MATRIX-ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY VALIDATION OF A PERIODONTAL *PREVOTELLA INTERMEDIA/NIGRESCENS* IDENTIFICATION SCHEME.

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MASTER OF SCIENCE

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ABSTRACT

Objectives: Prevotella intermedia and *Prevotella nigrescens* are two genetically-distinct, gram-negative, anaerobic rods associated with the subgingival microbiome of human periodontitis. The two species are frequently isolated from subgingival dental plaque biofilms in chronic periodontitis patients clinically experiencing progressive destructive disease activity. In anaerobically-incubated liquid or solid culture media, *P. intermedia* and *P. nigrescens* exhibit nearly identical phenotypic properties, with regard to their colony morphology features and biochemical properties, which differ from other subgingival *Prevotella* and non-*Prevotella* microbial species. As a result, rapid differentiation and identification of *P. intermedia/nigrescens* group organisms from other bacterial species in anaerobically-cultivated subgingival dental plaque biofilms has been based upon examination of culture isolates for a dark-pigmented colony appearance, presence of brick-red autofluorescence of colonies to long-wave ultraviolet light exposure, and biochemical testing demonstrating a lack of colony lactose fermentation. However, the accuracy of this phenotypic-based identification scheme for periodontal *P. intermedia/nigrescens* group species has yet to be validated with a broad-based reference method that encompasses testing for a wide array of microbial species.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry and associated analytic software, is recently approved for clinical microbiology diagnostic use in the United States by the Food and Drug Administration, and is capable of definitively identifying 4,970 different microbial species based on mass spectra of their bacterial proteins. To date, no performance evaluation has been carried out comparing the phenotypic-based identification scheme for periodontal *P. intermedia/*

nigrescens group species with definitive MALDI-TOF mass spectrometry identification of the organisms.

As a result, the purpose of this study was to assess with MALDI-TOF mass spectrometry the accuracy of the rapid phenotypic-based periodontal *P. intermedia/ nigrescens* group species identification scheme widely utilized since 1986 by clinical periodontal microbiology laboratories and periodontal microbiology culture-based research studies.

Methods: 84 fresh subgingival cultivable isolates from 23 chronic periodontitis patients were presumptively identified on anaerobically-incubated enriched Brucella blood agar primary isolation plates as *P. intermedia/nigrescens* group species based on their dark-pigmented colony morphology, presence of brick-red autofluorescence under long-wave ultraviolet light, and a negative MUG fluorescence test for lactose fermentation activity. Each of the putative *P. intermedia/nigrescens* clinical isolates were subjected to MALDI-TOF mass spectrometry analysis using a bench top mass spectrometer, Bruker FlexControl 3.0 software, and MALDI Biotyper 3.1 software (Bruker Daltonics, Billerica, MA, USA), which contains mass spectra for *P. intermedia* and *P. nigrescens* in its reference library of bacterial protein profiles. A MALDI Biotyper log score of ≥ 1.7 was required for reliable taxonomic classification of the clinical isolates, with scores of ≥ 2.0 representing more definitive species identification.

Results: A total of 60 (71.4%) of the putative *P. intermedia/nigrescens* clinical isolates were reliably identified with MALDI-TOF mass spectrometry as either *P. intermedia* (25 isolates, with eight isolates exhibiting MALDI Biotyper log scores of $\geq$ 2.0), or *P. nigrescens* (35 isolates, with nine isolates exhibiting MALDI Biotyper log

scores of ≥ 2.0). Among the 24 putative *P. intermedia/nigrescens* clinical isolates generating MALDI Biotyper log scores < 1.7, indicating a less reliable species identification, only *P. intermedia* (14 isolates) or *P. nigrescens* (10 isolates) were listed by the analytic software as the first choice among the most likely bacterial species. No other bacterial species other than *P. intermedia* or *P. nigrescens* were identified by MALDI-TOF mass spectrometry for any of the 84 tested putative *P. intermedia/ nigrescens* clinical isolates.

Conclusions: These findings document, for the first time with MALDI-TOF mass spectrometry, the relative accuracy of a rapid phenotypic-based periodontal *P. intermedia/nigrescens* identification scheme based on dark-pigmented colony morphology, presence of brick-red long-wave ultraviolet light autofluorescence, and a negative MUG test for lactose fermentation activity. 100% of the 84 presumptive *P. intermedia/nigrescens* clinical isolates tested were identified with MALDI-TOF mass spectrometry, with varying levels of reliability, as being only either *P. intermedia* or *P. nigrescens*. These findings provide validation for the continued use of this rapid phenotypic identification scheme for periodontal *P. intermedia/nigrescens*.

