EVALUATION OF ANTIBIOTIC DRUG SYNERGISMS AGAINST PERIODONTAL *AGGREGATIBACTER ACTINOMYCETEMCOMITANS.*

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by
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ABSTRACT

Objectives: Aggregatibacter actinomycetemcomitans is major putative bacterial pathogen in human periodontitis, particularly in aggressive periodontitis in younger-aged individuals. Systemic administration of certain antibiotics in combination have been demonstrated to exert synergistic antimicrobial effects against *A. actinomycetemcomitans*, and to markedly enhance elimination or suppression of *A. actinomycetemcomitans* from the subgingival dental plaque microbiome of periodontitis patients beyond that attained by periodontal mechanical debridement/surgery. However, studies on antibiotic synergisms against periodontal *A. actinomycetemcomitans* were conducted over 20 years ago, and only involved subgingival clinical isolates of the organism from periodontitis patients in Europe. Since temporal and geographic changes in antimicrobial susceptibility are documented among periodontitis-associated microorganisms, it is not known whether or not antibiotic synergisms against periodontal *A. actinomycetemcomitans* are present today among clinical isolates of the organism as recovered from periodontitis patients in the United States. As a result, the purpose of the present study was to assess the potential in vitro antimicrobial synergisms between amoxicillin plus metronidazole, between ciprofloxacin plus metronidazole, and between spiramycin and metronidazole, against clinical periodontal isolates of *A. actinomycetemcomitans* of United States origin.

Methods: Standardized cell suspensions, equivalent to a 1.0 McFarland turbidity standard, were prepared with four fresh clinical isolates of *A. actinomycetemcomitans*, each recovered from the subgingival microbiota of United States periodontitis subjects,

and plated onto to the surfaces of 150-mm diameter culture plates containing *Haemophilus* test medium. After drying, antibiotic-impregnated, quantitative, gradient diffusion strips (MIC Test Strip, Liofilchem s.r.l., Roseto degli Abruzzi, Italy) for amoxicillin, ciprofloxacin, spiramycin, and metronidazole were placed apart from each other onto the inoculated *Haemophilus* test medium surfaces, so that two test antibiotics per plate were employed against each *A. actinomycetemcomitans* clinical isolate for antibiotic susceptibility testing of individual antibiotic drugs. After 48 hours incubation in air + 5% CO_2, individual MIC values for each antibiotic against *A. actinomycetem-comitans* were read in µg/ml at the point where the edge of the bacterial inhibition ellipse intersected with the MIC Test Strip, with the occurrence of antibiotic resistance among the *A. actinomycetemcomitans* clinical isolates determined using *Haemophilus* species antibiotic resistance breakpoint concentrations established by the United States Clinical Laboratory Standards Institute (CLSI) and the Société Francaise de Microbiologie. In vitro synergy testing of amoxicillin plus metronidazole, ciprofloxacin plus metronidazole, and spiramycin plus metronidazole was performed after determination of individual antibiotic MIC testing by placing MIC Test Strips for each of the two test antibiotics per combination in a cross formation onto the surfaces of *A. actinomycetemcomitans*-inculated *Haemophilus* test medium agar so that there was a 90° angle between the two antibiotic strips at the point where their individual MIC values against *A. actinomycetem-comitans* intersected on their respective MIC interpretive scales. After 48 hours incubation in air + 5% CO_2, the MIC values for each antibiotic in combination against the *A. actinomycetemcomitans* clinical isolates was read in µg/ml where the edge of the

bacterial inhibition ellipses intersected with each of the MIC Test Strips. For each of the three antibiotic combinations tested in vitro against the four *A. actinomycetemcomitans* clinical isolates, the fractional inhibitory concentration (FIC) index was calculated per each antibiotic in combination. FIC index values of ≤ 0.5 indicated the presence of synergistic antimicrobial effects of the antibiotic combination against the test bacterial clinical isolates, whereas FIC index values of > 0.5, but ≤ 4.0 indicated indifference, and FIC values > 4.0 represented the presence of in vitro antimicrobial antagonism between the two antibiotics in combination.

Results: All of the four *A. actinomycetemcomitans* clinical isolates were susceptible in vitro to amoxicillin alone (all MIC values < 4 µg/ml), and to ciprofloxacin alone (all MIC values ≤ 1.0 µg/ml). In contrast, three of the four *A. actinomycetemcomitans* clinical isolates were resistant in vitro to metronidazole alone, with MIC values of resistant strains ranging between 24.0-48.0 µg/ml (resistance breakpoint threshold MIC value = ≥ 16 µg/ml), and all were resistant in vitro to spiramycin alone, with MIC values of > 32.0 µg/ml exhibited by each of the four tested periodontal *A. actinomycetemcomitans* strains. In synergy testing, markedly lower MIC values were found for amoxicillin and metronidazole, as well as with ciprofloxacin and metronidazole, when tested in combination together as compared to being tested alone. FIC index values for the combination of amoxicillin plus metronidazole were all ≤ 0.5, ranging from 0.110-0.344, which is indicative of a synergistic in vitro antimicrobial effect of the combination of amoxicillin plus metronidazole against all four periodontal *A. actinomycetemcomitans* clinical isolates tested. Similarly, FIC index values for the combination of ciprofloxacin

plus metronidazole were all ≤ 0.5, ranging from 0.105-0.355, which is also indicative of a synergistic in vitro antimicrobial effect of the combination of ciprofloxacin plus metronidazole against all four periodontal *A. actinomycetemcomitans* clinical isolates tested. FIC index values for the combination of spiramycin plus metronidazole were > 0.5 for three of the periodontal *A. actinomycetemcomitans* strains, ranging from 0.875-2.0, which is indicative of an indifferent in vitro antimicrobial interaction between spiramycin and metronidazole against the three periodontal *A. actinomycetemcomitans* clinical isolates. The FIC index value of the combination of spiramycin plus metronidazole against a single periodontal *A. actinomycetemcomitans* clinical isolate was ≤ 0.5 at a level of 0.427, which is indicative of a synergistic in vitro antimicrobial effect of the combination of spiramycin plus metronidazole against the single periodontal *A. actinomycetemcomitans* clinical isolate.

