

# In vitro Activity of Daptomycin, Linezolid and Rifampicin on *Staphylococcus epidermidis* Biofilms

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**Abstract** Owing to their massive use, *Staphylococcus epidermidis* has recently developed significant resistance to several antibiotics, and became one of the leading causes of hospital-acquired infections. Current antibiotics are typically ineffective in the eradication of bacteria in biofilm-associated persistent infections. Accordingly, the paucity of effective treatment against cells in this mode of growth is a key factor that potentiates the need for new agents active in the prevention or eradication of biofilms. Daptomycin and linezolid belong to the novel antibiotic therapies that are active against gram-positive cocci. On the other hand, rifampicin has been shown to be one of the most potent, prevalent antibiotics against *S. epidermidis* biofilms. Therefore, the main aim of this study was to study the susceptibility of *S. epidermidis* biofilm cells to the two newer antimicrobial agents previously mentioned, and compare the results obtained with the antimicrobial effect of rifampicin, widely used in the prevention/treatment of indwelling medical device infections. To this end the in vitro activities of daptomycin, linezolid, and rifampicin on *S. epidermidis* biofilms were accessed, using these antibiotics at MIC and peak serum concentrations. The results demonstrated that at MIC concentration, rifampicin was the

most effective antibiotic tested. At peak serum concentration, both strains demonstrated similar susceptibility to rifampicin and daptomycin, with colony-forming units (CFUs) reductions of approximately 3–4 log<sub>10</sub>, with a slightly lower response to linezolid, which was also more strain dependent. However, considering all the parameters studied, daptomycin was considered the most effective antibiotic tested, demonstrating an excellent in vitro activity against *S. epidermidis* biofilm cells. In conclusion, this antibiotic can be strongly considered as an acceptable therapeutic option for *S. epidermidis* biofilm-associated infections and can represent a potential alternative to rifampicin in serious infections where rifampicin resistance becomes prevalent.

## Introduction

*Staphylococcus epidermidis*, which are part of the normal microflora of the human skin and mucous membranes, have attracted considerable attention more recently as dangerous nosocomial pathogens [1]. This bacterium has the ability to adhere to implanted medical devices or damaged tissues, and form biofilms, which become the focus of persistent infections [2] and causes devastating complications which prolong disease and result in higher-morbidity and -mortality, especially in immuno-compromised patients [3]. Since bacteria in biofilm demonstrated a greater capacity to evade clearance by the host defences and by antimicrobial chemotherapy, this feature is recognized as a key virulence determinant of this pathogen [1, 2]. In addition to the reduced susceptibility of bacteria to antimicrobial agents when in this mode of life, the increasing occurrence of resistance has created a need for the development of new antimicrobial agents [4].

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Daptomycin and linezolid are newer antimicrobial agents that are active against gram-positive cocci [5, 6]. Daptomycin, a lipopeptide, has a spectrum of activity very similar to vancomycin [6]. Linezolid, has excellent activity against most staphylococci in systemic infections; nevertheless, resistance has begun to emerge [7–9]. Although rifampicin cannot be used as a single agent to treat infections because of the rapid selection of resistant mutants [10, 11], it has proven to be the most efficient antibiotic against *S. epidermidis* biofilm-associated infections [12, 13].

The aim of the present study was to evaluate the in vitro activity of two antibiotics of new generation, daptomycin and linezolid, against *S. epidermidis* biofilms and compare the results with the response to rifampicin, the most efficient of the traditional antibiotics for this purpose.

## Materials and Methods

### Bacterial Strains and Growth Conditions

In this study, two previously well-characterized biofilm-producing *S. epidermidis* strains were used: 9142 and 1457 [14]. These strains are clinical isolates and were stored at  $-80^{\circ}\text{C}$ . Tryptic soy broth (TSB) and tryptic soy agar (TSA) were prepared according to the manufacturer's instructions. All strains were inoculated into 15 ml of TSB from TSA plates not older than 2 days and grown for 18 ( $\pm 2$ ) h at  $37^{\circ}\text{C}$  in an orbital shaker at 130 rpm. Cells were harvested by centrifugation (for 10 min at  $9,500\times g$  and  $4^{\circ}\text{C}$ ), resuspended in TSB, and the suspension was adjusted to an optical density (640 nm) equivalent to  $1 \times 10^9$  cells  $\text{ml}^{-1}$  before being used in the subsequent assays. Each stock solution of daptomycin (with calcium supplementation at  $50 \text{ mg l}^{-1}$ ) [15, 16] and rifampicin was prepared in methanol. It was confirmed that methanol, at the concentration used, had no effect on the growth of the *S. epidermidis* strains studied. The stock solution of linezolid was prepared in Milli-Q water.

