PROTEOMIC COMPARISON OF AMOXICILLIN-RESISTANT AND SUSCEPTIBLE PERIODONTAL *PREVOTELLA INTERMEDIA* AND *PREVOTELLA NIGRESCENS* CLINICAL ISOLATES WITH MATRIX-ASSISTED LASER DESORPTION IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY.

A Thesis
Submitted to
the Temple University Graduate Board

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in Partial Fulfillment
of the Requirements for the Degree
MASTER OF SCIENCE

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by
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July, 2015

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ABSTRACT

Objectives: Prevotella intermedia and *Prevotella nigrescens* are dark-pigmented, non-motile, anaerobic rods regarded as important bacterial pathogens in the etiology and progression of human chronic periodontitis. Because the bacterial species may not be adequately suppressed in the subgingival microbiota of chronic periodontitis lesions by conventional mechanical root debridement when present in high cultivable proportions, systemic and/or local periodontal chemotherapy is often employed to reduce their subgingival numbers to levels compatible with periodontal health. However, many clinical isolates of *P. intermedia* and *P. nigrescens* may exhibit resistance to β-lactam antibiotics prescribed in periodontal therapy via expression of β-lactamase enzymes. Conventional methods of in vitro antibiotic susceptibility testing entail cultivation of targeted bacterial species, with evaluations of whether or not the organism is capable of growing in the presence of critical thresholds of test antibiotics, such as β-lactam antibiotics. Because such conventional in vitro antibiotic testing is time-consuming, expensive, and labor-intensive, there is an urgent clinical need for more rapid assays to determine the susceptibility or resistance of important periodontal pathogens, such as *P. intermedia* and *P. nigrescens*, to contemplated periodontal antimicrobial chemotherapy.

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. This methodology relies upon evaluations of bacterial protein profiles for microbial species identification. Since β-lactamase enzymes conferring bacterial resistance to β-

lactam antibiotics are proteins, it is possible that MALDI-TOF mass spectrometry may be able to reliably detect characteristic differences in protein profiles of microorganisms elaborating and not elaborating β-lactamase enzymes. Thus, the potential exists that protein profiles generated by MALDI-TOF mass spectrometry may be used to rapidly distinguish between antibiotic-resistant versus susceptible strains of bacterial species without employing time-consuming, expensive, and labor-intensive culture and in vitro drug testing. As a result, this study aimed carry out a proteomic comparison of amoxicillin-resistant and susceptible periodontal *P. intermedia* and *P. nigrescens* clinical isolates with MALDI-TOF mass spectrometry.

Methods: A total of 10 amoxicillin-resistant and two amoxicillin-susceptible fresh clinical subgingival isolates of *P. intermedia*, and 10 amoxicillin-resistant and one amoxicillin-susceptible clinical subgingival isolates of *P. nigrescens*, were recovered by culture and identified to species level with MALDI-TOF mass spectrometry from the subgingival dental plaque biofilms of adults with severe chronic periodontitis. The amoxicillin-resistant clinical isolates of *P. intermedia* and *P. nigrescens* exhibited growth on anaerobically-incubated enriched Brucella blood agar primary isolation culture plates supplemented with amoxicillin at 8 μg/ml, whereas no growth on these plates was found with amoxicillin-susceptible strains.

Normalized raw mass spectra, as well as normalized peak list spectrum representations, generated by MALDI-TOF mass spectrometry within a routine operating range of 2-20 kDa were visually compared for the amoxicillin-resistant and amoxicillin-susceptible *P. intermedia* and *P. nigrescens* clinical isolates, to see if there were consistently reproducible differences in their distribution of mass spectra peaks.

Results: Visual comparisons of normalized raw mass spectra, and normalized peak list spectrum, for amoxicillin-susceptible and amoxicillin-resistant subgingival clinical isolates of both *P. intermedia* and *P. nigrescens* failed to reveal consistently reproducible differences in their distribution of mass spectra peaks within a routine MALDI-TOF mass spectrometry operating range of 2-20 kDa.

Conclusions: Visual examination of raw mass spectra and mass spectra peaks generated by MALDI-TOF mass spectrometry within a routine operating range of 2-20 kDa failed to reveal noteworthy differences between amoxicillin-resistant and amoxicillin-susceptible subgingival clinical isolates of *P. intermedia* and *P. nigrescens*. These findings indicate that use of MALDI-TOF mass spectrometry employed within a routine operating range of 2-20 kDa may not provide sufficient differentiation in protein profiles between amoxicillin-resistant and amoxicillin-susceptible *Prevotella* species to be of rapid diagnostic use for assessing the species in vitro antibiotic susceptibility to β-lactam antibiotics. Additional evaluation of MALDI-TOF mass spectrometry employing higher kDa ranges beyond a routine upper operating range of 20 kDa is warranted to further evaluate its potential to accurately identify antibiotic-resistant versus susceptible strains of pathogenic microorganisms.

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CHAPTER 1

INTRODUCTION

Prevotella intermedia and *Prevotella nigrescens* are dark-pigmented, non-motile, anaerobic rods regarded as important bacterial pathogens in the etiology and progression of human chronic periodontitis (Bragd et al. 1987, Haffajee & Socransky 1994, Rams et al. 1996, Socransky et al. 1998, Colombo et al. 2012, Wade 2013). Based on a multitude of cross-sectional and longitudinal clinical studies, these two species are recognized as conferring a "moderate" risk in the pathogenesis of periodontitis (Haffajee & Socransky 1994), and are assigned to the "orange" microbial complex group (Socransky et al. 1998).

P. intermedia and *P. nigrescens* often elaborate β-lactamase enzymes, which can hydrolyze and degrade β-lactam class antibiotics (Luong et al. 2001, Fosse et al. 1999, Rams et al. 2013). This may compromise periodontal antimicrobial chemotherapy involving amoxicillin, which may lead to a clinical therapeutic failure in chronic periodontitis therapy (van Winkelhoff et al. 1996). Recent studies have found strains of subgingival species phenotypically-identified as belonging to the *Prevotella intermedia/nigrescens* group present in one-third of 400 chronic periodontitis patients which are able to grow in vitro in the presence of therapeutic threshold concentrations of 8 μg/ml of amoxicillin (Rams et al. 2014), which is indicative of antibiotic resistance by the organisms.

Conventional methods of antibiotic susceptibility testing of *P. intermedia* and *P. nigrescens* require their successful laboratory culture, and evaluations of whether or not

the species grow in the presence of test antibiotics, which are time-consuming, expensive, and labor-intensive procedures to perform (Rams et al. 2014, Veloo et al. 2015).

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 in the United States by the Food and Drug Administration. In MALDI-TOF mass spectrometry, a laser desorbs and ionizes microbial and matrix molecules from a target plate (Clark et al. 2013, Nomura 2015). The resulting cloud of ionized molecules is accelerated into a time-of-flight mass analyzer and toward a detector, with lighter molecules traveling faster, and heavier molecules traveling slower, over time. From this, a mass spectrum is generated, representing the number of ions hitting the detector over time (Clark et al. 2013, Nomura 2015). This methodology is capable of definitively identifying 4,970 different oral and non-oral microbial species based on mass spectra of their bacterial protein profiles, including *P. intermedia* and *P. nigrescens*.

In addition to bacterial species identification, MALDI-TOF mass spectrometry, which detects proteins, has also been suggested for detection of antibiotic resistance factors that are proteins, such as β-lactamase enzymes (Wybo et al. 2011, Pulido et al. 2013, Patel 2015). Thus, the potential exists that protein profiles generated by MALDI-TOF mass spectrometry may be used to rapidly distinguish between antibiotic-resistant versus susceptible strains of bacterial species without a need to carry out laboratory culture of the organisms and in vitro antibiotic testing. To date, this approach has been

used to study ampicillin-resistant versus susceptible oral *Fusobacterium nucleatum* clinical isolates (Al-Haroni et al. 2008), but not β-lactamase enzyme-positive *P. intermedia* or *P. nigrescens* periodontal clinical isolates.

As a result, this study aimed carry out a proteomic comparison of amoxicillin-resistant and susceptible periodontal *P. intermedia* and *P. nigrescens* clinical isolates with MALDI-TOF mass spectrometry to determine if there are consistently reproducible differences in their distribution of mass spectra peaks.

CHAPTER 2

MATERIALS AND METHODS

Laboratory Facilities

All laboratory procedures for this research 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).

Clinical Isolates of *P. intermedia* and *P. nigrescens*

A total of 10 amoxicillin-resistant and two amoxicillin-susceptible fresh clinical subgingival isolates of *P. intermedia*, and 10 amoxicillin-resistant and one amoxicillin-susceptible clinical subgingival isolates of *P. nigrescens*, were employed in this research study.

These fresh clinical periodontal isolates of *P. intermedia* and *P. nigrescens* were obtained in the spring of 2015 from subgingival dental plaque biofilm specimens from adults with severe chronic periodontitis submitted and processed by the OMTS Laboratory as part of their commercial diagnostic microbiology testing services, and designated to be discarded.

The 10 fresh clinical subgingival isolates of *P. intermedia* resistant in vitro to amoxicillin comprised a mean 10.4 ± 5.8 (standard deviation) % of the total cultivable subgingival microbiota per study patient (range: 0.3-14.3%), and were recovered from six adults (three male and three female), with a mean age of 58.0 ± 4.5 years (standard deviation), and an age range of 50-63 years. The one fresh clinical subgingival isolate of *P. intermedia* susceptible in vitro to amoxicillin was recovered as 3.6% of the total cultivable subgingival microbiota in one male, aged 56 years. None of the adults contributing fresh clinical subgingival isolates of *P. intermedia* were reported to have a current smoking habit.

The 10 fresh clinical subgingival isolates of *P. nigrescens* resistant in vitro to amoxicillin comprised a mean 5.6 ± 5.1 (standard deviation) % of the total cultivable subgingival microbiota per study patient (range: 0.1-15.6%), and were recovered from eight adults (four male and four female), with a mean age of 53.4 ± 8.7 years (standard deviation), and an age range of 44-66 years. One of these adults was reported to be a current smoker. The two fresh clinical subgingival isolate of *P. nigrescens* susceptible in vitro to amoxicillin were recovered as 15.6% of the total cultivable subgingival microbiota in one non-smoking male, aged 50 years.

The *P. intermedia* and *P. nigrescens* fresh clinical subgingival isolates originated from subgingival dental plaque biofilm specimens obtained by periodontists extramural to Temple University in private dental practice settings geographically distributed throughout the United States. 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 dental plaque biofilm specimens for microbial culture. The paper points were then placed together into a single glass vial containing 6-8 glass beads of 3.0 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 dental plaque biofilm 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).

Additional 0.1 ml aliquots of subgingival dental plaque biofilm sample dilutions were inoculated onto EBBA primary isolation plates supplemented with amoxicillin at 8 µg/ml. This antimicrobial concentration represents a non-susceptible/resistant breakpoint concentration against anaerobic bacteria for amoxicillin as recommended by the Clinical and Laboratory Standards Institute (2012).

Both non-antibiotic containing EBBA plates and amoxicillin-supplemented EBBA plates 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).

In vitro resistance to the antibiotic breakpoint concentration of amoxicillin (8 µg/ml) was recorded per patient when test species growth was noted on the amoxicillin-supplemented EBBA plates (Rams et al. 2014). *Bacteroides thetaiotaomicron* ATCC 29741, *Clostridium perfringens* ATCC 13124, and a multi-antibiotic-resistant clinical periodontal isolate of *F. nucleatum* were employed as positive and negative quality controls for antibiotic resistance testing on amoxicillin-supplemented EBBA plates.

After incubation, both antibiotic-supplemented and non-antibiotic-supplemented 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. Fresh clinical isolates presumptively identified as being either *P. intermedia* or *P. nigrescens* were identified as gram-negative, non-motile, anaerobic rods exhibiting circular, dome-shaped, dark-pigmented (black to brown), raised surface colonies, which displayed an autofluorescent brick-red color 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).

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.

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 *Prevotella 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 being either *P. intermedia* or *P. nigrescens*.

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

To obtain the identity of the putative *P. intermedia* and *P. nigrescens* clinical isolates, the clinical isolates, along with manufacturer-recommended BTS and non-*P. intermedia* and non-*P. 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.

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*, *F. nucleatum*, *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 *P. intermedia* and *P. nigrescens*.

Proteomic Comparison of Amoxicillin-Resistant and Susceptible *P. intermedia* and *P. nigrescens* Clinical Isolates

Raw mass spectra for the clinical isolates of *P. intermedia* and *P. nigrescens* were normalized using the MALDI Biotyper 3.1 software, and the distribution of mass spectra peaks visually compared between the amoxicillin-resistant and amoxicillin-susceptible strains (Wybo et al. 2011, Schaumann et al. 2012). A similar visual comparison of mass spectra peaks between the amoxicillin-resistant and amoxicillin-susceptible strains of *P.*

intermedia and *P. nigrescens* was carried out using graphical representations displaying the normalized peak list spectrum generated by MALDI-TOF mass spectrometry analysis. These graphical representations were printed onto transparency paper to permit overlay comparisons of the similarity or non-similarity of the distribution of mass spectra peaks between amoxicillin-resistant and amoxicillin-susceptible strains of *P. intermedia* and *P. nigrescens*.

Data Analysis

Data analysis was carried out by tabulating the distribution of Biotyper log scores for the test clinical isolates of *P. intermedia* and *P. nigrescens*. Additionally, tabulations were made of comparisons between the amoxicillin-resistant and amoxicillin-susceptible strains of *P. intermedia* and *P. nigrescens* relative to the similarity or non-similarity of their distribution of mass spectra peaks as determined by visual inspection of transparency overlays.

CHAPTER 3

RESULTS

Identification of Bacterial Strains

The manufacturer-recommended BTS control species was definitively identified as *E. coli* in MALDI-TOF mass spectrometry test runs, with MALDI Biotyper scores of ≥ 2.0, indicating appropriate performance of the MALDI-TOF mass spectrometry instrumentation and analytical software.

The negative control strains of *P. gingivalis*, *P. melaninogenica*, *P. denticola*, *F. nucleatum*, *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.

A total of 10 amoxicillin-resistant and two amoxicillin-susceptible fresh clinical subgingival isolates of *P. intermedia*, and 10 amoxicillin-resistant and one amoxicillin-susceptible clinical subgingival isolates of *P. nigrescens*, were identified by MALDI-TOF mass spectrometry in subgingival dental plaque biofilm samples from adults with severe chronic periodontitis.

Table 1 provides the distribution by patients and in vitro susceptibility to amoxicillin at 8 μg/ml the 10 amoxicillin-resistant and two amoxicillin-susceptible *P. intermedia* clinical isolates identified by MALDI-TOF mass spectrometry analysis.

Table 1. Identification of *P. intermedia* clinical isolates with MALDI-TOF mass spectrometry

Clinical isolate	Patient	Clinical isolate MALDI Biotyper score	Clinical isolate species identification	In vitro susceptibility to 8 µg/ml amoxicillin
1	1	2.152	*P. intermedia*	susceptible
2	1	2.086	*P. intermedia*	susceptible
3	2	1.880	*P. intermedia*	resistant
4	3	2.142	*P. intermedia*	resistant
5	3	2.106	*P. intermedia*	resistant
6	3	2.055	*P. intermedia*	resistant
7	4	2.115	*P. intermedia*	resistant
8	4	2.057	*P. intermedia*	resistant
9	4	1.946	*P. intermedia*	resistant
10	5	1.883	*P. intermedia*	resistant
11	6	2.008	*P. intermedia*	resistant
12	6	2.091	*P. intermedia*	resistant

Table 2 provides the distribution by patients and in vitro susceptibility to amoxicillin at 8 µg/ml the 10 amoxicillin-resistant and one amoxicillin-susceptible *P. nigrescens* clinical isolates identified by MALDI-TOF mass spectrometry analysis.

Table 2. Identification of *P. nigrescens* clinical isolates with MALDI-TOF mass spectrometry

Clinical isolate	Patient	Clinical isolate MALDI Biotyper score	Clinical isolate species identification	In vitro susceptibility to 8 µg/ml amoxicillin
1	1	1.974	*P. nigrescens*	susceptible
2	2	2.211	*P. nigrescens*	resistant
3	2	2.179	*P. nigrescens*	resistant
4	3	2.003	*P. nigrescens*	resistant
5	3	1.888	*P. nigrescens*	resistant
6	4	1.867	*P. nigrescens*	resistant
7	4	1.838	*P. nigrescens*	resistant
8	5	1.817	*P. nigrescens*	resistant
9	6	1.878	*P. nigrescens*	resistant
10	7	2.129	*P. nigrescens*	resistant
11	8	1.983	*P. nigrescens*	resistant

Proteomic Comparison of Amoxicillin-Resistant and Susceptible

P. intermedia and *P. nigrescens* Clinical Isolates

Visual comparison of normalized raw mass spectra for amoxicillin-susceptible (Figures 1 and 2) and amoxicillin-resistant (Figures 3-12) of *P. intermedia* failed to reveal consistently reproducible differences in their distribution of mass spectra peaks.

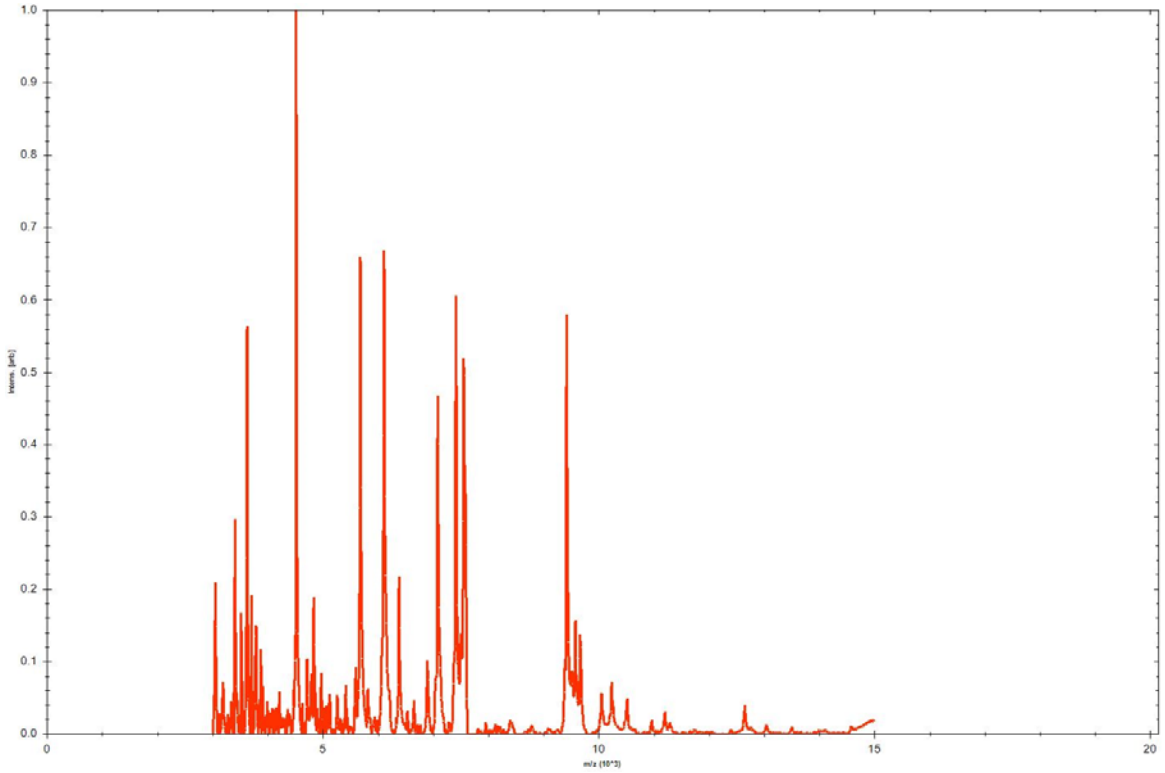

Figure 1. Normalized mass spectra for *P. intermedia* clinical isolate #1 susceptible in vitro to 8 μg/ml of amoxicillin.