Conclusions: Amoxicillin and ciprofloxacin individually were active against all *A. actinomycetemcomitans* clinical periodontal isolates, whereas most or all strains were resistant to metronidazole and spiramycin by themselves. Antimicrobial synergism was found for the combinations of amoxicillin plus metronidazole, and for ciprofloxacin plus metronidazole, against all four periodontal *A. actinomycetemcomitans* clinical isolates of United States origin. Spiramycin plus metronidazole generally failed to exhibit antimicrobial synergism against periodontal *A. actinomycetemcomitans*. These findings confirm and extend European synergism studies conducted in mid-1990s on antibiotic synergisms against periodontal *A. actinomycetemcomitans,* and are the first to demonstrate antibiotic synergism against United States periodontal *A. actinomycetem-*

comitans clinical isolates. Additional research studies are needed to determine whether these in vitro synergistic effects of amoxicillin plus metronidazole, and of ciprofloxacin plus metronidazole, also occur in vivo and significantly enhance subgingival elimination or suppression of subgingival *A. actinomycetemcomitans*.

TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

CHAPTER 1

INTRODUCTION

Aggregatibacter actinomycetemcomitans is a gram negative, facultatively anaerobic, capnophilic, non-motile rod (Zambon, 1985). *A. actinomycetemcomitans* has been shown to be a key microorganism in the development of localized aggressive periodontitis, although it can also be found in chronic periodontitis and periodontal health in much smaller concentrations (Slots & Listgarten, 1988). Studies have found that *A. actinomycetemcomitans* comprises less than 1% of the total cultivable subgingival microbiota in humans (Asikainen & Chen, 1999).

Slots (1976) has been credited as being the first to describe the role of *A. actinomycetemcomitans* in the development of periodontitis. To date there have been several different strains of *A. actinomycetemcomitans* described in the literature; however, six main serotypes labeled a through f are the most well described (Chen et al., 2010). The serotypes of *A. actinomycetemcomitans* are differentiated form each other based upon their genetic structure. For instance, the highly pathogenic JP-2 clone of *A. actinomycetemcomitans* serotype b is characterized by a 530-bp deletion in the promoter region of the leukotoxin operon (Haubek et al., 2004). Studies have shown serotype c is the dominant serotype, followed by serotypes a and b, with serotypes d, e, and f either not detectable or rare (Chen et al., 2012). However, the dominance and prevalence of various strains of *A. actinomycetemcomitans* appears to depend upon the population studied (Chen et al., 2012). The genetic differences seen in the various strains of *A. actino-mycetemcomitans* are mainly attributed to horizontal gene transfer, and acquisitions of

"*genomic islands"* (Hacker & Carniel, 2001), which, it is hypothesized, facilitate spread of virulence factors enhancing the pathogenicity of recipient microorganisms and adversely altering the host response (Chen et al., 2010).

Due to the ability of *A. actinomycetemcomitans* to invade host tissues, local periodontal disease treatment measures, such as scaling and root planing and/or surgical access flaps, do not typically remove all of these microorganisms (van Winkelhoff et al., 1996). As a result, the use of systemic antibiotics in treatment of *A. actinomycetemcomitans*-associated periodontal infections has been advocated (Slots, 2004). This has especially been the case in the treatment of aggressive forms of periodontitis as *A. actinomycetemcomitans* is increased significantly more in the subgingival dental plaque microbiota of aggressive periodontitis patients as compared to chronic periodontitis subjects (Slots & Listgarten, 1988). Localized aggressive periodontitis patients often do not respond well to standard periodontal therapy comprised of oral hygiene instructions and both surgical and/or non-surgical therapy (Christersson et al., 1985). It has been argued that the continuing loss of periodontal attachment in these patients is often due to the inability to eradicate *A. actinomycetemcomitans* (Christersson et al., 1985). Since the microorganism is able to invade periodontal tissues, it makes the bacterial species less accessible for mechanical removal, even with periodontal surgical resection of gingival soft tissues, which has only limited success in eradicating *A. actinomycetemcomitans* from periodontal pockets (Christersson et al., 1985).

Hence, the goal of systemic antibiotic therapy in the treatment of periodontitis, especially aggressive forms, is to suppress periodontal pathogens to levels that are

compatible with periodontal health (Slots, 2004). A number of studies have shown that the adjunctive use of systemic antibiotics can improve the results of initial periodontal treatment and can more predictably suppress periodontal pathogens in juvenile and adult periodontitis patients (van Winkelhoff et al., 1996, Slots, 2004). There have been several antibiotic medications which have been studied both as a monotherapy, and in combination therapy with another antibiotic. To date, several studies have demonstrated the superiority of a combination therapy between amoxicillin and metronidazole in the treatment of aggressive forms of periodontitis over other antibiotic regimens (van Winkelhoff et al., 1996, Slots, 2004). A combination of amoxicillin and metronidazole has shown to be an effective antibiotic regimen to combat subgingival *A. actinomycetem-comitans* (van Winkelhoff et al., 1989). In this regard, Pavicić et al. (1994) found that the adjunctive use of amoxicillin enhances the influx of metronidazole into *A. actino-mycetemcomitans* cells which may account for an observed synergic action of these two antibiotics against *A. actinomycetemcomitans* in vitro (Pavicić et al., 1991). It has been proposed that both metronidazole and its hydroxymetabolite act synergistically with amoxicillin against *A. actinomycetemcomitans*, which leads to significantly decreasing the antibiotic concentration needed to be effective against *A. actinomycetemcomitans* (Pavicić et al., 1991). de Graaff et al. (1989) looked at the presence of *A. actino-mycetemcomitans* following periodontitis treatment with the adjunctive use of antibiotics, such as tetracycline alone, and amoxicillin plus metronidazole together, and found that systemic amoxicillin combined with metronidazole eradicated subgingival *A. actino-*

mycetemcomitans completely in all cases, whereas systemic tetracycline is often ineffective.

