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Biofilm formation and antibiotic resistance patterns of *pseudomonas aerogenosa* isolated from different clinical sources

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Abstract : Aim: In the present study, we have assessed of methodological approach in microbial resistance to antibiotic, and evaluated of biofilm formation by thirty five clinical isolates of *pseudomonas aerogenosa* from different sources.
Method: the assays were performed to determine the antimicrobial sensitivity by the standard Kirby Bauer's disc diffusion method, using Mueller Hinton agar; the quantitative and qualitative biofilm formation assays were done in microtiter plate and tube method.
Results : Most isolated of *Pseudomonas aerogenosa* Were from the urine (28.57%), followed by wound and burn swab as (22.85 and 20)% respectively, so (94.3%) of *pseudomonas aerogenosa* isolates were form biofilm, but (5.7%) non- form biofilm by microtiter plate assay , as well as by tube method the 82.8% of *pseudomonas aerogenosa* isolates were form biofilm whilst 6 isolates (17.2%) were non- form biofilm As well as these bacteria were resistance to the most of tested antibioticsm which showed high resistance percentage (91.4%) to Ciprofloxacin also resistance (65.7%) to Amoxicillin/clavulanic acid; Cefotaxime and Nitrofurantoin, but high susceptible (97.1%) to both Norfloxacin and tobramycin, so 10 isolate of *P. aerogenosa* form strong biofilm and resistance to Amikacin, aw well as 31 and 22 isolate of pseudomonas form strong biofilm were resistance to Ciprofloxacin and Cefotaxime respectively , whilst only 2 isolate of *P. aerogenosa* form Moderate biofilm were resistance to Amikacin; Amoxicillin/clavulanic acid; Gentamycin and Nitrofurantoin respectively.
Conclusion: These conclusions indicated that resistance of antibiotics were higher among strain of *P. aerogenosa* that formed biofilm , as compared to non -form biofilm.
Key word: *pseudomonas aerogenosa*; biofilm; antibiotic resistance.

Introduction:

Pseudomonas aeruginosa is one most imported of opportunistic pathogens that capable to infections both plants and animals, and it's characterized by ability to form biofilm and high resistance antibiotic[(1)], so *Pseudomonas aeruginosa* was responsible about (10–15) % of the nosocomial infection worldwide [(2)]. So Often these infections are Harding to treat via the natural resistance of these species.

Also its remarkable ability of the acquiring mechanisms for resistance to multiple groups of antimicrobial agents[(3).]

Biofilms are a biologically active matrix of extra-cellular substances and cells in association with the solid surface[4].Biofilm also called city of microbes with extracellular polymeric substances (EPS), that represents 85% of total biofilm biomass, which play important role in virulence and their development [5], these EPS are composed of extracellular DNA (eDNA); exopolysaccharides ; biomolecules and polypeptides [6].

Methodology

Antibiotic sensitivity test of the diffusion method

The isolated bacteria were tested for antimicrobial susceptibility testing for nine antibiotics (Amikacin; Amoxicillin/clavulanic acid; Cefotaxime ; Ciprofloxacin; Gentamycin; Nalidixic acid; Nitrofurantoin ; Norfloxacin and Tobramycin,by the standard Kirby Bauer's disc diffusion method. The standard inoculums adjusted to 0.5 McFarland was swabbed on Mueller Hinton agar and allowed to soak for 2 to 5 minutes. After the antibiotic discs were placed on the surface of the media and pressed gently, Mueller agar plates were then incubated at 37 c for 24 h , the inhibition zone were measured and interpreted by the recommendation of clinical and laboratory standards institute(CLSI) in 2012 to determine the sensitive and resistant zone.

Quantitative biofilm formation assay (spectrophotometric method)

Working culture were prepared by inoculation on Columbia agar supplemented with 5% blood and incubated aerobically at 37c for 24 hr. the cultures were used to prepare bacterial suspension in sterile distilled water adjusted to a 0.5 McFarland standard . the suspensions obtained were inoculated into a brain-heart infusion broth ,after that poured into the wells of plastic microplates [7] .

The wells of sterile 96-well flat-bottomed plastic micro plates were filled with 250µl of the BHI broth. Negative control wells contained the broth only. Twenty µl of bacterial suspension was then added to each well. The plate were incubated at 37c for 24hr. following incubation , the content of each well was aspirated, and each well was washed three time with 300µl of sterile distilled water. The remaining attached bacteria were fixed with 200µl of methanol per well, and after 15 min the plates were emptied and left to air dry. After that the plates were stained for 5 min with 160µl per well of crystal violet used for gram stain. Excess stain was rinsed off by placing the microplate sunder running tap water. After the plates were air dried , the dye which was bound to the adherent cells was resolubilized with 160µl of 33% (v/v) glacial acetic acid per well. The optical density (OD) of each well was measured at 570nm [7]by Eliza reader.