### Minimum Inhibitory Concentration (MIC)

The MIC was determined by the microbroth dilution methodology, according to the CLSI. MIC determination of the tested agents and for both *S. epidermidis* strains (9142 and 1457) was performed by the serial twofold dilution method at concentrations ranging from 0.007 to  $16 \mu\text{g ml}^{-1}$ , using 96-well tissue culture plates (Sarstedt, Newton, NC, USA). The antibiotics were first diluted to the highest concentration to be tested, and then serial twofold dilutions were made. The final cell concentration used was approximately  $5 \times 10^5$  cells  $\text{ml}^{-1}$ . Plates were incubated for 24 h at  $37^{\circ}\text{C}$  on an orbital shaker (130 rpm). After

incubation, the MIC was determined as the lowest concentration required to inhibit bacterial growth.

The controls were the cells which were not exposed to the antimicrobial agents tested. All the experiments were carried out in triplicate and repeated three times.

### Biofilm Formation and Susceptibility Tests

Biofilms were formed in 96-well tissue culture plates (Sarstedt, Newton, NC, USA) containing  $200 \mu\text{l}$  of *S. epidermidis* cell suspension ( $1 \times 10^6$  cells  $\text{ml}^{-1}$ ) in TSB, supplemented with 0.25% of glucose per well to promote biofilm formation [12]. Plates were incubated at  $37^{\circ}\text{C}$  with orbital shaking at 130 rpm for 24 h. At the end, the planktonic cells were gently removed from the biofilm-containing well by pipetting off the liquid medium and then washing the well twice, each time by adding  $200 \mu\text{l}$  of saline solution [0.9% NaCl (Merck)], and by aspirating using a multichannel pipettor. The biofilms were incubated for 24 h, in fresh nutrient medium (TSB) containing daptomycin, linezolid, and rifampicin at the MIC and peak serum concentrations (Table 1). At time 0 (before exposure to antibiotics), the initial cellular concentration of biofilm ( $\sim 2 \times 10^8$  cells  $\text{ml}^{-1}$ ) was determined by CFU enumeration. To this end, the planktonic cells were carefully removed, and the biofilm-containing wells were washed with  $250 \mu\text{l}$  of saline solution. The wells were thoroughly scraped with a cell scraper (zellschaber/24 cm) and resuspended in 1 mL of 0.9% NaCl, followed by centrifugation for 10 min at  $9,500\times g$  and  $4^{\circ}\text{C}$ . The pellet was resuspended in 0.9% NaCl and washed twice, followed by sonication for 20 s at 22 W to homogenize the suspension. Viable cells were determined by performing tenfold serial dilutions in saline solution and plating in TSA. Colonies were counted after 24 h incubation at  $37^{\circ}\text{C}$ .

Crystal violet (CV) staining was used as indicator of total biofilm biomass. After exposure to the treatment agents, biofilms-containing wells were washed with  $250 \mu\text{l}$  of saline solution, and then  $250 \mu\text{l}$  of methanol was added to each well and allowed to react for 15 min. Afterward, methanol was removed using a multichannel pipettor. Then,  $250 \mu\text{l}$  of CV 1% (v/v) was added (5 min). The wells

**Table 1** MIC and peak serum concentrations of the antibiotics used in this study

Antibiotic	Peak serum ( $\mu\text{g ml}^{-1}$ )	<i>S. epidermidis</i> 9142 MIC ( $\mu\text{g ml}^{-1}$ )	<i>S. epidermidis</i> 1457 MIC ( $\mu\text{g ml}^{-1}$ )
DAP	95 [16]	0.25	0.50
LIN	18 [24]	1.00	1.00
RIF	10 [12]	0.015	0.015

DAP daptomycin, LIN linezolid, RIF rifampicin

were washed with distilled water and finally, acetic acid 33% (v/v) was added. The absorbance was read on an ELISA reader (Bio-Tek Instruments Inc., Vermont, USA) at 570 nm.

The number of viable cells after antibiotic treatment was assessed by CFU enumeration using the same protocol described above. A control was obtained with biofilm cells not exposed to the antibiotics tested. All the experiments were carried out in triplicate and repeated three times.

