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Efficacy of antibiotics to strains of periodontopathogenic bacteria within a single species biofilm – an in vitro study

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Abstract

Objectives: This study examined differences in the efficacy of antibiotics against a single strain of three periodontal pathogens grown in an artificial biofilm.

Methods: Single species biofilms were established with artificial saliva and one of the following bacterial strains: *Actinobacillus actinomycetemcomitans* Y4, *Streptococcus constellatus* 384b (a clinical isolate) and *Porphyromonas gingivalis* ATCC 33277. The efficacy of the antibiotics clindamycin, doxycycline, metronidazole, and moxifloxacin to these bacteria was determined using concentrations up to 100-fold minimal inhibitory concentration (MIC) to planctonic bacteria over 48 h.

Results: The ability of the bacteria to form a biofilm varied. The biofilms of *S. constellatus* 384b and *A. actinomycetemcomitans* Y4 contained more viable bacteria and showed a larger thickness in SEM photographs than those of *P. gingivalis* ATCC 33277. The antibiotics tested showed different efficacy for the different strains. Moxifloxacin was the most efficient antibiotic: onefold MIC was sufficient to eliminate *A. actinomycetemcomitans* Y4 and *P. gingivalis* ATCC 33277 after 48 h. However, only the 50-fold MIC completely eradicated *S. constellatus* 384b. SEM photographs underlined the damaging effect of moxifloxacin on the biofilm structure.

Conclusion: The complete removal of bacteria by the use of antibiotics alone seems to be impossible when taking into account MIC values and the level of antibiotics in gingival fluid.

Keywords: antibiotics; biofilm; periodontopathogenic species

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Biofilms are a main focus of research in many fields of microbiology and human disease. In the oral cavity, microbial-plaque communities are biofilms composed of numerous bacteria on host surfaces. The bacteria are embedded in exopolymers and slime layers (O'Toole et al. 2000). Dental plaque plays a key role in the pathogenesis of dental caries and periodontal disease. Most studies concerning biofilm investigated streptococci causing dental caries (Larsen & Fiehn 1996, Pratten et al. 1998) and only a few studies focused on the importance of biofilms in periodontitis (Ellen et al. 1997, Hansen et al. 2000).

In cases of severe forms of periodontitis antibiotics in combination with mechanical treatment are used. Common antibiotics are metronidazole, doxycycline and clindamycin (Bollen & Quirynen 1996, Walker & Karpinia 2002). In vitro resistance patterns also showed very low minimal inhibitory concentrations (MICs) of moxifloxacin on periodontopathogenic anaerobic and capnophilic species (Pfister et al. 2000). In vitro resistance patterns, however, only consider MICs that are relevant in patient serum in cases of systemically applied antibiotics. Higher concentrations of antibiotics in gingival fluid are achievable in topical application. Concentrations of over 100 μg/ml of clindamycin (Higashi et al. 1991), doxycycline (Stoller et al. 1998) and metronidazole (Stoltze 1992) have been measured over a few days. In general, it is postulated that the susceptibility of bacteria to antibiotics is reduced within biofilms (Sanz & Herrera 2001). A possibly different efficacy is mostly not included in recommendations. This pilot study aimed to obtain some