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CHAPTER 1

INTRODUCTION

In the search to determine the etiology of human periodontitis, numerous clinical studies have been conducted over the past several decades to examine the role of various bacterial species in periodontitis initiation and progression. Concomitant with these additional insights into clinical periodontal microbiology have been changes in bacterial taxonomy, with markedly different classifications for many bacterial species relative to their most appropriate genus and species designations (Shah et al. 2009).

The process of re-classifying existing bacterial species into multiple new species is a particularly vexing issue relative to periodontal microbiology. Early efforts at relating the subgingival microbial flora with periodontal health and periodontitis failed to find markedly different bacterial communities outside of a finding of elevated spirochete morphotypes seen microscopically, and greater numbers of microorganisms classified at that time as *Bacteroides melaninogenicus* (Socransky et al. 1963). It was later realized that *B. melaninogenicus* was heterogeneous in its composition, and was comprised of several different microbial species, which are known today as *Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens*, *Prevotella melaninogenica*, and other *Prevotella* organisms.

In 1992, *Prevotella intermedia* and *Prevotella nigrescens* were recognized as two genetically-distinct, gram-negative, anaerobic rods associated with the subgingival microbiome of human periodontitis (Shah & Gharbia 1992). Prior to 1992, the two organisms were considered to be two genotypes under the designation of *P. intermedia*.

In order to rapidly identify *P. intermedia* clinical isolates, and differentiate them from other *Prevotella* and *Porphyromonas* species in periodontal culture studies, Slots (1986) proposed a three-part identification scheme. This presumptive *P. intermedia* identification scheme was employed in a number of important clinical periodontal studies involving considerable numbers of patients, clinical measurements, and treatment outcomes (Bragd et al. 1987, Slots & Listgarten 1988, Listgarten et al. 1993, Rams et al. 1996, 1997, 2014). However, with changes since 1986 in taxonomic classification and recognition of new *Prevotella* species, it is not certain that this long-utilized phenotypic/biochemical-based identification scheme for identification of periodontal *P. intermedia/nigrescens* is valid exclusively for *P. intermedia/nigrescens*, or additionally identifies other bacterial species with the same criteria.

In this regard, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, and associated analytic software for the definitive identification *P. intermedia* and *P. nigrescens* in clinical specimens, has been approved for clinical microbiology diagnostic use in the United States by the Food and Drug Administration. To date, no performance evaluations have been carried out comparing the phenotypic/biochemical-based *P. intermedia/nigrescens* identification scheme proposed by Slots (1986) with definitive MALDI-TOF mass spectrometry identification of *P. intermedia* and *P. nigrescens*.

As a result, the purpose of this study was to to assess with MALDI-TOF mass spectrometry the accuracy of the phenotypic-based periodontal *P. intermedia/nigrescens*

identification scheme first proposed by Slots (1986), and widely utilized since then in periodontal microbiology culture-based research studies.

CHAPTER 2

MATERIALS AND METHODS

Laboratory Facilities

All laboratory procedures were performed in the Oral Microbiology Testing Service (OMTS) Laboratory, located in Room 365-A of Building 600, which is part of the Temple University Maurice H. Kornberg School of Dentistry on the Temple University Health Sciences Center campus in Philadelphia, Pennsylvania. The OMTS Laboratory facilities are inspected and licensed by the Pennsylvania Department of Health for high-complexity bacteriological analysis - Clinical Laboratory Permit No. 021872 - in meeting the same proficiency and quality control standards required of hospital medical microbiology laboratories. The OMTS Laboratory is also federally-certified by the United States Department of Health and Human Services - CLIA Certificate No. 39D0707385 - to be in compliance with Clinical Laboratory Improvement Amendments (CLIA)-mandated proficiency testing, quality control, patient test management, personnel requirements, and quality assurance standards required of clinical laboratories engaged in diagnostic testing of human specimens in the United States (Rauch & Nichols, 2007). All culture media preparation, specimen inoculation, and plate evaluations were carried out in a standardized fashion by the same OMTS Laboratory staff personnel for all of the study bacterial strains. Since the data for the present study was obtained from existing OMTS Laboratory bacterial strains that were otherwise being discarded, and was not obtained through intervention or interaction with living individuals, or through 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 (Department of Health and Human Services 2004).

