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ORIGINAL ARTICLE

Relation between Biofilm Formation, Quorum Sensing Genes (*Rh1I-LasI*) and Carbapenem Resistance Among *Pseudomonas Aeruginosa* Clinical Isolates

Shymaa A. El Askary*
Department of Medical Microbiology and Immunology, Faculty of Medicine, Menoufia University, Egypt

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Corresponding Author:
Shymaa A. El Askary
Department of Medical Microbiology and Immunology, Faculty of Medicine, Menoufia University, Egypt
Tel: 00201025538299
dr.shaimaaelaskary@yahoo.com

ABSTRACT

Background: *Biofilm has a vital role in the pathogenesis of P. aeruginosa infections that represent a major challenge for antimicrobial therapy.* ***Objectives:*** *This study was set out to investigate biofilm formation ability and its quantification in relation to the presence of Rh1I and lasI quorum sensing genes among P. aeruginosa clinical isolates. Furthermore, possible association between biofilm occurrence and carbapenemase activity was analyzed.* ***Methodology:*** *62 P. aeruginosa isolates from different clinical specimens were isolated and investigated for biofilm formation by tissue culture plate method (TCP) and PCR (RhI1 and LasI QS genes). Carbapenemase activity was also detected by Carba NP test and confirmed by PCR (**IMP** and VIM MβL genes).* ***Results:*** *Most of P. aeruginosa C.I.s (70.9%) were biofilm producers by TCP. The quorum sensing Rh1I and LasI genes were present in 67.7% and 48.4% of the isolates respectively. By Carba NP test, 64.5% of P. aeruginosa C.I.s were carbapenemase producers where each IMP and VIM gene presented alone in 33.9% and 24.2 % of the C.I.s respectively.* ***Conclusion****: There was a high tendency for biofilm formation by P. aeruginosa C.I.s that increased by the presence of QS. Non- significant relation between biofilm formation and carbapenem resistance.*

INTRODUCTION

Pseudomonas aeruginosa is generally known as an opportunistic invader rather than a cause of initial infection in healthy issues. It now represents an important hospital acquired pathogen in immunosuppressed patients and also leads to outbreaks of 9%–10% nosocomial infections with a high mortality rate [1]. Currently, *Pseudomonas aeruginosa* is a severe challenge for antimicrobial therapy [2]. A part from the trend of high antibiotic resistance seen in *P. aeruginosa*, they are equipped with many quorum-sensing genes that enable them to develop biofilm where Quorum-Sensing (QS) signal molecules termed autoinducers (AI) play a major role in the differentiation process and maintenance of biofilm by allowing the recognition of the population density, whatever the environmental condition in which the cells are found[3]. The quorum sensing is mainly mediated by three interconnected system, the *Las*, *Rh1* and *Pqs* system[4].

Biofilm has a vital role in pathogenesis of *P. aeruginosa* infections, it can promote and sustain infection due to restricted penetration of antimicrobials and also over expression of possible resistance genes with productivity of β-lactamases with no carbapenemase activity (AmpCs) or true carbapenemases as many carbapenemase genes are carried out by plasmids and are easily transferable among various bacterial species and genera [5]. So *P. aeruginosa* are not easily eradicated by bactericidal antibiotics [2].

In *Pseudomonas* spp., carbapenemases are mostly metallo-β-lactamase [6,3]. In the last decade, various classes A, B, and D β-lactamases have been discovered in *P. aeruginosa*[7]. The carbapenemases found are mostly different types of MBLs such as AIM, SME, GIM, IMP, NDM, SPM, and VIM, which their frequency has been increased in *P. aeruginosa* throughout the world[1]. The emergence of MβL-producing strains makes treatment hard and occasionally inefficient that cause high mortality[8].

In vitro identification of carbapenemase production using phenotype-based techniques, such as the modified Hodge test, that not highly sensitive and specific or -lactamase inhibitor-based techniques (boronic acid for KPC and EDTA for MBLs), which both require at least 24to72h to be performed[6].

