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SHORT COMMUNICATION

Slime Production by *Staphylococcus aureus* and *Staphylococcus epidermidis* Strains Isolated from Patients with Diabetic Foot Ulcers

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Abstract Slime production is a very important factor related to biofilm formation. The objective of the present study was to determine the frequency of slime production by *Staphylococcus aureus* and *Staphylococcus epidermidis* strains recovered from 50 patients with diabetic foot ulcers. Slime production was determined using the Congo red agar (CRA) method and compared with immunocytochemistry for the production of polysaccharide intercellular adhesin (PIA). Out of 55 *S. aureus* strains, 69% produced slime as shown by the CRA method. Of them, 84.2% also produced PIA. Of 17 CRA-negative strains, 70.6% produced PIA. Out of 20 *S. epidermidis* strains, 75% were CRA positive and 93.3% produced PIA. All CRA-negative *S. epidermidis* produced PIA. In conclusion, PIA production is a very common trait of *S. aureus* and *S. epidermidis* isolates obtained from diabetic foot ulcer patients.

Keywords Slime production · Staphylococci · Polysaccharide intercellular adhesin

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Introduction

Biofilm formation is one of the most important virulence factors. Bacteria form biofilms on indwelling medical devices and chronic wounds. Chronic wounds are ulcers that do not heal in an orderly way and in a predictable amount of time and they include diabetic foot, pressure, and venous leg ulcers. The most important factor contributing to chronicity of wound healing is bacterial colonization. Bacteria in a chronic wound have a long time to develop and achieve antibiotic resistance. Similarly, the prevalence of biofilms is observed in chronic wounds compared with acute wounds (James et al. 2008).

The production of slime, an extracellular substance which surrounds multiple cell layers, facilitates bacterial adherence. Staphylococcal slime is composed of different bacterial and host products (Götz 2002). A hallmark is the production of the slime-forming substance polysaccharide intercellular adhesin (PIA), initially described by Mack et al. (1996). PIA is a polysaccharide consisting of β1,6-linked *N*-acetylglucosamine residues produced by *Staphylococcus epidermidis* RP62A and encoded by the operon *ica* (Heilmann et al. 1996). A PIA-like substance, PNSG (poly-*N*-succinyl β1,6 glucosamine), produced by the *Staphylococcus aureus* MN8m strain was described by McKenney et al. (1999, 2000). Using multiple analytical techniques, Maira-Litrán et al. (2002) and Joyce et al. 2003) were able to demonstrate that the MN8m strain produced PIA/PNAG (poly-*N*-acetyl β1,6 glucosamine). These two polymers differed in terms of their molecular size, the degree of *N* acetylation, the degree of *O* succinylation, and immunogenicity. Preclinical studies showed that PIA/PNAG immunization was moderately protective in rabbit models of endocarditis and catheter-associated bacteremia as well as the murine Sidney

infection model. Antibodies to de-*N*-acetylated-PNAG (dPNAG) epitopes were opsonophagocytic to *S. aureus* and gave some protection to *S. aureus*-infected mice (Garcia-Lara and Foster 2009).

PIA is involved in the protection of staphylococci against innate host defenses. Phagocytosis by neutrophils is a major mechanism by which the innate immune system eliminates invading microorganisms and PIA is the first defined factor of the biofilm that protects bacteria from elimination by neutrophils (Kristian et al. 2008; Vuong et al. 2004). It was also found that biofilms increase bacterial resistance to antibiotics. Two main mechanisms contribute to biofilm resistance: (1) the prevention of an antibacterial substance from reaching its target, for example, by limited diffusion or repulsion, and (2) the specific physiology of the biofilm, which limits the efficacy of antibiotics, mainly of those that target active cell processes and which may also include specific subpopulations of resistant cells ("persisters") (Keren et al. 2004).

The objective of the present study was to determine the frequency of slime production by *S. aureus* and *S. epidermidis* strains isolated from patients with diabetic foot ulcers and compare two slime visualization methods: Congo red agar (CRA) and that based on an anti-PIA antiserum immunocytochemical assay.

