Microbial Pathogenesis 91 (2016) 54–60

Contents lists available at ScienceDirect

Microbial Pathogenesis

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

The degree of virulence does not necessarily affect MRSA biofilm strength and response to photodynamic therapy

Alaa A.Y. Abouelfetouh [a, *], Noha A. Nafee [b], Nihal K. Moussa [a]

[a] Department of Microbiology and Immunology, Faculty of Pharmacy, Alexandria University, Alexandria, Egypt
[b] Deparmtent of Pharmaceutics, Faculty of Pharmacy, Alexandria University, Alexandria, Egypt

ARTICLE INFO

Article history:
Received 31 August 2015
Received in revised form
5 November 2015
Accepted 9 November 2015
Available online 2 December 2015

Keywords:
Biofilm
MRSA
spa
cap
nuc
clfA
fnb
agr
Hypericin
PDT

ABSTRACT

Biofilm formation transforms infections from acute to chronic, increasing patient mortality and significantly increasing healthcare costs. We are studying the prevalence of some virulence genes among methicillin resistant *Staphylococcus aureus* (MRSA) isolates relative to biofilm formation and the potential of photoactivated hypericin to treat these infections. Isolates were collected from three Egyptian governorates over seven months in 2011, 100 isolates were identified as MRSA. Biofilm formation was established using crystal violet staining and 2,3,5-triphenyl tetrazolium chloride reduction. Twenty two percent of the isolates formed biofilms, of which 68.2% were moderate to strong. The virulence genes were detected using polymerase chain reaction. *spaX* (x-region of protein A) was most prevalent. All biofilm-formers lacked *cap5* (capsular polysaccharide 5), the other genes were: *nuc* (thermonuclease) > *clfA* (clumping factor) > *spaIgG* (IgG binding site of protein A), *fnbA* (fibronectin protein A), *cap8* (capsular polysaccharide 8), *agr* (accessory-gene-regulator locus) > *fnbB* (fibronectin protein B). *agr*-locus was only found in 22.22% of moderate biofilm-formers, the remaining genes were almost equally prevalent among biofilm-formers and negative controls. Photoactivated hypericin efficiently inhibited 92.2–99.9% of biofilm viability, irrespective of the number of virulence genes. To conclude, biofilm formation, and treatment might be affected by a myriad of virulence factors rather than a single gene, however, photoactivated hypericin remains a potential antibiofilm approach.

1. Introduction

Staphylococcus aureus is among the pathogens that are recovered the most from patients with nosocomial infections [1]. The reason behind the *S. aureus* increased prevalence is believed to be the high number of virulence factors it harbors [2]. The pathogen causes a wide variety of infections ranging from subcutaneous abscesses or furuncles to scalded skin syndrome, sepsis necrotizing pneumonia, and toxic shock syndrome (TSS) [3]. Such infections become more complicated when the strain is methicillin resistant, a phenotype that usually heralds a more generalized β-lactam resistance [4]. Published data about the prevalence of MRSA strains among Egyptian patients is not readily available, however a recently published study showed the prevalence between 2003 and 2005 to be 52% [5].

The ability of staphylococcal cells to grow in structured communities, adhering to biotic and abiotic surfaces and called biofilms, renders the infection significantly morbid and raises fatality rates [6,7]. *S. aureus* biofilm cells are sheathed in a self-produced extracellular matrix made of proteins, DNA and/or polysaccharides (referred to as the polysaccharide intercellular adhesion or PIA) [8–10]. This extracellular matrix prevents antimicrobials from reaching the biofilm cells making the biofilm more recalcitrant to killing, relative to planktonic cells [11,12]. A comparison in the response to antimicrobials between biofilm and planktonic cells reported a six-log increase in cell viability in *S. aureus* biofilms [13]. In addition, staphylococcal biofilms serve as a virulence factor allowing the pathogen to adhere to its target surfaces, including implanted devices and other foreign body materials [14,15].

The initial step of biofilm formation consists of attachment to the target surface. For this purpose *S. aureus* can express a myriad of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) that interact with host extracellular ligands. These MSCRAMMs comprise virulence factors such as fibronectin

* Corresponding author. Department of Microbiology and Immunology, Faculty of Pharmacy, Alexandria University, 1 Khartoum Sq., Azarita, 21521, Alexandria, Egypt.
E-mail address: alaa.abouelfetouh@pharmacy.alexu.edu.eg (A.A.Y. Abouelfetouh).

http://dx.doi.org/10.1016/j.micpath.2015.11.012
0882-4010/

binding proteins A and B (FnBPA, FnBPB), clumping factors A and B (ClfA, ClfB) [16], and staphylococcal protein A (Spa). Most of these proteins play a role in biofilm formation/accumulation in *S. Aureus* [17–19]. *S. aureus* also produces a number of exoenzymes that are considered virulence factors because they promote breakdown of host tissues supplying the pathogen with some nutrients to allow bacterial survival [20–22]. *S. aureus* uses accessory gene regulators including Agr, Sar, Sae and others to manage the production of these virulence factors, in response to some environmental signals, cell density (quorum sensing) and energy levels [23]. Previous studies implied that extracellular DNA (eDNA) supports biofilm formation in *S. aureus* and that nucleases, either endogenous or exogenously applied, negatively affect biofilm formation, at least under certain in vitro conditions [10].

