Antibacterial activity of moxifloxacin on bacteria associated with periodontitis within a biofilm

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The activity of moxifloxacin was compared with ofloxacin and doxycycline against bacteria associated with periodontitis within a biofilm (single strain and mixed population) in vitro. MICs and minimal bactericidal concentrations (MBCs) of moxifloxacin, ofloxacin and doxycycline were determined against single strains and mixed populations in a planktonic state. Single-species biofilms of two Porphyromonas gingivalis and two Aggregatibacter actinomycetemcomitans strains and a multispecies biofilm consisting of 12 species were formed for 3 days. The minimal biofilm eradication concentrations (MBECs) were determined after exposing the biofilms to the antibacterials (0.002–512 \( \mu \text{g ml}^{-1} \)) for 18 h, addition of nutrient broth for 3 days and subsequent subcultivation. Photographs were taken using confocal laser-scanning microscopy and scanning electron microscopy. The MICs and MBCs did not differ between ofloxacin and moxifloxacin against A. actinomycetemcomitans, whilst moxifloxacin was more active than the other tested antibacterials against anaerobes and the mixed population. The single-species biofilms were eradicated by moderate concentrations of the antibacterials, and the lowest MBECs were always found for moxifloxacin (2–8 \( \mu \text{g ml}^{-1} \)). MBECs against the multispecies biofilms were 128, >512 and >512 \( \mu \text{g ml}^{-1} \) for moxifloxacin, ofloxacin and doxycycline, respectively. In summary, moxifloxacin in a topical formulation may have potential as an adjunct to mechanical removal of the biofilms.

INTRODUCTION

Tooth loss is mostly a consequence of plaque-caused diseases such as periodontitis and caries, followed by endodontic infections. Oral microbial-plaque communities are biofilms composed of numerous bacteria on host surfaces. Streptococci and actinomycetes are the major initial colonizers. Fusobacteria play a central role as bridges that promote co-aggregation to anaerobic bacteria (Kolenbrander, 2000). Periodontal disease status impacts markedly on biofilm composition (Socransky & Haffajee, 2005). It is generally accepted that a small group of predominantly Gram-negative anaerobic or microaerophilic bacteria is associated with initiation and progression of periodontitis. Organisms strongly implicated as aetiological agents of periodontitis include Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Tannerella forsythia and Treponema denticola (Anonymous, 1996). The high proteolytic activity of Porphyromonas gingivalis, an asaccharolytic anaerobe, is mainly the result of gingipains (arginine- and lysine-specific cysteine proteases), which are considered to be one of the most important virulence factors (Guo et al., 2010). Virulence of Aggregatibacter actinomycetemcomitans is associated with synthesis of many toxins such as leukotoxin and cytotoxin (Henderson et al., 2003).

Controlling the total oral bacterial load and especially the periodontopathogenic bacteria is an essential component of any periodontitis treatment and is associated with an improvement and a stability of a post-therapeutic outcome. Plaque is removed mechanically by scaling and root planing (Van der Weijden & Timmerman, 2002; Apatzidou & Kinane, 2010). In particular cases, antibacterial chemotherapeutics might be useful when applied systemically (Herrera et al., 2008). Systematic reviews underline a probable positive effect for metronidazole combined with amoxicillin on clinical parameters in the treatment of aggressive and chronic periodontitis (Sgolastra et al., 2011, 2012). Recently, azithromycin has been tested to be effective in aggressive periodontitis patients (Haas et al.,