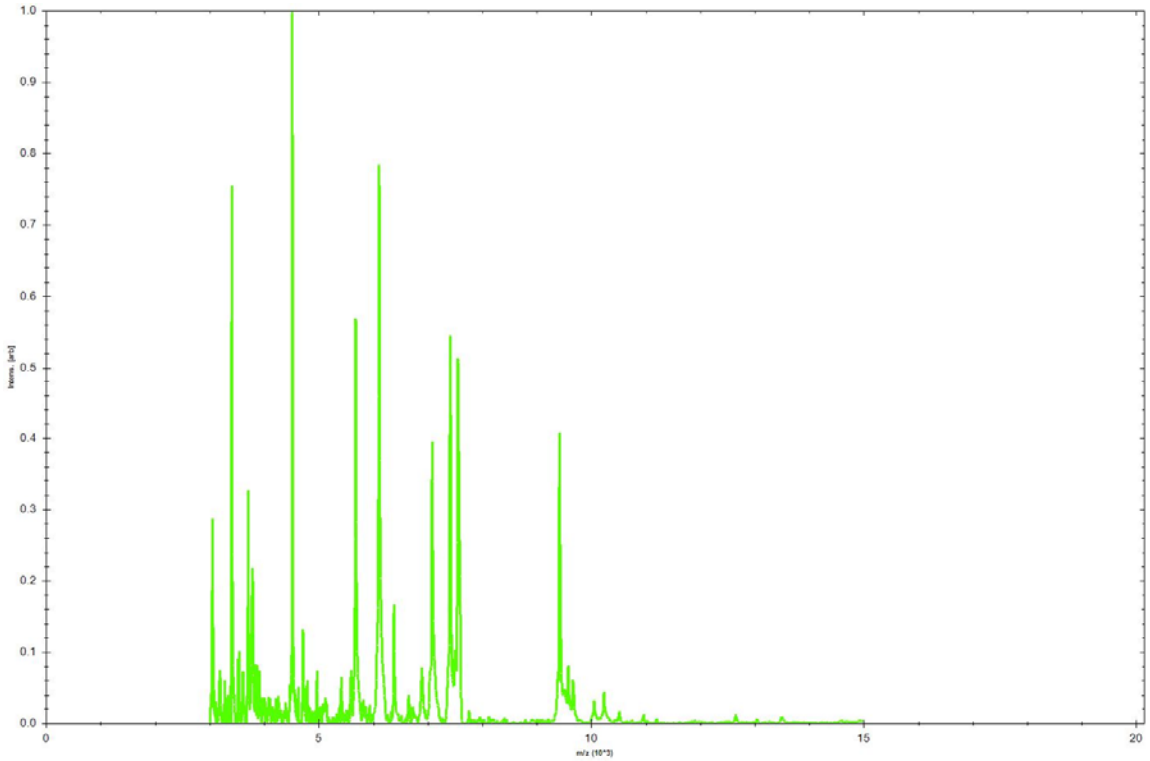

Figure 2. Normalized mass spectra for *P. intermedia* clinical isolate #2 susceptible in vitro to 8 μg/ml of amoxicillin.

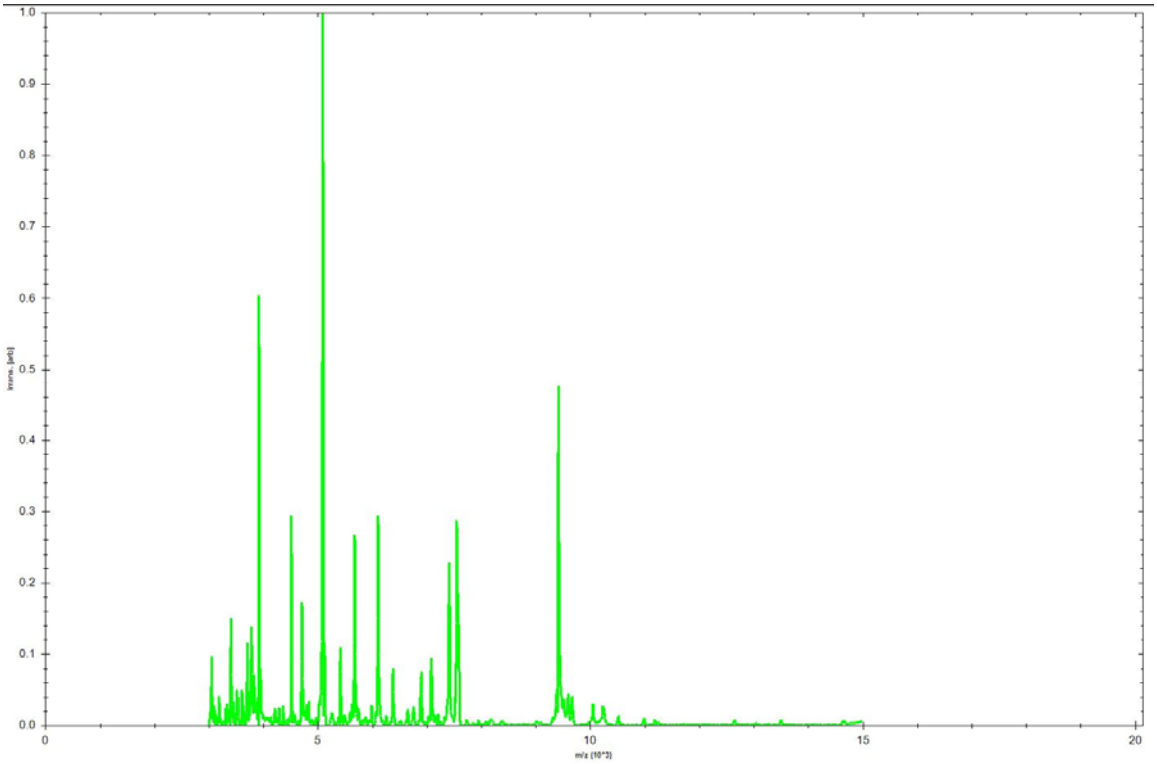

Figure 3. Normalized mass spectra for *P. intermedia* clinical isolate #3 resistant in vitro to 8 μg/ml of amoxicillin.

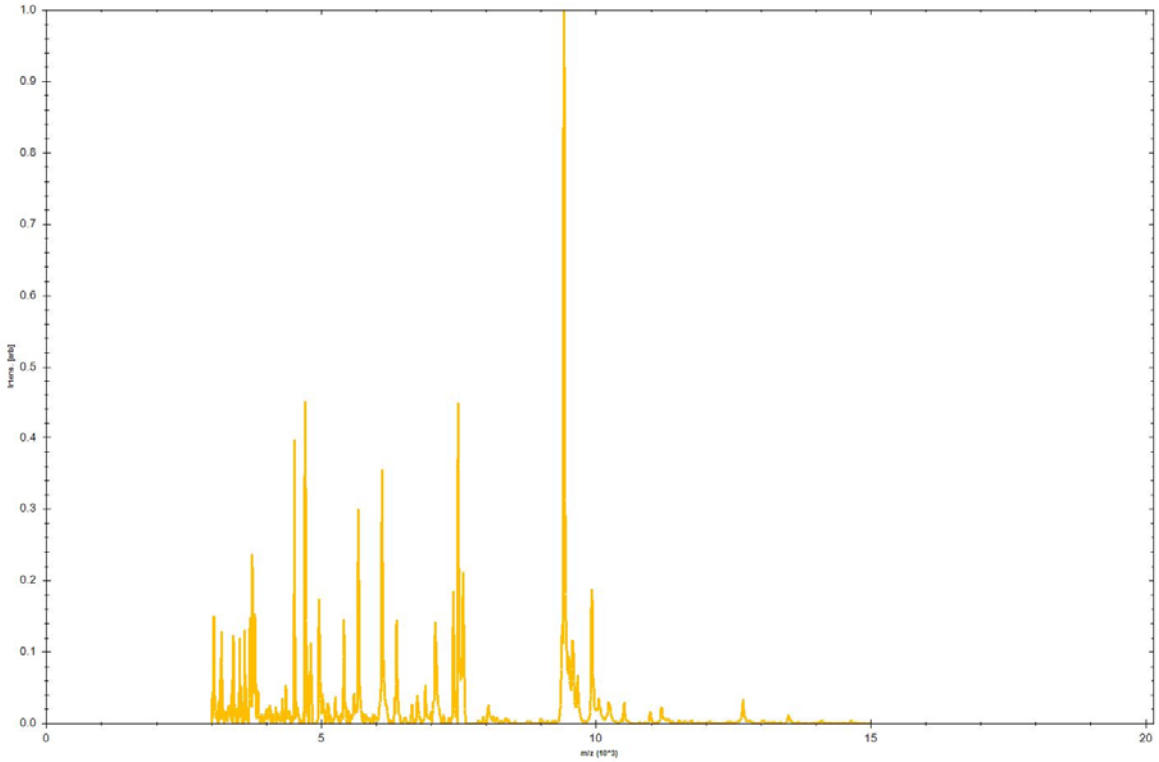

Figure 4. Normalized mass spectra for *P. intermedia* clinical isolate #4 resistant in vitro to 8 μg/ml of amoxicillin.

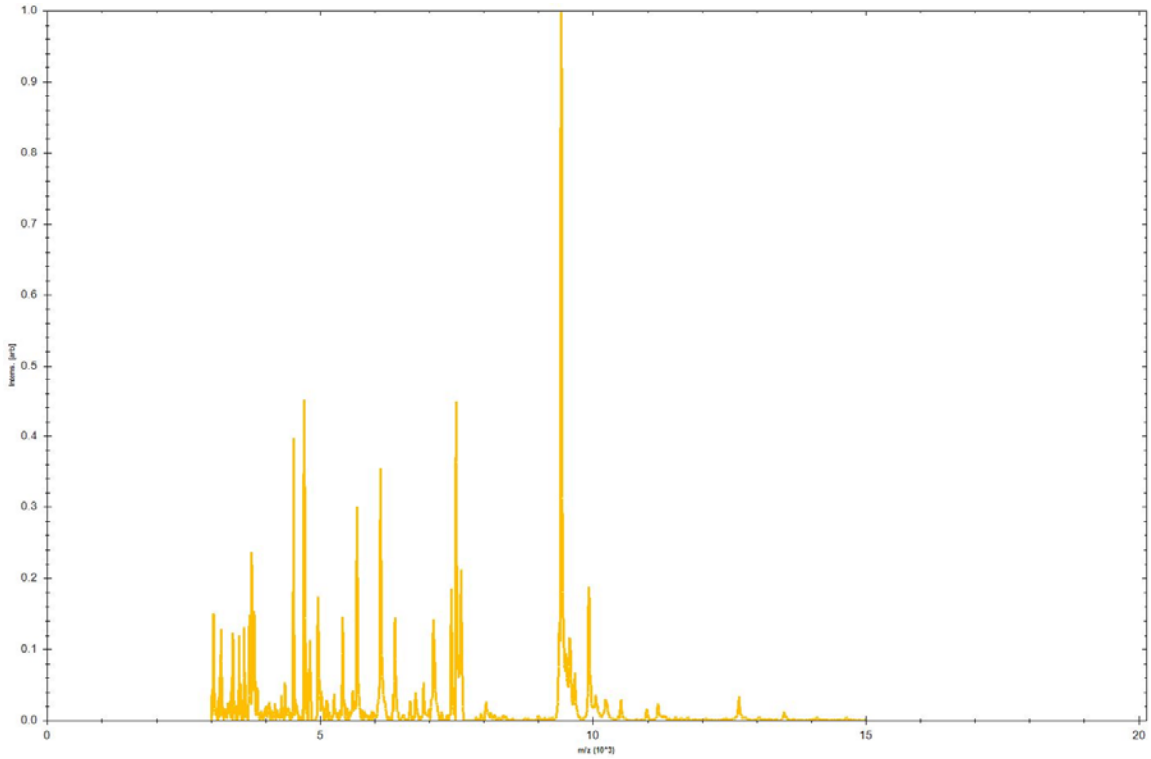

Figure 5. Normalized mass spectra for *P. intermedia* clinical isolate #5 resistant in vitro to 8 µg/ml of amoxicillin.

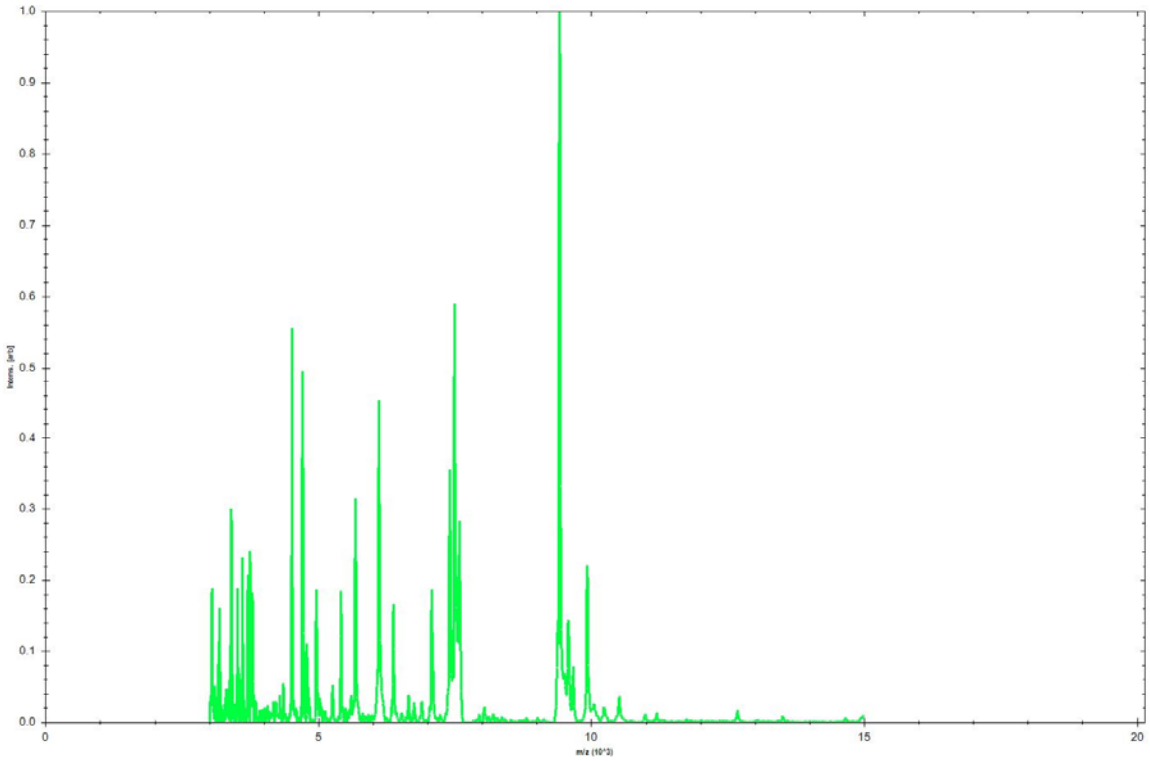

Figure 6. Normalized mass spectra for *P. intermedia* clinical isolate #6 resistant in vitro to 8 μg/ml of amoxicillin.

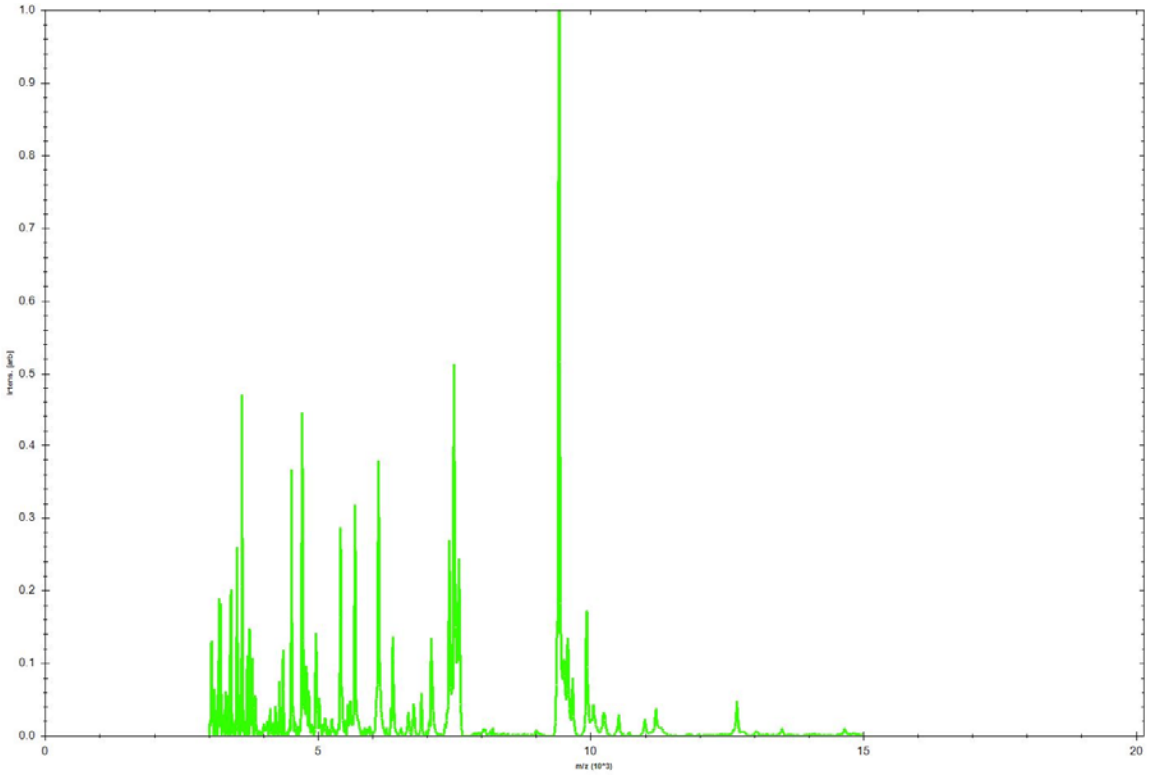

Figure 7. Normalized mass spectra for *P. intermedia* clinical isolate #7 resistant in vitro to 8 μg/ml of amoxicillin.

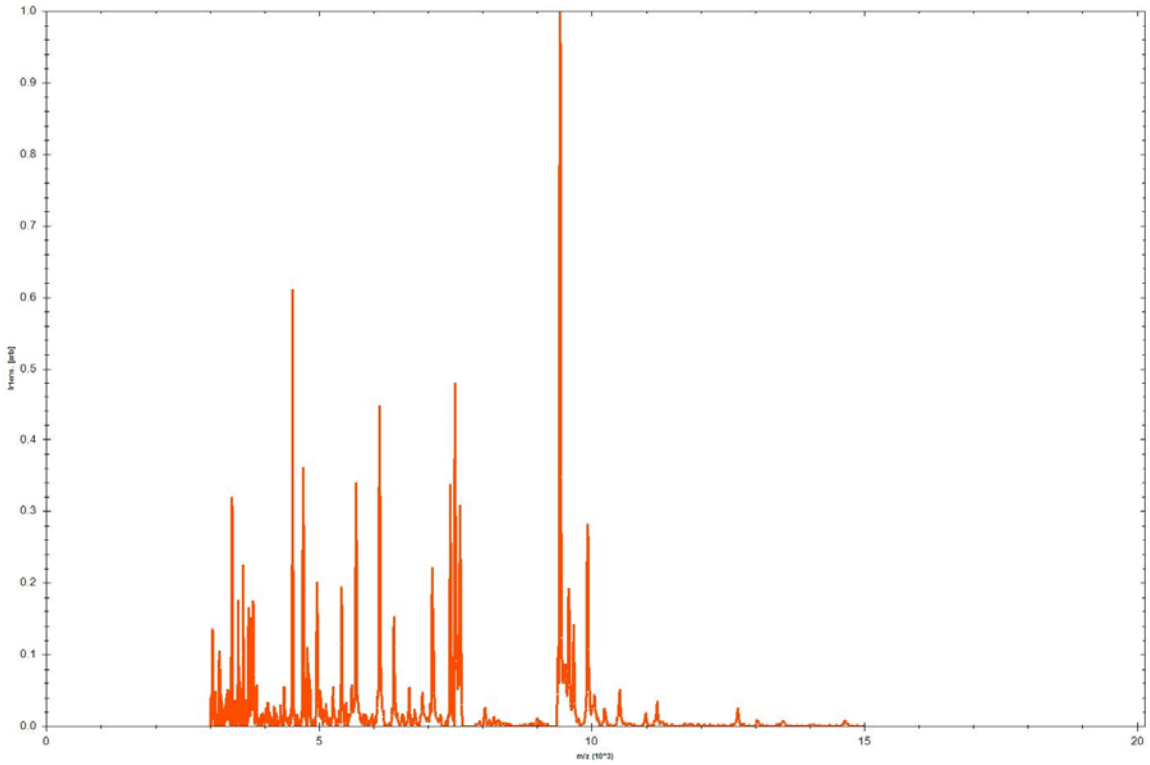

Figure 8. Normalized mass spectra for *P. intermedia* clinical isolate #8 resistant in vitro to 8 μg/ml of amoxicillin.

