



Research Article

# Association between Biofilm Formation and Susceptibility to Antibiotics in *Staphylococcus Lentus* Isolated from Urinary Catheterized Patients

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## Abstract

*Staphylococcus lentus* (*S. lentus*) is a coagulase negative gram positive cocci recognized as opportunistic pathogens and rarely forming biofilm; it has many virulence factors, but recently caused nosocomial and community infections. Biofilm formation of *Staphylococcus lentus* may be associated with the ability to resist antibiotics which leads to increase in mortality rate due to the difficulty in eradicate infections. To evaluate the biofilm forming capacity of *Staphylococcus lentus* and its susceptibility to antibiotics, phenotypic and genotypic assays were used. Among 28 biofilm bacteria, *Staphylococcus lentus* was isolated and identified from urine catheterized patients who were hospitalized in different departments of four Iraqi hospitals (Al-Diwaniyah Teaching, Al-Hilla Teaching, Al Qassim and Al Hashimiyah Hospitals). *Staphylococcus lentus* was examined for detection of biofilm formation by detecting *icaA* gene, the intercellular adhesion gene which expressed adhesion factor to form biofilm in staphylococci by using polymerase chain reaction (PCR) method and tested for antimicrobial susceptibility by disc diffusion method and VITEK2 system according to guidelines of the Clinical & Laboratory Standards Institute (CLSI). Three isolates of *Staphylococcus lentus* revealed the ability to form biofilm phenotypically which contained *icaA* gene with 100% antibiotics resistance to penicillin, carbenicillin, gentamicin, tobramycin, oxacillin, vancomycin, clindamycin, ciprofloxacin, and 0% antibiotics resistance to azithromycin. *icaA* genes are present in *Staphylococcus lentus* and responsible for biofilm formation which is considered as the indicator; biofilm formation is a strong cause of multidrug resistance in bacteria.

**Keywords:** Biofilm; *Staphylococcus lentus*; *icaA* gene; Antibiotics resistance; Urinary catheterized patients

## Introduction

*Staphylococcus lentus* (*S. lentus*) is recognized as opportunistic pathogens and rarely causing infections to human [1], but recently caused nosocomial and community infections [2]. *S. lentus* is a gram positive,

coagulase-negative staphylococci (CoNS); it is mostly infectious to animal species and may colonize humans, which has been reported to have been isolated from various human clinical specimens [3]. CoNS are considered to be an uncertain pathogenic in the urinary tract [4]. However, studies suggested that significance

of some CoNS as uropathogens may have been undervalued [5]. Conversely, *S. aureus* is a main cause of urinary tract infection (UTI) among *Staphylococcus* sp. [6].

Other studies detected that coagulase-negative staphylococci was isolated from biofilms on urinary catheter [7]. Biofilm formation of *Staphylococcus* sp. could simply be defined as the ability of bacterial cells to adhere with each other and to be surrounded by exopolysaccharide matrix, teichoic acid and extracellular DNA which was regulated by *ica* ADBC gene expression [8]. The significance of biofilm for catheter-associated urinary tract infection (CAUTI) is that a urinary catheter as a foreign body, entering a normally sterile, hydrated body site and connecting it to the external world will inescapably become colonized with microorganisms [9].

Biofilm-forming pathogens respond poorly to antibiotics to develop antibiotic resistance [10]. Antimicrobial resistance (AMR) has resulted from treatment failures to increase morbidity and mortality, in addition to the high cost of health care, leading to serious world problem according to WHO [11]. Especially, the biofilm production of staphylococci and methicillin resistant strains in addition to antibiotic resistance is one of the most challenging task for physicians and microbiologists [12]. Many local studies showed a high percentage of biofilm formation among *Staphylococcus* sp. especially CoNS with the ability of multidrug resistance (MDR) [13, 14], but there have been no studies about *S. lentus* exclusively.

The aim of the current study was to evaluate the biofilm-forming capacity of *S. lentus* isolated from urinary tract of catheterized patients and its ability of antibiotic resistance in biofilm forming bacteria by using phenotypic and genotypic assays.

## Experimental

### Collection of specimens

Twenty eight biofilm bacterial isolates were isolated from urine samples collected from catheterized patients from four Iraqi hospitals (Al Diwaniya Educational, Al Hilla Educational, Al Qassim and Al Hashimiya Hospitals) during the period from February to April 2017 in sterile tubes of 10 mL and then transferred to laboratory immediately.