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**Abbreviations**: CLSM, confocal laser-scanning microscopy; MBC, minimal bactericidal concentration; MBEC, minimal biofilm eradication concentration; SEM, scanning electron microscopy.
2008), whereas the results were contradictory in chronic periodontitis patients (Yashima et al., 2009; Han et al., 2012). Tetracyclines are mostly used as topical antibacterial chemotherapeutics (Bosco et al., 2009; Bland et al., 2010). Quinolones were only rarely included in clinical trials of periodontitis. Ciprofloxacin and ofloxacin were systematically applied to eradicate Aggregatibacter actinomycetemcomitans ( Müller et al., 1998; Kleinfelder et al., 2000). Moreover, ofloxacin was tested in a topical formulation (Yamagami et al., 1992). In vitro, moxifloxacin has a better activity towards anaerobes (including Porphyromonas gingivalis) than ofloxacin and ciprofloxacin (Behra-Miellet et al., 2002). In vivo, it shows superiority over doxycycline in systemic application (Guentsch et al., 2009). Tetracyclines are mostly used as topical antibacterial chemotherapeutics (Bosco et al., 2012). Moxifloxacin was tested in a topical formulation (Yamagami et al., 1992). In vitro, moxifloxacin has a better activity towards anaerobes (including Porphyromonas gingivalis) than ofloxacin and ciprofloxacin (Behra-Miellet et al., 2002). In vivo, it shows superiority over doxycycline in systemic application (Guentsch et al., 2008) and seems to be able to reduce pocket depths when applied topically (Flemming et al., 2011).

Our hypothesis was that moxifloxacin has a superior activity against bacteria associated with periodontitis in multispecies biofilm compared with doxycycline and ofloxacin. Thus, the aim of this in vitro study was to obtain more information about the antimicrobial activity of moxifloxacin in comparison with other antibacterial chemotherapeutics against selected bacteria in a planktonic form and within biofilms. These experiments were undertaken using monocultures and a mixed population consisting of 12 different species normally present in a periodontopathogenic biofilm to find differences among planktonic bacteria and within biofilms as well as between monocultures and mixed cultures.

**METHODS**

**Antibacterial chemotherapeutics.** Moxifloxacin (Bayer Innovation GmbH), ofloxacin (Sigma-Aldrich) and doxycycline (Bayer Innovation) were tested. A stock solution of 1024 µg ml⁻¹ in deionized water was prepared and diluted twice with deionized water down to a concentration of 0.004 µg ml⁻¹. These dilutions were always added in a ratio of 1:1 to the nutrient broth. Thus, the final tested concentrations ranged from 512 µg ml⁻¹ to 0.002 µg ml⁻¹. deionized water served as the negative control.

**Micro-organisms.** For the biofilm experiments, two Aggregatibacter actinomycetemcomitans strains (Y4 and clinical isolate J7) were chosen. Two Porphyromonas gingivalis strains (ATCC 33277 and clinical isolate M5-1-2) were also included. Additionally, a mixed population consisting of 12 species (Streptococcus gordonii ATCC 10558, Actinomyces naeslundii ATCC 12104, Fusobacterium nucleatum ATCC 25586, Campylobacter rectus ATCC 33238, Eubacterium mundi ATCC 33099, Eikenella corrodens ATCC 23834, Prevotella intermedia ATCC 25611, Parvimonas micra ATCC 33270, Porphyromonas gingivalis ATCC 33277, Tannerella forsythia ATCC 43037, Treponema denticola ATCC 35405, Aggregatibacter actinomycetemcomitans Y4) was used.

All the strains were pre-cultivated for 24–72 h prior to the experiments. Modified tryptic soy agar (Ferres et al., 2002) was used except for Treponema denticola ATCC 35405. Here, the medium was mycoplasma broth (BD) with the addition of 1 mg glucose ml⁻¹, 400 µg niacinamide ml⁻¹, 150 µg spermine tetrahydrochloride ml⁻¹, and 20 µg sodium isobutyrate ml⁻¹ and enriched by 1 mg cysteine ml⁻¹ and 5 µg cocarboxylase (Sigma-Aldrich) ml⁻¹. Cultivation was always carried out at 37 °C. The atmosphere was 5% CO₂ for the Aggregatibacter actinomycetemcomitans strains and for S. gordonii ATCC 10558. The other strains were incubated anaerobically.

From all cultures, a microbial suspension of approximately McFarland standard 0.5 (~1.5 × 10⁸ micro-organisms) was prepared. Bacterial suspension (1 ml) was added to 15 ml nutrient broth. For the mixed population, 25 µl of S. gordonii ATCC 10558 suspension, 50 µl of Actinomyces naeslundii ATCC 12104 suspension and 100 µl of the suspensions of the other strains were mixed and pipetted into 15 ml nutrient broth.