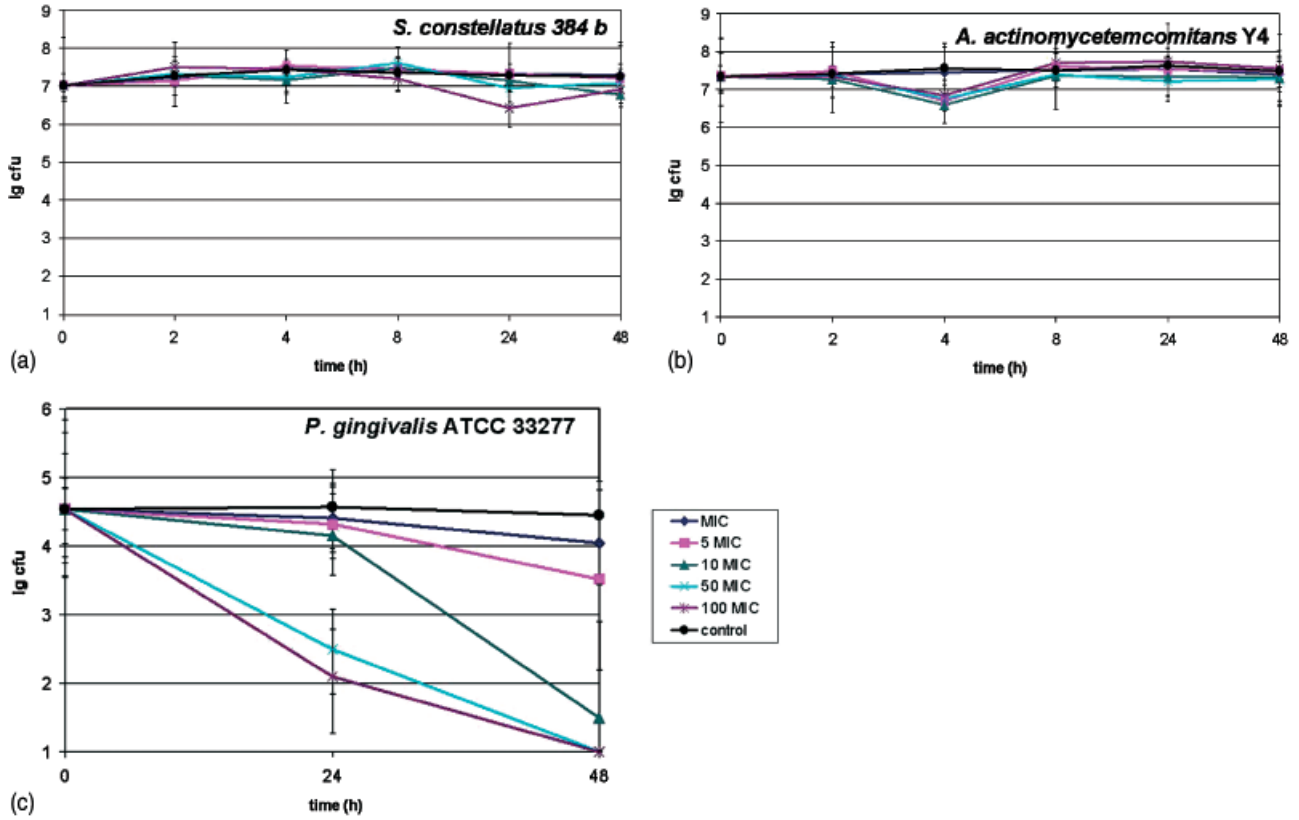

Fig. 1. Number of colony-forming units (CFU) of *S. constellatus* 384b, *A. actinomycetemcomitans* Y4 and *P. gingivalis* ATCC 33277 in a biofilm after exposure to different clindamycin MICs over a period of 48 h (results expressed as medians including range).

knowledge about the differences in the efficacy of antibiotics on single strains of periodontopathogenic bacteria within a biofilm.

Material and Methods

Bacterial strains

Three different bacterial strains were used. *Streptococcus constellatus* J384b is a clinical isolate obtained from a patient with a severe, recurrent form of chronic periodontitis. *Porphyromonas gingivalis* ATCC 33277 represents the type strain. *Actinobacillus actinomycetemcomitans* Y4 is a strain well known for its synthesis of leukotoxin. The strains were subcultivated to log phase on appropriate solid media 16 h before the test assay.

Antibiotics

The following antimicrobial agents were studied: clindamycin (ratiopharm, Ulm, Germany); doxycycline (ratiopharm, Ulm, Germany); metronidazole (Braun, Melsungen, Germany); and moxifloxacin (Bayer-Vital, Wuppertal, Germany). Antibiotic MICs for the selected planctonic bacteria were determined using the bouillon dilution technique. Mueller–Hinton broth for *S. constellatus* J384b and Wilkins–Chalgren-broth enriched with 5% lysed sheep blood for *A. actinomycetemcomitans* Y4 and *P. gingivalis* ATCC 33277 were used as test media. Tubes were incubated for 48 h in a capnophilic or anaerobic atmosphere (*P. gingivalis* ATCC 33277). The MIC of an antibiotic was defined as the lowest concentration without visible turbidity of the broth.

Biofilm assay

Commercially available slides and cuvettes were used for the biofilm assay. First, the slides were soaked in artificial saliva (ISO 10993) for 1 h (1 l contained 0.7 g sodium chloride, 0.26 g disodium phosphate, 0.33 g potassium thiocyanate, 1.2 g potassium dihydrogen phosphate, 1.5 g sodium hydrogen carbonate and 1.2 g potassium chloride). This solution was supplemented with 4 g porcine mucin and 50 g albumin for 1 h. The slides were then transferred to a new cuvette containing 120 ml nutrient broth (Schaedler broth with 5% sheep blood and 0.1 ml bacterial suspension (MacFarland 4)). The bacteria were allowed to attach to the slide surface and to form an artificial biofilm. Subsequently the slides were transferred once more to another cuvette. This cuvette contained Mueller–Hinton broth or Wilkins–Chalgren-broth, respectively, with 5% lysed sheep blood and the antibiotic in the appropriate concentration. The concentrations tested were: control without antibiotic, onefold MIC, fivefold MIC, 10-fold MIC, 50-fold MIC and 100-fold MIC. Two slides each were removed at 0, 2, 4, 8, 24 and 48 h except for *P. gingivalis* ATCC 33277 (0, 24 and 48 h) and dipped in PBS for 30 sec to remove non-adherent bacteria. Colony-forming units (CFU) per slide were determined after ultrasonic treatment (160 W for 1 min), plating on the appropriate media and cultivation. All experiments were carried out in duplicate so that at least four single data per each concentration of an antibiotic and per bacterial strain were included for the medians presented in the results.

