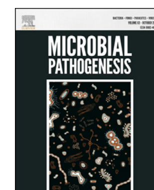
Microbial Pathogenesis xxx (2014) 1–6

Contents lists available at ScienceDirect

Microbial Pathogenesis

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



Adhesive properties and extracellular enzymatic activity of *Staphylococcus aureus* strains isolated from oral cavity

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A R T I C L E I N F O

Article history:
Received 28 February 2014
Received in revised form
2 May 2014
Accepted 5 May 2014
Available online xxx

Keywords:
Staphylococcus aureus
Hydrolytic enzymes
Biofilm
Orthodontic biomaterials

A B S T R A C T

Staphylococcus aureus is one of prominent bacterial pathogen that occurs in oral region. In this study, 21 strains of *S. aureus* isolated from the oral cavity of Tunisian patients were investigated for slime production using Congo red agar method (CRA) and adherence assay. Biofilm formation of oral isolates on orthodontic biomaterials (Bis-GMA and PMMA) was also evaluated by MTT reduction assay. In addition, the production of hydrolytic enzymes by *S. aureus* strains was analyzed and the presence of protease, lipase and β-hemolysin genes (*sspA*, *sspB*, *geh*, *hlb*) was achieved by polymerase chain reaction (PCR).

Qualitative biofilm production tested on CRA revealed that 91% of strains were slime producers. The result of OD570 showed that five strains isolated from the oral cavity were highly biofilm positive. The metabolic activity of *S. aureus* biofilm formed on Bis-GMA and PMMA did not differ between tested strains. The atomic force micrographs demonstrated that biofilm formed by *S. aureus* strains was organized in typical cocci cells attached to each other through production of exopolymeric substances. The production of hydrolytic enzymes showed that all *S. aureus* strains were protease positive. Lipase (77%) and beta hemolytic (59%) activities were also detected. Among the tested strains, 17 were positive for *sspA*, *sspB* and *hlb* genes. While only ten *S. aureus* strains harbor the *geh* gene (48%).

These data highlight the importance of evaluation of biofilm formation and exoenzyme production in oral *S. aureus* isolates to investigate the role of this pathogen and its impact in oral pathology.

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1. Introduction

The human oral cavity contains more than 500 species of bacteria that interact among themselves and with their host tissues [1,2]. Oral cavity is considered as a reservoir of opportunistic pathogens that played a role in the onset of oral diseases [3]. *Staphylococcus aureus* is known as the leading cause of nosocomial infections. It is responsible for a wide range of human diseases, including endocarditis, food poisoning, septicemia and skin infections [4]. Furthermore, several studies underlined the prevalence of *S. aureus* in the oral cavity [5]. In fact, it was isolated from root carious lesions [6], periodontal pockets [7], dental abscesses [8], as well as from saliva and supragingival plaque of healthy adults [9].

To survive and colonize the oral cavity, *S. aureus* should develop many strategies such as biofilm formation and extracellular enzyme secretion. Indeed, this pathogen has the capacity to form multicellular communities that grow embedded in a self-produced extracellular matrix, referred to as biofilms. The biofilm matrix, composed of exopolysaccharides, proteins, nucleic acids and lipids, plays a well-known role as a defense structure, protecting bacteria from the host immune system and antimicrobial therapy [10].

Adhesion ability is an important factor in bacterial pathogenicity since it precedes penetration of the microorganisms in the host tissues promoted by the production of toxins [11]. *S. aureus* can form biofilms on many host tissues and implanted medical devices often causing chronic infections [12,13]. The adherence of this bacterium to an either biotic or abiotic surface is the critical first event in the establishment of *S. aureus* infection. Indeed, it harbors

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http://dx.doi.org/10.1016/j.micpath.2014.05.002
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a variety of proteins compounds that mediate attachment to a multitude of host factors, such as extracellular matrix and plasma proteins and human host cells, or intercellular adhesion, which is essential for biofilm accumulation [14].

This bacterium produces a wide array of cell surface and extracellular proteins involved in virulence [15]. It secreted enzymes that degrade host components and hemolysins that damage the host tissue [16]. Two major extracellular proteases, namly serine protease (V8 protease; SspA), and cysteine protease (SspB) are produces by *S. aureus* and encoded within the same operon [17]. Moreover, this pathogen secreted lipase enzyme, which encoded by *geh* gene [15] enable it to invade and destroy host tissue. In addition to enzymes production, *S. aureus* secretes multiple toxins acting in the immediate area of infection, such as alpha, beta, gamma, and delta toxins [18].

To date, few studies have analyzed the prevalence of *S. aureus* carriage on human oral mucosa [3]. Therefore, the role of *S. aureus* in several diseases of the oral cavity merits further investigation, and the characterization of a potential *S. aureus* virulence factor became a necessity to understand their subsistence in oral cavity.

The aim of this study was to evaluate the slime production of oral *S. aureus* strains and their ability to adhere to polystyrene and orthodontic biomaterials and to investigate their potency to secrete exoenzymes and hemolytic factor.

