

AZITHROMYCIN IN VITRO ANTIMICROBIAL ACTIVITY AGAINST
SELECTED PERIODONTAL BACTERIAL PATHOGENS

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by
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ABSTRACT

Objectives: Azithromycin is a second-generation macrolide active against a wide range of bacteria, including obligate anaerobes implicated as bacterial pathogens in human periodontitis. Clinical studies indicate short-term systemic azithromycin therapy to be beneficial in the treatment of acute periodontal abscesses and as an adjunct to mechanical periodontal therapy of periodontitis patients. Only sparse recent data is available on the susceptibility or resistance of putative periodontal bacterial pathogens to azithromycin, particularly among subgingival isolates from periodontitis patients residing in the United States. Thus, the present degree to which major periodontal bacterial pathogens in United States periodontitis patients exhibit resistance to azithromycin is not known. As a result, the purpose of the present study was to determine the prevalence of in vitro resistance to azithromycin, as compared to metronidazole, among selected red/orange complex periodontal pathogens isolated from severe periodontitis patients in the United States.

Methods: A retrospective record review was performed on a total of 100 consecutively established and processed patient records in the year 2021 from pre-existing archived data in the Oral Microbiology Testing Service (OMTS) Laboratory at Temple University School of Dentistry, Philadelphia, Pennsylvania. Patient record inclusion criteria required that 1.) the patient was culture-positive with one or more of the following red/orange complex periodontal pathogens: *Porphyromonas gingivalis*, *Tannerella forsythia*, *Prevotella intermedia/nigrescens*, *Parvimonas micra*, *Fusobacterium nucleatum*, *Campylobacter rectus*, or *Streptococcus constellatus*, in subgingival biofilm samples obtained pre-treatment from at least 3 periodontal sites

having ≥ 6 mm periodontal probing depths, 2.) the patient was between the ages of 35-88 years, 3.) the patient was diagnosed by a periodontist as having severe periodontitis, and 4.) the subgingival biofilm samples were subjected to in vitro antibiotic resistance testing with azithromycin and metronidazole, at concentrations of 4 mg/L and 16 mg/L, respectively. Data on patient age, gender, and current smoking status, as well as the occurrence and proportional cultivable recovery of the evaluated red/orange complex test species, and their in vitro resistance to 4 mg/L of azithromycin and/or 16 mg/L of metronidazole, was extracted from the identified patient records by the OMTS Laboratory Director and entered without any unique patient identifiers into a Microsoft Excel spreadsheet. Independently performed descriptive analysis of the de-identified data tabulated patient demographics on age, gender, and smoking status, as well as the occurrence and proportional cultivable recovery of red/orange complex test species per patient. Red/orange complex test species growing on culture media supplemented with either 4 mg/L of azithromycin or 16 mg/L of metronidazole were considered resistant to the incorporated antibiotic concentration. Based on these designated breakpoint thresholds, the prevalence and subgingival proportions of azithromycin- and metronidazole-resistant test species were determined and tabulated per patient. Fisher's exact test was used to statistically evaluate the prevalence among patients of red/orange complex periodontal pathogens with in vitro resistance to azithromycin as compared to metronidazole. Logistic regression statistical modeling also assessed odds ratio relationships between the presence of azithromycin-resistant red/orange complex periodontal pathogens and patient age group, gender, and current smoking status. A 2-tailed P -value of ≤ 0.05 was required for all tests of statistical significance.

Results: The 100 identified severe periodontitis patients (43 male, 57 female; mean age = 58.6 years) included 16 current smokers and 33 persons who were aged 65 years or older. A total of 82 (82%) of the severe periodontitis patients yielded one or more red/orange complex periodontal pathogens resistant in vitro to azithromycin, as compared to only 13 (13%) patients with metronidazole-resistant test species (6.3-fold difference, $P < 0.001$, Fisher's exact test). *Parvimonas micra*, *Tannerella forsythia*, and *Prevotella intermedia/nigrescens* were test species most frequently resistant to 4 mg/L of azithromycin, whereas *Streptococcus constellatus* was most often found resistant to 16 mg/L of metronidazole. The presence of one or more red/orange complex periodontal pathogens resistant in vitro to azithromycin was significantly more frequent in older severe periodontitis patients aged 65 years or greater as compared to younger-aged patients (odds ratio = 5.0; 95% confidence interval = 1.1, 23.5; $P = 0.041$), as determined in logistic regression analysis controlling for gender and current smoking status.

Conclusions: A high prevalence of in vitro resistance to azithromycin, which was significantly greater than detected to metronidazole and significantly more frequent in patients aged 65 years or greater, was found among red/orange complex periodontal pathogens in severe periodontitis patients in the United States. These findings question the potential usefulness of azithromycin in treatment of United States periodontitis patients who frequently harbor azithromycin-resistant periodontal pathogens.

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CHAPTER 1

INTRODUCTION

Human periodontitis is likely triggered by pathogenic bacteria and certain lytic herpesviruses (Chen et al. 2020), and mediated by hyper-inflammatory host immune responses, leading to connective tissue attachment loss on teeth and resorption of surrounding supporting alveolar bone (Kinane et al. 2017). Bacterial species belonging to red and orange complex microbial clusters in subgingival biofilms are most strongly associated with severe forms of periodontitis (Socransky et al. 1998). Red complex species include *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*, and orange complex species include *Prevotella intermedia*, *Prevotella nigrescens*, *Parvimonas micra*, *Fusobacterium nucleatum* group species, *Fusobacterium periodonticum*, *Streptococcus constellatus*, *Eubacterium nodatum* and several *Campylobacter* species (Socransky et al. 1998).