**Determination of MICs and minimal bactericidal concentrations (MBCs) of planktonic bacteria.** MICs and MBCs (against planktonic bacteria) of the agents were determined for the monocultures of all single strains and for the mixed population. Microtitre plates were used and 100 µl of the antibacterial dilutions was added well by well. Thereafter, 100 µl of bacteria-containing broth was added. The broth used was double-concentrated Wilkins Chalgren broth (Oxoid) supplemented with 5% sheep blood (and 5 µg cocarboxylase ml⁻¹ for the mixed culture). After careful mixing, the microtitre plates were incubated in the appropriate atmosphere overnight (18 h). The plates were then checked for turbidity and each 1 µl medium was subcultivated on modified tryptic soy agar for 3 days. The MIC was defined as the lowest concentration without visible turbidity of the broth confirmed by subcultivation on agar plates. For the assessment of MICs and MBCs of the antibacterial chemotherapeutics against Treponema denticola, the antibacterials were added to the cultivation medium. The MBC was the lowest concentration without growth of the subcultivations on the agar plates (equivalent to a reduction of 99.9% of the initial inoculum). All experiments were carried out in independent duplicates.

**Determination of minimal biofilm eradication concentrations (MBECs).** MBECs were evaluated for the two Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis strains and the 12-species mixed population. The wells of flat-bottomed microtitre plates were first covered with 10 µl of 25% (v/v) inactivated human serum (Sigma-Aldrich) per well for 1 h. Next, 200 µl of bacterial suspension was added. The medium used was brain–heart infusion broth (Oxoid) with 5% (v/v) blood (and 5 µg cocarboxylase ml⁻¹ for the mixed population). The microtitre plates were incubated in the appropriate atmosphere (5% CO₂ for the Aggregatibacter actinomycetemcomitans single-species biofilms, and anaerobic for the others). After 48 h, the medium was carefully exchanged. In the case of the mixed biofilm, Porphyromonas gingivalis ATCC 33277, Tannerella forsythia ATCC 43037 and Treponema denticola ATCC 35405 were again added to the nutrient medium before application to the wells. Further addition of selected bacterial strains guaranteed a sufficient number of these species within the biofilms. After an additional incubation for 24 h (day 3), the medium was removed, the biofilms were carefully washed and 100 µl of antibacterial dilution mixed with 100 µl of doubled-concentrated Wilkins Chalgren broth and supplemented with 5% sheep blood (and 5 µg cocarboxylase ml⁻¹ for the mixed population) was added. The microtitre plates were incubated in the appropriate atmosphere overnight (18 h). The following day (day 4), the medium was removed, the biofilms were carefully washed and fresh antibacterial-free nutrient broth (brain–heart infusion broth with 5% blood (and 5 µg cocarboxylase ml⁻¹) was added for 72 h. Finally (day 7), after removing the medium and washing, the biofilm was scraped and mixed by pipetting, and 10 µl was subcultivated on modified tryptic soy agar for 3 days. The MBEC was the lowest concentration without any growth after subcultivation. These experiments were carried out in two independent experiments, each in duplicate.

In preliminary experiments, the bacterial counts within the control biofilms (without the addition of antibacterials) were evaluated by
enumeration of c.f.u. for the included species at day 3 (time before addition of antibacterials in test biofilms), day 4 (time after removal of antibacterials in test biofilms) and day 7 (end of the experiments). To confirm the c.f.u. counts of most of the species and to enumerate Treponema denticola, real-time PCR was carried out using primers and a protocol described previously (Ashimoto et al., 1996; Eick et al., 2011).

**Confocal laser-scanning microscopy (CLSM) and scanning electron microscopy (SEM).** CLSM and SEM photographs were taken to visualize the multispecies biofilm results. Biofilms were formed on glass slides in 24-well plates as described above. The biofilms were exposed to 4, 32 and 256 μg antibacterial chemotherapeutics ml⁻¹. dH₂O was used as negative control. The medium was changed 18 h later for an antibacterial-free medium and the plates were incubated for a further 3 days. The photographs for CLSM were prepared by using live/dead staining (Live/dead BacLight Bacterial Viability kit; Invitrogen) according to the manufacturer’s description. The samples were examined with a Zeiss LSM510 Exciter confocal microscope (Carl Zeiss NTS). For SEM, samples were fixed in 2% glutaraldehyde in cacodylate buffer for 30 min, washed twice with cacodylate buffer and dehydrated using a graded ethanol series (10 min for each concentration). Following critical-point drying, samples were sputter coated with gold and examined with a ZEISS LEO-1530 Gemini scanning electron microscope (Carl Zeiss NTS) equipped with a field emission electron gun at 10 keV.

