

IN VITRO ANTIMICROBIAL ACTIVITY OF POVIDONE-IODINE
AGAINST SELECTED HUMAN RED AND ORANGE COMPLEX
PERIODONTAL PATHOGENS.

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Chander Gupta, DMD
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Thesis Approvals:

Thomas E. Rams, DDS, MHS, PhD, Thesis Advisor, Department of Periodontology
and Oral Implantology, Temple University School of Dentistry
Lawrence R. Page, DDS, PhD, Department of Periodontology and Oral Implantology,
Temple University School of Dentistry
Eugene J. Whitaker, DMD, PhD, Department of Restorative Dentistry, Temple
University School of Dentistry

ABSTRACT

Objectives: Successful treatment of severe human periodontitis lesions has been shown to be highly dependent upon adequate suppression or eradication of key bacteria, most often species classified as red and orange complex periodontal pathogens, in the subgingival microbiome of diseased periodontal sites. Multiple clinical studies have reported superior therapeutic outcomes when conventional mechanical root debridement of severe periodontitis lesions is supplemented with professional periodontal pocket delivery of a povidone-iodine antiseptic solution, which offers antimicrobial effects against red and orange complex periodontal pathogens.

However, many questions involving the clinical application of povidone-iodine in periodontal therapy remain unresolved, such as which concentration of povidone-iodine is preferred, and how long of a contact time period is needed between povidone-iodine and targeted bacteria in periodontal pockets. Previous *in vitro* studies on the effects of povidone-iodine on periodontal bacterial pathogens employed 5-minute or longer contact times and most often tested laboratory stock strains of microorganisms, even though inflamed periodontal pockets undergo a rapid washout of introduced fluids, and bacterial stock collections frequently develop altered properties and decreased virulence in comparison to freshly-recovered wild-type clinical isolates.

To address some of these issues, the objective of this study was to further explore the *in vitro* effects of povidone-iodine on periodontal bacterial pathogens by employing a subgingival biofilm species eradication assay to test the antimicrobial effects of a 60-second *in vitro* exposure of 5% and 10% povidone-iodine on freshly-isolated red and orange complex periodontal pathogens from severe human periodontitis lesions.

Methods: Paper point subgingival biofilm samples from 22 adults with severe periodontitis that were to be discarded after microbiological analysis at the Oral Microbiology Testing Service Laboratory at Temple University School of Dentistry were secondarily employed in this study. Dilution aliquots from each subgingival specimen were mixed with either 10% or 5% povidone-iodine for a 60-second in vitro contact time period, and then neutralized with 3% sodium thiosulfate. The mixtures were then inoculated onto enriched Brucella blood agar plates, and incubated anaerobically for 7 days at 37°C. Bacterial species growing subsequent to the 60-second povidone-iodine contact time were considered to be resistant to that concentration of povidone-iodine. Total viable counts in povidone-iodine exposed subgingival specimens were quantitated, and established phenotypic criteria employed to identify the following red and orange complex periodontal pathogens: *Porphyromonas gingivalis*, *Tannerella forsythia*, *Prevotella intermedia/nigrescens*, *Parvimonas micra*, *Campylobacter rectus*, *Fusobacterium nucleatum* group species, and *Streptococcus constellatus*. Other cultivable isolates recovered from povidone-iodine exposed subgingival specimens were identified using matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry and Bruker MALDI Biotyper analytic software.

Subgingival sample dilution aliquots not exposed to povidone-iodine were similarly processed as controls for comparison with povidone-iodine exposed specimens, and were additionally inoculated onto enriched Brucella blood agar plates supplemented with either metronidazole at 16 mg/L, doxycycline at 4 mg/L, amoxicillin at 8 mg/L, or clindamycin at 4 mg/L, which represent recognized non-susceptible drug breakpoint concentrations for each of the antibiotics, followed by anaerobic incubation for 7 days at

37°C. In vitro antibiotic resistance was noted when any of the evaluated red and orange complex periodontal pathogens displayed growth on one or more of the antibiotic-supplemented enriched Brucella blood agar plates.

Paired t-tests compared mean total subgingival viable counts, and mean total subgingival proportions of the evaluated anaerobic red and orange complex periodontal pathogens per patient, between subgingival biofilm samples exposed and not exposed in vitro to 10% or 5% povidone-iodine, with a P -value of ≤ 0.05 required for statistical significance. Comparisons were also made between antibiotic-resistance and susceptible strains of the evaluated red and orange complex periodontal pathogens to assess their in vitro sensitivity to 10% and 5% povidone-iodine.

Results: Subgingival specimens exposed in vitro for 60-seconds to either 10% or 5% povidone-iodine yielded significantly lower total subgingival viable counts per patient than those not exposed to povidone-iodine ($P < 0.001$, paired t-test), with no statistically significant differences found between the two povidone-iodine concentrations ($P = 0.125$, paired t-test).

All evaluated red and orange complex periodontal pathogens were suppressed below detection levels after 10% povidone-iodine in vitro exposure, except for one *F. nucleatum* strain in one patient specimen. As a result, 21 (95.5%) of the patient subgingival biofilm samples were culture-negative for red and orange complex periodontal pathogens after 10% povidone-iodine in vitro exposure. A similar suppression of red and orange complex periodontal pathogens was found following 5% povidone iodine in vitro exposure, where only one patient sample yielded *P. intermedia/nigrescens*, and two other samples *F. nucleatum*. This resulted in 19 (86.4%)

of patient subgingival biofilm samples being devoid of red and orange complex periodontal pathogens after 5% povidone-iodine in vitro exposure. Total subgingival proportions of red and orange complex periodontal pathogens averaged 0.5% per patient in subgingival specimens exposed in vitro to 10% povidone-iodine, and 0.7% per patient in those exposed to 5% povidone-iodine, which were both significantly lower than 14.8% mean proportions detected in subgingival biofilms not exposed to povidone-iodine ($P < 0.0001$, paired t-test). No statistically significant differences were found between 10% and 5% povidone-iodine relative to suppression of total red and orange complex periodontal pathogen proportions ($P = 0.743$, paired t-test).

Both povidone-iodine concentrations were similarly active against red and orange complex periodontal pathogens which were resistant in vitro to breakpoint levels of doxycycline, amoxicillin, or clindamycin, as compared to antibiotic-susceptible clinical isolates.

Various *Streptococcus* species, particularly *Streptococcus oralis*, were the most frequently recovered microorganisms in subgingival biofilm specimens after 60-second in vitro exposure to povidone-iodine, indicative of their in vitro resistance to both 10% and 5% povidone-iodine.

Conclusions: Povidone-iodine exerted marked antimicrobial effects against both antibiotic-resistant and susceptible fresh clinical isolates of red and orange complex periodontal pathogens, and total viable counts, in subgingival biofilm specimens from severe periodontitis patients after only a brief (60-second) in vitro contact time, with no statistically significant differences found between 10% and 5% concentrations of povidone-iodine.

The profound suppression of red and orange complex periodontal pathogens, and concurrent predominance of periodontal health-associated *Streptococcus* species, that occurs in subgingival biofilm specimens following in vitro exposure to povidone-iodine, further supports the clinical use of povidone-iodine in periodontal therapy as an adjunct to mechanical root debridement in altering pathogenic subgingival microbial populations towards one compatible with periodontal health.