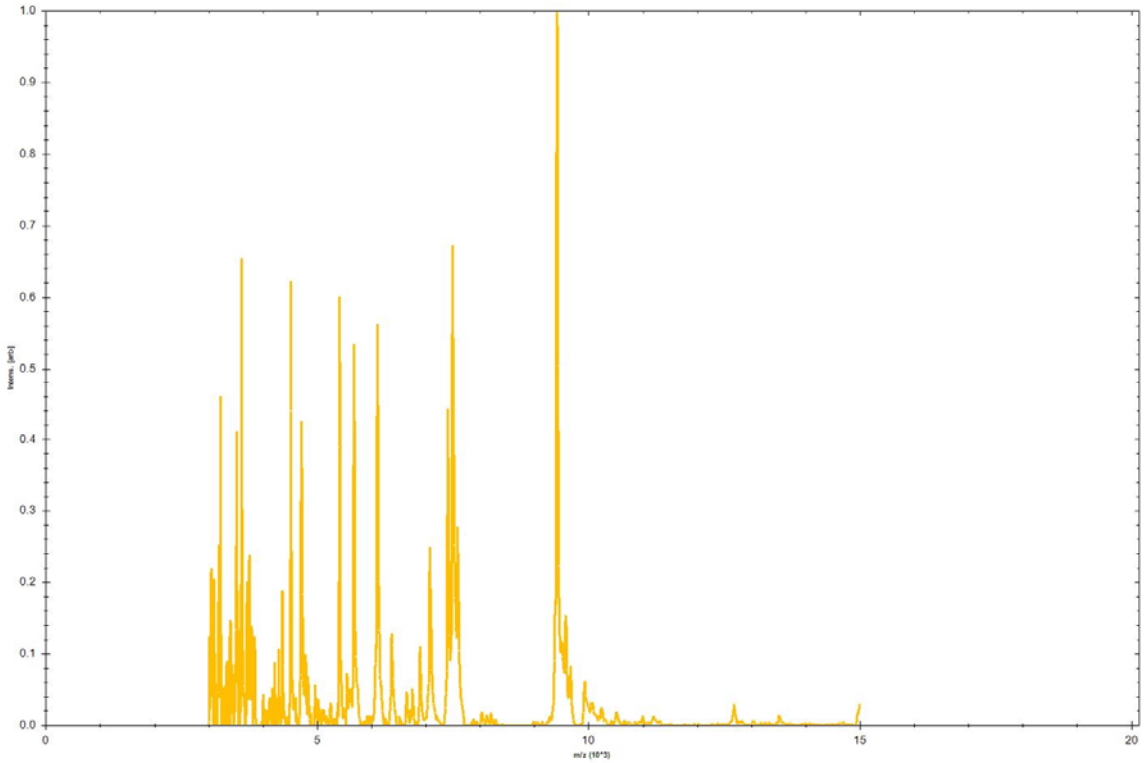

Figure 9. Normalized mass spectra for *P. intermedia* clinical isolate #9 resistant in vitro to 8 μg/ml of amoxicillin.

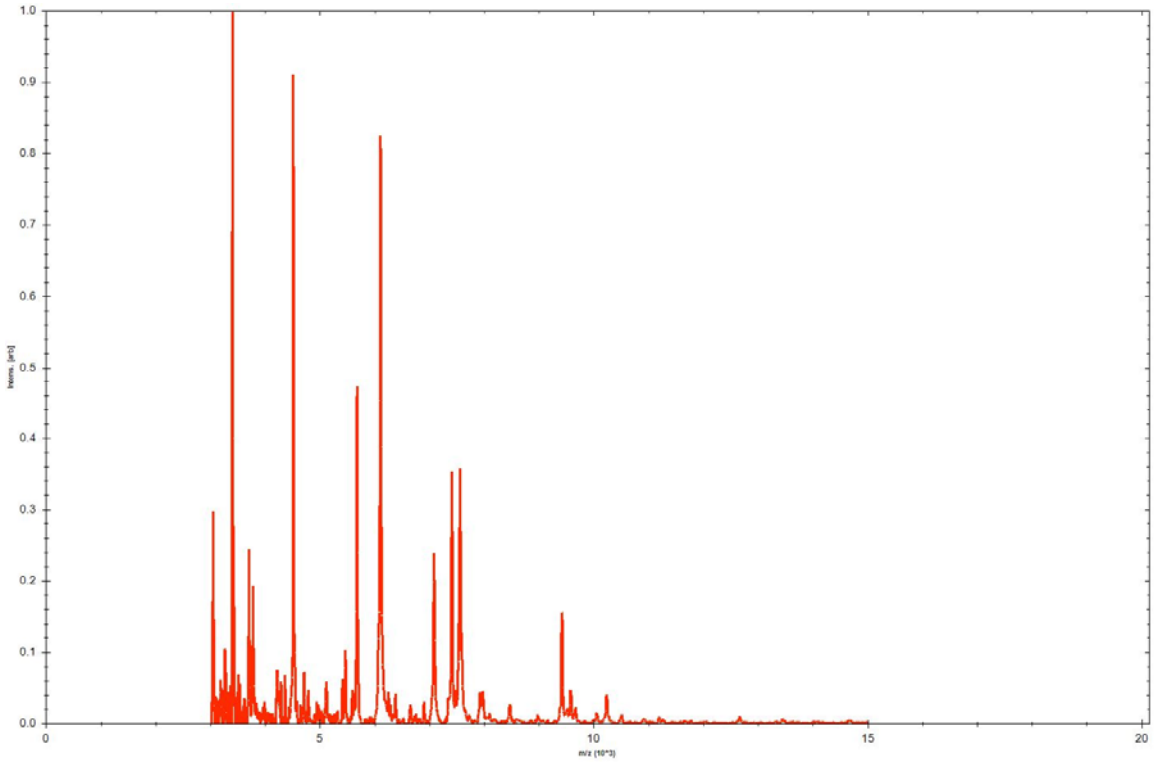

Figure 10. Normalized mass spectra for *P. intermedia* clinical isolate #10 resistant in vitro to 8 μg/ml of amoxicillin.

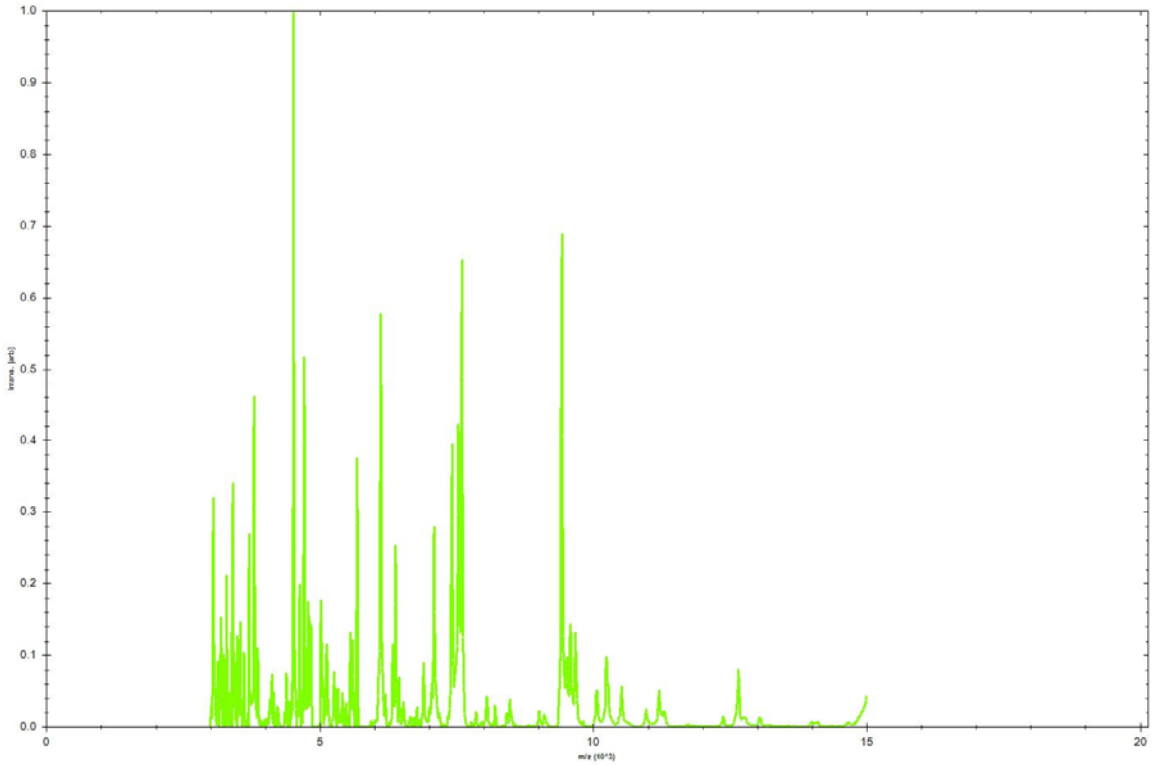

Figure 11. Normalized mass spectra for *P. intermedia* clinical isolate #11 resistant in vitro to 8 μg/ml of amoxicillin.

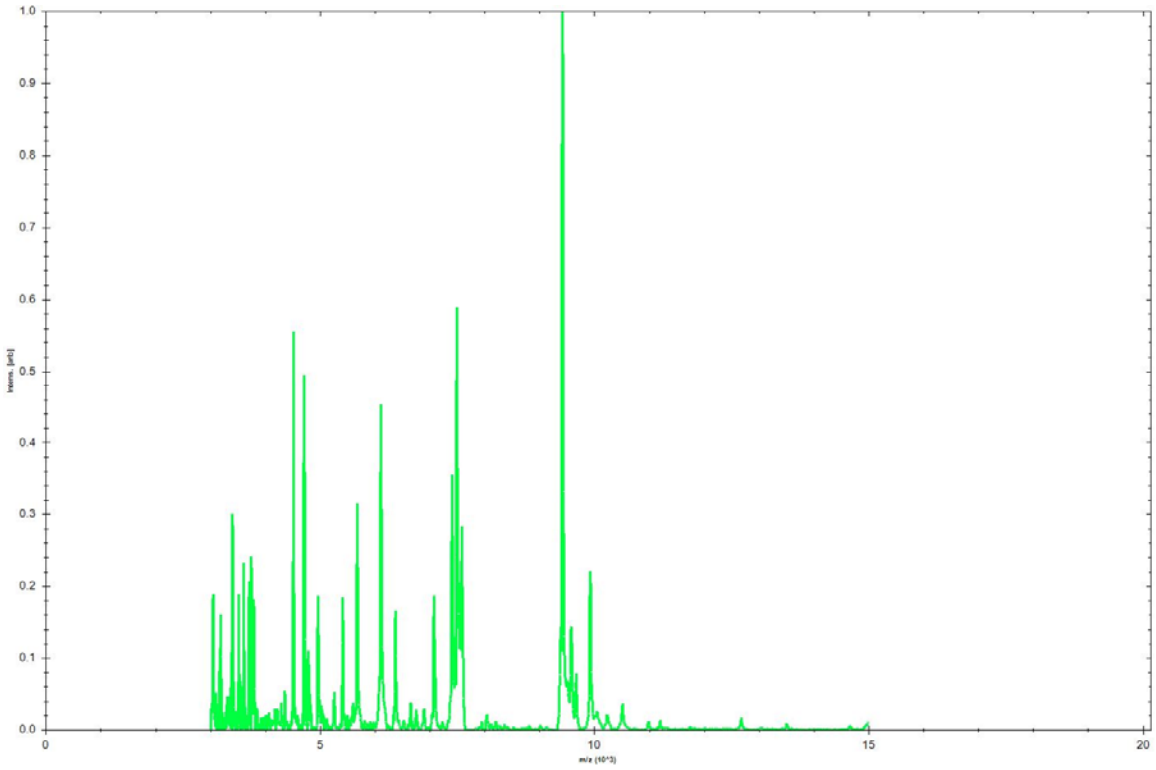

Figure 12. Normalized mass spectra for *P. intermedia* clinical isolate #12 resistant in vitro to 8 μg/ml of amoxicillin.

Visual comparison of normalized peak list spectrum representations for amoxicillin-susceptible (Figures 13 and 14) and amoxicillin-resistant (Figures 15-24) of *P. intermedia* similarly failed to reveal consistently reproducible differences in their distribution of mass spectra peaks.

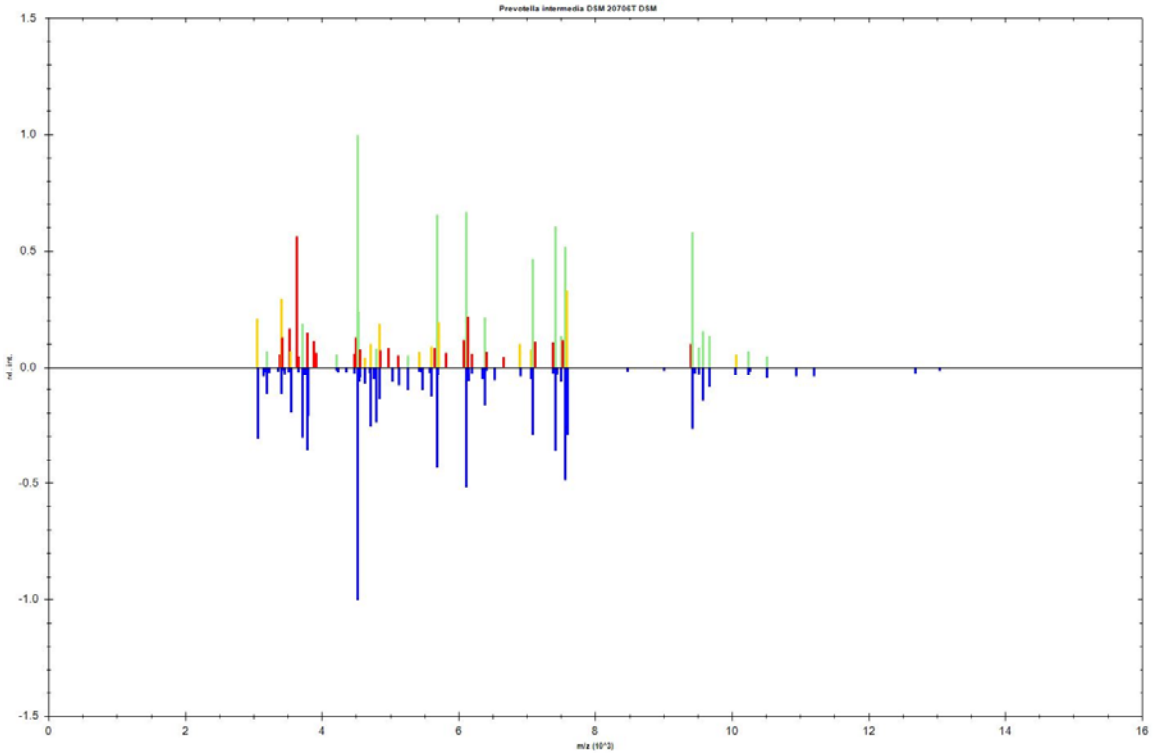

Figure 13. Normalized peak list spectrum for *P. intermedia* clinical isolate #1 susceptible in vitro to 8 μg/ml of amoxicillin (top, with green peaks representing a match, yellow a partial match, and red no match to mass spectra peaks of *P. intermedia*, bottom, in Biotyper reference library).

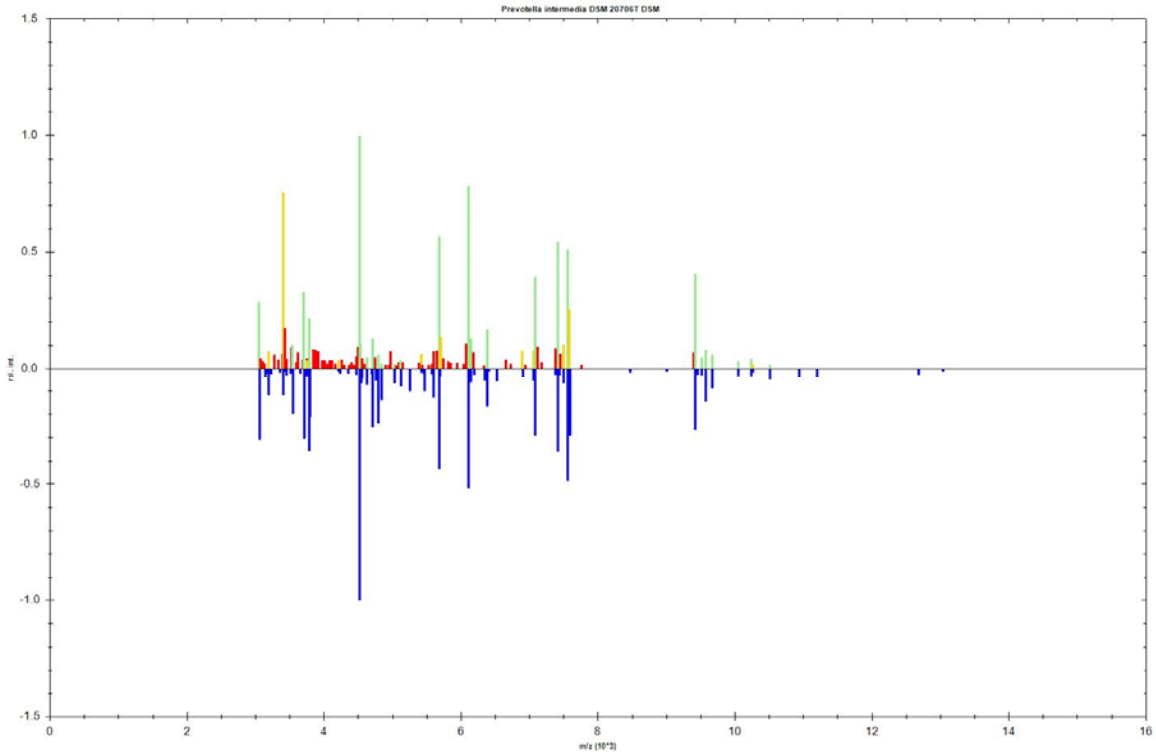

Figure 14. Normalized peak list spectrum for *P. intermedia* clinical isolate #2 susceptible in vitro to 8 µg/ml of amoxicillin (top, with green peaks representing a match, yellow a partial match, and red no match to mass spectra peaks of *P. intermedia*, bottom, in Biotyper reference library).