### Statistical Analysis

The data from all the assays were compared using one-way analysis of variance (ANOVA), by applying Tukey's test with all the calculations carried out using SPSS software (Statistical Package for the Social Sciences). Differences at a confidence level of 95% were considered significant.

## Results and Discussion

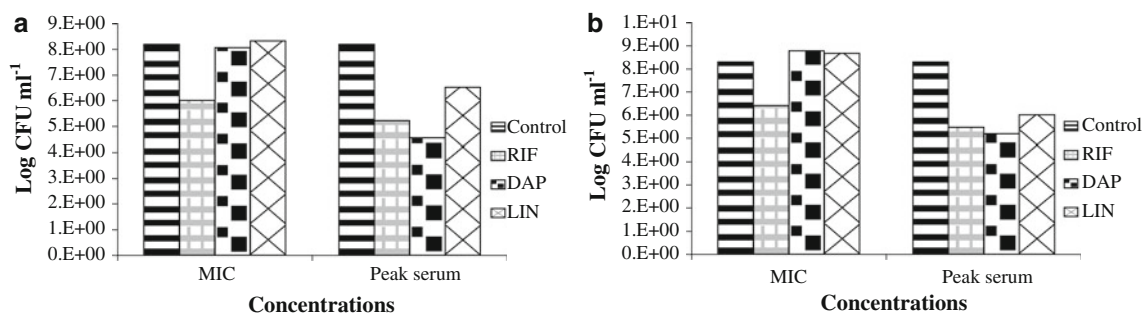
It is well-known that bacteria in biofilms are able to survive in the presence of high concentrations of antimicrobial agents. Bacterial survival in biofilms may be determined by multiple factors, among which the presence of a surface film composed of lipid components similar to those in bacterial membranes that cover the biofilm and form a barrier impairing the penetration of some antibiotics [2]. The inherent tolerance of biofilm cells and the antibiotic resistance among pathogenic bacteria is a well-documented phenomenon with severe consequences for the treatment of nosocomial infections. Current antibiotics have been proving inadequate to meet these challenges. Therefore, new antibiotics are being developed aiming to replace existing antibiotics that succumb to the rising tide of resistance. In this decade, two antibacterials that belong to new classes—daptomycin (a lipopeptide) and linezolid (an oxazolidinone)—were approved and have offered new options for the treatment of, e.g., complicated skin and skin

structure infections caused by resistant gram-positive pathogens [17]. These facts triggered our interest in the possible use of these two novel antibiotics in the treatment of *S. epidermidis* biofilm-related infections.

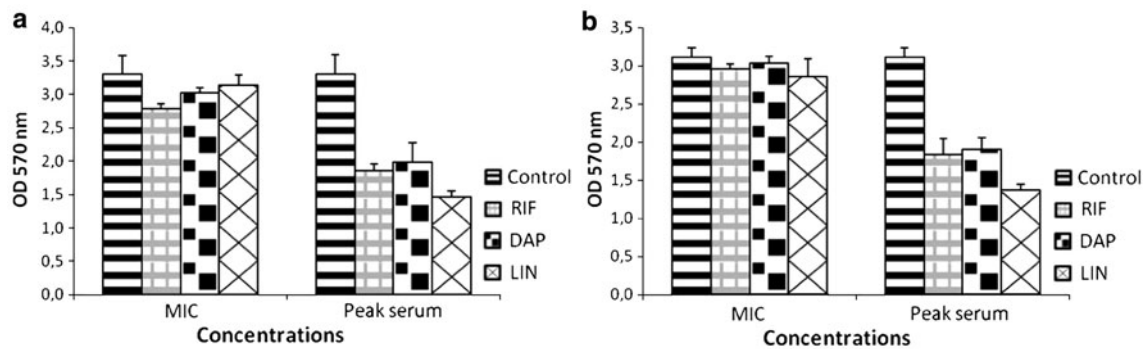
Biofilms can be more than 1000 times more resistant to antimicrobial agents, because of their inherent tolerance [18]. In fact, a high antimicrobial effect against planktonic cells is not a proof of high efficacy against the corresponding cells in biofilm forms [19, 20]. Taking this into consideration, the present study attempts to evaluate the *in vitro* activity of these two antibiotics against *S. epidermidis* biofilms, considering the potential use of these antimicrobial agents as possible alternatives to current antibiotics (such as rifampicin), commonly used in the combat of infections caused by *S. epidermidis*.