**RESULTS**

**MICs and MBCs against planktonic bacteria**

In monocultures, the *Aggregatibacter actinomycetemcomitans* strains were equally susceptible to both fluoroquinolones studied, whereas the other species were inhibited by lower concentrations of moxifloxacin than ofloxacin. The MICs of moxifloxacin ranged from 0.032 to 2 μg ml⁻¹, whilst those of ofloxacin and doxycycline ranged from 0.032 to 16 and 0.125 to 128 μg ml⁻¹, respectively. In general, the MBCs were one to six titration steps higher for moxifloxacin, one to eight titrations steps higher for ofloxacin, and zero to five titration steps higher for doxycycline.

The MBCs were generally one step higher than the MICs with the exception of doxycycline where the MBCs were the same as the MICs. The MIC and MBC of doxycycline were higher against the ATCC strain in comparison with the clinical isolates. Moxifloxacin was more active against the *Porphyromonas gingivalis* strain in comparison with ofloxacin. All MBCs were higher than the MICs.

Against the multispecies mixture, the MIC of moxifloxacin was as high as the highest one against a single strain involved in the mixture, i.e. 2 μg ml⁻¹. The MICs of ofloxacin (4 μg ml⁻¹) and doxycycline (4 μg ml⁻¹) were lower than the highest MIC against a single strain. In the case of ofloxacin, for two strains a higher MIC (up to two steps) was determined in comparison with the mixture. All MBCs were several steps higher than the MICs. Doxycycline was not able to eliminate all bacteria up to a concentration of 512 μg ml⁻¹.

All MICs and MBCs were reproducible. The MICs and MBCs are given in Table 1.

**Biofilms**

In preliminary experiments, evaluation of the biofilm mode of growth was carried out specifically in so far as biofilm-grown bacteria were in contact with the antibacterials for 18 h only on day 3 of the experiment and that viable counts were quantified on day 7.

The c.f.u. counts of the single-species biofilms were in the range of 10⁶–10⁷ per well at each time point. The numbers of bacteria within the mixed population (c.f.u. except for real-time PCR of *Treponema denticola*) were 7.13 ± 0.23 log₁₀ c.f.u. per well at day 3, 7.31 ± 0.15 log₁₀ c.f.u. per well at day 4 and 7.07 ± 0.03 log₁₀ c.f.u. at day 7. The bacteria counts for *Eubacterium nodatum*, *Prevotella intermedia* and *Eikenella corrodens* were about 4 log₁₀ c.f.u. per well, and for the other species were between 5 and 6 log₁₀ c.f.u. per well. Viable counts did not differ among the time points (Fig. 1).

**MBECs**

The MBECs determined in two independent experiments varied by two titration steps. As expected, MBECs of biofilm-grown bacteria were up to seven doubling dilutions higher than the respective MBCs for planktonic bacteria.

The quinolones eradicated *Aggregatibacter actinomycetemcomitans* within a biofilm at moderate concentrations. Moxifloxacin was more active than ofloxacin against *Aggregatibacter actinomycetemcomitans* within a biofilm. The MBEC of doxycycline was higher than those of the quinolones, in accordance with higher MBCs against planktonic bacteria.

The MBECs against *Porphyromonas gingivalis* biofilms were higher than the respective MBCs against planktonic bacteria; the differences between the MBEC₅₀ and the MBCs were three to four doubling dilution steps for doxycycline and twofold dilution steps for the quinolones. Associated with the lower MBCs, moxifloxacin was the most active agent of the tested antibacterials against *Porphyromonas gingivalis* within a biofilm.

The MBECs against the multispecies biofilms were extremely high. Only moxifloxacin in a high concentration (≥ 128 μg ml⁻¹) was able to eradicate the bacteria within the biofilm (Table 2).