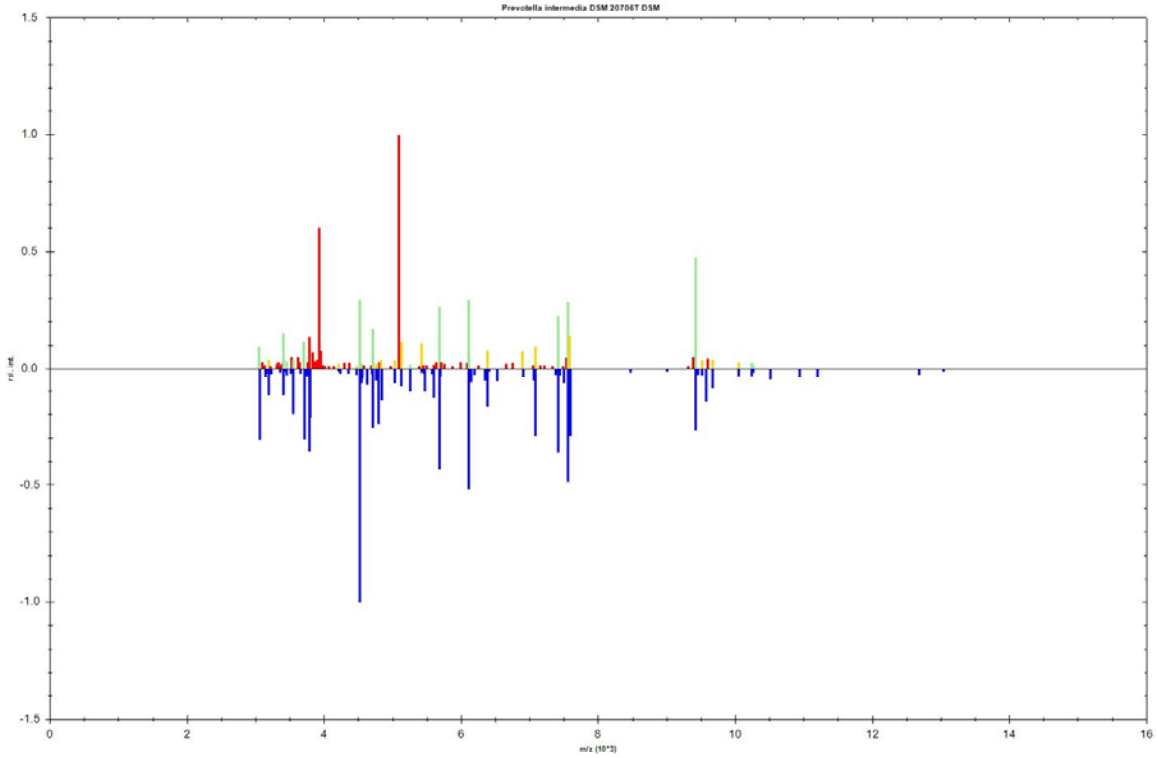

Figure 15. Normalized peak list spectrum for *P. intermedia* clinical isolate #3 resistant in vitro to 8 μg/ml of amoxicillin (top, with green peaks representing a match, yellow a partial match, and red no match to mass spectra peaks of *P. intermedia*, bottom, in Biotyper reference library).

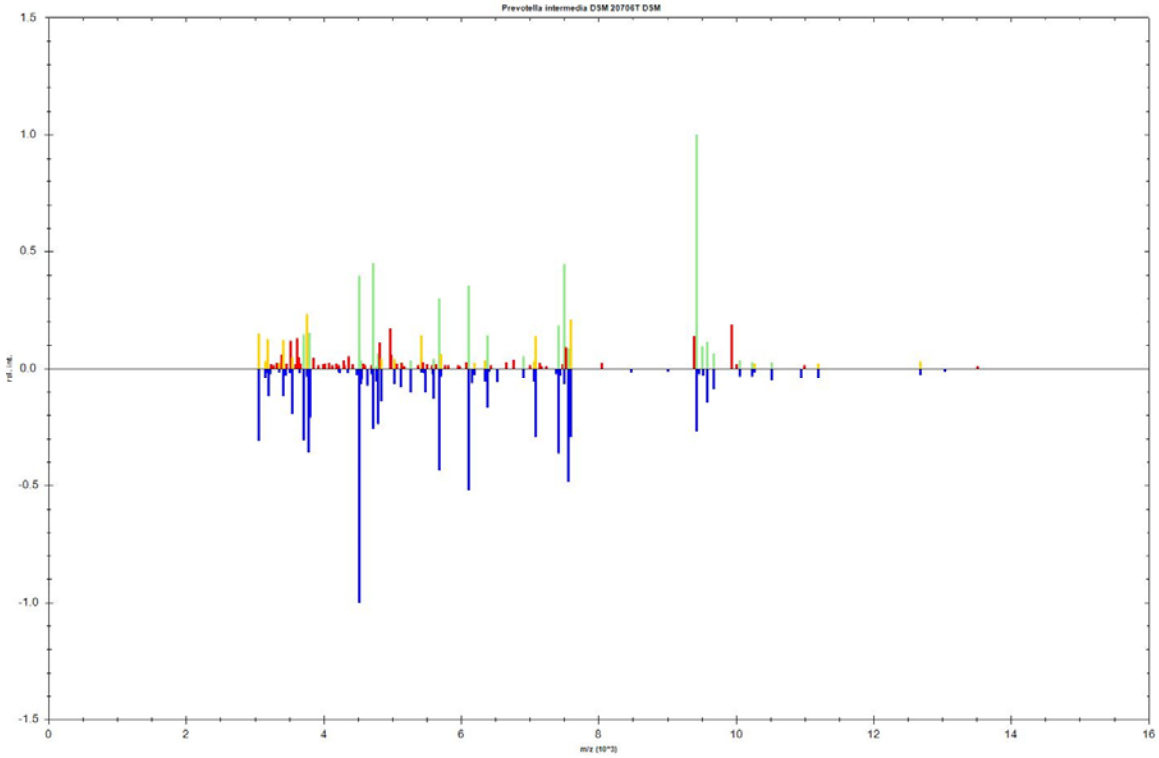

Figure 16. Normalized peak list spectrum for *P. intermedia* clinical isolate #4 resistant in vitro to 8 μg/ml of amoxicillin (top, with green peaks representing a match, yellow a partial match, and red no match to mass spectra peaks of *P. intermedia*, bottom, in Biotyper reference library).

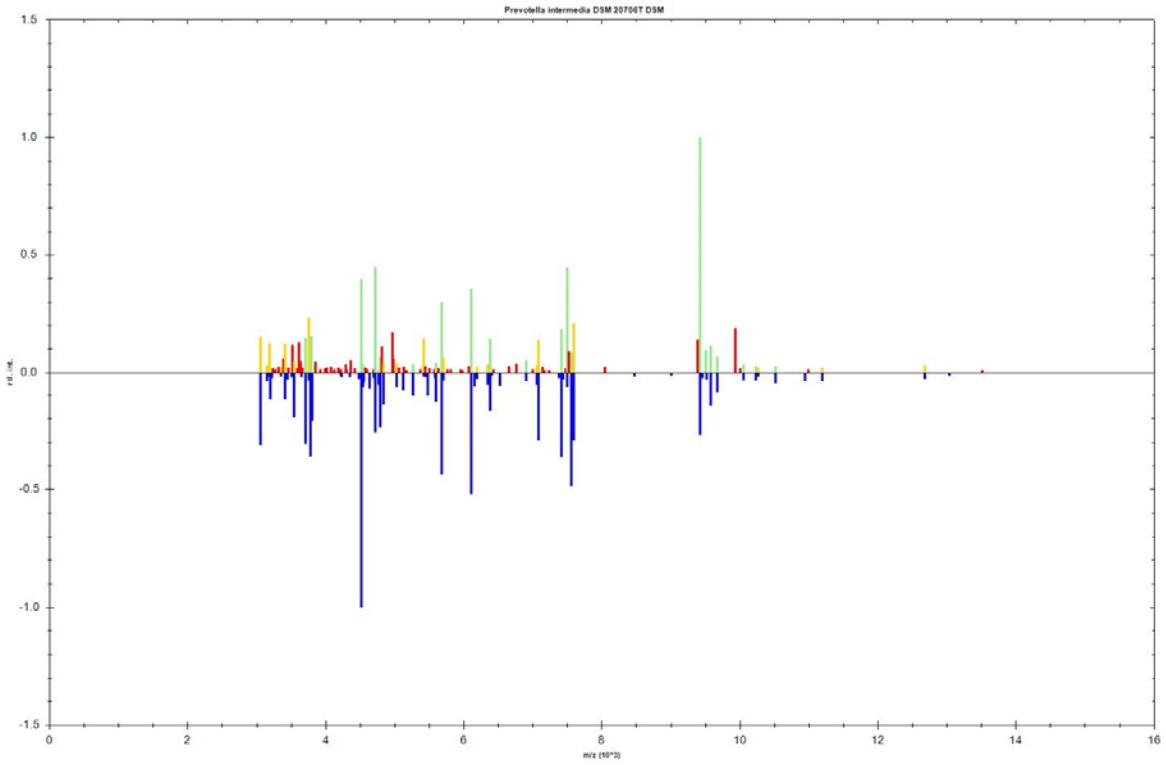

Figure 17. Normalized peak list spectrum for *P. intermedia* clinical isolate #5 resistant in vitro to 8 µg/ml of amoxicillin (top, with green peaks representing a match, yellow a partial match, and red no match to mass spectra peaks of *P. intermedia*, bottom, in Biotyper reference library).

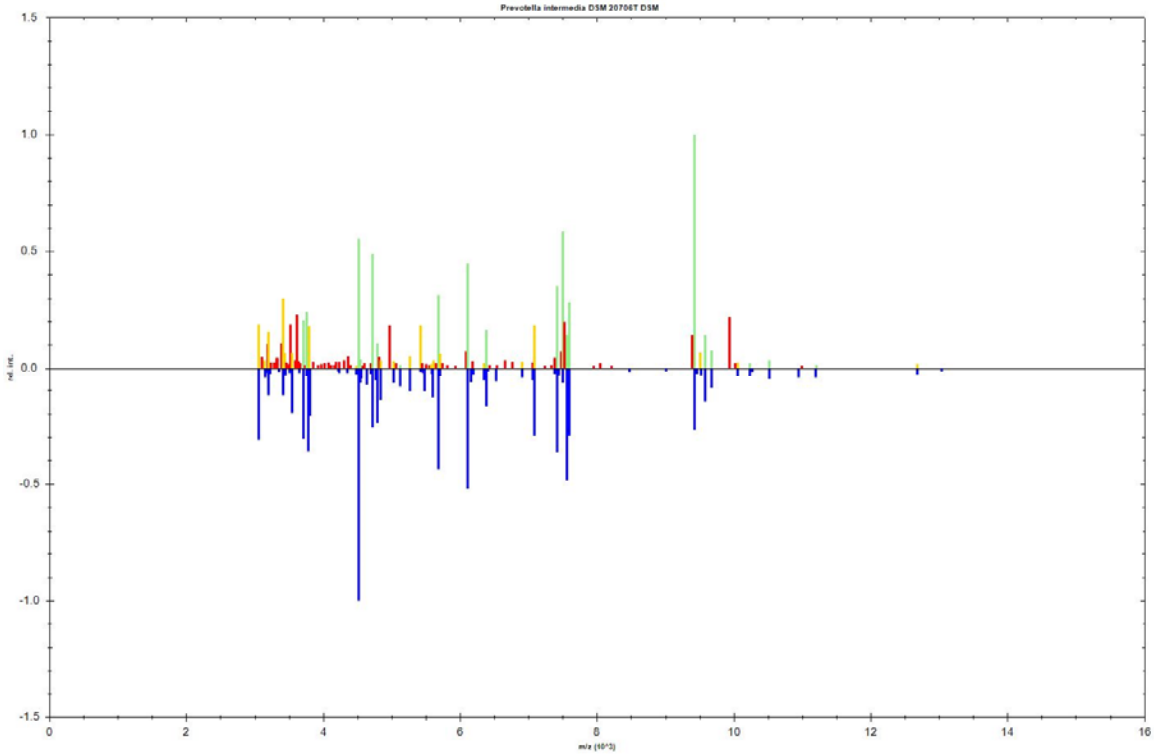

Figure 18. Normalized peak list spectrum for *P. intermedia* clinical isolate #6 resistant in vitro to 8 μg/ml of amoxicillin (top, with green peaks representing a match, yellow a partial match, and red no match to mass spectra peaks of *P. intermedia*, bottom, in Biotyper reference library).

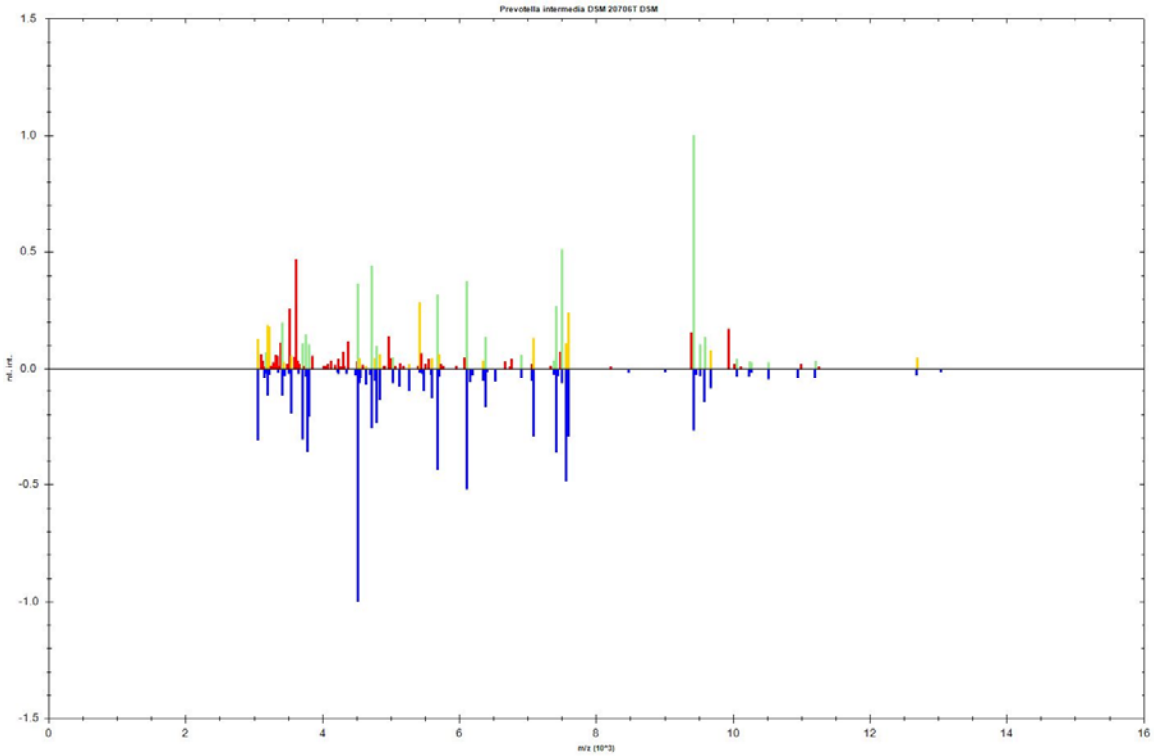

Figure 19. Normalized peak list spectrum for *P. intermedia* clinical isolate #7 resistant in vitro to 8 µg/ml of amoxicillin (top, with green peaks representing a match, yellow a partial match, and red no match to mass spectra peaks of *P. intermedia*, bottom, in Biotyper reference library).

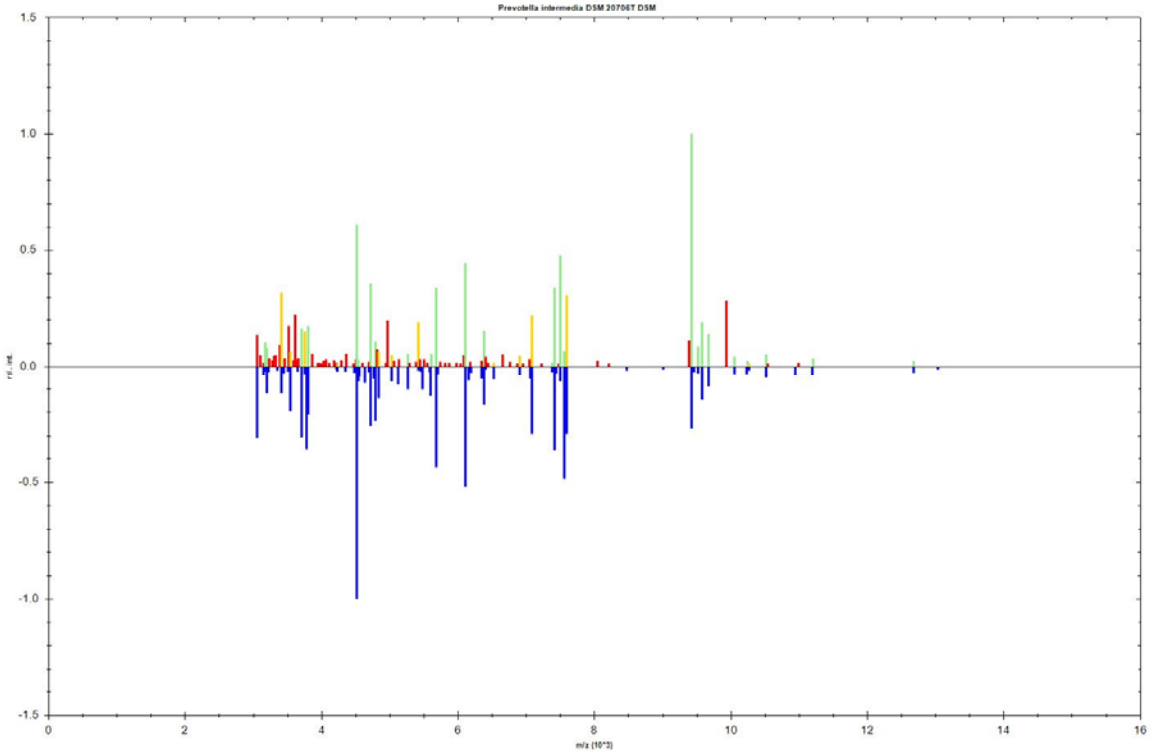

Figure 20. Normalized peak list spectrum for *P. intermedia* clinical isolate #8 resistant in vitro to 8 µg/ml of amoxicillin (top, with green peaks representing a match, yellow a partial match, and red no match to mass spectra peaks of *P. intermedia*, bottom, in Biotyper reference library).

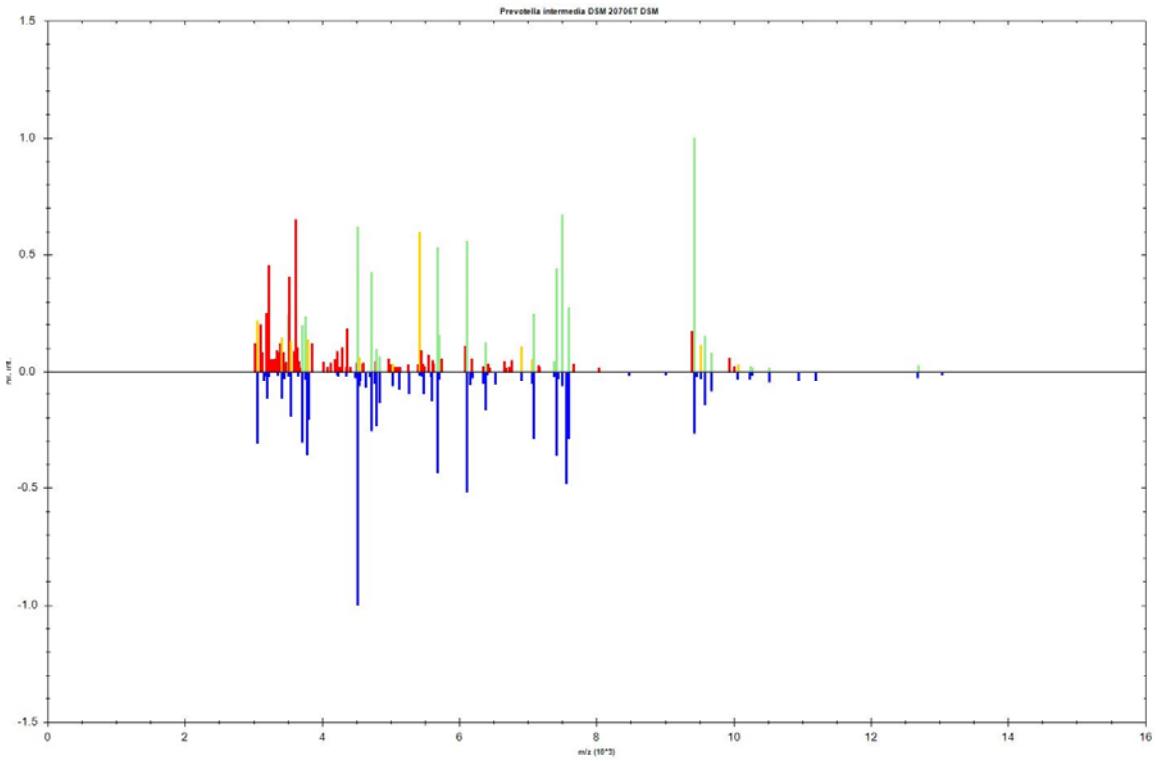

Figure 21. Normalized peak list spectrum for *P. intermedia* clinical isolate #9 resistant in vitro to 8 µg/ml of amoxicillin (top, with green peaks representing a match, yellow a partial match, and red no match to mass spectra peaks of *P. intermedia*, bottom, in Biotyper reference library).

