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RESEARCH ARTICLE

ANALYSIS OF THE ANTIBIOGRAM PROFILES OF BIOFILM FORMING *STAPHYLOCOCCUS AUREUS* AND *ESCHERICHIA COLI*

Amal Ali Bahakim[1], Eidha Ali Bin-Hameed[1,2]*
[1]*Biology Department, Faculty of Sciences, Hadhramout University, Yemen.*
[2]*Health Science Department, Faculty of Medicine and Health Science, University of Science and Technology, Hadhramout, Yemen.*

ABSTRACT

Background and Objectives: Bacteria attach to the surfaces and produce polymeric matrix resulting in the biofilms formation that are involved in a wider range of human infections. Biofilms forming that produced by *Staphylococcus aureus* and *Escherichia coli* are considered to be highly antibiotics resistant. This study was aimed to analysis the antibiogram profile of biofilm forming *S. aureus* and *E. coli* isolates of Mukalla city, Hadhramaut, Yemen.

Methods: Sixty clinical isolates of *S. aureus* and *E. coli* were isolated from different clinical samples, and identified by standard bacteriological methods, then subjected to biofilm formation 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 analyze the data and *p* value< 0.05 was taken as significant.

Results: Among the total isolates *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 of *S. aureus* developed significantly higher degrees of antibiotic resistance of amoxicillin/clavulanic acid 100%, ceftazidime 95.8%, cefotaxime 62.5%, cefadroxil 45.8%, ciprofloxacin 41.7% and ceftriaxone 25% with a significant statistics correlation the resistance of amoxicillin/clavulanic acid and ceftazidime and bacterial biofilm production (*p-value*< 0.05). The rates of antibiotics resistance biofilm *E. coli* were 100%, 91.7%, 75%, 70.8%, 66.7%, 62.5% and 33.3% for amoxicillin/clavulanic acid, cefadroxil, cefotaxime, ceftazidime, ceftriaxone, ciprofloxacin and co-trimoxazole respectively with statistically significant correlation of cefadroxil resistance (*p*-value < 0.05).

Conclusion: TCP method showed that *S. aureus* and *E. coli* isolates have a high degree of biofilm forming ability. A high antibiotics resistance found in biofilm producers isolates than non-biofilm producers.

Keywords: Biofilm formation, *Escherichia coli*, multi-drug resistance, *Staphylococcus aureus*, tissue culture plate

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Address for Correspondence:
Dr. Eidha A. Bin-Hameed, Biology Department, Faculty of Sciences, Hadhramout University, Yemen. Health Science Department, Faculty of Medicine and Health Science, University of Science and Technology, Hadhramout, Yemen.
Tel- +967 773568330; E-mail: *eidha6@gmail.com*

INTRODUCTION

Bacterial biofilm is defined as an organized bacterial community embedded in extracellular polymeric matrix attached to biotic or abiotic surfaces[1]. Bacterial biofilm is usually pathogenic and cause infection. Among microbes and chronic infections, about 65% are associated with the formation of biofilm[2], whereas biofilm protects the organism from host defenses and impedes the delivery of antibiotics which may cause impairment in the healing of wound[3].The ability of bacterial aggregation and biofilm formation is strictly related to the capacity of producing the extracellular mucoid substance such as the slime layer whose main the component of polysaccharide nature and consists of glycosaminoglycans[4]. The extracellular polymeric matrix can block the diffusion of substances and binding to the antibiotics, and this will provide the effective resistance for biofilm bacterial cells[5]. Biofilm formation also helps in the spread of antibiotic-resistant traits in bacterial pathogens by increasing the rates of mutation and by the exchange of genes that are responsible for antibiotics resistance[6].

Staphylococcus aureus (*S. aureus*) and *Escherichia coli* (*E. coli*) are considered the most common etiological agent causing both community and hospital acquired infections[7,8]. *E. coli* infections leading to serious secondary health issues worldwide and tends to form

micro colonies in mucosa lining the urinary bladder known as biofilm[8]. These biofilms make the bacterium to resist the immune response of the host, more virulent and lead to the evolution of antibiotics resistance by enclosing them in the extracellular biochemical matrix[9]. *S. aureus* is able to form biofilm and considered to be a major virulence factor influencing its survival and persistence in both the environment and the host[10]. The biofilms forming by *S. aureus* have been associated with a variety of persistent infections which respond poorly to traditional antibiotics therapy[11]. The most of previous studies in Yemen focused on the prevalence of antibiotics resistant bacteria among the clinical samples and neglected the evaluation of biofilm-producing bacteria resistant to antibiotics[12,13,14]. Only one study was conducted at Ibb city by Al-Hobiashy *et al.*,[15] they reported that 49.3% of isolated uropathogenic bacteria was biofilm producer. Therefore, this study aimed to analysis the antibiogram profile of biofilm forming *S. aureus* and *E. coli* in Mukalla city, Hadhramaut, Yemen.

SUBJECTS AND METHODS

Study design and area

This is a cross-sectional study that conducted at the National Center for Public Health Laboratories which located in Mukalla city, Hadhramaut, Yemen, during the period of December 2018 to May 2019. The patients suffered from wounds and urinary tract infections were enrolled in this study.