Test Bacterial Strains

A total of 84 fresh subgingival cultivable isolates of *P. intermedia/nigrescens* from 23 chronic periodontitis patients, each recovered during the spring of 2015 by the OMTS Laboratory as part of their commercial diagnostic microbiology testing services and otherwise being discarded, were utilized in the present study.

The *P. intermedia/nigrescens* clinical isolates were recovered from subgingival microbial samples obtained by extramural periodontists in private dental practice settings. The periodontists were instructed by the OMTS Laboratory, as part of its standard sampling procedure recommendations, to remove supragingival plaque from 3-5 periodontal sites per patient exhibiting moderate (5-6 mm) to deep periodontal probing depths (≥ 7 mm) and gingival inflammation, and to isolate 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 advanced with sterile forceps into each isolated periodontal site for approximately 10 seconds in order to collect subgingival plaque specimens 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 prereduced, anaerobically sterilized and stored Möller's VMGA III transport media (Möller 1966), which possesses a high preservation capability for oral microorganisms during transit after sampling to the laboratory (Möller 1966, Dahlén et al. 1989, Dahlén et al. 1993).

The collected pooled subgingival plaque samples were then transported to the OMTS Laboratory via overnight delivery services for processing within 24 hours.

Upon arrival at the OMTS Laboratory, the VMGA III vials were warmed to 35ºC for 10 minutes in order to liquefy the gelatin in the VMGA III transport medium. The sampled plaque organisms 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 prereduced, anaerobically sterilized 0.25% tryptose-0.25% thiotone E peptone-0.5% NaCl (Möller 1966). Using a sterile bent glass rod, 0.1 ml aliquots of appropriate dilutions were plated onto pre-reduced, enriched Brucella blood agar (EBBA), comprised of 4.3% Brucella agar (BBL Microbiology Systems, Cockeysville, MD) supplemented with 0.3% bacto-agar, 5% defibrinated sheep blood, 0.2% hemolyzed sheep red blood cells, 0.0005% hemin, and 0.00005% menadione (Slots et al. 1988). EBBA plates inoculated with 10^{-5} to 10^{-6} specimen dilutions were incubated at 37ºC in a 25-cubic foot upright heated incubator (Caron, Marietta, OH USA) for 7 days in anaerobic jars containing an 85% N_2-10% H_2-5% CO_2 atmosphere introduced by an Anoxomat™ Mark II automatic jar evacuation-replacement system (Advanced Instruments, Inc., Norwood, MA USA) (Brazier & Smith 1989).

Phenotypic Identification of Periodontal *P. intermedia/nigrescens*

After incubation, EBBA plates were visually examined with a 2.25x ring-light Luxo Taskmaster magnifying loupe (Lighting Specialists, Buffalo, Grove, IL, USA), and an Olympus SZX2 dissecting research stereomicroscope (Olympus America, Center

Valley, PA, USA) with a Fostec Ace I fiberoptic light source. *P. intermedia/nigrescens* was presumptively identified as gram-negative, non-motile, anaerobic rods exhibiting circular, dome-shaped, dark-pigmented (black to brown), raised surfaces colonies (Figure 1), which displayed an autofluorescent brick-red color (Figure 2) under long-wave ultraviolet light exposure with a Wood's lamp at a wavelength of 365 nm (Slots & Reynolds 1982), and had a negative MUG fluorescence test for lactose fermentation activity (Alcoforado et al. 1987).

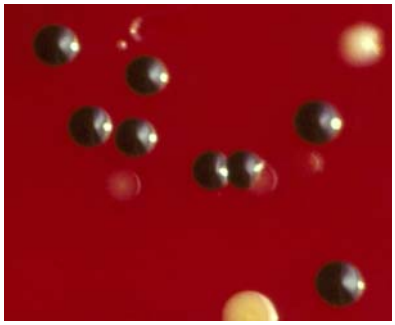

Figure 1. Colony appearance of *P. intermedia/nigrescens* (black colonies) on EEBA primary isolation plate.

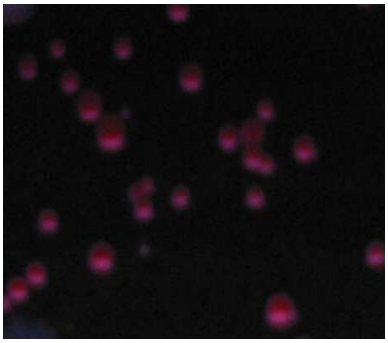

Figure 2. Autofluorescent brick-red color of *P. intermedia/nigrescens* clinical isolates under long-wave ultraviolet light on EEBA primary isolation plate.

