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Assessment of adhesion, invasion and cytotoxicity potential of oral *Staphylococcus aureus* strains

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ABSTRACT

The oral cavity is regarded as a relevant site for *Staphylococcus aureus* colonization. However, characterization of virulence mechanisms of oral *S. aureus* remains to be uncovered. In this study, twenty one *S. aureus* strains isolated from the oral cavity of Tunisian patients were screened for adherence, invasion and cytotoxicity against HeLa cells. In addition, the presence of adhesins (*ica*A, *ica*D, *can*, *fnb*A and *fnb*B) and α-hemolysin (*hla*) genes in each strain was achieved by polymerase chain reaction (PCR).

Our finding revealed that oral *S. aureus* strains were able to adhere and invade epithelial cells, with variable degrees ($P < 0.05$). Moreover they exhibited either low (23.8%) or moderate (76.2%) cytotoxic effects. In addition 76.2% of strains were *ica*A and *ica*D positive and 90.5% harbor both the *fnb*A and the *fnb*B gene. While the *cna* gene was detected in 12 strains (57.2%). Furthermore, the *hla* gene encoding the α-toxin was found in 52.4% of the isolates. All these virulence factors give to *S. aureus* the right qualities to become a redoubtable pathogen associated to oral infections.

1. Introduction

Staphylococcus aureus is an opportunistic pathogen that can cause a variety of self-limiting to life-threatening diseases in humans [1]. This bacterium presents an astounding arsenal of virulence factors that allow them to conquer many different niches throughout the course of infection. In the oral cavity, *S. aureus* has been isolated from a wide range of infective oral conditions, such as angular cheilitis and parotitis [2], carious lesions and gingival pockets [3,4], periodontitis and peri-implantitis [5,6]. Reported isolation rates of *S. aureus* vary with the population studied, with reported carriage rates of 24%–84% in healthy adult dentate oral cavities [7,8]. Particularly, this bacterium was isolated frequently from the oral cavity of some patient groups such as children [9], elderly [10], terminally ill patients [11], rheumatoid arthritis patients [12], and patients with hematological malignancies [13]. Thus, the role of *S. aureus* in oral health and disease remains contentious and needs more investigation.

Generally, bacterial virulence is a multi-factorial process that requires the use of a variety of components, many of which are coordinately regulated to allow the organism to adapt to the host environment and become successful pathogens [14]. More specifically, the pathogenicity of *S. aureus* is a complex process involving a diverse array of extracellular and cell wall components that are coordinately expressed during different stages of infection [15,16]. *S aureus* microbial surface is mainly composed of proteins implicated in adhesion to and invasion of host cells and tissues, evasion of immune responses and biofilm formation [17]. Cell wall-anchored proteins are essential virulence factors for the survival of *S. aureus* in the commensal state and during invasive infections [18]. *S. aureus* harbored series of adhesins among them, two fibronectin-binding proteins, (FnbA and FnbB), and a collagen-binding protein (Cna) that have been proved a significant

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contributor to tissue colonization in various pathological conditions [19]. Moreover, in *S. aureus*, the adhesins exist with other surface components such as the polysaccharide intercellular adhesins (PIA), which are implicated in biofilm formation [20,21] and encoded by icaADBC genes [22,23]. The pathogenicity and virulence of *S. aureus* is also associated with the capacity of this organism to produce several virulence factors including enterotoxins, toxic shock syndrome toxin-1, cytolytic toxins (alpha and beta hemolysins), exfoliative toxins, Panton–Valentine leukocidin (PVL), protein A, and several enzymes [24]. Hemolysin alpha is encoded by the *hla* gene and represented the most potent membrane-damaging toxin which plays an important role in the pathogenesis of *S. aureus* infections [25].

The present study was undertaken to explore the virulence mechanisms such as adherence, invasion and cytotoxicity of *S. aureus* strains isolated from oral cavity and to investigate the prevalence of adhesins and α-toxin genes.

2. Materials and methods

2.1. Bacterial strains

Bacteria used in this study were: *S. aureus* ATCC 6538 as a reference strain and twenty one *S. aureus* strains isolated from the oral cavity of a Tunisian patient suffering of pyogenic granulomas, abscesses and dental caries, as previously published [26].

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. Stock cultures were maintained in BHI containing 25% glycerol at −80 °C.

2.2. Cell adhesion and invasion assay

Human epithelial adenocarcinoma cells (HeLa Cell Line, ATCC No. CCL-2) were used in this study. HeLa cells were maintained in Eagle's minimum essential medium (MEM; Gibco) supplemented with 10% Fetal bovine serum (FBS; Gibco), 100 U/ml penicillin and 2 mM L-Glutamine at 37 °C in 5% CO2 and subcultured every 2–3 days. HeLa cells were seeded into 24-well tissue culture plates at a density of 5×10^4 cells per well. Once monolayers reached 80% confluence (approx. 2×10^5 cells per well), cells were washed and incubated in MEM medium for use in adhesion and invasion assay.