In addition, SEM photographs were taken. A 24-well tissue culture plate was

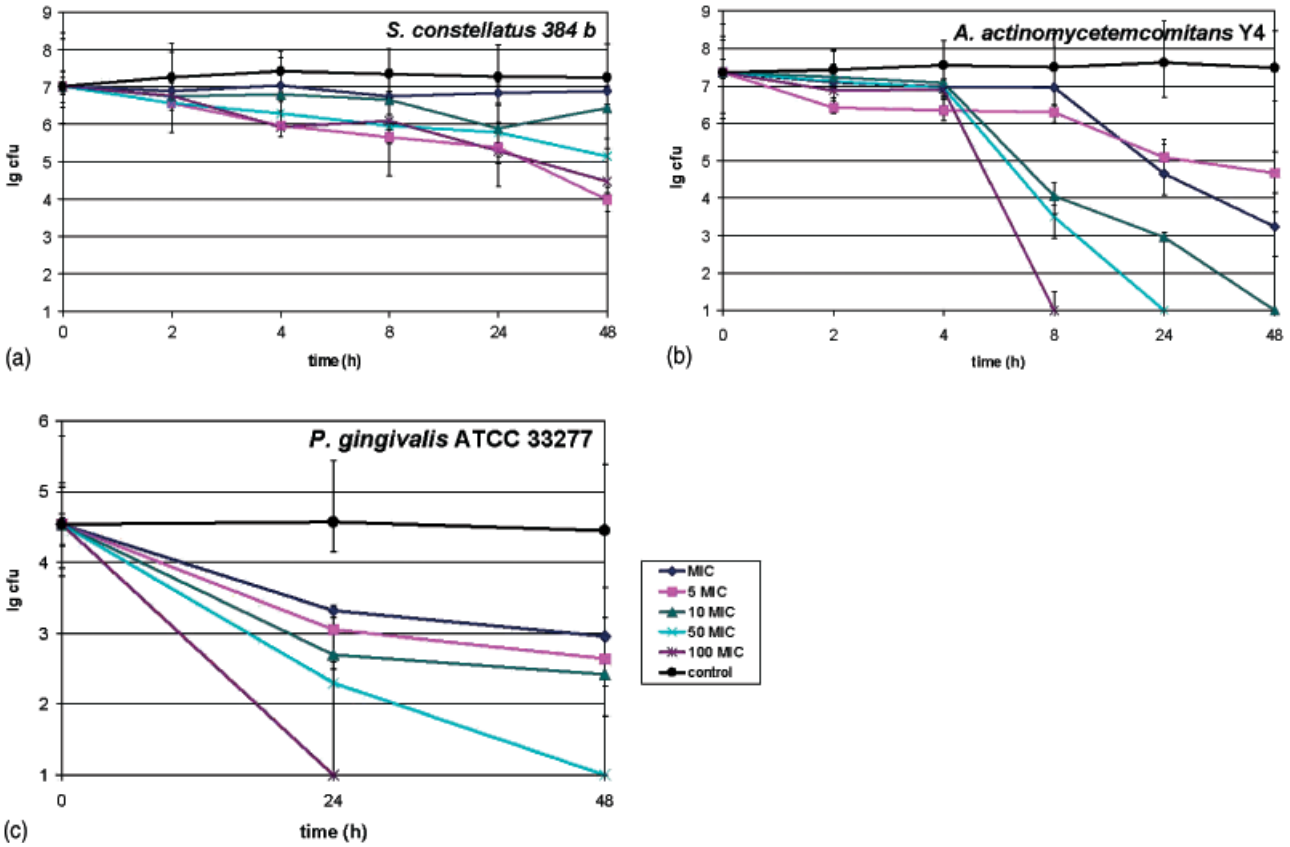

Fig. 2. Number of colony-forming units (CFU) of *S. constellatus* 384b, *A. actinomycetemcomitans* Y4 and *P. gingivalis* ATCC 33277 in a biofilm after exposure to different doxycyclin MICs over a period of 48 h (results expressed as medians including range).