MUG testing was performed using a Thomas Micro Atomizer (Thomas Scientific, Philadelphia, PA, USA) to spray a 1.0% concentration of the fluorogenic compound 4-methylubelliferyl-β-D-galactoside (MUG) (Sigma Chemical Co., St. Louis, MO USA) dissolved in dimethyl sulfoxide (Figure 3).

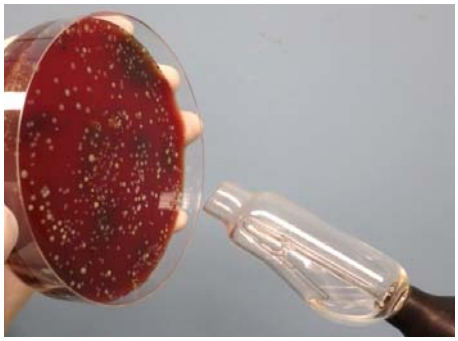

Figure 3. Application of MUG reagent with micro atomizer to colonies of dark-pigmented subgingival clinical isolates on EEBA primary isolation plate.

Because bacterial lactose fermentation is dependent upon the action of the enzyme β-galactosidase, the MUG reagent forms 4-methylumbelliferone, which is brightly fluorescent under long-wave ultraviolet light, when it is hydrolyzed by β-galactosidase produced by lactose-positive bacterial species (Alcoforado et al. 1987). *P. intermedia* and *P. nigrescens* are MUG fluorescent test-negative, since they do not ferment lactose, in contrast to MUG fluorescent-positive dark-pigmented *Prevotella* species such as *P. melaninogenica*, *Prevotella loeschii,* and *Prevotella denticola* (Alcoforado et al. 1987). A positive MUG test was found when dark-pigmented EBBA surface colonies suspected of belonging to the *Prevotella* genus exhibited a bright blue fluorescence after being sprayed with the 1% MUG test reagent and exposed to a Wood's lamp at a wavelength of 365 nm in a darkroom. The absence of such a bright blue

fluorescence reaction among dark-pigment anaerobic rod colonies also yielding an autofluorescent brick-red color under long-wave ultraviolet light exposure with a Wood's lamp at a wavelength of 365 nm were used to presumptively identify clinical isolates on EBBA primary isolation plates as *P. intermedia/nigrescens*. No additional phenotypic, biochemical or genetic characterization of the presumptive *P. intermedia/nigrescens* clinical isolates was performed.

Control Bacterial Strains

A manufacturer-recommended bacterial test standard (BTS), comprised of the gram-negative, facultative rod *Escherichia coli*, was prepared according to manufacturer instructions, and employed as a positive test control verifying proper MALDI-TOF mass spectrometry analysis. Negative controls in the MALDI-TOF mass spectrometry analysis included clinical strains of *P. gingivalis*, *P. melaninogenica*, *P. denticola*, *Eubacterium brachy*, *Fusobacterium nucleatum*, and *Parvimonas micra*, which were recovered from severe chronic periodontitis subjects by the OMTS Laboratory, using similar subgingival sampling, microbial transport, and culture methods as were employed for recovery of periodontal *P. intermedia/nigrescens*.

MALDI-TOF Mass Spectrometry Identification of Periodontal *P. intermedia/nigrescens*

Each of the putative *P. intermedia/nigrescens* clinical isolates, along with manufacturer-recommended BTS and the non-*P. intermedia/nigrescens* control bacterial strains, were subjected to MALDI-TOF mass spectrometry analysis using a Bruker Microflex LT bench top mass spectrometer (Bruker Daltonics, Billerica, MA, USA),

Bruker FlexControl 3.0 software, and MALDI Biotyper 3.1 software (Bruker Daltonics, Billerica, MA, USA).

Using a sterile toothpick, a single colony of each test and control bacterial strain was smeared onto the surface of a polished steel MALDI-TOF mass spectrometry target plate into an individual circular spot, and allowed to dry at room temperature. Then, a 1.0 µl overlay of a 98% formic acid solution was placed and allowed to air dry over the colony smears to facilitate on-plate extraction of cellular 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, which was prepared following manufacturer's instructions, and also allowed to dry at room temperature. The manufacturer-recommended BTS control was also spotted onto the MALDI-TOF mass spectrometry target plate and overlaid with the formic acid and matrix solutions, similar to test and control bacterial strains. Other control spots contained only the dried matrix solution without any bacterial specimen, and one without anything on it.

