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ANTIBIOTIC RESISTANCE AND *NDVB* GENE EXPRESSION AMONG BIOFILM PRODUCING *PSEUDOMONAS AERUOGINOSA* ISOLATES

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ABSTRACT

A novel antibiotic resistant mechanism among biofilms is glucan-mediated sequestration in which *ndvB* gene encodes a glucosyltransferase involved in the formation of this glucans. We studied the biofilm formation and antibiotic susceptibility pattern of *P. aeruginosa* isolated from clinical samples, and measured the expression of *ndvB* gene among biofilm forming isolates and their planktonic counterparts. The study was conducted on 92 *P. aeruginosa* isolates. Biofilm was measured using tissue culture plate method. Antibiotic susceptibility of biofilm positive isolates and planktonic counterparts for ciprofloxacin, tobramycin and gentamycin was tested using tube microdilution method. Expression of *ndvB gene* was measured using Syper green real time PCR. We found that 44 isolates (47.8%) of *P. aeruginosa* were biofilm positive. The biofilm formation was high among urine, endotracheal tube aspirate and burn isolates compared to isolates of wound specimens, with statistically non-significant differences. None of biofilm forming isolates was susceptible to the 3 antibiotics compared to the presence of susceptible isolates among the planktonic counterpart (18/40.9% for ciprofloxacin, 12/27.3% for tobramycin and 13/29.5% for gentamycin). Expression of *ndvB* gene was significantly high in biofilm isolates than their corresponding counterpart, with significant correlations with minimal biofilm inhibitory concentration (MBIC) values of cibrofloxacin (r=+ 0.65, $p<0.001$), tobramycin (r= + 0.54 $p<0.001$) and gentamycin (r=+ 0.77, $p<0.001$).From this study we concluded that biofilm formation is an important character of *P. aeruginosa* that is a main cause of antibiotic resistance especially in isolates from catheterized urine , wound and endotracheal tube aspirate. *NdvB* gene expression is a mechanism of resistance to antibiotics in *P. aeruginosa* biofilms.

Key Words: *P. aeruginosa*, biofilm, antibiotic resistance and *ndvB* gene.

LA RESISTANCE AUX AINTIBIOTIQIQUESET L'EXPRESSION GENIQUE *NDVB* PARMI LES BIOFILMS PRODUISANT *PSEUDOMONAS AERUGINOSA* ISOLAT

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RESUME

Un mécanisme de résistance aux antibiotiques chez les nouveaux films biologiques est glucane médiation par séquestration dans laquelle le gène codant pour la glycosyltransferase *ndvB* impliqués dans la formation de ces glucanes. Nous avons étudié la formation des biofilms et le profil de la sensibilité de *P.aeruginosa* aux antibiotiques isolé d'échantillons clinique et on a mesuré l'expression de gène de *ndvB* parmi les isolats qui forment les biofilms et leurs homologues planctoniques. L'étude a été menée sur 92isolats de *P.aeruginosa*. Biofilm a été mesuré en utilisant la méthode du tissu plaque de culture. Sensibilité aux antibiotiques des biofilms isolats positifs et homologues planctoniques pour la ciprofloxacine, la tobramycine, et la gentamycine a été teste en utilisant la méthode tube de micro dilution. L'expression de *ndvB* a été mesurée en utilisant la PCR en temps réel vert super. Nous avons trouvé que 44 isolats (47.8%) de *P.aeruginosa*étaient biofilm positifs. La formation de biofilm était élevé parmi les urines, sonde endotrachéale aspirée, et isolats brulure par rapport aux isolats de spécimens

de plaies avec des différences statistiquement non – significatives. Aucun des isolats formant des biofilms était sensible aux trois antibiotiques par rapport à la présence des isolats sensibles parmi les homologues planctoniques (18/40,9%pour ciprofloxacine, 12/27,3% pour tobramycine et 13/29,5% pour gentamycine). L'expression de gène de *ndvB* était significativement plus élevée chez les isolats biofilm que leurs homologues correspondants, avec des corrélations significatives avec un minimum biofilm concentration inhibitrice (CMI) des

valeurs de ciprofloxacine (r= + 0,65, p<0,05), tobramycine (r= + 0,54 p<0,05) et gentamycine (r= + 0,77 p < 0,001). De cette étude nous avons conclu que la formation des biofilms est un caractère important de p. aeruginosa qui est une cause principale de la résistance aux antibiotiques en particulier dans les isolats provenant de l'urine cathétérisées, plaie et sonde endotrachéaleaspirée. L'expressiondugène de*ndvB*est un mécanisme de résistance antibiotique chez biofilms de *P.aeruginosa*.
Mots clés : *P.aeruginosa*, biofilms, résistance aux antibiotiques, etgène de *ndvB*.