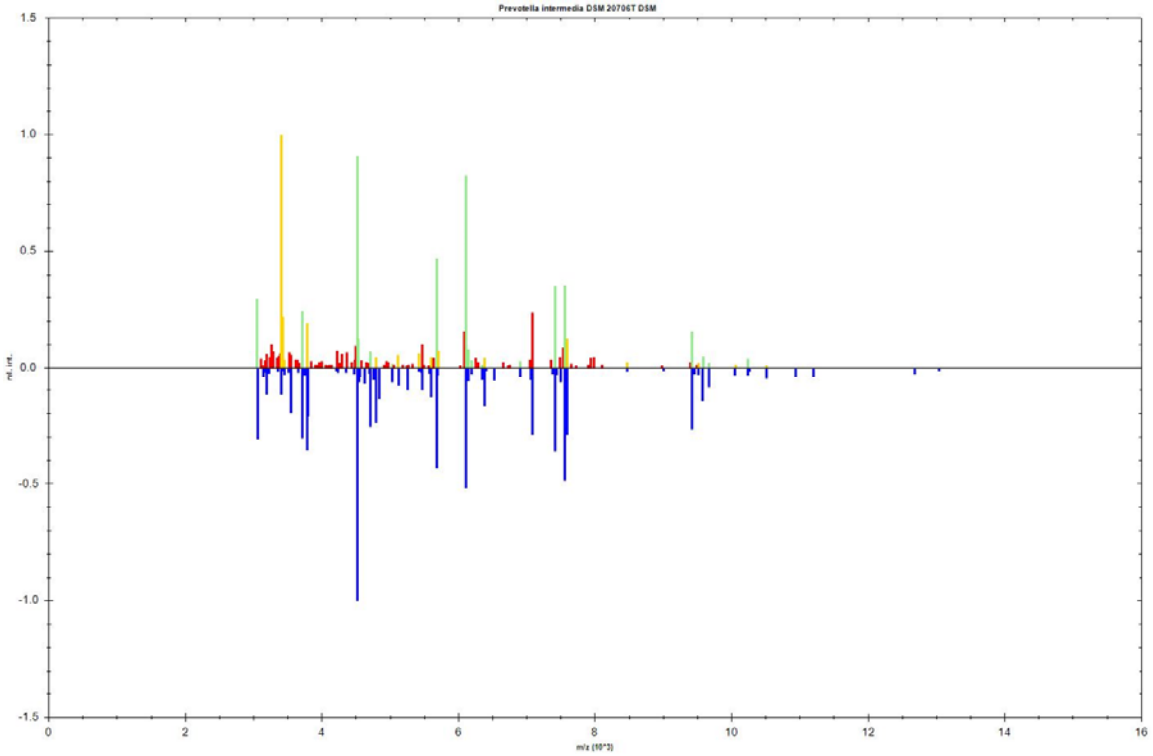

Figure 22. Normalized peak list spectrum for *P. intermedia* clinical isolate #10 resistant in vitro to 8 µg/ml of amoxicillin (top, with green peaks representing a match, yellow a partial match, and red no match to mass spectra peaks of *P. intermedia*, bottom, in Biotyper reference library).

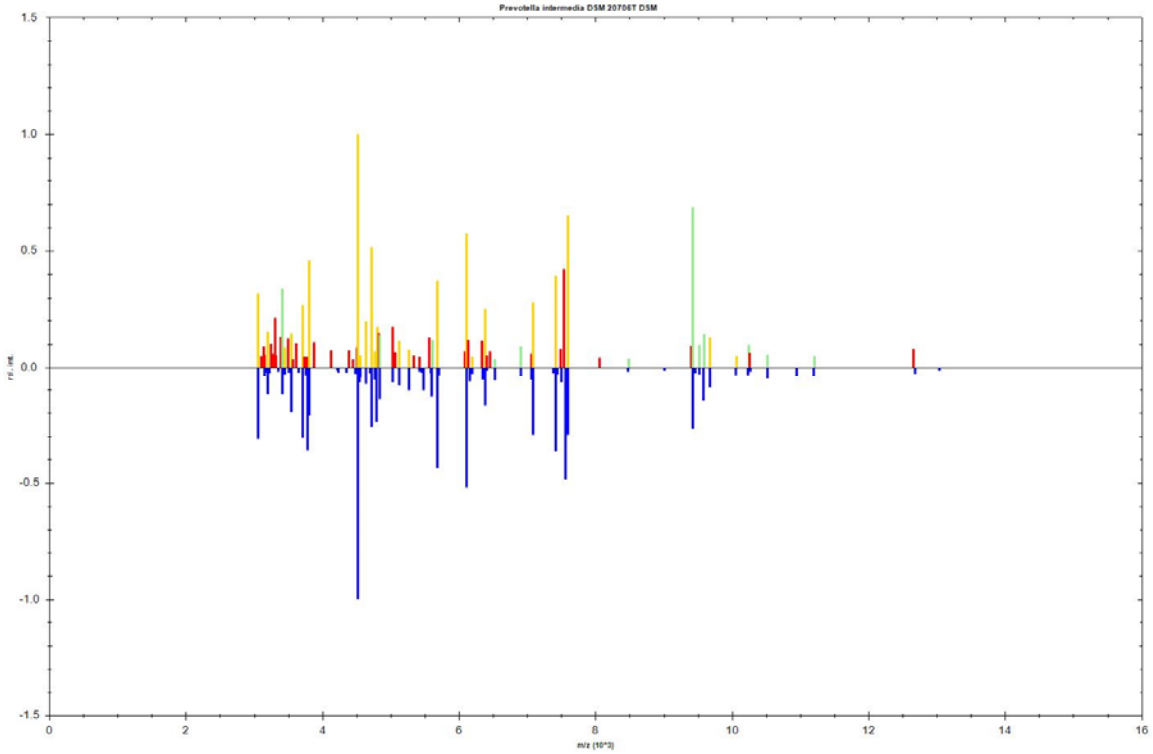

Figure 23. Normalized peak list spectrum for *P. intermedia* clinical isolate #11 resistant in vitro to 8 μg/ml of amoxicillin (top, with green peaks representing a match, yellow a partial match, and red no match to mass spectra peaks of *P. intermedia*, bottom, in Biotyper reference library).

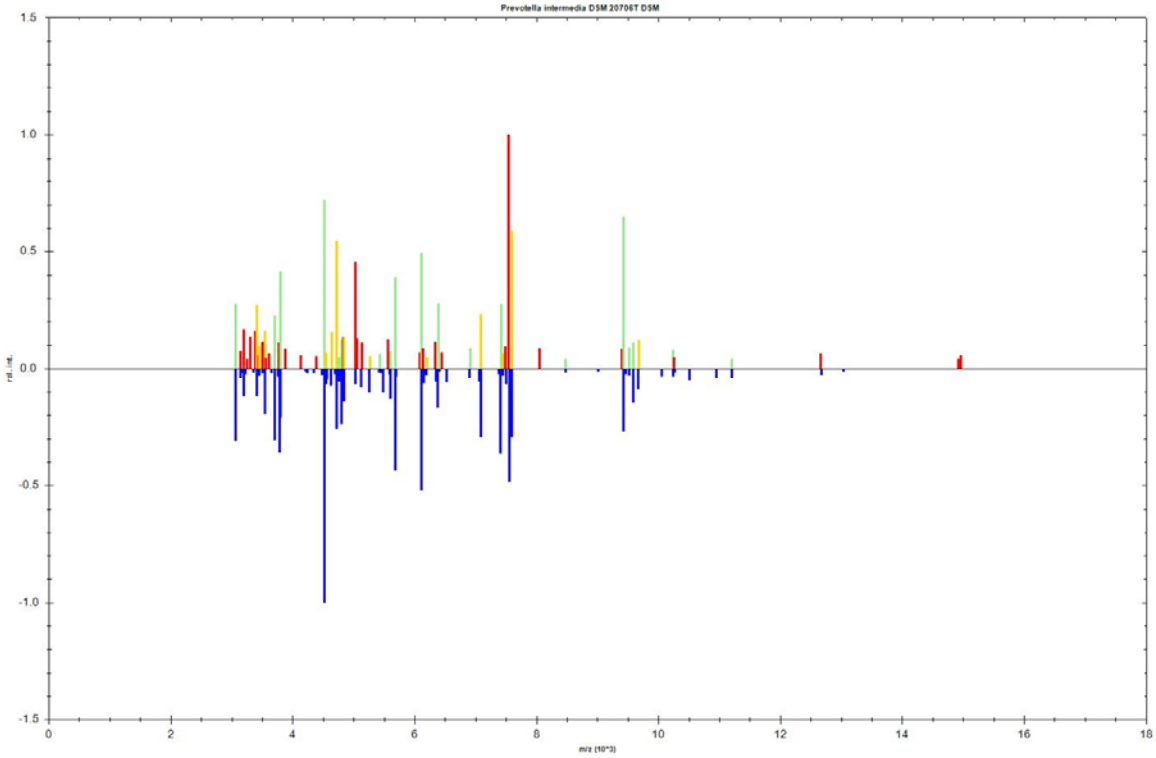

Figure 24. Normalized peak list spectrum for *P. intermedia* clinical isolate #12 resistant in vitro to 8 μg/ml of amoxicillin (top, with green peaks representing a match, yellow a partial match, and red no match to mass spectra peaks of *P. intermedia*, bottom, in Biotyper reference library).

Consistent with these findings for *P. intermedia*, visual comparison of normalized raw mass spectra for amoxicillin-susceptible (Figure 25) and amoxicillin-resistant (Figures 26-35) of *P. nigrescens* also failed to reveal consistently reproducible differences in their distribution of mass spectra peaks.

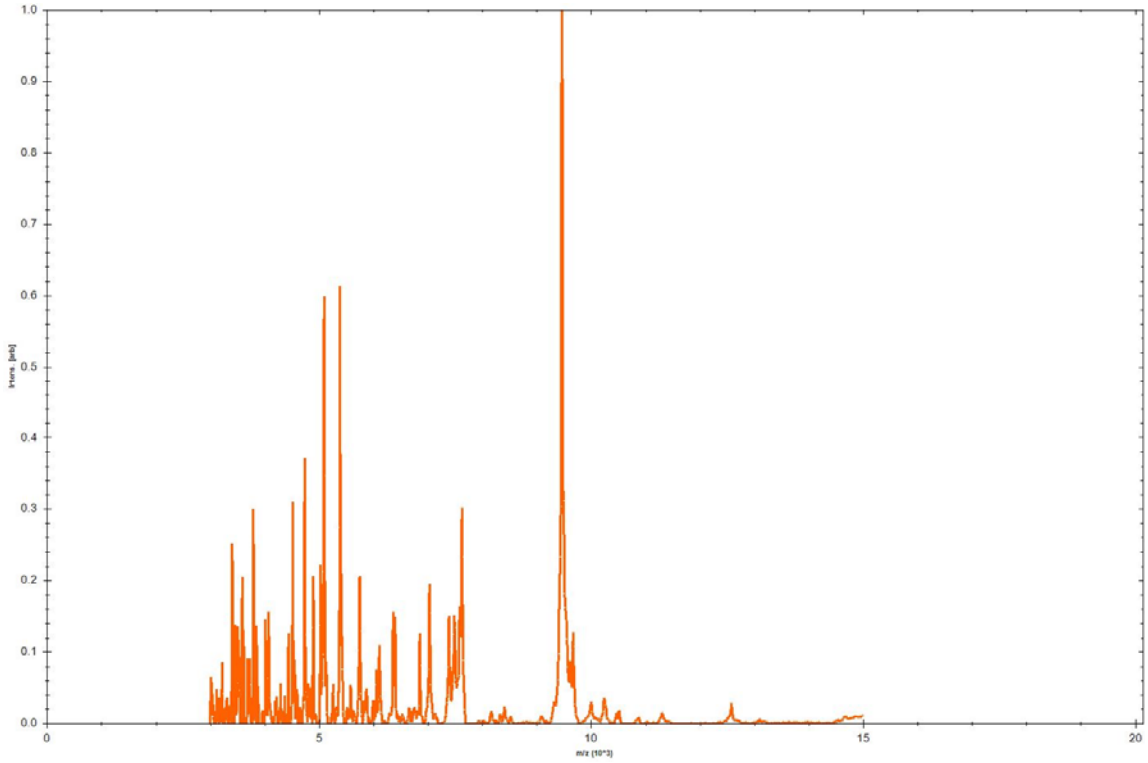

Figure 25. Normalized mass spectra for *P. nigrescens* clinical isolate #1 susceptible in vitro to 8 μg/ml of amoxicillin.

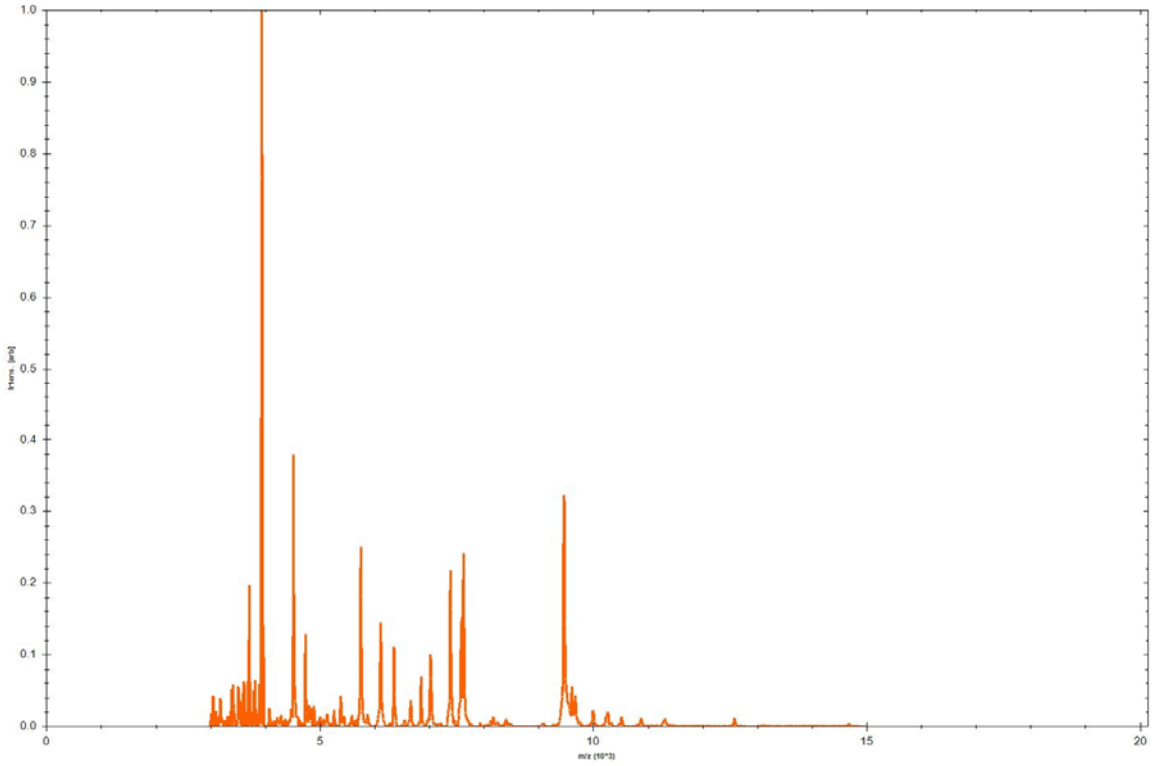

Figure 26. Normalized mass spectra for *P. nigrescens* clinical isolate #2 resistant in vitro to 8 µg/ml of amoxicillin.

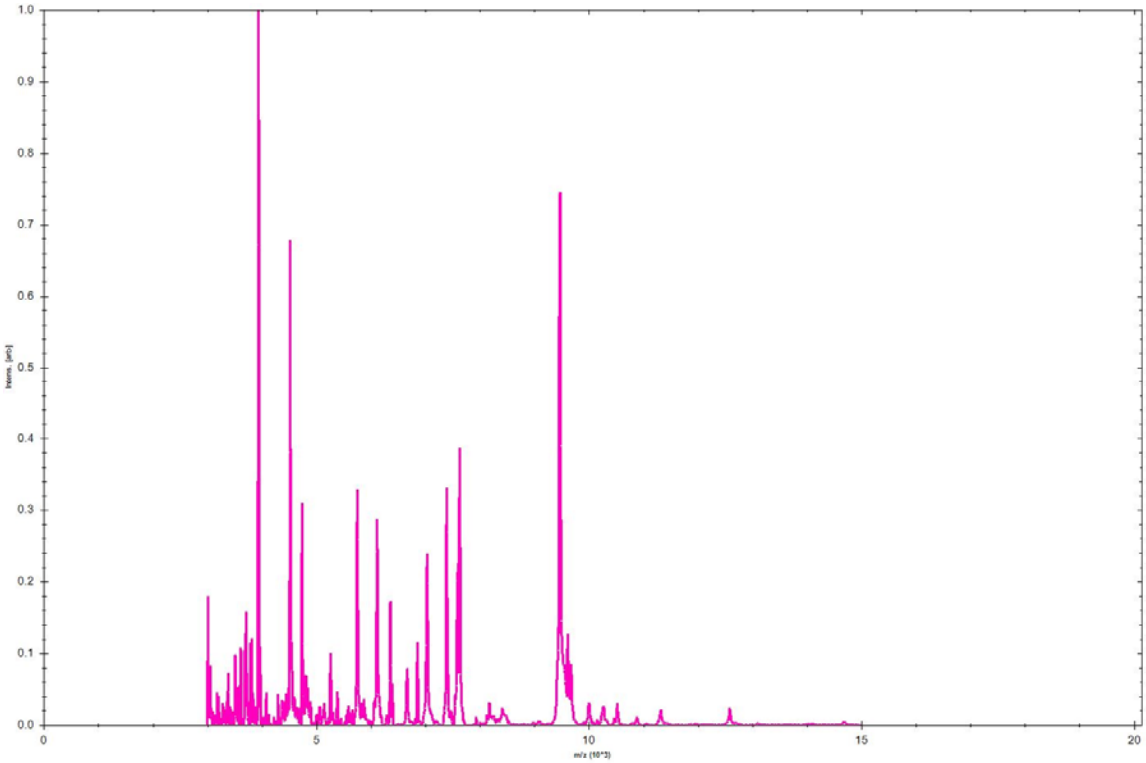

Figure 27. Normalized mass spectra for *P. nigrescens* clinical isolate #3 resistant in vitro to 8 μg/ml of amoxicillin.

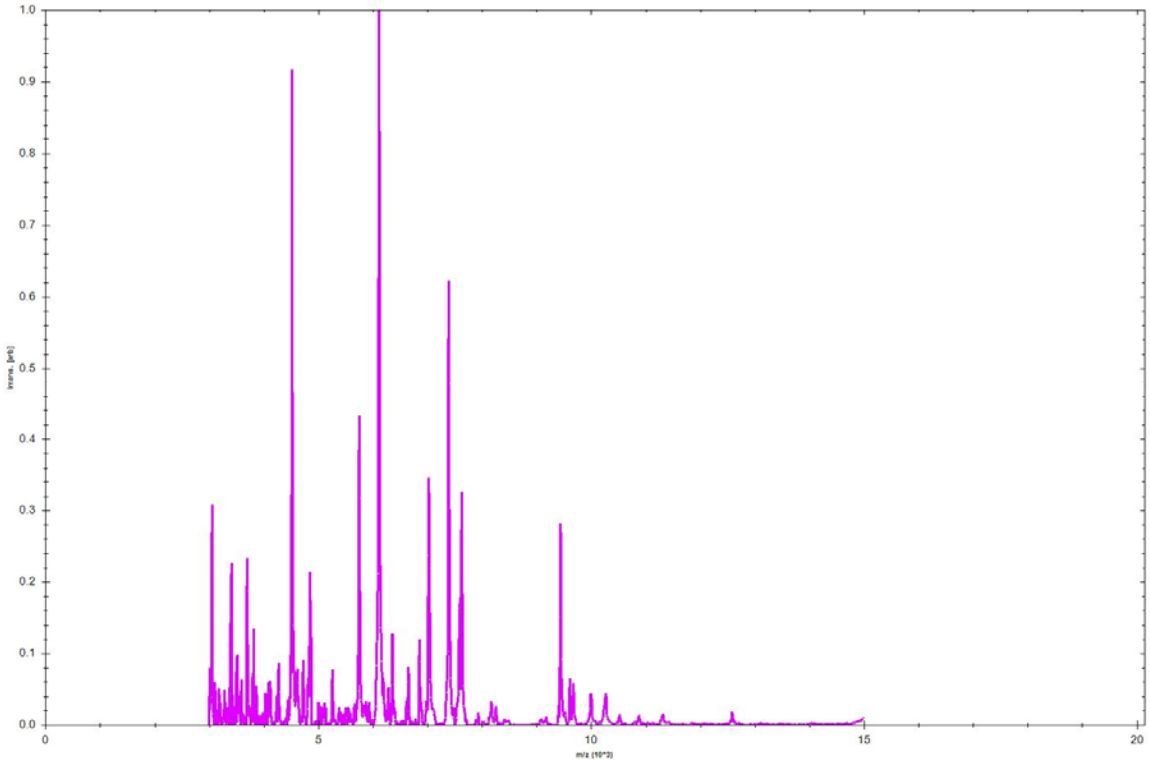

Figure 28. Normalized mass spectra for *P. nigrescens* clinical isolate #4 resistant in vitro to 8 μg/ml of amoxicillin.