**CLSM and SEM analysis**

The live/dead staining showed a high percentage of viable bacteria. No dead bacteria were visible in the controls and following exposure to any of the antibacterials at 4 μg ml⁻¹. In addition, no dead bacteria could be seen after exposure to ofloxacin at a concentration of up to 256 μg ml⁻¹. Doxycycline and moxifloxacin showed a concentration-dependent activity (Fig. 2).
SEM photographs clearly underlined the fact that the antibacterials were not able to destroy the multispecies biofilm, although more extracellular matrix was visible in the untreated biofilms. Ruffling of cell walls as a possible sign of dead bacteria was especially visible after exposure of the biofilms to the quinolones (Fig. 3).

**DISCUSSION**

The aim of the present study was to evaluate and to compare the activity of moxifloxacin with ofloxacin and doxycycline against bacterial species associated with periodontitis within a biofilm.

Moxifloxacin is a fourth-generation synthetic fluoroquinolone (8-methoxyquinolone) with improved activity against Gram-positive bacteria as well as against microaerophiles and anaerobes in comparison with earlier fluoroquinolones, such as ofloxacin (Barman Balfour & Wiseman, 1999; Ackermann et al., 2000; Hardy et al., 2000; Speciale et al., 2002). Moxifloxacin shows favourable pharmacokinetic properties, a high bioavailability, safety, a long half-life time allowing a once-daily dosage, high secretion in gingenal crevicular fluid and saliva, and good penetration into tissues and cells, e.g. polymorphonuclear granulocytes and epithelial cells (Stass et al., 1998; Pascual et al., 1999; Dalhoff & Schmitz, 2003). In dentistry, moxifloxacin has been shown to be more effective than clindamycin as the antibacterial in the treatment of inflammatory infiltrates and as effective as clindamycin in the treatment of odontogenic abscesses (Cachovan et al., 2011). Ofloxacin is a second-generation fluoroquinolone; it is available in a topical formulation recommended in ophthalmology (Ta et al., 2006) and in the treatment of otitis externa (Torum et al., 2004). Considering the recently published EUCAST (2013) clinical breakpoints, Gram-positive (non-spore-forming) and Gram-negative anaerobes are listed as poor targets for ofloxacin, whereas for moxifloxacin the evidence is insufficient against these species.

The results of the quinolones were compared further with doxycycline as a well-established agent in topical antibacterial treatment of periodontitis (Tomasi & Wennström, 2011; Tonetti et al., 2012).

Aggregatibacter actinomycetemcomitans was equally sensitive to moxifloxacin and ofloxacin, and the MICs and MBCs of moxifloxacin against the anaerobes were lower compared with ofloxacin. Thus, the good *in vitro* activity of moxifloxacin against both oral anaerobes and *Aggregatibacter actinomycetemcomitans* (Ardila et al., 2010) was confirmed. As far as a mixed population of the bacteria was concerned, the MICs were in the range of the individual MICs. Recently, it has been shown that a combination of two strains might be more or less sensitive than the MICs of the single strains (Mouratidou et al., 2011). MBCs were equal or higher than

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Mono-culture</th>
<th>Mixed population*</th>
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<tr>
<td><strong>Table 1. MICs and MBCs of the three antibiotics against monocultures of selected bacterial strains associated with periodontitis and a mixed population consisting of 12 species in a planktonic state</strong></td>
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<tr>
<td><strong>Bacterial strain</strong></td>
<td><strong>MIC (µg ml&lt;sup&gt;-1&lt;/sup&gt;)</strong></td>
<td><strong>MBC (µg ml&lt;sup&gt;-1&lt;/sup&gt;)</strong></td>
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<td><strong>Moxifloxacin</strong></td>
<td><strong>Doxycycline</strong></td>
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<td>0.032</td>
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<tr>
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<td>0.032</td>
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<tr>
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<tr>
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<td>Tannerella forsythia ATCC 43037</td>
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<tr>
<td>Treponema denticola ATCC 35405</td>
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<td>2</td>
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<tr>
<td><strong>Mixed population</strong></td>
<td>4</td>
<td>2</td>
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</table>

*Among the 14 strains tested in this study, only strains Aggregatibacter actinomycetemcomitans J7 and Porphyromonas gingivalis M5-1-2 were not used in the mixed population.
the single MBCs. The quinolones were bactericidal at a concentration of 256 \, \mu{g} \, ml^{-1}, and doxycycline was not able to eliminate all bacteria up to 512 \, \mu{g} \, ml^{-1}. A l lt es t u d i es are well known for good tissue penetration (Kuck et al., 1998; Beringer et al., 2012), but these concentrations are much higher than the levels within tissues after systemic administration.

