International Journal of Antimicrobial Agents xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: http://www.elsevier.com/locate/ijantimicag

Inefficacy of vancomycin and teicoplanin in eradicating and killing *Staphylococcus epidermidis* biofilms in vitro

J. Claessens[a,*], M. Roriz[a], R. Merckx[a], P. Baatsen[b], L. Van Mellaert[c], J. Van Eldere[a]

[a] *KU Leuven—University of Leuven, Department of Microbiology and Immunology, Laboratory of Clinical Bacteriology and Mycology, UZ Leuven Campus Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium*
[b] *Electron Microscopy Facility (EMoNe) of the VIB BIO Imaging Core at KU Leuven—University of Leuven, Center for Human Genetics, Herestraat 49, B-3000 Leuven, Belgium*
[c] *KU Leuven—University of Leuven, Department of Microbiology and Immunology, Laboratory of Molecular Bacteriology, Rega Institute for Medical Research, Herestraat 49, B-3000 Leuven, Belgium*

ARTICLE INFO

Article history:
Received 3 May 2014
Accepted 24 November 2014

Keywords:
Vancomycin
Teicoplanin
Biofilm biomass
Killing efficacy
Staphylococcus epidermidis
Biofilm

ABSTRACT

Biofilm-associated bacteria display a decreased susceptibility towards antibiotics. Routine assessment of antibiotic susceptibility of planktonic bacteria therefore offers an insufficient prediction of the biofilm response. In this study, in vitro biofilms of eight clinical *Staphylococcus epidermidis* strains were subjected to treatment with vancomycin, teicoplanin, oxacillin, rifampicin and gentamicin. In addition, the biofilms were subjected to combinations of an antibiotic with rifampicin. The effects on the biofilms were assessed by crystal violet staining to determine the total biofilm biomass, staining with XTT to determine bacterial cell viability, and microscopy. Combining these methods showed that treatment of *S. epidermidis* biofilms with glycopeptides increased the total biofilm biomass and that these antibiotics were not effective in killing bacteria embedded in biofilms. The decreased killing efficacy was more pronounced in biofilms produced by strains that were classified as 'strong' biofilm producers. Rifampicin, oxacillin and gentamicin effectively killed biofilm-associated bacteria of all tested strains. Combining antibiotics with rifampicin increased the killing efficacy without influencing the total biofilm biomass. When vancomycin or teicoplanin were combined with rifampicin, the increase in biofilm biomass was neutralised and also the killing efficacy was influenced in a positive way. We conclude that the combined methodology used in this study showed that glycopeptides were not effective in eradicating *S. epidermidis* biofilms but that combination with rifampicin improved the killing efficacy in vitro.

1. Introduction

Determination of the minimum inhibitory concentration (MIC) of an antibiotic is the standard method to determine bacterial susceptibility towards antibiotics. This method is based on the inhibition of planktonic growth of bacteria. However, in many types of infections, bacteria with a sessile lifestyle resulting in biofilms are involved [1,2]. A biofilm is defined as a multicellular aggregate of micro-organisms attached to a surface and embedded in a self-produced extracellular matrix. Bacteria growing in biofilms are characterised by increased resistance towards antibiotics and the host's immune response [2]. This is attributed to: (i) a different phenotype of bacteria growing in a biofilm compared with planktonic bacteria, with decreased activity of the basic metabolic pathways and increased expression of stress response genes [3]; (ii) the extracellular matrix, which creates a micro-environment that interferes with antibiotic activity by decreasing penetration of the antibiotic, sequestering it or by the presence of modifying enzymes [4]; and (iii) the facilitated exchange of antibiotic resistance genes when bacteria are growing in high numbers close to each other [5].

