CULTIVABLE *FUSOBACTERIUM* SPECIES IN CHRONIC PERIODONTITIS MICROBIOTA IDENTIFIED WITH MATRIX-ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY.

A Thesis
Submitted to
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MASTER OF SCIENCE

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by
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ABSTRACT

Objectives: Fusobacteria are prominent participants in the maturation of subgingival dental plaque biofilms in humans. A number of various species belonging to the *Fusobacterium* genus have been recovered from the subgingival microbiota of chronic periodontitis patients. However, conventional *Fusobacterium* species identification is labor-intensive, time-consuming, and complicated by shortcomings in phenotypic-based classification schemes, where many fusobacteria display overlapping and non-distinguishing morphologic features and biochemical properties. In addition, molecular identification of fusobacteria is plagued with difficulties of validating the specificity of nucleic acid probes and primers to various *Fusobacterium* species that have closely-related interspecies genetic profiles.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, and its associated analytic software, was recently approved for clinical microbiology diagnostic use by the United States Food and Drug Administration. MALDI-TOF mass spectrometry has the potential to rapidly identify cultivable clinical isolates to a species level for 4,970 different bacterial species based on mass spectra of their bacterial protein profiles, including many *Fusobacterium* species.

The purpose of this study was to use MALDI-TOF mass spectrometry to rapidly identify the patient distribution of fusobacteria isolated from the subgingival microbiota of chronic periodontitis patients.

Methods: A total of 34 chronic periodontitis patients provided 96 fresh subgingival cultivable fusobacteria isolates (one to seven isolates per patient), which were presumptively identified by their chartreuse-positive colony autofluorescence under

long-wave ultraviolet light on anaerobically-incubated, non-selective, enriched Brucella blood agar primary isolation plates. Each of the presumptive fusobacteria 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 a variety of fusobacteria in its reference library of bacterial protein profiles. Each clinical isolate underwent on-target plate formic acid protein extraction, and was taxonomically classified with MALDI-TOF mass spectrometry within an approximately 30-45 minute time period from the point of colony harvesting from primary isolation culture plates. A MALDI Biotyper log score of ≥ 1.7 was required for reliable taxonomic classification of the clinical fusobacteria isolates.

Results: A majority (58.8%) of the chronic periodontitis patients yielded two or three different species of subgingival *Fusobacterium* on non-selective enriched Brucella blood agar primary isolation plates. *Fusobacterium naviforme* was identified by MALDI-TOF mass spectrometry analysis in 14 (41.2%) chronic periodontitis study patients, *Fusobacterium nucleatum* subspecies *vincentii* in 13 (38.2%) patients, *Fusobacterium nucleatum* subspecies *polymorphum* in 9 (26.5%) patients, *Fusobacterium nucleatum* and *Fusobacterium* species each in 6 (17.6%) patients, *Fusobacterium nucleatum* subspecies *nucleatum* in 4 (11.8%) patients, and *Fusobacterium nucleatum* subspecies *animalis* in 3 (8.8%) patients. Three patients additionally yielded subgingival isolates of *Fusobacterium canifelinum*, normally an inhabitant of the oral cavity of dogs and cats. 52 (54.2%) of the fusobacteria clinical isolates revealed MALDI Biotyper log scores of ≥ 1.7, the threshold for reliable taxonomic classification, while in comparison,

44 (45.8%) had log scores < 1.7, indicating a less reliable species identification. No other microbial species, other than one of the *Fusobacterium* species, was listed by the MALDI-TOF mass spectrometry analytic software as the most likely organism for the tested clinical isolates.

Conclusions: These findings indicate that a variety of *Fusobacterium* species may be rapidly identified with MALDI-TOF mass spectrometry in the subgingival microbiota of chronic periodontitis patients. *F. naviforme* and *F. nucleatum* subspecies *vincentii* were the most frequently isolated subgingival fusobacteria species in the evaluated study patients. Three chronic periodontitis patients also unexpectedly revealed subgingival isolates of the animal species *F. canifelinum*, which is normally in the oral cavity of dogs and cats. MALDI-TOF mass spectrometry may facilitate rapid identification of cultivable fusobacteria in human subgingival dental plaque biofilms, and enhance understanding of bacterial community structure in periodontal pockets.

