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Original Article

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Biofilm-Producing and Specific Antibiotic Resistance Genes in *Pseudomonas aeruginosa* Isolated from Patients Admitted to a Tertiary Care Hospital, Bangladesh

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***Pseudomonas aeruginosa* is one of the organisms well-known for producing biofilm. Biofilms are responsible for persistent infections and antimicrobial resistance. The aim of this was to investigate *P. aeruginosa* for its ability to form biofilm. Genes that were responsible for the production of biofilms and biofilm-specific antimicrobial resistance were detected. The association between antibiotic resistance and biofilm was investigated. This cross-sectional study was conducted from July 2017 to December 2018. A total of 446 samples (infected burns, surgical wounds, and ETA) were collected from admitted patients at Dhaka Medical College and Hospital, Bangladesh. *P. aeruginosa* was isolated and identified by biochemical tests and PCR. Biofilm production by the tissue culture plate (TCP) method was followed by the detection of biofilm-producing genes (*pqsA*, *pslA*, *pslD*, *pslH*, *pelA*, *lasR*) and biofilm-specific antibiotic resistance genes (*ndvB*, PA1874, PA1876, PA1877) by PCR. The antibiotic susceptibility test was carried out by the disc diffusion method; for colistin agar dilution, the MIC method was followed. Among 232 (52.02%) positive strains of *P. aeruginosa*, 24 (10.30%) produced biofilms in TCPM. Among biofilm-producing genes, *pqsA* was found the highest number of isolates (79.17%), which was followed by *pslA* and *pelA* (70.83%). Other were found in lesser extent. Among the biofilm-specific antibiotic resistance genes, 16.67% of the isolates had *ndvB*, and 8.33% had *PA*1874 and *PA*1877. Biofilm-forming strains were significantly resistant to colistin in comparison to non-biofilm-forming ones. In conclusion, detection of biofilm-forming genes may be a good tool for the evaluation of biofilm production, which will help in prompt and better management of chronic or device-associated infections.**

Keywords: antibiotic resistance, biofilm, *Pseudomonas aeruginosa*

Introduction:

Pseudomonas aeruginosa (*P. aeruginosa*) has various factors that help to adhere to and damage cells and mucosal tissues of the host, elicit inflammation, and impair defense mechanisms[1]. Biofilm is one of the factors that helps in the establishment of the organism on different host tissues, especially in immunocompromised patients, patients with implanted devices, and burn wounds[2,3].

Biofilm is a network of multilayered cell clusters that acts as a protective barrier against the host immune system and antibiotic therapy [4]. *P. aeruginosa* produces three extracellular polysaccharides (EPS): alginate, polysaccharide (Psl), and pellicle (Pel), that help in biofilm formation[5]. Quorum sensing (QS), a cell-to-cell signaling system, aids in the production of different virulent factors causing chronic infection [6]. The pqs QS system uses the *Pseudomonas* quinolone signal (PQS) as the signal molecule acting as an ideal anti-virulence drug target [7]. In the Las QS system, the LasR (elastase) gene acts as a transcriptional activator that encodes virulence factors like protease, elastase, hydrogen cyanide, and phenazines [8].

Tolerance to killing by antimicrobials is the hallmark of biofilm[9]. *P. aeruginosa* uses a dual resistance mechanism: reduced penetration and active drug efûux [10,11]. Moreover, bacteria embedded in the biofilm develop tolerance to high antibiotic concentrations[12]. The *ndvB* gene encodes glucosyltransferase, which is involved in the synthesis of cyclic glucagon situated in the periplasm. *P. aeruginosa* cyclic glucans interact with antibiotics and sequester them in the periplasm [13]. Moreover, *P. aeruginosa* also contains an ATP-binding cassette (ABC) transport system [14]. The ABC transport system includes PA1874, PA1875, PA1876, and PA1877 (PA1874-77), which remove antibiotics from the cells within the biofilm [9].

Young biofilms are more susceptible to antibiotics compared to more developed biofilms[15]. Thus, early intervention could be useful regarding the management of intractable biofilm-associated persistent infections. There was no study on the genes responsible for biofilm formation and biofilm-associated antimicrobial resistance among clinical isolates of *P. aeruginosa* in Bangladesh. Therefore, this study was conducted to detect biofilm and genes responsible for biofilm and antibiotic resistance among *P. aeruginosa.*

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Materials and Methods

Sample collection

This cross-sectional study was conducted in the Department of Microbiology of Dhaka Medical College (DMC), Dhaka, Bangladesh, from July 2017 to December 2018. A total of 232 clinical isolates of *Pseudomonas aeruginosa* from the infected burn, surgical wounds, and endotracheal aspirate (ETA) were included.