Materials and Methods

Bacterial Strains, Culture Conditions

The study was done on 75 staphylococci strains obtained from curettage of the diabetic ulcer base and skin biopsies from the ulcer edge and toe web swabs of 50 patients with DFU (Galkowska et al. 2009). Cotton swabs moistened with 0.9% saline obtained from toe webs and samples from curettage of the ulcer base and edge were placed in transport medium (Copan, Brescia, Italy) and transferred to the microbiology department. The samples were then plated for culture on blood agar and Chapman agar plates selective for staphylococci (Oxoid, Basingstoke, UK). The API Staph system (bioMerieux, Poland Ltd.) was applied for the identification of staphylococcal species. The SLIDEX Staph-Kit (bioMerieux Poland Ltd.) was used to discriminate *S. aureus* strains from coagulase-negative staphylococci. The frozen bacterial isolates, stored at –80°C on cryobeads (Cryobank Mix, Mast Diagnostica, Reinfeld, Germany), were subcultured in brain-heart infusion broth (BHI, bioMerieux, Poland Ltd.) at 37°C for 8 h and then plated on trypticase soy agar plates (bioMerieux, Poland Ltd.) for 20 h.

CRA Test

The ability to form slime was tested by the standard CRA method according to Krukowski et al. (2008). Bacteria from one colony were cultured on a CRA plate in two replicates. The CRA medium was composed of 37 g/l brain-heart infusion broth (BHI, bioMerieux, Poland Ltd.), 50 g/l sucrose, 10 g/l agar no. 1 (Oxoid), and 0.8 g/l Congo red (Sigma, Poland). The Congo red stain was prepared as a concentrated aqueous solution, autoclaved (121°C for 15 min) separately, and added to the agar growth medium cooled to 55°C. The CRA plates with bacteria were incubated aerobically for 24 h at 37°C. Strains that produced slime formed black colonies on CRA. Negative results were indicated by pink colonies (Freeman et al. 1989). The score system for CRA was + (black colonies) and – (pink colonies).

PIA Production Test

The ability of the strains to produce PIA was tested by the immunocytochemical method according to Kristian et al. (2004) with some modifications. Bacteria were suspended in 0.9% saline to the 0.5 MacFarland standard. For immunocytochemistry, 100-µl volumes of the bacterial suspensions were placed on Superfrost® Plus slides and dried at room temperature. Then the slides were fixed in cold acetone for 10 min, treated with blocker (Dual Endogenous Enzyme Block, DakoCytomation, Poland), and washed two times with TBS (TRIS-buffered saline, pH 7.6) supplemented with 0.1% Tween-20. After blocking, the slides were subjected to 2% normal goat serum for 20 min at room temperature, then washed two times with TBS/Tween and incubated overnight at 4°C with diluted 1:250 rabbit PIA-specific antiserum (a generous gift from Michael Otto, Laboratory of Human Bacterial Pathogenesis, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA). After incubation the slides were washed two times with TBS/Tween, treated with goat anti-rabbit IgG (Biotinylated Link, DakoCytomation LSAB2 System-HRP) for 15 min at room temperature, washed two times with TBS/Tween, incubated for 15 min at room temperature with goat anti-rabbit IgG (Streptavidin-HRP, DakoCytomation LSAB2 System-HRP), and washed two times with TBS/Tween. Then the slides were treated with DAB (3,3′-diaminobenzidine) substrate (Sigma, Poland). The DAB solution consisted of 8 ml of TBS/Tween, 25 mg of DAB, and three drops of peroxide solution and was freshly mixed and kept in the dark before use. Slides with the DAB substrate were incubated for 10 min at room temperature. The development of a brown color was observed under a

Table 1 Slime and PIA production by *S. aureus* and *S. epidermidis* isolates

Species/numbers (*n*)	CRA test (*n*)	Score for immuno-cytochemistry	PIA-producing isolates	
			CRA+ *n* (%)	CRA– *n* (%)
S. aureus (55)	CRA+ (38)	+	21 (55.3)	2 (11.8)
	CRA– (17)	±	11 (28.9)	10 (58.8)
		–	6 (15.8)	5 (29.4)
S. epidermidis (20)	CRA+ (15)	+	14 (93.3)	5 (100)
	CRA– (5)	±	0 (0)	0 (0)
		–	1 (6.7)	0 (0)

microscope and immediately stopped by removing the DAB. Then the slides were rinsed with dH_20 for 1 min, counterstained with hematoxylin for 90 s, washed with tap water, and subjected to the mounting procedure. To control non-specific background staining, one slide was not exposed to the primary antibody. All strains were studied in duplicate. Slime-producing *S. epidermidis* RP62A ATCC35984 was used as a positive control. The score system used for immunocytochemistry assay was – (no staining, 0% brown cells), ± (weak staining, 25–50% brown cells), and + (moderate to strong staining, >50–100% brown cells).

