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Comparison of virulence factors and biofilm formation among *Staphylococcus aureus* strains isolated from human and bovine infections

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ABSTRACT

The aim of this study was to find different prevalence of genes involved in the biofilm formation process and to assess the phenotypic and genotypic markers of biofilm formation among *Staphylococcus aureus* strains isolated from human and bovine infections. In this study, 215 *S. aureus* strains were collected from human and dairy cow's infections. The biofilm forming capacity of the strains was evaluated using a colorimetric microtiter plate assay. The genes encoding microbial surface components, recognizing adhesive matrix molecules (MSCRAMMs) (*ebpS*, *eno*, *fib*, *fnb*A, *fnb*B, *cna* and *bap*), and the intracellular adhesion (*ica*) genes (*icaA*, and *icaD*) were targeted by polymerase chain reaction (PCR)-based method.

Approximately 70% of the isolates produced biofilm. Among these, 59.3% were producers of weakly adherent biofilms while 34.8% and 5.8% produced moderate and strong biofilms, respectively. The most prevalent gene was *icaD* found in 88.4% of the isolates, followed by *icaA*, *fib* and *eno* found in 87.9%, 75.8% and 75.3% of the isolates, respectively. The *bap* gene was not detected in any of the isolates. The prevalence of *ebpS* and *fnb*A genes among bovine isolates were significantly higher than those in human isolates, whilst the prevalence of *cna* gene was significantly higher in the human isolates.

In this study, a high prevalence of biofilm production was found among *S. aureus* strains isolated from human and bovine infections. Most biofilm producing isolates were positive for MSCRAMM, *icaA*, and *icaD* genes.

1. Introduction

Biofilm is a community of microorganisms adhering to each other on a surface. These adherent cells are surrounded by a self-produced matrix of extracellular polymers [1,2]. Biofilm formation is considered to be a two-step process in which the bacteria first adhere to the surface by adhesin factors, followed by growth, multiplication and cell aggregation to form multilayered cell clusters encased within a slimy matrix [3,4]. The importance of biofilm is well recognized in medical and veterinary contexts. Bacteria in biofilm display elevated resistance to antibiotics and disinfectants [5,6]. Biofilm also contributes to the evasion of immunological defenses as well as to the difficulty of pathogen eradication, often resulting in persistent infections [6].

Staphylococcus aureus is an important etiologic agent causing infections in human and animals [7,8]. Most studies, concerning biofilm formation in animals, have focused on *S. aureus* strains isolated from bovine mastitis. Biofilm formation is believed to play an important role in the pathogenesis of staphylococcal infections [9]. The adherence stage of *S. aureus* is mediated by a protein family of staphylococcal microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) such as fibronectin binding proteins (FnbA and FnbB), collagen binding protein (Cna), laminin binding protein (Eno), elastin binding protein (EbpS), fibrinogen binding protein (Fib) and biofilm associated protein (Bap) [4,10,11], while the aggregation stage is conducted, under certain conditions,

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by the synthesis of polysaccharide intercellular adhesin (PIA) molecule [4,12]. It has been found that the intracellular adhesion (*ica*) operon is essential for the control of biofilm production [3,13]. The *ica* locus, consisting of the gene *ica*ADBC, encodes the proteins mediating the synthesis of polysaccharide intercellular adhesin (PIA) molecule [3,14,15]. The aim of this study was to find different prevalence of genes involved in the biofilm formation process and to assess the phenotypic and genotypic markers of biofilm formation among *S. aureus* strains isolated from human and bovine infections.

2. Material and methods

2.1. Bacterial strains

A total of 215 *S. aureus* strains were isolated from human and dairy cows, in the same geographical area, between April 2009 and September 2010. Human strains were isolated from wound infections in two different hospitals in Tehran, and animal samples were isolated from dairy cows with subclinical mastitis infections from 5 different farms in Tehran. Subclinical mastitis was diagnosed by California mastitis Test (CMT). The isolates were identified to the species level using standard biochemical methods including Gram stain, catalase test, tube coagulase, DNase, and mannitol fermentation [16]. To confirm the identity of the isolate as *S. aureus*, the *nuc*A gene was amplified by a PCR-based method, using primers *nucA*-F 5′-CTGGCATATGTATGGCAATTGTT-3′ and *nucA*-R 5′- TAT TGA CCT GAA TCAGCG TTG TCT-3′ [17].