Azithromycin is a second-generation macrolide active against a number of bacteria implicated as periodontal pathogens, including several red/orange complex species (van Winkelhoff et al. 2005). The antibiotic is well-absorbed into the bloodstream after oral drug administration, and concentrates for extended periods in gingival crevicular fluid and gingival tissues of periodontitis patients (Gomi et al. 2007, Lai et al. 2011). These properties result in azithromycin having minimal side-effects relative to gastrointestinal distress frequently associated with poor absorption of first-generation macrolides, and the ability to administer the drug once-a-day for relatively short time periods (Firth & Prathapan 2020). Additionally, azithromycin concentrates in

body sites exhibiting inflammation via its uptake by neutrophils and macrophages (Blumer 2005), and exerts anti-inflammatory effects (Firth & Prathapan 2020). Clinical studies indicate azithromycin beneficial in treatment of periodontal abscesses and as an adjunct to mechanical periodontal therapy of periodontitis patients (Zhang et al. 2016).

However, no recent data is available on azithromycin susceptibility of putative periodontal bacterial pathogens freshly-isolated from the subgingival microbiota of severe periodontitis patients in the United States. It is not known whether major putative periodontal bacterial pathogens in these patients, such as red/orange complex subgingival species, have changed in their antibiotic resistance to azithromycin as a result of increased human exposure over time to antibiotics, such as from antibiotic over-prescription and overconsumption, livestock and fish farming practices, and other environmental sources. In this regard, subgingival *P. micra*, isolated from severe periodontitis patients in the United States, was recently documented to exhibit a 37.7-fold increase in the prevalence of laboratory resistance to doxycycline, and a 23.7-fold increase in the prevalence of laboratory resistance to clindamycin, over a 10-year time between 2006 and 2016, whereas in vitro *P. micra* resistance to amoxicillin and metronidazole remained low and statistically unchanged over the same time period (Rams et al. 2020). Since azithromycin antibiotic resistance testing was not included in the study, it is undetermined as to whether the antibiotic resistance of putative periodontal bacterial pathogens to azithromycin has adversely changed or remained stable in recent years in United States periodontitis patients.

The purpose of the present study was to examine in a retrospective record review the prevalence of in vitro resistance of fresh clinical subgingival isolates of selected human red/orange complex periodontal pathogens from United States severe periodontitis patients to azithromycin as compared to metronidazole.

CHAPTER 2

MATERIALS AND METHODS

Microbiological Data

A retrospective record review was performed on a total of 100 consecutively established and processed patient records in the year 2021 from pre-existing archived data in the Oral Microbiology Testing Service (OMTS) Laboratory at Temple University School of Dentistry, Philadelphia, Pennsylvania. Approval for the retrospective record review was granted by the Temple University Human Subjects Institutional Review Board (Protocol # 29149).

The OMTS Laboratory is state-licensed for high-complexity bacteriological analysis by the Pennsylvania Department of Health (Clinical Laboratory Permit No. 021872) as an oral microbiology reference laboratory, and is also federally certified by the US Department of Health and Human Services to be in compliance with Clinical Laboratory Improvement Amendments (CLIA) regulations (CLIA Certificate No. 39D0707385). The OMTS Laboratory receives subgingival biofilm samples taken from patients with periodontitis from dental professionals practicing around the United States. The subgingival biofilm specimens are cultured for major periodontal bacterial pathogens, which are additionally subjected to *in vitro* antibiotic resistance testing. A resulting laboratory microbiology report is returned to the treating dentists, which is intended to provide guidance on the clinical management of the patient, particularly in regard to the potential use of adjunctive periodontal chemotherapy with antibiotics.

As previously described (Rams et al. 2014a), subgingival biofilm samples from periodontitis patients collected with sterile paper points, pooled into Möller's VMGA III transport media (Möller 1966), and shipped overnight to the OMTS Laboratory, are mechanically dispersed and plated in serial 10-fold dilutions onto pre-reduced, enriched Brucella blood agar (EBBA) medium, composed of 4.3% Brucella agar (BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with 0.3% bacto-agar, 5% defibrinated sheep blood, 0.2% hemolyzed sheep red blood cells, 0.0005% hemin, and 0.00005% menadione. The inoculated EBBA plates are then incubated in the OMTS Laboratory at 37 °C for 7 days in an upright heated incubator (Caron, Marietta, OH, USA) in anaerobic jars, with an 85% N₂-10% H₂-5% CO₂ anaerobic atmosphere introduced into the jars using an Anoxomat™ Mark II automatic jar evacuation-replacement system (Advanced Instruments, Inc., Norwood, MA, USA) (Brazier & Smith 1989). Additional dilution aliquots of the subgingival biofilm samples are also inoculated onto EBBA culture plates supplemented with various individual antibiotics (all obtained as pure powder from Sigma-Aldrich, St. Louis, MO, USA), including 4 mg/L of azithromycin and 16 mg/L of metronidazole, followed by anaerobic incubation at 37 °C for seven days.