After insertion of the prepared target plate into the Bruker Microflex LT bench top mass spectrometer, mass spectra for each spotted bacterial isolate was acquired with the instrument in a linear positive mode within a 2-20 kDa range, with ion source 1.0 at 20 kV, ion source 2.0 at 18.05 kV, the lens at 6.0 kV, and the linear detector at 2,560 V. Each mass spectra was analyzed and compared with the MALDI Biotyper 3.1 software database, comprised of 4,970 distinct bacterial species, to determine the most likely microbial genus and 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). Log scores of ≥ 2.0 were considered to represent more definitive species identification. Scores of < 1.7 were considered to provide less reliable bacterial identification.

Data Analysis

Data analysis was carried out by tabulating the distribution of microbial genus and species identification provided by the MALDI-TOF mass spectrometry analysis for Biotyper log score levels of ≥ 2.0, ≥ 1.7, and < 1.7 among the tested presumptive *P. intermedia/nigrescens* clinical isolates.

CHAPTER 3

RESULTS

Control Bacterial Strains

The manufacturer-recommended BTS control species was definitively identified as *E. coli* in duplicate MALDI-TOF mass spectrometry test runs, with MALDI Biotyper scores of 2.178 and 2.303, indicating appropriate performance of the MALDI-TOF mass spectrometry instrumentation and analytical software.

The negative control strains of *P. gingivalis*, *P. melaninogenica*, *P. denticola*, *E. brachy*, *F. nucleatum*, and *P. micra* were reliably identified with MALDI Biotyper scores of ≥ 1.7. The control spot containing only the dried matrix solution without any bacterial specimen, and the one left empty, did not give any mass spectra peaks or bacterial identification.

Test Bacterial Strains

A total of 60 (71.4%) of 84 putative *P. intermedia/nigrescens* clinical isolates were reliably identified (log score ≥ 1.7) with MALDI-TOF mass spectrometry. A total of 17 of the 60 reliably identified clinical isolates exhibited log scores of ≥ 2.0, which indicates a more definitive species identification of the clinical isolate. All of the 60 putative *P. intermedia/nigrescens* clinical isolates reliably identified by the Biotyper software database were classified as being either *P. intermedia* (25 isolates, with 8 having log scores of ≥ 2.0), or *P. nigrescens* (35 isolates, with 9 having log scores of ≥ 2.0) (Table 1).

Table 1. Bacterial identification of 60 presumptive periodontal *P. intermedia/nigrescens* clinical isolates with MALDI Biotyper scores of ≥ 1.7

Clinical isolate	MALDI Biotyper score	Species identification
15-56a	2.086	*P. intermedia*
15-56b	2.152	*P. intermedia*
15-54	1.873	*P. nigrescens*
15-58c	1.911	*P. nigrescens*
15-58d	1.856	*P. nigrescens*
15-58e	1.971	*P. nigrescens*
15-58f	1.837	*P. nigrescens*
15-58g	2.024	*P. nigrescens*
15-58g	1.883	*P. nigrescens*
15-59	1.880	*P. intermedia*
15-60b	2.055	*P. intermedia*
15-60c	2.142	*P. intermedia*
15-60d	2.106	*P. intermedia*
15-60e	2.003	*P. intermedia*
15-60f	1.872	*P. intermedia*
15-60g	1.919	*P. intermedia*

15-60h	1.861	*P. intermedia*
15-60i	1.941	*P. intermedia*
15-60j	1.778	*P. intermedia*
15-61b	1.946	*P. intermedia*
15-61d	1.743	*P. intermedia*
15-61e	1.875	*P. intermedia*
15-61f	1.795	*P. intermedia*
15-61g	1.848	*P. intermedia*
15-61h	1.947	*P. nigrescens*
15-61i	1.904	*P. intermedia*
15-61j	2.057	*P. intermedia*
15-61k	2.115	*P. intermedia*
15-61l	1.742	*P. intermedia*
15-61m	1.781	*P. intermedia*
15-61n	1.736	*P. intermedia*
15-63a	1.883	*P. intermedia*
15-63b	1.859	*P. intermedia*
15-66	1.974	*P. nigrescens*
15-90	1.830	*P. nigrescens*
15-92a	2.179	*P. nigrescens*
15-92b	1.981	*P. nigrescens*