Carba NP test is a novel test, that identify carbapenemase producers with high sensitivity, specificity, and rapidity (less than 2 h) and adjustable to any laboratory worldwide based on a procedure designed to discover the hydrolysis of the b-lactam ring of a carbapenem[9].

Identification of genes coding for carbapenem resistance by PCR is usually reliable and accurate despite its limited application due to the cost[2].

Assessment of phenotypic and genotypic CRPA C.I.s and their association with biofilm formation would be necessary for understanding the resistance mechanisms and its potential spread.

The current study was set out to investigate biofilm formation ability and its quantification with presence of *Rh1I* and *lasI* quorum sensing genes among *P. aeruginosa* C.I.s. Furthermore, possible association between biofilm occurrence and carbapenem resistance was analyzed.

METHODOLOGY

Collection of Samples:

A total of 62 *P. aeruginosa* C.I.s were collected from different samples (tracheal aspirate, blood, urine, wound, catheter, and peritoneal fluid) from patients admitted to Menoufia University Hospitals (MUH) during the period of Augaust 2018 to February 2019. The study design was approved by the ethical committee, faculty of medicine, Menoufia University.

Bacterial Isolation and Identification:

All clinical samples obtained were cultured and identified according to standard microbiological methods[10]. Identification of *P.aeruginosa* was done by biochemical tests (Gram-negative bacilli, citrate positive, non fermentative, triple sugar iron Alk/Alk, motile, hydrogen sulfide (H2S) negative, urease) and by API system (bioMèrieux).

Carbapenem Resistance:

P.aeruginosa C.I.s were tested for carbapenems susceptibility using the Carba NP (Carbapenemase Nordmann-Poirel) test that is based on in vitro hydrolysis of a carbapenem (imipenem) by a bacterial lysate, which is detected by changes in pH values using the phenol red indicator (red to yellow/orange)[9].

Procedures:

Enzymatic extraction from the bacteria:

One calibrated loop (10µl) of the tested strain directly recovered from the Mueller-Hinton agar plate was resuspended in 100 µl of a 20 mmol/L. Tris-HCl lysis buffer, vortexed for 1 min, and further incubated at room temperature for 30min.This bacterial suspension was centrifuged at 10,000 X g at room temperature for 5 min then the supernatant was used for testing for carbapenemase enzyme [9, 11].

Solution R (the revelation solution) was prepared by adding 2 ml concentrated red phenol solution 0.5%w/v to 16.6 ml distilled water then pH adjusted at 7.8by adding drops of NaOH solution.

Solution A was prepared by adding 180 µl of ZnSO4 10 mM to solution R to obtain a final concentration of 0.1 mM.

Thirty microliters of the supernatant, corresponding to the enzymatic bacterial suspension was added to each of two 1.5 ml eppendorf tubes (A and B). One hundred µl of solution A was added to **tube A** and 100 µl solution A + imipenem 6 mg/ml to **tube B**. All were incubated at 37°C for a maximum of 2 hours[9].

Biofilm detection by Microtiter Plate method (MTP):

The biofilm quantification for *P. aeruginosa* C.I.s were assayed on microtiter plates and interpreted as described previously by Christensen et al[12]. Bacteria subcultured onto trypticase soy agar (Oxoid) plus 5% glucose then transferred to trypticase soy broth plus 5% glucose, then were inoculated in wells of polystyrene plate. Negative control wells contained sterile BHI broth. After incubation for 48h., the plates were shaken then fixed with methanol for 10 min. The attached bacterial material was stained by adding 150 ml crystal violet for 20 min. The optical density was measured and interpreted with an ELISA reader at a wavelength 570nm[12]. And classified into four categories, according to the mean optical densities (OD) in relation to the ODc results. The mean OD value obtained from negative control well was deducted from the average test OD values to obtain the OD value of each test strain. The categories were based on the following criteria: non-adherent if ODi ≤ ODc; weakly adherent (+) if ODc < ODi ≤ 2 x ODc; moderately adherent (++) if 2 x ODc < ODi ≤ 4 x ODc; or strongly adherent (+++) if 4x ODc < ODi [4].

