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Prevalence of Panton-Valentine leucocidin and phenotypic and genotypic characterization of biofilm formation among *Staphylococcus aureus* strains isolated from children with adenoid hypertrophy

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ABSTRACT

Adenoids as a first line of host defense against respiratory microbes play an important role in majority of upper airway infectious and noninfectious illnesses. Bacterial pathogen can colonize on the adenoid tissue and probably act as a reservoir for them. To determine phenotypic and genotypic characterization of biofilm forming capacity of *Staphylococcus aureus isolates from* children with adenoid hypertrophy and prevalence of Panton-Valentine leukocidin (PVL) gene we collected 17 consecutive, clinically significant *S. aureus* isolates from children with adenoid hypertrophy undergoing adenoidectomy with one or more of the upper airway obstruction symptoms, nasal obstruction, mouth breathing, snoring, or sleep apnea. Biofilm formation was evaluated by colorimetric microtiter plate's assay. Gene encoding PVL and adhesion- or biofilm formation-encoding genes were targeted by polymerase chain reaction (PCR) assay. According to the results, all strains produced biofilm. Seven (41.2%) isolates produced strong biofilm whereas 7 (41.2%) isolates produced week and 3 (17.6%) isolates produced medium biofilm. Regarding the adhesion- or biofilm formation-encoding genes, 16 (94.1%) isolates were positive for the gene *eno*, 13(76.4%) for *icaA*, 13 (76.4%) for *icaD*, 10 (58.8%) for *fib*, 10 (58.8%) for *fnb*B, 4(23.5%) for *can*, and 1(5.8%) for *fnbA*. The high prevalence of genes encoding biofilms and adhesins and phenotypic ability to form a biofilm by *S. aureus* strains emphasizes the pathogenic character of strains isolated from children with adenoid hypertrophy.

1. Introduction

It is believed that adenoids as a first line of host defense against respiratory microbes play an important role in several upper airway infectious and noninfectious illnesses [1]. Bacterial pathogen can colonize on the adenoid tissue which may later serve as a reservoir for pathogens [2]. In children with recurrent infections, biofilm formation by bacteria has been detected on adenoid and tonsil surface [3–5]. Bacterial biofilms are defined as a complex aggregation of microorganisms in an exopolysaccharide matrix [6]. It is proposed that biofilm formation lead to the antibiotic therapy failure for chronic infections caused by *Moraxella catarrhalis, Streptococcus pneumoniae, Haemophilus influenzae* and *Staphylococcus aureus* [7]. There are several studies in which *S. aureus* has been isolated from adenoid of children with adenoid hypertrophy [8,9]. Some strains of *S. aureus* produce biofilm and help to the virulence of this bacterium [10]. Expressions of specific surface-associated proteins such as polysaccharide intercellular adhesin (PIA) in *S. aureus* facilitate the interaction between organism and extracellular matrix proteins in host cell such as fibronectin and fibrinogen [11]. The PIA has an important role in biofilm formation by *S. aureus*. Structurally, PIA is made up of a linear 1, 6-linked glycosaminoglycan, which is encoded by the intercellular adhesion (*ica*) locus [12]. Among the four genes in the *icaADBC* loci of *S. aureus*, *icaA* and *icaD* have been reported to play a significant role in biofilm formation [13]. Although the expression of the *icaA* gene alone induce low enzyme activity but co-expression of *icaA* and

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icaD genes leads to the full phenotypic expression of the capsular polysaccharide [14]. Several studies have been shown that fibronectin-binding proteins (FnbA and FnbB), fibrinogen binding protein (Fib), and collagen-binding protein (Cna) in Staphylococci are able to bind to a variety of relevant mammalian extracellular proteins [10–16].

A key virulence factor of interest for *S. aureus* infections especially skin and soft-tissue infections as well as more severe symptoms in a wide spectrum of infections is the Panton-Valentine leukocidin (PVL); a two-component leukolytic toxin is known as a virulence factor associated with tissue necrosis [17,18].

To date, there are limited studies about the characteristic of *S. aureus* strains isolated from the adenoid tissue of children with adenoid hypertrophy primarily regarding biofilm characterization and virulence factors. The aim of the current study was phenotypic and genotypic characterization of biofilm forming capacity of *S. aureus isolates from* children with adenoid hypertrophy *as well as* the frequency of PVL-production among studied isolates.

2. Material and methods

2.1. S. aureus isolates

We collected 17 consecutive, clinically significant *S. aureus* isolates from children aged 1–12 years old who were admitted at the department of otolaryngology of two teaching hospitals of Tehran University of Medical Sciences. All of selected patients had adenoid hypertrophy and suffered with either one or more of the upper airway obstruction symptoms, nasal obstruction, mouth breathing, snoring, or sleep apnea and they were candidate for adenoidectomy. The present study was approved by the University Ethics Committee.

S. aureus isolates were identified by standard microbiological methods including Gram stain, catalase test, tube coagulase, DNase and fermentation of mannitol at the microbiology laboratory of Tehran University of Medical Sciences [19]. Amplification of the *femA* gene was used for molecular confirmation of *S. aureus* [20].