INTRODUCTION

Pseudomonas aeruginosa is a key opportunistic pathogen characterized by high-level antibiotic resistance and biofilm formation (1).Biofilm is a structured community of bacterial cells enclosed in a self-produced polymeric matrix adherent to an inert or living surface. Biofilm-producing organisms are more antimicrobial resistant than organisms without biofilm. In some extreme cases, the concentrations of antimicrobials required to kill biofilm positive organisms can be three- to four-fold higher than for biofilm negative bacteria, depending on the species and drug combination (2). Biofilms have great importance for public health as they are the main cause of nosocomial infections, especially implant-based and chronic infections (3).

Antibiotic resistance in biofilms is due to a combination of many factors that act together to result in a level of resistance that is much higher than that of planktonic bacteria (4,5). One factor of these is the altered expression of specific genes involved in the biofilm. Some of these genes may function to protect biofilm cells from antibiotics. N*dvB gene,* encodes the glucosyltransferase enzyme that responsible for the formation of cyclic glucans **(6).** The glucans are cyclic polymers of 12 to 15 β- (1 → 3) -linked glucose molecules with phosphoglycerol substitutions (7). Inactivation of *ndvB* gene blocked glucan synthesis without affect the kinetics of biofilm formation, the architecture of the biofilms or growth of bacteria **(6).** However, *ndvB* mutants of PA14 strains exhibited increased sensitivity to the aminoglycosides tobramycin and gentamicin and the fluoroquinolone ciprofloxacin (6,7). This study aimed to detect the biofilm formation and antibiotic susceptibility pattern of *Pseudomonas aeruginosa* strains isolated from clinical samples, and to measure the expression of *ndvB* gene among biofilm forming isolates.

MATERIALS AND METHODS

Bacterial strains

They were isolated from patients hospitalized in surgery , burn units, and intensive care unit, at Zagazig University Hospitals, during the period from February 2015 –August 2015. Isolates were obtained from 274 specimens including catheterized urine, endotracheal tube aspirates (ETAs) and exudates of burn and septic wound. The specimens were analysed in the Lab. of Microbiology& Immunology Department, Zagazig University Hospitals .The isolates were identified as *P. aeruoginosa* by *API 20NE* and stored at -70°C. The study was approved after receiving permission from Zagazig University Ehical Committee and a written consent was taken from each patient or his relative.

Quantitation of biofilm

It was done by tissue culture plate method as described previously (8) using sterile 96 well-flat bottom polystyrene tissue culture plate with a lid (Sigma, USA). Methicillin –sensitive *staphylococcus aureus (MSSA)* ATCC-25923 and *P. aeruginosa* ATCC-27853 were used as negative and positive control organisms , respectively (9). Sterile broth was inoculated in negative control wells. The absorbance at 570 nm was determined using Microtiter plate reader (Spectra III, SIT measurement, Australia). All samples were tested in triplicate and the results were averaged. The interpretation for biofilm production was done according to specific criteria (10).

Antibiotic susceptibility testing

Antimicrobial agents

They included gentamycin, tobramycin and ciprofloxacin, provided as standard powder from Sigma Company, USA,. Antibiotics were serially diluted in cation- adjusted Muller Hinton broth (CAMHB) according to Clinical Laboratory Standard Institute (CLSI) (11).

Biofilm susceptibility assay

It was performed as previously described **(**12**)** with certain modifications to make the procedure compatible with our clinical microbiology laboratory. Biofilm positive strains were subcutured twice on tryptic soy agar (TSA, Oxoid) with 5% sheep blood after retrieval from -70°C storage and then gown overnight in CAMHB. After dilution of this culture to 0.5 McFarland, 100 µl was transferred to all except the well No 12 of a round-bottom 96-well microtiter plate with a

