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Phenotypic Profile And Multi-Drug Resistance Of Biofilm Producing *Staphylococcus Aureus* And *Escherichia Coli*

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Abstract: **Background:** Microbes attach to the surfaces and produce extracellular polymer matrix of biofilms are involved in a wide range of human 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 producing biofilms causing nosocomial infections and considered to be highly antibiotics resistance and multi-resistance drugs (MDR). **Objective:** To detect the phenotypic prophile of biofilm formation *S. aureus* and *E. coli* and determine the susceptibility patterns of antibiotics in Mukalla city, Hadhramaut, Yemen. **Material and methods:** Sixty clinical isolates of *S. aureus* and *E. coli* were isolated from UTIs and SSIs samples and identified by standard bacteriological procedures, then subjected to biofilm detection by tissue culture plate (TCP) method. Disc diffusion method was used to determine the antibiotics susceptibility patterns. **Findings:** TCP method detected 33(55%) strong, 15(25%) moderate and 12(20%) weak/non-biofilm producers of *S. aureus* and *E. coli*. Biofilm forming *S. aureus* showed higher degree of resistance against the antibiotics amoxiclav 100%, ceftazidime 95.8%, cefotaxime 62.5%, cefadroxil 45.8%, ciprofloxacin 41.7% and ceftriaxone 25% with statistically significant correlation of amoxyclav and ceftazidime resistance and bacterial biofilm production (*P-value* <0.05). The rate of antibiotics 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). MDR showed in *S. aureus* and *Esch. coli* isolates for more than three antibiotics belonged to three or more different classes. **Conclusion:** The study revealed that *S. aureus* and *E. coli* isolated from nosocomial UTIs and SSIs have high

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degree of biofilm forming ability by TCP method. Antibiotics resistance and MDR was observed in *S. aureus* and *E. coli* isolates of biofilm producers than non-biofilm producers.

Keywords: Biofilm, *Escherichia Coli*, Multi-Drug Resistance, *Staphylococcus Aureus*, Phenotypic Profile, Tissue Culture Plate

1. Introduction

Bacterial biofilm forming and multi-drug resistance (MDR) are major reservoirs for transmission of infections. 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 [1]. The extracellular polymeric matrix can block the diffusion of substances and binding to the antibiotics, and this will provide the effective resistance for bacterial cells forming biofilm [2]. Among microbes causing chronic infections, 65% are associated with biofilms formation [3], whereas biofilm protects the microbes from host defenses and impedes the delivery of antibiotics which may cause impairment in the healing of wounds [4]. Biofilm formation also helps in the spread of antibiotics-resistant traits in bacterial pathogens by increasing the rates of mutation and exchange of genes responsible for antibiotics resistance [5].

Staphylococcus aureus (*S. aureus*) and *Escherichia coli* (*E. coli*) are considered the most common etiological agents causing both community and hospital acquired infections [6][7]. *E. coli* infections leading to serious secondary health issues worldwide and tends to form microcolonies in mucosa lining the urinary bladder known as biofilms [7]. 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 [8]. 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 [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 tretment [11]**.** Biofilm producing *S. aureus* is known to be more difficult to control, having greater resistance to antibiotics than *S. aureus* not embedded in biofilm [12]. Detachment of matured *S. aureus* forming biofilm is a prerequisite for the dissemination of wounds infections [13][14].

In Yemen, the most previous studies focused on the prevalence of antibiotics resistant bacteria among the clinical samples. Nevertheless, the evaluation of biofilm-producing bacterial species resistant to antibiotics was neglected [15][16][17][18]. Bacteria producing biofilm which colonize the surgical wounds and the urinary tract showed higher resistance to traditional antibiotics used for the treatment of surgical sites infections (SSIs) and urinary tract infections (UTIs), and 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. This study was aimed to determine the phenotypic profile and multi-drug MDR resistance of biofilm forming *S. aureus* and *E. coli* strains isolated from nosocomial SSIs and UTIs in Mukalla city, Hadhramaut, Yemen.

2. Materials and Methods

2.1 Study design

This cross-sectional study was conducted at the National Center for Public Health Laboratories (Mukalla, Hadhramout) in a period from December 2018 to May 2019. Clinical sampling collection was provided by Ibn Sina Authority Teaching Hospital and the University Hospital for GYNOBST and Pediatrics at Mukalla city Hadhramout, Yemen.