Because of the decreased sensitivity of biofilm-associated bacteria to antibiotics, methods to evaluate antibiotic susceptibility should, in the case of biofilm-related infections, be supplemented with tests conducted on in vitro biofilms [6]. Different methods have been proposed to do this, including determination of the minimum antibiotic concentrations that inhibit biofilm growth (biofilm MIC), reduce the bacterial load of the biofilm [biofilm minimum bactericidal concentration (MBC)] or completely eradicate the biofilm [minimum biofilm eradication concentration (MBEC)] [7]. However, the resulting antibiotic concentrations exceeded physiologically achievable concentrations [7]. Another way to determine

* Corresponding author. Tel.: +32 16 346 274; fax: +32 16 346 275.
E-mail addresses: jolien.cl@hotmail.com, jolien.claessens@med.kuleuven.be (J. Claessens).

http://dx.doi.org/10.1016/j.ijantimicag.2014.11.011
0924-8579/

antibiotic response consists of assessing the effects on the bacterial viability and the extracellular matrix separately [6]. In the present study, the effects of antibiotics on in vitro *Staphylococcus epidermidis* biofilms were evaluated using XTT staining as a measure for bacterial viability [8] and crystal violet (CV) to determine the total biofilm biomass [9]. These methods were combined with microscopic techniques to visualise the observed effects. The applied antibiotic concentrations corresponded to the peak serum concentrations, which is different from previously published studies that used subinhibitory antibiotic concentrations [10,11].

2. Materials and methods

2.1. Bacterial strains and growth conditions

Table 1 summarises the *S. epidermidis* strains used in this study. All strains, except *S. epidermidis* 567, were isolated from bloodstream infections associated with central venous catheters (CVCs). *S. epidermidis* 567 was isolated from a urinary tract catheter infection; this strain was kindly provided by Prof. Dr W. Ziebuhr (University of Würzburg, Würzburg, Germany) [12]. *S. epidermidis* 1457 is a type strain often used in biofilm research [13], and *S. epidermidis* 10b was isolated from a confirmed case of catheter infection in the University Hospital Leuven (Belgium) [14]. In addition to the laboratory strains, clinical isolates from proven biofilm-related infections were used. These strains are designated *S. epidermidis* 12c, 13c, 16c, 17c and 22c (Table 1).

Tryptone soya broth (TSB) (Oxoid Ltd., Basingstoke, UK) was prepared according to the manufacturer's instructions. Growth conditions were adjusted to optimise biofilm formation and detection (Table 1). Because of the high biofilm formation capacities of *S. epidermidis* 1457, 10b and 22c, biofilm formation of these strains was allowed to occur in 10% TSB in phosphate-buffered saline (PBS) (pH 7.2). This was done to adjust the total biofilm biomass to the upper detection limit of the enzyme-linked immunosorbent assay (ELISA) reader (VICTOR3^{TM} Multilabel Plate Reader; PerkinElmer Singapore Pte Ltd., Singapore). *S. epidermidis* 567 was characterised as a biofilm-negative, *ica*-positive strain; biofilm formation was induced by adding 4% NaCl to the culture medium [15].

The antibiotics used to treat the biofilms were all purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Antibiotic stock solutions, except rifampicin, were prepared in sterilised Milli-Q® water (Merck Millipore, Carrigtwohill, Ireland) and were kept at −20 °C. The rifampicin stock was prepared in methanol and were kept at −20 °C.

2.2. Minimum inhibitory concentration determination

The MICs of antibiotics towards planktonic bacteria were determined by the broth dilution method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [16]. Bacterial growth was assessed by measurement of the optical density at 590 nm using a VICTOR3^{TM} Multilabel Plate Reader.

2.3. In vitro biofilm formation and treatment with antibiotics

Starting from overnight cultures on blood agar plates, several colonies were re-suspended in saline (0.9% w/v NaCl) to reach an optical density equal to 0.5 McFarland standard (Cobas Inocheck; Roche Diagnostics GmbH, Mannheim, Germany). This suspension was diluted 100 times in the appropriate growth medium used for biofilm formation (Table 1). Then, 200 μL of the bacterial suspension was applied to each well of a flat-bottomed 96-well microtitre plate (Cellstar®; Greiner Bio-One, Frickenhausen, Germany). Plates were incubated statically for 24 h at 37 °C. Subsequently, the supernatants were discarded and the formed biofilms were washed once with 250 μL of PBS. Biofilms were treated with 200 μL of antibiotic solution (in 1% TSB, i.e. 100 times diluted in PBS) for 24 h at 37 °C. The applied concentrations of the antibiotics were 40 μg/mL for vancomycin, 50 μg/mL for teicoplanin, and 10 μg/mL for oxacillin, rifampicin and gentamicin.