TABLE OF CONTENTS

	Page
ABSTRACT.....	ii
LIST OF TABLES	viii
LIST OF FIGURES	ix
CHAPTER	
1. INTRODUCTION.....	1
2. MATERIALS AND METHODS	7
Laboratory Facilities.....	7
Subgingival Biofilm Specimens.....	7
Subgingival Biofilm Species Eradication Assay with Povidone-Iodine.....	9
Control Specimens and In Vitro Antibiotic Resistance Testing.....	10
Bacterial Identification.....	11
Data Analysis.....	15
3. RESULTS.....	17
Subgingival Biofilm Species.....	17
In Vitro Antibiotic Resistance Among Test Bacterial Species.....	18
Povidone-Iodine In Vitro Effects on Subgingival Biofilm Species.....	20
Povidone-Iodine Effects on Antibiotic-Resistant and Susceptible Red and Orange Complex Periodontal Pathogens	25
Other Subgingival Biofilm Species Resistant In Vitro to Povidone-Iodine	25
4. DISCUSSION.....	28
5. CONCLUSIONS.....	32
REFERENCES CITED.....	33

LIST OF TABLES

Table	Page
1. Presence and proportional recovery of selected periodontal pathogens in subgingival biofilm specimens without exposure to povidone-iodine	17
2. In vitro resistance of selected periodontal pathogens to breakpoint concentrations of four antibiotics.....	19
3. Other subgingival biofilm species resistant in vitro to povidone-iodine.....	26

LIST OF FIGURES

Figure	Page
1. Molecular structure of povidone-iodine.....	3
2. Flow chart for subgingival biofilm species eradication assay for 10% and 5% povidone-iodine	15
3. Average total viable counts in subgingival biofilm specimens exposed and not exposed in vitro to povidone-iodine	21
4. Average total proportions of red and orange complex periodontal pathogens in subgingival biofilm specimens exposed and not exposed in vitro to povidone-iodine.....	23
5. Example #1 of a subgingival biofilm specimen exposed and not exposed in vitro to povidone-iodine.....	24
6. Example #2 of a subgingival biofilm specimen exposed and not exposed in vitro to povidone-iodine.....	24

CHAPTER 1

INTRODUCTION

Successful treatment of severe human periodontitis lesions has been shown to be highly dependent upon adequate suppression or eradication of key bacteria, most often species classified as red and orange complex periodontal pathogens, in the subgingival microbiome of diseased periodontal sites. Socransky et al. (1998) studied 13, 261 subgingival plaque biofilm samples from 185 periodontally-healthy and diseased adults with multiple cluster and community ordination statistical analysis. Three bacterial species, *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*, were designated as members of a red complex group statistically associated with the most severe forms of human periodontitis. Another group of 12 species, including *Prevotella intermedia*, *Prevotella nigrescens*, *Parvimonas micra*, *Fusobacterium nucleatum* group species, *Streptococcus constellatus*, and several *Campylobacter* species, were grouped into an orange complex associated with less severe forms of destructive periodontal disease (Socransky et al. 1998).

In clinical studies of severe periodontitis treatment, subgingival persistence of *P. gingivalis* after extensive non-surgical root instrumentation and supragingival plaque control occurred in 59% of 17 chronic periodontitis patients (Mombelli et al. 2000). This continued *P. gingivalis* presence was significantly related to a poorer clinical treatment outcome, resulting in greater numbers of non-resolving ≥ 5 mm post-treatment periodontal pockets (Mombelli et al. 2000). Another clinical study also found persistence of subgingival *P. gingivalis* after nonsurgical root instrumentation to be associated with

an increased risk of progressive alveolar bone loss (odds ratio = 31.9; positive predictive value = 84%) (Chaves et al. 2000). In contrast, post-treatment radiographic alveolar bone gain was found when *P. gingivalis* was absent from the subgingival microbiota of study patients after the completion of periodontal therapy (Chaves et al. 2000). Persistence of increased numbers of *P. gingivalis* and *T. denticola* in post-treatment samples of subgingival biofilm was associated with a 62% and 130% excess risk, respectively, for subsequent clinical periodontal breakdown occurring 3 months later in treated periodontitis patients receiving systematic periodontal maintenance care (Byrne et al. 2009). Post-periodontal therapy presence of elevated levels of one or more of the three red complex species, or the presence of at least two of the orange complex periodontal pathogens *P. intermedia*, *C. rectus* and/or *F. nucleatum*, were statistically related to significant odds ratio relationships of 39.3 and 61.9, respectively, with marked clinical periodontal attachment loss and increased probing depths within a 2-year period in treated chronic periodontitis patients (Charalampakis et al. 2013). In multivariate analysis, Rams et al. (1996) reported a 2.5 (150%) excess relative risk for periodontitis recurrence within 12 months post-treatment for treated chronic periodontitis presenting with elevated post-treatment subgingival levels of either *P. gingivalis*, *P. micra*, *C. rectus*, *P. intermedia/nigrescens* and/or *Aggregatibacter actinomycetemcomitans*. In contrast, Colombo et al. (2012) found in periodontitis patients favorably responding to periodontal therapy with mean post-treatment clinical periodontal attachment gains, and no periodontal sites with progressive periodontal attachment loss of ≥ 2.5 mm from baseline values, both red and orange complex periodontal pathogens to be longer

dominant in subgingival biofilm analysis, where instead a variety of non-anaerobic bacteria, including various *Streptococcus* species, were major colonizers.

In therapeutic efforts to adequately suppress red and orange complex periodontal pathogens in subgingival sites of severe periodontitis patients, multiple clinical studies have supplemented conventional mechanical root debridement with professional periodontal pocket delivery of a povidone-iodine antiseptic solution. Povidone-iodine is classified as an iodophor, where molecular iodine is combined with the synthetic polymer polyvinylpyrrolidone (Figure 1), a solubilizing agent that increases the water solubility and stability of povidone-iodine, and reduces some adverse properties associated with molecular iodine alone, such as staining and irritation of human soft tissues (Cooper 2007).

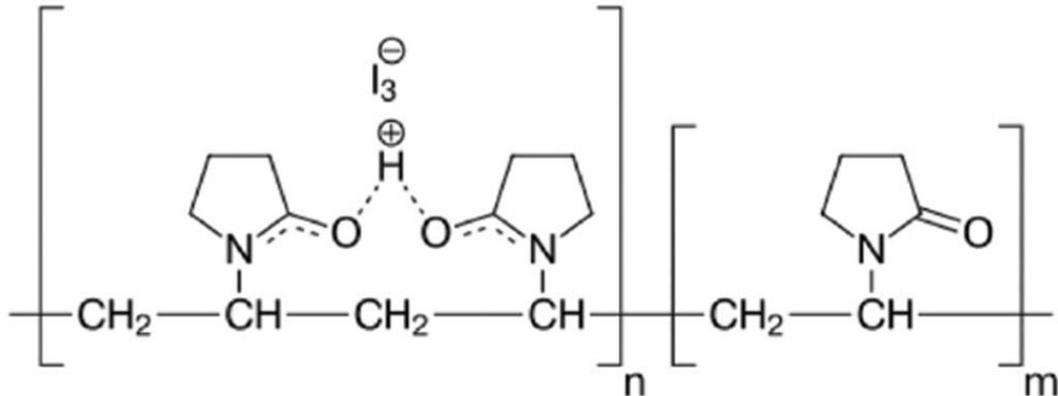


Figure 1. Molecular structure of povidone-iodine. Adapted from Zamora (1986).

