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Research Article

Molecular Study of Intracellular Adhesion Genes (ica) and Fibronectin Binding Protein Genes (FnB) in Clinical Isolates of *Staphylococcus aureus* Isolated from Patients Under Chemotherapy

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Abstract

Background and objectives: *Staphylococcus aureus* (*S. aureus*) is common hospital acquired pathogen. Biofilm formation capacity of *S. aureus* increases its pathogenicity. Biofilm formation is controlled by several factors among which are intracellular adhesion genes (ica) and fibronectin binding protein Genes (FnB). The aim of the present study is to evaluate the genotypic and phenotypic capacity of clinically isolated *S. aureus* strains for formation of biofilms.

Materials and methods: The study was conducted on 182 consecutive *S. aureus* isolates obtained from patients with hospital acquired infections. The isolates were subjected to antibiotics study by disc diffusion method, biofilm detection by microtitre plate method. Molecular studies for icaR, icaA, icaB, icaD and FnbA and FnbB was carried out by polymerase chain reaction.

Results: The frequency of biofilm formation among *S. aureus* was 35.5%. The most frequent detected genes among *S. aureus* were icaR (68.2%), icaC (63.6%), ica D (60.6%), fibA 56.1%, fib (53.0%, icaB, 51.5% and icaA 30.3%. Twenty one isolates of *S. aureus* had no detected genes of the studied seven genes.

Conclusion: The present study reveals that biofilm formation was common our clinical isolates of *S. aureus*. The prevalence of icaABCDR genes and fib A, B were common among these isolates with predominance of icaR gene. Biofilm formation was associated with significant antibiotics resistance. Longitudinal studies are required to evaluate biofilm formation and its genetic mechanisms.

Keywords: *S. aureus*; Biofilm; ica genes; FnB genes

Introduction

Staphylococcus aureus (*S. aureus)* is a common gram positive cocci that lead to serious hospital acquired infections. The invading *S. aureus* is either removed by host innate immune responses or resists immune eliminations by several mechanisms [1]. Among factors associated with pathogenesis is the adhesion to host extracellular matrix proteins and formation of a biofilm [2].

S. aureus virulent strains associated with hospital acquired infections with increase resistance to antibiotics and biofilm formation has increasing incidence due the increase in multiple invasive medical devices. The increase rates with infections with these strains are associated with prolonged hospital stay, increase in the medical services costs, development of chronic infections and mortality up to 25% as reported in US [3-5].

A biofilm is a multiple bacterial cells that are attached to each other, to foreign body as medical devices or a substratum and embedded in a matrix of extracellular polymeric substance. Biofilm has a specific culture, gene expression and protein production [6].

Pathogenesis of *S. aureus* is related to many factors such as antibiotics resistance and biofilm formation. The association of biofilm formation and antibiotics resistance were previously described [7-11].

S. aureus production of biofilm is multilayer embedded in a glycoclyx or slime layer. Glycolysis formed essentially from teichoic acids (80%) and staphylococcal cells and host proteins. Moreover, there is specific polysaccharide antigen named Polysaccharide Intercellular Antigen (PIA) was isolated. PIA is composed of b1, 6 linked N-acetyl glucosamine residues (80-85%) and an anionic fraction with a lower content of non N acetylated D glucosaminyl residues that contains phosphate and ester linked succinate (15-20%) [2].

PIA is produced is produced through the control of intercellular adhesion (ica) locus. The ica locus components are icaR (regulatory) and ica ADBC (biosynthetic) genes. This locus is associated with tendency for biofilm formation, virulence of *S. aureus* and anaerobic growth which are ultimate conditions for biofilm formation [12,13]. Other protein that can be associated with *S. aureus* biofilm formation is a fibronectin binding proteins (FnBPs) that attributes to biofilm formation through an essential role by the major autolysin and sig B regulation [14]. Fibronectin binding proteins (FnBPs) are the products of two genes fibronectin A (FnbA) and fibronectin B (fnbB).

The aim of the present study is to evaluate the genotypic and phenotypic capacity of clinically isolated S. *aureus* strains for formation of biofilms. Genes studied in association with biofilm were icaR, icaA, icaB, icaD and FnbA and FnbB.

Materials and Methods

The study was conducted at Mansoura University Hospital, Egypt. *S. aureus* isolated from different clinical samples from patients with different types of cancer under chemotherapy from March 2015 till March 2017. The study was approved by Mansoura Faculty of medicine ethical committee and written consent was obtained from each participating. Primary Isolates were sub cultured on blood agar (oxoid) at 37°C for 48 hours. Colonies were identified by gram stain, coagulase test and catalase test. Further identification was performed by Micro scan system.