lid (Sigma, USA) followed by incubation at 37°C for 20-24 h with no movement. After incubation, the microtiter plate was rinsed by sterile distilled water and 100 µl of CAMHB with serial two fold dilutions of antibiotics (512 - 1µg/ml for gentamycin and tobramycin and 128 - 0.25µg/ml for ciprofloxacin) were added to corresponding wells from 1-10 in each row. In each row, well No 11 was used as growth control while well No 12 was used as sterility control. Each row represents an antibiotic susceptibility test of one antibiotic against one isolate of the organism. The plate was incubated at 37°C for 20-24 h. After antibiotic incubation, the microtiter plate was again rinsed three times in sterile water then 100 µl antibiotic-free CAMHB was added to each well. Each plate was sonicated at room temperature for 5 min The optical density at 650 nm (OD_{650}) was measured on microtiter plate reader before and after incubation at 37°C for 6 h. Adequate biofilm growth for growth control wells was defined as a mean OD650 difference that is ≥0.05**.** The biofilm inhibitory concentration (MBIC) was the lowest concentration of antibiotic that resulted in an OD_{650} difference ≤ 10% of the mean of two readings of growth control wells (12)**.**

Planktonic susceptibility assay Conventional minimum inhibitory concentration, MIC, of the planktonic bacterial population was measured using standard broth microdilution method according to CLSI guidelines (11). MIC was the lowest concentration of antibiotic at which there is no visible growth.

Interpretations: In the statistical analysis MIC (or MBIC) values at ≤ 1 µg/ml for tobramycin or gentamicin, and ≤ 0.25 µg/ml for ciprofloxacin were considered as 1 µg/ml and 0.25 µg/ml respectively and which have MIC (or MBIC) values at > 512 µg/ml for tobramycin or gentamicin, and > 128 µg/ml for ciprofloxacin were considered as 1024 µg/ml and 256 µg/m respectively . The susceptible strains have tobramycin, gentamicin or ciprofloxacin MIC (or MBIC) values at ≤ 4µg/ml, ≤ 4 µg/ml, and ≤ 1 µg/ml respectively and resistant strains have MIC (or MBIC) values at ≥ 8 µg/ml, ≥ 8 µg/ml, and ≥ 2 µg/ml respectively (13).

Real- Time polymerase reaction: It was done to measure the expression of antibiotic resistance *ndv B* gene in 18 biofilm forming *P. aerurginosa* strains and their planktonic counterpart. Total bacterial RNA was isolated from the biofilm bacteria and planktonic counterpart. Briefly, the planktonic cells were cultivated in LB broth at 37°C to early stationary phase that corresponds to optical density of 0.7 at 600 nm , while the biofilm cells were grown on M63 agar plates overnight at 37°C followed by another night at room temperature as described previously (14). RNA was extracted using IQeasy TM plus CTB RNA Extraction Mini Kit (iNtRON Biotechnology, Inc, Korea) according to manufacturer instructions. Reverse Transcription was done using Maxime RT PreMix Kit (iNtRON Biotechnology, Inc, Korea) in which 0.1 µg template RNA and distilled water were added into the Maxime RT PreMix tubes that contain random primer to a total volume of 20µl. Complementary DNA synthesis was performed at 45° C for 60 min and RTase inactivation step at 95 °C for 5 min, using PCR Thermal cycler (Biometra, Germany) .

Complementary DNA was measured with SYBR green real-time PCR using superReal premix plus kit (TIANGEN, China). To measure *the ndvB* gene expression, a pair of primers specific for *ndvB* gene (5′-GGCCTGAA CATCTTCTTCACC- 3′ [forward]) and (5′-GATCTTGCCGACCTTGAAGAC -3′ [reverse]) was used to amplify and quantify cDNA corresponding to its mRNA. For a control, the primers (5′-GATCCGGAACAGGTGGAAGAC-3′ [forward]) and (5′-TCAGCAGTTCC ACGGTACCC-3′ [reverse]) were used to amplify and quantify mRNA of the constitutively expressed *bacterial rpoD* gene (15)**.** Each 20µl quantitative real-time PCR mixture contained 10µl SuperRealPreMix Plus, 0.5 µl Rox dye , 100µM of each primer and 2 µl cDNA (1µg cDNA) . The cycler (Stratagene Mx3000P qPCR real-time PCR) was programmed as following; 95 °C for 15 min, and 40 cycles each cycle consists of 95°C for 60 sec., 56°C for 30 sec. and 72° C for 30 sec.

Statistical analysis: Continuous variables were expressed as the median (Range), and the categorical variables were expressed as a number (percentage). Paired data were analyzed using the **Mann-Whitney U (MWU)** test. Percent of categorical variables were compared using Chi-square test (χ^2) with Fisher's exact correction. Spearman's rank correlation coefficient (r) was calculated, (+) sign was indicator for direct relationship & (-) sign was indicator for inverse relationship. All tests were two sided, *p*-value <0.05 was considered significant. All statistics were performed using SPSS 22.0.