Molecular study:

- ***DNA extraction*:** was performed as follows: the isolates were inoculated into 5ml of trypticase soy broth and incubated for 24h at 37°C. Cells from 1.5ml of an overnight culture were harvested by centrifugation for 5min at 10,000 rpm where the pellet was re-suspended in 500µl of distilled water. The cells were lysed by heating them at 95°C for 10min and cellular debris was removed by centrifugation for 5min .The supernatant was used as a source of template DNA for amplification [13].
- ***Genotypic detection of IMP and VIM genes coding for carbapenem resistance:*** Done by using multiplex PCR assay where each PCR reaction mixture (50 µl) consisted of 25µl Taq green PCR Master Mix, 1µl for each IMP-F (5′- GAG TGG CTT AAT TCT CRA TC-3′), IMP-R (5′AAC TAY CCA ATA YRT AAC -3′) 120bp gene primer, VIM-F (5′-GTT TGG TCG CAT ATC GCA AC-3′) and VIM-R (5′-AAT GCG CAG CAC CAG GAT AG-3′) 382bp gene primer (Qiagen, Germany),1µl template DNA and 20µl nuclease-free water[13]. The PCR program was performed in a thermal cycler (Biometra-Germany) and it consisted of an initial incubation of 10 min at 37°C and an initial denaturation step at 94°C for 5 min, followed by 30 cycles of DNA denaturation at 94°C for 1 min, primer annealing at 54°C for 1 min, and primer extension at 72°C for 1.5 min. Finally, an extended 72°C step for 10min was recorded to ensure that all of the products are full-length After the last cycle, the products were stored at 4°C[13].

- ***Genotypic detection of RhlI and LasI quorum sensing genes coding for biofilm formation:***
  For ***RhlI*** gene, amplification reaction mixtures (25 µl) containing 12.5 µl of Taq green PCR Master Mix, 2.5 µl for each RhlI-F(5′- CTC TCT GAA TCG CTG GAA GG-3′) and RhlI-R(5′- GCG AAG ACT TCC TTG AGC AG-3′) 245 gene primer (Qiagen, Germany), 5 µl template DNA and 2.5 µl nuclease-free water. Amplification was performed in a DNA thermal cycler (Biometra-Germany), and it consist of an initial denaturation at 94°C for 3 min, and 30 cycles of denaturation at 95°C for 30 s, annealing at 55.5°C for 30 s, extension at 72°C for 1 min and a final extension at 72°C for 5 min[3].
  For ***LasI*** gene, amplification reaction mixtures (25 µl) containing 12.5µl of Taq green PCR Master Mix, 1.25 µl of LasI-F(5′- CGT GCT CAA GTG TTC AAG G-3′) and LasI-R(5′- TAC AGT CGG AAA AGC CCAG-3′) 295 gene primer (Qiagen, Germany), 2 µl template DNA and 8 µl nuclease-free water was added[14]. Amplification was performed in a DNA thermal cycler (Biometra-Germany), with the following cycling program 30 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 1.5 min[14].
- ***Detection of amplified DNA products***: on 1.5% agarose gels using ethidium bromide staining (Sigma, USA). A DNA ladder (100-1000bp) (Fermentas, Germany) was used to estimate allele sizes in base pairs (bp) for the gel.

Statistical analysis:

The data were analyzed using SPSS version 20.0 on IBM compatible computer (SPSS Inc., Chicago, IL, USA). The data was described as frequency and percentage for qualitative data. Analysis was done using Chi-square test for comparing more than 2 groups of qualitative data. Z test for comparing two proportions in two independent groups. P value was considered significant at a value less than (0.05).

RESULTS

In the current study, biofilm formation by *P. aeruginosa* C.I.s and its correlation with quorum sensing genes (*Rh1I* and *LasI*) and carbapenem resistance were investigated.