An alternative antibiotic that can be used in treatment of periodontal diseases is ciprofloxacin, which is a synthetic antibiotic in the fluoroquinolone drug family (van Winkelhoff et al., 1996). It exerts antimicrobial activity against bacteria by interfering with synthesis of DNA and proteins. *A. actinomycetemcomitans* has been found to be highly susceptible in vitro to ciprofloxacin, and systemic use of ciprofloxacin may be a useful alternative antibiotic in combination with metronidazole for the treatment of *A. actinomycetemcomitans*-associated periodontitis in cases of penicillin allergy (Pavicić et al., 1992). To date, synergy data for ciprofloxacin is limited analysis of *A. actino-mycetemcomitans* strains of European origin (Pavicić et al., 1992).

Another antibiotic combination, involving spiramycin plus metronidazole, is available in Canada and Europe, and is used to treat periodontal diseases (van Winkelhoff et al., 1996). Spiramycin is a 16-membered ring macrolide antibiotic often used to treat toxoplasmosis and respiratory infections, but it is not yet approved by the United States Food & Drug Administration for routine use in the United States. Spiramycin works by irreversibly binding to the 50S subunit of the ribosome, and thus, blocks protein synthesis. Spiramycin has shown efficacy against putative periodontal microorganisms such as *Actinomyces, Porphyromonas, Prevotella*, and some type of spirochetes (Quee et al., 1983, Rams et al., 2011). Spiramycin alone exhibits a high resistance rate of about 49% against various oral pathogens, but has shown a high efficacy when used in combination with metronidazole in non-*A. actinomycetemcomitans* infections (Rams et

al., 2011). The spiramycin and metronidazole combination is widely used in Europe (and also less frequency in Canada) on the basis of several different factors. First, the complementary antibacterial spectrum of both agents, second, the potential in vitro and in vivo synergy against periodontal pathogen bacteria, and finally, the ability to concentrate in tissues at the site of periodontal infection (Laufer et al., 1973). It has been suggested that spiramycin activity against *Actinomyces* species is enhanced in the presence of metronidazole (Quee et al., 1983). Limited data is currently available on the synergism of spiramycin plus metronidazole against different strains of *A. actinomycetemcomitans.*

Thus, the extent to which *A. actinomycetemcomitan*s clinical isolates recovered from periodontitis lesions are susceptible to various antibiotics is clinically relevant and an important issue that remains to be determined. The purpose of this research study was to determine in vitro the minimal inhibitory concentrations of metronidazole, amoxicillin, ciprofloxacin, and spiramycin alone, and in combinations comprised of amoxicillin plus metronidazole, spiramycin plus metronidazole, and ciprofloxacin plus metronidazole, against four periodontal *A. actinomycetemcomitans* clinical isolates from United States periodontitis patients. From this data, it was determined the extent to which amoxicillin plus metronidazole, spiramycin plus metronidazole, and ciprofloxacin plus metronidazole, exert synergistic antimicrobial activity against four periodontal *A. actinomycetemcomitans* clinical isolates.

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, PA. 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 principal investigator and the same OMTS Laboratory staff personnel for all of the study bacterial strains.

Test Bacterial Strains

Four fresh clinical isolates of *A. actinomycetemcomitans*, each recovered during 2012 from the subgingival microbiota of one of four United States periodontitis subjects by the OMTS Laboratory and present in their collection of periodontal bacterial isolates, were utilized in the present studies. Pure cultures of each *A. actinomycetemcomitans* clinical isolate were grown in vitro on pre-reduced trypticase soy-bacitracin-vancomycin (TSBV) agar, a selective medium for *A. actinomycetemcomitans* (Slots, 1982), which was incubated at 35ºC for three days in air + 5% CO_2. Visual examination of incubated TSVB plates was performed using a 2.25x ring-light Luxo Taskmaster magnifying loupe (Lighting Specialists, Buffalo, Grove, IL, USA), as well as a Meijo Techno RZ 75x dissecting stereomicroscope with a Fostec Ace I fiberoptic light source. On the selective TSBV medium, *A. actinomycetemcomitans* was identified as circular, convex, translucent, adherent, glistening colonies with slightly irregular edges and an inner star-shaped morphology (Figure 1), which had a catalase-positive bubbling reaction upon 3% hydrogen peroxide application (Slots, 1982). No additional phenotypic, biochemical or genetic characterization of the *A. actinomycetemcomitans* clinical isolates was performed.

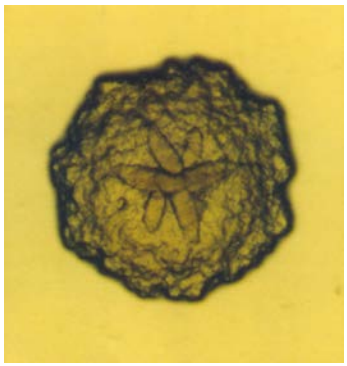

Figure 1. Typical *A. actinomycetemcomitans* colony appearance on TSBV culture media at a magnification of approximately 10x.

In Vitro Individual Antibiotic Susceptibility Testing

Inocula of each of the test bacterial strains were obtained from pure *A. actino-mycetemcomitans* colonies scraped off of the TSBV medium, and standardized cell suspensions were prepared 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), equivalent to a 1.0 McFarland turbidity standard, which provided approximately 3×10^8 of the *A. actinomycetemcomitans* organisms/ml. Using a sterile cotton-tip swab, the *A. actinomycetemcomitans* suspensions for each of the four clinical isolates were separately applied to the surfaces of 150-mm diameter culture plates containing *Haemophilus* test medium (BBL Microbiology Systems, Cockeysville, MD, USA). After drying, antibiotic-impregnated, quantitative, gradient diffusion strips (MIC Test Strip, Liofilchem s.r.l., Roseto degli Abruzzi, Italy) were placed apart from each other onto the inoculated *Haemophilus* test medium surfaces, so that two test antibiotics

per plate were employed against each *A. actinomycetemcomitans* clinical isolate for antibiotic susceptibility testing of individual antibiotic drugs (Figure 2).