2.2 Cultivationa of clinical samples

Samples of wound swabs and midstream urine (MSU) were collected from SSIs and UTIs in strict aseptic conditions, and cultured into blood agar (Himedia, India), MacConkey agar (Deben Diagnostics Ltd., England) and Eosine methylene blue (EMB) agar (TM media, India) media. The inoculated media were incubated aerobically at 37°C for 24 hours, then the plates were examined for bacterial growth [19].

2.3 Identification of bacterial isolates

All bacterial isolates of *S. aureus* and *E. coli* were identified by different diagnostic phenotypic culture characteristics, Gram staining, and biochemical testing methods [20].

2.4 Detection of biofilm formation by TCP method

Briefly, TCP method was done as described by Yadav et al. [21] as the following; sub-cultures of *S. aureus* and *E. coli* isolates from fresh nutrient agar were inoculated in 10mL of trypticase soy broth with 1% glucose added, then incubated for 24 hours at 37ºC. The cultures were diluted 1:100 with fresh medium, then the individual wells of sterile 96 polystyrene microtiter plates were filled with 0.2ml 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 with gentle tapping and washed with 0.2mL phosphate buffer saline (pH 7.3) three times to remove free floating planktonic bacteria. 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, then the plates were kept for drying. Quantitative analysis of biofilm production was performed by adding 150µl of 95% ethanol to destain each well. Optical density (OD) of stained adherent biofilm was obtained after 30 minutes using microtiter plate ELISA reader at wave length 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: $OD \leq ODc$ no biofilm producer; $ODc < OD \leq 2 \times ODc$ weak biofilm producer; $2 \times ODc < OD \leq 4 \times ODc$ moderate biofilm producer; $4 \times ODc < OD$ strong biofilm producer.

2.5 Antibiotics susceptibility testing

Antibiotics susceptibility testing procedures was done using the disc diffusion (Kirby-Bauer method) for bacterial isolates on Mueller Hinton agar (Deben Diagnostics Ltd., England) according to the guidelines of the Clinical Laboratory Standard Institute for 8 commonly used antibiotics [22].

2.6 Statistical analysis

The software Statistical Package for Social Sciences (SPSS) version 25 (IBM SPSS Statistics for Windows, IBM Corp., Released 2015, Armonk, NY, USA) was used for data statistical analysis. The association between categories of bacterial isolates and its biofilm formation, distribution and changes in antibiotics resistance and MDR patterns were calculated and compared using Pearson Chi-square test (χ^2). The level of statistical significance was set at *P-value* <0.05.

3. Results and Discussion

In the present study, we processed surgical wound swabs and MSU samples and screened in vitro the ability of bacterial isolates to form biofilms by phenotypic TCP method because they can be performed in most laboratories settings. A total of 60(19.4%) clinical isolates of *S. aureus* and *E. coli* were isolated from nosocomial UTIs and SSIs. Thirty isolates of *S. aureus* were isolated from wound swabs 12% and MSU 5.5%, while 30 isolates of *E. coli* were isolated from wound swabs 4% and MSU 20.2% as given in **Table 1**.

Table 1: Frequencies of *S. aureus* and *E. coli* isolated from clinical samples

Type of clinical sample	No.	Bacterial isolates No.(%)	
		S. aureus	*E. coli*
Wound swabs	200	24(12.0)	8(4.0)
Midstream urine	109	6(5.5)	22(20.2)
Total	309	30(9.7)	30(9.7)

TCP method detected biofilm formation of *S. aureus* and *E. coli* isolates in 33(55%) strong, 15(25%) moderate and 12(20%) 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 producers, 9/30 isolates were moderate biofilm producers, and weak/non-biofilm producers isolates identified in 6/30 isolates. There was no statistically significant difference of TCP method for screening biofilm production (*P-value* = 1.000) as presented in **Table 2**.

Table 2: Biofilm formation of *S. aureus* and *E. coli* by TCP method

Bacterial isolates	Biofilm formation by TCP method No. (%)			χ^2 test value	*P-value*
	Strong	Moderate	Weak/non		
S. aureus	18(30)	6(10)	6(10)		
E. coli	15(25)	9(15)	6(10)	0.00	1.000
Total	33(55)	15(25)	12(20)		

Similar study was conducted at Ibb city, Yemen by Al-Hobiashy *et al*. [23] reported that 49.3% of isolated uropathogenic bacteria was biofilm producers. Other study revealed TCP method detected 81% bacterial isolates biofilm producers [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 producers [26]. While differences in the observations showed in other study that TCP detected 27% as bacterial biofilm producers [27]. Other study reported biofilm producers identified by TCP method 22% [28].