Povidone-iodine appears to exert marked bactericidal effects through creation of pores in cell walls of microorganisms, leading to leakage of intercellular contents and cell death (Schreier et al. 1997).

A number of clinical studies have reported favorable outcomes with adjunctive periodontal pocket delivery of povidone-iodine into severe periodontitis sites. A meta-analysis published in 2010 concluded that adjunctive professional applications of povidone-iodine during periodontal root instrumentation significantly increased post-treatment periodontal pocket depth reductions (Sahrman et al. 2010). When diluted povidone-iodine (0.5%) was applied as a coolant during ultrasonic scaler pocket debridement of initially deep (≥ 7 mm) periodontal pockets, a significantly greater proportion of these sites experienced a gain of clinical periodontal attachment of ≥ 2 mm at 12 months post-treatment (79%) as compared to that attained with water as the ultrasonic scaler coolant (56%) (Rosling et al. 1986). This beneficial treatment outcome was replicated in a follow-up clinical study with a similar investigative design (Christersson et al. 1988). In a different 13-year clinical trial, diluted povidone-iodine (0.1%) applied into periodontal pockets as an ultrasonic scaler coolant during initial periodontal therapy and periodontal maintenance care appointments induced significantly greater gains of clinical periodontal attachment in initially deep periodontal sites (≥ 6 mm) at one-year post-treatment in comparison to water-cooled ultrasonic root debridement without povidone-iodine, with this statistically significant therapeutic advantage holding up over the remaining 12-year length of the clinical study (Rosling et al. 2001). Importantly, markedly less recurrent periodontal breakdown occurred in the povidone-iodine treated group, where among study patients experiencing dramatic clinical periodontal loss of ≥ 2 mm on ≥ 4 teeth each year of the study, nine of them were treated with povidone-iodine applications, and 31 were among patients treated with water

as the ultrasonic scaler coolant (Rosling et al. 2001). More recently, subgingival application of a 10% povidone-iodine solution as an ultrasonic scaler coolant, along with pocket placement of a 10% povidone-iodine gel ointment, as compared to water as an ultrasonic scaler coolant, resulted in significantly greater periodontal probing depth reductions at 3 months post-treatment, and better suppression of subgingival *P. gingivalis* during the first one-month post-treatment time period (Sahrman et al. 2014). Additionally, a single pocket irrigation with 10 ml of 10% povidone-iodine solution, as compared to irrigation with physiologic saline, for 60 seconds after periodontal root instrumentation on deep periodontal interproximal posterior pockets (> 6 mm) in 20 adults with chronic periodontitis resulted in significantly greater probing depth reductions (3.9 mm for povidone-iodine irrigation vs. 2.7 mm physiologic saline irrigation) and greater gains of CAL (4.0 mm for povidone-iodine irrigation vs. 2.7 mm for physiologic saline irrigation) at 6 months post-treatment (Denez et al. 2016).

Despite these favorable clinical findings, many questions involving the application of povidone-iodine in periodontal therapy remain unresolved, such as which concentration of povidone-iodine is preferred, and how long of a contact time period is needed between povidone-iodine and targeted bacteria in periodontal pockets. Previous in vitro studies on the effects of povidone-iodine on periodontal bacterial pathogens employed 5-minute or longer contact times (Caufield et al. 1987, Maruniak et al. 1992, Bercy & Lasserre 2007), and most often tested laboratory stock strains of microorganisms (Caufield et al. 1987, Maruniak et al. 1992, Higashitsutsumi et al. 1993, Nakagawa et al. 2006, Bercy & Lasserre 2007, Hosaka et al. 2012), even though inflamed

periodontal pockets undergo a rapid washout of introduced fluids (Goodson 2003), and bacterial stock collections frequently develop altered properties and decreased virulence in comparison to freshly-recovered wild-type clinical isolates (Rosan et al. 1988).

To address some of these issues, the objective of this study was to further explore the in vitro effects of povidone-iodine on periodontal bacterial pathogens by employing a subgingival biofilm species eradication assay to test the antimicrobial effects of a 60-second in vitro exposure of 5% and 10% povidone-iodine on freshly-isolated red and orange complex periodontal pathogens from severe human periodontitis lesions.

CHAPTER 2

MATERIALS AND METHODS

Laboratory Facilities

All procedures in this study were performed using the facilities of the Oral Microbiology Testing Service (OMTS) Laboratory located at the Temple University Maurice H. Kornberg School of Dentistry on the Temple University Health Sciences Center campus in Philadelphia, Pennsylvania. Since the present study was non-clinical and laboratory-based, with study data obtained through secondary use of subgingival plaque biofilm samples without any intervention or interaction with living individuals, and not involving any identifiable private information, the research activity did not involve human subjects, as defined by United States Department of Health and Human Services regulations at 45 CFR part 46.116(f), and did not require a human subjects institutional review board approval, per a written determination issued by the Temple University Human Subjects Protections Institutional Review Board.

Subgingival Biofilm Specimens

Subgingival biofilm specimens were used from 22 adults with severe periodontitis from whom subgingival samples were submitted to the OMTS Laboratory for microbiological analysis and antibiotic resistance testing by subscribing private practicing periodontists extramural to Temple University. The subgingival specimens were normally discarded by the OMTS Laboratory after completion of the requested microbiological testing, but were additionally used in this study after removal of all unique patient identifiers. As a result, other than knowledge that the patients were 35

years of age or older, and identified by their treating periodontist as having severe periodontitis untreated at the time of the microbiological sampling, no additional information is available for the patients, such as their exact age, gender, systemic health history, medications, smoking status, or nature of their clinical/radiographic periodontal parameters beyond their overall periodontal diagnosis.

The subgingival specimens were obtained by the treating periodontists, as instructed by the OMTS Laboratory, by removing supragingival plaque on the patients from 3-5 periodontal sites exhibiting moderate (5-6 mm) to deep periodontal probing depths (≥ 7 mm) and gingival inflammation, and isolating them with cotton rolls and air drying to avoid saliva contamination in the microbial samples. Following these steps, one to two sterile paper points were then advanced with sterile forceps into each isolated periodontal site for approximately 10 seconds in order to collect subgingival biofilm for microbial culture. The paper points were then placed together into a single glass vial containing 6-8 glass beads of 3 mm in diameter, and 2.0 ml of pre-reduced, anaerobically sterilized and stored Möller's VMGA III transport media (Möller 1966), which possesses a high preservation capability for oral microorganisms after sampling during transit to the laboratory (Möller 1966, Dahlén et al. 1989, Dahlén et al. 1993).

The pooled subgingival biofilm samples were delivered within 24 hours to the OMTS Laboratory in Philadelphia, Pennsylvania, which has been in continuous operation since its founding in 1991. 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. The OMTS

Laboratory 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), and as a result, adheres to all of the 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 (Rauch & Nichols, 2007).

Upon arrival at the OMTS Laboratory, the VMGA III vials were warmed to 37°C for 10 minutes prior to processing in order to liquefy gelatin in the VMGA III transport medium. The sampled microorganisms were then mechanically dispersed in the medium with a Vortex mixer at the maximal setting for 45 seconds. Serial 10-fold dilutions of the dispersed bacteria were carried out in Möller's VMG I anaerobic dispersion solution, comprised of pre-reduced, anaerobically sterilized 0.25% tryptose-0.25% thiotone E peptone-0.5% NaCl (Möller 1966).