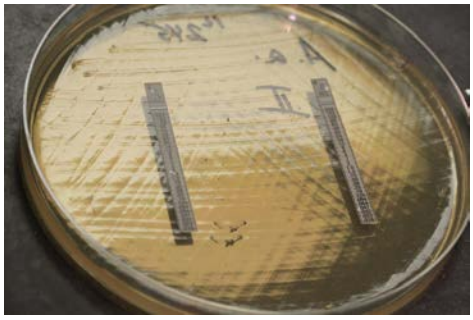

Figure 2. Placement of antibiotic-containing test strip for metronidazole (left) and amoxicillin (right) on *A. actinomycetemcomitans*-inoculated plate.

A Nema C88 vacuum pen (bioMérieux, Inc., Durham, NC, USA) was used to apply MIC Test Strips onto inoculated agar plates. The applicator pen has a suction cup, vacuum level regulator, and switch control. After adjusting the vacuum level to facilitate efficient pick-up and release of the antibiotic-containing strips, the applicator was held like a pen, with the evacuation hole covered with a fingertip to create suction in order to lift up the antibiotic strip, position it onto the agar plates, and release it into place by removal of the fingertip from the pen evacuation hole.

Liofilchem MIC Test Strips are comprised of special paper impregnated with a predefined antibiotic gradient immobilized across 15 two-fold dilutions on one side, and a minimal inhibitory concentration (MIC) interpretive scale printed on the other side. The antibiotic-containing surface was applied down onto inoculated agar, permitting the

preformed exponential gradient of antimicrobial agent to diffuse into the *Haemophilus* test medium agar matrix. After 48 hours incubation in air + 5% CO_2, a symmetrical inhibition ellipse centered along the strip was noted when test antibiotic inhibition of the *A. actinomycetemcomitans* clinical isolates occurred. The MIC value associated with the bacterial inhibition was read in μg/ml directly from the MIC Test Strip scale at the point where the edge of the bacterial inhibition ellipse intersected with the MIC Test Strip. The MIC values were scored following directions of the manufacturer, and well as guidelines previously described (Pajukanta et al., 1992). The individual antibiotics tested for MIC value determination per *A. actinomycetemcomitans* clinical isolate were amoxicillin, ciprofloxacin, spiramycin, and metronidazole.

The occurrence of antibiotic resistance among the four *A. actinomycetemcomitans* clinical isolates to the four individual test antibiotics was determined using antibiotic resistance breakpoint concentrations established by the United States Clinical Laboratory Standards Institute (CLSI) for *Haemophilus* species (Clinical and Laboratory Standards Institute, 2012) as follows: ≥ 4 ug/ml for amoxicillin, > 1 ug/ml for ciprofloxacin, and ≥ 16 ug/ml for metronidazole. A resistance breakpoint concentration ≥ 4 μg/ml of spiramycin was used in accordance with guidelines for macrolide antibiotics recommended by the Société Francaise de Microbiologie (Comité de l'Antibiogramme de la Société Française de Microbiologie, 2010).

Quality control testing of the Liofilchem MIC Test Strips was performed for each antibiotic batch used in the present study. For amoxicillin-containing MIC Test Strips, the MIC value against *Streptococcus pneumoniae* ATCC strain 49619 was determined on

Mueller Hinton agar with 5% sheep blood incubated aerobically at 36°C for 18-24 hours. For ciprofloxacin-containing MIC Test Strips, the MIC values against *Staphylococcus aureus* ATCC strain 29213, *Pseudomonas aeruginosa* ATCC strain 27853, *Enterococcus faecalis* ATCC strain 29212, and *Escherichia coli* ATCC strain 25922 were determined on Mueller Hinton agar incubated aerobically at 36°C for 18-24 hours. For metronidazole-containing MIC Test Strips, the MIC values against *Bacteroides fragilis* ATCC strain 25285 and *Bacteroides thetaiotaomicron* ATCC strain 29741 were determined on Schadler K agar with 5% sheep blood incubated microaerophilically at 36°C for 18-24 hours. For specially-fabricated spiramycin-containing MIC Test Strips, MIC values against *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 were determined on Mueller Hinton agar incubated aerobically at 36°C for 18-24 hours, and against *S. pneumoniae* ATCC 49619 on Mueller Hinton agar with 5% sheep blood incubated microaerophilically at 36°C for 18-24 hours.

The MIC values for each of the quality control test organisms for amoxicillin, ciprofloxacin, and metronidazole MIC Test Strips were compared to standard MIC value ranges recognized by the CLSI as meeting acceptable clinical laboratory performance standards in the United States (Clinical and Laboratory Standards Institute, 2012). No comparisons to CLSI performance standards could be made with MIC quality control values for the specially-manufactured spiramycin-containing MIC Test Strips, since standard in vitro MIC value ranges for spiramycin have not been issued to date by the CLSI.

In Vitro Antibiotic Synergy Testing

In vitro synergy testing of three antibiotic combinations against the four *A. actinomycetemcomitans* clinical isolates was performed using methodology previously described (White et al., 1996). The antibiotic combinations tested in vitro were amoxicillin plus metronidazole, ciprofloxacin plus metronidazole, and spiramycin plus metronidazole. Synergy testing was performed after determination of individual antibiotic MIC testing by placing MIC Test Strips for each of the two test antibiotics per combination in a cross formation onto the surfaces of *A. actinomycetemcomitans*-inculated *Haemophilus* test medium agar so that there was a 90° angle between the two strips at the point where their individual MIC values against *A. actinomycetemcomitans* intersected on their respective MIC interpretive scales (Figure 3).

