Microbial Pathogenesis 50 (2011) 1–5

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

XTT assay for evaluating the effect of alcohols, hydrogen peroxide and benzalkonium chloride on biofilm formation of *Staphylococcus epidermidis*

Kamel Chaieb [a,b,*,1], Tarek Zmantar [a,1], Yosra Souiden [b], Kacem Mahdouani [b], Amina Bakhrouf [a]

[a] Laboratoire d'Analyses, Traitement et Valorisation des Polluants de l'Environnement et des Produits, Faculté de Pharmacie, Rue Avicenne 5000 Monastir, Tunisia
[b] Laboratoire de Biologie Moléculaire, Hôpital Régionale de Kairouan, Tunisia

ARTICLE INFO

Article history:
Received 4 July 2010
Received in revised form
7 November 2010
Accepted 9 November 2010
Available online 18 November 2010

Keywords:
Staphylococcus epidermidis
Alcohols
Hydrogen peroxide
Benzalkonium chloride
Biofilm
XTT

ABSTRACT

To analyze the degree of biofilm formation on three *ica*-positives *Staphylococcus epidermidis* as a function of biocides, the medium was supplemented with increasing concentrations of isopropanol, ethanol, and methanol at 0, 1, 4, 6, 8, 10, 12 and 14% (v/v), hydrogen peroxide (0, 0.125, 0.25, 0.5, 1, 2, 3, 4 and 5% v/v) and benzalkonium chloride (0, 0.125, 0.25, 0.5, 1, 2, 3, 4 and 6 $\mu g\,ml^{-1}$).

In biocide-free biofilms, the results showed that two strains (*S. epidermidis* CIP106510 and E24) were strongly biofilm positive displaying a high oxidative activity (1.254 and 0.855, respectively) in comparison with the non-adherent one (S22). In addition biofilm formation was induced with 1% alcohol (isopropanol and ethanol) supplementation. The three studied strains cultured in TSB supplemented with 2% methanol displayed a strong oxidative activity ($P = 0.008$).

Moreover wells with 0.125% hydrogen peroxide enhanced increasing oxidative activity of *S. epidermidis* CIP106510 and S22. A significant induction of biofilm was noted after treatment with 1 $\mu g\,ml^{-1}$ of benzalkonium chloride.

This study suggests that some biocides currently used in hospitals are ineffective against nosocomial pathogens growing in biofilms when used at weak concentration and fail to control this reservoir for hospital-acquired infection.

1. Introduction

Staphylococcus epidermidis is a major cause of medical device-associated sepsis. It is frequently associated with catheter-related bloodstream infections [1]. Biofilms are responsible for hospital infections by growing on implants, catheters, and other medical devices [2–4]. As a member of human skin microflora, *S. epidermidis* can easily contaminate medical surfaces following surgical procedures [5]. It has been demonstrated that its ability to form biofilms on polymer surfaces greatly contributes to its spread [6]. Biofilms are notoriously tolerant to conventional chemical disinfectants [7]. These high tolerances may be caused by slow diffusion of these compounds through the extracellular polymeric matrices [8]. Some studies have reported that slimes make *S. epidermidis* highly resistant to antibiotics and host defences [2]. In clinical practice, disinfection is the most important measures to prevent bacterial spread. Over the years, the use of biocides for disinfection purposes has increased [9] inducing the emergence of antiseptic resistant staphylococci which has been reported in many countries [10]. Alcohol-based skin disinfectants are frequently used, resulting in a high extent of bacterial elimination [11]. Recently, ethanol washings were shown to induce biofilms formation [12].

Biocides are used in clinical medicine for preventing the dissemination of nosocomial infections. It is important to evaluate the susceptibility of clinical strains to these compounds in order to assess the efficiency of preventive measures currently used in hospitals. The routine use of alcohol-based skin disinfectants in hospitals, have more clinical attention to get rid against their effect on *S. epidermidis* biofilm formation.

In this study, the effects of commonly used hospital alcohols (isopropanol, ethanol and methanol), hydrogen peroxide and benzalkonium chloride were tested for biofilm formations by clinical *S. epidermidis* isolated from haemodialysis service in Tunisia. The XTT colorimetric assay was used to determine the percentage of viable cells in biofilms following each treatment.

* Corresponding author. Laboratoire d'Analyse, Traitement et Valorisation des Polluants de l'Environnement et des Produits, Faculté de Pharmacie, rue Avicenne 5000 Monastir, Tunisia. Tel.: +216 73461000; fax: +216 73461830.
E-mail address: chaieb_mo@yahoo.fr (K. Chaieb).
[1] Kamel Chaieb and Tarek Zmantar contributed equally to this manuscript.

0882-4010/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.micpath.2010.11.004

2. Material and methods

2.1. Bacterial strains

Bacteria used in this study were: *S. epidermidis* CIP106510, biofilm positive (E24) and biofilm negative (S22) strain which were isolated from haemodialysis biomaterial and carried the intercellular adhesion gene (*ica*) as previously published [13].

2.2. Adherence assay of S. epidermidis cells as a function of biocides supplementation

Biofilms formation by *S. epidermidis* strains grown in Trypticase Soy broth (TSB, Bio-Rad, France) was determined using a colorometric assay on 96-well plates (Nunc, Roskilde, Denmark), as described previously with slight modification [6,14].