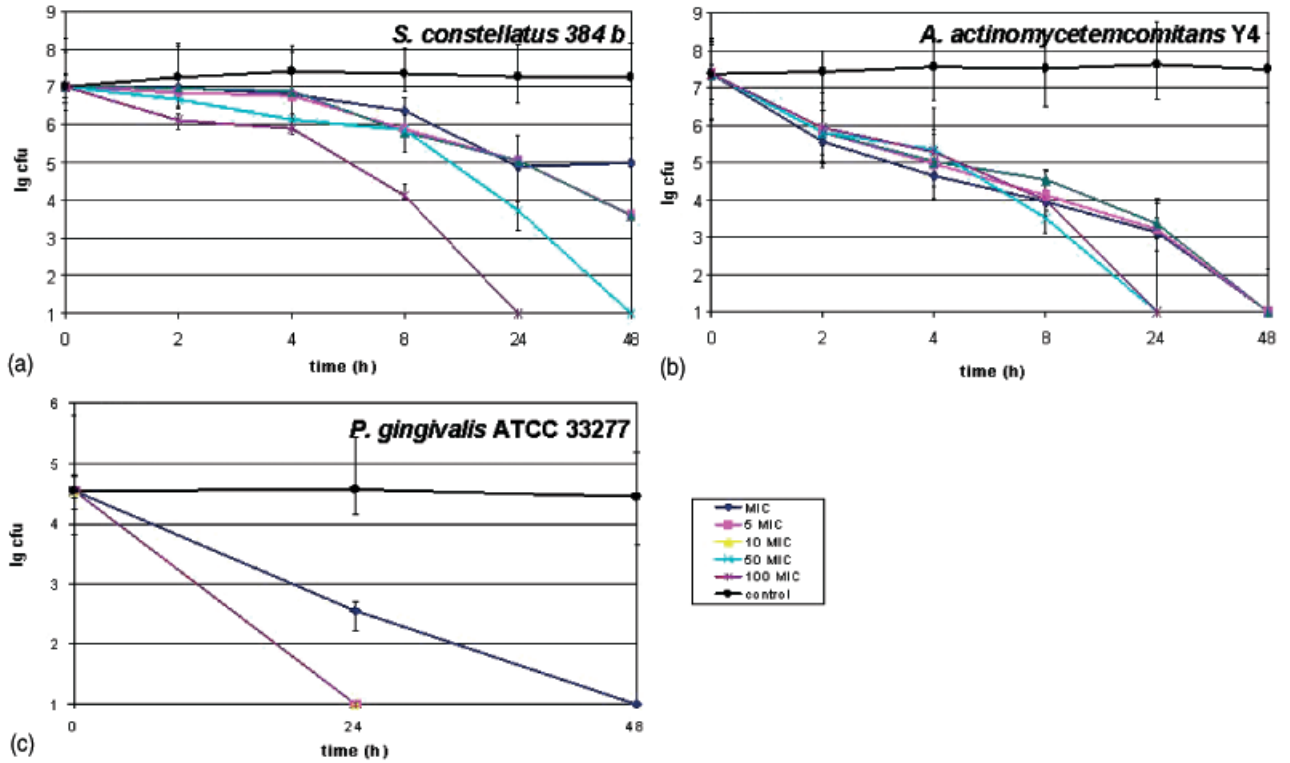

Fig. 3. Number of colony-forming units (CFU) of *S. constellatus* 384b, *A. actinomycetemcomitans* Y4 and *P. gingivalis* ATCC 33277 in a biofilm after exposure to different moxifloxacin MICs over a period of 48 h (results expressed as medians including range).

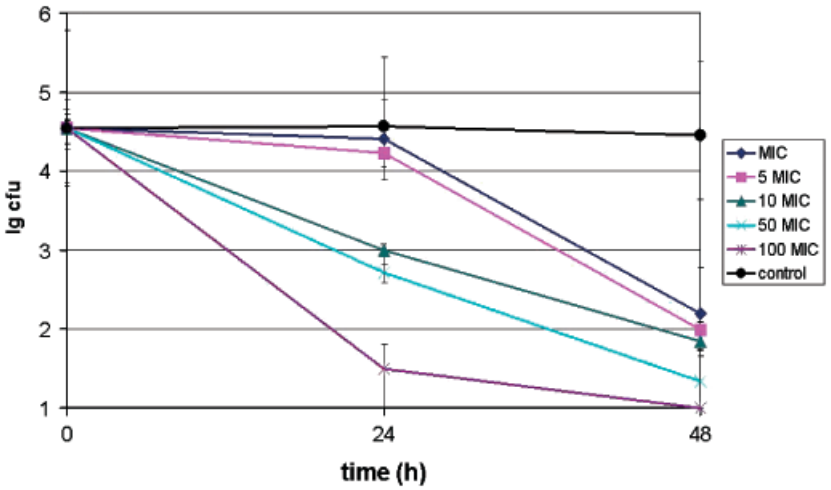

Fig. 4. Number of colony-forming units (CFU) of *P. gingivalis* ATCC 33277 in a biofilm after exposure to different metronidazole MICs over a period of 48 h (results expressed as medians including range).

Table 1. Minimal inhibitory antibiotic concentrations (MIC) for planctonic bacterial strains; determined by bouillon dilution technique

Bacterial strain	MIC (μg/ml)			
	Clindamycin	Doxycycline	Metronidazole	Moxifloxacin
S. constellatus J384b	1	16	>256	0.25
A. actinomycetemcomitans Y4	2	2	>256	0.25
P. gingivalis ATCC 33277	0.016	0.25	1	0.032

Table 2. Minimal inhibitory antibiotic concentration for bacterial strains tested in an artificial biofilm (after 48 h exposure)

Bacterial strain	MIC (μg/ml)			
	Clindamycin	Doxycycline	Metronidazole	Moxifloxacin
S. constellatus J384b	>100	>1600	>256	12.5
A. actinomycetemcomitans Y4	>200	20	>256	0.25
P. gingivalis ATCC 33277	0.8	12.5	100	0.032

filled with cover slips. The slips were rinsed with 200 μl artificial saliva in each case for 1 h and thereafter 1 ml nutrient broth supplemented with the bacterial suspension was added. After an incubation time of 24 h in the appropriate atmosphere, the medium was removed, the slips were carefully washed with PBS and the test media (10-fold MIC of each antibiotic including a control without antibiotic) were added and incubated again for 24 h. After removal of the media and washing, the samples were initially fixed in 2% glutaraldehyde in cacodylate buffer for 30 min, then washed twice with cacodylate buffer and dehydrated for 10 min using a graded ethanol series. A critical point drying procedure followed and the specimens were then sputter-coated with gold. Samples were examined with LEO 1450 VP (Carl Zeiss Company, Germany).

Results

MICs for planctonic bacteria

The MICs for the planctonic bacteria are presented in Table 1. Clindamycin and moxifloxacin showed sufficient efficacy for the strains selected. Doxycycline MIC for *S. constellatus* J384b was high (16 μg/ml). *S. constellatus* and *A. actinomycetemcomitans* were highly resistant to metronidazole. Its efficacy in a biofilm was therefore only studied in *P. gingivalis* ATCC 33277.