After incubation, culture plates in 2021 were evaluated by the OMTS Laboratory staff for total anaerobic viable counts, and the presence and levels of number of putative periodontal bacterial pathogens, including selected red/orange complex periodontal bacterial pathogens *P. gingivalis*, *T. forsythia*, *P. intermedia/nigrescens*, *P. micra*, *F. nucleatum*, *C. rectus*, and *S. constellatus*. *P. gingivalis* was identified based on its colony

morphology and brown-black pigmentation, lack of autofluorescence with long-wave ultraviolet light (Slots & Reynolds 1982), and a positive CAAM test for trypsin-like activity (Slots 1987). *T. forsythia* isolates were 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 positive for trypsin-like enzyme activity (Rams & van Winkelhoff 2005). *P. intermedia/nigrescens*, which represented clinical isolates of either *Prevotella intermedia* and/or *Prevotella nigrescens* due to the inability of phenotypic identification methods to reliably differentiate between them (Rams et al. 2018), was recognized as autofluorescent red-positive, black-pigmented colonies exhibiting lactose MUG-test negative (Alcoforado et al. 1987) and trypsin CAAM test-negative reactions. *P. micra* was identified as small (minute to 1.0 mm in diameter), shiny, non-hemolytic, mainly opaque white, circular, convex surface colonies on anaerobically incubated EBBA (Rams et al. 1992). *C. rectus* was identified as previously described (Rams et al. 1993) on the basis of its motility and colony/cellular morphology. *F. nucleatum* was identified, as specified by Jousimies-Somer et al. (2002), as gram-negative, non-motile, anaerobic, slender, fusiform rods with pointed cell ends (needle-shaped morphology), exhibiting circular, entire, raised, catalase-negative, non-pigmented, non-agar pitting, “bread crumb”-like or speckled colonies under a magnification loupe, and demonstrating an autofluorescent chartreuse (pale yellow-green) colony color when exposed in a dark room to long-wave ultraviolet light (Brazier 1986). *S. 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 (Rams et al. 2011). Proportional recovery of these organisms was calculated as the percent recovery of each of the species colony forming units (CFU) among total cultivable subgingival anaerobic viable counts on non-antibiotic-supplemented EBBA culture plates.

On EBBA culture plates supplemented with 4 mg/L of azithromycin or 16 mg/L of metronidazole, growth by any of the evaluated red/orange complex species indicated their in vitro resistance to the antibiotic concentration. These antibiotic concentrations represented non-susceptible or resistant breakpoint values for azithromycin as recommended by the French Society for Microbiology for bacteria where no species-specific drug breakpoint concentrations are available (Antibiogram Committee of the French Society of Microbiology 2010), and for metronidazole against anaerobic bacteria as recommended by the Clinical and Laboratory Standards Institute (CLSI) (Clinical and Laboratory Standards Institute 2020).

Extraction of Microbiological Data

Consecutively established and processed patient records in 2021 were reviewed from existing archived records in the OMTS Laboratory to identify 100 patient records where the following qualifying criteria were met: 1.) the patient was culture-positive with one or more of the following red/orange complex putative periodontal pathogens: *P. gingivalis*, *T. forsythia*, *P. intermedia/nigrescens*, *P. micra*, *F. nucleatum*, *C. rectus*, or *S. constellatus* in subgingival biofilm samples obtained pre-treatment from at least 3 periodontal sites having ≥ 6 mm periodontal probing depths, 2.) the patient was between the ages of 35-88 years, 3.) the patient was diagnosed by a periodontist as having severe

periodontitis, and 4.) the patient's birth year, gender, and smoking status at time of microbial sampling were reported on the laboratory test request form. Due to patient privacy concerns, OMTS Laboratory Director Dr. Thomas E. Rams carried out the record review and data extraction, as approved by the Temple University Human Subjects Institutional Review Board.

When one or more of the above qualifying criteria were not met, then no data was extracted or recorded from the OMTS Laboratory record. When all of the above qualifying criteria were identified on an OMTS Laboratory record, the following were entered by Dr. Rams into a PC computer-based data spreadsheet (Microsoft Excel 2010, Microsoft Corporation, Redmond, WA, USA) which was held in a password-protected computer file:

1. "age" - The patient age in years was calculated prior to data entry by subtracting with a hand-held calculator the patient's birth year from the year of the laboratory report.
2. "male?" - A score of "1" was entered onto the data spreadsheet when the patient gender was identified as male, with a score of "0" entered for females.
3. "smoker?" - A score of "1" was entered into the data spreadsheet when the patient was identified as a current smoker, with a score of "0" otherwise entered.
4. "% Pg" - The percent cultivable *P. gingivalis* recovered in the subgingival biofilm specimen was entered into the data spreadsheet.
5. "R-az-Pg" - A score of "1" was entered into the data spreadsheet when the letter "R" was listed for *P. gingivalis* strains under the response to azithromycin at 4 mg/L, with a score of "0" when the letter "S" was listed.

6. “R-me-Pg” - A score of “1” was entered into the data spreadsheet when the letter “R” was listed for *P. gingivalis* strains under the response to metronidazole at 16 mg/L, with a score of “0” when the letter “S” was listed.
7. “% Tf” - The percent cultivable *T. forsythia* recovered in the subgingival biofilm specimen was entered into the data spreadsheet.
8. “R-az-Tf” - A score of “1” was entered into the data spreadsheet when the letter “R” was listed for *T. forsythia* strains under the response to azithromycin at 4 mg/L, with a score of “0” when the letter “S” was listed.
9. “R-me-Tf” - A score of “1” was entered onto the data spreadsheet when the letter “R” was listed for *T. forsythia* strains under the response to metronidazole at 16 mg/L, with a score of “0” when the letter “S” was listed.
10. “% Pi” - The percent cultivable *P. intermedia/nigrescens* recovered in the subgingival biofilm specimen was entered into the data spreadsheet.
11. “R-az-Pi” - A score of “1” was entered into the data spreadsheet when the letter “R” was listed for *P. intermedia/nigrescens* strains under the response to azithromycin at 4 mg/L, with a score of “0” when the letter “S” was listed.
12. “R-me-Pi” - A score of “1” was entered into the data spreadsheet when the letter “R” was listed for *P. intermedia/nigrescens* strains under the response to metronidazole at 16 mg/L, with a score of “0” when the letter “S” was listed.
13. “% Pm” - The percent cultivable *P. micra* recovered in the subgingival biofilm specimen was entered into the data spreadsheet.