A total of 21 selected *S. aureus* clinical isolates, as well as the ATCC 6538 reference strain, were grown to early exponential phase in Brain Heart Infusion (BHI) broth and harvested by centrifugation at 4500 g for 15 min, washed in PBS and adjusted to an appropriate density in MEM to give an M.O.I. (multiplicity of infection, i.e. ratio of bacteria per host cell) of 100. Host cells were washed with MEM and bacterial suspensions were added to wells in triplicate and incubated for 2 h at 37 °C in 5% CO2. A 1 ml sample of bacterial suspension was incubated in parallel during this infection period to control for any bacterial growth or death during the experiment. Following incubation, HeLa cells were washed three times with MEM to remove non adhered bacteria and then treated with lysis solution (0.1% TritonX100 in PBS) for 15 min at 37 °C and the total number of associated bacteria (adherent and internalized) was assessed by the colony counting method in agar plates. Plates were then incubated aerobically for 24 h at 37 °C. To quantify the number of internalized bacteria, extracellular bacteria and those adhered to the epithelial cell surface were killed by incubation with gentamicin (100 μg/ml in MEM) for 1 h. Gentamicin was removed by washing three times with PBS. Host cells were then lysed and followed by serial dilution and spotting onto agar plates for bacterial cultured viable count. From these data, the number of c.f.u. recovered ml^{-1} was calculated. The number of adherent but non-invading bacteria was calculated by subtracting the number that had been internalized (c.f.u. after gentamicin treatment) from the total cell-associated number (c.f.u. before gentamicin treatment) [27].

2.3. Confocal laser scanning microscopy

HeLa cells were seeded on glass coverslips placed in 24-well plates. Adhesion and invasion experiments were performed as described in section 2.2. Infected cells were washed three times with MEM, and coverslips were removed from the culture medium and washed with physiologically relevant buffer and the submerged in the staining solution of Red CellMask™ plasma membrane stain 1X (1/1000) for 5–10 min at 37 °C. After staining cells were fixed with warm 4% Formaline (sigma) and incubated at room temperature for 10 min. Cover slips were then rinsed with PBS and cells were permeabilized with 0.1% Triton-X100 for 5 min and incubated in FBS (1/100 PBS) for 30 min at room temperature before staining with DAPI (1/2000) solution for 5 min at room temperature. Images of adherent and invasive bacteria to HeLa cells were performed with LSM780 confocal microscope (Zeiss), installed on an Axio Observer Z1 and equipped with four lasers. For each fluorochrome, the bandwidth of the emitted signal detection is adjusted with Quasar Detection Unit. Optical sectioning was managed with the Zen software (Zeiss) with a step of 400 nm between 2 slices. The total thickness of the sample was between 5 and 8 μm leading to the acquisition of 20–25 slices. The orthogonal view was made using Zen software (Zeiss). Images were acquired by Zen software and analyzed in the ImageJ software.

2.4. Cytotoxicity assay

S. aureus isolates were inoculated in tryptic soy broth (TSB, Bio-Rad, France) and incubated at 37 °C for 18–24 h. At the end of incubation, the flask contents were transferred to sterile 50 ml test tubes and centrifuged at 3000 rpm for 15 min. The supernatant was filtered through a 0.22 μm pore size filter membrane (Millipore, Germany). Confluent monolayers of HeLa cells cultured in 96-well tissue culture plates were washed with PBS and 50 μL aliquots of MEM Earl's was added to each well. Then, 50 μl of bacterial filtrates for each strain was added to HeLa cell monolayers, previously washed in PBS, and incubated at 37 °C in 5% CO2 for 24 h [28]. Wells containing only MEM Earl's were served as a control.

To determine the cell viability, the MTT assay was carried out as described by Saliba et al. [29]. After washing with PBS, cells were exposed to 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) at 5 mg/ml in PBS and incubated for 1 h at 37 °C. Supernatants were then removed and cells were treated with 100 μL DMSO to dissolve the formazan crystals formed in the viable metabolically active cells. The eluates of each well of each tested isolate were collected and their absorbance was measured using microplate reader at 540 nm (GIO. DE VITA E C, Rome, Italy). The percentage cytotoxicity was calculated using the following formula [30]:

$$\%\,\text{Cytotoxicity} = (1 - (\text{A540 of infected culture} / \text{A540 of control})) \times 100$$

Strains with cytotoxicity level greater than 85% were considered as highly cytotoxic; from 85% to 50% as moderately cytotoxic and those with cytotoxicity lower than 50% as weakly cytotoxic [14].

2.5. Flow cytometry analysis (FACS)

HeLa cells (5×10^4 cells) were grown in 6-well tissue culture

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