Table 1 presents the MIC and peak serum concentration values of daptomycin, linezolid, and rifampicin used in this study. The reductions in biofilm cells after 24 h of treatment with the three antibiotics at MIC concentration are presented in Fig. 1, where it is seen that, in such condition neither the daptomycin nor the linezolid displayed an antimicrobial effect against *S. epidermidis* biofilms. On the other hand, rifampicin showed a statistically significant ( $P < 0.05$ ) effect against both the strains of *S. epidermidis* biofilm cells. However, although rifampicin was the most active antibiotic tested, the CFU reduction promoted was only slightly above 2 log<sub>10</sub> and did not attain the 3 log<sub>10</sub> threshold, to be considered bactericidal.

As described by several authors, antibiotics at concentrations of 10<sup>2</sup> to 10<sup>4</sup> times the MIC cause no killing effect on bacteria in biofilm communities [21]. Taking this into consideration, and with the aim of testing higher concentrations than MIC, we determined the susceptibility of *S. epidermidis* biofilms to daptomycin, linezolid, and rifampicin at the respective peak serum concentrations, since such concentration is the highest level of drug that can be obtained in the blood, usually following the administration of multiple doses. In this case, rifampicin and daptomycin showed a bactericidal effect against both strains of



**Fig. 1** Effects of rifampicin (RIF), daptomycin (DAP), and linezolid (LIN), at MIC and peak serum concentrations, on the number of biofilm cells of *S. epidermidis* 9142 (a) and 1457 (b), after 24 h of treatment assessed by CFU enumeration. Error bars represent standard deviation



**Fig. 2** Effects of rifampicin (RIF), daptomycin (DAP), and linezolid (LIN), at MIC and peak serum concentrations, on total biofilm biomass of *S. epidermidis* 9142 (a) and 1457 (b), after 24 h of treatment, expressed as CV absorbance. Error bars represent standard deviation

*S. epidermidis* in biofilm form ( $P < 0.05$ ), while linezolid effect was slightly smaller and more strain dependent (Fig. 1).

Figure 2 depicts the effect of the antimicrobial agents tested on total biofilm biomass of *S. epidermidis* biofilms, assessed by CV staining. At MIC concentration, none of the antibiotics caused any significant effect on biofilm biomass of *S. epidermidis* biofilms. However, at peak serum concentrations, the three antibiotics were able to reduce biofilm biomass, with linezolid causing the highest reduction ( $P < 0.05$ ) on total biofilm biomass, although not much higher than the others.

This slightly higher reduction in total biofilm biomass promoted by linezolid is expected to be because of a decrease in the extracellular matrix, taking into account that it was the agent that reduced the number of cells in a lesser extent. The possible reduction in the extracellular matrix caused by linezolid may be a potential advantage, since the biofilm structure become weaker, and the cells present in the biofilm can be more susceptible to human immune system and other antimicrobial agents. Therefore, this mechanism of action can potentiate the inhibitory effect of other antimicrobial agents, promoting synergistic interactions. As an example, the combination of *N*-acetylcysteine (NAC) and tigecycline was reported to work in synergy [22]. A possible explanation to this increased-effect may be due to the degradation of the extracellular polysaccharide biofilm matrix by NAC, causing the biofilm-associated cells more susceptible to the action of tigecycline [22].

These results demonstrated that rifampicin and daptomycin at peak serum had a very similar bactericidal effect on *S. epidermidis* biofilms. However, taking into consideration the rapid emergence of rifampicin resistance when used as monotherapy [23], it that the use of daptomycin in the treatment of *S. epidermidis*-related infections is more feasible. This is supported by other researchers who reported that, upon sometime of exposure, daptomycin was

the most active agent in eradicating *S. aureus* [23], exhibiting very rapid bactericidal activity against this pathogen [15]. As both *S. aureus* and *S. epidermidis* are now among the major nosocomial pathogens, their similar response to these antibiotics is very favorable from the clinical point of view because it is possible to define a relatively broad spectrum therapy.

The overall results obtained us led to conclude that daptomycin at peak serum concentration was the most active antimicrobial agent tested against *S. epidermidis* biofilm cells. This novel antibiotic can be strongly considered as a potential alternative to rifampicin as monotherapy. However, regarding the therapeutic use in biofilm control, future studies using animal models have to be performed to determine the in vivo response of *S. epidermidis* cells to this antimicrobial agent.

Although rifampicin also demonstrated to be highly active against *S. epidermidis* biofilms either at MIC or PS concentration, it should only be considered for enhanced anti-Staphylococcal activity but in combination with other antibiotics, to avoid the rapid emergence of resistance.

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