14. "R-az-Pm" - A score of "1" was entered into the data spreadsheet when the letter "R" was listed for *P. micra* strains under the response to azithromycin at 4 mg/L, with a score of "0" when the letter "S" was listed.
15. "R-me-Pm" - A score of "1" was entered into the data spreadsheet when the letter "R" was listed for *P. micra* strains under the response to metronidazole at 16 mg/L, with a score of "0" when the letter "S" was listed.
16. "% Fuso" - The percent cultivable *F. nucleatum* recovered in the subgingival biofilm specimen was entered into the data spreadsheet.
17. "R-az-Fn" - A score of "1" was entered into the data spreadsheet when the letter "R" was listed for *F. nucleatum* strains under the response to azithromycin at 4 mg/L, with a score of "0" when the letter "S" was listed.
18. "R-me-Fn" - A score of "1" was entered into the data spreadsheet when the letter "R" was listed for *F. nucleatum* strains under the response to metronidazole at 16 mg/L, with a score of "0" when the letter "S" was listed.
19. "% Sc" - The percent cultivable *S. constellatus* recovered in the subgingival biofilm specimen was entered into the data spreadsheet.
20. "R-az-Sc" - A score of "1" was entered into the data spreadsheet when the letter "R" was listed for *S. constellatus* strains under the response to azithromycin at 4 mg/L, with a score of "0" when the letter "S" was listed.
21. "R-me-Sc" - A score of "1" was entered into the data spreadsheet when the letter "R" was listed for *S. constellatus* strains under the response to metronidazole at 16 mg/L, with a score of "0" when the letter "S" was listed.

22. “% Cr” - The percent cultivable *C. rectus* recovered in the subgingival biofilm specimen analyzed was entered into the data spreadsheet.
23. “R-az-Cr” - A score of “1” was entered into the data spreadsheet when the letter “R” is listed for *C. rectus* strains under the response to azithromycin at 4 mg/L, with a score of “0” when the letter “S” was listed.
24. “R-me-Cr” - A score of “1” was entered into the data spreadsheet when the letter “R” was listed for *C. rectus* strains under the response to metronidazole at 16 mg/L, with a score of “0” when the letter “S” was listed.

The process by which Dr. Rams carried out the OMTS Laboratory patient record reviews was as follows:

1. The above qualifying criteria were evaluated to determine if an OMTS Laboratory patient record met the required inclusion criteria.
2. For OMTS Laboratory patient records that did not meet one or more of the qualifying criteria, no data was recorded from the OMTS Laboratory patient record into the data spreadsheet. When an OMTS Laboratory patient record met all of the required qualifying criteria, then data for the 24 variables listed above was extracted from the patient record by Dr. Rams and entered directly into the computer data spreadsheet.
3. This process was repeated by Dr. Rams until the first 100 consecutively-established and processed patient records starting from January 1, 2021 in the OMTS Laboratory were identified as meeting the qualifying criteria and reviewed.

Data Analysis

Data analysis was carried out by Regina Vayner, DMD, MPH. Descriptive analysis of the de-identified OMTS Laboratory patient record data tabulated patient demographics on age, gender, and smoking status, as well as the occurrence and proportional cultivable recovery of red/orange complex test species per patient. The prevalence and subgingival proportions of azithromycin- and metronidazole-resistant test species were determined and tabulated per patient, with Fisher's exact test used to statistically evaluate the prevalence among patients of red/orange complex periodontal pathogens with in vitro resistance to azithromycin as compared to metronidazole. Logistic regression statistical modeling assessed odds ratio relationships between the presence of azithromycin-resistant red/orange complex periodontal pathogens and patient age group, gender, and current smoking status. A 2-tailed *P*-value of ≤ 0.05 was required for all tests of statistical significance. Microsoft Excel 2010 spreadsheet software (Microsoft Corporation, Redmond, WA, USA) and the PC-based STATA/SE 16.1 for Windows (StataCorp PL, College Station, TX, USA) 64-bit statistical software package were used in the data analysis.

CHAPTER 3

RESULTS

Patients

Table 1 provides available demographic features of the 100 severe periodontitis study patients.

Table 1. Demographic Features of Severe Periodontitis Study Patients

<u>Demographic Feature:</u>	
No. of Patients	100
Age Range, Years	35-88
Mean Age \pm SD, Years	58.6 \pm 13.4
No. of Patients \geq 65 Aged Years	33
No. of Males/Females	43/57
No. of Current Smokers	16

All of the severe periodontitis study patients were adults \geq 35 years old, with one-third of them 65 years or older in age. The majority of the study patients were female, with only a relatively small percentage (16%) having a current smoking habit.

Subgingival Biofilm Species

Table 2 lists various red and orange complex periodontal pathogens isolated by microbial culture from subgingival biofilms removed from the 100 severe periodontitis study patients.

