de behandeling van parodontitis en peri-implantitis. Hiermee kan de kans op therapie falen worden verkleind. In dit hoofdstuk worden klinische aanbevelingen gedaan hoe deze nieuwe informatie in de praktijk kan worden geïmplementeerd. Tevens worden voorstellen gedaan voor toekomstig onderzoek op het gebied van antibiotica voor de behandeling van parodontale en peri-implantaire infecties.

Chapter 11

Curriculum Vitae

Personal Information

Thomas Edwin Rams
Birthdate: July 24, 1955, Columbus, Ohio, USA
Citizenship: USA, Canada

Education

- 1974 - Bachelor of Science in Environmental Health
George Washington University, Washington, DC
- 1974 - Para-Medical Certificate in Environmental Health
George Washington University School of Medicine and Health Sciences,
Washington, DC
- 1977 - Master of Health Science in Environmental Health Sciences
Johns Hopkins University School of Public Health, Baltimore, MD
- 1980 - Doctor of Dental Surgery
University of Maryland School of Dentistry, Baltimore, MD
- 1982 - Postgraduate Fellowship Certificate in Clinical Dentistry
National Institute of Dental Research, National Institutes of Health, Bethesda, MD
- 1987 - Postgraduate Specialty Certificate in Periodontics
New York Veterans Administration Medical Center, New York, NY
- 1994 - Postgraduate Specialty Certificate in Dental Public Health
National Institute of Dental Research, National Institutes of Health, Bethesda, MD

Licensure

- 1980 - District of Columbia dental license
- 1986 - Pennsylvania dental license
- 1995 - Clinical Laboratory Director Permit (Bacteriology),
Pennsylvania Department of Health

Specialty Certification

- 1991 - Diplomate, American Board of Periodontology
- 2006 - Diplomate, International Congress of Oral Implantologists

Professional Career

- 1980-1982 - Dental Staff Fellow, National Institute of Dental Research,
National Institutes of Health, Bethesda, MD
- 1983-1985 - Private practice of general dentistry, Washington, DC
- 1984-1986 - Clinical Assistant Professor of Community Dentistry and Microbiology,
Georgetown University Schools of Dentistry and Medicine, Washington, DC

- 1985-1987 - Postgraduate specialty resident in periodontics,
New York Veterans Administration Medical Center, New York, NY
- 1987-present - Private practice of periodontics, Washington, DC
- 1987-1995 - Clinical Assistant Professor of Periodontics,
University of Pennsylvania School of Dental Medicine, Philadelphia, PA
- 1991 - Fellowship, American College of Dentists.
- 1992-1999 - Medical Staff Consultant (Periodontics),
Warren Grant Magnuson Clinical Center,
National Institutes of Health, Bethesda, MD
- 1993-1994 - Staff Fellow (dental public health postgraduate specialty resident),
National Institute of Dental Research,
National Institutes of Health, Bethesda, MD
- 1995-1996 - Associate Professor of Dental Medicine & Surgery,
Director, Oral Microbiology Testing Service Laboratory,
Medical College of Pennsylvania and Hahnemann University,
Philadelphia, PA
- 1996-present - Professor of Periodontology and Oral Implantology,
Director, Oral Microbiology Testing Service Laboratory,
Temple University School of Dentistry, Philadelphia, PA
- 1996-2010 - Chairman, Department of Periodontology and Oral Implantology,
Temple University School of Dentistry, Philadelphia, PA
- 1997-present - Professor, Temple University Graduate School, Philadelphia, PA
- 1999-2004 - Associate Dean for Advanced Education and Research,
Temple University School of Dentistry, Philadelphia, PA
- 2003-2013 - The Paul H. Keyes Term Professorship in Periodontology,
Temple University School of Dentistry, Philadelphia, PA
- 2004-2008 - Senior Associate Dean,
Temple University School of Dentistry, Philadelphia, PA
- 2005 - Fellowship, International College of Dentists.
- 2007 - Christian R. and Mary F. Lindback Foundation Award for Distinguished Teaching
at Temple University, Philadelphia, PA
- 2010-present - Professor of Microbiology and Immunology,
Temple University School of Medicine, Philadelphia, PA

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Chapter 12

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Daddy and Mama several years after my birth in 1955.