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CHAPTER 1

INTRODUCTION

Fusobacteria in human periodontal pockets are gram-negative, non-spore forming, non-motile, obligate anaerobic, spindle-shaped, bacterial rods that produce butyric acid as a major fermentation product from their metabolism of glucose and peptone (Bolstad et al. 1996). Members of the *Fusobacterium* genus that colonize the human oral cavity are taxonomically comprised of a variety of closely related and phenotypically-similar bacterial species (Moore & Moore 1994), including *Fusobacterium nucleatum*, its recognized subspecies *nucleatum*, *polymorphum*, *fusiforme*, *vincentii* and *animalis* (Dzink et al. 1990, Gharbia & Shah 1992), *Fusobacterium periodonticum* (Gmür et al. 2008), *Fusobacterium gonidiaformans*, *Fusobacterium naviforme*, *Fusobacterium necrophorum*, and *Fusobacterium* species oral taxons 203, 205, and 370 (Dewhirst et al. 2010).

Fusobacterium species are frequently found as a constituent of human dental plaque biofilms, both in low levels in the presence of periodontal health, and in higher subgingival proportions in persons with gingivitis and periodontitis (Moore et al. 1985). *F. nucleatum* was classified in a cluster analysis of 13,261 subgingival dental plaque samples in 185 humans as one of the "orange complex" bacterial species moderately associated with severe periodontal attachment loss (Socransky et al. 1998). In one longitudinal clinical study, *Fusobacterium* species are reported as the most frequently detected cultivable subgingival dental plaque bacteria in human disease-active periodontitis sites undergoing marked progression of clinical periodontal attachment loss,

as compared to clinically-quiescent and stable periodontal sites (Dzink et al. 1988). More recent molecular-based clinical studies employing 16S rRNA gene amplicon sequencing also reveal fusobacteria to be abundant in the subgingival microbiota of periodontitis patients who smoke (Bizzarro et al. 2013).

Fusobacterium species also play a key role in the maturation of dental plaque biofilms by providing binding sites for interbacterial adherence or "bridging" between various dental plaque bacterial species not capable of direct co-aggregation with each other (Nagayama et al. 2001). Moreover, *Fusobacterium* species have the capability to enhance attachment of the major periodontal bacterial pathogen, *Porphyromonas gingivalis*, to human fibroblasts (Metzger et al. 2009), which may contribute to the pathogenesis of periodontal tissue destruction.

Conventional *Fusobacterium* species identification from cultivation is labor-intensive, time-consuming, and complicated by shortcomings in phenotypic-based classification schemes, where many fusobacteria display overlapping and non-distinguishing morphologic features and biochemical properties (Shah et al. 2009). In addition, molecular identification of fusobacteria is plagued with difficulties of validating the specificity of nucleic acid probes and primers to various *Fusobacterium* species that have closely-related interspecies genetic profiles (Park et al. 2013).

As an alternative, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, and its associated analytic software, was recently approved for clinical microbiology diagnostic use by the United States Food and Drug Administration. MALDI-TOF mass spectrometry has the potential to rapidly identify

cultivable clinical isolates to a species level for 4,970 different bacterial species based on mass spectra of their bacterial protein profiles, including many *Fusobacterium* species.

The purpose of this study was to use MALDI-TOF mass spectrometry to rapidly identify the patient distribution of fusobacteria isolated from the subgingival microbiota of chronic periodontitis patients.

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 96 fresh subgingival cultivable isolates of presumptive fusobacteria from 34 chronic periodontitis patients (one to seven isolates per patient), each recovered during the spring of 2015 by the OMTS Laboratory as part of their microbiology testing services and otherwise being discarded, were utilized in the present study.

The fusobacteria 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 Fusobacteria

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. Fusobacteria was presumptively identified by their chartreuse-positive (pale yellow-green) colony autofluorescence (Figure 1) under long-wave ultraviolet light exposure with a Wood's lamp (Figure 2) at a wavelength of 365 nm (Brazier 1986).

