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Combinatorial effects of amoxicillin and metronidazole on selected periodontal bacteria and whole plaque samples

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ARTICLE INFO

Article history:
Accepted 18 March 2014

Keywords:
Aggregatibacter (*Actinobacillus*) *actinomycetemcomitans*
Eikenella corrodens
Amoxicillin
Metronidazole
Etest
Combinatorial effect

ABSTRACT

Objective: The aim of the present study was to analyze *in vitro* the combinatorial effects of the antibiotic combination of amoxicillin plus metronidazole on subgingival bacterial isolates.

Design: *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans*, *Prevotella intermedia/nigrescens*, *Fusobacterium nucleatum* and *Eikenella corrodens* from our strain collection and subgingival bacteria isolated from patients with periodontitis were tested for their susceptibility to amoxicillin and metronidazole using the Etest. The fractional inhibitory concentration index (FICI), which is commonly used to describe drug interactions, was calculated.

Results: Synergy, *i.e.* FICI values ≤ 0.5, between amoxicillin and metronidazole was shown for two *A. actinomycetemcomitans* (FICI: 0.3), two *F. nucleatum* (FICI: 0.3 and 0.5, respectively) and one *E. corrodens* (FICI: 0.4) isolates. Indifference, *i.e.* FIC indices of >0.5 but ≤ 4, occurred for other isolates and the 14 *P. intermedia/nigrescens* strains tested. Microorganisms resistant to either amoxicillin or metronidazole were detected in all samples by Etest.

Conclusion: Combinatorial effects occur between amoxicillin and metronidazole on some strains of *A. actinomycetemcomitans*, *F. nucleatum* and *E. corrodens*. Synergy was shown for a few strains only.

1. Introduction

Periodontal therapy aims in a destruction of the pathogenic subgingival biofilm by means of mechanical debridement. Most patients respond to this well-established treatment and periodontal health may be maintained in the long term.[1] There are, however, certain conditions when systemic antibiotics are indicated as adjunct to mechanical therapy.[2–4] The combination of amoxicillin plus metronidazole has been initially introduced for the adjunctive treatment of *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans* associated periodontitis.[5] Subsequently, this antibiotic combination has been shown to be clinically successful in patients with aggressive and/or advanced forms of chronic periodontitis, and superior to adjunctive amoxicillin or metronidazole alone.[6–10]

Combinatorial effects between the two antibiotics might explain the demonstrated efficacy. In a combination

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http://dx.doi.org/10.1016/j.archoralbio.2014.03.007
0003–9969/

treatment, antibiotics can be more effective displaying either an additive effect, *i.e.* an effect equal to the sum of the treatments, or a synergistic effect, *i.e.* an effect greater than the sum of the treatments. A combination of two antibiotics can also be antagonistic, with the effect of the combination treatment being less than the effect of the respective single-drug treatments.[11] Synergistic interactions between metronidazole and amoxicillin or amoxicillin and the hydroxymetabolite of metronidazole has been shown for the suspected periodontal pathogen *A. actinomycetemcomitans* only.[12,13] In addition, a recent analysis of whole subgingival plaque samples suggests a combinatorial effect of the combination of amoxicillin and metronidazole.[14] Using this approach, a differentiation between additive and synergistic effects is not possible and therefore, a testing of individual isolates of oral bacteria seems to be indicated.

Various methods exist to test for *in vitro* synergy including checkerboard titrations and time-kill methods, which are the most commonly applied techniques.[15] Recently, Etest combination testing methods have been introduced to evaluate the synergy of antimicrobials.[15–19]

Another Etest technology is based on the application of Etest strips directly on mono- or polymicrobial samples obtained from either the lower respiratory tract or sputum without first cultivating individual microorganisms.[20,21] In addition to a visualization of the antibiotic concentrations that will inhibit all microorganisms present in a polymicrobial sample, different bacterial populations with different levels of resistance as well as subpopulations of resistant bacteria inside the ellipse of inhibition can be detected. Recently, the Etest was used to compare the antibiotic susceptibilities of pure cultures and definite co-cultures of suspected oral pathogens.[22] However, whole subgingival plaque samples have not been tested so far.

Therefore, the aims of the present study were (i) to assess the *in vitro* combinatorial effects of subgingival bacterial isolates and (ii) to analyze the potential of the Etest for synergy testing on whole plaque samples.