15-92c	2.211	*P. nigrescens*
15-92d	1.784	*P. nigrescens*
15-92e	1.783	*P. nigrescens*
15-92f	1.822	*P. nigrescens*
15-92g	2.119	*P. nigrescens*
15-92h	2.027	*P. nigrescens*
15-92i	1.874	*P. nigrescens*
15-92j	2.168	*P. nigrescens*
15-92k	1.851	*P. nigrescens*
15-92l	2.074	*P. nigrescens*
15-92m	1.947	*P. nigrescens*
15-96a	1.983	*P. nigrescens*
15-96b	2.003	*P. nigrescens*
15-96c	1.888	*P. nigrescens*
15-98a	1.755	*P. nigrescens*
15-98b	1.867	*P. nigrescens*
15-98c	1.841	*P. nigrescens*
15-98d	1.757	*P. nigrescens*
15-118a	2.004	*P. nigrescens*
15-125a	1.788	*P. nigrescens*
15-125b	1.956	*P. nigrescens*

15-135	1.786	*P. nigrescens*
15-151	1.974	*P. nigrescens*

Another 24 (28.6%) of the 84 putative *P. intermedia/nigrescens* clinical isolates had log scores < 1.7, indicating less reliable species identification (Table 2). However, the only species listed by the MALDI Biotyper analytic software as the first choice among the most likely bacterial species for these clinical isolates were *P. intermedia* (14 isolates) and *P. nigrescens* (10 isolates).

Table 2. Bacterial identification of 24 presumptive periodontal *P. intermedia/nigrescens* clinical isolates with MALDI Biotyper scores of < 1.7

Clinical isolate	MALDI Biotyper score	First choice listed by Biotyper software of most likely species
15-58a	1.556	*P. nigrescens*
15-58b	1.562	*P. nigrescens*
15-60a	1.562	*P. intermedia*
15-601	1.613	*P. nigrescens*
15-602	1.641	*P. nigrescens*
15-603	1.600	*P. intermedia*
15-604	1.646	*P. intermedia*

15-611	1.586	*P. intermedia*
15-612	1.604	*P. intermedia*
15-613	1.520	*P. intermedia*
15-614	1.548	*P. intermedia*
15-615	1.529	*P. intermedia*
15-616	1.604	*P. intermedia*
15-617	1.542	*P. intermedia*
15-62	1.514	*P. intermedia*
15-94a	1.578	*P. nigrescens*
15-94b	1.555	*P. nigrescens*
15-94c	1.612	*P. nigrescens*
15-135b	1.527	*P. nigrescens*
15-137	1.632	*P. intermedia*
15-138	1.647	*P. nigrescens*
15-139	1.594	*P. intermedia*
15-140	1.684	*P. intermedia*
15-152	1.382	*P. nigrescens*

An example of a mass spectra typical of subgingival isolates reliably identified as *P. intermedia* in MALDI-TOF mass spectrometry analysis is presented in Figure 4.

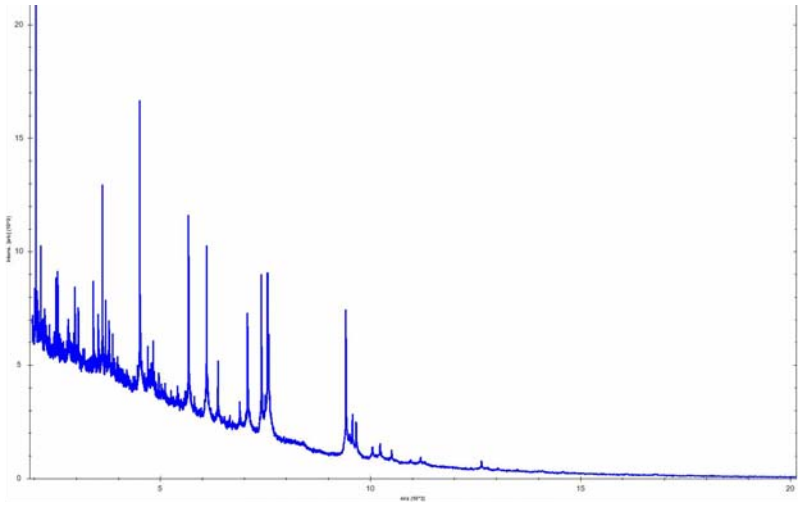

Figure 4. Mass spectra for presumptive *P. intermedia/nigrescens* clinical isolate identified as *P. intermedia* by MALDI-TOF mass spectrometry analysis.