Results

The results of the examination of the slime-forming ability of the *S. aureus* and *S. epidermidis* strains are given in Table 1. Figure 1 presents a visualization of PIA produced by staphylococci and slime production on CRA. Of the 55 *S. aureus* isolates, 69% (38 strains) produced slime as shown by the CRA method. Of these CRA + isolates, 84.2% (32 strains) also produced PIA and 15.8% of them (6 strains) did not. Of the 17 CRA-negative *S. aureus* isolates, 70.6% (12 strains) produced PIA and 29.4% of them (5 strains) did not. Of the 20 *S. epidermidis* strains, 75% (15 strains) produced slime as shown by the CRA method. Of these CRA + isolates, 93.3% (14 strains) also produced PIA and only 6.7% (1 strain) did not. Of the 5 CRA-negative *S. epidermidis* isolates, all (100%) produced PIA.

Discussion

The CRA test is both sensitive and specific to biofilm detection when applied to *S. aureus* as well as to *S. epidermidis* (Jain and Agarwal 2009). However, the immunocytochemical assay we used seems to be more sensitive since even CRA-negative staphylococcal isolates produced PIA. The detection of PIA producers among colonizing and invasive staphylococci strains would allow the early application of proper treatment, especially in chronic non-healing wounds.

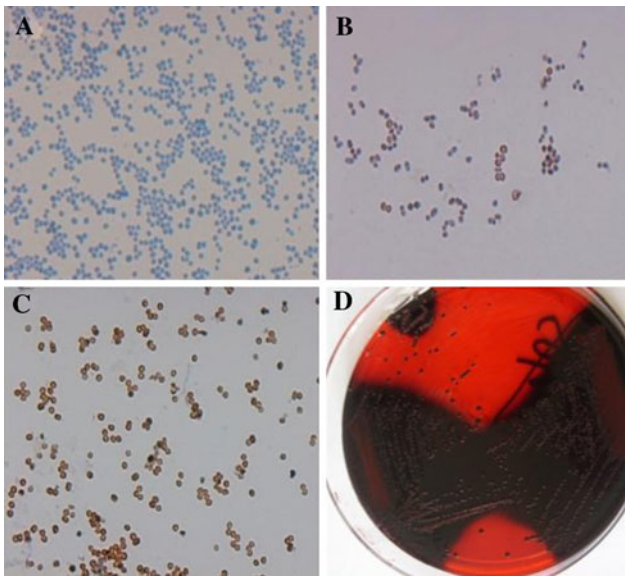

Fig. 1 Detection of PIA-producing *S. aureus* cells and slime formation on CRA medium. **a** *Blue* Negative for PIA *S. aureus* cells, **b** PIA-producing *S. aureus* cells (*blue* and *brown* cells, 50% positive), **c** PIA-producing *S. aureus* isolates (*brown* cells, 100% positive), **d** *Black* colonies of a slime-producing *S. aureus* strain growing on CRA

Our understanding of how staphylococci form biofilm is far from complete and many questions remain unanswered. Despite PIA/PNAG production, there are PIA-independent mechanisms of biofilm formation. Teichoic acids, autolysins, adhesins, and Bap and Aap proteins can be essential for both initial adherence and intercellular accumulation during biofilm development (Götz 2002; O'Gara 2007; Vuong and Otto 2002). Recently, extracellular DNA was demonstrated to be a structural component of staphylococcal biofilm (Izano et al. 2008). Biofilm formation can be influenced by many environmental factors such as anaerobic conditions (Cramton et al. 2001), glucose, urea and global regulators such as Agr, Sar, SigB, and RsBU (Götz 2002), and other systems such as LuxS and Rbf (O'Gara 2007). The existence of these *ica* operon-independent mechanisms could explain the discrepant results we

obtained in the CRA test and the assay with anti-PIA serum.

In conclusion, on the basis of the obtained results it may be stated that PIA production is a very common trait of *S. aureus* and *S. epidermidis* isolates obtained from diabetic foot ulcer patients.

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