Antibiotics susceptibility was determined by disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) [15]. Isolated strains were subculture on Muller-Hinton agar and the discs used were imipenem (10 µg/disk), amikacin (30 µg), amoxicillin/clavulinic acid (30 µg), ampicillin (10 µg), ciprofloxacin (10 µg), cefotaxime (30 µg), ceftazidime (10 µg), cefoxitin (10 µg), trimethoprim/sulfamethaxone (25 µg) erythromycin (15 µg) (Oxoid-

Thermo Fisher Scientific- Thermo Fisher Scientific 168 Third Avenue, Waltham, MA USA 02451).

Microtiter plate assay for biofilm

Assessment of biofilm production was performed by the use of microtitre plate method. Briefly, isolated colonies of *S. aureus* were subculture on 10 ml glucose enriched tryptic soya broth and incubated for 48 hours at 37°C. Later on, 1:100 dilution of the broth was performed and 200 microns of growth was put on well on microtitre plate. Negative control was obtained by adding 200 microns of non-culture tryptic soya broth to one well. The plate was incubated for 24 hours at 37°C. Then, washed with distilled water for three times and stained with crystal violet stain 1% for 10 minutes. Then, wash the plate for two times and add 250 microns of 95% ethanol solution in each well. Measure the Optical Density (OD) at 570 wave length. The calculation of OD was performed by subtracting of OD of negative control from OD for each strain. The OD was considered to be high positive at ≥ 0.300, intermediate between 0.200 and 0.299, and negative at ≤ 0.100 [16].

Strains of *S. aureus* that had the capacity to produce biofilm were further studied for ica genes and fibn genes by PCR.

PCR detection of *S. aureus* genes

DNA extraction: *S. aureus* was cultured on blood agar plate at 37°C overnight. Pure colonies were used for DNA extraction by the use of Qiagen extraction kit for DNA (Qiagen) according to the manufacturer's recommendations. Extracted DNA was kept frozen at -20°C till amplification procedures.

PCR amplification: Primers used for the amplifications of the studied genes were shown in Table 1. PCR amplifications were performed according to previous publication [17]. Multiplex PCR was used for amplification of fnbA and *fnb*B [18].

The PCR reactions were performed using Qiagen amplification complete kit as previously reported by Arciola et al. [17] using 50 ng DNA of *S. aureus* and 10 pmol for each primer. The PCR was performed using a DNA thermal cycler. The amplicons were stained with ethidium bromide and electrophoresed in 1.5% agarose gel at 80 V for 30 min. PCR products were visualized and the products were compared against a 100 bp DNA marker (Fermentas, Germany).

Statistical analysis

Data was analysed by SPPS24. Descriptive statistical was expressed as percentage. Comparison between percentages was performed by the use of Chi-Square test. P was considered significant at P<0.05.

Results

The study included 182 *S. aureus* strains obtained from clinical samples from Mansoura University hospital. Isolates were common from blood culture (54.3%) followed by wound swabs culture (32.2%) and urine culture (12.1%) (Figure 1).

More than half of the isolates were resistant to cefoxitin 59.3% and considered as MRSA, 65.9% to amoxicillin/clavulinic acid, 63.2% to cefotaxime, 58.2% to ceftazidime. The least resistance was for ciprofloxacin 32.9% (Figure 2).

The frequency of biofilm formation among *S. aureus* was 35.5%. The majority had high tendency to produce biofilm by *in vitro* study (54.5%), and intermediate biofilm production (48.5%) (Tables 2 and 3).

The comparison between antibiotics resistance of *S. aureus* positive for biofilm production and those strains negative for biofilm production reveals that the first strains had highly significant resistance for all used antibiotics, P=0.0001 (Table 4).

The most frequent detected genes among *S. aureus* were icaR (68.2%), icaC (63.6%), ica D (60.6%), fibA 56.1%, fib (53.0%, icaB, 51.5% and icaA 30.3%).

Twenty one isolates of *S. aureus* had no detected genes of the studied seven genes, data not shown. Figures 3 and 4 demonstrates biofilm formation by microtitre plate.

Discussion

Biofilm formation is a virulence factor associated with *S. aureus*. It is governed by several factors genetic and non-genetic. In the present study we thought to detect the prevalence of ica genes group and fibronactein genes prevalence among *S. aureus* with capacity to form biofilm.