An example of proteomic spectral fingerprinting performed by Biotyper analytic software resulting in a match of the above *P. intermedia* clinical isolate mass spectra to the most appropriate species present in the software database of bacterial species-specific spectra is presented in Figure 5.

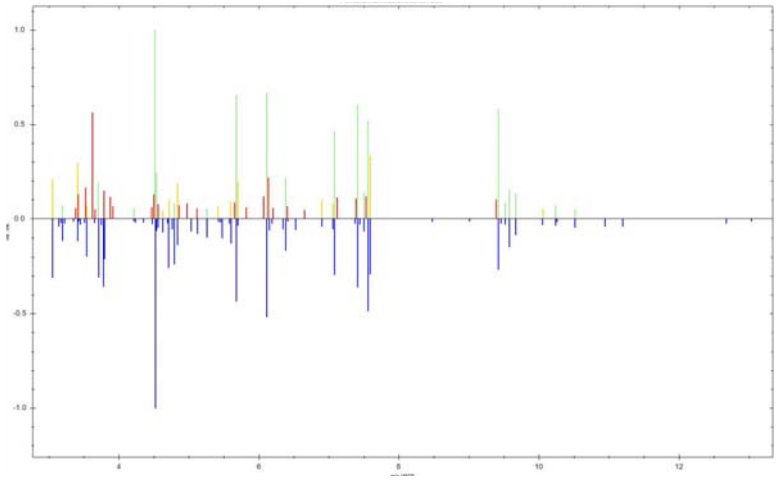

Figure 5. Peaks of mass spectra for presumptive *P. intermedia/nigrescens* clinical isolate (top, with green peaks representing a match) matched to peaks of mass

spectra of *P. intermedia* (bottom) in Biotyper reference library.

An example of a mass spectra typical of subgingival isolates reliably identified as *P. nigrescens* in MALDI-TOF mass spectrometry analysis is presented in Figure 6.

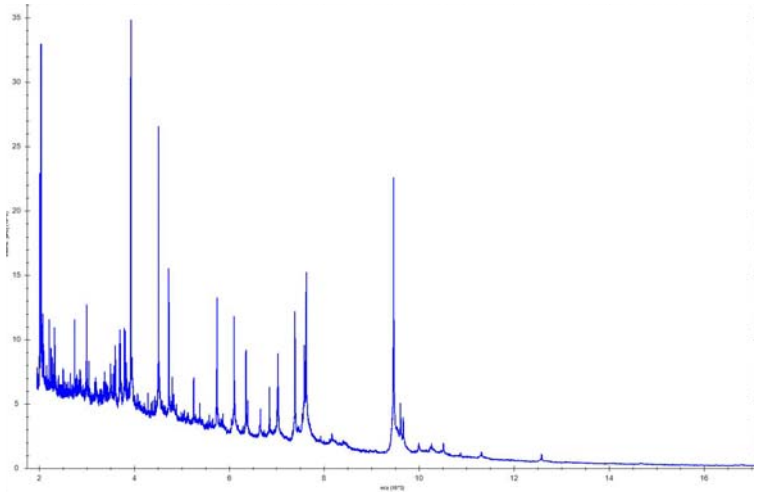

Figure 6. Mass spectra for presumptive *P. intermedia/nigrescens* clinical isolate identified as *P. nigrescens* by MALDI-TOF mass spectrometry analysis.

An example of proteomic spectral fingerprinting performed by Biotyper analytic software resulting in a match of the above *P. nigrescens* clinical isolate mass spectra to the most appropriate species present in the software database of bacterial species-specific spectra is presented in Figure 7.

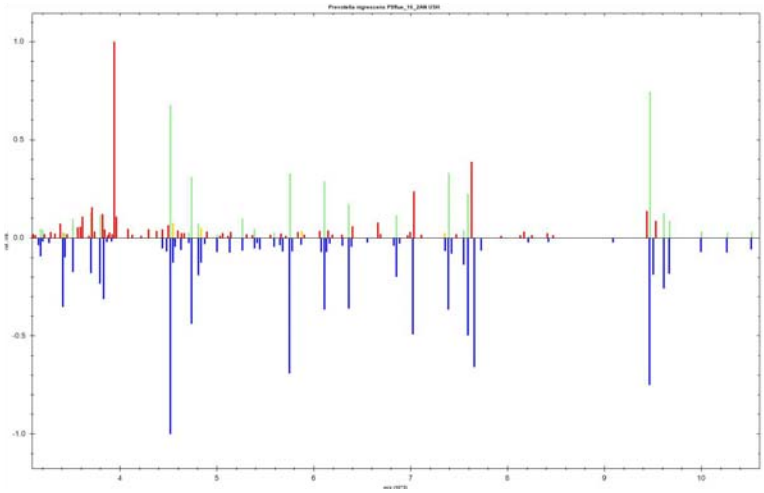

Figure 7. Peaks of mass spectra for presumptive *P. intermedia/nigrescens* clinical isolate (top, with green peaks representing a match) matched to peaks of mass spectra of *P. nigrescens* (bottom) in Biotyper reference library.

