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Research Article

Biofilm Formation by *Staphylococcus aureus* and *Escherichia coli* Isolated from Patients with Suspected Nosocomial Infections and their Association with Antibiotics Resistance

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Abstract

Background: Bacteria attach to surfaces and produce polysaccharides resulting in the formation of biofilms which are involved in a wide range of human nosocomial infections such as urinary tract infections (UTIs) and surgical sites infections (SSIs). *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) are the most common bacteria causing nosocomial infections leading to serious health issues. Bacterial biofilms are considered to be highly resistant to antimicrobial agents.

Objectives: This study was aimed to performing *in vitro* detection of biofilm formation of *S. aureus* and *E. coli* strains isolated from patients with suspected nosocomial UTIs and SSIs and determine their susceptibility patterns to antibiotics in Mukalla city, Hadhramaut, Yemen during the period from December 2018 to May 2019.

Methodology: A total of 60 clinical isolates of *S. aureus* and *E. coli* were isolated and identified by standard bacteriological procedures, then subjected to biofilm detection by tissue culture plate (TCP) method. The antibiotics susceptibility test was performed by Kirby-Bauer disc diffusion method. Chi-square test was used to assess the statistical significance of observed antibiotic sensitivity. A value of $P < 0.05$ was taken as significant.

Results: Of the total clinical isolates of *S. aureus* and *E. coli*, TCP method detected 33(55%) as strong, 15 (25%) as moderate and 12 (25%) as weak/non-biofilm producers. Biofilm forming *S. aureus* developed significantly higher degree of resistance towards antibiotics drugs for amoxiclav 100%, ceftazidime 95.8%, cefotaxime 62.5%, cefadroxil 45.8%, ciprofloxacin 41.7% and ceftriaxone 25% with statistically significant correlation of amoxycillin/clavulanic acid and ceftazidime resistance and bacterial biofilm production (*P-value* < 0.05). The rate of antibiotic resistance biofilm forming *E. coli* were 100% for amoxiclav, cefadroxil 91.7%, cefotaxime 75%, ceftazidime 70.8%, ceftriaxone 66.7%, ciprofloxacin 62.5% and co-trimoxazole 33.3% with statistically significant correlation of cefadroxil resistance and bacterial biofilm production (*P-value* < 0.05).

Conclusion: TCP method showed that *S. aureus* and *E. coli* isolated from suspected nosocomial UTIs and SSIs have high degree of biofilm forming ability. A high antibiotics resistance and multi-drug resistance was observed in biofilm producers than non-biofilm producers.

Recommendation: Of recommended antibiotics therapies for UTIs and SSIs, amoxycillin/clavulanic acid, cefadroxil, cefotaxime and ceftazidime were the least active antibiotics, whereas co-trimoxazole and amikacin were found as the most effectual for *S. aureus* and *E. coli* biofilm producer.

Keywords: *Biofilm; Escherichia coli; Multi-Drug Resistance; Staphylococcus aureus; Tissue Culture Plate*

Abbreviations

CLSI: Clinical Laboratory Standard Institute; *E. coli; Escherichia coli:* EMB; Eosin Methylene Blue: MDR: Multi-Drug Resistance; OD: Optical Density; *S. aureus; Staphylococcus aureus*: SSIs: Surgical Sites Infections; TCP: Tissue Culture Plate; UPEC: Uropathogenic *E. coli*: UTIs: Urinary Tract Infections

Introduction

Bacterial biofilm defined as an organized bacterial community embedded in an extracellular polymeric matrix attached to biotic or abiotic surfaces [1], the extracellular polymeric matrix that can restrict the diffusion of substances and binding to antibiotics and this will provide effective resistance for biofilm cells [2]. Bacterial biofilms are usually pathogenic and cause nosocomial infections. Among all microbial and chronic infections, about 65% are associated with biofilm formation [3]. Biofilm protects the microorganism from host defenses and impedes delivery of antibiotics which may contribute to impairment in wound healing [4].