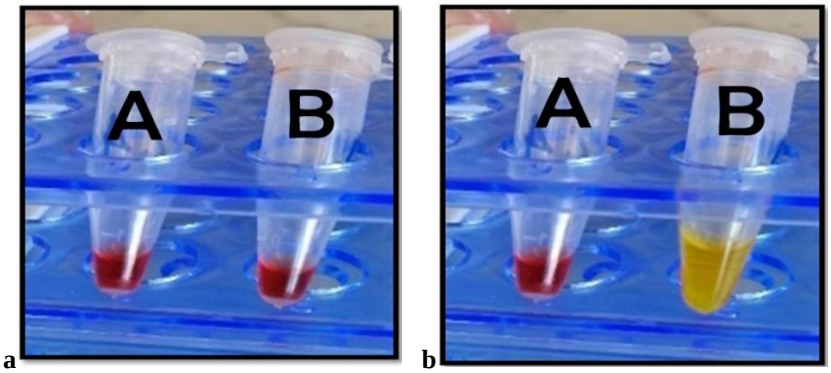
In our study, a total of 62 *P. aeruginosa* C.I.s were recovered from different clinical specimens where the highest percentage was from catheter specimens (27.5%); followed by urine (20.9%), blood (19.3%), wound (17.7%), tracheal aspirates (11.4%), and lastly peritoneal fluid specimens (3.2%). (table 1).

In the current work, by tissue culture plate method (TCP), biofilm was observed in 70.9% of *P. aeruginosa* C.I.s. With respect to isolation site, 85.7% of tracheal aspirate C.I.s were biofilm producers, followed by urine (76.9%), catheter specimens (70.6%), blood (66.7%), wound (63.6%) and peritoneal fluid C.I.s (50.0%) (table1).

Table1: **Relation between *P. aeruginosa* biofilm formation, carbapenemase production, QS, MβL genes and specimen types.**

***P. aeruginosa* C.I.s (No= 62)**	**Specimen types**						
	Tracheal aspirate 7(11.4%)	**Blood 12 (19.3%)**	**Urine 13 (20.9)**	**Wound 11 (17.7%)**	**Catheter 17 (27.5%)**	**Peritoneal fluid 2(3.2%)**	**Total N = 62**
TCP testing							
Biofilm non producer	1(14.3%)	4(33.3%)	3(23.1%)	4(36.4%)	5(29.4.0%)	1(50.00%)	18(29.1%)
Biofilm producer	6(85.7%)	8(66.7%)	10(76.9%)	7(63.6%)	12(70.6%)	1(50.0%)	44(70.9%)
Carba NP test							
+Ve	4(57.1%)	5(41.7%)	10(69.9%)	7(63.6%)	12(70.6%)	2(100%)	40(64.5%)
-Ve	3(42.9%)	7(58.3%)	3(23.1%)	4(36.4%)	5(29.4%)	0(0.0%)	22 (35.5%)
QS genes							
1- RhlI gene only+ve	1(14.3%)	4(33.3%)	6(46.1%)	4(36.4%)	8(47.1%)	1(50.0%)	24(38.7%)
2-LasI gene only+ve	2(28.6%)	3(25.0%)	4(30.7%)	1(9.0%)	3(17.6%)	0(0.0%)	13(20.9%)
3- *RhlI* + *LasI* genes +ve	2(28.6%)	4(33.3%)	2(15.4%)	3(27.3%)	6(35.3%)	0(00.0%)	17(27.4%)
4- Non *RhlI* or *LasI* genes	2(28.6%)	1(8.4%)	1(7.8%)	3(27.3%)	0(0.0%)	1(50.0%)	8(12.9%)
5- Total+ve *Rh1I* gene	3(42.8%)	8(66.6%)	8(61.5%)	7(63.6%)	14(82.4%)	2(100.0%)	42(67.7%)
6- Total +ve *LasI* gene	4(57.2%)	7(58.3%)	6(46.2%)	4(36.4%)	9(52.9%)	0(0.0%)	30(48.4%)
MβL genes							
1- *IMP* gene only +ve	2 (28.6%)	3 (25.0%)	5 (38.5)	3 (27.3%)	7 (41.2%)	1 (50.0%)	21 (33.9)
2- *VIM* gene only +ve	1(14.3%)	2 (16.7%)	3(23.1)	5 (54.5%)	3 (17.6%)	1 (50.0%)	15 (24.2)
3- *IMP* & *VIM* genes +ve	1(14.3%)	1 (8.3%)	2 (15.4%)	3 (27.3%)	3 (17.6%)	0 (0.0%)	10 (16.1)
4- *IMP* & *VIM* genes -ve	3 (42.9%)	6 (50.0%)	3 (23.1%)	0 (0.0%)	4 (23.5%)	0 (0.0%)	16 (25.8)
5- Total *IMP* gene +ve	3 (42.9%)	4 (33.3%)	7 (53.8%)	6 (54.5%)	10 (58.8%)	1 (50.0%)	31 (50.0%)
6- Total *VIM* gene +ve	2 (28.6%)	3 (25.0%)	5 (38.5%)	8 (72.7%)	6 (35.3%)	1 (50.0%)	25 (40.3%)