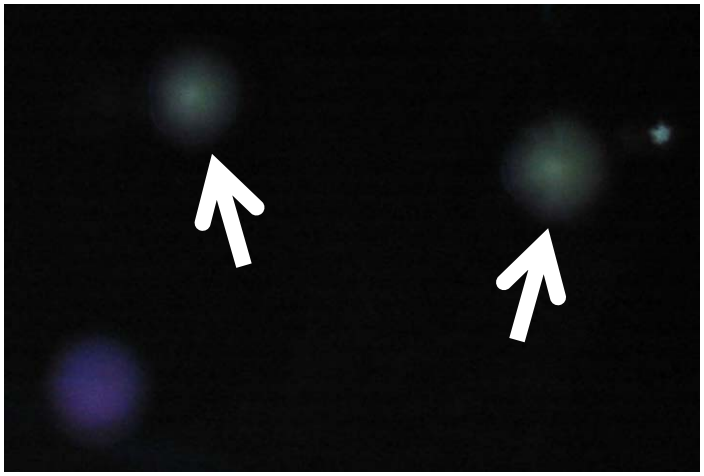

Figure 1. Colonies of *Fusobacterium* species display an autofluorescent chartreuse (pale yellow-green) color under long-wave ultraviolet light (arrows).

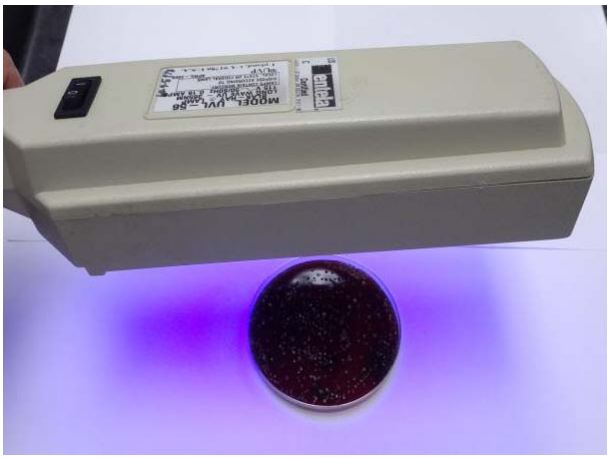

Figure 2. Use of a Wood's lamp for autofluorescent testing of possible subgingival fusobacteria clinical isolates on EBBA primary isolation plate.

No additional phenotypic, biochemical or genetic characterization of the presumptive subgingival fusobacteria clinical isolates was performed prior to MALDI-TOF mass spectrometry testing. Selected clinical isolate colony photographs were taken.

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 *Porphyromonas gingivalis*, *Prevotella melaninogenica*, *Prevotella denticola*, *Eubacterium brachy*, 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 fusobacteria.

MALDI-TOF Mass Spectrometry Identification of Periodontal Fusobacteria

Each of the putative fusobacteria clinical isolates, along with manufacturer-recommended BTS and the non-fusobacteria 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 (Figure 3).

Figure 3. Individual fusobacteria clinical isolates picked off of EBBA primary isolation plates (left) are smeared onto polished steel target plate (right).

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 (Figure 4).

Figure 4. Placement of formic acid onto smeared fusobacteria clinical isolates.

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 blank target plate spot.

After insertion of the prepared target plate into the Bruker Microflex LT bench top mass spectrometer (Figure 5), 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.

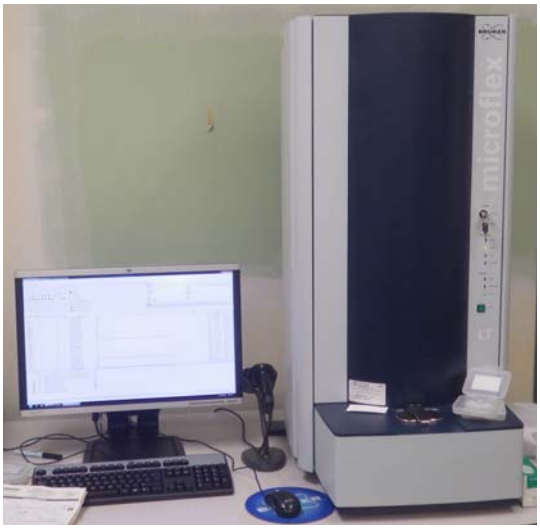

Figure 5. MALDI-TOF mass spectrometry instrument.

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). Scores of < 1.7 were considered to provide less reliable bacterial identification. The taxonomic classification with MALDI-TOF mass spectrometry occurred within an approximately 30-45 minute time period from the point of colony harvesting from primary isolation culture plates.