Results and Discussion

Table 1: Distribution of *pseudomonas aerogenosa* according to the type of specimen.

Bacterial isolate	***Pseudomonas aerogenosa***					
Type of sample	urine	wound	Burn	blood	sputum	ear
Number and percent	10	8	7	5	2	5
	28.57%	22.85%	20%	14.28%	5.7%	14.28%
Total	35					

In table 1 showed most isolated of *Pseudomonas aerogenosa* were from the urine (28.57%), followed by wound and burn swab as (22.85 and 20) % respectively , whilst (14.28%) from both bloodspecimen and ear swab and low percentage (5.7%) from sputum specimen .

Table 2: Biofilm formation by *pseudomonas aerogenosa* isolates using tube method and microtiter plate assay

Type of method	No. and percent of positive isolates	No. and percent of negative isolate	C.S
Tube method	29	6	*Chi-square statistic =0.258* *DF=1* **P=0.6115*
	82.8%	17.2%	
Microtiter plate assay	33	2	
	94.3%	5.7%	
Total	35		

NS*: P≥0.05 non-significant

In table -2 showed thirty - three out of 35 of *pseudomonas aerogenosa* isolates (94.3%) were form biofilm, but only 2 strain (5.7%) were non- form biofilm by microtiter plate assay , as well as 82.8% of *pseudomonas aerogenosa* isolates were form biofilm whilst 6 isolates (17.2%) were non- form biofilm by tube method.

Table 3: Antibacterial resistance patterns for*P. aerogenosa*isolates

Antibiotic type	Sensitivity				C.S
	Resistance (R)		Sensitive (S)		
	No.	%	No.	%	***P≥ 1.00* **P=0.0000* *DF=8* *Post-hoc t-test:* **P= 0.0000*
*Amikacin	12	34.3	23	65.7	
**Amoxicillin/clavulanic acid	23	65.7	12	34.3	
**Cefotaxime	23	65.7	12	34.3	
*Ciprofloxacin	32	91.4	3	8.6	
*Gentamycin	18	51.4	17	48.6	
*Nalidixic acid	18	51.4	17	48.6	
*Nitrofurantoin	23	65.7	12	34.3	
*Norfloxacin	1	2.9	34	97.1	
*Tobramycin	1	2.9	34	97.1	

The Antibacterial resistance patterns of *P. aerogenosa* isolatesare presented in table -3, These bacteria were resistance to the most of tested antibiotics, which showed high resistance percentage (91.4%) to Ciprofloxacin also resistance (65.7%) to Amoxicillin/clavulanic acid; Cefotaxime and Nitrofurantoin , but high susceptible (97.1%) to both Norfloxacin and tobramycin, whilst in same time its susceptible to (97.1%) were but low resistance (2.9%) to both Norfloxacin and tobramycin ,also in same time its susceptible to Amikacin (65.7%) and low percentage (48.6%) to both Gentamycin and Nalidixic acid .

Table 4: Relationship between antibiotics resistance and biofilm formation by *P. aerogenosa*

Biofilm formation	No. of								
	Amik	Amox/clavu	Cefot	Cipro	Genta	Nalid	Nitro	Nor	Tobra
Strong biofilm	10	21	22	31	16	17	21	0	1
Moderate biofilm	2	2	1	1	2	1	2	1	0
No biofilm	0	0	0	0	0	0	0	0	0
Total resistance	12	23	23	32	18	18	23	1	1
C.S	*P-value = 0.001 " highly significant difference"* *F= 17.269*								

In table -4 from 33 isolate of *P. aerogenosa* form biofilm, consisted of (no. = 10) strong form biofilm were resistance to Amikacin, also isolate of pseudomonas (no. = 31, 22) form strong biofilm were resistance to Ciprofloxacin and Cefotaxime respectively , whilst only 2 isolate of *P. aerogenosa* form Moderate biofilm were resistance to Amikacin; Amoxicillin/clavulanic acid; Gentamycin and Nitrofurantoin respectively , so only 1 isolate of pseudomonas form Moderate biofilm were resistance to Nalidixic acid; Norfloxacin and Tobramycin respectively .