Subgingival Biofilm Eradication Assay with Povidone-Iodine

A subgingival biofilm eradication assay was employed to test the antimicrobial effects of a 60-second in vitro exposure of 10% and 5% povidone-iodine on whole subgingival biofilm specimens from severe periodontitis patients, similar to the in vitro method described by Pozhitkov et al. (2015) for testing the effects of diluted bleach on subgingival microorganisms.

One ml aliquots of 10^{-6} subgingival biofilm dilutions from each study patient were mixed with either 0.05 ml of 10% povidone-iodine (Povidone-Iodine 10% USP, CVS Health, Woonsocket, RI, USA), or 0.05 ml of 5% povidone-iodine (created by diluting

0.025 ml of 10% povidone-iodine with 0.025 ml of sterile water). After a 60-second contact time period, the povidone-iodine in each mixture was neutralized with 0.05 ml of a 3% sodium thiosulfate solution (Selwyn & Bakhtiar 1988, Goldenheim & Inger 1988, Asanaka et al. 1988). Using a sterile bent glass rod, the mixtures were then plated onto pre-reduced, enriched Brucella blood agar (EBBA), comprised 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 EBBA inoculated plates were incubated at 37°C for 7 days in a upright heated incubator (Caron, Marietta, OH, USA) in jars containing an 85% N₂-10% H₂-5% CO₂ anaerobic atmosphere introduced by an Anoxomat™ Mark II automatic jar evacuation-replacement system (Advanced Instruments, Inc., Norwood, MA, USA) (Brazier & Smith 1989). Bacterial species growing on EBBA plates subsequent to the 60-second in vitro povidone-iodine exposure and neutralization were considered to be resistant to that concentration of povidone-iodine.

Control Specimens and In Vitro Antibiotic Resistance Testing

One ml aliquots of 10⁻⁶ specimen dilutions not exposed to povidone-iodine were similarly processed as controls for comparison with povidone-iodine exposed samples, and were additionally inoculated onto EBBA plates supplemented with either metronidazole at 16 mg/L, doxycycline at 4 mg/L, amoxicillin at 8 mg/L, or clindamycin at 4 mg/L (all antibiotics obtained as pure powder from Sigma-Aldrich, St. Louis, MO, USA), and incubated anaerobically for 7 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) (Clinical and Laboratory Standards Institute, 2012), and for doxycycline as recommended by the French Society for Microbiology (French Society of Microbiology Antibigram Committee 2010). In vitro resistance to the antibiotic breakpoint concentrations was recorded when test species growth was noted on the respective antibiotic-supplemented EBBA plates (Slots et al. 1988, Feres et al. 1999, Rams et al. 2011, Rams et al. 2014). *Bacteroides thetaiotaomicron* ATCC 29741, *Clostridium perfringens* ATCC 13124, and a multi-antibiotic-resistant clinical periodontal isolate of *Fusobacterium nucleatum* were used as positive and negative quality controls for all antibiotic resistance testing on drug-supplemented EBBA plates.

Two of the subgingival biofilm samples were also mixed with 0.05 ml of 3% sodium thiosulfate alone, and similarly processed, to evaluate the potential effect of the povidone-iodine neutralization agent by itself on in vitro subgingival bacterial growth.

Bacterial Identification

For all EBBA plates, total viable counts were quantitated, and established phenotypic criteria employed to identify the following red and orange complex periodontal pathogens: *Porphyromonas gingivalis*, *Tannerella forsythia*, *Prevotella intermedia/nigrescens*, *Parvimonas micra*, *Campylobacter rectus*, *Fusobacterium nucleatum* group species, and *Streptococcus constellatus*.

P. gingivalis identification was based on 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). Presumptive *C. rectus* species isolates were identified as previously described (Rams et al. 1993) on the basis of motility, colonial and cellular morphology from other organisms as short, straight, motile rods forming small (< 1.5 mm in diameter), glistening black, convex colonies with entire edges. *F. nucleatum* group isolates were presumptively 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 on anaerobically incubated EBBA, 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 subject recovery of these test bacterial species was calculated as the percent recovery of each test species colony forming units (CFU) among the total cultivable subgingival anaerobic viable count as determined on EBBA plates where the inoculated subgingival specimen was not exposed in vitro to povidone-iodine.

Other cultivable isolates recovered from povidone-iodine exposed subgingival specimens were identified using matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry and Bruker MALDI Biotyper analytic software (Bruker Daltonics, Billerica, MA, USA), following procedures previously described (Rams et al. 2016, 2018). In brief, a single colony of the bacterial isolate was picked with a sterile wooden toothpick from the surface of EBBA medium plates, direct-spotted as a thin film into individual circles on the surface of a polished steel MALDI-TOF mass spectrometry, and allowed to dry at room temperature. A 1.0 µl overlay of a 98-100% formic acid solution was placed and allowed to air dry over the colony smears to facilitate on-plate extraction of bacterial cell proteins. Each spot was then subjected to a second overlay solution with 1.0 µl of a matrix mixture, comprised of alpha-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid, and allowed to dry at room temperature. A bacterial test standard, containing *Escherichia coli* DH 5 alpha strain ribosomal proteins, was prepared according to manufacturer instructions, and processed similar to the other bacterial isolates. Negative control spots on the target plate were left blank or with the dried matrix solution alone.

Mass spectra for each isolate was acquired using a Microflex LT benchtop mass spectrometer equipped with a 20-Hz nitrogen laser (Bruker Daltonics). The instrument was operated with Bruker Daltonics FlexControl software (version 3.4) in a linear positive mode within a 2-20 kDa mass range, with voltage settings of 20 kV for ion source one, 18.05 kV for ion source two, 6.0 kV for the lens, and 2,560 V for the linear detector. Mass spectra were automatically obtained using an average 500 laser shots, and analyzed with Bruker Daltonics MALDI Biotyper 3.1 software and the MBT 6903 MSP Library, to determine the most likely microbial species identification. A MALDI Biotyper score, generated as a level of probability by the software, of ≥ 1.7 was utilized as a threshold for reliable species identification, as recommended for assessment of anaerobic bacteria (Hsu & Burnham 2014). Scores of < 1.7 were considered to provide less reliable bacterial identification. A MALDI Biotyper log score ≥ 2.0 was considered to represent definitive species identification.

All laboratory culture media preparation, specimen inoculation, and culture analysis were carried out in a standardized fashion by a single oral microbiology laboratory technician (Jackie Sautter), with oversight and review by the laboratory director (Dr. Thomas E. Rams). Dr. Rams carried out all MALDI-TOF mass spectrometry laboratory procedures.

Figure 2 summarizes how the subgingival biofilm species eradication assay was carried out in the present study.