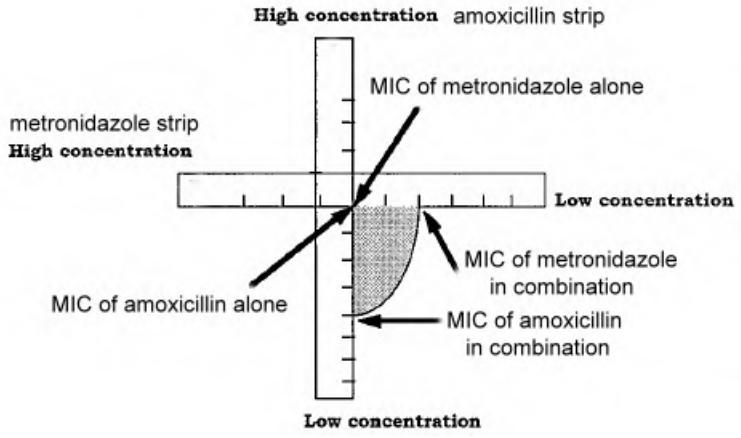

Figure 3. Example of MIC Test Strip placement for in vitro amoxicillin plus metronidazole synergy testing (diagram modified from White et al., 1996).

After 48 hours incubation in air + 5% CO_2, the MIC values for each antibiotic in combination against the *A. actinomycetemcomitans* clinical isolates was read in µg/ml where the edge of the bacterial inhibition ellipses intersected with each of the MIC Test Strips, similar to the assessments made with individual antibiotic susceptibility testing.

For each of the three antibiotic combinations tested in vitro against the four *A. actinomycetemcomitans* clinical isolates, the fractional inhibitory concentration (FIC) index was calculated per each antibiotic in combination, as previously described (White et al., 1996). In brief, the FIC index was calculated as the sum of the FIC of antibiotic #1 tested in combination plus the FIC of antibiotic #2 tested in combination against *A. actinomycetemcomitans*. The FIC of antibiotic #1 was determined by dividing the MIC of antibiotic #1 against *A. actinomycetemcomitans* in combination with antibiotic #2 by the MIC of antibiotic #1 alone against *A. actinomycetemcomitans*. Similarly, The FIC of antibiotic #2 was determined by dividing the MIC of antibiotic #2 against *A. actinomycetemcomitans* in combination with antibiotic #1 by the MIC of antibiotic #2 alone against *A. actinomycetemcomitans*.

Interpretative FIC index guidelines by White et al. (1996) were used to assess the presence of in vitro antibiotic combination synergism, indifference, or antagonism against periodontal *A. actinomycetemcomitans*. FIC index values of ≤ 0.5 indicated the presence of synergistic antimicrobial effects of the antibiotic combination against the test bacterial clinical isolates. FIC index values of > 0.5, but ≤ 4.0 indicated indifference between the two antibiotics in their antimicrobial effects, and FIC values > 4.0 represented the

presence of in vitro antimicrobial antagonism between the two antibiotics in combination (White et al., 1996).

Data Analysis

Other than descriptive observations and calculation of the FIC index values for each of the three antibiotic combinations against the four *A. actinomycetemcomitans* clinical isolates, no additional data analysis or statistical hypothesis testing was performed in the present study.

CHAPTER 3

RESULTS

Quality Control Testing of MIC Test Strips

For amoxicillin-containing MIC Test Strips, a quality control MIC value = 0.03 µg/ml was found against *S. pneumoniae* ATCC strain 49619, which is within the acceptable CLSI clinical laboratory performance standard MIC range of 0.03-0.12 µg/ml.

For ciprofloxacin-containing MIC Test Strips, quality control MIC value = 0.19 µg/ml against *S. aureus* ATCC strain 29213, MIC value = 0.25 µg/ml against *P. aeruginosa* ATCC strain 27853, MIC value = 2.0 against *E. faecalis* ATCC strain 29212, and MIC value = 0.012 µg/ml against *E. coli* ATCC strain 25922, were within the acceptable CLSI clinical laboratory performance standard MIC ranges of 0.12-0.5 µg/ml, 0.25-1.0 µg/ml, 0.25-2.0 µg/ml, and 0.004-0.015 µg/ml, for each of the test quality control organisms, respectively.

For metronidazole-containing MIC Test Strips, quality control MIC values of 0.25 µg/ml against *B. fragilis* ATCC strain 25285, and 0.5 µg/ml against *B. thetaiotaomicron* ATCC strain 29741, were within the acceptable CLSI clinical laboratory performance standard MIC ranges of 0.25-1.0 µg/ml, and 0.5-2.0 µg/ml, for each of the test quality control organisms, respectively.

For spiramycin-containing MIC Test Strips, MIC values found were 1.0 µg/ml against *S. aureus* ATCC 29213, 0.75 µg/ml against *E. faecalis* ATCC 29212, and 0.125 µg/ml against *S. pneumoniae* ATCC 49619.

In Vitro Individual Antibiotic Susceptibility Testing

MIC values for each of the four test antibiotics individually against the four *A. actinomycetemcomitans* clinical isolates (identified as OMTS 245, OMTS 308, OMTS 309, and OMTS 324) is presented in Table 1.

Table 1. In Vitro MIC Values (µg/ml) of Four Test Antibiotics Against Four Periodontal *A. actinomycetemcomitans* Clinical Isolates

	A. actinomycetemcomitans clinical isolates			
Antibiotic	OMTS 245	OMTS 308	OMTS 309	OMTS 324
Amoxicillin	1.0	0.75	1.5	1.0
Ciprofloxacin	0.016	0.023	0.064	0.064
Spiramycin	> 32.0	> 32.0	> 32.0	> 32.0
Metronidazole	8.0	48.0	24.0	48.0

All of the four *A. actinomycetemcomitans* clinical isolates were susceptible in vitro, according to CLSI standards, to amoxicillin alone (all MIC values < 4 µg/ml), and to ciprofloxacin alone (all MIC values ≤ 1.0 µg/ml). In contrast, three of the four *A. actinomycetemcomitans* clinical isolates were resistant in vitro to metronidazole alone, with MIC values of resistant strains ranging between 24.0-48.0 µg/ml (resistance

breakpoint threshold MIC value = ≥ 16 µg/ml). All *A. actinomycetemcomitans* clinical isolates were resistant in vitro to spiramycin alone, with MIC values of > 32.0 µg/ml exhibited by each of the four tested periodontal *A. actinomycetemcomitans* strains.