2.2. Quantification of biofilm production

The biofilm forming capacity of the strains was assessed using a colorimetric microtiter plate assay described by Peeters et al. [18]. Briefly, *S. aureus* isolates were grown on Trypticase Soy Broth (TSB) overnight at 37 °C (Merck, Darmstadt, Germany) and the cultures were then diluted 1:100 in TSB medium. Subsequently, 150 μl of this cell suspension was used to inoculate sterile flat-bottomed 96-well polystyrene microtiter plates. After 24 h incubation at 37 °C, without shaking, wells were gently washed three times with 200 μl of phosphate buffered saline (PBS; Sigma–Aldrich, USA) and dried in an inverted position. For fixation of biofilms, 100 μl of 99% methanol was added and after 15 min, supernatants were removed and the plate was air-dried. In the next step, 100 μl of crystal violet (CV; HiMedia, India) 1% was added to all the wells. The excess CV was removed, after 20 min, by washing the plate under running tap water. The bound CV was, finally, released by adding 150 μ of 33% acetic acid. The optical density (OD) of each well was measured at 590 nm using a microtiter plate reader. All the tests were repeated three times.

The uninoculated medium was used, as control, to determine the background OD. The cut-off OD (ODc) was defined as three standard deviations above the mean OD of the negative control and the final OD value, of a tested strain, was defined as the average OD of the strain reduced by the ODc value. The adherence ability of the tested strain was classified into four categories based on the OD [19]: non-adherent (OD < ODc), weakly adherent (ODc < OD < 2XODc), moderately adherent (2XODc < OD < 4XODc), and strongly adherent (4XODc < OD).

2.3. Detection of MSCRAMM and ica genes

Genomic DNA was extracted as described by Fatholahzadeh et al. [20]. The primers used to detect the genes icaA, icaD, ebpS, eno, fib, fnbA, fnbB, cna and bap, conditions of PCR and the size of the amplified products were as described by Vancraeynest et al. [21]. Amplified products were analyzed by electrophoresis on 1% agarose gel in 0.5× TBE (Tris base, boric acid and EDTA) buffer at 110 V.

2.4. Statistical analysis

SAS software, 2001, was used for statistical analysis. Differences in the prevalence of genes between *S. aureus* strains isolated from human and bovine infections were calculated using the chi-square test for each gene. A P value of ≤ 0.05 was considered as statistically significant. Nonparametric Kruskal–Wallis test was used to compare the significant difference in the prevalence of MSCRAMM and *ica* genes between biofilm producing groups and non biofilm producing groups.

3. Results

Out of the 215 *S. aureus* strains, 123 (57.2%) were isolated from human infections and 92 (47.8%) from bovine mastitis.

3.1. Detection of MSCRAMM and ica genes

The prevalence rate of MSCRAMM and *ica* genes are summarized in Table 1. The most prevalent gene was *icaD* found in 88.4% of the isolates, followed by *icaA*, *fib* and *eno* found in 87.9%, 75.8% and 75.3% of the isolates, respectively. The *bap* gene was not detected in any of the isolates. The prevalence of *ebpS* and *fnb*A genes among the bovine isolates were significantly higher than those in the human isolates ($P < 0.0001$), whilst the prevalence of the *cna* gene was significantly higher in the human isolates ($P < 0.0001$).

3.2. Biofilm formation

Approximately 70% (155/215) of the isolates produced biofilm. Among these, 59.3% (92/155) were weakly adherent biofilm producers, while 34.8% (54/155) and 5.8% (9/155) produced moderate and strong biofilms, respectively. Also *58.4% (73/125) of S. aureus* strains isolated from human and 91.11% (82/90) *S. aureus* strains isolated from bovine infections produced biofilm and there is a significant differences between biofilm producers in two *S. aureus* groups ($P < 0.0001$).

Out of the 155 biofilm producing isolates, 5.2% (6/115), which were all weakly and moderately adherent biofilm producers, were negative for both *iac*A and *ica*D genes while 2.6% (3/115) were positive only for the *ica*A gene. Among the 60 non biofilm producing isolates, 70% (42/60) were positive for both *iac*A and *ica*D genes but negative for most MSCRAMM genes.

Statistical analysis, using the Kruskal–Wallis test, showed a significantly different prevalence rate of MSCRAMM and *ica* genes between the isolates with no biofilms (median = 3) and those with weak, moderate and strong biofilms (median = 6). However, no difference was seen in the prevalence rate of these genes among different groups.

3.3. Comparison of biofilm production between humans and animals

No significant difference was found in the prevalence of *eno*, *fnbB*, *bap*, *fib*, *icaA* and *icaB* genes among *S. aureus* strains isolated from humans and dairy cow infections. A comparison between the biofilm producing and non-biofilm producing isolates showed that a high significant difference was present in the prevalence of the MSCRAMM and *ica* genes, but not the *bap* and *can* genes, among these 2 groups (Table 2).

In the isolates obtained from humans, there is a systematic trend