Bacterial isolation

Samples were collected aseptically, inoculated in blood agar and MacConkey agar media, and then aerobically incubated for 48 hours at 37°C and 42°C. By using standard procedures, *P. aeruginosa* was isolated and identified using colony morphology, Gram staining, and biochemical testing[16]. Identification of *P. aeruginosa* was confirmed by PCR from the culture with specific primers.

Antimicrobial susceptibility testing

Isolates were tested for antimicrobial susceptibility using the Kirby Bauer modified disc diffusion method and the agar dilution method of MIC[17]. Antibiotic susceptibility was interpreted following the CLSI guidelines[18].

Method of detection of biofilm

Congo red agar (CRA) method: CRA medium was prepared with brain heart infusion broth 37 g/L, sucrose 50 g/L, agar No. 1 10 g/L, and Congo Red indicator 8 g/L. Congo Red stain and brain heart infusion agar with sucrose were autoclaved separately, followed by mixing at 55ºC. CRA plates were inoculated with test organisms and incubated at 37ºC for 24 h aerobically. Black colonies with a dry, crystalline consistency indicated biofilm production[19]. The experiment was performed in triplicate and repeated three times.

Tube method

A loop-full of test organisms was inoculated in 10 mL of trypticase soy broth (TSB) with 1% glucose in test tubes. The tubes were incubated at 37ºC for 24 h. After incubation, tubes were decanted, and washed with phosphate buffered saline (PBS) (pH 7.3), and dried. The tubes were then stained with crystal violet (0.1%). The excess stain was washed with deionized water and dried in an inverted position. The scoring for the tube method was done according to the results of the control strains. Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube. The amount of biofilm formed was scored as 1-weak/none, 2-moderate, and 3-high/strong. The experiment was performed in triplicate and repeated three times [20].

Tissue culture plate method (TCP)

Cultures were transferred into a fresh medium ~~in~~ at a dilution of 1:100. Ninety-six well flat-bottom polystyrene tissue culture-treated plates (Sigma Aldrich, Costar, USA) were filled with 200 µL of diluted cultures. Control organisms were also incubated, diluted, and added to the tissue culture plate. The sterile broth was taken as a negative control. After incubation at 37ºC for 24 h, the contents of each well were removed by gentle tapping and washed with 0.2 mL of PBS four times to remove free-floating bacteria. Biofilm formed by bacteria adherent to the wells was fixed with 2% sodium acetate and stained with crystal violet (0.1%). The excess stain was removed with deionized water and dried. The optical density (OD) of stained adherent biofilm was obtained using a micro-ELISA auto reader (model 680, Biorad, UK) at a wavelength of 570 nm. The experiment was performed in triplicate and repeated three times [21].

Calculation of OD values

The average OD values were calculated for all tested strains and negative controls since all tests were performed in triplicate and repeated three times. Second, the cut-off value (ODc) was established. It was defined as three standard deviations (SD) above the mean OD of the negative control: ODc = average OD of the negative controls + (3× SD of the negative control)[22].

Table 1: *Interpretation of biofilm production*

Average OD value	Biofilm production
OD ≤ODc	None
ODc< OD d" 2ODc	Weak
2ODc < OD d" 4ODc	Moderate
4ODc < OD	High

Polymerase chain reaction

DNA was extracted by boiling method. Bacterial pellets were mixed with 300 ìl of distilled water, followed by boiling at 100°C for 10 minutes in a block heater (DAIHA Scientific, Seoul, Korea). After cooling on the ice pack, the mixture was centrifuged at four degrees Celsius at 13,500 g for 10 minutes. The extracted DNA was then kept at -20°C[23]. Amplification of the extracted DNA was carried out by adding of primers and DNA template to 2x master mix. PCR was performed in a DNA thermal cycler (Eppendorf AG, Mastercycler gradient, Hamburg, Germany).

The amplified DNA was analyzed by 1.5% agarose gel-electrophoresis at 100 volts for 35 minutes, stained with 1% ethidium bromide, and visualized under a UV transilluminator (Gel Doc, Major Science, Taiwan).

Data analysis

Data analysis was done using the 'Microsoft Office Excel 2010' program. The test of significance was calculated using the chi-square test, and a p value <0.05 was taken as the minimal level of significance.

Ethics

This study was approved by the Research Review Committee (RRC), the Department of Microbiology, and the Ethical Review Committee (ERC) of DMC. Informed written consent was obtained from each patient or authorized legal guardian before sample collection. Anonymity of the patients and confidentiality of information were maintained strictly.