Representative MIC Test Strip outcomes with individual antibiotics against periodontal *A. actinomycetemcomitans* strains are presented in Figure 4.

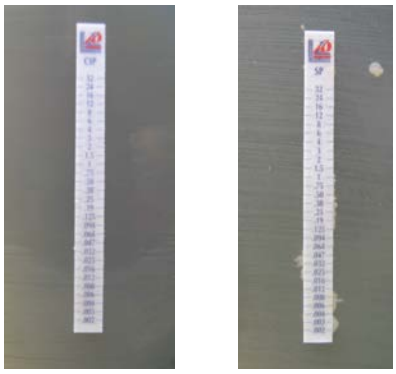

Figure 4. Examples of MIC Test Strip outcomes of individual antibiotics tested in vitro against periodontal *A. actinomycetemcomitans*. An MIC of 0.016 µg/ml is found for ciprofloxacin against *A. actinomycetemcomitans* clinical isolate OMTS 245 (left side), whereas no inhibition of the same clinical isolate was attained by 32 µg/ml or less of spiramycin (right side).

In Vitro Antibiotic Synergy Testing

In vitro synergy testing results of amoxicillin plus metronidazole in combination against the *A. actinomycetemcomitans* clinical isolates is presented in Tables 2 and 3.

Table 2. In Vitro MIC Values (µg/ml) of Amoxicillin and Metronidazole Alone and In Combination Against Periodontal *A. actinomycetemcomitans* Clinical Isolates

	A. actinomycetemcomitans clinical isolates			
Antibiotic	OMTS 245	OMTS 308	OMTS 309	OMTS 324
Amoxicillin				
Alone	1.0	0.75	1.5	1.0
Metronidazole				
Alone	8.0	48.0	24.0	48.0
Amoxicillin in combination with metronidazole	0.19	0.125	0.250	0.047
Metronidazole in combination with amoxicillin	1.0	6.0	4.0	3.0

Table 3. Fractional Inhibitory Concentration (FIC) Index Values of Amoxicillin and Metronidazole Alone and In Combination Against Four Periodontal *A. actinomycetemcomitans* Clinical Isolates

	A. actinomycetemcomitans clinical isolates			
FIC Value	OMTS 245	OMTS 308	OMTS 309	OMTS 324
FIC of amoxicillin	0.190	0.167	0.167	0.047
FIC of metronidazole	0.125	0.125	0.167	0.063
FIC of amoxicillin plus metronidazole	0.315	0.292	0.334	0.110
Antimicrobial synergism of amoxicillin plus metronidazole	Yes	Yes	Yes	Yes

Among the four periodontal *A. actinomycetemcomitans* clinical isolates, markedly lower MIC values were found for amoxicillin when tested in combination with metronidazole as compared to being tested alone. In combination with metronidazole, amoxicillin MIC values against periodontal *A. actinomycetemcomitans* ranged from 0.047 to 0.25 µg/ml, whereas MIC values of 0.75-1.5 µg/ml were found with amoxicillin alone. Metronidazole MIC values were similarly decreased against periodontal *A. actinomycetemcomitans* when tested in combination with amoxicillin, as compared to being tested alone. MIC values for metronidazole when tested in combination with amoxicillin ranged from 1.0-6.0 µg/ml, in comparison to MIC values of 8.0-48.0 µg/ml when tested alone.

FIC index values for the combination of amoxicillin plus metronidazole were all $\leq$ 0.5, ranging from 0.110-0.344, which is indicative of a synergistic in vitro antimicrobial effect of the combination of amoxicillin plus metronidazole against all four periodontal *A. actinomycetemcomitans* clinical isolates tested.

In vitro synergy testing results of ciprofloxacin plus metronidazole in combination against the *A. actinomycetemcomitans* clinical isolates is presented in Tables 4 and 5.

Table 4. In Vitro MIC Values (µg/ml) of Ciprofloxacin and Metronidazole Alone and In Combination Against Periodontal *A. actinomycetemcomitans*

	A. actinomycetemcomitans clinical isolates			
Antibiotic	OMTS 245	OMTS 308	OMTS 309	OMTS 324
Ciprofloxacin				
Alone	0.016	0.023	0.064	0.064
Metronidazole				
Alone	8.0	48.0	24.0	48.0
Ciprofloxacin in combination with metronidazole	0.002	0.004	0.012	0.004
Metronidazole in combination with ciprofloxacin	0.5	6.0	4.0	2.0

Table 5. Fractional Inhibitory Concentration (FIC) Index Values of Ciprofloxacin and Metronidazole Alone and In Combination Against Four Periodontal *A. actinomycetemcomitans* Clinical Isolates

	A. actinomycetemcomitans clinical isolates			
FIC Value	OMTS 245	OMTS 308	OMTS 309	OMTS 324
FIC of ciprofloxacin	0.125	0.174	0.188	0.063
FIC of metronidazole	0.063	0.125	0.167	0.042
FIC of ciprofloxacin plus metronidazole	0.188	0.299	0.355	0.105
Antimicrobial synergism of ciprofloxacin plus metronidazole	Yes	Yes	Yes	Yes

Among the four periodontal *A. actinomycetemcomitans* clinical isolates, lower MIC values were found for ciprofloxacin when tested in combination with metronidazole as compared to being tested alone. In combination with metronidazole, ciprofloxacin MIC values against periodontal *A. actinomycetemcomitans* ranged from 0.002-0.012 µg/ml, whereas MIC values of 0.016-0.064 µg/ml were found with ciprofloxacin alone. Metronidazole MIC values were similarly decreased against periodontal *A. actinomycetemcomitans* when tested in vitro in combination with ciprofloxacin, as compared to being tested alone. MIC values for metronidazole when tested in combination with ciprofloxacin ranged from 0.5-6.0 µg/ml, in comparison to MIC values of 8.0-48.0 µg/ml when tested alone.