### Bacterial isolate

*S. lentus* was isolated from urine of hospitalized

catheterized patients which were identified by microscopical characteristics, biochemical tests and novobiocin resistance (novobiocin disc at 5 µg) [15], and then was confirmed by the automated system VITEK 2 (bioMerieux, Marcy l'Etoile, France) to achieve final diagnostics and identification of the species level. In addition, non-biofilm CoNS was used as control.

### Phenotypic detection of biofilm

The biofilm formation was detected by using two methods:

Congo red agar method (quality method) according to Freeman et al. [16]. Black crystalline colonies were considered as strong biofilm producers, dark colonies without dry crystalline colonies as moderate biofilm producers and dark pink colonies as non-biofilm producers.

Tissue culture plate method (TCP) (semi quantity method) according to Christensen et al. [17]. The mean of absorbance value from replicate wells was read; the biofilm degree was calculated according to the following equation:

Biofilm degree =

Mean OD<sub>630</sub> of tested bacteria – Mean OD<sub>630</sub> of control.

### Genotypic detection of biofilm

PCR technique was performed for biofilm formation genes (*icaA* gene) in (*Staphylococcus* sp.). The PCR primers were designed by using National Center for Biotechnology Information (NCBI) gene sequence data base and primer 3 plus design. This primer was provided by Bioneer, South Korea (Table 1).

**Table 1** PCR primers and their sequence

Primer	Sequence (5→3)	Amplicon size
icaA gene	F 5-TGGATGTTGGTGCCTGAAAC-3	77 bp
	R AGTACTTCATGCCACCTTGAG	

Note: Genbank: *icaA*: DQ836167.1; F: forward; R: reversed.

### DNA extraction

DNA extraction was done according to manufactured instructions of commercial DNA extraction kit (Presto Mini-DNA Bacteria Kit. Geneaid Biotech Ltd. USA). Then, extracted DNA was estimated by nanodrop device at 260/280 nm.

### PCR master mix preparation

PCR master mix was prepared from AccuPower®PCR-PreMix-Kit master mix reagent according to the company directions (Table 2).

The PCR mixture revealed in the table above was placed in AccuPower PCR-PreMix that contained all PCR components (Taq DNA polymerase, dNTPs, and 10 PCR buffers). Then, all the PCR tubes were transferred into vortex vibration for 3 min and transferred into thermocycler apparatus (MyGene, Bioneer, Korea).

### PCR thermocycler conditions

Conditions of the PCR thermocycler are listed in Table 3.

**Table 2** Company instructions of PCR master mix

PCR master mix	Volume
DNA template	5 µL
Forward primer (10 pmol)	1.5 µL
Reverse primer (10 pmol)	1.5 µL
PCR water	12 µL
Total volume	20 µL

**Table 3** PCR thermocycler conditions

PCR step	Temperature	Time	Repeat cycle
Initial denaturation	95 °C	5 min	1
Denaturation	95 °C	30 sec	30
Annealing	58 °C	30 sec	30
Extension	72 °C	1 min	30
Final extension	72 °C	5 min.	1
Hold	4 °C	Forever	–

### PCR product analysis

The DNA product was examined by electrophoresis apparatus in a 1% agarose substance by using buffer, then stained with ethidium bromide, and observed under the UV transilluminator.

### Antimicrobial susceptibility testing

The test was carried out by the following two methods.

#### Disc diffusion method

According to Baur et al. [18], antibiotics discs containing penicillin 10 µg, carbenicillin 100 µg, gentamicin 10 µg, tobramycin 10 µg, oxacillin 1 µg, vancomycin 10 µg, azithromycin 15 µg and

ciprofloxacin 5 µg were obtained from Bioanalysis, India.

#### Minimum inhibitory concentration (MIC)

The testing was done by VITEK 2 AST system for antibiotics including benzylpenicillin, oxacillin, gentamicin, tobramycin, levofloxacin, moxifloxacin, erythromycin, clindamycin, teicoplanin, vancomycin, tetracycline, nitrofurantoin, rifampicin, and trimethoprim/sulfamethoxazole.

The results in both methods were regarded as sensitive, intermediate or resistant based on the Clinical and Laboratory Science Institute (CLSI) standard guidelines, M100, 2017 (19).

### Statistical analysis

All experiments were carried out in triplicate to validate the reproducibility of the experiments. Statistical analysis was done by using one way ANOVA at p-value 0.05 by SPSS Statistics 24.0 software.