Table 2. Presence and Proportional Recovery of Subgingival Red and Orange Complex Periodontal Pathogens From Severe Periodontitis Study Patients

Species	No. of positive patients	% recovery in species-positive patients \pm SD	Range %
<u>Red complex species:</u>			
<i>P. gingivalis</i>	14	4.2 \pm 3.3	0.6-10.0
<i>T. forsythia</i>	47	2.1 \pm 1.0	0.1-4.8
<u>Orange complex species:</u>			
<i>P. intermedia/nigrescens</i>	89	6.2 \pm 5.9	0.9-32.0
<i>P. micra</i>	100	8.7 \pm 5.6	1.5-39.1
<i>C. rectus</i>	44	0.1 \pm 0.1	0.1-0.9
<i>F. nucleatum</i>	97	10.9 \pm 6.5	2.8-45.0
<i>S. constellatus</i>	17	10.1 \pm 10.7	0.9-46.2

The severe periodontitis study patients most frequently yielded three orange complex periodontal pathogens in their subgingival biofilms: *P. micra* in all 100 patients at an average 8.7% of total subgingival anaerobic viable counts, *F. nucleatum* in 97 patients at a mean 10.9%, and *P. intermedia/nigrescens* in 89 patients at 6.2% average proportional levels. *S. constellatus* was less frequently recovered from only 17 patients, but in some species-positive patients composed relatively high levels of the cultivable subgingival microbiota (up to 46.2%). Among red complex periodontal pathogens, *T.*

forsythia was recovered from 47 patients at a mean 2.1% of subgingival viable counts, whereas *P. gingivalis* was isolated from 14 patients at an average 4.2% of subgingival cultivable bacteria.

In Vitro Bacterial Antibiotic Resistance

Table 3 reveals the prevalence of in vitro resistance to 4 mg/L of azithromycin, and to 16 mg/L of metronidazole, among the recovered subgingival red/orange complex periodontal pathogens in the 100 severe periodontitis study patients.

Table 3. In Vitro Resistance of Red/Orange Complex Periodontal Pathogens to 4 mg/L of Azithromycin and 16 mg/L of Metronidazole

Bacterial Species (No. of species-positive patients)	Azithromycin 4 mg/L ^a	Metronidazole 16 mg/L
<u>Red complex species:</u>		
<i>P. gingivalis</i> (14)	0 ^c	0
% recovery ^b	0	0
<i>T. forsythia</i> (47)	25 (53.2%)	0
% recovery	2.0 ± 0.9	0
<u>Orange complex species:</u>		
<i>P. intermedia/nigrescens</i> (89)	29 (32.6%)	1 (1.1%)
% recovery	7.3 ± 5.6	6.0 ± 0.0
<i>P. micra</i> (100)	72 (72.0%)	0
% recovery	8.5 ± 6.1	0

Table 3 (continued)

<i>C. rectus</i> (44)	0	0
% recovery	0	0
<i>F. nucleatum</i> (97)	21 (21.7%)	0
% recovery	11.6 ± 9.2	0
<i>S. constellatus</i> (17)	4 (23.5%)	12 (70.6%)
% recovery	15.5 ± 20.5	12.0 ± 12.2

^a breakpoint concentration of antibiotic used in vitro; ^b mean ± SD percentage levels of antibiotic-resistant bacterial strains in subgingival biofilms of patients with antibiotic-resistant species; ^c No. (%) of species-positive patients with species resistant in vitro to antibiotic.

P. micra, *T. forsythia*, and *P. intermedia/nigrescens* were bacterial species most frequently resistant in vitro to 4 mg/L of azithromycin, with their antibiotic-resistance prevalence among species-positive patients found to 72%, 53.2%, and 32.6%, respectively. *S. constellatus* and *F. nucleatum* clinical isolates were less frequently azithromycin-resistant, with 23.5% and 21.7% of patient strains growing on azithromycin-supplemented EBBA culture plates. All subgingival isolates of *P. gingivalis* and *C. rectus* were sensitive in vitro to 4 mg/L of azithromycin.

In comparison, *S. constellatus* was most often found resistant in vitro to 16 mg/L of metronidazole, with 70.6% of patient isolates exhibiting metronidazole resistance. In contrast, all clinical subgingival isolates of *P. gingivalis*, *T. forsythia*, *P. micra*, *F.*

nucleatum, and *C. rectus*, and 88 out of 89 patient strains of *P. intermedia/nigrescens*, were sensitive in vitro to 16 mg/L of metronidazole.

Figure 1 graphically presents the prevalence of in vitro resistance to 4 mg/L of azithromycin among the evaluated red/orange complex periodontal pathogens.