In the present study, The most frequently detected genes among *S. aureus* were icaR (68.2%), icaC (63.6%), ica D (60.6%), fibA (56.1%), fib (53.0%), icaB (51.5%) and icaA (30.3%). Ica genes are the most

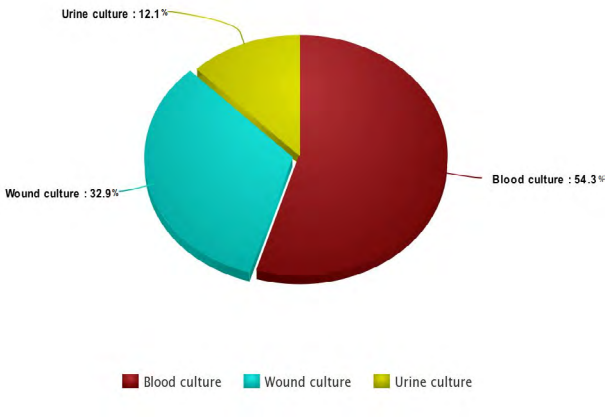

Figure 1: Distribution of isolated *Staphylococcus aureus* among different types of culture.

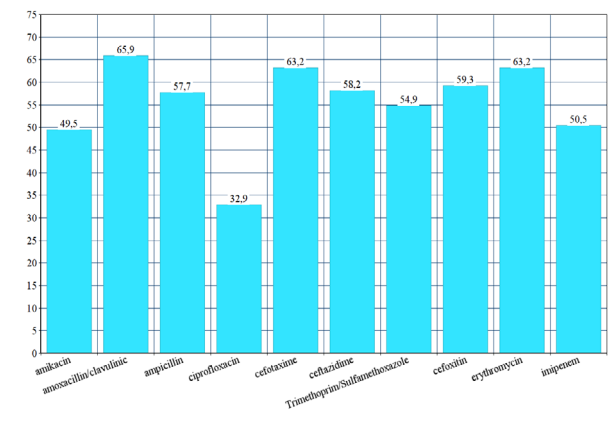

Figure 2: Antibiotics resistance among isolated *S. aureus*.

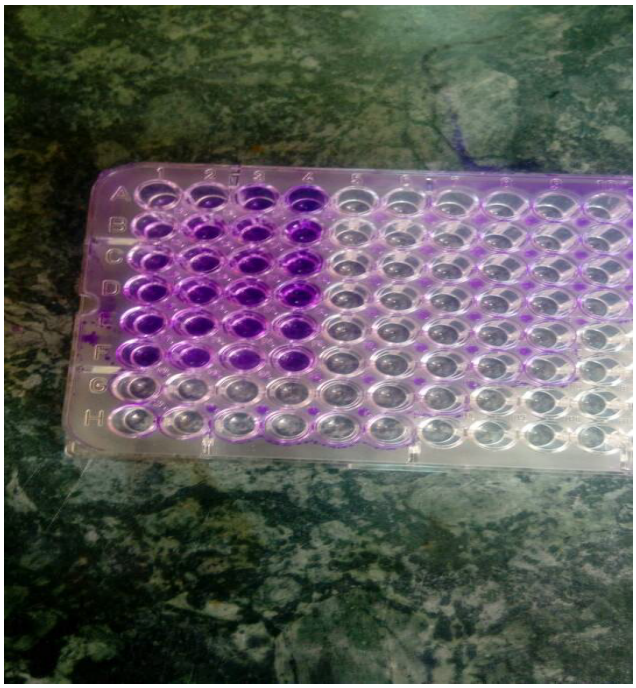

Figure 3: Biofilm formation by microtitre plate.

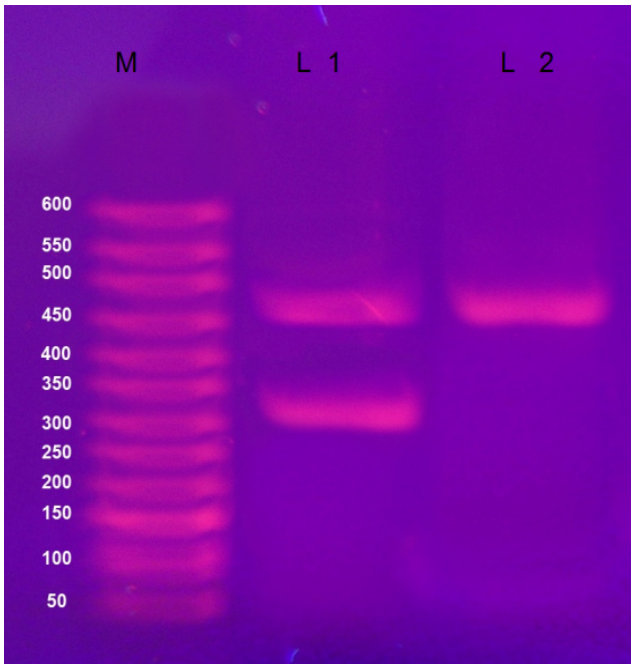

Figure 4: PCR products. M: marker; L1: Positive sample for icaR and icaB; L2: Positive sample for fbB.