In this study, by Carba NP test, 64.5% of *P. aeruginosa* C.I.s were carbapenemase producers. Where 100%, 70.6%, 69.9%, 63.6%, 57.1% and 41.7% of *P. aeruginosa* isolated from peritoneal fluid, cathter, urine, wound, tracheal aspirate, and blood specimens showed carbapenemase activity (table 1 & fig.1).



a b

Fig 1: Shows that **tube A** without adding antibiotic, **tube B** with imipenem adding. (a): **red** colour of **tube A and B** indicate **negative** carbapenemase production; (b) **red** colour of **tube A** and **yellow** colour of **tube B** indicate **positive** carbapenemase production.

In this study we used PCR for detection of quorum sensing genes (*LasI* and *Rh1I*) as indicators for biofilm formation ability (table1& fig.2a,b) and MβL genes (*IMP* and *VIM)* for carbapenem resistance detection among different specimen types (table 1& fig.3).

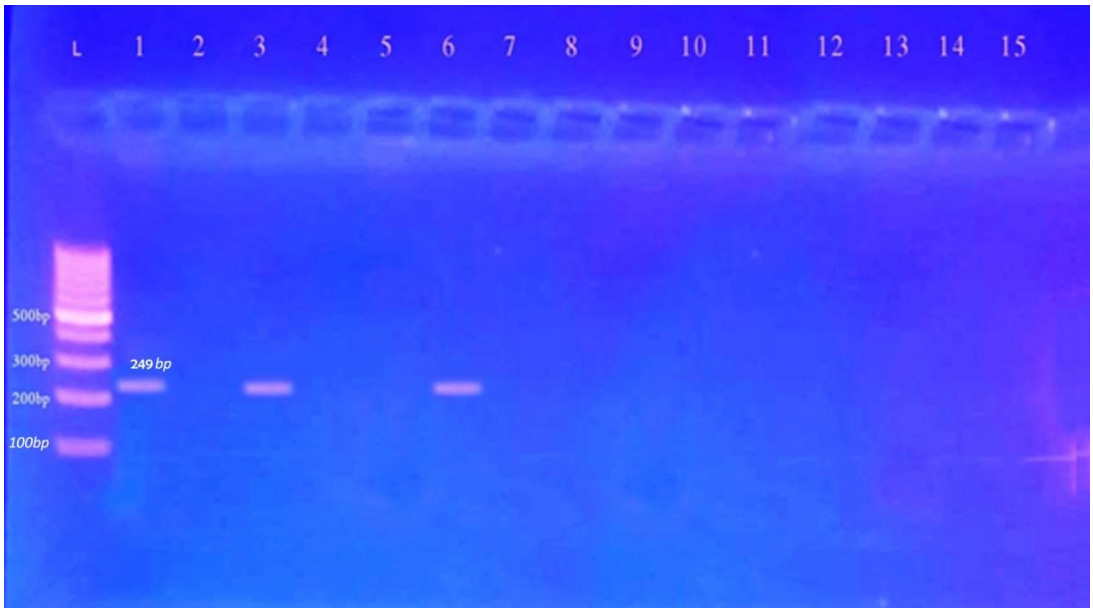


Fig.2 a: Gel electrophoresis of PCR amplification product of ***RhlI*** gene. Lane L; DNA ladder (100-1000bp), lanes 1, 3 and 6 + ve ***RhlI*** gene (249bp).

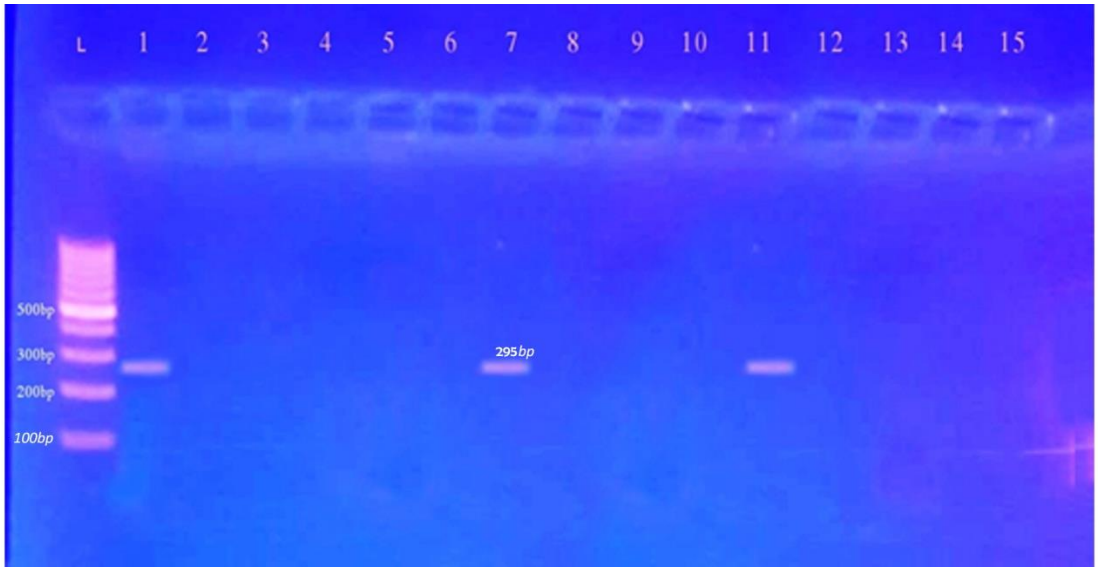


Fig.2b: Gel electrophoresis of PCR amplification product of ***LasI*** gene. Lane L; DNA ladder (100-1000bp), lanes 1, 7 and 11+ ve ***LasI*** gene (295bp).