As well as 32 strains that were form biofilm and resistance to Ciprofloxacin, also 23 strains of *P. aerogenosa* that were form biofilm and resistance to Amoxicillin/clavulanic acid; Cefotaxime and Nitrofurantoin (table -4).

These conclusions indicated that resistance of antibiotics were higher among strain of *P. aerogenosa* that formed biofilm , as compared to non -form biofilm

Discussion:

Most isolated of *Pseudomonas aerogenosa*were from the urine, followed by wound and burn swab, whilst and low percentage from both bloodspecimen and ear swab, the results of this study vary with the findings of Al-Marzoqi1 and Al Taee ,2013 where the results showed mostly isolated of *Pseudomonas aerogenosa*were high percentage (22.46%) from Wound followed (22.11%) urine and (18.6%) from the Swab [(8)].

Thirty - three out of 35 of *pseudomonas aerogenosa* isolates form biofilm, but only 2 strain were non-form biofilm by microtiter plate assay, 6 isolates were non- form biofilm by tube method.

A recent study indicated extracellular DNA play important role in an initial establishment of biofilms of *P. aeruginosa*[(9)] as well as these bacteria have ability to synthesize many alternative polysaccharides which play important role in matrix of biofilm , because of their intrinsic resistance to many antimicrobial agents , biofilms are significant in both industry and medicine [(10)] , the Antibacterial resistance patterns of *P. aerogenosa* isolatesare deferent, in india , Smitha etal., 2005 showed in his study amikacin was high sensitivity against *P. aeruginosa* ,because the amikacin was conceder as poor substrate to enzymes[(11)] , whilst Chamber*setal.*, 2006. Explained the increased resistance of *P. aeruginosa* because has limited using of other classes of antibiotics as chloramphenicol ;tetracyclines and fluoroquinolones[(12).]

Al-Muhannak ,2010 reported that the P. *aerogenosa* isolates were less percentage resistant (40%) to Ciprofloxacin whilst high percentage resistant (89.8%) to Ceftazidime [(13)]and agree with result of Mohammed ,2012 who showed *P. aerogenosa* isolates were resistant (54.6%) to Ciprofloxacin, so 60% to Gentamicin, and (82.6%) to Ceftazidime[(14).]

So Kiffer *etal.*, 2005 showed in his study resistance to imipenem and (49 and 49.8) % respectively and 64% to meropenem whilst (63.8 and 63.4) % to piperacillin/tazobactam and amikacin as well as 55.8% to ceftazidime[(15)] .

These resistance *of* Antibiotics *in the Pseudomonas.aeruginosa* adueproducing many mechanisms: enzymatic inactivation, efflux, impermeability , low permeability of its cell wall.and mutants as well as combination of these deferent mechanisms[(16)]also the resistance *of* Antibiotics was partly causing by form biofilms because the biofilm conceders as physical barrier to penetration of antibiotics[(17).]

Bano*etal.*previously in his reported that for *P. aerogenosa*, biofilm-forming isolates were less frequently resistant to imipenem and ciprofloxacin[(18)], indicating that these strains are not as dependent on antimicrobial resistance as non-biofilm-forming strains for survival [(19)] ,which is consistent with our result. Moreover, exposure to sub-MIC levels of certain antibiotics promotes biofilm formation of *Staphylococcus aureus*, indicating that biofilms tend to be stronger when resistance is challenged [(19)]. In this study, biofilm-forming capacity was measured in the absence of antibiotic-mediated stress.

In *P. aeruginosa* the percentage about 1% of the genes that related to the virulence factors and resistance of antibiotic which its differential expression [(20)].

Different factors as the slow penetration of the various antibiotics via biofilm ;persister formation [21] and the extracellular matrix that inducted the lipid modification operon [22], These factors can combination and act together which causing greater level of antibiotic resistance in biofilm[23].

Conclusion

1. Most isolated of *Pseudomonas aerogenosa* from urine, followed by wound and burn swab .
2. High percentage of *pseudomonas aerogenosa* isolates were form biofilm by microtiter plate assay .
3. These bacteria were resistance to the most of tested antibiotics , which showed high resistance to Ciprofloxacin ; Amoxicillin/clavulanic acid; Cefotaxime and Nitrofurantoin , but high susceptible to both Norfloxacin and tobramycin.
4. Microtiter plate assay (MtP) were higher sensitivity when comparing with the tube method which used to determine the biofilm producers.
5. Most isolate of *P. aerogenosa* form strong biofilm have higher resistance of antibiotics compared to non -form biofilm.

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