Biofilm Production	No	%
Positive	66	35.50%
Negative	120	64.50%
Total	186	100%

Table 1: Frequency of biofilm formation among isolated *S. aureus*.

High	**36**	54.50%
Intermediate	**30**	48.50%
Total	**66**	100%

Table 2: Biofilm titer among *Staphylococcus aureus*.

	Biofilm Positive *S. aureus*	Biofilm Negative *S. aureus*	P
Amikacin	50 75.6%	40 34.5%	P=0.001
Amocacillin/Clavulinic	65 98.8%	55 47.4%	P=0.001
Ampicillin	65 98.8%	40 34.5%	P=0.001
ciprofloxacin	40	20	P=0.001
Cefotaxime	65 98.8%	50 43.1%	P=0.001
Ceftazidime	56 84.8%	40 43.1%	P=0.001
Trimethoprim/Sulfamethoxazole	60 90.9%	40 43.1%	P=0.001
cefoxitin	65 98.8%	43 37.1%	P=0.001
Erythromycin	65 98.8%	50 43.1%	P=0.001
Imipenem	62 93.9%	30 25.9%	P=0.001
Total	66 100%	116 100%	P=0.001

Table 3: Comparison between antibiotic resistances in *S. aureus* according to biofilm formation.

Genes	No. %
icaA	20 30.3%
icaB	34 51.5%
ica C	42 63.6%
icaD	40 60.6%
icaR	45 68.2%
fibA	37 56.1%
fib B	35 53.0%

Table 4: Frequency of genes studied among *S. aureus* positive for biofilm formation.

frequently reported genes associated with biofilm formation as reported by previous studies [19,20]. Interestingly, twenty one isolates with biofilm formation capacity had no any genes of the seven genes studied. This may be explained by the involvement of other genes or other mechanisms in formation of biofilm [21].

In contrary to previous studies [22-24], icaR was detected by higher frequency than other genes. This may be explained by the role played by icaR genes as a regulatory gene for biofilm production as mentioned previously [25,26].

In the present study, more than half of the isolates were resistant to cefoxitin 59.3% and considered as MRSA, 65.9% to amoxicillin/clavulinic acid, 63.2% to cefotaxime, and 58.2% to ceftazidime. The least resistance was for ciprofloxacin 32.9%.

Multiple antibiotics resistance are common with *S. aureus* isolated from clinical samples [27-29]. MRSA was reported as a common nosocomial pathogen [22]. Therefore, careful study of antibiotics resistance pattern is recommended for antibiotics decision in infected patients.

Interesting finding in the present study was the significant high resistance pattern (P=0.0001) among biofilm forming *S. aureus* strains compared to those with negative strains. It was reported previously that biofilm growth of *S. aureus* favour antibiotics resistance by horizontal plasmid transfer of antibiotics resistance [27].

The use of micro plate method for detection of biofilm revealed that 35.5% of isolated *S. aureus* had the capacity to produce biofilm. The majority had high tendency to produce biofilm by *in vitro* study (54.5%), and intermediate biofilm production (48.5%).

There are many laboratory techniques that can be used to measure biofilm formation such as tube method, Congo red agar method and microtitre plate [28]. Microtitre plate method is simple, accurate method and was described as a standard laboratory method for measurement of biofilm formation [29].

Biofilm formation depends upon multiple factors such as genetic and environmental. The frequency of biofilm formation among clinical *S. aureus* varies among different studies from 57.8% up to 100%, and the high tendency of biofilm formation varies from 14.4% up to 38.7% [21,30,31]. The differences in frequency of biofilm formation may be attributed to the difference in *S. aureus* clinical origin, difference in the genetic factors and environmental conditions.

The present study reveals that biofilm formation was common our clinical isolates of *S. aureus*. The prevalence of ica ABCDR genes and fibA and B were common among these isolates with predominance of icaR gene. Biofilm formation was associated with significant antibiotics resistance. Longitudinal studies are required to evaluate biofilm formation and its genetic mechanisms.

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