Escherichia coli (*E. coli*) is the most common etiological agent causing both community and hospital-acquired urinary tract infections (UTIs), leading to serious secondary health issues worldwide and tends to form microcolonies in mucosa lining of urinary bladder known as biofilm [5]. These biofilms make the bacterium to resist the host immune response, more virulent and lead to the evolution of antibiotics resistance by enclosing them in an extracellular biochemical matrix [6]. Currently, recurrent UTI is a serious health problem may be due to bacterial virulence factors exhibited by uropathogenic *E. coli* (UPEC) which enable colonization of the bacteria and help the bacterium overcome host defenses and invade the urinary tract [7].

Staphylococcus aureus (*S. aureus*) is one of the most virulent bacterial pathogens cause nosocomial and community acquired infections [8]. The ability of *S. aureus* to form biofilm is considered to be a major virulence factor influencing its survival and persistence in both the environment and the host [9]. Biofilm forming multi-drug resistance (MDR) *S. aureus* are major reservoirs for transmission of infections. The ability of bacteria to aggregate and form biofilm is strictly related to the capacity of producing an extracellular mucoid substance such as the slime whose main component of polysaccharide nature and consists of glycosaminoglycans [10]. *S. aureus* biofilms have been associated with a variety of persistent infections which respond poorly to conventional antibiotic therapy [11]. Biofilm formations also help in the spread of antibiotic resistant traits in nosocomial pathogens by increasing mutation rates and by the exchange of genes which are responsible for antibiotic resistance. So, biofilm-producing *S. aureus* is known to be more difficult to control, having greater resistance to antibacterial agents than *S. aureus* not embedded in biofilm [12]. Detachment of matured biofilm of *S. aureus* is a prerequisite for the dissemination of wound infection [4,13].

Biofilm-producing bacteria, which colonize surgical wounds and the urinary tract show higher resistance to standard antibiotics used for the treatment of surgical sites infections (SSIs) and UTIs. This leads to the development of recurrent infections in the affected population. Most studies conducted previously focus on either biofilm production by a single microbe causing SSIs and UTIs or biofilm formation in hospitalized patients.

Aim of the Study

This study was aimed to performing *in vitro* detection of biofilm formation of *S. aureus* and *E. coli* strains isolated from patients with suspected nosocomial SSIs and UTIs and determine their susceptibility patterns to antibiotics in Mukalla city, Hadhramaut, Yemen.

Materials and Methods

Study design

This retrospective study was carried out in the National Center for Health Laboratories (Mukalla, Hadhramout) between December 2018 and May 2019. Data were collected and the identification of bacterial strains provided by the Ibn Sina Authority Teaching Hospital and the University Hospital for GYNOBST and Pediatrics at Mukalla city Hadhramout, Yemen.

Bacteriological testing

Samples of wound swabs and midstream urine were collected from surgical sites infections and urinary tract infections in strict aseptic conditions, and then cultured into blood agar, MacConkey agar and Eosine methylene blue (EMB) agar media. The inoculated media were incubated aerobically at 37°C for 24 hours, then the plates examined for bacterial growth [14].

Bacteria identification

S. aureus and *E. coli* isolates were identified according to the conventional methods for the identification of bacterial culture growth, all bacterial isolates were identified by different diagnostic phenotypic culture characteristics, Gram staining, and biochemical testing methods [15].

Antibiotics susceptibility testing

The disc diffusion (Kirby-Bauer method) of antibiotics susceptibility procedures has been performed for bacterial isolates according to the guidelines of the Clinical Laboratory Standard Institute for 8 commonly used antibiotics [16].