## Results and Discussion

Out of 28 biofilm formation bacteria isolated from urine samples of catheterized hospitalized patients, 3 (10.7%) of *S. lentus* isolates were recovered and the other 25 (89.3%) were biofilm formation gram negative bacteria. The results were higher than other studies which isolated *S. lentus* from urine of hospitalized patients at 0.9% [20] and 0.18% [5].

Because of fewer studies about *S. lentus*, it was chosen as the model of gram positive bacteria to study their ability to form biofilm at phenotypic and genotypic levels with relation to antibiotic resistance.

*Staphylococcus* sp. is the main cause of CAUTI [21]. Several investigators have reported the isolation of *S. lentus* from urine [5]. Bose et al. found that coagulase negative Staphylococci was generally the main cause of biofilm forming on indwelling medical devices [22]. This capability is due to that coagulase negative Staphylococci is capable to produce urease which increases the pH of urine and effects the formation of calcium and magnesium phosphate crystals which predispose to form biofilm [23]. Biofilm is an assemblage of microbial cells attached to a surface and encapsulated in a film or slime layer of extracellular polymers and can be formed by a wide variety of microorganisms such as both gram-positive and negative bacteria [7].

Bacterial isolates that produced biofilm on Congo red media (Fig. 1) where the black color indicated the ability of Congo red dye to stain the polysaccharide matrix which was formed during the biofilm forming process [22].

In TCP method, the bacterial isolates were shown as strong, moderate, weak or non-biofilm production (Fig. 2). The Results were interpreted according to Christensen et al. [17], as shown in Table 4.

The TCP results revealed that *S. lentus* isolates had the ability of strong and moderate degree of biofilm formation with a significant differences between each

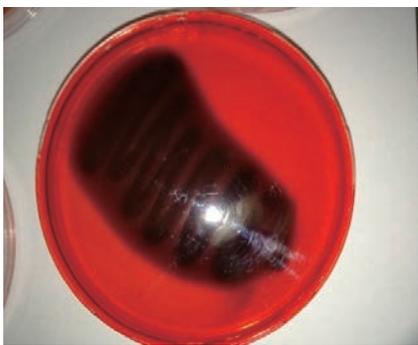


Fig. 1 Congo red agar indicating the biofilm of *S. lentus*.

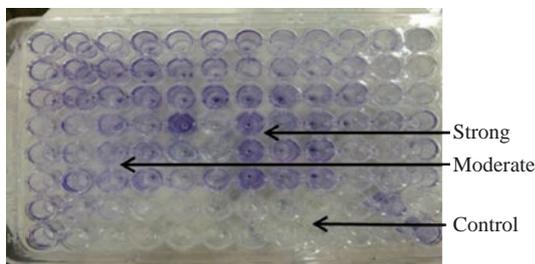


Fig. 2 TCP method indicating the biofilm degree of *S. lentus*.

Table 4 Classification of biofilm formation according to optical density (OD) values at 630 nm

Mean OD value	Adherence	Biofilm formation
< 0.120	Non	Non/weak
0.120-0.240	Moderate	Moderate
> 0.240	Strong	Strong

Table 5 Optical density value of *S. lentus* biofilm degree according to TCP method

Bacteria	Optical density (630 nm)	Biofilm degree
<i>S. lentus</i> 1	0.246 ± 0.00041 A	Strong
<i>S. lentus</i> 2	0.151 ± 0.00108 B	Moderate
<i>S. lentus</i> 3	0.125 ± 0.00204 C	Moderate

Note: Values represent mean ± S.E. Different capital letters mean significant differences ( $p \leq 0.05$ ) between different optical densities

other at  $p$ -value 0.05 (Table 5).

*Staphylococcus* sp. mostly can be introduced easily by contaminated medical advices and cause biofilm associated infections via commensal *Staphylococcus* sp. on skin and mucous surface [12] also *S. lentus*.

The biofilm formation process was controlled at the genetic level and by environmental signals regulation. The current study focused on the gene listed in Table 1 as the main regulatory gene of bacterial biofilms in the selected strain.