MICs for bacteria within a biofilm

All bacterial strains were able to form biofilms in this test assay. The numbers of viable bacteria remained constant over the test period of 48 h. Nevertheless, the numbers of *S. constellatus* 384b and *A. actinomycetemcomitans* Y4 were two log stages higher than those of *P. gingivalis* ATCC 33277. Clindamycin concentrations up to 100-fold MIC had no effect on *S. constellatus* J384b and *A. actinomycetemcomitans* Y4. The number of viable *P. gingivalis* ATCC 33277 was reduced dependent on the antibiotic concentration, but only 50-fold MIC was able to kill all the bacteria in a biofilm after 48 h (Fig. 1).

Doxycycline inhibited the growth of the bacterial strains in relation to time and concentration. Complete inactivation of *S. constellatus* J384b, however, was not achieved by any of the applied concentrations. A 10-fold MIC was required to kill *A. actinomycetemcomitans* Y4 after 48 h and 50-fold MIC eliminated this strain after 24 h. *P. gingivalis* ATCC 33277 was killed by 50-fold MIC after 48 h (Fig. 2).

Moxifloxacin reduced the number of viable bacteria in all concentrations tested. *S. constellatus* was completely eradicated by 50-fold MIC after 48 h. Onefold MIC for planctonic bacteria eliminated both *A. actinomycetemcomitans* and *P. gingivalis* after 48 h; higher concentrations were effective after a shorter period of time (Fig. 3).

Metronidazole was only studied in *P. gingivalis*. This antibiotic also showed a concentration-dependent effect on the bacterium, although only 100-fold MIC could eliminate this anaerobic species within a biofilm (Fig. 4).

Considering the MICs for planctonic bacteria and the conditions of the artificial biofilm, concentrations above 100 μg/ml for the antibiotics with the exception of moxifloxacin were required for *S. constellatus* 384b. For *A. actinomycetemcomitans* Y4, 20 μg/ml doxycycline and >200 μg clindamycin were necessary for elimination (Table 2). *P. gingivalis* ATCC 33277 was only killed by 100 μg/ml metronidazole (Table 2).

SEM photographs

SEM photographs (Figs. 5–7) underline the results obtained by CFU counting. *S. constellatus* 384b and *A. actinomycetemcomitans* Y4 formed thick biofilms with many bacteria visibly attached to