FIC index values for the combination of ciprofloxacin plus metronidazole were all ≤ 0.5, ranging from 0.105-0.355, which is indicative of a synergistic in vitro antimicrobial effect of the combination of ciprofloxacin plus metronidazole against all four periodontal *A. actinomycetemcomitans* clinical isolates tested.

In vitro synergy testing results of spiramycin plus metronidazole in combination against the *A. actinomycetemcomitans* clinical isolates is presented in Tables 6 and 7.

Table 6. In Vitro MIC Values (µg/ml) of Spiramycin and Metronidazole Alone and In Combination Against Periodontal *A. actinomycetemcomitans*

	A. actinomycetemcomitans clinical isolates			
Antibiotic	OMTS 245	OMTS 308	OMTS 309	OMTS 324
Spiramycin				
Alone	> 32.0	> 32.0	> 32.0	> 32.0
Metronidazole				
Alone	8.0	48.0	24.0	48.0
Spiramycin in combination with metronidazole	> 32.0	3.0	12.0	16.0
Metronidazole in combination with spiramycin	8.0	16.0	12.0	24.0

Table 7. Fractional Inhibitory Concentration (FIC) Index Values of Spiramycin and Metronidazole Alone and In Combination Against Four Periodontal *A. actinomycetemcomitans* Clinical Isolates

	A. actinomycetemcomitans clinical isolates			
FIC Value	OMTS 245	OMTS 308	OMTS 309	OMTS 324
FIC of spiramycin	1.0	0.094	0.375	0.500
FIC of metronidazole	1.0	0.333	0.500	0.500
FIC of spiramycin plus metronidazole	2.0	0.427	0.875	1.0
Antimicrobial synergism of spiramycin plus metronidazole	No	Yes	No	No

For three of the four periodontal *A. actinomycetemcomitans* clinical isolates, lower MIC values were found for spiramycin when tested in combination with metronidazole as compared to being tested alone. In combination with metronidazole, spiramycin MIC values against three periodontal *A. actinomycetemcomitans* strains ranged from 3.0-16.0 µg/ml, whereas MIC values of > 32.0 µg/ml were found with spiramycin alone. With the fourth periodontal *A. actinomycetemcomitans* clinical isolate, no change in the spiramycin MIC value was detected when tested in combination with metronidazole as compared to alone (MIC > 32.0 µg/ml in both situations). Metronidazole MIC values were also lower against three of the four periodontal *A. actinomycetemcomitans* when tested in vitro in combination with spiramycin, as compared to being tested alone. MIC values against three periodontal *A. actino-mycetemcomitans* strains for metronidazole when tested in combination with spiramycin ranged from 12.0-24.0 µg/ml, in comparison to MIC values of 24.0-48.0 µg/ml when tested alone.

FIC index values for the combination of spiramycin plus metronidazole were > 0.5 for three of the periodontal *A. actinomycetemcomitans* strains, ranging from 0.875-2.0, which is indicative of an indifferent in vitro antimicrobial interaction between spiramycin and metronidazole against the three periodontal *A. actinomycetemcomitans* clinical isolates. The FIC index value of the combination of spiramycin plus metro-nidazole against a single periodontal *A. actinomycetemcomitans* clinical isolate was ≤ 0.5 at a level of 0.427, which is indicative of a synergistic in vitro antimicrobial effect of the

combination of spiramycin plus metronidazole against the single periodontal *A. actino-mycetemcomitans* clinical isolate.

Representative MIC Test Strip outcomes demonstrating synergisms between combinations of antibiotics against periodontal *A. actinomycetemcomitans* strains are presented in Figure 5.

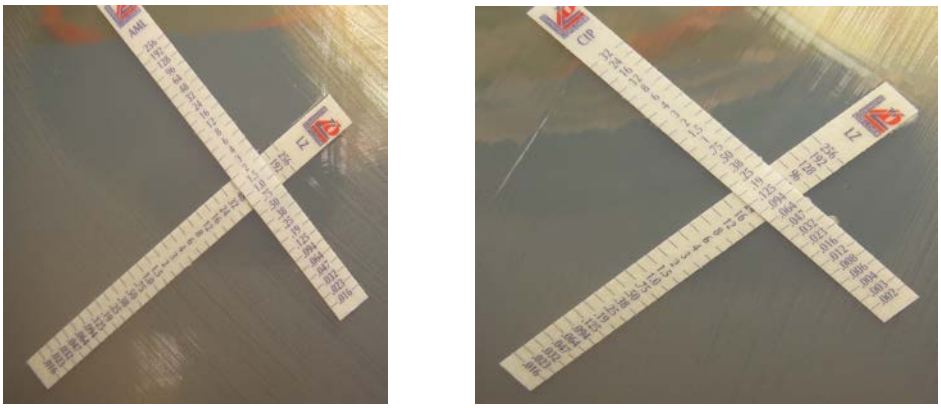

Figure 5. Examples of MIC Test Strip outcomes demonstrating synergisms between combinations of antibiotics tested in vitro against periodontal *A. actinomycetemcomitans*. Lower MIC values for amoxicillin (0.125 µg/ml versus 0.75 µg/ml) and metronidazole (6.0 µg/ml versus 48.0 µg/ml) are found against *A. actinomycetemcomitans* OMTS strain 308 when the two antibiotics are tested in combination as compared to alone (left side). Lower MIC values for ciprofloxacin (0.012 µg/ml versus 0.064 µg/ml) and metronidazole (4.0 µg/ml versus 24 µg/ml) are found against *A. actinomycetemcomitans* OMTS strain 309 when the two antibiotics are tested in combination as compared to alone (right side).