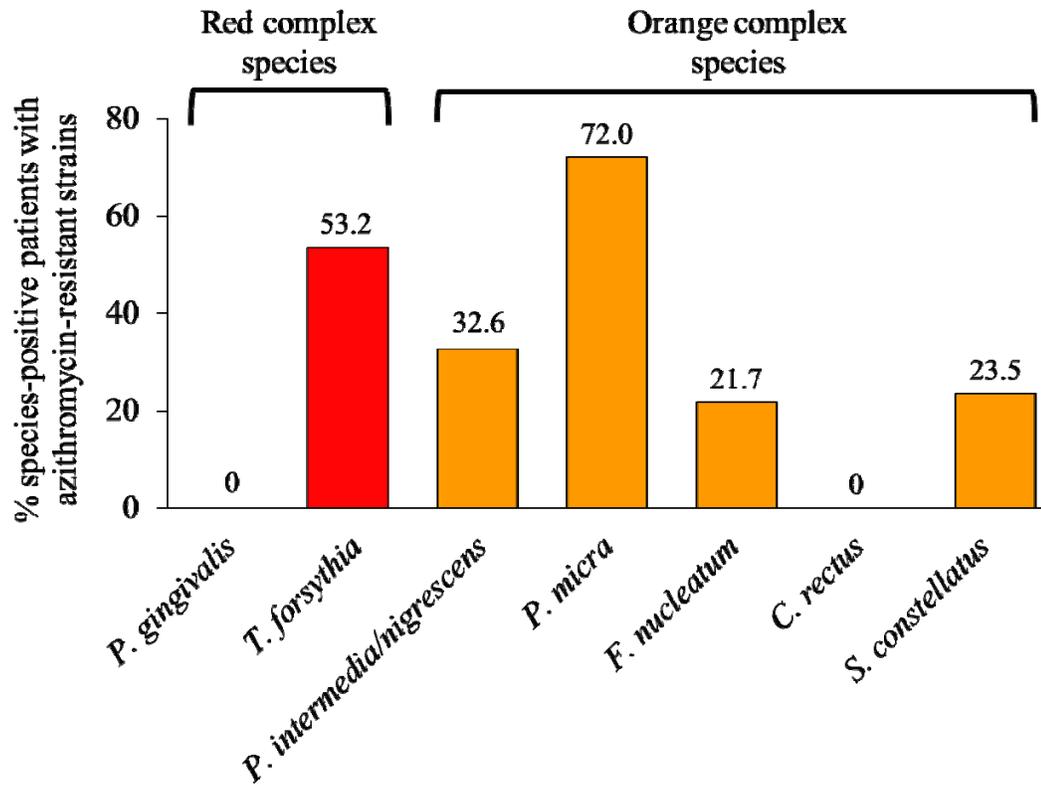


Figure 1. Prevalence of in vitro resistance to 4 mg/L of azithromycin among red/orange complex periodontal pathogens.

Figure 2 displays the distribution of in vitro resistance to 16 mg/L of metronidazole among the evaluated red/orange complex periodontal pathogens.

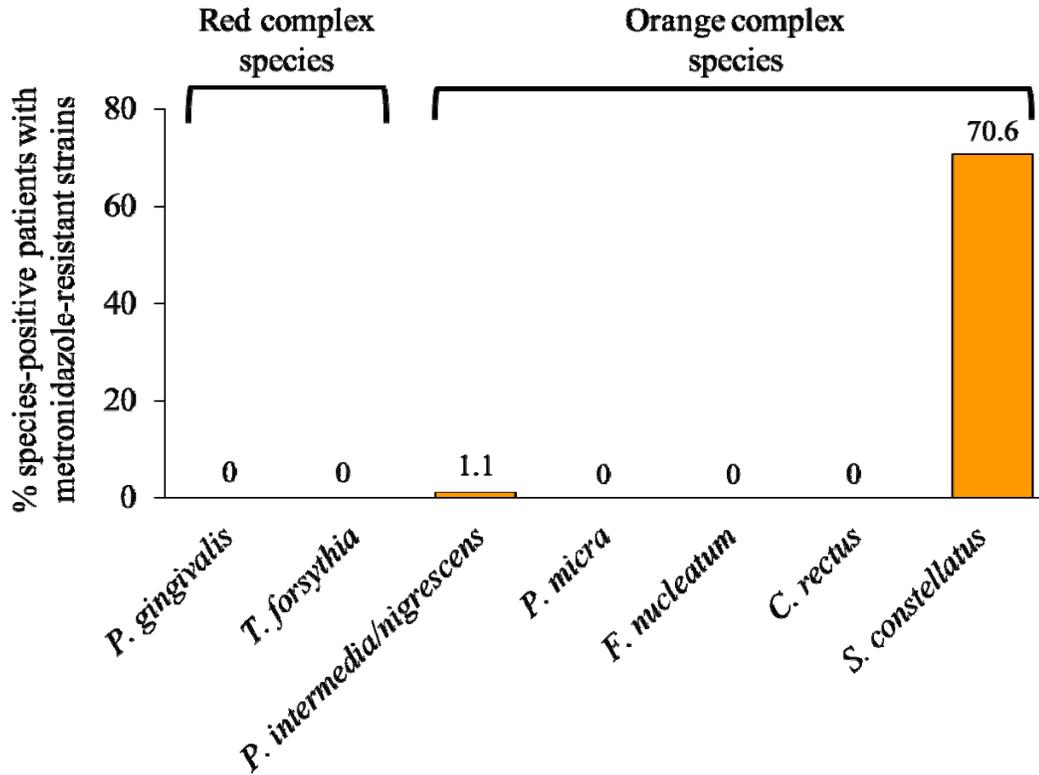


Figure 2. Prevalence of in vitro resistance to 16 mg/L of metronidazole among red/orange complex periodontal pathogens.

At a patient level, 82 (82%) of the severe periodontitis study patients yielded one or more red/orange complex periodontal pathogens resistant in vitro to 4 mg/L of azithromycin, as compared to only 13 (13%) patients with red/orange complex periodontal pathogens resistant in vitro to 16 mg/L of metronidazole (6.3-fold difference, $P < 0.001$, Fisher's exact test).

In logistic regression analysis where gender and current smoking status were statistically controlled for, the presence of one or more red/orange complex periodontal pathogens resistant in vitro to 4 mg/L of azithromycin was significantly more frequent in older severe periodontitis patients aged 65 years or greater as compared to younger-aged patients (odds ratio = 5.0; 95% confidence interval = 1.1, 23.5; $P = 0.041$).