All 3 strains of *S. lentus* were tested for the presence of *icaA* gene and were found to be positive for it (Fig. 3), similar to the findings by Gad et al. that this gene was present in other *Staphylococcus* sp. (*S. aureus*, *S. epidermidis*) [21]. Many researches indicated that *ica* genes had an important role as the virulence factor of staphylococcal infections associated with urinary catheter [21, 24]. The *icaA* gene among *ica* genes has a significant role in biofilm formation in *Staphylococcus* sp. [25], encoding N-acetylglucosaminyltransferase which is involved in the synthesis of polysaccharide intercellular adhesion (PIA) and  $\beta$ -1-6-linked poly-N-acetylglucosamine polymer (PNAG) that lead to expressing adhesion and capsular polysaccharide phenotypic character [24, 26]. That may explain the ability of *Staphylococcus* sp. to colonize artificial materials and assist adsorption on biomaterial and solid surface [27]. Other researches showed no biofilm formation by *Staphylococcus* sp. when negative for *icaA* gene, which might be due to the lack of the entire *ica* *ADBC* operon [24, 28]. The production of biofilm have several significant advantages, including increased resistance to antibiotics and invading the

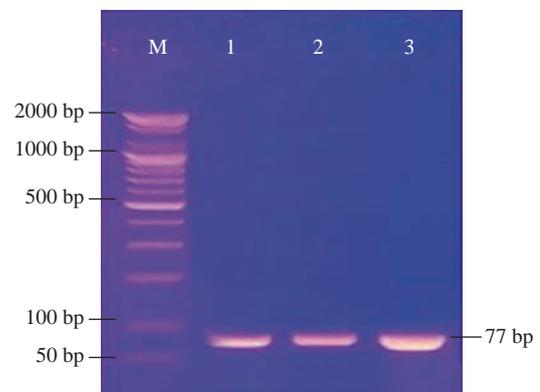


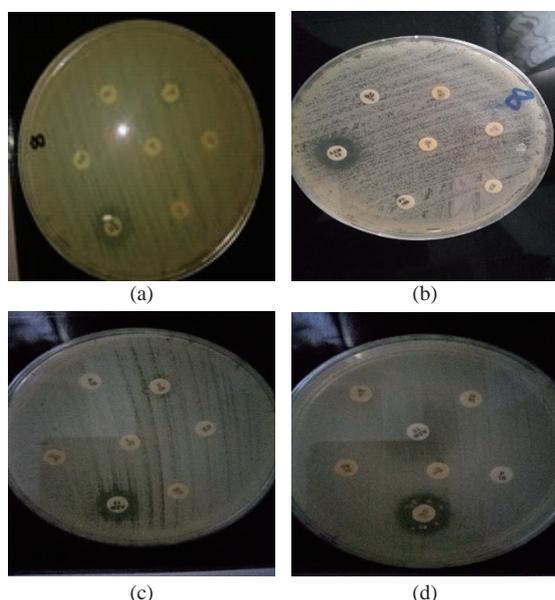
Fig. 3 Agarose gel electrophoresis of PCR assay showed the positive results of biofilm formation of *icaA* gene in *Staphylococcus* sp. positive isolates. Lane (M): DNA marker (2000-100 bp); Lanes (1-3): Positive *icaA* at 77 bp PCR product size.

immune system which provide a typical environmental for many bacteria [29].

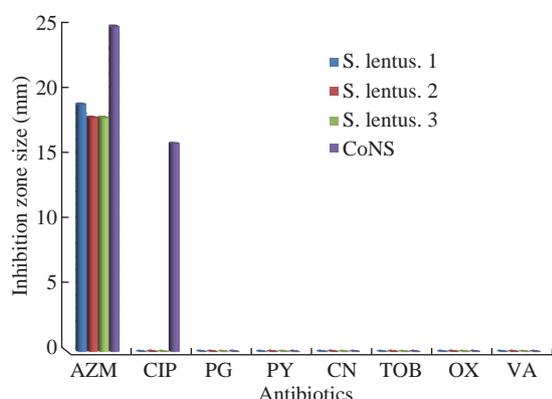
Antibiotic resistance patterns in biofilm forming pathogens were a protective mode to avoid antimicrobial action of traditional antibiotics that aid to persist infection in UTI. The current study focuses on determining and evaluating antibiotic resistance in biofilm forming urobacteria.