Biofilm formation by tissue culture plate (TCP) method

This quantitative TCP method was performed as described by Yadav., *et al* [17]. Briefly, cultures of the isolates from fresh nutrient agar were inoculated in 10mL of trypticase soy broth with 1% glucose. After incubated for 24 hours at 37ºC, the cultures were diluted 1:100 with fresh medium. Individual wells of sterile 96 polystyrene microtiter plates were filled with 0.2 ml aliquots of the diluted cultures. Negative control wells were maintained by adding broth without culture. After incubation for 24 hours at 37ºC, the wells were removed by gentle tapping and washed with 0.2 mL phosphate buffer saline (pH 7.3) three times to remove free floating planktonic bacteria. Then the wells were dried for 1 hour and stained with crystal violet (0.1% w/v) and the excess stains were removed using deionized water and the plates were kept for drying. Quantitative analysis of biofilm production was performed by adding 150 µl of 95% ethanol to destain each well. After 30 min, optical density (OD) of stained adherent biofilm was obtained by using microtiter plate ELISA reader at wavelength 630 nm. The experiment was performed in triplicate and repeated three times. Optical density cut-off value (ODc) calculated as average OD of negative control + 3x standard deviation (SD) of negative control. The bacterial species tested were classified into four categories as follows: OD ≤ ODc no biofilm producer; ODc < OD ≤ 2 x ODc weak biofilm producer; 2 x ODc < OD ≤ 4 x ODc moderate biofilm producer; 4 x ODc < OD strong biofilm producer.

Statistical analysis

Data statistical analysis were conducted using the software of Statistical Package for Social Sciences SPSS version 25 (IBM SPSS Statistics for Windows, IBM Corp., Released 2015, Armonk, NY, USA). A Chi-square test was used to study distribution and changes in antibiotics resistance patterns. Statistical significance was determined at P-value < 0.05.

Results

Clinical isolates of bacteria

In this study, 60 clinical isolates of *S. aureus* and *E. coli* were isolated from suspected nosocomial UTIs and SSIs.

Biofilm detection by tissue culture plate (TCP) method

Tissue culture plate method detected biofilm formation of *S. aureus* and *E. coli* isolates in 33 (55%) as strong, 15 (25%) as moderate and 12 (25%) as weak/non-biofilm producers. Among *S. aureus* isolates, 18/30 were strong biofilm producers, 6/30 isolates were moderate biofilm producers and 6/30 isolates were weak/non-biofilm producers. Of *E. coli* isolates showed 15/30 were strong biofilm produc-

ers, 9/30 isolates were moderate biofilm producers, and weak/non-biofilm producers isolates identified in 6/30 isolates. There was no significant statistical difference of TCP method for screening biofilm production (*P-value* = 1.000) as shown in table 1.

Biofilm formation by TCP method		χ^2 test value	*P-value*
Result	No. (%)		
Strong	33 (55)	0.00	1.000
Moderate	15 (25)		
Weak/None	12 (20)		
Total	60 (20)		

Table 1: *Screening of S. aureus and E. coli for biofilm formation by tissue culture plate (TCP) method.*

Relationship of antibiotics resistance pattern with biofilm and non-biofilm producing *S. aureus* and *E. coli*

Among the 60 *S. aureus* and *E. coli* isolates, biofilm producers isolates using TCP method showed high resistance rates to antibiotics used compared to non-biofilm producers isolates as show in table 2 and 3. *S. aureus* biofilm producing isolates were found highly resistant to amoxycillin/clavulanic acid, ceftazidime, cefotaxime, cefadroxil, ciprofloxacin and ceftriaxone in a rate of 100%, 95.8%, 62.5%, 45.8%, 41.7% and 25% respectively. There was significant statistical correlation of antibiotic resistance of amoxycillin/clavulanic acid and ceftazidime and bacterial biofilm production (*P-value* < 0.05). Biofilm producing *E. coli* isolates had increased resistance pattern of the antibiotics amoxycillin/clavulanic acid, cefadroxil, cefotaxime, ceftazidime, ceftriaxone, ciprofloxacin and co-trimoxazole, 100%, 91.7%, 75%, 70.8%, 66.7%, 62.5% and 33.3% respectively with significant statistical correlation of antibiotic resistance of cefadroxil (*P-value* < 0.05).