Data Analysis

Data analysis was carried out by tabulating the distribution by patients of the microbial genus and species identified by the MALDI-TOF mass spectrometry analysis among the tested subgingival fusobacteria clinical isolates, as well as their associated Biotyper log scores. For clinical isolates with Biotyper log scores < 1.7, the first choice listed by Biotyper analytic software of most likely species was noted.

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*, 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 blank target spot, did not give any mass spectra peaks or bacterial identification.

Test Bacterial Strains

A majority (58.8%) of the chronic periodontitis patients yielded two or three different species of subgingival *Fusobacterium* on non-selective enriched Brucella blood agar primary isolation plates.

Table 1 provides the distribution by patients of the microbial genus and species identified by the MALDI-TOF mass spectrometry analysis among the tested subgingival fusobacteria clinical isolates, as well as their associated Biotyper log scores (for clinical isolates with Biotyper log scores < 1.7, the first choice listed by Biotyper analytic software of most likely species is provided).

Table 1. Bacterial identification of presumptive periodontal fusobacteria clinical isolates with MALDI-TOF mass spectrometry

Patient	Clinical isolate MALDI Biotyper score	Clinical isolate species identification
1	2.141	*F. naviforme*
1	2.142	*F. naviforme*
1	1.554	*F. naviforme*
2	1.937	*F. naviforme*
2	2.225	*F. naviforme*
2	1.836	*F. naviforme*
2	2.245	*F. naviforme*
2	2.258	*F. naviforme*
2	2.103	*F. naviforme*
2	2.284	*F. naviforme*
3	2.020	*F. nucleatum spp. animalis*
3	1.647	*F. nucleatum spp. polymorphum*
4	1.969	*F. nucleatum spp. vincentii*
4	1.822	*F. naviforme*
4	1.762	*F. naviforme*

4	1.736	*F. naviforme*
4	1.859	*F. naviforme*
4	1.829	*F. naviforme*
5	1.712	*F. nucleatum spp. nucleatum*
5	1.663	*F. nucleatum spp. nucleatum*
5	1.437	*F. nucleatum spp. nucleatum*
5	1.741	*F. nucleatum spp. polymorphum*
6	1.795	*F. naviforme*
6	1.726	*F. naviforme*
6	1.719	*F. naviforme*
6	1.708	*F. nucleatum spp. nucleatum*
7	1.934	*F. nucleatum spp. polymorphum*
7	1.901	*F. naviforme*
7	1.838	*F. nucleatum spp. nucleatum*
7	1.876	*F. nucleatum spp. polymorphum*
7	1.569	*F. nucleatum spp. polymorphum*
8	1.817	*F. nucleatum*
8	1.452	*F. nucleatum*
8	1.584	*F. nucleatum spp. vincentii*
8	1.552	*F. nucleatum spp. vincentii*
8	1.495	*F. nucleatum*

9	1.482	*F. nucleatum spp. vincentii*
9	1.575	*Fusobacterium* species
9	1.440	*Fusobacterium* species
10	1.640	*F. nucleatum spp. polymorphum*
10	1.588	*F. nucleatum*
11	1.369	*F. nucleatum*
12	1.386	*F. nucleatum spp. vincentii*
13	1.654	*F. nucleatum spp. polymorphum*
13	1.270	*F. nucleatum spp. nucleatum*
14	1.994	*F. naviforme*
15	1.582	*F. canifelinum*
16	1.713	*F. naviforme*
16	1.529	*F. nucleatum spp. polymorphum*
16	1.861	*F. nucleatum*
17	1.913	*F. nucleatum spp. vincentii*
17	1.603	*F. nucleatum spp. vincentii*
17	1.960	*F. nucleatum spp. polymorphum*
17	1.725	*F. nucleatum spp. polymorphum*
18	1.750	*F. nucleatum spp. vincentii*
19	1.296	*F. nucleatum spp. polymorphum*
20	1.990	*F. nucleatum*