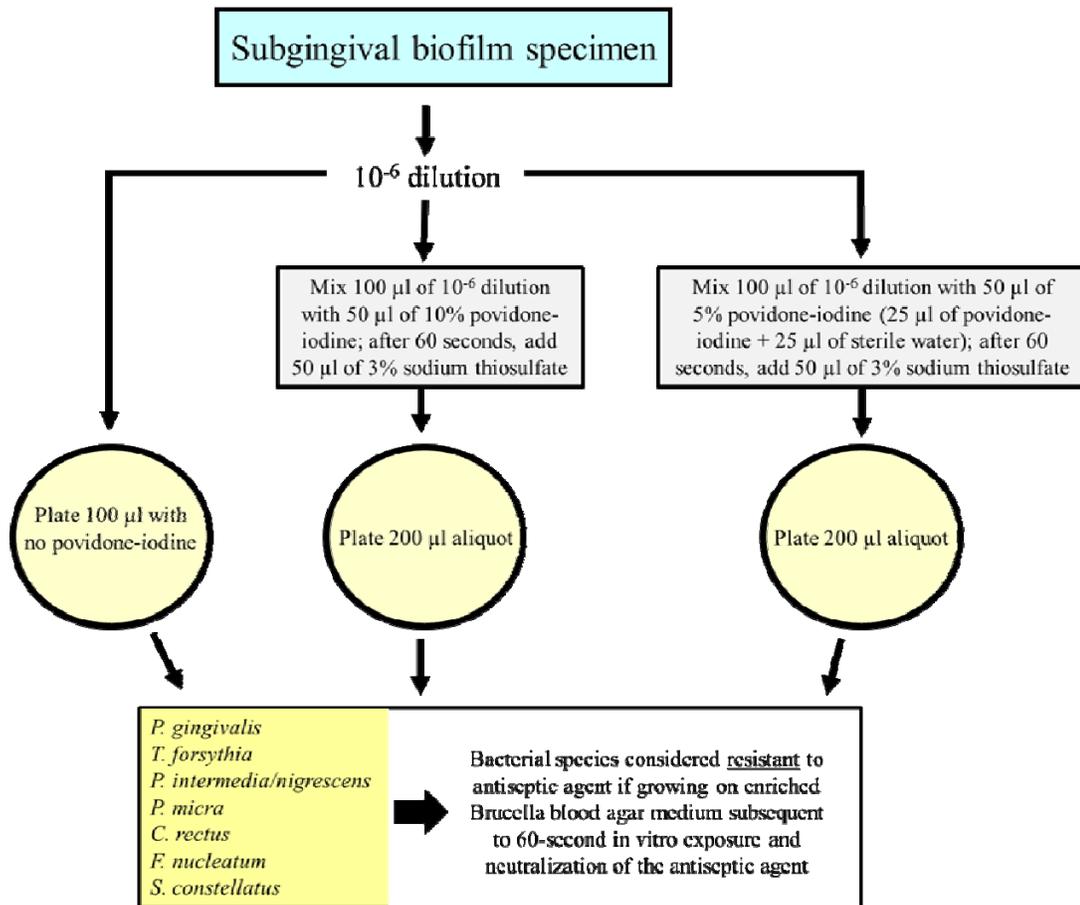


Figure 2. Flow chart for subgingival biofilm species eradication assay for 10% and 5% povidone-iodine.

Data Analysis

Mean total subgingival viable counts were calculated and transformed to \log_{10} values for each study patient subgingival sample exposed and not exposed in vitro to povidone-iodine. For each of the test bacterial species, the number and proportion of organism-positive patients was determined, along with the organism's mean subgingival proportional recovery and standard deviation (SD) or standard error (SE), as well as the number and proportion of patients with bacterial species resistant to either 5% or 10%

povidone-iodine in vitro exposure. Total subgingival proportions of red and orange complex periodontal pathogens (Carvalho et al. 2005, Page & Rams 2013, McCawley et al. 2018) were determined by summing together individual species data for each patient, and then calculating total mean values across all patients.

A paired t-test compared mean total subgingival viable counts, and mean total subgingival proportions of red and orange complex periodontal pathogens per patient, between patient subgingival samples exposed and not exposed in vitro to either 5% or 10% povidone-iodine, with a *P*-value of ≤ 0.05 required for statistical significance. Comparisons were also made between antibiotic-susceptible and antibiotic-resistant strains of test bacterial species for their in vitro sensitivity to 5% and 10% povidone-iodine exposure. The PC-based STATA/SE 14.2 for Windows (StataCorp PL, College Station, TX, USA) 64-bit statistical software package was used in the data analysis.

CHAPTER 3

RESULTS

Subgingival Biofilm Species

Table 1 lists subgingival bacterial species recovered in the 22 study patient specimens not exposed in vitro to either 10% or 5% povidone-iodine.

Table 1. Presence and Proportional Recovery of Selected Periodontal Pathogens in Subgingival Biofilm Specimens Without Exposure to Povidone-Iodine

Species	No. (%) of positive patients	% recovery in species-positive patients \pm SD	Range %
<u>Red complex species:</u>			
<i>P. gingivalis</i>	4 (18.2)	5.3 \pm 7.9	1.1-17.2
<i>T. forsythia</i>	6 (27.3)	2.8 \pm 0.6	1.8-3.4
<u>Orange complex species:</u>			
<i>P. intermedia/nigrescens</i>	13 (59.1)	4.1 \pm 4.6	0.2-14.8
<i>P. micra</i>	20 (90.9)	5.5 \pm 4.1	0.2-13.8
<i>C. rectus</i>	9 (40.9)	0.1 \pm 0.03	0.1-0.2
<i>F. nucleatum</i>	18 (81.8)	6.3 \pm 4.3	0.5-15.7
<i>S. constellatus</i>	1 (4.6)	1.4 \pm 0.0	1.4

Orange complex periodontal pathogens were the most predominant test species in the 22 subgingival biofilm specimens from severe periodontitis patients not exposed to

povidone-iodine. *P. micra* and *F. nucleatum* were the most frequently isolated test species, with 90.9% and 81.8% of study subject samples yielding the species, and averaging 5.5% and 6.3% of *P. micra* and *F. nucleatum*, respectively. *P. intermedia/nigrescens* was recovered from 59.1% of the study patient specimens, with mean subgingival proportions of 4.1% in species-positive samples. Only low subgingival levels of *C. rectus* averaging 0.1% were found in 40.9% of patient samples. Only one specimen yielded *S. constellatus*, which comprised 1.4% of the cultivable subgingival microbiota from the patient.

Among red complex periodontal pathogens, *T. forsythia* was found to average 2.8% of subgingival isolates in 27.3% of the study patient samples. *P. gingivalis* was recovered in only 18.2% of samples, and averaged 5.3% of cultivable subgingival organisms in species-positive samples.

Total subgingival proportions of red and orange complex periodontal pathogens averaged 14.8 ± 2.8 (SE) % per patient in subgingival biofilm specimens not exposed to povidone-iodine.

In Vitro Antibiotic Resistance Among Test Bacterial Species

For red and orange complex periodontal pathogens recovered by culture from subgingival biofilms specimens not exposed to povidone-iodine, Table 2 lists the distribution of their in vitro resistance to breakpoint concentrations of four antibiotics frequently employed in periodontal disease treatment regimens.