*S. lentus* displayed resistance to multi-antibiotics and was tested in two methods: disc diffusion (Fig. 4 and 5); MIC in VITEK2 AST (Table 7)). In disc diffusion method, the results displayed that 3 isolates of *S. lentus* were resistant to most antibiotics tested in the

experiment. *S. lentus* isolates revealed a resistance rate of 100% to each penicillin, ciprofloxacin, carbenicillin, gentamycin and tobramycin, except azithromycin which was effected on *S. lentus* at a resistance rate of 0% with mean inhibition zone of 18 mm in contrast to non-biofilm control (CoNS). *S. lentus* isolates also revealed antibiotic resistance but with high sensitivity to azithromycin at 25 mm and intermediate to ciprofloxacin at 16 mm inhibition zone (Fig. 4 and 5) with a significant difference between *S. lentus* isolates and control at p-value 0.05 (Table 6). Many studies have shown that biofilm forming bacteria are more resistant to antibiotics than planktonic bacteria [28, 30, 31]. Azithromycin is a member of the macrolide group, although there are only limited data available about anti-gram positive biofilm properties of macrolides [32], macrolide antibiotics and especially azithromycin have recently been found to have antibiofilm activity as shown by restrict bacterial quorum sensing, and inhibition of polysaccharide synthesis to prevent biofilm formation and its relation to the *ica* genes of *Staphylococcus* sp. [33].



**Fig. 4** Disc diffusion method testing of antibiotic resistance to *S. lentus* isolates (a) *S. lentus* 1, (b) *S. lentus* 2 and (c) *S. lentus* 3; (d) CoNS as control.



**Fig. 5** Zone of inhibition (mm) of different antibiotics in disc diffusion method against *S. lentus* and CoNS as control. (AZM = azithromycin; CIP = ciprofloxacin; PG = penicillin G; PY = carbenicillin; CN = gentamycin; TOB = tobramycin; OX = oxacillin; VA = vancomycin.)

**Table 6** Statistical analysis of susceptibility of *S. lentus* and control to azithromycin according to inhibition zone

Bacteria	Inhibition zone (mm)
<i>S. lentus</i> 1	18.00 ± 0.527 A
<i>S. lentus</i> 2	18.00 ± 0.020 A
<i>S. lentus</i> 3	18.00 ± 0.333 A
CoNS	23.00 ± 2.888 B

Note: Values represent mean ± S.E. Different capital letters mean significant differences ( $p \leq 0.05$ ) between different inhibition zones.

In VITEK AST results (Table 7), *S. lentus* was resistant to all antibiotics. According to CLSI guidelines, the cefoxitin screen which was tested for *Staphylococcus* isolates predicted results for *mecA*-mediated oxacillin resistance [19]. The *mecA* gene in *Staphylococcus* sp. is responsible for antibiotic resistance [34]. VITEK AST revealed that *S. lentus* was positive for the tested, that is, *S. lentus* isolates were methicillin-resistant, which was confirmed by oxacillin disc test in disc diffusion test, in addition to its resistance to vancomycin also to be methicillin resistant coagulase negative staphylococci (MRCoNS) and Vancomycin resistant coagulase staphylococci (VRCoNS). Methicillin-resistant staphylococci resisted

**Table 7** VITEK2 antibiotic sensitivity test (AST) system results

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Beta-lactamase	NEG	–	Erythromycin	≥ 8	R
Cefoxitin screen	POS	+	Clindamycin	≥ 8	R
Benzylpenicillin	≥ 0.5	R	Teicoplanin	≥ 32	R
Oxacillin	≥ 4	R	Vancomycin	≥ 32	R
Gentamicin	≥ 16	R	Tetracycline	≥ 16	R
Tobramycin	≥ 16	R	Nitrofurantain	256	R
Levofloxacin	≥ 8	R	Rifampicin	≥ 32	R
Moxifloxacin	≥ 8	R	Trimethoprim/Sulfamethoxazole	≥ 320	R
Inducible clindamycin resistance	NEG	–	–	–	–

Note: R = resistant

all b-lactam antibiotics (cephalosporins, penicillins) and aminoglycosides, which has been considered as multi-drug resistance (MRD) [19, 35].

In addition to the reasons above, many factors lie beneath the ability of bacteria to resist antibiotics, one of which is the biofilm. In this study, the biofilm-forming bacteria displayed significantly high antibiotic resistance, which was correspondent to other studies [30, 31]. In the biofilm state, the antibiotics were more difficult to diffuse into the bacteria, and the compounds of the matrix bound to the antibiotics also increased the difficulty [36]. In addition to their grade of nutrients and oxygen viability, different metabolic states also depend on their depth inside the biofilm layers which affect bacterial susceptibility to antibiotics [30].

## Conclusions

From this study, it was found that the *icaA* genes present in *S. lentus* were responsible for biofilm formation as the cause genes and could be considered as the indicator of biofilm. The biofilm formation was a strong cause of multi-drug resistance in bacteria.

## Conflict of Interests

The authors declare that no competing interest exists.

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