CHAPTER 4

DISCUSSION

The present study first revealed that amoxicillin and ciprofloxacin individually were active against all four *A. actinomycetemcomitans* clinical periodontal isolates, whereas most or all strains were resistant to metronidazole and spiramycin by themselves. All amoxicillin MIC values against the periodontal *A. actinomycetemcomitans* stains studied were < 4 μg/ml, and all ciprofloxacin MIC values were ≤ 1.0 μg/ml. In contrast, three of the four *A. actinomycetemcomitans* clinical isolates were resistant in vitro to metronidazole alone, with MIC values of resistant strains ranging between 24.0-48.0 μg/ml (resistance breakpoint threshold MIC value = ≥ 16 μg/ml). All four *A. actinomycetemcomitans* clinical periodontal isolates were also resistant in vitro to spiramycin alone, with MIC values for each > 32.0 μg/ml. These findings are consistent with previous in vitro susceptibility evaluations of various antibiotics against periodontal *A. actinomycetemcomitans* (Madinier et al., 1999), where amoxicillin and ciprofloxacin were found to exhibit marked in vitro antimicrobial activity against periodontal *A. actinomycetemcomitans*, while inconsistent or no in vitro antimicrobial activity was revealed by metronidazole and spiramycin.

Secondly, this study found markedly lower MIC values for amoxicillin and metronidazole, as well as with ciprofloxacin and metronidazole, when the drugs were tested in combination together against periodontal *A. actinomycetemcomitans* as compared to being tested alone. FIC index values for the combination of amoxicillin plus metronidazole were all ≤ 0.5, ranging from 0.110-0.344, which was indicative of a

synergistic in vitro antimicrobial effect of the combination of amoxicillin plus metronidazole against all four periodontal *A. actinomycetemcomitans* clinical isolates tested. Similarly, FIC index values for the combination of ciprofloxacin plus metronidazole were also all ≤ 0.5, ranging from 0.105-0.355, which was indicative of a synergistic in vitro antimicrobial effect of the combination of ciprofloxacin plus metronidazole against all four periodontal *A. actinomycetemcomitans* clinical isolates tested. In contrast, FIC index values for the combination of spiramycin plus metronidazole were > 0.5 for three of the periodontal *A. actinomycetemcomitans* strains, ranging from 0.875-2.0, which was indicative of an indifferent in vitro antimicrobial interaction between spiramycin and metronidazole against the three periodontal *A. actinomycetemcomitans* clinical isolates. The FIC index value of the combination of spiramycin plus metronidazole against a single periodontal *A. actinomycetemcomitans* clinical isolate was ≤ 0.5 at a borderline level of 0.427, which suggested a synergistic in vitro antimicrobial effect of the combination of spiramycin plus metronidazole against the single periodontal *A. actinomycetemcomitans* clinical isolate.

In previous studies, synergistic effects were found for amoxicillin plus metronidazole, and for ciprofloxacin plus metronidazole, against European clinical periodontitis isolates of *A. actinomycetemcomitans* (Pavicić et al., 1991, 1992), which are in accord with the present study findings on *A. actinomycetemcomitans* isolated from United States periodontitis patients. Also consistent with the present study findings, Mouton et al. (1984) failed to show any synergistic effect of the combination of

spiramycin plus metronidazole against 65 periodontal bacterial strains, including isolates of *A. actinomycetemcomitans*.

The present study is the first in the dental field to use antibiotic synergism testing methodology described by White et al. (1996) on oral microorganisms. Prior antibiotic synergy testing against periodontal bacterial species was performed with a checkerboard microdilution plate assay system (Pavicić et al., 1991, 1992), instead of the cross-placement of gradient diffusion strips on inoculated agar plates as employed in the present study. The present study is also the first in dentistry to use the newly-available MIC Test Strips, which are made with paper strips, as an alternative to widely-employed and more expensive E-test strips, which are made of plastic strips (Pajukanta et al., 1992).

Moreover, the present study is the first in the world to utilize specially-manufactured spiramycin gradient diffusion strips, which have not been available prior to the conduct of this study. The lack of availability of gradient diffusion strips containing spiramycin has hindered in vitro susceptibility testing with spiramycin in clinical periodontal microbiology laboratories, as well in diagnostic medical microbiology laboratories located in countries where spiramycin is approved for human use.

Finally, it is important that additional research studies be conducted to determine whether the in vitro synergistic effects of amoxicillin plus metronidazole, and of ciprofloxacin plus metronidazole, also occur in vivo and significantly enhance subgingival elimination or suppression of subgingival *A. actinomycetemcomitans*.

CHAPTER 5
CONCLUSIONS

The present study aimed to assess the potential in vitro antimicrobial synergisms between amoxicillin plus metronidazole, between ciprofloxacin plus metronidazole, and between spiramycin and metronidazole, against four clinical periodontal isolates of *A. actinomycetemcomitans* of United States origin.

Amoxicillin and ciprofloxacin individually were active against all four *A. actinomycetemcomitans* clinical periodontal isolates, whereas most or all strains were resistant to metronidazole and spiramycin by themselves. Antimicrobial synergism was found for the combinations of amoxicillin plus metronidazole, and for ciprofloxacin plus metronidazole, against all four periodontal *A. actinomycetemcomitans* clinical isolates studied. Spiramycin plus metronidazole generally failed to exhibit antimicrobial synergism against periodontal *A. actinomycetemcomitans*, with indifferent antimicrobial effects found against three of the four *A. actinomycetemcomitans* clinical isolates.

These findings confirm and extend European synergism studies conducted in mid-1990s on antibiotic synergisms against periodontal *A. actinomycetemcomitans*, and are the first to demonstrate antibiotic synergism against United States periodontal *A. actinomycetemcomitans* clinical isolates.

Additional research studies are needed to determine whether these in vitro synergistic effects of amoxicillin plus metronidazole, and of ciprofloxacin plus

metronidazole, also occur in vivo and significantly enhance subgingival elimination or suppression of subgingival *A. actinomycetemcomitans*.

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