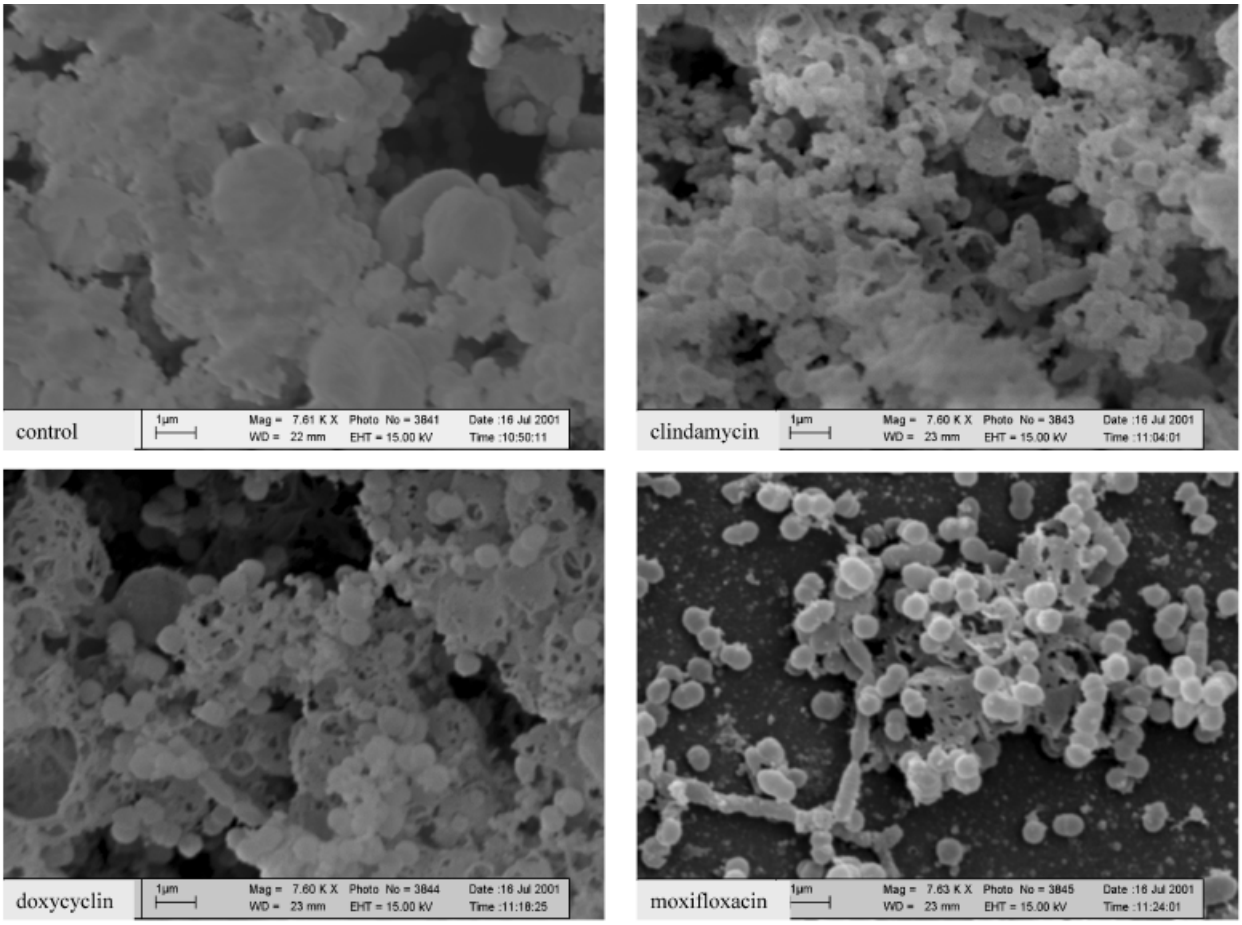

Fig. 5. SEM photographs of *S. constellatus* 384b biofilm after 24 h exposure to 10-fold MIC of clindamycin, doxycycline and moxifloxacin.

the surfaces and coaggregated among themselves. *P. gingivalis* ATCC 33277 formed a thinner layer of biofilm. It adhered to the saliva-coated surface of the slide and to erythrocytes. After the addition of clindamycin, SEM photographs showed only minor deviations of the bacterial surface and the matrix of the biofilm; the number of bacteria appeared not to be reduced. Doxycycline changed the structure of the biofilm, and fewer bacteria were visible in comparison to the controls. Moxifloxacin showed the most striking results, with the matrix apparently partially disappearing and the number of bacteria being remarkably reduced. Bacterial cells such as *A. actinomycetemcomitans* Y4 became elongated and showed a damaged surface. Metronidazole clearly had no influence on the biofilm of *P. gingivalis* ATCC 33277.

Discussion

The main purpose of this pilot study was to show differences in the efficacy of four different antibiotics on a single strain of each three test bacterial species grown in an artificial biofilm. Although biofilms in the oral cavity (plaque) can contain hundreds of bacterial species, only individual bacterial strains were tested in a biofilm in this in vitro study. One has to conclude that the efficacy of the antibiotics is substantially reduced in vivo because of the many different species, coaggregation among bacterial species and the more complex structure of the dental plaque. The bacteria communicate through coaggregations and metabolic interactions and contacts induce the expression of surface proteins (Kolenbrander 2000). Recently, some reviews were published about antibiotic resistance within a biofilm (Lewis 2001, Mah & O'Toole 2001, Stewart & Costerton 2001). In general, the following statements were made: the antibiotics penetrate slowly or incompletely into a biofilm; local accumulation of microbial nutrients and waste products alter the local environment; the decreasing growth rate of bacteria leads to a decreasing sensitivity to antibiotics; a few bacteria differentiate into a protected state, they become persisters. In vitro models of biofilm formation that successfully included two species such as *P. gingivalis* and *Actinomyces viscosus* or *S. gordonii* have been described (Ellen et al. 1997, Cook et al. 1998), but it is difficult to establish a more complex model with *P. gingivalis.* It has not been possible to detect this anaerobic species in an artificial microcosm after application of 11 species (Saunders & Greenman 2000).

In our study, a simple technique simulating the conditions for biofilm formation in the oral cavity was used. All bacterial strains included in the study were able to colonise within a biofilm. Furthermore, the number of viable bacteria within a biofilm remained constant, although only artificial saliva including mucin supplemented with albumin were given to the media for the first 12 h. Albumin represents the essential protein of the gingival fluid (Makela et al. 1991).