Table 2: *Primers for biofilm forming genes*

Gene	Sequence (5'- 3')	Size (bp)	Reference
lasR- F*lasR*- R	AAGTGGAAAATTGGAGTGGAGGTAGTTGCCGACGACGATGAAG	130	[24]
pqsA- F*pqsA*- R	CCCGATACCGCCGTTTATCAAACCCGAGGTGTATTGCAGG	448	[25]
pelA- F*pelA*- R	CCTTCAGCCATCCGTTCTTCTTCGCGTACGAAGTCGACCTT	118	[26]
pslA- F*pslA*- R	TGGGTCTTCAAGTTCCGCTCATGCTGGTCTTGCGGATGAA	119	[25]
pslD- F*pslD*- R	CTCATGAAACGCACCCTCCTTGCGACCGATGAACGGATAG	295	[25]
pslH- F*pslH*- R	CAGATGCTGGTCTGGGAGTGGGAACGAAGCCTTGCCATTC	719	[25]

Table 3: *Primers for biofilm- specific antimicrobial resistance genes*

Gene	*Sequence (5'- 3')	Size (bp)	Reference
ndvB- F*ndvB*- R	GAGGTGGCAAAATGGGCAAGCATGCAGGCAAGAATCGACG	781	[27]
PA1874- FPA1874- R	GGCCATTACACGATCCACTCGGCTGTATGCAGACCGAAC	183	[28]
PA1876- FPA1876- R	GATTGTCGGAGGGTCAGAAACGACACCAGTTGCAGAAATG	200	[28]
PA1877- FPA1877- R	GCCACAAAATCGAGGAAAAGCGCCAATCGTTGTGATGTAG	186	[28]

*- PA1874, PA1876 and PA1877 are orthologous of BPSL1661, BPSL1664 and BPSL1665 respectively.[28] Gene sequences are used accordingly.

Results

A total of 446 samples were collected, among which 232 (52.02%) yielded growth of *Pseudomonas aeruginosa*. The detection of biofilm production in different methods by these *P. aeruginosa* from different samples is shown in Table 4.

Considering TCP as the gold standard method, comparisons of diagnostic parameters (sensitivity, specificity, positive and negative predictive value, and accuracy) between the TM and CRA methods ~~is~~ are demonstrated in Table 5.

In Table 6, the distribution of biofilm-forming genes among 24 biofilm-producing isolates in the TCP method is shown. *pqsA* was the most common gene isolated from biofilm-forming *P. aeruginosa* isolated from both burn and ETA samples, followed by *pslA* and *pelA*.

N = total number of samples, n= number of *P. aeruginosa* having respective biofilm producing genes, ETA= endotracheal aspirate.

Among the 24 biofilm-producing isolates ~~in~~ produced by the tissue culture plate method, four (16.67%) were positive for *ndvB*, two (8.33%) for PA1874, and two (8.33%) for PA1877. All were isolated from burn wound samples. No PA1876 was detected.

The antibiotic resistance pattern of all the isolated *P. aeruginosa* (N=232), biofilm-forming (n=24), and non-biofilm-forming (n=208) is demonstrated in Table 4. Though a higher percentage of resistance was observed to most antibiotics among biofilm-forming stains, resistance to cefotaxime was statistically significant in non-biofilm-forming isolates, whereas resistance to colistin was statistically significant among biofilm-forming *P. aeruginosa* (Table 7).

Table 4: *Detection of biofilm production in different methods by P. aeruginosa from different samples (n = 232).*

Samples	Methods of biofilm formation		
	TM, n (%)	TCP method, n (%)	CRA method, n (%)
Burn wound (N=126)	33 (26.19)	21 (16.67)	12 (9.52)
Surgical/traumatic wound (N= 95)	0	0	0
ETA (N=11)	3 (27.27)	3 (27.27)	0 (0.00)
Total (N=232)	36 (15.51)	24 (10.34)	12 (5.17)

N = total number of samples, n= number of bacteria forming biofilm in respective method.
ETA= endotracheal aspirate, TM= Tube method, TCP= Tissue Culture Plate, CRA= Congo Red Agar

Table 5: *Diagnostic parameters of TM and CRA method for biofilm detection.*

Screening method	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative protective value (%)	Accuracy (%)
TM	100%	94%	66.67%	100%	94%
CRA	50%	100%	100%	94.55%	94.83%

TM = Tube method, CRA= Congo Red Agar

Table 6: *Proportion of biofilm forming genes among biofilm forming P. aeruginosa detected by TCP method.*