No other bacterial species other than *P. intermedia* or *P. nigrescens* were identified by MALDI-TOF mass spectrometry for any of the 84 tested putative *P. intermedia/nigrescens* clinical isolates.

CHAPTER 4

DISCUSSION

These study findings document, for the first time with MALDI-TOF mass spectrometry, the relative accuracy of the rapid phenotypic periodontal *P. intermedia/ nigrescens* identification scheme of Slots (1986), which is based upon the finding of a dark-pigmented colony morphology, presence of a brick-red autofluorescence of the dark-pigmented colony under long-wave ultraviolet light, and a negative MUG fluorescence test for lactose fermentation.

The major study finding is that 100% of 84 presumptive *P. intermedia/nigrescens* clinical isolates were identified with MALDI-TOF mass spectrometry, with varying levels of reliability, as being only either *P. intermedia* or *P. nigrescens*. A total of 60 (71.4%) of 84 putative *P. intermedia/nigrescens* clinical isolates were reliably identified (log score ≥ 1.7) with MALDI-TOF mass spectrometry as being either *P. intermedia* (25 isolates, with 8 having log scores of ≥ 2.0), or *P. nigrescens* (35 isolates, with 9 having log scores of ≥ 2.0.

A total of 24 putative *P. intermedia/nigrescens* clinical isolates had log scores < 1.7, indicating less reliable species identification. However, the only species listed by the analytic software as the first choice among the most likely bacterial species were either *P. intermedia* (14 isolates) or *P. nigrescens* (10 isolates).

It is not clear why between one-fourth and one-third of the presumptive *P. intermedia/nigrescens* clinical isolates had less reliable Biotyper log scores. One possible source for diminished reliability may have been the EBBA primary isolation plates from

where the presumptive *P. intermedia/nigrescens* were harvested for evaluation by MALDI-TOF mass spectrometry. Due to the heavy mixed bacterial populations on many of the plates, it is possible that when presumptive *P. intermedia/nigrescens* colonies were being picked for smearing on the MADI-TOF polished steel target plate, cells from other microorganisms unrelated to *P. intermedia/nigrescens* may have been mistakenly included in the tooth pick sample because of their close proximity or overlying growth. This would serve to reduce the reliability of the MALDI-TOF mass spectrometry identification as compared to pure colonies or cells of *P. intermedia/nigrescens*. Additional research is needed to ascertain the source of the uncertainty for the subset of tested *P. intermedia/nigrescens* clinical isolates that demonstrated less reliable MALDI-TOF identification log scores. Nevertheless, it is important to note that in all cases where the MALDI-TOF log score was < 1.7, no other bacterial species outside of *P. intermedia* or *P. nigrescens* was listed by the MALDI-TOF mass spectrometry as the most likely species identification.

These findings give renewed confidence in the accuracy and specificity of prior periodontal microbiology data on *P. intermedia/nigrescens* (Bragd et al. 1987, Slots & Listgarten 1988, Listgarten et al. 1993, Rams et al. 1996, 1997, 2014), and strongly suggests the continued usefulness of such data in periodontal disease research.

CHAPTER 5

CONCLUSIONS

These findings document, for the first time with MALDI-TOF mass spectrometry, the relative accuracy of a rapid phenotypic-based periodontal *P. intermedia/nigrescens* identification scheme based on dark-pigmented colony morphology, presence of brick-red long-wave ultraviolet light autofluorescence, and a negative MUG test for lactose fermentation activity. 100% of the 84 presumptive *P. intermedia/nigrescens* clinical isolates tested were identified with MALDI-TOF mass spectrometry, with varying levels of reliability, as being only either *P. intermedia* or *P. nigrescens*. These findings provide validation for the continued use of this rapid phenotypic identification scheme for periodontal *P. intermedia/nigrescens*.

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