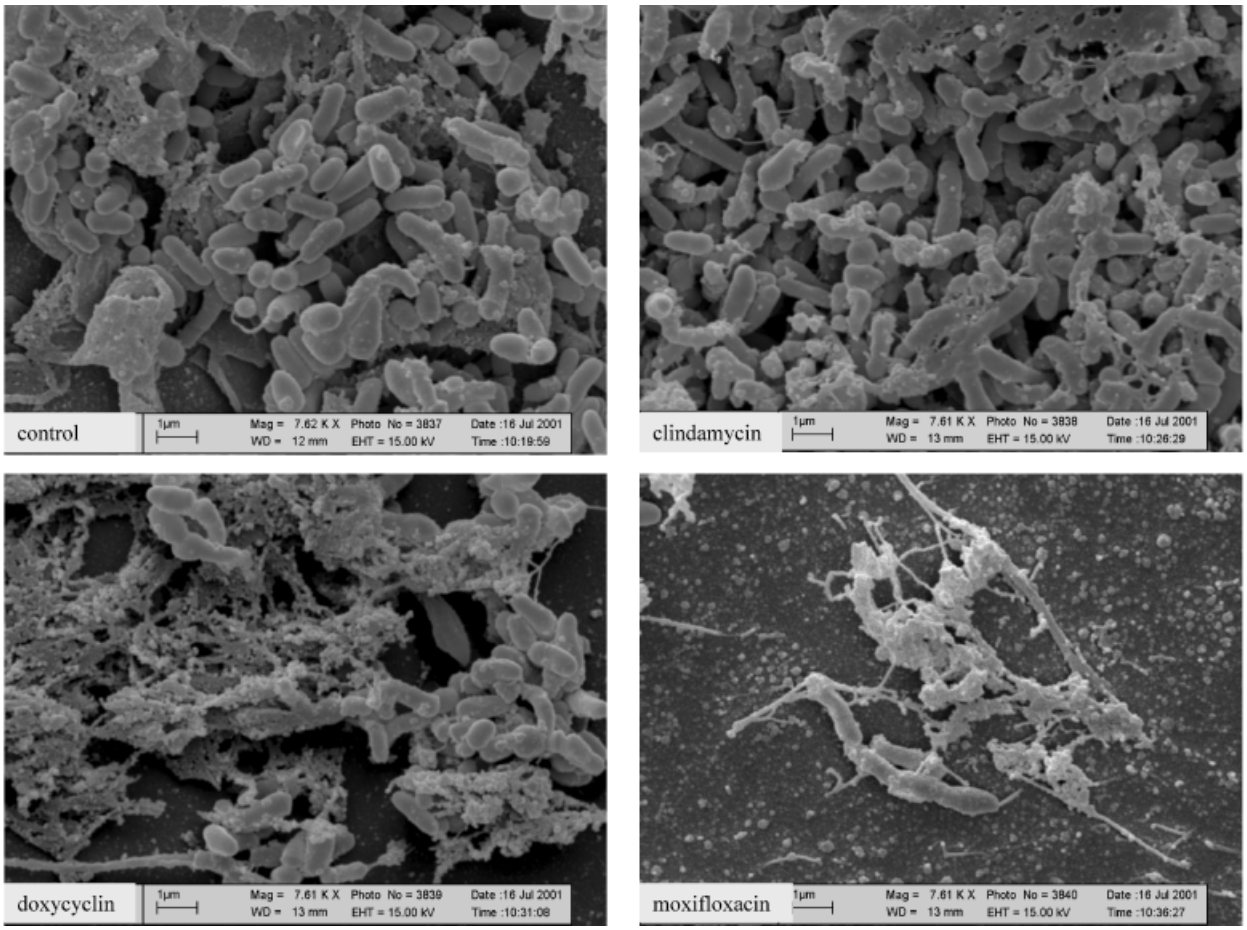

Fig. 6. SEM photographs of *A. actinomycetemcomitans* Y 4 biofilm after 24 h exposure to 10-fold MIC of clindamycin, doxycycline and moxifloxacin.

This study investigated periodontopathogenic bacteria. *A. actinomycetemcomitans* and *P. gingivalis* represent two important periodontal pathogens (Genco et al. 1996); *S. constellatus* was chosen as a streptococcal strain. Streptococci are known for their ability to form biofilms. Oral streptococci are usually indicators of a healthy periodontium. Only *S. constellatus* and the related *S. intermedius* were found in higher quantities in special forms of periodontitis (Socransky et al. 2000). The quantity of colonised bacteria within the biofilm varied between the strains. The amount of *S. constellatus* J384b and also *A. actinomycetemcomitans* Y4 was high whereas the number of *P. gingivalis* ATCC 33277 was much lower. Fimbriae of *P. gingivalis* can bind to statherin, proline-rich proteins and proline-rich glycoproteins of the saliva (Amano et al. 1999), but the strain usually belongs to the late colonisers of dental plaque and it adheres to already established microorganisms of this community (Kolenbrander 1993).

Clindamycin up to 100-fold MIC had no effect on *S. constellatus* J384b and *A. actinomycetemcomitans* Y4 and showed lower efficacy in *P. gingivalis* ATCC 33277 compared with doxycycline and moxifloxacin. This low efficacy in a biofilm is surprising because clindamycin is well known to influence the formation of a biofilm (Ichimiya et al. 1994). The MIC of doxycycline for *S. constellatus* J384b was high. Fiftyfold MICs ($50 \times 0.25\ \mu g/ml = 12.5\ \mu g/ml$) for the elimination of *P. gingivalis* ATCC 33277 and 10-fold MICs ($10 \times 2\ \mu g/ml = 20\ \mu g/ml$) for *A. actinomycetemcomitans* are the required concentrations in gingival fluid. This finding emphasises that a complete elimination of periodontopathogenic species in a biofilm by doxycycline systemically applied might be questionable. *S. constellatus* J384b and *A. actinomycetemcomitans* Y4 as capnophilic species were resistant to metronidazole. Therefore only *P. gingivalis* was tested. Even 50 MIC of metronidazole was not able to kill all the bacteria in a biofilm after 48 h. This result is similar to Wright et al. (1997) who reported a concentration of 160 MIC for *P. gingivalis* within a biofilm after 4 h. The planctonic *P. gingivalis* strain tested had an MIC of 1 μg/ml. Our finding implies that at least 100 μg/ml (100-fold MIC) are required to be effective in a biofilm and this strain has to be considered resistant. The serum level of metronidazole is about 10 μg/ml if the antibiotic is systemically applied (Galmier et al. 1998). Although locally applied, metronidazole offers benefits over scaling and root planing, a treatment regimen of scaling and root planing in combination with a tetracycline gave a greater reduction in probing depth (Kinane & Radvar 1999, Salvi et al. 2002).