2.4. Evaluation of the biofilms

The supernatants were discarded and the biofilms were washed twice with 250 μL of PBS. The plates were stained with CV according to the method proposed by Stepanović et al. [9]. After fixing the biofilms with ethanol (96%), the plates were dried and stained for 15 min with 230 μL of Hucker's CV solution [0.5 mg CV (Sigma-Aldrich) dissolved in 5 mL of ethanol 100%, and combined with 45 mL of an aqueous solution containing 1% ammonium oxalate (Sigma-Aldrich)]. The plates were washed to remove excess stain and were subsequently dried. Bound CV was eluted by adding 200 μL of 5% acetic acid to each well. After 30 min of incubation at room temperature, 150 μL of the eluate was transferred to a new 96-well plate and the absorbance of the eluate was measured at 590 nm using a VICTOR3^{TM} Multilabel Plate Reader.

For staining biofilms with XTT, the method described by Cerca et al. [8] was used. The XTT solution (0.2 mg/mL XTT, 0.02 mg/mL phenazine methosulphate; Sigma-Aldrich) was prepared in 1% TSB, and incubation of the biofilms with the staining solution lasted for 2 h at 37 °C in the dark. Absorbance of the supernatant was measured at 490 nm using a VICTOR3^{TM} Multilabel Plate Reader.

The results after antibiotic treatment of the biofilms were expressed relative to the staining result after treatment of the biofilm with the negative control (1% TSB).

2.5. Scanning electron microscopy (SEM)

Biofilms were grown on glass coverslip disks (13 mm diameter; Assistent, Glaswarenfabrik Karl Hecht, Sondheim, Germany) for 24 h and were treated with antibiotics as mentioned previously. Biofilms on the coverslip disks were rinsed once with 1 mL of PBS and were fixed for 2 h with glutaraldehyde (2.5%) in sodium cacodylate buffer (0.1 M, pH 7.4) at room temperature. Afterwards, biofilms were washed with sodium cacodylate buffer and post-fixed for 2 h with osmium tetroxide (1% in Milli-Q water) at 4 °C, protected from light. The fixed samples were dehydrated with ethanol in ascending concentrations [30–50–70–90% (v/v)] for 5 min and 100% (v/v) ethanol for 3 × 5 min. For the final dehydration step, hexamethyldisilazane (HMDS) (Sigma-Aldrich) was used for 2 × 15 min. After overnight drying in a vacuum desiccator, the samples were sputter-coated with platinum for 120 s (Automatic sputter coater; Agar Scientific, Elektron Technology UK Ltd., Stansted, UK). Imaging was conducted on a JSM 7401F scanning electron microscope (JEOL, Tokyo, Japan). In each sample, three different areas were observed at magnifications of 1000×, 4000× and 10 000×.

2.6. Statistical analysis

Data were processed with Microsoft Excel (Microsoft Corp., Redmond, WA) and statistical analysis was conducted with IBM SPSS Statistics for Windows v.22.0 (IBM Corp., Armonk, NY). Differences between treatments were analysed by one-way analysis of variance (ANOVA), and differences between strains were characterised by two-way ANOVA. ANOVA analysis assumes that the data have a normal distribution and equal variances. Normality of the data was tested with the Shapiro–Wilk test and evaluation of the Q–Q plots. Equality of variances was analysed with the Levene's test. If the variances were not equal, Welch ANOVA analysis was applied.

Please cite this article in press as: Claessens J, et al. Inefficacy of vancomycin and teicoplanin in eradicating and killing *Staphylococcus epidermidis* biofilms in vitro. Int J Antimicrob Agents (2015), http://dx.doi.org/10.1016/j.ijantimicag.2014.11.011