Bacteria in oral cavities mostly form biofilms. Subgingival plaque consists of hundreds of different taxa (Shchipkova et al., 2010), many different co-aggregations occur, and bacteria communicate and transfer DNA (Kolenbrander et al., 2010). One general phenomenon of biofilms is that antibiotics are not as active as against planktonic bacteria. The higher resistance is associated with limited growth, protein synthesis and metabolic activity and an increased mutation frequency as well as with a polymer matrix around microcolonies (Høiby et al., 2010).

Single- and multispecies biofilms were studied. Recently, a study determined the activity of antibacterials in concentrations found after systemic application in gingival crevicular fluid on a multispecies biofilm in vitro; a significant reduction in bacterial counts was not found (Belibasakis & Thurnheer, 2013). Our study design tried to mimic the in vivo situation, when an antibacterial is topically placed into a periodontal pocket. Concentrations within gingival crevicular fluid are much higher after topical application compared with a systemic one, and doxycycline concentrations of more than 1000 \, \mu{g} \, ml^{-1} were measured for 1 day after topical application, whereas after systemic application the levels did not exceed 3 \, \mu{g} \, ml^{-1} (Stoller et al., 1998). Gingival crevicular fluid is known for its high turnover. The flow rate is about 44 \, \mu{l} \, h^{-1} with a resting volume of 1.5 \, ml in severe periodontitis (Goodson, 2003). Delivery devices are used to maintain a high level of the antibacterial at the site of placement (Zilberman & Elsner, 2008). Here, the antibacterial was applied at the concentration to be tested for 18 h; later, nutrient broth was added to mimic the high turnover of gingival crevicular fluid. This also allowed replication of non-killed bacteria.

In single-species biofilms, the MBECs were several steps higher than the corresponding MBCs to planktonic antibacterials are well known for good tissue penetration (Kuck et al., 1998; Beringer et al., 2012), but these concentrations are much higher than the levels within tissues after systemic administration.

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Fig. 1. Counts of bacteria per well in single-species (a) and multispecies (b) biofilms without exposure to the antibacterials at day 3 (time before addition of antibiotics), day 4 (time after removal of the antibacterials) and day 7 (end of the experiments). A actinom., Aggregatibacter actinomycetemcomitans.

Table 2. MBEC_{so} and range of MBECs of the three antibacterials against the Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis single-species biofilms and a multispecies biofilm consisting of 12 species

MBEC_{so} was the value at which 50% of bacteria the biofilms were not cultivable in comparison with the controls without exposure to antibacterials.

<table>
<thead>
<tr>
<th>Biofilm</th>
<th>MBEC_{so} (\mu{g} , ml^{-1})</th>
<th>Range (\mu{g} , ml^{-1})</th>
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<tr>
<td></td>
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<td>2</td>
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<td>2</td>
</tr>
<tr>
<td>Porphyromonas gingivalis ATCC 33277</td>
<td>16</td>
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</tr>
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<tr>
<td>Multispecies</td>
<td>&gt;512</td>
<td>128</td>
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bacteria. Moxifloxacin was most efficient, confirming an earlier study where clindamycin, doxycycline, metronidazole and moxifloxacin were tested (Eick et al., 2004b). In an Aggregatibacter actinomycetemcomitans biofilm model comparing β-lactam antibiotics, tetracyclines, erythromycin, levofloxacin and ofloxacin, ofloxacin was the only one that exerted an inhibitory activity in mature biofilms (Takahashi et al., 2007). Moxifloxacin was shown to reduce the biomass of Staphylococcus aureus biofilms (Di Bonaventura et al., 2004). It induced slime disruption of biofilms produced by selected respiratory specimens (Roveta et al., 2007). In contrast to single-species biofilms of periodontopathogens (Eick et al., 2004b), moxifloxacin seemed not to be able to destroy the structure of a multispecies biofilm, although SEM photographs showed damaged bacterial cells and less extracellular matrix.