Moxifloxacin was the most efficient antibiotic to all tested bacterial strain within a biofilm. The MIC was sufficient for elimination of *P. gingivalis* ATCC 33277 and *A. actinomycetemcomitans* Y4 after 48 h. *S. constellatus* J384b was also completely eradicated

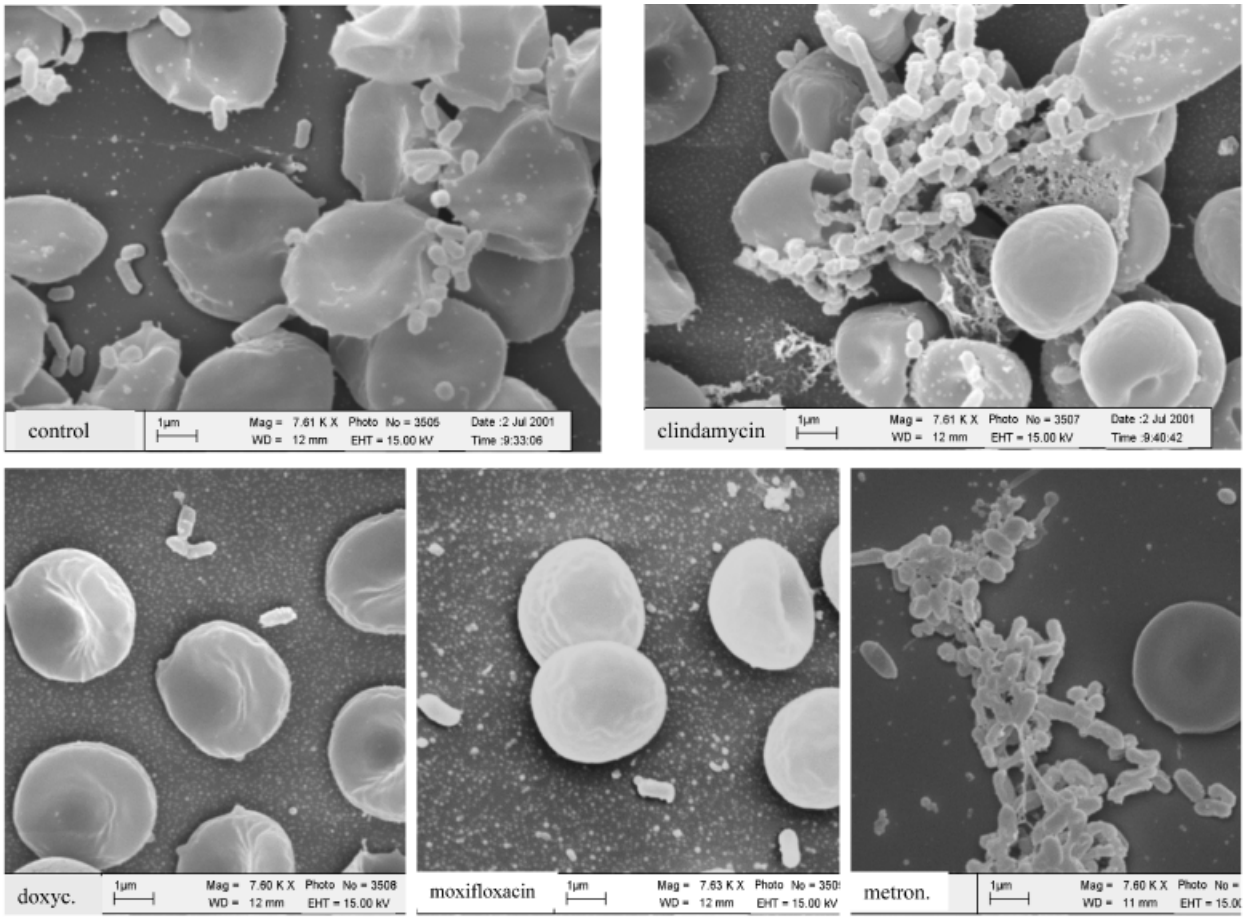

Fig. 7. SEM photographs of *P. gingivalis* ATCC 33277 biofilm after 24 h exposure to 10-fold MIC of clindamycin, doxycycline, moxifloxacin, and metronidazole.

by the highest tested concentration of 50 MIC. Considering the MIC of 0.25 μg/ml and the achievable level of 4.73 μg/ml moxifloxacin in serum (Stass et al. 1998) or gingival fluid, this *S. constellatus* strain cannot be eliminated by this antibiotic. The good efficacy of new fluorquinolones to bacteria is well known. Compared with macrolides, newer fluorquinolones such as moxifloxacin show greater efficacy in *Staphylococcus aureus* strains within a biofilm (Dasgupta et al. 1997).

The present study clearly demonstrated antibiotic-specific effects. The efficacy of the antibiotics, however, depended also on the bacterial strain tested. *S. constellatus* 384b showed the lowest sensitivity to all antibiotics tested. A study by Larsen & Fiehn (1996) even found a strain-specific effect. They assessed the efficacy of doxycycline in two *Streptococcus sanguis* strains. One strain was eliminated by fivefold MIC and the other one needed 500-fold MIC.

The efficacy of the antibiotics tested is different within a biofilm. The good result for moxifloxacin might be an additional indication for testing this antibiotic in clinical trials. However, considering the MIC and achievable levels, elimination of species such as *S. constellatus* is questionable. The results of our study underline that MICs to planctonic bacteria cannot be easily transferred to bacteria within a biofilm.

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