20	1.776	*F. canifelinum*
21	1.570	*F. nucleatum spp. polymorphum*
21	1.738	*F. canifelinum*
21	1.640	*F. canifelinum*
21	1.647	*F. canifelinum*
22	1.862	*F. nucleatum*
23	1.459	*F. nucleatum spp. polymorphum*
23	2.082	*F. nucleatum spp. animalis*
23	1.509	*F. naviforme*
24	1.435	*F. naviforme*
24	1.698	*F. nucleatum spp. vincentii*
24	1.344	*F. nucleatum spp. vincentii*
24	1.838	*F. naviforme*
25	1.439	*F. nucleatum spp. vincentii*
26	1.284	*F. nucleatum spp. vincentii*
26	1.805	*F. naviforme*
27	1.660	*Fusobacterium* species
27	1.705	*Fusobacterium* species
27	1.716	*Fusobacterium* species
27	1.465	*Fusobacterium* species
27	1.519	*Fusobacterium* species

28	2.209	*Fusobacterium* species
29	1.507	*F. naviforme*
29	1.636	*F. naviforme*
29	1.701	*F. naviforme*
29	1.874	*F. nucleatum spp. vincentii*
30	1.493	*F. naviforme*
31	2.117	*F. nucleatum spp. animalis*
32	1.289	*Fusobacterium* species
32	1.832	*F. nucleatum spp. vincentii*
32	1.722	*F. naviforme*
32	1.238	*Fusobacterium* species
33	1.332	*F. nucleatum spp. vincentii*
33	1.762	*F. nucleatum spp. vincentii*
33	1.847	*F. nucleatum spp. vincentii*
33	1.592	*F. naviforme*
34	1.124	*Fusobacterium* species
34	2.080	*F. nucleatum spp. vincentii*
34	1.846	*F. nucleatum spp. vincentii*

Fusobacterium naviforme was identified by MALDI-TOF mass spectrometry analysis in 14 (41.2%) chronic periodontitis study patients, *Fusobacterium nucleatum*

subspecies *vincentii* in 13 (38.2%) patients, *Fusobacterium nucleatum* subspecies *polymorphum* in 9 (26.5%) patients, *Fusobacterium nucleatum* and *Fusobacterium* species each in 6 (17.6%) patients, *Fusobacterium nucleatum* subspecies *nucleatum* in 4 (11.8%) patients, and *Fusobacterium nucleatum* subspecies *animalis* in 3 (8.8%) patients. Three patients additionally yielded subgingival isolates of *Fusobacterium canifelinum.*

52 (54.2%) of the fusobacteria clinical isolates revealed MALDI Biotyper log scores of ≥ 1.7, the threshold for reliable taxonomic classification, while in comparison, 44 (45.8%) had log scores < 1.7, indicating a less reliable species identification. 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 with log scores < 1.7 were fusobacteria.

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

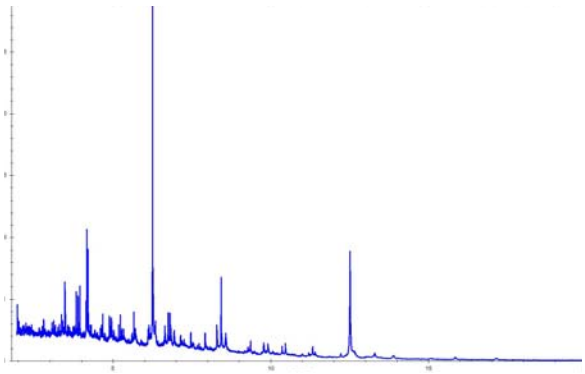

Figure 6. Mass spectra for presumptive fusobacteria clinical isolate identified as *F. naviforme* by MALDI-TOF mass spectrometry analysis.