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 [29]. Also, bacterial biofilms are most of the time associated with long-term persistence of bacteria in various environmental conditions [30]. TCP 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 [31][32][33]. In contrast, statistical analysis of biofilm formation indicated that TCP method was the most sensitive and specific method for screening biofilm production [34].

In this study, we analyzed the antibiotics resistance patterns of biofilm and non-biofilm producing of all *S. aureus* and *E. coli* isolates. *S. aureus* and *E. coli* biofilm producers isolates showed high resistance rates to antibiotics used (**Tables 3 and 4**). *S. aureus* biofilm producing isolates were found highly resistant to amoxyclav 100%, ceftazidime 95.8%, cefotaxime 62.5%, cefadroxil 45.8%, ciprofloxacin 41.7% and ceftriaxone 25%. There was statistically significant correlation of antibiotics resistance of amoxyclav and ceftazidime and bacterial biofilm production (*P-value* < 0.05). Biofilm producing *E. coli* isolates had increased resistance pattern of the antibiotics amoxyclav 100%,

cefadroxil 91.7%, cefotaxime 75%, ceftazidime 70.8%, ceftriaxone 66.7%, ciprofloxacin 62.5% and co-trimoxazole 33.3% with statistically significant correlation of antibiotic resistance of cefadroxil (*P-value* < 0.05).

Table 3: Antibiotics susceptibility patterns of biofilm and non-biofilm producing *S. aureus*

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
Amoxyclav	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*

*Significant P-value, (S) Sensitive, (M) Intermediate sensitive, (R) Resistant

Table 4: Antibiotics susceptibility patterns of biofilm and non-biofilm producing *E. coli*

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
Amoxyclav	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

*Significant P-value, (S) Sensitive, (M) Intermediate sensitive, (R) Resistant

This pattern of *S. aureus* 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. [35] showed biofilm producing *S. aureus* resistant to ciprofloxacin and co-trimoxazole 83.3% and 28.6% respectively. Other study found that the Gram-positive bacteria had high resistance to ciprofloxacin 40% and co-trimoxazole 30% [31]. Neopane et al. [4] reported that resistance toward erythromycin and co-trimoxazole was increased due to the excessive used of these drugs for the treatment of both minor and more serious staphylococcal infections.

The pattern of *E. coli* resistance was agreed with the study findings reported high resistant biofilm producing *E. coli* to amoxyclav 77.61%, ceftriaxone 71.48%, ciprofloxacin 71.48% and amikacin 7.58% [36], another study showed biofilm producing *E. coli* isolates were resistance to ceftaxime, ceftriaxone, and amoxyclav 65.6%, 50% and 40.6% respectively [37]. While other study showed lesser resistance of biofilm producing *E. coli* to co-trimoxazole 47.4%, ciprofloxacin 47% and ceftaxime 42.5% [38]. In other study, Gram negative bacteria had high resistance to ciprofloxacin, co-trimoxazole, amikacin and ceftriaxone 95%, 90%, 64% and 58% respectively [31], another study found resistance of biofilm forming *E. coli* isolates to ciprofloxacin 95% and amikacin 65% [39].

Bacteria in biofilm display dramatically increased resistance to antibiotics [30]. So, 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 biofilms and hinders the activity of antibiotics against the bacterial cells [4][37].

In this study, MDR showed in *S. aureus* and *Esch. coli* isolates to three antibiotics or more belonged to three or more different classes. Among 48 biofilm producers isolates of *S. auerus* and *E. coli*, 40(83.3%) isolates were MDR, 5(41.7%) were non-producer and MDR. There was statistically significant association between biofilm formation and MDR isolates (*P-value* = 0.006), as presented in **Table 5**.

Table 5: Relationship the biofilm production *S. aureus* and *E. coli* and MDR

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

*Significant P-value

These results were agreed to the different findings reported by various studies [1][28][40][41]. In the contrary, another study reported no significant association between MDR and biofilm formation [37][30]. 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].

4. Conclusion

TCP 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 MDR were observed in biofilm producers than non-biofilm producers. Detection of bacterial 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.

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