Sample collection and bacteriological testing

Three hundred and nine clinical samples (200 wound swabs and 109 midstream urine) were subjected to culture processing. *S. aureus* and *E. coli* were isolated and identified by the standard methods for bacterial culture growth, Gram staining and biochemical tests[16].

Antibiotics susceptibility testing

Antibiotics susceptibility testing was done using Kirby-Bauer disc diffusion method according to the guidelines of the Clinical Laboratory Standard Institute (CLSI)[17]. The antibiotics were used in this study included; Ciprofloxacin (5µg), Co-trimoxazole (25µg), Ceftriaxone (30µg), Cefotaxime (30µg), Amoxicillin/ clavulanic acid (30µg), Amikacin (30µg), Cefadroxil (30µg), and Ceftazidime (30µg).

Biofilm formation detection by tissue culture plate (TCP) method

TCP as quantitative method was performed as described by Yadav *et al.*,[18]. In briefly, subcultures of bacterial isolates on nutrient agar were inoculated in 10mL of trypticase soy broth with added 1% glucose and incubated overnight at 37ºC, then the cultures were diluted 1:100 with fresh medium. The wells of sterile 96 polystyrene microtiter plate were filled with 2mL aliquots of the diluted cultures. Negative control wells were maintained by adding broth without culture. After overnight incubation at 37ºC the wells were removed by gentle tapping and washed with 0.2mL phosphate buffer saline (pH 7.3) three times to remove free floating planktonic bacteria[18].

The wells then 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. Analysis of biofilm production was performed by adding 150µl of 95% ethanol to destain each well. After 30 min, optical density (OD) of stained adherent biofilm was obtained using a microtiter plate ELISA reader at wave length 630 nm. The experiment was done in triplicate and repeated three times. Optical density cut-off value (ODc) calculated as average OD of the negative control + 3x standard deviation (SD) of negative control. The tested bacterial species were classified into four categories: OD≤ODc no biofilm producer; ODc<OD≤ 2x ODc weak biofilm producer; 2xODc< OD≤4xODc moderate biofilm producer; 4xODc<OD strong biofilm producer.

Statistical analysis

Data analysis was conducted using the software of Statistical Package for Social Sciences (SPSS) version 25. Chi-square test was used to study the distribution and changes in antibiotics resistance patterns. Statistical significance was determined at *p*-value <0.05.

Table 1: Frequencies of bacterial growth results of clinical samples

Type of sample	No.	Culture growth results No.(%)			
		S. aureus	*E. coli*	Other isolates	No growth
Wound swabs	200	24(12.0)	8(4.0)	112(56.0)	56(28.0)
Midstream urine	109	6(5.5)	22(20.2)	68(62.4)	13(11.9)
Total	309	30(9.7)	30(9.7)	180(58.3)	69(22.3)

RESULTS

Data of samples distribution and bacterial isolates results

A total of 60(19.4%) isolates of *S. aureus* and *E. coli* were identified. Thirty isolates of *S. aureus* were isolated from wound swabs 12% and midstream urine 5.5%, while 30 isolates of *E. coli* were isolated from wound swabs 4% and midstream urine 20.2% as given in Table 1.

Biofilm detection by tissue culture plate (TCP) method

The present result revealed that TCP method was detected biofilm formation in 33(55%) of isolates as strong, 15(25%) as moderate and 12(25%) as weak/non-biofilm producers. There was no significant statistical analysis of TCP method for screening biofilm production (*p*-value=1.000) (Table 2). Among *S. aureus* isolates, 18(30%) were strong biofilm producers, 6(10%) were both moderate and weak/non-biofilm producers of *E. coli* isolates showed 15(25%) were strong biofilm producers, 9(15%) isolates were moderate biofilm producers, and weak/non-biofilm producers isolates identified in 6(10%) isolates (Table 3).

Table 2: Biofilm formation by TCP method.

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)		

Relationship the antibiogram profiles with biofilm and non-biofilm producing *S. aureus* and *E. coli*

Among 60 *S. aureus* and *E. coli* isolates, biofilm producers isolates by TCP method given high rates resistance of antibiotics used compared to non-biofilm producers isolates. *S. aureus* biofilm producing isolates showed highly resistant to amoxicillin/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 amoxicillin/clavulanic acid and ceftazidime and bacterial biofilm production (p<0.05) as show in Table 4. Biofilm producing by the isolates of *E. coli* had increased resistance profiles of the antibiotics amoxicillin/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 statistically correlation of cefadroxil resistance (p-value<0.05) as presented in Table 5.

Table 3: *S. aureus* and *E. coli* biofilm formation by TCP method.

Bacterial isolates	Producer		Non-producer
	Strong	Moderate	Weak/None
	No.(%)	No.(%)	No. (%)
S. aureus	18(30.0)	6(10.0)	6(10.0)
E. coli	15(25.0)	9(15.0)	6(10.0)
Total	33(55.0)	15(25.0)	12(20.0)

Table 4: Antibiogram profiles of biofilm and non-biofilm producing *S. Aureus.*

Antibiotic	Biofilm producer 24(80%)			Non-biofilm producer 6(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
Amoxicillin/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*

* p-value<0.05 is considered statistically significant.