CHAPTER 4

DISCUSSION

The major finding from this study was the unexpectedly high prevalence of azithromycin in vitro resistance among red/orange complex periodontal pathogens sampled from subgingival biofilms of severe periodontitis patients in the United States. A very high percentage (82%) of United States severe periodontitis patients yielded one or more red/orange complex periodontal pathogens resistant in vitro to 4 mg/L of azithromycin, with *P. micra*, *T. forsythia*, and *P. intermedia/nigrescens* among evaluated bacterial species most frequently exhibiting in vitro azithromycin resistance.

P. micra was particularly resistant to azithromycin, with 72% of *P. micra*-positive severe periodontitis patients from the United States harboring azithromycin-resistant *P. micra* strains. While the present cross-sectional prevalence study was not designed to assess temporal changes in azithromycin resistance among subgingival microorganisms, there is evidence that that *P. micra* resistance to azithromycin may not be of recent origin. Over 20 years ago, Feik & Rams (2002) found considerable variable in azithromycin susceptibility among 44 clinical periodontal *P. micra* isolates, with minimum inhibitory concentrations (MIC) of azithromycin against *P. micra* ranging from < 0.016 mg/L to > 256 mg/L, with the MIC₉₀ (denoting inhibition of ≥ 90% of the tested isolates) of azithromycin against *P. micra* determined to be an exceedingly high > 256 mg/L.

Additional orange complex periodontal pathogens often resistant to azithromycin in the present study were *S. constellatus* and *F. nucleatum*, where approximately one in 5

species-positive severe periodontitis study patients had strains of these bacteria resistant in vitro to 4 mg/L of azithromycin. For these two microbial species, rather high subgingival proportions of the organisms were found in patients with azithromycin-resistant strains, with *S. constellatus* composing an average 15.5% of total subgingival anaerobic counts when resistant to azithromycin, and *F. nucleatum* a mean 11.6% of the cultivable subgingival microbiota. The subgingival levels for azithromycin-resistant *S. constellatus* are in excess of subgingival *S. constellatus* proportions (> 2.4%) associated with refractory forms of periodontitis in adults where a poor clinical response is found to conventional mechanical-surgical periodontal therapy augmented with systemic tetracycline therapy (Colombo et al. 1999), and where other types of systemic antibiotic therapy, like azithromycin, are most frequently considered for use (Slots 2004). Moreover, the 4 of 17 (23.5%) *S. constellatus* strains resistant to 4 mg/L of azithromycin in the present 2021 study suggests a higher level of *S. constellatus* azithromycin resistance than was reported nearly a decade earlier, where only 2 of 33 (6.1%) periodontal *S. constellatus* strains from United States periodontitis patients were resistant to 2 mg/L of azithromycin (Rams et al. 2014b).

The emergence of azithromycin resistance among red/orange complex periodontal pathogens may be a function of repeated use and exposure to azithromycin and other macrolide antibiotics over a lifetime. Unfortunately, data on past antibiotic use and exposure was not available for the severe periodontitis study patients in the present study to analyze relative to azithromycin resistance in their cultivable subgingival microbiota. However, elderly age increases the risk of infection with drug-resistant bacteria, such as

the greater prevalence of penicillin and other drug-resistant *Streptococcus pneumoniae* infections in persons 65 years of age and older (Niederman & Ahmed 2003). Consistent with this, the subgingival presence of one or more red/orange complex periodontal pathogens resistant in vitro to 4 mg/L of azithromycin was found with logistic regression analysis in the present study to be significantly more frequent in older severe periodontitis patients aged 65 years or greater as compared to younger-aged patients (odds ratio = 5.0), even after taking into account the effects of gender and current smoking status. This apparent increased risk of subgingival carriage of azithromycin-resistant red/orange complex periodontal pathogens in elderly persons with severe periodontitis raises important therapeutic questions about the appropriateness of empiric use of azithromycin in periodontal therapy administered to older-aged patients. Inappropriate antimicrobial treatment, which is use of an antimicrobial agent to which the targeted pathogen microorganism is resistant, is associated with increased morbidity and mortality outcomes in a number of medical diseases (Davey & Marwick 2003), and may also lead to therapeutic failures in periodontal disease management, although documentation of this phenomena is limited to a small number of patient care reports (Fine 1994).

In contrast to the relatively high prevalence of azithromycin resistance among many red/orange complex periodontal pathogens, all 14 evaluated patient strains of *P. gingivalis*, perhaps the most virulent human periodontal pathogen that potentially contributes to a wide range of extra-oral medical diseases (Hajishengallis & Diaz 2020, Peng et al. 2022), were found in this study to be sensitive in vitro to 4 mg/L of

azithromycin. The absence of azithromycin resistance in *P. gingivalis* strains originating from United States periodontitis patients is in agreement with reports of excellent antimicrobial activity of azithromycin against *P. gingivalis* isolates in Finland, where all 82 *P. gingivalis* strains were inhibited in vitro by ≤ 1 mg/L of azithromycin (Pajukanta 1993); data from Iran, where all 50 periodontal *P. gingivalis* isolates were sensitive to azithromycin (Japoni et al. 2011); data from Moroccan periodontitis patients, where 30 *P. gingivalis* strains were uniformly sensitive to azithromycin (Mínguez et al. 2019); and periodontitis patients in Switzerland, where all 56 *P. gingivalis* strains were susceptible to azithromycin (Kulik et al. 2019). In contrast to these findings, 21.3% of *P. gingivalis*, as well as 21.0% of *T. forsythia*, subgingival isolates from periodontitis patients in Columbia, where over-the-counter antibiotic availability leads to greater antibiotic use and exposure, exhibited in vitro azithromycin resistance (Ardila & Bedoya-García 2020).