Table 2. In Vitro Resistance of Selected Periodontal Pathogens to Breakpoint Concentrations of Four Antibiotics

Species (No. of positive patients)	MET 16 mg/L ^a	AMOX 8 mg/L	CLIN 4 mg/L	DOX 4 mg/L
<u>Red complex species:</u>				
<i>P. gingivalis</i> (4)	0 ^b	0	0	0
<i>T. forsythia</i> (6)	0	1 (16.7)	1 (16.7)	0
<u>Orange complex species:</u>				
<i>P. intermedia/nigrescens</i> (13)	0	4 (30.8)	5 (38.5)	8 (61.5)
<i>P. micra</i> (20)	0	0	8 (40.0)	3 (15.0)
<i>C. rectus</i> (9)	0	0	0	0
<i>F. nucleatum</i> (18)	0	0	0	0
<i>S. constellatus</i> (1)	1 (100)	1 (100)	1 (100)	1 (100)

^a non-susceptible breakpoint concentration of antibiotic used in vitro.

^b No. (%) of organism-positive patient samples demonstrating in vitro resistance of organism to non-susceptible breakpoint concentration of antibiotic.

Key to table: MET = metronidazole, AMOX = amoxicillin, CLIN = clindamycin,

DOX = doxycycline

Metronidazole inhibited in vitro growth of all test periodontal pathogens except for one resistant strain of *S. constellatus*. In vitro resistance to amoxicillin was found for 30.8% of *P. intermedia/nigrescens* patient isolates, and one strain each of *T. forsythia* and *S. constellatus*. In vitro resistance to clindamycin was detected for 40.0% of *P. micra*, and 38.5% of *P. intermedia/nigrescens*, along with one strain each of *T. forsythia* and *S. constellatus*. In vitro resistance to doxycycline occurred for a majority of *P. intermedia/nigrescens* (61.5%), and to a lesser extent among *P. micra* (15.0%) isolates and one strain of *S. constellatus*.

At a study patient level, 1 (4.5%), 9 (40.9%), 6 (27.3%), and 11 (50.0%) of patients, had strains of red and/or orange complex periodontal pathogens resistant to either metronidazole, doxycycline, amoxicillin, or clindamycin, respectively.

Povidone-Iodine In Vitro Effects on Subgingival Biofilm Species

Total subgingival viable counts in subgingival biofilm patient specimens not exposed to povidone-iodine averaged 1.98 ± 0.08 (SE) $\log_{10} \times 10^6$ CFU per patient. In comparison, total subgingival viable counts in specimens exposed in vitro to 10% povidone-iodine averaged 0.63 ± 0.16 (SE) $\log_{10} \times 10^6$ CFU per patient, while those exposed in vitro to 5% povidone-iodine averaged 0.80 ± 0.14 (SE) $\log_{10} \times 10^6$ CFU per patient. In paired test-test analysis, subgingival specimens exposed in vitro to either povidone-iodine concentration yielded significantly lower total subgingival viable counts per patient than those not exposed to povidone-iodine ($P < 0.001$). However, no statistically significant differences in mean total subgingival viable counts were found for

samples exposed to 10% povidone-iodine as compared to 5% povidone-iodine ($P = 0.125$, paired t-test) (Figure 3).

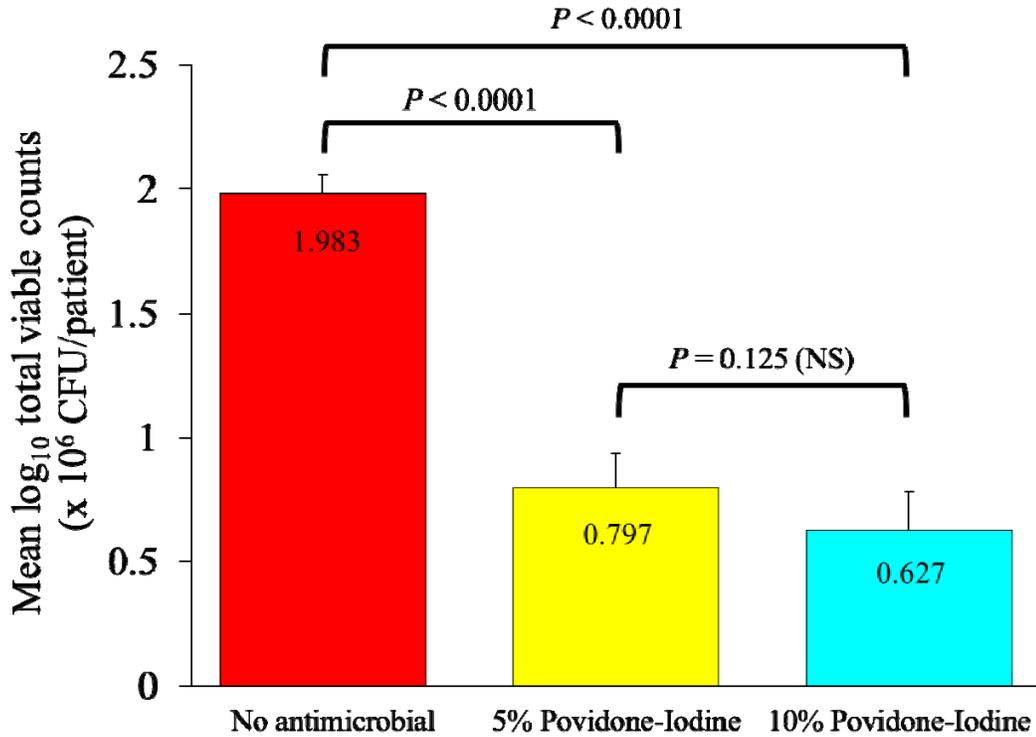


Figure 3. Average total viable counts in subgingival biofilm specimens exposed and not exposed in vitro to povidone-iodine.

All evaluated red and orange complex periodontal pathogens were suppressed below detection levels after 10% povidone-iodine in vitro exposure, except for one *F. nucleatum* strain in one patient specimen. As a result, 21 (95.5%) of the 22 patient subgingival biofilm samples were culture-negative for red and orange complex periodontal pathogens after 10% povidone-iodine in vitro exposure.

A similar suppression of red and orange complex periodontal pathogens was found following 5% povidone iodine in vitro exposure, where only one patient sample

yielded *P. intermedia/nigrescens*, and two other samples *F. nucleatum*, resulting in 19 (86.4%) of patient subgingival biofilm samples being devoid of red and orange complex periodontal pathogens after 5% povidone-iodine in vitro exposure.

Total subgingival proportions of red and orange complex periodontal pathogens in subgingival specimens exposed in vitro to 10% povidone-iodine averaged 0.5 ± 0.5 (SE) % per patient, and to 5% povidone-iodine 0.7 ± 0.3 (SE) % per patient. Both of these proportions were significantly lower than the mean 14.8% levels per patient found for red and orange complex periodontal pathogens in subgingival biofilms not exposed to povidone-iodine ($P < 0.0001$, paired t-test). However, no statistically significant differences in mean total subgingival proportions of red and orange complex periodontal pathogens were found between samples exposed to 10% povidone-iodine as compared to 5% povidone-iodine ($P = 0.743$, paired t-test) (Figure 4).

Figures 5 and 6 provide representative views of EBBA culture plates displaying the effects of in vitro 10% and 5% povidone iodine exposure on total viable microbial counts and red and orange complex periodontal pathogens in subgingival biofilm specimens from severe human periodontitis lesions.

The two subgingival biofilm samples were mixed with 0.05 ml of 3% sodium thiosulfate alone yielded virtually identical bacterial counts and recovered species as non-exposed sample dilutions (data not shown), indicating that sodium thiosulfate alone exerted negligible effects on in vitro subgingival biofilm growth.