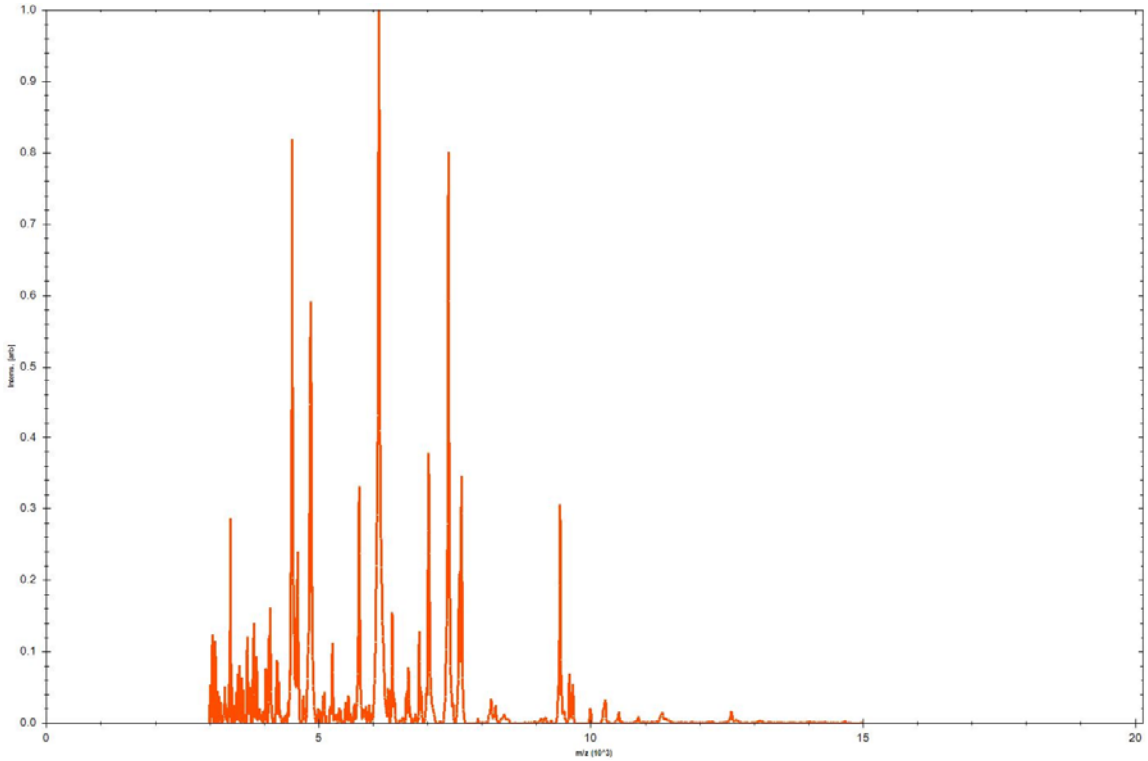

Figure 29. Normalized mass spectra for *P. nigrescens* clinical isolate #5 resistant in vitro to 8 μg/ml of amoxicillin.

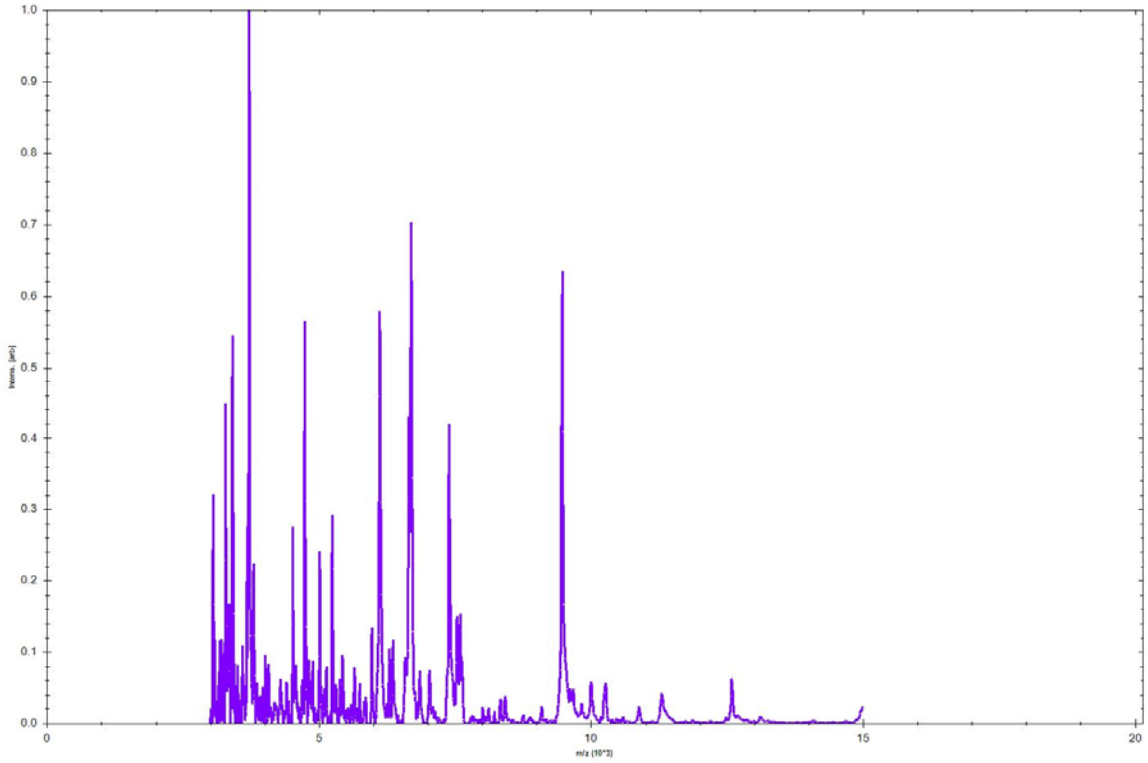

Figure 30. Normalized mass spectra for *P. nigrescens* clinical isolate #6 resistant in vitro to 8 μg/ml of amoxicillin.

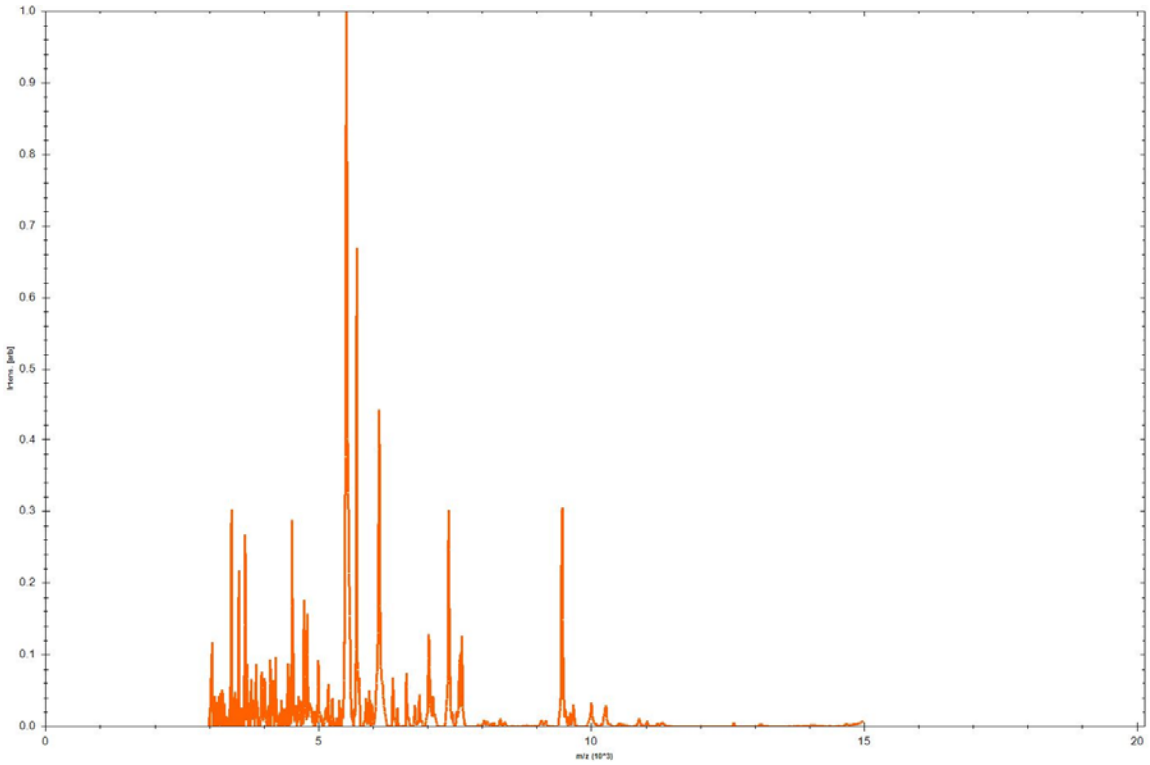

Figure 31. Normalized mass spectra for *P. nigrescens* clinical isolate #7 resistant in vitro to 8 μg/ml of amoxicillin.

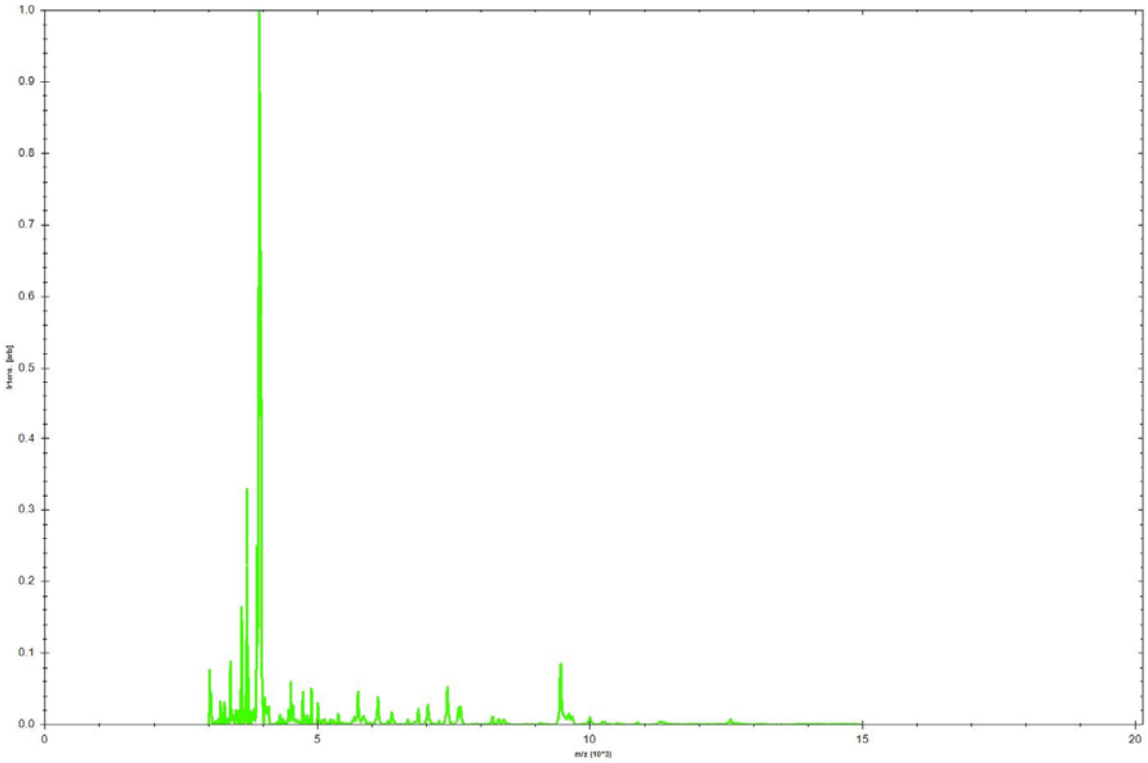

Figure 32. Normalized mass spectra for *P. nigrescens* clinical isolate #8 resistant in vitro to 8 μg/ml of amoxicillin.

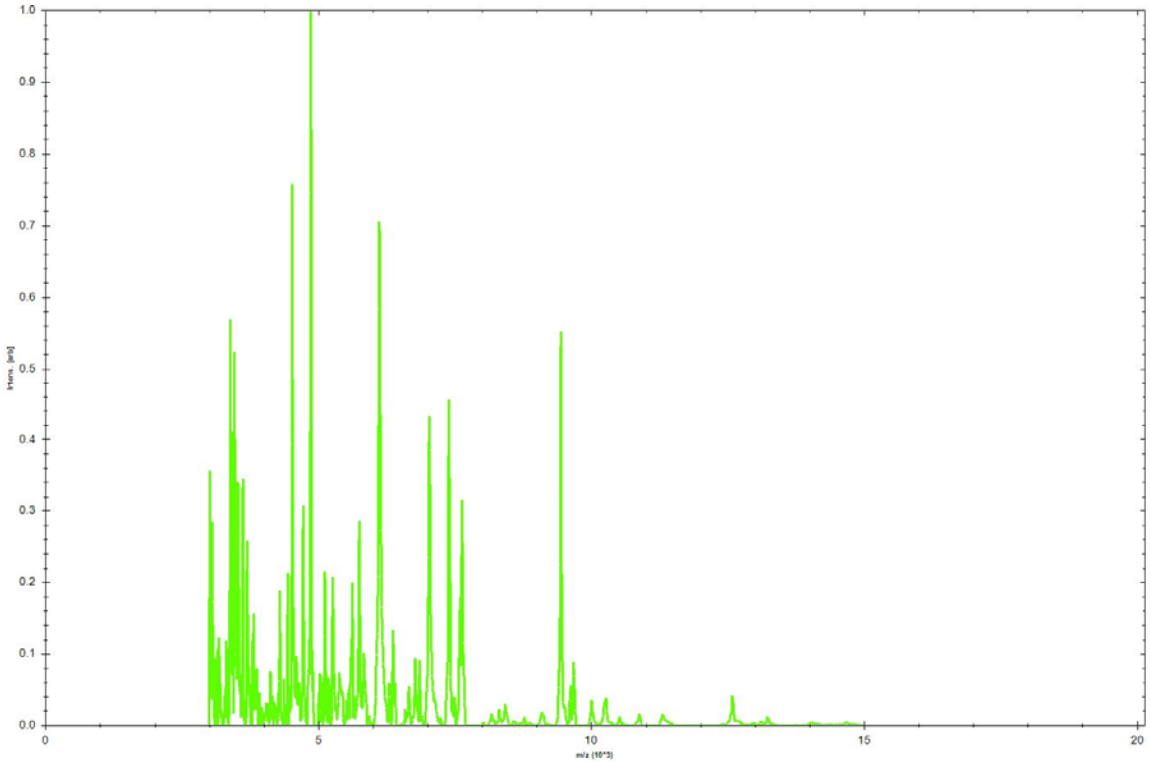

Figure 33. Normalized mass spectra for *P. nigrescens* clinical isolate #9 resistant in vitro to 8 μg/ml of amoxicillin.

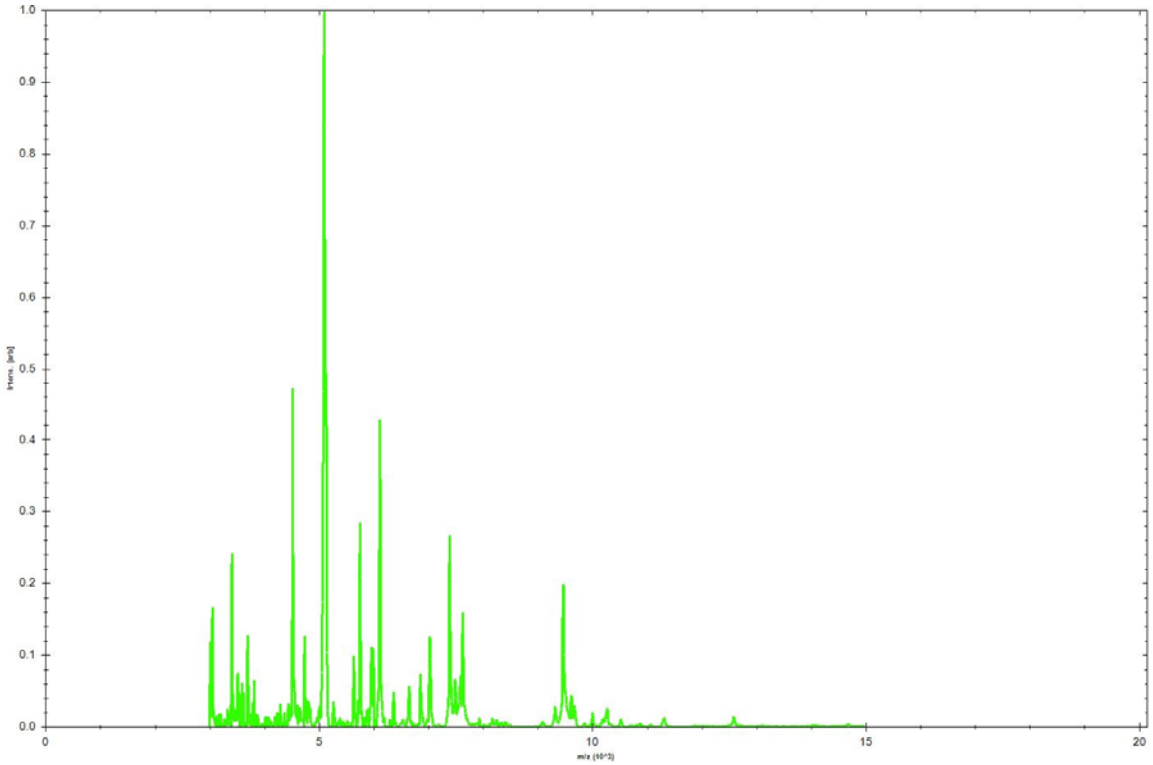

Figure 34. Normalized mass spectra for *P. nigrescens* clinical isolate #10 resistant in vitro to 8 μg/ml of amoxicillin.

