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High potential of adhesion to biotic and abiotic surfaces by opportunistic *Staphylococcus aureus* strains isolated from orthodontic appliances

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ABSTRACT

Orthodontic and other oral appliances act as reservoir of opportunistic pathogens that can easily become resistant to antibiotics and cause systemic infections. The aim of this study was to investigate the ability of *Staphylococcus aureus* strains isolated from healthy patients with orthodontic appliances, to adhere to biotic (HeLa cells) and abiotic surfaces (polystyrene and dental alloy). Adhesive ability to polystyrene was tested by crystal violet staining and quantitative biofilm production on dental alloy surfaces was evaluated by MTT reduction assay. In addition, the presence of *ica*A and *ica*D genes was achieved by polymerase chain reaction (PCR). Qualitative biofilm production revealed that 70.6% of strains were slime producers. The metabolic activity of *S. aureus* biofilms on dental alloy surfaces was high and did not differ between tested strains. Moreover, all the isolates were adhesive to HeLa cells and 94% of them harbor *ica*A and *ica*D genes. Considerable adhesion and internalization capacity to the epithelial HeLa cells and strong biofilm production abilities together, with a high genotypic expression of *ica*A/*ica*D genes are an important equipment of *S. aureus* to colonize orthodontic appliances and eventually to disseminate towards other body areas.

1. Introduction

Orthodontic appliances are known to severely hamper biofilm control in the oral cavity. They have various negative side-effects related to biofilms such as the white spot lesions and the increase in the volume of dental plaque and gingivitis. One of these effects is manifested particularly by bacterial adhesion to biomaterial surfaces constituting the bracket-adhesive enamel junction which represents a growing problem in orthodontics [32].

Increase in detectable opportunistic bacteria in the oral cavity of orthodontic patients was revealed in many reports [1,26]. For instance, *Staphylococcus aureus* is one of the prominent human pathogen frequently found in the implant-associated infections [22]. This bacterium is known to adhere and form biofilms on materials and cells and it was detected in high frequencies when the immune response is reduced [1]. It has been shown that insertion of dentures increased the regular recovery of *S. aureus* both in healthy subjects [31] and in institutionalized elderly subjects [21]. Therefore, monitoring and controlling the dissemination of this opportunistic bacterium is one of the key factors for reducing nosocomial infections.

S. aureus is able to develop biofilm as a leading cause of associated chronic infections. This biofilm is initiated when bacterial cells attach and adhere to the surfaces of implants or host tissues [8]. Moreover, *S. aureus* produces a large variety of virulence factors that mediate cell and tissue adhesion, contribute to tissue damage and protect bacteria against the host immune defense system [15,16].

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S. aureus can produce a multilayered biofilm embedded within a glycocalyx or slime layer with heterogeneous protein expression throughout. A specific polysaccharide antigen named polysaccharide intercellular antigen (PIA) responsible for biofilm formation has been isolated and characterized [3]. PIA is produced from UDP-N-acetylglucosamine via products of the intercellular adhesion (ica) locus [13]. The genes and products of the ica locus [icaR (regulatory) and icaADBC (biosynthetic) genes] have been demonstrated to be involved in biofilm formation and virulence of *S. aureus* [14].

The aim of this study was to assess the adhesion ability of oral *S. aureus* strains isolated from orthodontic appliances to biotic (epithelial HeLa cells) and abiotic surfaces (polystyrene and dental alloy), and to investigate the prevalence of *ica*A/*ica*D genes in tested isolates.

2. Materials and methods

2.1. Patients and bacterial strains

The study included on 46 healthy patients from the dental clinic of Monastir, Tunisia. The subjects were 23 males and 23 females, wearing orthodontic appliances and without periodontal infections. The mean age of patients was 17.75 ± 3.3. Ethical clearance was taken prior to the commencement of study. All clinical procedures were approved by the Ethical Committee of the Faculty of Dentistry of Medicine, University of Monastir, Tunisia.

1.0 ml of non-stimulated saliva was collected from each patient and plaque was removed from the areas adjacent to the brackets and wires. Removed appliances from each patient were also taken for investigation of *S. aureus* adherent bacteria. After incubation of all samples in brain heart infusion (BHI) medium during 24 h, 10 μl of each medium 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).

After isolation and identification, a stock culture of each isolate was maintained in BHI containing 25% glycerol at −80 °C. Prior to experiments, strains were cultured in trypticase soy broth (TSB), grown at 37 °C for 24 h and cell concentrations were adjusted by optical density at 600 nm.

2.2. Phenotypic characterization of bacteria-producing slime

Qualitative detection of slime producer strains was studied by culturing the isolates on Congo red agar plate (CRA) made by mixing 36 g saccharose (Sigma Chemical Company, St. Louis, MO) with 0.8 g Congo red in one liter of brain heart infusion agar (Biorad, USA) as previously described [17]. 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 [45].

2.3. Cell surface hydrophobicity

The hydrophobicity of isolated strains was evaluated by the microbial adhesion to hexadecane (MATH) test [7]. It consisted in evaluating the affinity of the cells towards apolar solvents (hexadecane). For the experiments, bacterial cells were harvested by centrifugation at 7000× g for 5 min and resuspended to Abs600 nm = 0.4 (10^8 CFU/ml) (Abs1) in 0.01 M potassium phosphate buffer (pH 6.5). This bacterial suspension was mixed with a solvent in the proportion of 1:6 (0.4/2.4 v/v) by vortexing for 90 s in order to form an emulsion. This mixture was then left for 20 min until the separation of two phases. Aqueous phase absorbance was measured (Abs2) and the percentage of adhesion was expressed as: % adhesion = (1- Abs2/Abs1) × 100 [28]. Cells with hydrophobicity percentage greater than 70% as highly hydrophobic; from 70 to 30% as weakly hydrophobic and those with hydrophobicity lower than 30% as highly hydrophilic [9].

2.4. Semi quantitative adherence assay

Biofilm production by *S. aureus* strains grown in BHI (Bio-Rad, France) was determined using a semi quantitative adherence assay on 96-well tissue culture plates (Nunc, Roskilde, Denmark) as described previously [12,30]. 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, Rome, Italy). Biofilm formation was interpreted as highly positive (OD570≥1), low grade positive (0.1 ≤ OD570 < 1), or negative (OD570<0.1) [48]

2.5. Biofilm formation on dental alloy

2.5.1. Preparation of strips

In the present study, metallic samples Co—Cr (Adentatec GmbH, Germany) were made by the casting process, 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.

2.5.2. MTT metabolic assay

The specimens (n = 18) 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 [11]. 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 [41]. 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. 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, Rome, Italy). A higher absorbance indicates a higher formazan concentration, which in turn indicates more metabolic activity in the biofilm on the material [11].

2.5.3. Biofilm visualization by atomic force microscopy (AFM)

To visualize the biofilm formed on orthodontic appliance surface and to study the morphological changes in the cells during biofilm production, *S. aureus* ATCC 6538 strain was used as a positive