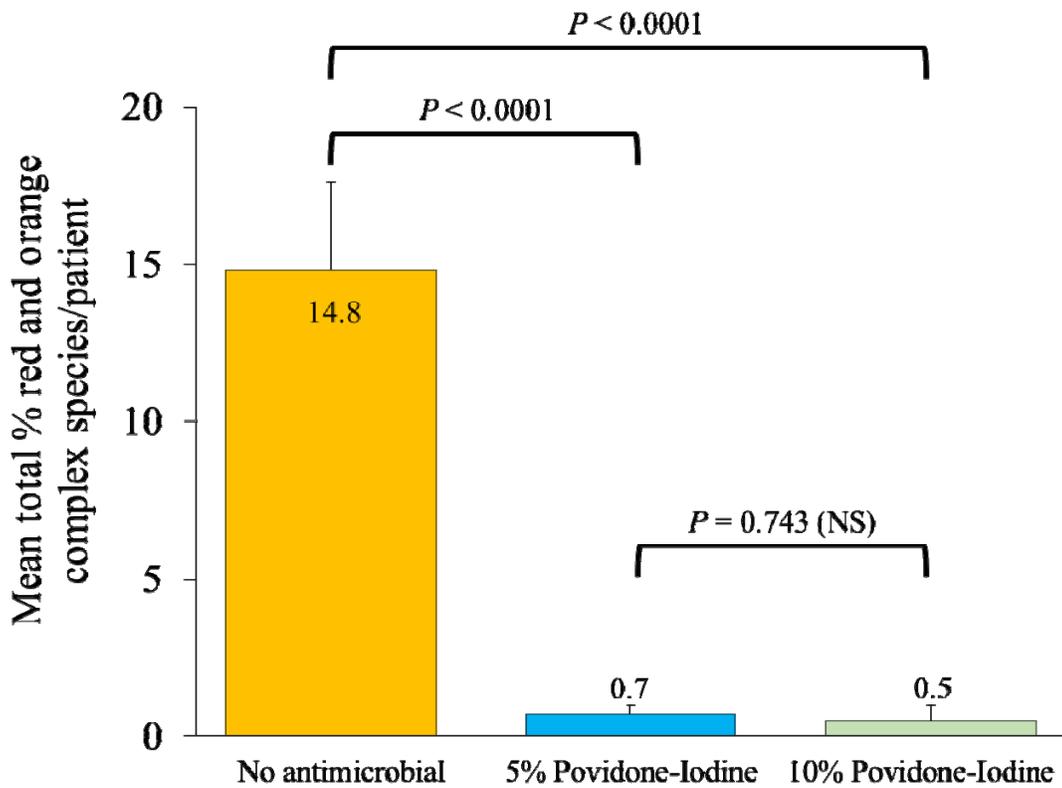


Figure 4. Average total proportions of red and orange complex periodontal pathogens in subgingival biofilm specimens exposed and not exposed in vitro to povidone-iodine. Paired t-tests assessed mean differences between subgingival specimen types. NS = not statistically significant.

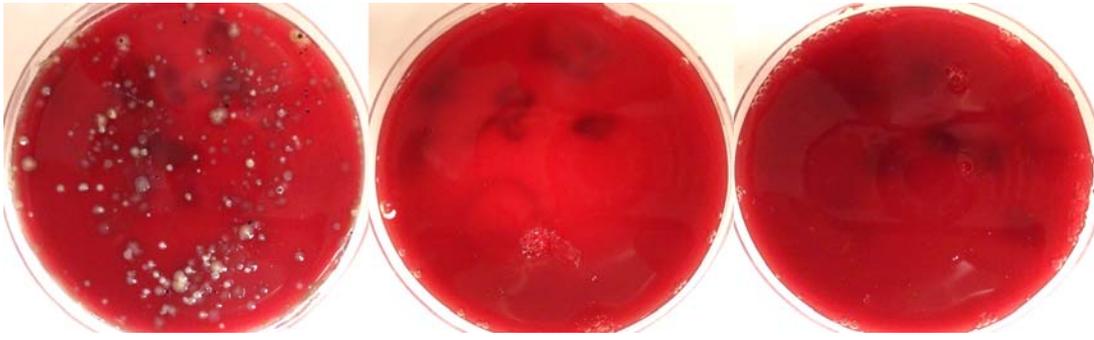


Figure 5. Example #1 of a subgingival biofilm specimen exposed and not exposed in vitro to povidone-iodine. Non-exposed specimen aliquot (left) yielded 2.8% *T. forsythia*, 14.8% *P. intermedia/nigrescens*, 10.2% *P. micra* (resistant in vitro to doxycycline), and 15.7% *F. nucleatum*. Specimen aliquots exposed to either 5% (center) or 10% povidone-iodine (right) revealed no growth.



Figure 6. Example #2 of a subgingival biofilm specimen exposed and not exposed in vitro to povidone-iodine. Non-exposed specimen aliquot (left) grew 0.7% *P. intermedia/nigrescens* (resistant in vitro to amoxicillin), 2.4% *P. micra* (resistant in vitro to clindamycin), 0.1% *C. rectus*, and 4.9% *F. nucleatum*. Specimen aliquots exposed to 5% povidone-iodine yielded only *Streptococcus oralis* (center), with only one *S. oralis* CFU recovered after 10% povidone-iodine exposure (right).

Povidone-Iodine Effects on Antibiotic-Resistant and Susceptible

Red and Orange Complex Periodontal Pathogens

All red and orange complex periodontal pathogens that were resistant in vitro to one or more of the test antibiotics (Table 2) were inhibited by 10% povidone-iodine in vitro exposure. Only one study patient sample yielded a *F. nucleatum* strain resistant to 10% povidone-iodine, but the strain did not exhibit in vitro resistance to any of the four test antibiotics.

Among subgingival biofilm specimens exposed in vitro to 5% povidone-iodine, one patient sample had a *P. intermedia/nigrescens* isolate resistant in vitro to 5% povidone-iodine, as well as to doxycycline, amoxicillin, and clindamycin. Two other patient samples each demonstrated a *F. nucleatum* strain resistant in vitro to 5% povidone-iodine, but not to any of the test antibiotics.

Overall, both 5% and 10% povidone-iodine were similarly active against red and orange complex periodontal pathogens which were resistant in vitro to breakpoint levels of doxycycline, amoxicillin, or clindamycin, as compared to antibiotic-susceptible clinical isolates.

Other Subgingival Biofilm Species Resistant In Vitro to Povidone-Iodine

Various *Streptococcus* species, particularly *Streptococcus oralis*, were the most frequently recovered microorganisms identified with MALDI-TOF mass spectrometry in subgingival biofilm specimens after 60-second in vitro exposure to povidone-iodine, indicative of their in vitro resistance to both 10% and 5% povidone-iodine (Table 3).