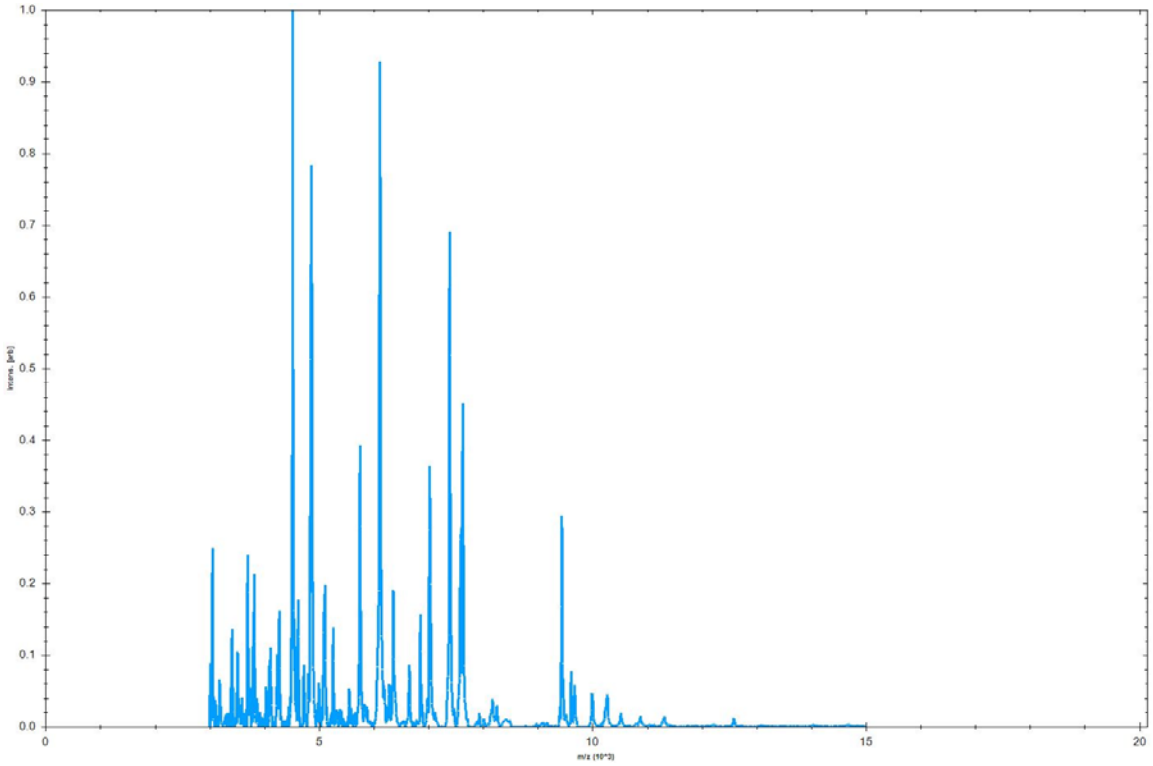

Figure 35. Normalized mass spectra for *P. nigrescens* clinical isolate #11 resistant in vitro to 8 μg/ml of amoxicillin.

Visual comparison of normalized peak list spectrum representations for amoxicillin-susceptible (Figure 36) and amoxicillin-resistant (Figures 37-46) of *P. nigrescens* also failed to reveal consistently reproducible differences in their distribution of mass spectra peaks.

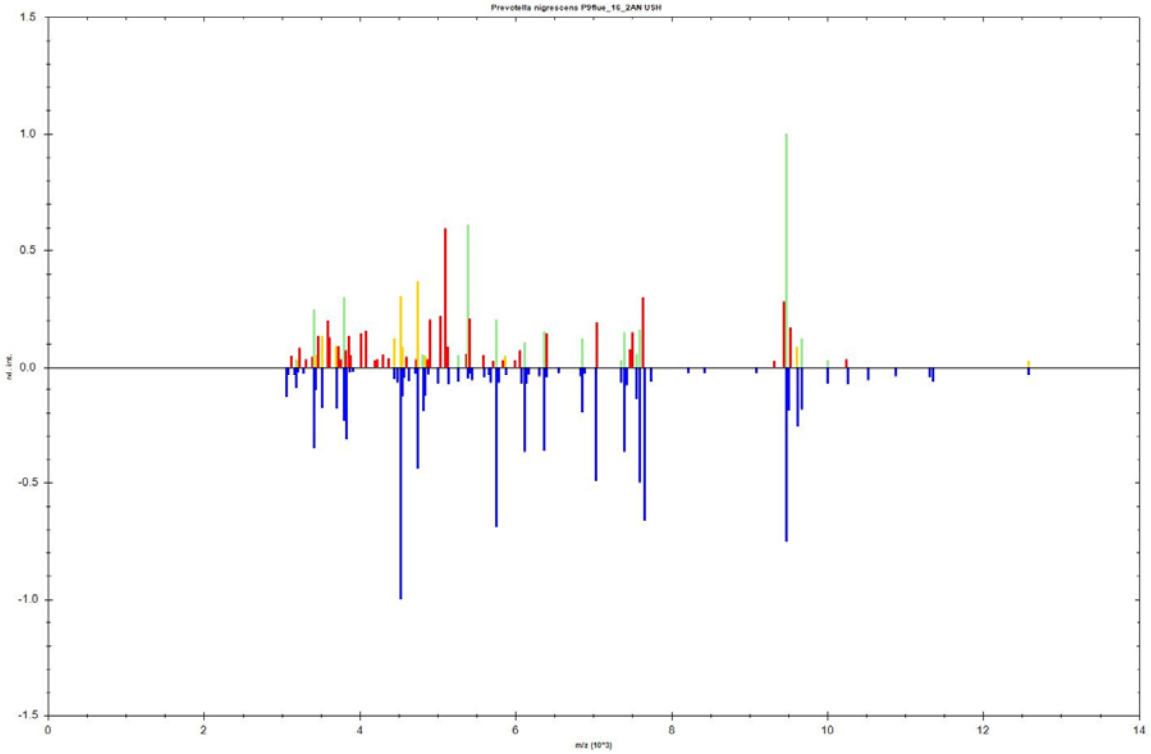

Figure 36. Normalized peak list spectrum for *P. nigrescens* clinical isolate #1 susceptible in vitro to 8 µg/ml of amoxicillin (top, with green peaks representing a match, yellow a partial match, and red no match to mass spectra peaks of *P. nigrescens*, bottom, in Biotyper reference library).

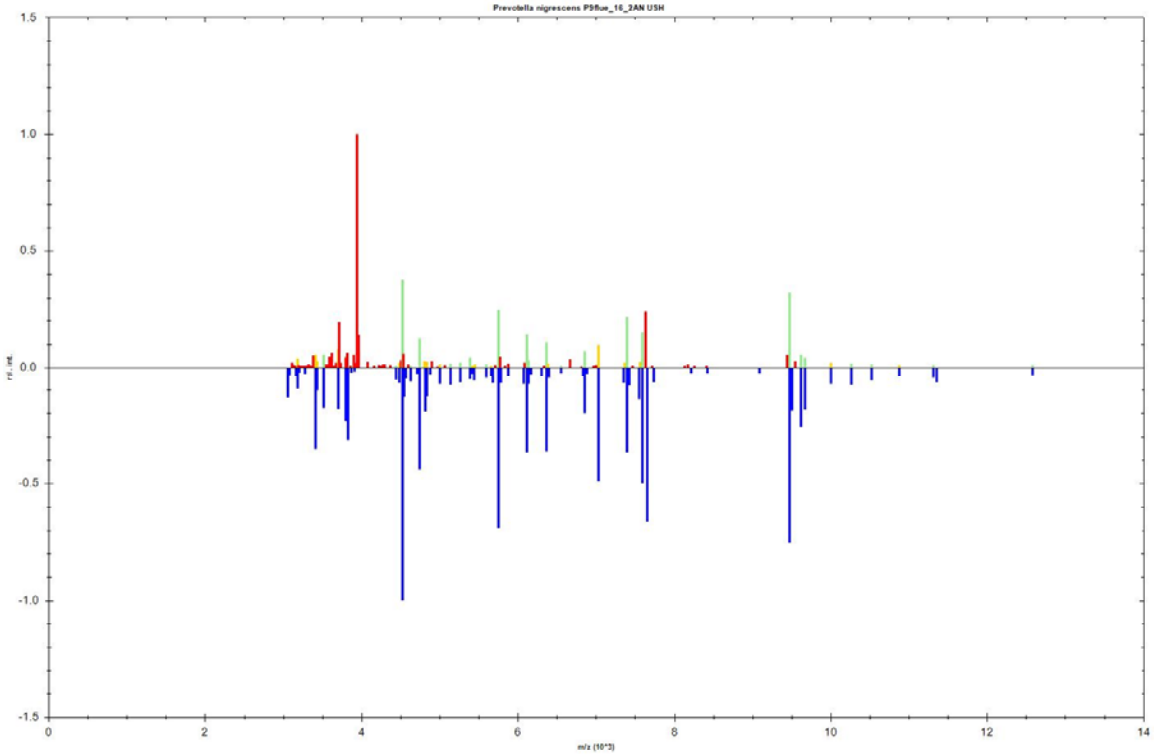

Figure 37. Normalized peak list spectrum for *P. nigrescens* clinical isolate #2 resistant in vitro to 8 µg/ml of amoxicillin (top, with green peaks representing a match, yellow a partial match, and red no match to mass spectra peaks of *P. nigrescens*, bottom, in Biotyper reference library).

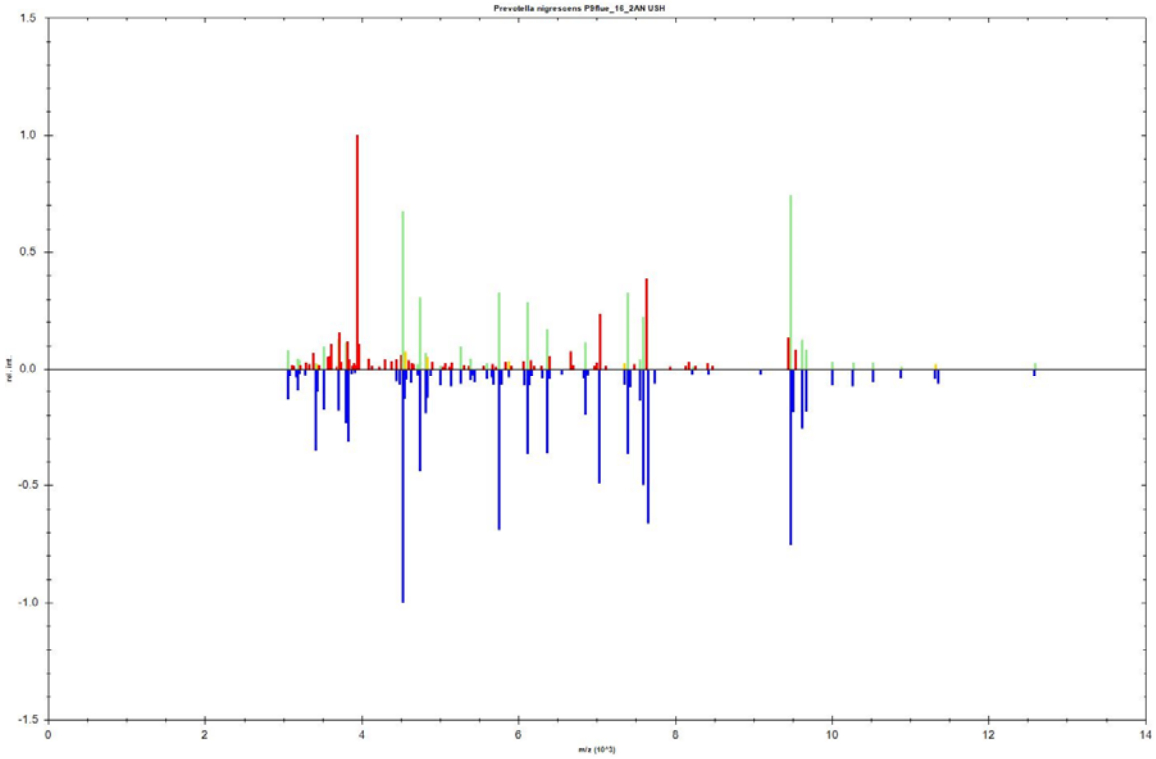

Figure 38. Normalized peak list spectrum for *P. nigrescens* clinical isolate #3 resistant in vitro to 8 μg/ml of amoxicillin (top, with green peaks representing a match, yellow a partial match, and red no match to mass spectra peaks of *P. nigrescens*, bottom, in Biotyper reference library).

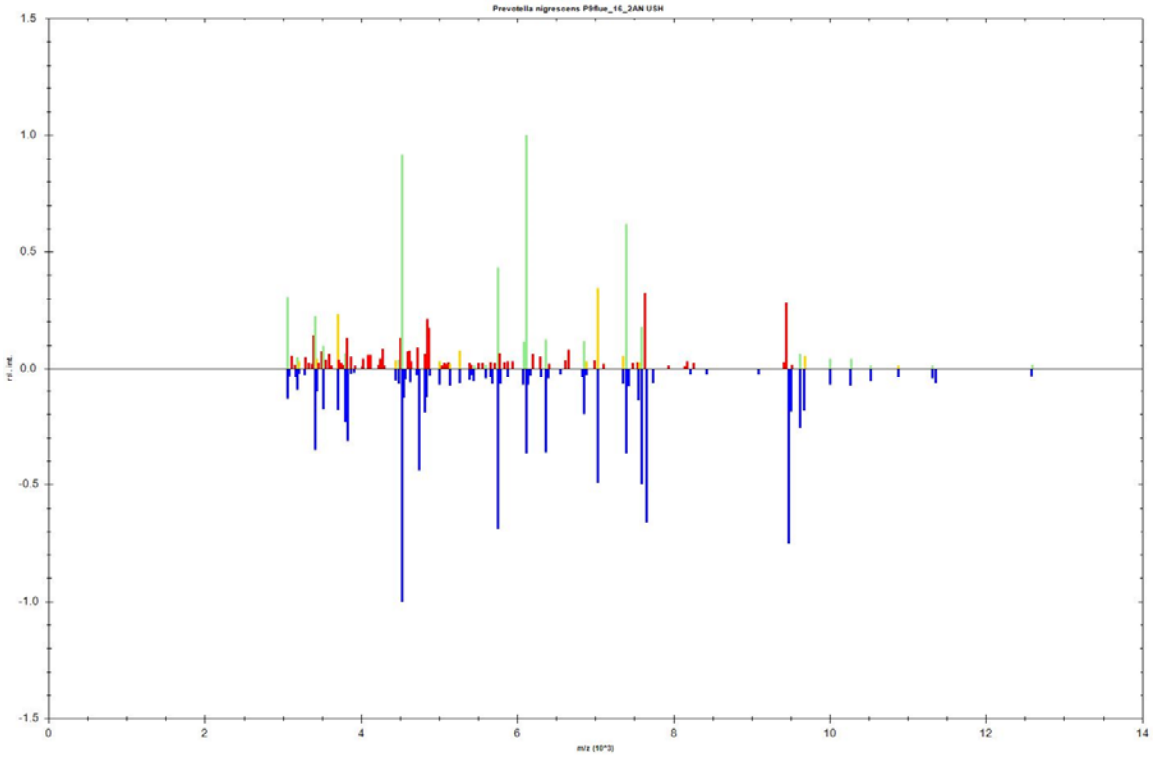

Figure 39. Normalized peak list spectrum for *P. nigrescens* clinical isolate #4 resistant in vitro to 8 µg/ml of amoxicillin (top, with green peaks representing a match, yellow a partial match, and red no match to mass spectra peaks of *P. nigrescens*, bottom, in Biotyper reference library).

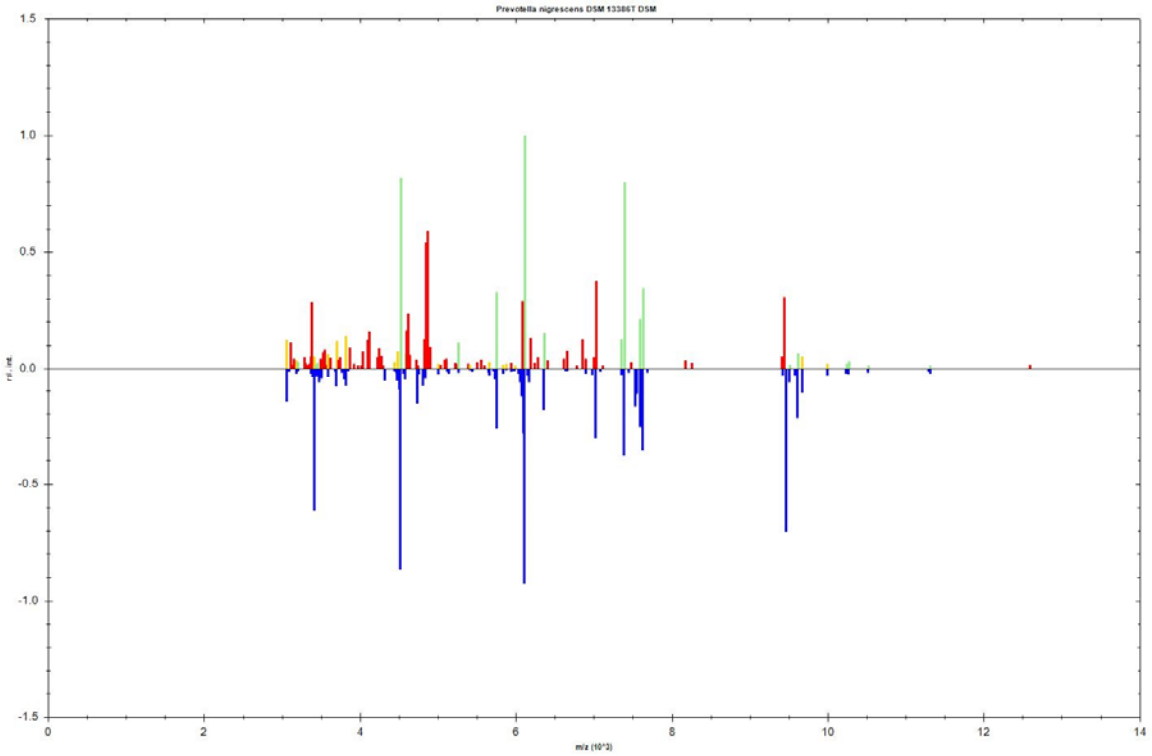

Figure 40. Normalized peak list spectrum for *P. nigrescens* clinical isolate #5 resistant in vitro to 8 µg/ml of amoxicillin (top, with green peaks representing a match, yellow a partial match, and red no match to mass spectra peaks of *P. nigrescens*, bottom, in Biotyper reference library).

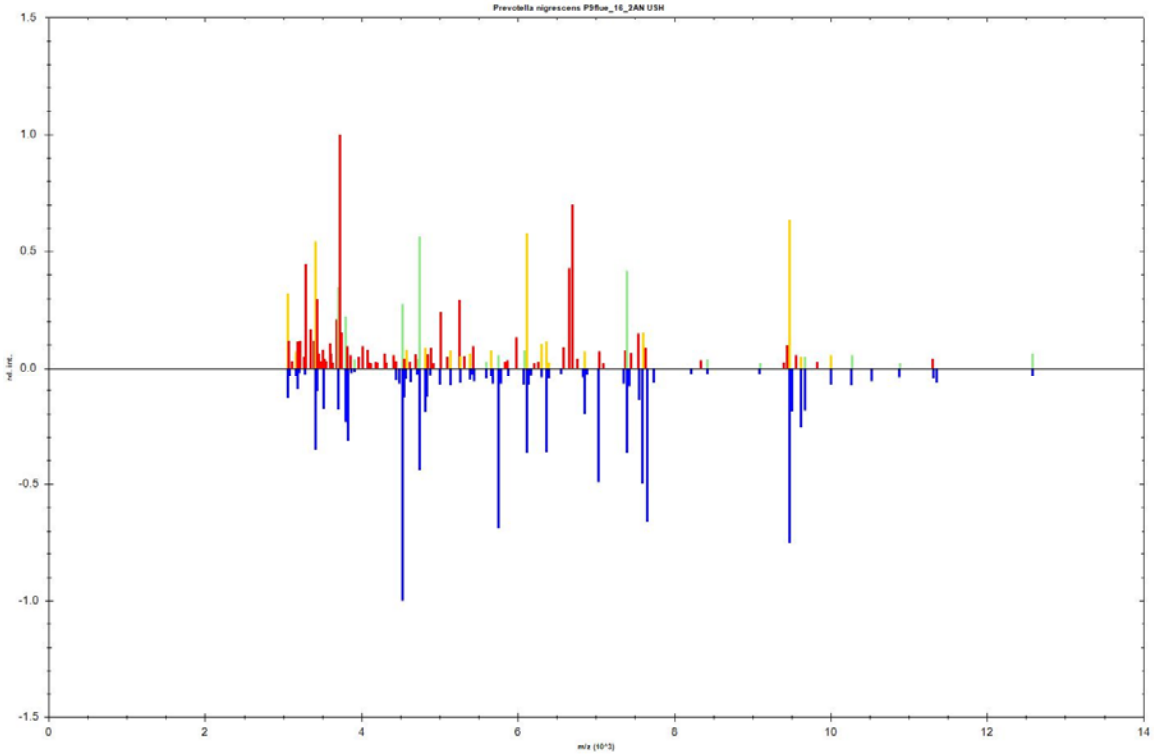

Figure 41. Normalized peak list spectrum for *P. nigrescens* clinical isolate #6 resistant in vitro to 8 μg/ml of amoxicillin (top, with green peaks representing a match, yellow a partial match, and red no match to mass spectra peaks of *P. nigrescens*, bottom, in Biotyper reference library).