Antibiotic	Biofilm producer 24/30 (80%)			Non-biofilm producer 6/30 (20%)			χ^2 test value	P-value
	S	I	R	S	I	R		
Ciprofloxacin	14	0	10	4	0	2	0.139	0.709
Co-trimoxazole	22	0	2	6	0	0	0.536	0.464
Ceftriaxone	8	10	6	3	2	1	0.590	0.745
Cefotaxime	2	7	15	2	3	1	4.766	0.092
Amoxycillin/clavulanic acid	0	0	24	1	0	5	4.138	0.042*
Amikacin	19	2	3	6	0	0	1.500	0.472
Cefadroxil	5	8	11	3	1	2	2.149	0.342
Ceftazidime	0	1	23	2	0	4	8.704	0.013*

Table 2: *Antibiotics susceptibility test results of biofilm and non-biofilm producing S. aureus.*
**P-value < 0.05 is considered statistically significant.*
Key: (S) Sensitive, (M) Intermediate sensitive, (R) Resistant.

Antibiotic	Biofilm producer 24/30 (80%)			Non-biofilm producer 6/30 (20%)			χ^2 test value	P-value
	S	I	R	S	I	R		
Ciprofloxacin	8	1	15	4	0	2	2.304	0.316
Co-trimoxazole	16	0	8	4	0	2	0.00	0.694
Ceftriaxone	6	2	16	3	1	2	2.222	0.329
Cefotaxime	5	1	18	2	1	3	1.875	0.392
Amoxycillin/clavulanic acid	0	0	24	0	0	6	-	-
Amikacin	18	4	2	4	1	1	0.379	0.827
Cefadroxil	2	0	22	4	0	2	10.208	0.007*
Ceftazidime	5	2	17	2	1	3	0.967	0.617

Table 3: *Antibiotics susceptibility test results of biofilm and non-biofilm producing E. coli.*
**P-value < 0.05 is considered statistically significant.*
Key: (S) Sensitive, (M) Intermediate sensitive, (R) Resistant.

Relationship of biofilm production *S. aureus* and *E. coli* with multi-drug resistance

Among 48 biofilm producers isolates of *S. aureus* and *E. coli*, 40/48 (83.3%) isolates were multi-drug resistant, 5/12 (41.7%) were non-producer and multi-drug resistant. Statistically, there was significant association between biofilm formation and multi-drug resistance isolates (*P-value* = 0.006) (Table 4).

Bacterial biofilm	Multi-drug resistance			χ^2 test value	P-value
	Yes	No	Total		
Producer	40 (83.3)	8 (16.7)	48 (100.0)	8.889	0.006*
Non-producer	5 (41.7)	7 (58.3)	12 (100.0)		
Total	45 (75.0)	15 (25.0)	60 (100.0)		

Table 4: *Relationship of biofilm production S. aureus and E. coli and multi-drug resistance.*

**P-value < 0.05 is considered statistically significant.*

Discussion

More than 50% of all microbial infections have now been associated with the biofilm formation and several bacterial surface structures are known to be involved in biofilm creation [18]. Also, bacterial biofilms are most of the time associated with long-term persistence of bacteria in various environmental conditions [19]. Tissue culture plate was most reliable and easy method for detection of biofilm and it can be used as a general screening method for detection of bacterial producing biofilm [20-22]. In contrast, statistical analysis of biofilm formation indicated that TCP method was the most sensitive and specific method for screening biofilm production [23].

In the present study, we processed surgical wound swabs and midstream urine samples of suspected nosocomial infections and investigated the ability of bacterial isolates to form biofilm *in vitro* by phenotypic TCP method because they can be performed in most laboratories settings. Tissue culture plate method detected biofilm formation in 80% of cases with no significant statistics among the severe, intermediate and weak biofilm producers (*P-value* = 1.000). Similar study revealed TCP method detected 81% bacterial isolates biofilm producer [24]. Another study showed that 76% were bacterial biofilm producers detected by TCP method [25]. Other study found that TCP detected 64% as bacterial biofilm producer [26]. While differences in the observations showed in other study that TCP detected 27% as bacterial biofilm producers [27]. Other study reported biofilm producer identified by TCP method 22% [28].