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

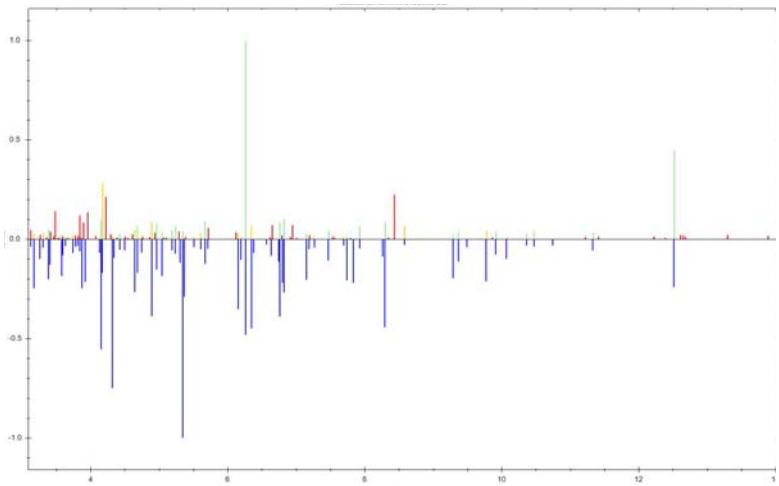

Figure 7. Peaks of mass spectra for presumptive fusobacteria clinical isolate (top, with green peaks representing a match) matched to peaks of mass spectra of *F. naviforme* (bottom) in Biotyper reference library.

In comparison, an example of a mass spectra typical of subgingival Fusobacterium isolates reliably identified as *F. nucleatum* subspecies *polymorphum* in MALDI-TOF mass spectrometry analysis is presented in Figure 8.

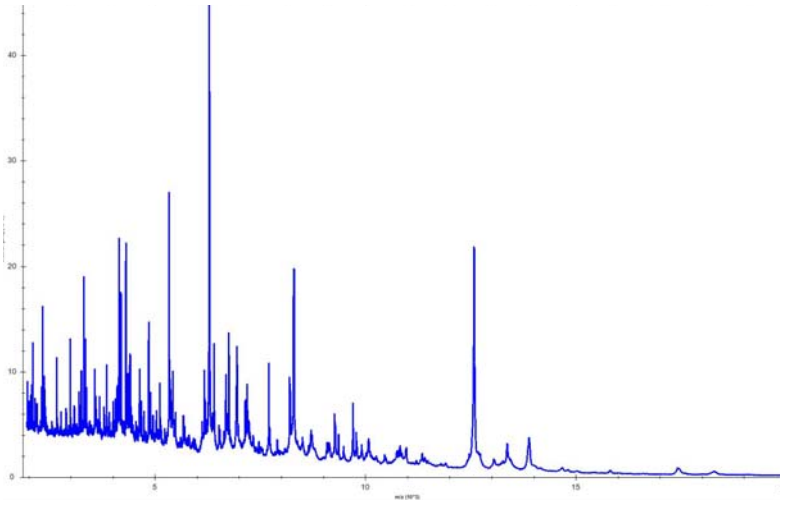

Figure 8. Mass spectra for presumptive fusobacterium clinical isolate identified as *F. nucleatum* subspecies *polymorphum* by MALDI-TOF mass spectrometry analysis.

An example of proteomic spectral fingerprinting performed by Biotyper analytic software resulting in a match of the above *F. nucleatum* subspecies *polymorphum* clinical isolate mass spectra to the most appropriate species present in the software database of bacterial species-specific spectra is presented in Figure 9.

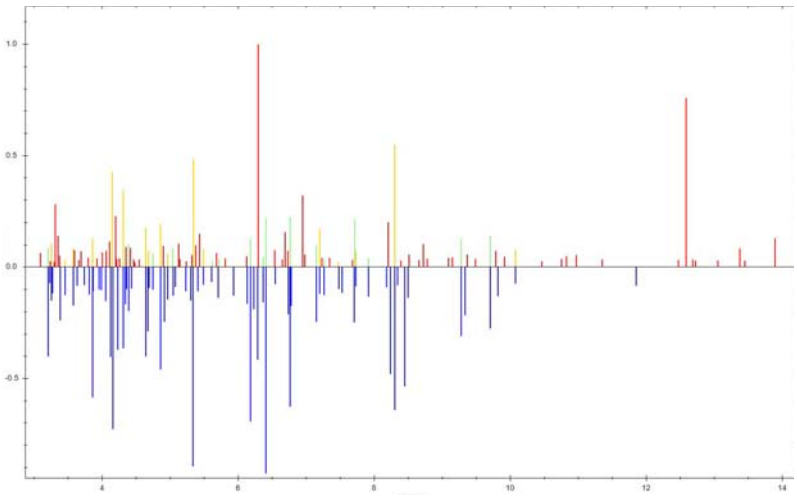

Figure 9. Peaks of mass spectra for presumptive fusobacteria clinical isolate (top, with green peaks representing a match) matched to peaks of mass spectra of

F. nucleatum subspecies *polymorphum* (bottom) in Biotyper reference library.

Selected colony photographs of clinical isolates identified by MALDI-TOFF mass spectrometry as *F. naviforme* are presented in Figure 10.