RESULTS

During the study period, 92 *p. aeruginosa isolates* were identified from 274 clinical specimens. They were recovered from 24 (33.3%) out of 72 wound exudates, 16 (44.4%) out of 36 burn exudate, 33 (29.5%) out of 112 urine and 19 (35.2 %) out of 54 ETA specimens. 44(47.8%) out of these 92 isolates were biofilm positive by tissue culture plate method. The biofilm formation was high among urine, ETA and burn isolates compared to isolates of wound specimens, however with statistically non-significant difference, $P>0.05$ (Table 1). In urine specimens, 15 biofilm positive and 4 biofilm negative *P. aerruoginosa* strains were isolated from patients catheterized for more than 4 days, while 5 biofilm positive and 9 negative were from paients catheterized for less than or equal to 4 days ($p< 0.05$)). In ETA , 10 biofilm positive and 3 biofilm negative *P. aerruoginosa* strains were isolated from intubated patients for more than 4 days, while 1 biofilm positive and 5 negative were from ETA with intubation of less than or equal to 4 days ($p< 0.05$) .

After measuring MIC/ MBIC in µg /ml of the 44 biofilm positive *P. aeruginosa* isolates and their planktonic counterpart to the 3 antibiotics, it was found that none of biofilm form were susceptible to ciprofloxacin, tobramycin and gentamycin while 18 strains (40.9%), 12 strains (27.3%) and 13 strains (29.5%) of the planktonic counterpart were susceptible to the 3 antibiotics respectively with high statistic significant differences between biofilm form and their planktonic counterpart , $P<0.001$ for each. The differences between median (range) of biofilm positive cells MBIC and those of their planktonic counterpart MIC for the 3 antibiotics were statistically significant (Table 2). .

The expression of *ndvB* gene was measured in 18 biofilm isolates by SYBR green real time PCR It was higher among biofilm form in contrast to negligible expression in their planktonic counterpart and the difference between their median (range) of their expression was statistically significant (Table 3). According to our findings of statistical analysis for evaluating the association between the expression of *ndvB* gene and the antibiotic resistance in biofilm forming isolates, there were significant correlations between *ndvB* gene expression and MBIC values of ciprofloxacin (r=+ 0.65, $P< 0.001$), tobramycin (r= + 0.54 ,$P<$ 0.05) and gentamycin (r=+ 0.77 ,$P< 0.001$).

TABLE (1): DISTRIBUTION OF BIOFILM +VE AND BIOFILM –VE *P. AERUGINOSA ISOLATES* IN EACH CLINICAL SPECIMEN

Source of specimen	N	Biofilm negative (N=48)		Biofilm positive (N=44)	
		No.	%	No.	%
Wound	24	17	70.8%	7	29.2%
Urine	33	13	39.4%	20	60.6%
ETA	19	8	42.1%	11	57.9%
Burn	16	10	62.5%	6	37.5%
χ^2		6.96			
*p-value		0.073			

Qualitative data are presented as number (%),χ^2: Chi-square test, *$p>0.05$ is non-significant.

TABLE (2): COMPARISON BETWEEN PLANKTONIC BIOFILM +VE MIC AND BIOFILM +VE MBIC OF CIPROFLOXACIN, TOBRAMYCIN AND GENTAMYCIN (MG/ML)

Antibiotic	Planktonic MIC (N=44)		Biofilm +ve MBIC (N=44)		MWU*	
	Median	(Range)	Median	(Range)	Z	#p-value
Ciprofloxacin	16	(0.25 – 128)	96	(32 – 256)	-5.92	<0.001
Tobramycin	96	(1.0 – 512)	512	(64 – 1024)	-4.80	<0.001
Gentamycin	128	(1.0 – 1024)	512	(8 – 1024)	-4.14	<0.001

***MWU: Mann-Whitney U Test; #p< 0.001 is highly significant.**

TABLE (3): RELATIVE EXPRESSION OF NDVB GENE IN RELATION TO CONTROL GENE IN BIOFILM +VE ISOLATES AND PLANKTONIC COUNTERPARTS

	Planktonic (N=18)		Biofilm +ve (N=18)		*MWU test	
	Median	(Range)	Median	(Range)	Z	#p-value
Relative cDNA conc.	0.0008	(0.0006 – 0.0329)	5.1060	(2.0110 – 7.4370)	-5.11	<0.001