Bacteria in biofilm display dramatically increased resistance to antibiotics [19]. In this study, we analyzed the antibiotics resistance pattern of biofilm and non-biofilm producing of all isolates *S. aureus* and *E. coli*. Biofilm forming isolates demonstrated increased resistance to the commonly used antibiotics compared to non-biofilm producers. *S. aureus* isolates biofilm producing in our study were found to be highly resistant to amoxycillin/clavulanic acid, ceftazidime, cefotaxime, cefadroxil, ciprofloxacin and ceftriaxone in rate of 100%, 95.8%, 62.5%, 45.8%, 41.7% and 25% respectively with significant statistical correlation of antibiotic resistance of amoxycillin/clavulanic acid and ceftazidime and bacterial biofilm production (*P-value* < 0.05). This pattern of resistance coincides with the study findings which reported biofilm producing *S. aureus* highly resistant to co-trimoxazole 66.7% and ciprofloxacin 60% [4]. Manandhar., *et al*. [29] showed biofilm producing *S. aureus* resistant to ciprofloxacin and co-trimoxazole 83.3% and 28.6% respectively. Also, Neopane., *et al*. [4] reported that resistance toward erythromycin and co-trimoxazole was increased due to the excessive use of these drugs for the treatment of both minor and more serious staphylococcal infections. Other study found that the Gram-positive bacteria had high resistance to ciprofloxacin 40% and co-trimoxazole 30% [20]. Our study results revealed that biofilm producing *E. coli* isolates had increased resistance pattern of the antibiotics amoxiclav 100%, cefadroxil 91.7%, cefotaxime 75%, cetazidime 70.8%, ceftriaxone 66.7%, ciprofloxacin 62.5% and co-trimoxazole 33.3% with significant statistical correlation of antibiotic resistance of cefadroxil (*P-value* < 0.05). This pattern of resistance agreed with the study findings reported high resistant biofilm producing *E. coli* to amoxycillin/clavulanic acid, ceftriaxone, ciprofloxacin and amikacin 77.61%, 71.48%, 71.48% and 7.58% respectively [30], another study showed biofilm producing *E. coli* were

resistance to ceftaxime, ceftriaxone, and amoxycillin/clavulanic acid 65.6%, 50% and 40.6% respectively [31]. While other study showed lesser resistance of biofilm producing *E. coli* to co-trimoxazole, ciprofloxacin and ceftaxime 47.4%, 47% and 42.5% respectively [32]. Gram negative bacteria had high resistance to ciprofloxacin, co-trimoxazole, amikacin and ceftriaxone 95%, 90%, 64% and 58% respectively [20], another study found resistance of biofilm forming *E. coli* isolates to ciprofloxacin and amikacin 95% and 65% respectively [33]. The increased antibiotics resistance among bacterial biofilm producers is due to slow growth rate and the presence of the protective covering of exopolysaccharide which alters the penetration of antibiotics through the biofilm and hinders the activity of antibiotics against the bacterial cells [4,32].

Considering MDR, 83.3% of biofilm producers *S. aureus* and *E. coli* isolates were found to be resistant to more than two of the antibiotics employed in the study and statistical analysis proved a significant correlation between MDR and biofilm formation (*P-value* = 0.006), which is in accordance to the findings reported by various studies [10,28,34,35]. In the contrary, another study reported no significant association between MDR and biofilm formation [32]. The mechanism of MDR in biofilm-forming bacteria is described as a direct result of close cell to cell contact in the biofilm, which facilitates easy transfer of plasmids containing MDR genes among one another [4].

Conclusion

Tissue culture plate method showed that *S. aureus* and *E. coli* isolated from nosocomial UTIs and SSIs have high degree of biofilm forming ability. A high antibiotics resistance and multi-drug resistance were observed in biofilm producers than non-biofilm producers. Of the recommended antibiotics therapies for UTIs and SSIs, amoxycillin/clavulanic acid, cefadroxil, cefotaxime and ceftazidime were the least active, whereas co-trimoxazole and amikacin were found as the most effectual for *S. aureus* and *E. coli* biofilm producer. Therefore, detection of biofilms is recommended for all patients with chronic or recurrent nosocomial infections. Further studies are needed for the development of effective preventive and treatment strategies of biofilm associated UTIs and SSIs to avoid infection recurrence and persistence.

Ethics Approval and Consent to Participate

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Conflict of Interest Statement

The author declares no conflict of interest.

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