An overnight culture grown at 37 °C was diluted to 1:100 in TSB medium with 2% (w/v) glucose to maximise *ica* operon induction, as reported elsewhere [15].

A total of 200 μl of each bacterial suspension were transferred in a *U*-bottomed 96-well microtiter plate (Nunc, Roskilde, Denmark). Wells with sterile TSB alone were served as negative controls. *S. epidermidis* CIP106510 was used as positive control. The plates were incubated aerobically at 37 °C for 24 h. Each strain was tested in triplicate.

To analyze the degree of biofilms formation as a function of biocides, the TSB medium was supplemented with increasing concentrations; 0, 1, 4, 6, 8, 10, 12 and 14% (v/v) of isopropanol, ethanol and methanol. Hydrogen peroxide was added as following: 0, 0.125, 0.25, 0.5, 1, 2, 3, 4 and 5% (v/v) and benzalkonium chloride were used at 0, 0.125, 0.25, 0.5, 1, 2, 3, 4 and 6 μg ml^{-1}. Each test was done in triplicate.

2.3. Quantification of viable cells in the biofilm by XTT assay

The XTT assay was used to quantify the number of viable cells in each of the wells following biocides supplementation in comparison with disinfectant-free controls. This method has been used extensively for the quantification of bacterial biofilm [16]. It measures the reduction of a tetrazolium salt (2,3-bis[2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT)) by metabolically active cells to a coloured water soluble formazan derivative that can be easily quantified colorimetrically [16,17].

Briefly, XTT solution (1 mg/ml) was prepared in phosphate-buffered saline (7 mM Na2HPO4, 3 mM NaH2PO4 and 130 mM NaCl at pH 7.4), sterilized through a 0.22 μm pore size filter (Millipore, Sartorius Minisart CE 0297, Germany) and stored at −70 °C. Menadione (Sigma–Aldrich, Switzerland) solution (0.4 mM) was prepared in acetone and sterilized instantaneously before each assay.

Following biocide exposure, the plate was rinsed three times with PBS to remove loosely attached cells, dried in an inverted position, and then 180 μl of PBS and 20 μl of the XTT-menadione solution (12.5 volume of XTT solution was mixed with 1 volume of menadione solution) were added to each prewashed and control wells and the plate was incubated at 37 °C in the dark for 3 h [17]. Reduction of XTT (oxidative activity) was then measured at 492 nm using automated Multiskan reader (GIO, Rome, Italy).

2.4. Statistical analysis

Each analysis was performed using the SPSS 17.0 statistics package for Windows. The differences in the degree of biofilm formation as a function of biocide supplementation were examined by the Friedman test, followed by the Wilcoxon signed ranks test. $P < 0.05$ were considered significant.

3. Results

3.1. Biofilm production under standard conditions

The XTT assay showed in biocide-free biofilms (TSB 2% glucose w/v) two strains (*S. epidermidis* CIP106510 and E24) were strongly biofilm-producers and displayed a high oxidative activity (1.254 and 0.855 respectively) in comparison with the non-adherent strain (0.196).

3.2. Effect of biocides on biofilm production

In the present study, XTT assay were performed for evaluating biofilm formation by three *ica*-positive *S. epidermidis* strains under standard growth conditions and in the presence of alcohols (isopropanol, ethanol and methanol), hydrogen peroxide and benzalkonium chloride supplementation.

Our data revealed that the addition of alcohols (1%) to TSB medium resulted in induction of biofilm formation in the non-

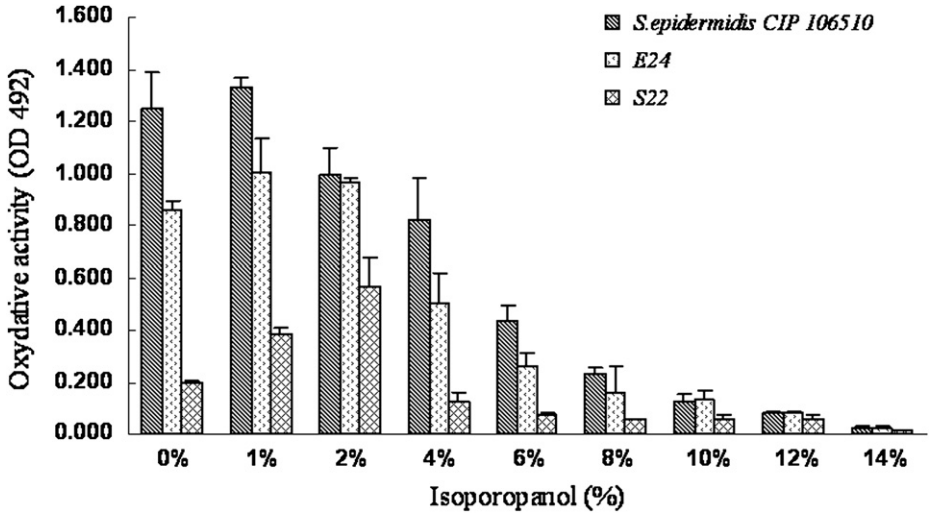

Fig. 1. Biofilm oxidative activity of the three *S. epidermidis* at various isopropanol supplementation.