DISCUSSION

In the present study, we investigated the ability of *S. aureus* and *E. coli* isolates to produce biofilm *in vitro* using phenotypic TCP method because they can be performed in most laboratories' settings. Bacterial biofilms are most of the time associated with the long-term persistence of bacterial species in various environmental conditions[19]. More than 50% of microbial infections have now been associated with biofilm formation, and several bacterial cell surface structures are known to be involved in the biofilm creation[20]. TCP was the most reliable and easy method for the detection of bacterial biofilm and it can be used as a general screening method for the detection of biofilm producing[21,22,23]. In contrast, statistical analysis of biofilm formation indicated that the TCP method was the most sensitive, specific, and accurate method for the biofilm production screening[24]. In this study, among all isolates *S. aureus* and *E. coli* TCP method detected biofilm formation 80% with no significant statistics (p-value=1.000).

Table 5: Antibiogram profiles of biofilm and non-biofilm producing *E. coli*

Antibiotic	Biofilm producer 24(80%)			Non-biofilm producer 6(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
Amoxicillin/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

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

According to these results, similar researches revealed that TCP method detected 81%of bacterial isolates were biofilm producer[25]. Another study found that TCP detected 64% as bacterial biofilm producers[26], whereas another study showed that TCP detected 27% as bacterial biofilm producers[27]. A study revealed that 76% were bacterial biofilm producers detected by TCP method[28]. Another study reported biofilm producer identified by TCP method 22%[29]. Also, several studies showed similar results for the detection of biofilm production[30,31].

Bacterial biofilm display dramatically increased resistance to antibiotics[19]. In this study, it was analyzed that the antibiotics resistance profiles of biofilm and non-biofilm producing of the isolates *S. aureus* and *E. coli*. The biofilm forming of bacterial isolates demonstrated increased resistance to the commonly used antibiotics compared to non-biofilm producers. *S. aureus* isolates biofilm producing in our study were found highly resistant to amoxicillin/clavulanic acid, ceftazidime, cefotaxime, cefadroxil, ciprofloxacin and ceftriaxone in a rate of 100%, 95.8%, 62.5%, 45.8%, 41.7% and 25% respectively with a significant statistical correlation of antibiotic resistance of amoxicillin/clavulanic acid and ceftazidime and bacterial biofilm production. These profiles of resistance coincide with the study findings reported highly resistant biofilm produced by *S. aureus* to the antibiotics co-trimoxazole 66.7% and ciprofloxacin 60%[3]. Another study showed resistance to ciprofloxacin and co-trimoxazole 83.3% and 28.6% respectively[32]. Other research reported that resistance toward erythromycin and co-trimoxazole was increased due to the extensive use of these drugs for the treatment of minor and serious staphylococcal infections[3]. Another study found that the Gram-positive bacteria had high resistance to ciprofloxacin 40% and co-trimoxazole 30%[21]. The current study results revealed that biofilm producing *E. coli* isolates had increased resistance profiles 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. This profile of resistance agreed with the study findings reported high resistant biofilm producing *E. coli* to amoxicillin/clavulanic acid, ceftriaxone, ciprofloxacin and amikacin 77.61%, 71.48%, 71.48% and 7.58% respectively[33,34,35]. However, other studies showed biofilm producing *E. coli* were resistance to ceftaxime, ceftriaxone, and amoxicillin/clavulanic acid 65.6%, 50% and 40.6% respectively[36]. While other study showed less rate resistance of biofilm producing *E. coli* to co-trimoxazole, ciprofloxacin and ceftaxime 47.4%, 47% and 42.5%respectively[37]. In the present study showed Gram negative bacteria had high resistance to antibiotics ciprofloxacin, co-trimoxazole, amikacin and ceftriaxone 95%, 90%, 64% and 58% respectively[21]. Another study found resistance of biofilm forming *E. coli* isolates to ciprofloxacin and amikacin 95% and 65% respectively[30]. The increased of resistance antibiotics among bacterial biofilm producers is due to the slow growth rate and the presence of protective covering of exopolysaccharide that alters the penetration of antibiotics through the biofilm and hinders the activity of antibiotics against the bacterial cells[3,37]. So, we believed that the variability observed in the antibiotics susceptibility patterns reflects the different protocols and panels of antibiotics being used in different hospitals and differences in the geographical locations from where these isolates have been obtained.

CONCLUSION

S. aureus and *E. coli* isolates have a high degree of biofilm forming ability detection by TCP method. Highly resistance of antibiotics was observed in the biofilm producers than non-biofilm producers. Antibiotics therapies recommended are amoxicillin/ clavulanic acid, cefadroxil, cefotaxime and ceftazidime were less active antibiotics, whereas co-trimoxazole and amikacin found as the most effective for *S. aureus* and *E. coli* biofilm producers.

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CONFLICT OF INTEREST

No conflict of interest associated with this work.

AUTHOR'S CONTRIBUTION

The manuscript was prepared, written and approved in collaboration with all authors.

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