2.2. Biofilm formation

Biofilm formation was evaluated by colorimetric microtiter plates assay as described previously [6,10]. Briefly, *S. aureus* colonies were grown overnight at 37 8C in Trypticase Soy Broth (TSB; Merck, Darmstadt, Germany) for 24 h, then the bacterial suspensions were diluted (10^{-2}) in a new TSB medium and 150 μl of this dilution was used to inoculate the sterile flat-bottomed 96-well polystyrene microtiter plates. Subsequent to an incubation period of 24 h at 37 8C without shaking, wells were gently washed three times with 200 μl of with sterile phosphate buffered saline (PBS). For fixation of the biofilms, 100 μl of 99% methanol was added and, after 15 min, the solutions were removed and the plate was air-dried. In the next step, 150 μl of crystal violet 1% (CV) was added to all wells for 20 min. After removing the dye, the bound CV was released with adding 150 μl of 33% acetic acid. The optical density (OD) of each well was measured at 590 nm using a microtiter plate reader. As a control, uninoculated medium was used to determine background OD. The cut-off OD (ODc) was defined as three standard deviations above the mean OD of the negative control. In term of biofilm production, considering the results of microtiter plate test, the isolates were classified into four following categories based on the optical density: non-biofilm (OD test < ODc), weak biofilm (ODc < OD < 2X ODc), moderate biofilm (2X ODc < OD < 4X ODc), and strong biofilm producers (4X ODc < OD).

2.3. Detection of the pvl gene and the adhesion- or biofilm formation-encoding genes

Gene encoding the PVL (*pvl*) was targeted by PCR using specific primers (*pvl*-F, 5′-ATC ATT AGG TAA AAT GTC TGG ACA TGA TCCA -3′; *pvl*-R, 5′- GCA TCA AST GTA TTG GAT AGC AAA AGC-3. The primers were used to detect *ebps*, *eno, can*, *fnbA*, *fnbB*, *fib, icaA* and *icaD* genes; conditions of polymerase chain reaction (PCR); and size of the amplified products were as our previous report [10].

2.4. Statistical analyses

Statistical comparisons were carried out by SPSS (Version 14.SPSS Inc, USA). The correlation between possession of the adhesion- or biofilm formation-encoding genes and the biofilm phenotypes was made using Fisher's exact tests. *P-value* less than 0.05 was considered statistically significant.

3. Results

The prevalence of biofilm capacity, biofilm associate genes and gene encoding PVL among *S. aureus* isolates are shown in Table 1. Quantitative biofilm determination using the microtiter assay revealed that all strains (100%) produced biofilm. Of isolates, 7 (41.2%) produced strong biofilm whereas 7 (41.2%) produced week and 3 (17.6%) produced medium biofilm. Regarding the adhesion- or biofilm formation-encoding genes, 16 (94.1%) isolates were positive for the *eno* gene, 13(76.4%) for *icaA*, 13 (76.4%) for *icaD*, 10 (58.8%) for *fib*, 10 (58.8%) for *fnb*B, 4(23.5%) for *can*, and 1(5.8%) for *fnbA*. The most isolates (70.5%) with different biofilm phenotypes carried both *icaA* and *icaD*. Coexistence of the adhesion- or biofilm formation-encoding genes was found among 14 (82.3%) isolates. There was no correlation in the distribution of biofilm density and the adhesion- or biofilm formation-encoding genes. The *pvl* gene was only detected in 1 (5.8%) isolates.

4. Discussion

In the present study all of isolated *S. aureus* from adenoid of children with adenoid hypertrophy produced biofilm. Since bacteria living in biofilms are resistant to antimicrobials and also to immune system clearance [21–23], thereby biofilms significantly affect medical treatment and infection control. Biofilm formation includes two critical steps: the attachment and accumulation. An important group of *S. aureus* virulence factors that initiate attachment and colonization known as the staphylococcal microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) such as FnbA, FnbB, Cna, Fib, laminin binding protein (Eno), elastin binding protein (EbpS), and biofilm associated protein (Bap) protein that encoded by different genes [10,24]. In the current study, as in many reports [10,23,24], the *eno* was the most prevalent MSCRAMMs genes flowed by *fib* and *fnb*B, encountered in more than 91%, 58.8% and 58.8% of all *S. aureus* isolates respectively. However, the prevalence of the adhesion encoding genes vary greatly among *S. aureus* strains isolated from different clinical samples or sources [25–28] which may reflect differences in type of infection, tissue tropism, origin of the isolates and other factors. About 70.5% of the isolates were positive for both *icaA* and *icaD*. Numerous reports have demonstrated these genes among *S. aureus* isolates [14,24–28]. The coexistence of genes encoding biofilms and adhesins may was useful in the understanding of the pathogenicity of upper airway illnesses caused by *S. aureus*. We found association between adhesion- or biofilm formation-encoding genes among *S. aureus* isolates, as did Atshan et al. [24], Salaberry et al. [25] and Seo et al. [27]. However, in the present study there