2. Materials and methods

2.1. Patients and bacterial strains

2.1.1. Patients

The study was done on 105 patients from the dental clinic of Monastir, Tunisia. The subjects were 50 males and 55 females, with dental caries, pyogenic granulomas or abscesses. The mean age was 45.84 ± 15.82. Ethical clearance was taken prior to the commencement of study. Written informed consent was obtained from all participants. All clinical procedures were approved by the Ethical Committee of the Faculty of Medicine, University of Monastir, Tunisia. Medical data and dental history were obtained from each patient. The criteria for inclusion were: no antibiotic treatment during sampling, no use of mouth rinses or any other preventive measure that might involve exposure to antimicrobial agents.

2.1.2. Media and growth conditions

Samples were taken from dental abscess, caries and saliva of each patient with a sterile swab. After incubation in brain heart infusion (BHI) medium during 24 h, the swab was plated aerobically on sheep blood agar plates containing 4% NaCl, for 24 h at 37 °C. Suspected colonies of *S. aureus* were confirmed by their positive Gram stain, catalase and DNAse positive reaction and the presence of free plasma coagulase using rabbit plasma (Bio-Merieux, France). Specie identification was performed using API 20 Staph strips (Bio-Merieux, France) according to the manufacturer's recommendation and the results were read using an automated microbiological mini-API (Bio-Merieux, France).

2.2. Phenotypic characterization of bacteria-producing slime

Qualitative detection of slime producer strains was studied by culturing the isolates on Congo red agar (CRA) plate made by mixing 36 g saccharose (Sigma Chemical Company, St. Louis, MO) with 0.8 g Congo red in 1 L of brain heart infusion agar (Biorad, USA) as previously described [19]. The strains were incubated at 37 °C for 24 h under aerobic conditions. Slime-producing strains gave black colonies with a rough surface against red colonies with a smooth surface for non-producing strains. Variable phenotype strains gave colonies with a black center and red outline, or red center and black outline were considered as positive slime producers [20].

2.3. Semi-quantitative adherence assay

Biofilm production by *S. aureus* strains grown in BHI (Biorad, France) was determined using a semi-quantitative adherence assay on 96-well tissue culture plates (Nunc, Roskilde, Denmark) as described previously [21,22]. Adherent bacteria were fixed with 95% ethanol and stained with 100 mL of 1% crystal violet (Merck, France) for 5 min. The microplates were air-dried and the optical density of each well was measured at 570 nm (OD570) using an automated Multiskan reader (GIO. DE VITA E C, ome, Italy). Biofilm formation was interpreted as highly positive (OD570 $\geq$ 1), low grade positive (0.1 $\leq$ OD570 $<$ 1), or negative (OD570 $<$ 0.1).

2.4. Biofilm formation on biomaterials

2.4.1. Preparation of strips

In the present study, the denture base materials used is the bisphenol A glycidyl methacrylate (Bis-GMA) composite resins and the polymethyl methacrylate acrylic (PMMA). The Bis-GMA and PMMA strips were made according to the method used in the laboratory of Biomaterials and Biotechnology in the Faculty of Dentistry of Monastir (Tunisia).

All strips were cut into 1 cm^2 squares. They were disinfected by dipping in 70% alcohol for 30 min and washed with sterile distilled water. They were then ultrasonicated for 20 min to remove any contaminants and artifacts from the surfaces, washed again in sterile distilled water, dried and used for the biofilm assay.

2.4.2. MTT metabolic assay

The composite disks were placed in a 24-well plate, inoculated with 1.5 mL of the inoculation medium, and cultured for 24 h. Each disk was transferred to a new 24-well plate for the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) assay. It is a colorimetric assay that measures the enzymatic reduction of MTT, a yellow tetrazole, to formazan [23]. This kind of indirect viability assay is based on the formation of insoluble purple formazan due to the reduction of MTT by (respiratory) reductases of living staphylococcal cells [24]. Briefly, 1 mL of MTT dye (0.5 mg/ml MTT in PBS) was added to each well and incubated at 37 °C for 1 h. After 1 h, the disks were transferred to a new 24-well plate, 1 mL of dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystals, and the plate was incubated for 20 min with gentle mixing at room temperature in the dark. A total of 200 μL of the DMSO solution from each well was transferred to a 96-well plate, and the absorbance at 540 nm (optical density OD540) was measured via a microplate reader (GIO. DE VITA E C, ome, Italy). A higher absorbance indicates a higher formazan concentration, which in turn indicates more metabolic activity in the biofilm on the composite.

2.4.3. Biofilm visualization by atomic force microscopy (AFM)

To visualize the biofilm formed on Bis-GMA and PMMA surfaces and to study the morphological changes in the cells during biofilm production, *S. aureus* ATCC 6538 was used as a positive control. After biofilm formation on strips, the surfaces were fixed on the round cover slide and were examined by AFM [25].

2.5. Characterization of the enzymatic activity

The activities of various enzymes were determined after inoculation of cultures onto TSA-1 to which the following substrates had been added: 1% [wt/vol] skim milk for caseinase, 1% [wt/vol]

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Please cite this article in press as: Meghrni A, et al., Adhesive properties and extracellular enzymatic activity of *Staphylococcus aureus* strains isolated from oral cavity, Microbial Pathogenesis (2014), http://dx.doi.org/10.1016/j.micpath.2014.05.002

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