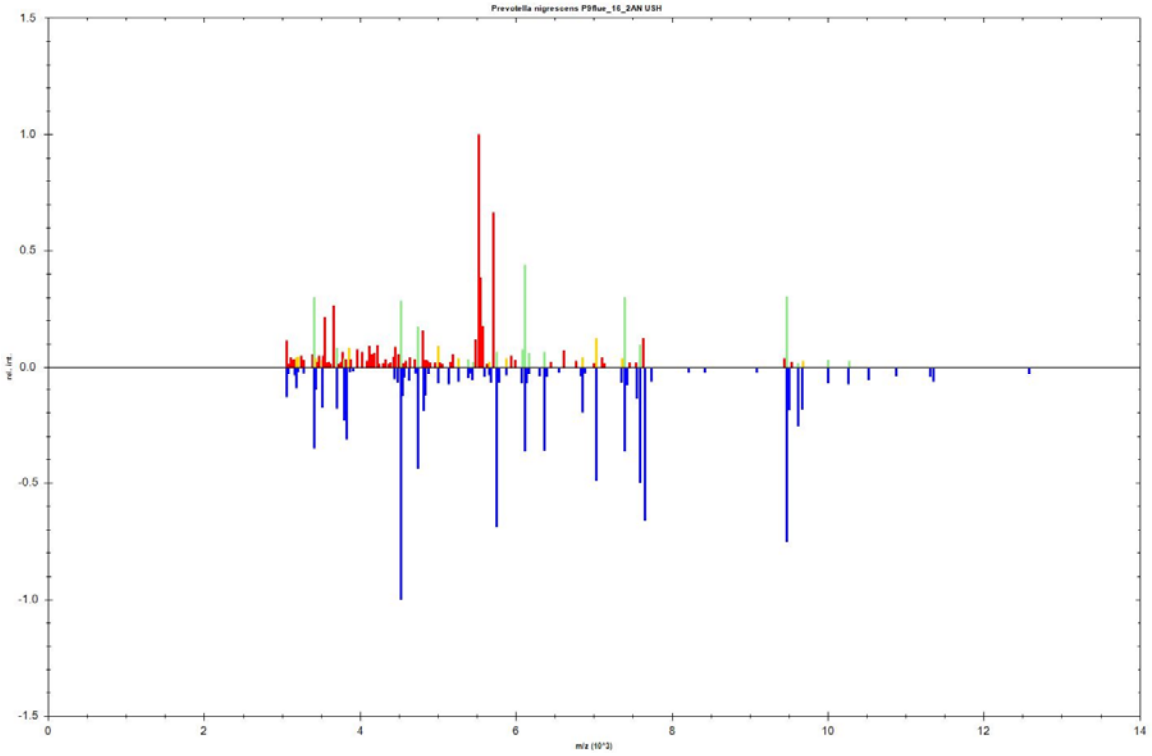

Figure 42. Normalized peak list spectrum for *P. nigrescens* clinical isolate #7 resistant in vitro to 8 µg/ml of amoxicillin (top, with green peaks representing a match, yellow a partial match, and red no match to mass spectra peaks of *P. nigrescens*, bottom, in Biotyper reference library).

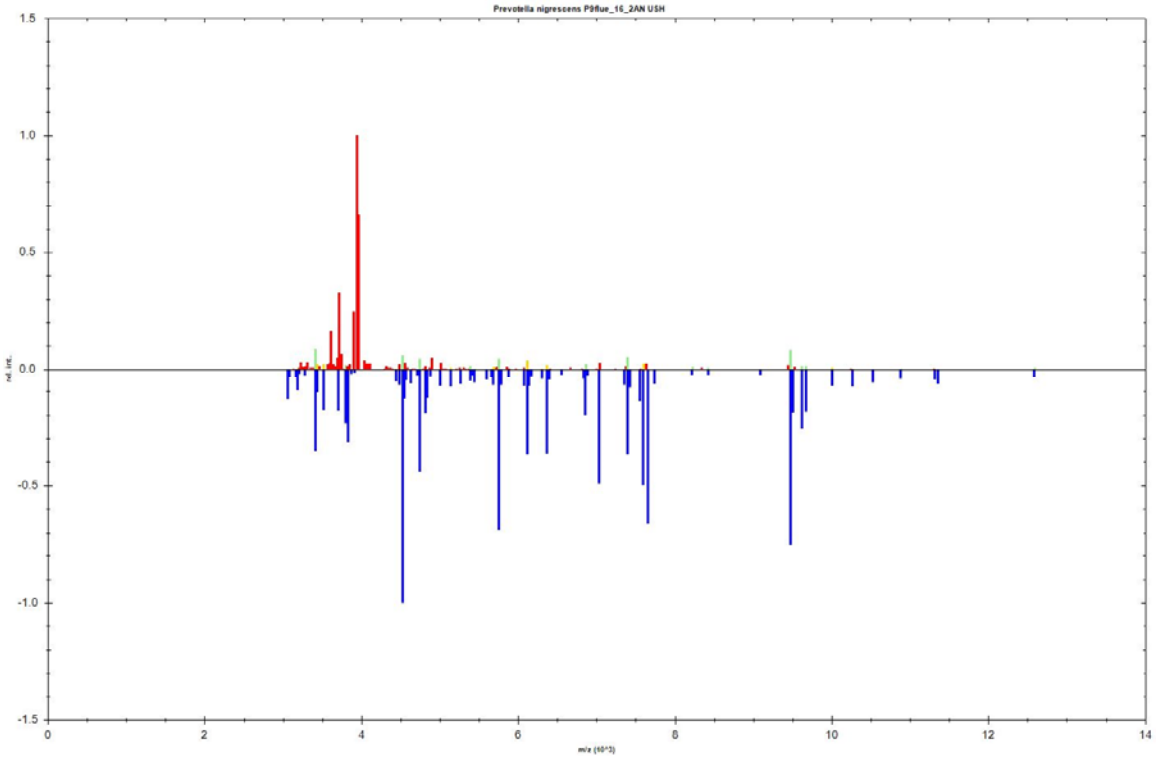

Figure 43. Normalized peak list spectrum for *P. nigrescens* clinical isolate #8 resistant in vitro to 8 μg/ml of amoxicillin (top, with green peaks representing a match, yellow a partial match, and red no match to mass spectra peaks of *P. nigrescens*, bottom, in Biotyper reference library).

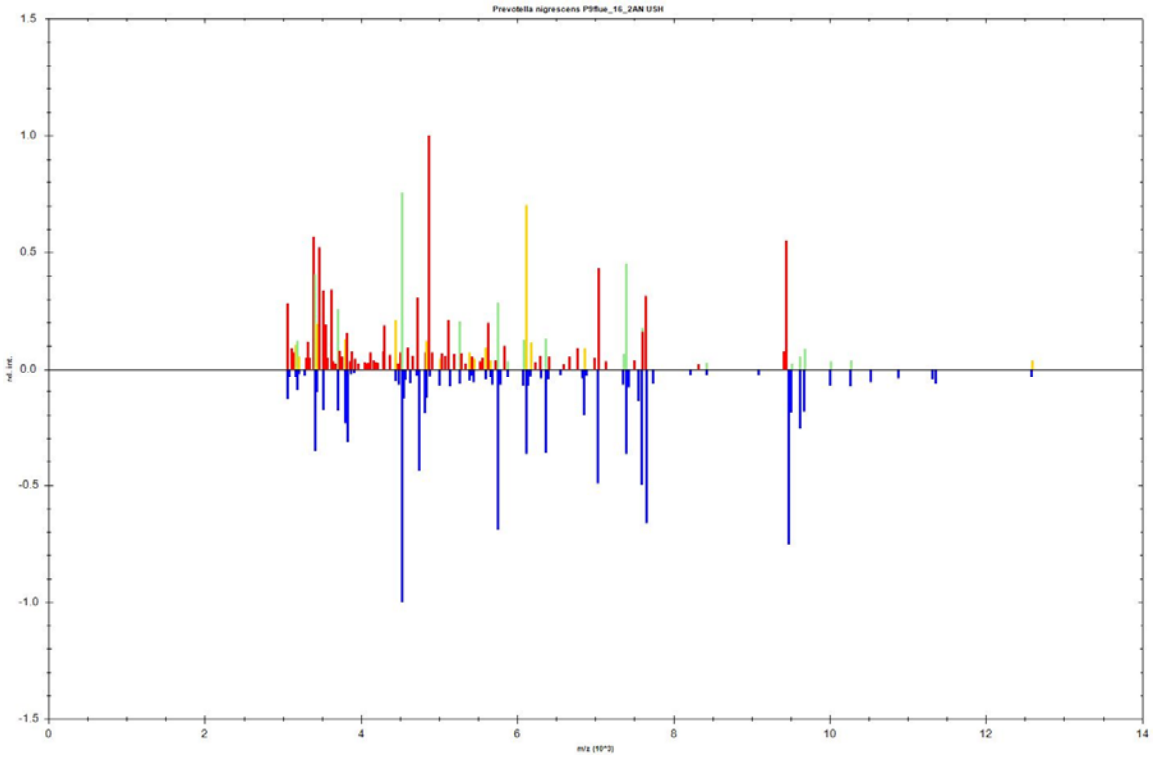

Figure 44. Normalized peak list spectrum for *P. nigrescens* clinical isolate #9 resistant in vitro to 8 µg/ml of amoxicillin (top, with green peaks representing a match, yellow a partial match, and red no match to mass spectra peaks of *P. nigrescens*, bottom, in Biotyper reference library).

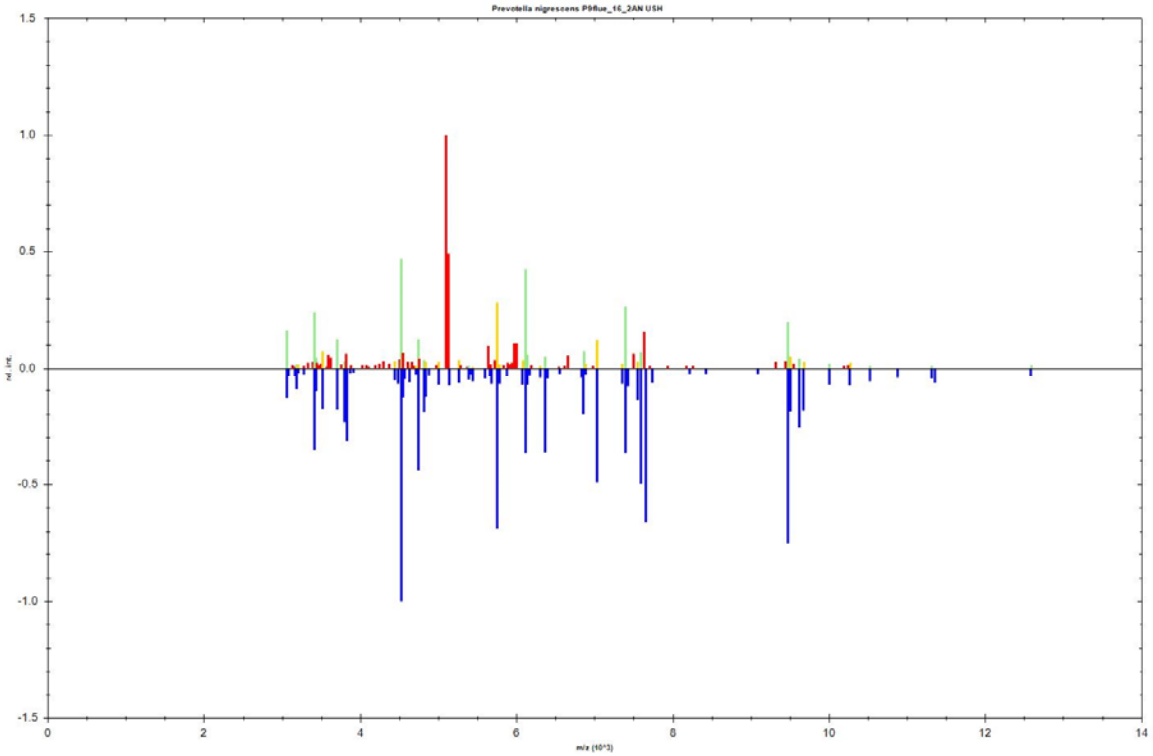

Figure 45. Normalized peak list spectrum for *P. nigrescens* clinical isolate #10 resistant in vitro to 8 µg/ml of amoxicillin (top, with green peaks representing a match, yellow a partial match, and red no match to mass spectra peaks of *P. nigrescens*, bottom, in Biotyper reference library).

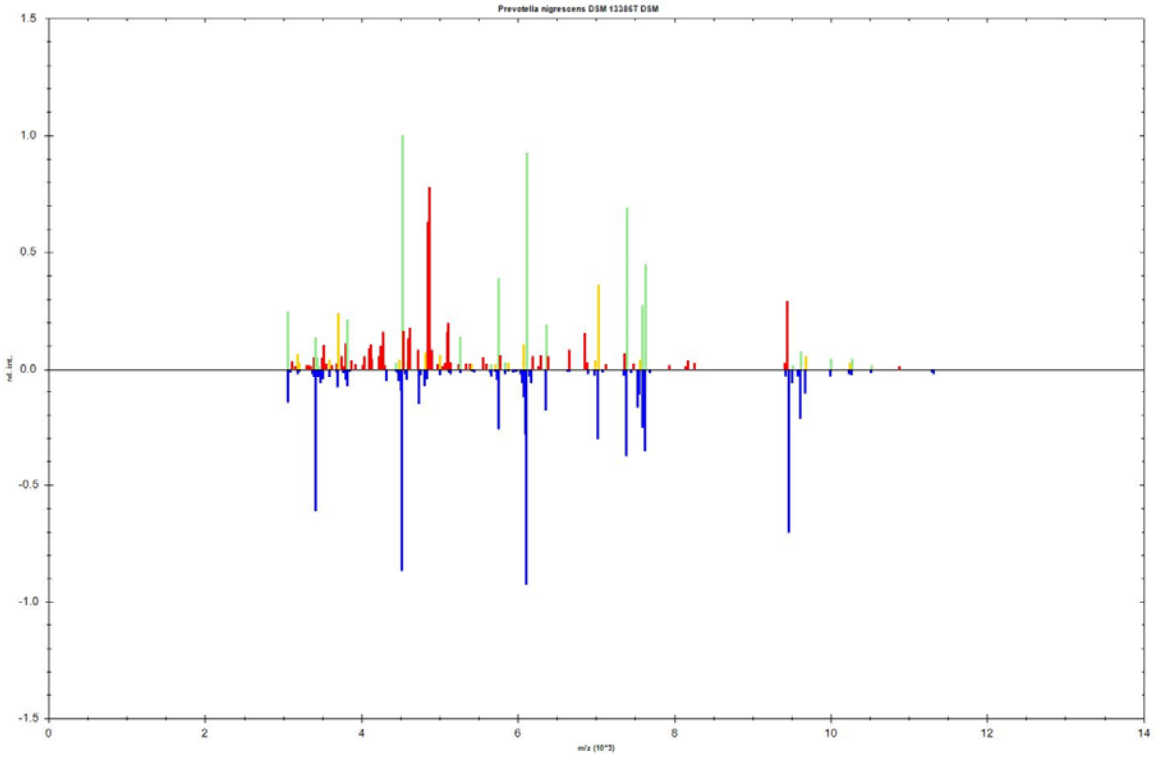

Figure 46. Normalized peak list spectrum for *P. nigrescens* clinical isolate #11 resistant in vitro to 8 µg/ml of amoxicillin (top, with green peaks representing a match, yellow a partial match, and red no match to mass spectra peaks of *P. nigrescens*, bottom, in Biotyper reference library).

CHAPTER 4

DISCUSSION

In this study, visual examination of raw mass spectra and mass spectra peaks generated by MALDI-TOF mass spectrometry within a routine operating range of 2-20 kDa failed to reveal noteworthy differences between amoxicillin-resistant and amoxicillin-susceptible subgingival clinical isolates of *P. intermedia* and *P. nigrescens*. This suggests that MALDI-TOF mass spectrometry employed within a routine operating range of 2-20 kDa may not provide sufficient differentiation in protein profiles between amoxicillin-resistant and amoxicillin-susceptible *Prevotella* species to be of rapid diagnostic use for assessing the species in vitro antibiotic susceptibility to β-lactam antibiotics. These observations are in contrast to those reported by Wybo et al. (2011), who found marked mass spectra differences between *cfi*A-negative and *cfi*A-positive (which encodes for a class B metallo-β-lactamase enzyme) isolates of *Bacteroides fragilis* belonging to two genotypically distinct groups. In their report, the mass spectra protein profiles differed at approximately 10 mass spectra peaks between the two genotypic bacterial groups (Wybo et al. 2011).

There are several reasons why no consistently reproducible differences were visually found in the mass spectra peaks of amoxicillin-susceptible and amoxicillin-resistant subgingival clinical isolates of *P. intermedia* and *P. nigrescens*. First, unlike findings with *B. fragilis*, where multiple mass spectra peaks were found to differ between β-lactamase-positive and negative clinical strains (Wybo et al. 2011), amoxicillin-susceptible and amoxicillin-resistant subgingival clinical isolates of *P. intermedia* and *P.*

nigrescens exhibit nearly identical mass spectra peaks, and if differences occurred, they were not consistently found among the clinical isolates studied. This may be due to differences in clonality among amoxicillin-resistant *P. intermedia* and *P. nigrescens*, where different β-lactamase encoding genes may be present and expressed.

Second, the low concentrations of β-lactamase enzymes, and their similar molecular weights to other bacterial proteins (Patel 2015), may have led to their masking in mass spectra among the multitude of other bacterial proteins present in the clinical isolates tested.

Third, the mass spectra examined were generated using the MALDI-TOF mass spectrometry within a routine operating range of 2-20 kDa. Since some antibiotic resistance factors have molecular weights that range from 72-75 kDa (Muroi et al. 2012), it is possible that if bacterial protein differences occurred between the amoxicillin-resistant and amoxicillin-susceptible *P. intermedia* and *P. nigrescens* periodontal clinical isolates, they may not have been able to be reliably detected by MALDI-TOF mass spectrometry used within a routine operating range of 2-20 kDa. As a result, additional evaluation of MALDI-TOF mass spectrometry employing higher kDa ranges beyond a routine upper operating range of 20 kDa is warranted to further evaluate its potential to accurately identify antibiotic-resistant versus susceptible strains of pathogenic microorganisms.

Fourth, the introduction of contaminating microbial species from ungloved skin, non-sterile wooden toothpicks, or other carriers onto the polished steel target plate used

in MALDI-TOF mass spectrometry, will alter mass spectra patterns, and potentially mask consistent differences between amoxicillin-resistant and sensitive test bacterial species.

CHAPTER 5

CONCLUSIONS

Visual examination of raw mass spectra and mass spectra peaks generated by MALDI-TOF mass spectrometry within a routine operating range of 2-20 kDa failed to reveal noteworthy differences between amoxicillin-resistant and amoxicillin-susceptible subgingival clinical isolates of *P. intermedia* and *P. nigrescens*. These findings indicate that use of MALDI-TOF mass spectrometry employed within a routine operating range of 2-20 kDa may not provide sufficient differentiation in protein profiles between amoxicillin-resistant and amoxicillin-susceptible *Prevotella* species to be of rapid diagnostic use for assessing the species in vitro antibiotic susceptibility to β-lactam antibiotics. Additional evaluation of MALDI-TOF mass spectrometry employing higher kDa ranges beyond a routine upper operating range of 20 kDa is warranted to further evaluate its potential to accurately identify antibiotic-resistant versus susceptible strains of pathogenic microorganisms.

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