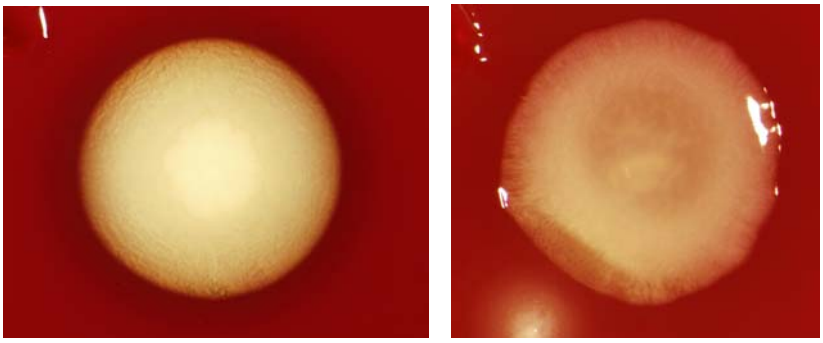

Figure 10. Two clinical isolates identified as *F. naviforme*.

Selected colony photographs of clinical isolates identified by MALDI-TOFF mass spectrometry as *F. nucleatum* subspecies *polymorphum* are presented in Figure 11.

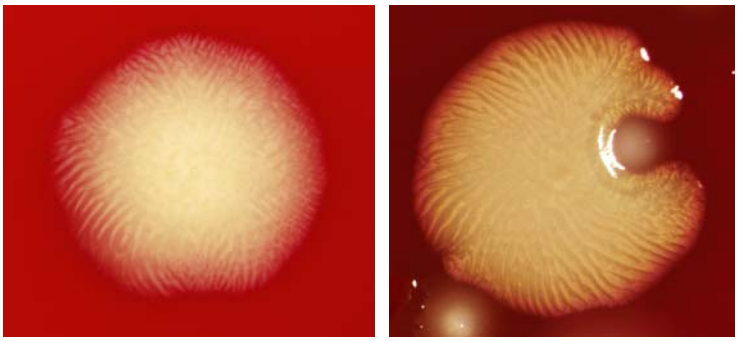

Figure 11. Two clinical isolates identified as *F. nucleatum* subspecies *polymorphum*.

Selected colony photographs of clinical isolates identified by MALDI-TOFF mass spectrometry as *F. nucleatum* subspecies *vincentii* are presented in Figure 12.

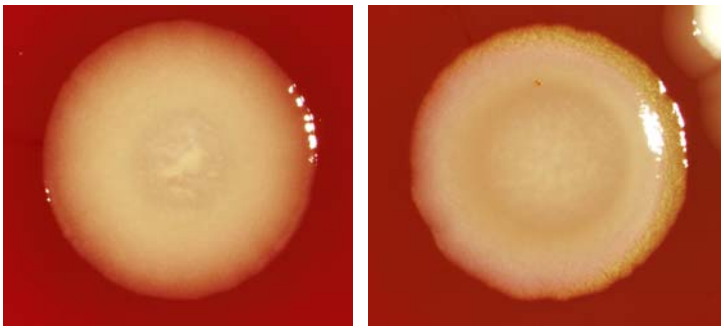

Figure 12. Two clinical isolates identified as *F. nucleatum* subspecies *vincentii*.

A colony photograph of a clinical isolates identified by MALDI-TOFF mass spectrometry as *F. canifelinum* is presented in Figure 13.

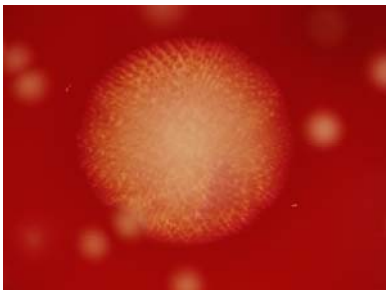

Figure 13. Clinical isolate identified as *F. canifelinum*.