***MWU: Mann-Whitney U Test; #p< 0.001 is highly significant.**

DISCUSSION

Our finding that 47.8% of isolated *P. aeruginosa* strains were biofilm positive is in consistent with Hassan *et al* (16**)** and contrasting with Gupta *et al* (17) who found that all *P. aeruginosa* isolates were biofilm producers. In our study, biofilm production was 29.2% from wound, 37.5%from burn, 60.6% from urine and 57.9% from ETA specimens . We didn't find any statistically significant differences in biofilm production among the *P. aeruginosa* strains recovered from different specimens, which comes in agreement with the result of Mikucionyte *et al* (18).

However, different clinical results were reported by different authors. It was100% or 95% among isolates from ETAs in ICUs (19,20)**,** 76.4% among urine isolates of catheterized patients (21)and 40% or 60 % among chronic infected wound isolates (22,23**).**

Microorganisms must adhere to exposed surfaces of devices for long period to be irreversibly attached, once irreversibly attached, it produce extracellular polysaccharide matrix to develop a biofilm (24). In our study, instrumentation either by urinary catheter or endotracheal tube for more than 4 days considered as risk factor as it increased the risk for acquiring biofilm formation than in instrumented patients less than 4 days, which agrees with many reports from different studies (24-26).

None of 44 biofilm cells were susceptible to ciprofloxacin, tobramycin and gentamycin while 18, 12 and 13 strains of the planktonic counterparts were susceptible to the 3 antibiotic respectively. This result is more or less in agreement with a previous study carried out in ICU of Zagazig University Hospitals (27). In the same direction, Sepandj *et al* (28) found that, out of 8 strains, by using MIC results 8, 6 and 7 isolates were sensitive to ciprofloxacin, tobramycin and gentamycin respectively while, by measuring minimal biofilm eliminating concentration (MBEC), they found that sensitive isolates were one for each antibiotic.

In previous studies, several *P.aeruginosa* genes were identified that contribute to biofilm-specific antibiotic resistance by screening for mutants with increased antibiotic sensitivity when growing in biofilms (6,29,30). One of These genes, *ndvB,* encodes a glucosyltransferase enzyme involved in the formation of cyclic glucans (6**).**

In our study, the expression of *ndvB* gene was significantly higher among biofilm +ve *P. aeruginosa* isolates than their planktonic counterparts. There were also significant correlations between the gene expression and MBIC values of ciprofloxacin, tobramycin and gentamycin .These results are in consistent with the results reported on mutants of *P. aeruginosa* growing in biofilm (6,7,15) where **Mah *et al* (6)** reported that biofilm form of *P. aeruginosa* PA 14 had increased antibiotic resistance especially to the antibiotic tobramycin, compared to the isogenic *ndvB* mutant. Those authors proposed that this resistance was due to the ability of crude periplasmic carbohydrate extracts to interact physically with tobramycin forming molecular complex and prevent antibiotic from reaching to its site of action. The proposed mechanism is in agreement with the known properties of cyclodextrins, cyclic glucans derived from starch, as good chelating agents forming inclusion complexes with a wide variety of hydrophobic guest molecules (31) **.**

Furthermore, other studies have reported increased sensitivity of double mutant *ndvB/pa1874-1877* and *ndv B/PA0756-0757* to gentamycin and tobramycin (1,29). The relation between increased *ndvB* gene expression and synthesis of glucans in planktonic and biofilm cells have been studied by direct chemical analysis (7), and it was found that total amount of glucans was higher in biofilm culture which was in agreement with up-regulation of *ndvB* gene expression. The chemical structure of *ndvB* – dependent *P. aeruginosa* glucans was identified and it was able to interact with the aminoglycoside kanamycin, most probably by forming molecular complexes stabilized by ionic interaction. Importantly the cyclic nature and

the high negative charge of the cyclic B-(1...3)-glucans is in a good agreement with their properties to bind to positively charged antibiotics (7).

Conclusion: *P. aeruginosa*is has the ability to form biofilm that is the a cause of antibiotic resistance. Increasesd *NdvB* gene expression is an important mechanism of resistance in *P. aeruginosa* biofilm , and increased MBIC of biofilm positive *P. aeruginosa isolates to ciprofloxacin , tobramycin and gentamycin was associated with increased expression* of this gene.

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