In multispecies biofilms, only moxifloxacin was able to kill all bacteria at a concentration of 128 μg ml⁻¹, whilst it was not possible to eliminate all bacteria with ofloxacin and doxycycline at concentrations of up to 512 μg ml⁻¹.

CLSM photographs showed a concentration-dependent activity on the percentage of dead bacteria analysed by live/dead staining. Nevertheless, they did not confirm the results determined by cultivation; the percentage of viable bacteria was always extremely high (Fig. 1). The live/dead combined staining (red/green) method used is based on membrane integrity. The SYTO 9 (green fluorescence) labels bacteria with intact membranes and propidium iodide (red fluorescence) enters bacteria with damaged membranes. It can be said that viable stained bacteria are not cultivable anymore. On the other hand, the 100% viability in many biofilms suggests in part false-positive results for vital bacteria. By comparing different staining methods for the vitality determination of planktonic streptococci, the highest vitality rates have been found for this kind of staining (Decker, 2001). In addition, biofilm components might prevent an uptake of propidium iodide in dead bacteria.

Single-species biofilms were less resistant against the action of the antibacterial. The activity of antibacterials was tested against a 12-species biofilm. Antimicrobials against multispecies biofilms have rarely been tested (Badet & Quero, 2011; Hofer et al., 2011; Belibasakis & Thurnheer, 2013). Nevertheless, the model used in this study represents only a small part of the complexity of biofilms. Thus, it might be suggested that the efficacy of antibacterials in vivo is much less than that shown in this in vitro model.

Moxifloxacin may have potential as a topically applied drug in the treatment of residual pockets in periodontitis. Previously, it has been shown that it is active in vitro against an intracellular infection (Eick & Pfister, 2004).

Fig. 2. CLSM photographs of the effect of the three antibacterials at different concentrations against 12-species biofilms: (a) 4 μg ml⁻¹; (b) 32 μg ml⁻¹; (c, d) 256 μg ml⁻¹. In (a–c), staining is shown for both live (green) and dead (red) bacteria; in (d), a replica of (c), only dead bacteria are stained.
Moreover, in a randomized clinical trial, it was most effective as an adjunct to scaling and root planing, with regard to the clinical outcomes and microbiological and immunological parameters in comparison with adjunctive doxycycline use and scaling and root planing alone (Guentsch et al., 2008). Flemmig et al. (2011) showed recently additional pocket depth reduction following local application of moxifloxacin as an adjunct to scaling and root planing in chronic periodontitis patients with residual pockets. Data about the level of moxifloxacin within gingival crevicular fluid are not available, but similar levels as measured for ofloxacin, as an example of another fluoroquinolone, might be assumed. Evaluation of a controlled-release insert consisting of 11% ofloxacin showed concentrations of 1700 μg ml⁻¹ at 3 h after insertion, which decreased to 10 μg ml⁻¹ after 1 day (Higashi et al., 1989). A general problem associated with antibacterials use is the development of resistance. In vitro, it has been shown that Porphyromonas gingivalis is able to develop resistance (Eick et al., 2004a); however, in a clinical study (Guentsch et al., 2008), these resistant strains were not found (unpublished data). Nevertheless, application of antibacterials should be limited to selected cases. Antibacterial resistance depends on the general usage of antibacterial chemotherapeutics, e.g. resistance of periodontopathogens is much more prevalent in Spain as a country with a high consumption of antibacterials in comparison with the Netherlands where the application of antibacterials is restricted (van Winkelhoff et al., 2005).

In conclusion, moxifloxacin was more active against anaerobes than ofloxacin and also exerted activity against bacteria within a biofilm. Among the tested antibacterials, it was the only one that was able to eradicate bacteria in a multispecies biofilm, although the concentrations required were high. In combination with mechanical removal of the biofilms, moxifloxacin might be a favourable topical antibacterial chemotherapeutic for selected cases of periodontitis.

Fig. 3. SEM photographs of the effect of the antibacterials against 12-species biofilms without antibiotics (a) and after exposure to 256 μg doxycycline ml⁻¹ (b), 256 μg ofloxacin ml⁻¹ (c) and 256 μg moxifloxacin ml⁻¹ (d). Bars, 1 μm.
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REFERENCES