Biofilm forming genes	Burn wounds (N=21)n (%)	ETA (N=3)n (%)	Total (N=24)n (%)
pqsA	17 (80.95)	2 (66.67)	19 (79.17)
pslA	16 (76.19)	1 (33.33)	17 (70.83)
pslD	11 (52.38)	0 (0)	11 (45.83)
pslH	9 (42.86)	0 (0)	9 (37.5)
pelA	11 (52.38)	1 (33.33)	12 (50)
lasR	8 (33.33)	1 (33.33)	9 (37.5)

Table 7: *Comparison of antibiotic resistance patterns between biofilm positive (N= 24) and biofilm negative (N= 208) P. aeruginosa.*

Antimicrobial agents	Resistance pattern in biofilm positive strains n (%)	Resistance pattern in biofilm negative strainsn (%)	*p* value
Imipenem	7 (29.17)	65 (30.28)	>0.05
Piperacillin/Tazobactam	13 (54.17)	92 (44.23)	>0.05
Amikacin	20 (83.33)	172 (82.69)	>0.05
Gentamicin	22 (91.67)	185 (88.94)	>0.05
Netilmicin	16 (66.67)	149 (71.64)	>0.05
Aztreonam	18 (75)	155 (74.52)	>0.05
Ciprofloxacin	22 (91.67)	187 (89.90)	>0.05
Cefotaxime	21 (87.5)	202 (97.11)	<0.05**
Ceftazidime	17 (70.83)	142 (68.27)	>0.05
Colistin	4 (16.67)	12 (5.77)	<0.05*

**Resistance to Cefotaxime is statistically significant in biofilm negative *P. aeruginosa*.

Discussion

Biofilm-related infections are subject to developing recurrent and chronic wound infections and device-related infections. Besides, biofilm-related bacterial infection increases the mortality rate in burn patients[29].

Considering the tissue culture plate (TCP) method as the gold standard, the tube method showed a cent percent negative predictive value, and the Congo red agar (CRA) method displayed a 100% positive predictive value. Sultan and Nabiel reported higher sensitivity and specificity in the tube method, which contradicted our study[30]. Sultana found higher percentage biofilm production in DMCH (61.54% in TCP, 50% in TM, and 11.84% in the CRA method)[31]. There is no clear explanation for such variations in these studies, but it can be due to subjective judgment because TM and CRA are qualitative methods.

Biofilms adhere to the human cell surface as a community of microorganisms. These organisms are embedded in the matrix of EPS, which is self-produced by these adherent cells. pel and psl operons biosynthesize the EPS and help in the interactions between cells during biofilm formation[32]. In the current study, the biofilm-forming gene *pqsA* (79.17%) was the most prevalent in *Pseudomonas aeruginosa* isolated from both burn wound and ETA samples. The presence of *pslA* (70.83%), *pslD* (45.83%), and *pslH* (37.5%) in biofilm-forming *P. aeruginosa* isolated from burn wound samples was statistically significant. Though biofilm formation is a multistage process that is attributed to many factors, these genes may act as markers for screening biofilm-forming bacteria.

The resistance in biofilms is multifactorial, including the diffusion barrier and efflux transporter in *P. aeruginosa*[33]. Zhang *et al.* described mutations within the *ndvB* gene of *P. aeruginosa*, which encodes a glucosyltransferase, that results in increased sensitivity of *P. aeruginosa* bioûlms to several antibiotics[34]. In this study, 16.67% of biofilm-forming *P. aeruginosa* were positive for the biofilm-specific antimicrobial resistance gene *ndvB*, followed by 8.33% for PA1874 and PA1877, which are not substantial. Besides, resistance to cefotaxime was statistically

significant in non-biofilm-forming *P. aeruginosa* compared to biofilm-forming strains. On the other hand, colistin resistance among biofilm-forming *P. aeruginosa* isolates was statistically significant ($p < 0.05$) compared to non-biofilm-forming isolates. So, it was inconclusive whether there was an association between biofilm formation and antibiotic resistance in *P. aeruginosa* in this study. Because the resistance patterns of other antibiotics among biofilm-forming and non-biofilm-forming *P. aeruginosa* isolated in this study were almost similar. A lesser number of biofilm-forming isolates could be responsible in this matter.

Conclusion

Biofilm-forming *Pseudomonas aeruginosa* was detected in infected burn wounds and ETA samples by the TCP method. Detection of biofilm-forming genes can be helpful in screening biofilm-producing bacteria. Both biofilm-forming and non-biofilm-forming *P. aeruginosa* were resistant to common antibiotics. *ndvB,* PA1874, PA1876, and PA1877 may have roles in antibiotic resistance, but biofilm-specific antibiotic resistance encompasses multiple mechanisms. Biofilm detection in routine laboratory tests could minimize nosocomial infections. It will help the clinicians select the proper antibiotics for appropriate patients.

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