Most clinical *S. aureus* isolates possess a capsule that is mainly polysaccharide in nature, with capsular polysaccharide (CP) serotypes 5 and 8 being the most abundant in humans. CP5 and CP8 increase *S. aureus* virulence and protect the organism from phagocytosis [24]. The expression of CP5 or CP8 can be affected by certain global regulators e.g. *agr*, *arlRS*, *saeRS*, and *sarA* [25–29].

The architecture of the biofilms makes them resist the host defenses as well as protecting the bacterial cells from the action of many antibiotics [30]. This fact necessitates the search for alternative approaches to treat biofilm related infections. Antimicrobial photodynamic therapy (aPDT) is one example. It involves the use of a photosensitizer that is then activated by light [31,32]. Photosensitizers are usually photoactive pigments that generate singlet oxygen when irradiated by visible light at a wavelength corresponding to their absorption spectrum. These reactive oxygen species are toxic to several cell structures, including DNA, lipids and enzymes. The action of aPDT is selective as it targets microbial cells killing them more effectively than host cells via apoptosis or necrosis [33]. Hypericin is a naturally occurring potent photosensitizer extracted from *Hypericum perforatum* (commonly known as St. John's Wort). The antimicrobial effect of hypericin was assessed against different bacterial and fungal strains [34]. It also showed biofilm inhibition potential against staphylococcus biofilms [35].

This work is investigating whether the ability of some staphylococcal isolates, obtained from hospitalized patients in three Egyptian governorates, to form a biofilm and the biofilm strength are affected by the presence of some virulence genes. In addition, the antimicrobial effect of hypericin is tested against some biofilm-forming isolates.

2. Materials and methods

2.1. Clinical isolates

A total of 100 MRSA clinical isolates were identified in this study. They were isolated between April and October 2011, from the clinical microbiology laboratories affiliated with El Meery tertiary teaching hospital (n = 44), El Mowasah hospital (n = 15) and Medical Research Institute hospital (n = 18) in Alexandria, Kafr El Sheikh public hospital in Kafr El Sheikh (n = 14) and Assiut University hospital in Assiut (n = 9). The clinical specimens were collected from blood (n = 42), sputum (n = 22), pus (n = 21), urine (n = 13) and bronchoalveolar lavage (n = 2).

2.2. Bacterial identification

The isolates were identified according to the conventional methods, based on their morphological, tinctorial, cultural and biochemical characteristics including growth on mannitol salt agar, and production of catalase and coagulase. Methicillin (β-lactam) resistance was determined based on the susceptibility of the isolates to oxacillin and cefoxitin using the disc diffusion method following the Clinical and Laboratory Standards Institute guidelines [36] for susceptibility checking. Frozen glycerol stocks (30%) of the identified isolates were stored at −70 °C.

2.3. Assessment of the biofilm biomass (crystal violet staining assay)

Sterile nutrient broth was distributed in 96-well microtiter plates in 90 μl aliquots. Equal volumes of nutrient broth inoculated with the test isolates were transferred to the wells of the plates to yield about 5×10^7 CFU ml^{-1}. The plates were wrapped in aluminium foil sheets and incubated overnight at 37 °C. Following incubation, the planktonic cultures were discarded and the plates were washed with sterile saline. Each well then received 100 μl of 1% crystal violet (CV) to stain the attached biofilm and the plates were incubated at room temperature for 15 min. The dye was discarded and the plates were washed with saline to remove any excess dye then left to dry. The optical density of the stained plates was measured at 630 nm using the absorbance microplate reader (Biotek) as described previously [37]. An average of three readings was calculated and used to draw the histograms. An isolate was termed a biofilm-former if its OD630 was ≥0.09.

2.4. Determination of the metabolic activity of the isolates biofilms (2,3,5-triphenyl tetrazolium chloride reduction assay)

The metabolic (respiratory) activity of the biofilm cells was determined using the modified 2,3,5-triphenyl tetrazolium chloride (TTC) reduction assay as described elsewhere [38]. TTC is a redox indicator used to differentiate between metabolically active and inactive cells; the colorless compound is metabolically reduced to red TPF (1,3,5-triphenylformazan) in living cells due to the activity of various dehydrogenases. Briefly, biofilms were formed as described above, and following washing, 150 μl of 1.5% v/v TTC (Sigma Aldrich) in nutrient broth were distributed in the wells. The plates were wrapped in aluminium foil sheets and incubated for 2 h, and the optical density was measured at 450 nm. The experiment was done in triplicates and average readings was calculated and used to draw the histograms.

The biofilm strength was decided as a function of the biofilm biomass, as determined by CV staining assay and biofilm metabolic activity, as determined by TTC reduction assay. A combination of OD630 ≥ 0.09 in the CV staining assay and OD450 ≥ 0.4 in TTC reduction assay was required to describe a strain as a strong biofilm-former. Moderate biofilm-formers were those that showed an OD630 ≥ 0.09 and OD450 < 0.4. Any isolate that failed to show consistent biofilm formation in the different replications was considered a weak biofilm-former and was excluded from further testing.

2.5. PCR method to detect the virulence genes

All strong and moderate biofilm-formers, in addition to the selected negative controls, were tested for the presence of the virulence genes; *clfA*, *spa (IgG-binding)*, *spa (X-region)*, *nuc*, *fnbA*, *fnbB*, *cap5*, *cap8* and *agr*-locus, using PCR as previously described [39,40]. The primers sequence and PCR conditions are detailed under Table 2.

2.6. Determination of the antimicrobial photoactivity of hypericin against planktonic S. aureus

A stock solution of hypericin, isolated from *H. perforatum*, purity >98% HPLC (Planta Natural Products GmbH, Vienna, Austria), was