CHAPTER 4

DISCUSSION

These study findings demonstrate that that a variety of *Fusobacterium* species may be rapidly identified with MALDI-TOF mass spectrometry from the subgingival microbiota of chronic periodontitis patients. The MALDI-TOF mass spectrometry species identification was able to be obtained within an approximately 30-45 minute time period from the point of colony harvesting from the EBBA primary isolation culture plates. This is a substantially shorter time period than what is commonly encountered using conventional biochemical and phenotypic fusobacteria classification schemes.

In the present study, *F. naviforme* and *F. nucleatum* subspecies *vincentii* were the most frequently isolated subgingival fusobacteria species in the evaluated patients. *F. naviforme* was found in 14 (41.2%) of the 34 chronic periodontitis study patients, whereas *F. nucleatum* subspecies *vincentii* was identified in 13 (38.2%) patients. Overall, *F. nucleatum* group species, including all of its subspecies, were recovered from the subgingival microbiota of 28 (82.3%) of the chronic periodontitis patients evaluated, illustrating the frequent occurrence of this bacterial group of species in chronic periodontitis.

An unexpected and surprising finding in the present study was the subgingival isolation of the animal species *F. canifelinum* from three of the chronic periodontitis patients. *F. canifelinum* is a normal inhabitant of the oral cavity of dogs and cats (Conrads et al. 2004). It is not known how or why this animal species became a member of the subgingival microbiome of the three chronic periodontitis patients. No information

was available for any of the three patients relative to their age, habits, medications, pets or history of periodontitis. However, recent evidence suggests the potential for household pets to transfer their oral microbiota to their owners as a result of close environmental contact leading to an exchange of oral fluids, such as may occur with kissing (Booij-Vrieling et al. 2010). Reports of *F. canifelinum* in the human oral cavity are uncommon, but Moon et al. (2014) recently found *F. canifelinum* to show an inverse relationship with smokers with periodontitis in a Korean population group. *F. canifelinum* was one of 11 microbial species to have a ≥ 2-fold lower abundance in the subgingival microbiome of smokers with chronic periodontitis, as compared to non-smokers with chronic periodontitis.

A total of 52 (54.2%) of the fusobacteria clinical isolates in the present study had MALDI Biotyper log scores of ≥ 1.7, the threshold for reliable taxonomic classification, whereas 44 (45.8%) had log scores < 1.7, indicating a less reliable species identification. This lower proportion than expected of reliable identification log scores may be due to possible inclusion of cells from more than one bacterial species during colony harvesting and spotting on the MALDI-TOF steel target, particularly since cultures of subgingival dental plaque are heavily colonized by an enormous number of different species ((Dewhirst et al. 2010). The less than reliable log scores may also reflect a lack of marked differences in protein profiles between the various species in the *Fusobacterium* genus, which would complicate the process of successfully differentiating between multiple fusobacteria species. The occurrence of less reliable bacterial species identification in slightly less than one-half of the tested subgingival fusobacteria reduces

the overall potential application of MALDI-TOF mass spectrometry with the presently-constituted analytic database.

However, it is important to point out that when MALDI Biotyper log scores < 1.7 were recorded for chartreuse-positive (pale yellow-green) autofluorescence surface colonies on EBBA primary isolation plates of subgingival biofilm specimen, only fusobacteria species were listed by the MALDI Biotyper analytic software as the first choices among the most likely bacterial species in the clinical isolates. Additional modification and refinement of the MALDI Biotyper database is needed to attain more reliable identification of fusobacteria in subgingival plaque biofilms.

CHAPTER 5

CONCLUSIONS

These findings indicate that a variety of *Fusobacterium* species may be rapidly identified with MALDI-TOF mass spectrometry in the subgingival microbiota of chronic periodontitis patients. *F. naviforme* and *F. nucleatum* subspecies *vincentii* were the most frequently isolated subgingival fusobacteria species in the evaluated study patients. Three chronic periodontitis patients also unexpectedly revealed subgingival isolates of the animal species *F. canifelinum*, which is normally in the oral cavity of dogs and cats. MALDI-TOF mass spectrometry may facilitate rapid identification of cultivable fusobacteria in human subgingival dental plaque biofilms, and enhance understanding of bacterial community structure in periodontal pockets. However, additional modification and refinement of the MALDI Biotyper database is needed to attain more reliable identification of fusobacteria in subgingival plaque biofilms.

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