2. Materials and methods

2.1. Bacterial strains and culture conditions

All bacterial strains were routinely maintained on Columbia blood agar plates (Columbia Agar Base [BBL Becton Dickinson, Allschwil, Switzerland] supplemented with 4 mg/l hemin, 1 mg/l menadione, and 50 ml/l human blood) under anaerobic conditions (10% CO_2, 10% H_2, 80% N_2) at 36 °C. Respective isolates were selected either from a previous study,[23] from our strain collection of clinical isolates or from fresh subgingival plaque samples as described below.

2.2. Combination resistance testing of selected oral bacteria by Etest

A necessary requirement for detecting potential combinatorial effects by Etest strips is that the minimal inhibitory concentration (MIC) values for the individual antibiotics are above 0.016 mg/l, as the Etest strips for amoxicillin and metronidazole have antibiotic concentration ranges of 0.016–256 mg/l.

From a previous study, eight strains of *A. actinomycetemcomitans* and six strains of *Prevotella intermedia/nigrescens* could be selected which met these prerequisites.[23] The respective *A. actinomycetemcomitans* isolates had MIC values between 0.25 mg/l and 0.27 mg/l for amoxicillin and between 0.75 mg/ml and 24 mg/l for metronidazole, and the *P. intermedia/nigrescens* isolates had MIC values between 1 mg/l and 8 mg/l for amoxicillin and between 0.063 mg/l and 0.25 mg/l for metronidazole. However, no *Porphyromonas gingivalis* isolate met this requirement.[23]

Furthermore, 21 *Fusobacterium nucleatum* and 36 *Eikenella corrodens* strains were selected from our strain collection of clinical isolates. All strains have been isolated from various intraoral sites and stored at −70 °C (Microbank, Chemie Brunschwig, Basel, Switzerland). The MIC values of these bacterial species against amoxicillin and metronidazole were determined by the Etest (Axonlab, Baden, Switzerland) method as described by Kulik et al.[23] The breakpoints for susceptibility of anaerobes to the antibiotics were applied as recommended by the Clinical and Laboratory Standards Institute.[23–25] From these strains, isolates were selected which had MIC values for the individual antibiotics above 0.016 mg/l in order to be able to detect potential combinatorial effects by Etest strips.

Additionally, individual colonies of *P. gingivalis*, *P. intermedia/nigrescens*, *A. actinomycetemcomitans*, *F. nucleatum* and *E. corrodens* were isolated from fresh subgingival plaque samples and tested for comparison. Also here, isolates were selected which had MIC values for the individual antibiotics above 0.016 mg/l in order to be able to detect potential combinatorial effects by Etest strips.

The combination testing was done according to the MIC/MIC Ratio Method (Etest application sheet EAS 023; AB BIODISC, Solna, Sweden). Briefly, an amoxicillin Etest strip was placed on the inoculated agar surface and left for 1 h at room temperature under anaerobic conditions (10% CO_2, 10% H_2, 80% N_2). The position corresponding to the MIC value obtained before for amoxicillin was marked on the agar surface with a sterile needle on both sides of the strip and the amoxicillin strip was removed.[19] A metronidazole Etest strip was then placed on top of the imprint of the first Etest strip using a Nema C88 vacuum pen (AB BIODISC, bioMérieux, Geneva, Switzerland) so that the MICs for amoxicillin and metronidazole overlapped at the same position. The metronidazole strip was left on the agar plate and the plates were incubated under anaerobic conditions (10% CO_2, 10% H_2, 80% N_2) for one week. The results were interpreted by two examiners and performed at least twice.

2.3. Isolation of bacteria from subgingival plaque samples

Fresh subgingival plaque samples of three randomly selected otherwise healthy patients with advanced periodontitis (one female, two males) were taken for antibiotic resistance analysis by pooled paper points according to standard protocols.

Briefly, supragingival plaque was removed, the sampling site (PPD > 6 mm) was isolated using cotton rolls and gently dried with air. A sterile paper point was inserted to the bottom of the pocket, left in place for 20 s and placed in 0.5 ml of thioglycolate broth (bioMérieux, Geneva, Switzerland).[14,26] Immediately after sampling, the paper points were vortexed