In addition to its antimicrobial activity, azithromycin at subinhibitory concentrations inhibits *P. gingivalis* fimbriae production, which is considered essential to *P. gingivalis* intraoral colonization (Lo Bue et al. 1997, Kan et al. 2019). Azithromycin further acts against *P. gingivalis* internalized within gingival epithelial cells and gingival fibroblasts, working equally as effective as the antibiotic combination of metronidazole plus amoxicillin against intraepithelial *P. gingivalis*, and better than the drug combination against *P. gingivalis*-infected gingival fibroblasts (Lai & Walters 2016). Thus, the present study findings augment knowledge on the potential value of azithromycin relative

to treating not only *P. gingivalis* in periodontal pockets, but also *P. gingivalis* infections at non-oral body sites.

A limitation of the present study was the absence of any molecular assessment of microbial genes associated with the observed in vitro azithromycin bacterial resistance. Additional studies are likely to find azithromycin-resistant red/orange complex periodontal pathogens carrying *erm* genes, which code for MLS_B resistance (resistance to macrolides, lincosamides, and streptogramin B (Miklasińska-Majdanik 2021). In vitro resistance to azithromycin has been associated with *erm*(F) genes in periodontal *Prevotella* clinical isolates from adults with periodontitis (Arredondo et al. 2019).

The present study findings represent the most current point estimate for the prevalence of azithromycin resistance in selected cultivable red/orange complex periodontal pathogens from United States periodontitis patients. Lower levels of such azithromycin resistance have been reported in data from other countries, particularly those where antibiotic use and exposure is more limited than in the United States. Subgingival biofilms from periodontitis patients in the Netherlands, where there is low antibiotic consumption and exposure (European Centre for Disease Prevention and Control et al. 2017), revealed no growth of *P. gingivalis*, and ≤ 4.5% of antibiotic-resistant growth of *P. micra*, *T. forsythia* and *P. intermedia/nigrescens* isolates plated onto microbial culture media containing 2 mg/L of azithromycin (van Winkelhoff et al. 2000). However, 96.7% of Dutch *F. nucleatum* strains were resistant in vitro to 2 mg/L of azithromycin (van Winkelhoff et al. 2000). A similar low level of azithromycin

resistance in cultivable red/orange complex periodontal pathogens from German periodontitis patients was noted over a 7-year time period (Jepsen et al. 2021).

In contrast, similar subgingival biofilm specimens from periodontitis patients in Spain, where antibiotic use is markedly higher than in the Netherlands and the northern part of western Europe (European Centre for Disease Prevention and Control et al. 2017), 20% of *P. gingivalis* strains were azithromycin-resistant, along with 25% of *T. forsythia* strains, 17.4% of *P. intermedia/nigrescens* strains, 10.5% of *P. micra* strains, and interestingly, only 6.5% of *F. nucleatum* strains (van Winkelhoff et al. 2000). It is likely that azithromycin resistance levels in both Dutch and Spanish periodontitis patients would be lower if a 4 mg/L threshold concentration of azithromycin was used to determine species drug resistance, as was employed in the present study, instead of 2 mg/L of azithromycin.

Sensitivity of the evaluated red/orange complex periodontal pathogens to 16 mg/L of metronidazole was employed by the present study as a comparison or control study arm. As expected with the inherent susceptibility of anaerobic bacteria to metronidazole (Leitsch 2019), all clinical subgingival isolates of *P. gingivalis*, *T. forsythia*, *P. micra*, *F. nucleatum*, and *C. rectus*, and 88 out of 89 patient strains of *P. intermedia/nigrescens*, were sensitive in vitro to 16 mg/L of metronidazole, similar to findings of previous studies of United States periodontitis patients (Rams et al. 2011, 2014a, 2020). In comparison, *S. constellatus*, a Gram-positive facultative cocci, was less sensitive to metronidazole, with 70.6% of species-positive severe periodontitis patients harboring metronidazole-resistant *S. constellatus*, consistent with prior studies (Rams et al. 2014).

Overall, 82 (82%) of the evaluated severe periodontitis study patients yielded one or more red/orange complex periodontal pathogens resistant in vitro to 4 mg/L of azithromycin, as compared to only 13 (13%) patients with red/orange complex periodontal pathogens resistant in vitro to 16 mg/L of metronidazole (6.3-fold difference, $P < 0.001$). This alarming level of azithromycin in vitro resistance in red/orange complex periodontal pathogens among severe periodontitis patients in the United States raises clinical doubt about appropriateness of empiric use of azithromycin therapy in periodontal disease management when no microbiological analysis with antibiotic susceptibility testing is performed.

CHAPTER 5

CONCLUSIONS

A high prevalence of in vitro resistance to azithromycin, which was significantly greater than detected to metronidazole and significantly more frequent in patients aged 65 years or greater, was found among red/orange complex periodontal pathogens in the subgingival microbiota of severe periodontitis patients in the United States.

These findings question the potential usefulness of azithromycin in treatment of United States periodontitis patients who frequently harbor azithromycin-resistant subgingival red/orange complex periodontal pathogens.

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