Table 3. Other Subgingival Biofilm Species Resistant In Vitro to Povidone-Iodine

Species:	No. of samples with in vitro resistant species	
	5% povidone-iodine	10% povidone-iodine
<i>Streptococcus</i> species	6	5
<i>Streptococcus oralis</i>	4	4
<i>Streptococcus mitis</i>	1	1
<i>Streptococcus salivarius</i>	1	1
<i>Streptococcus vestibularis</i>	1	2
<i>Streptococcus anginosus</i>	1	1
<i>Streptococcus intermedius</i>	4	0
<i>Staphylococcus</i> species	1	0
<i>Staphylococcus aureus</i>	1	0
<i>Actinomyces oris</i>	1	0
<i>Capnocytophaga granulosa</i>	1	0
<i>Cutibacterium acnes</i>	2	1

Among the 22 patient subgingival biofilm specimens exposed in vitro to 10% povidone-iodine, 8 (36.4%) exhibited no growth in the 10^{-6} sample dilution aliquot, and 14 (63.6%) yielded only various *Streptococcus* species (listed in Table 3). In subgingival samples exposed in vitro to 5% povidone-iodine, 5 (22.7%) exhibited no growth in the 10^{-6} sample dilution aliquot, and 9 (40.9%) yielded only various *Streptococcus* species (listed in Table 3). Other organisms resistant in vitro to 5% povidone-iodine and

appearing in the remaining 8 subgingival biofilm samples included *Staphylococcus* species, *Staphylococcus aureus*, *Actinomyces oris*, *Capnocytophaga granulosa*, and *Cutibacterium acnes*.

CHAPTER 4

DISCUSSION

The major findings from this study are that povidone-iodine exerted marked antimicrobial effects against total viable counts, and against clinical isolates of red and orange complex periodontal pathogens, in subgingival biofilm specimens from severe periodontitis patients after only a brief (60-second) in vitro contact time. Additionally, no statistically significant differences were found between 10% and 5% concentrations of povidone-iodine in their antimicrobial effects on subgingival microorganisms, with antibiotic-resistant and antibiotic-susceptible red and orange complex periodontal pathogens both equally susceptible to the in vitro povidone-iodine exposure. Among the 22 severe periodontitis subgingival specimens tested, 21 (95.5%) were culture-negative for red and orange complex periodontal pathogens after 10% povidone-iodine in vitro exposure, and 19 (86.4%) after 5% povidone-iodine in vitro exposure.

These findings of a profound antibacterial impact by povidone-iodine against microbial species associated with periodontitis are in agreement with prior in vitro studies (Caufield et al. 1987, Maruniak et al. 1992, Higashitsutsumi et al. 1993, Nakagawa et al. 2006, Bercy & Lasserre 2007, Hosaka et al. 2012), and a prior in vivo investigation of povidone-iodine subgingival irrigation into severe periodontitis lesions (Hoang et al. 2003). In the latter human study, subgingival irrigation with 10% povidone-iodine as a supplement to mechanical root debridement of deep periodontal pockets induced significantly greater probing depth reductions than conventional mechanical periodontal

therapy alone at five weeks post-treatment, and better reduced total cultivable periodontal pathogen microbiological counts (Huang et al. 2003).

However, relative to the present study findings, no prior studies have tested povidone-iodine on fresh subgingival clinical isolates from severe periodontal pockets without their subculture, as was done in the present study, nor have any prior studies evaluated the potential influence of antibiotic resistance among periodontal microorganisms on the antimicrobial effectiveness of povidone-iodine. The antimicrobial effects of povidone-iodine against antibiotic-resistant red and orange complex periodontal pathogens may facilitate reduction of bacterial biofilms in periodontal pockets as a potential source for antibiotic resistance genes in the human microbiome (Olsen 2015).

The finding of primarily *Streptococcus* species as the predominant bacteria surviving in cultivable subgingival biofilms after povidone-iodine in vitro exposure is also unique to the present study. It may be inferred that povidone-iodine introduction into severe periodontal sites may provide an ecologic advantage to post-treatment recolonization of periodontal pockets with streptococci of low to negligible periodontopathic potential (Slots 1977), and contribute to improved clinical periodontal outcomes.

A 60-second povidone-iodine in vitro exposure period was used in the present study to mimic the peak contact time of solutions in inflamed periodontal pockets prior to their rapid clearance by increased gingival crevicular fluid flow (Goodson 2003). This issue is particularly important to consider for antiseptics agents lacking substantivity for

oral tissues after their non-sustained delivery into periodontal pockets (Rams & Slots 1996), which applies to subgingival irrigation of povidone-iodine solutions.

Limitations of the present study need to be appreciated. Only laboratory-based testing of povidone-iodine against selected periodontal pathogens was conducted. It is not known if these in vitro findings will be paralleled if applied into an in vivo clinical situation. No detailed patient data was available on study patient gender, age, systemic health history, medications, smoking status, or their clinical and radiographic periodontal parameters. Only selected anaerobic red and orange complex periodontal pathogens were initially detected in the subgingival plaque biofilm specimens prior to povidone-iodine exposure, without assessment of additional periodontal pathogens were likely present. Only phenotypic criteria were employed to identify the targeted anaerobic red and orange complex periodontal pathogens, instead of potentially more precise molecular or biochemical methods. However, recent validation studies confirmed that the phenotypic methods used in the present study to identify *P. gingivalis* and *P. intermedia/nigrescens* clinical isolates highly correlate to species identification obtained via proteomic spectral fingerprinting of bacterial ribosomal protein profiles by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Rams et al. 2016, 2018).

Potential clinical application of povidone-iodine in periodontal therapy additionally requires clinical consideration of possible drug toxicity to the host, specifically to patient thyroid functions. Ferguson et al. (1978) found that two-minute mouth rinsing by humans with a 50% diluted (5%) povidone-iodine solution four times per day for two weeks led to significant adverse changes in thyroid function, including

the development of above-normal serum iodide levels. Another longitudinal human study of 5% povidone-iodine daily rinsing over a six-month time period reported significant systemic iodine absorption in the form of elevated serum total and inorganic iodine levels, and elevated urinary iodine excretion values, but no changes in serum T3 and T4 concentrations, and free T4 index levels (Ader et al. 1988). However, significant rise in serum TSH concentrations was measured during the povidone-iodine rinse period, with all values remained within a normal range (Ader et al. 1988). Because of these potential systemic risks to patients from povidone-iodine exposure, it is important to limit periodontal use of povidone-iodine to professionally-applied treatments in a dental office setting, where use of high-speed suction during periodontal pocket povidone-iodine applications can help reduce systemic adsorption of the antiseptic. The present study findings of a marked antimicrobial effect of 5% povidone-iodine, which was not significantly different from that attained by a 10% povidone-iodine solution, provide additional support for using a less concentrated povidone-iodine concentration in clinical settings, not only due to its similar antimicrobial activity as 10% povidone-iodine, but also because it more safely exposes the patient to a lower drug dose and lower potential systemic adsorption of povidone-iodine.

CHAPTER 5

CONCLUSIONS

Povidone-iodine exerted marked antimicrobial effects against both antibiotic-resistant and susceptible fresh clinical isolates of red and orange complex periodontal pathogens, and total viable counts, in subgingival biofilm specimens from severe periodontitis patients after only a brief (60-second) in vitro contact time, with no statistically significant differences found between 10% and 5% concentrations of povidone-iodine.

The profound suppression of red and orange complex periodontal pathogens, and concurrent predominance of periodontal health-associated *Streptococcus* species, that occurs in subgingival biofilm specimens following in vitro exposure to povidone-iodine, further supports the clinical use of povidone-iodine in periodontal therapy as an adjunct to mechanical root debridement in altering pathogenic subgingival microbial populations towards one compatible with periodontal health.

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