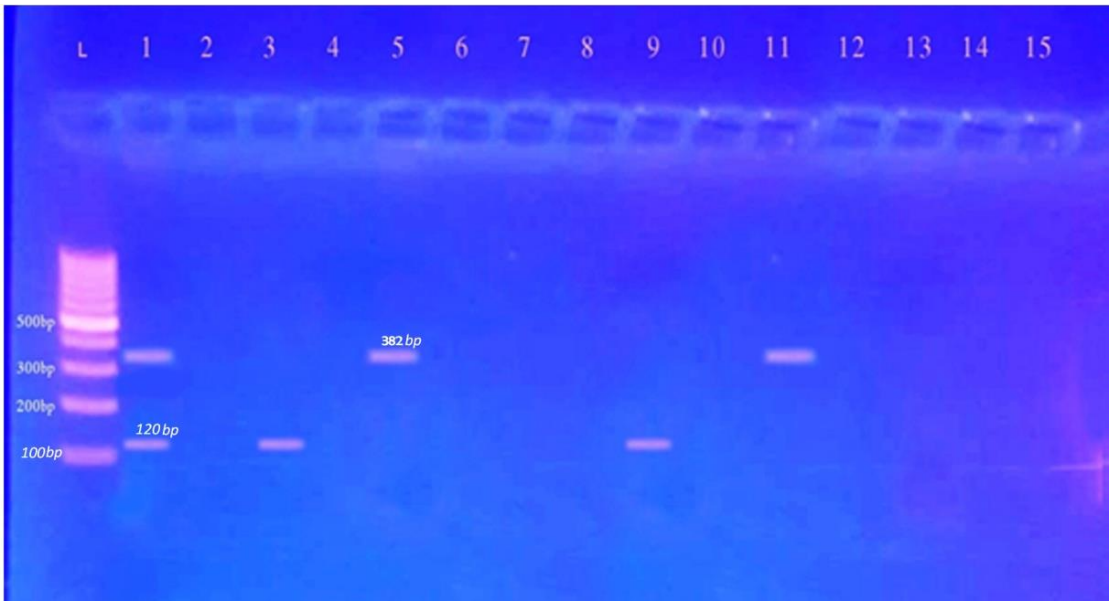


Fig. 3: Gel electrophoresis of PCR amplification product of *IMP* and *VIM* MβLs genes. Lane L; DNA ladder (100-1000bp), lanes 1, 3 and 9; + ve *IMP* gene (120bp), lanes 1, 5 and 11; + ve for *VIM* gene (382).

In our study, most of biofilm forming *P. aeruginosa* C.I.s (36/44) showed strong adherence (table2).

In the current work, there was a highly significant relation between biofilm quantification and the presence of *RhlI* gene ($P< 0.001$), *LasI* gene ($P=0.02$) and both of them ($P< 0.001$) (table2).

The TCP method is considered a reliable method for biofilm detection in comparison with PCR method (table2).

Table 2: Relation between biofilm quantification and QS genes.

***P. aeruginosa* C.I.s (N=62)**					**Total 62(100%)**	**Z test (p value)**
QS genes	**Biofilm quantification by TCP**					
	Non adherent 18 (29.1%)	Weakly adherent 2 (4.5%)	Moderately adherent 6(13.6%)	strongly adherent 36(81.9%)		
1- RhlI gene only	3(12.5%)	1(4.2%)	5(20.8%)	15(62.5%)	24(38.7%)	20.4 (<0.001)
2-LasI gene only	4(30.8%)	1(7.6%)	1(7.6%)	7(53.8%)	13(20.9%)	9.69 (0.02)
3- *RhlI + LasI* genes	3(17.6%)	0(0.0%)	0(0.0%)	14(82.4%)	17(27.4%)	15.0 (<0.001)
4-Non *RhlI* or *LasI* genes	8(100%)	0(0.0%)	0(0.0%)	0(0.0%)	8(12.9%)	Ref.
Total	18(29.1%)	2(3.2%)	6 (9.7)	36 (58.1%)	62(100%)	
Biofilm detection by TCP	**QS genes**					
	+ve 54 (87.1%)		**-ve 8(12.9%)**			Total 62(100%)
Biofilm non producer 18 (29.1%)	44 (81.5%)		0 (0.0%)			18 (29.9%)
Biofilm producers 44(70.9%)	10 (18.5%)		8 (100%)			44 (70.9%)
Sensitivity	81.5%					
Specificity,	100%					
Positive predictive value,	100%					
Negative predictive value	44.4%					
Accuracy	83.9%					

Ref. = Reference category.

In our study, there was a highly significant relation between the presence of MβL genes**,** *IMP* and *VIM*, and positive Carba NP testing (p<0.001). And the Carba NP test is a golden test for carbapenem resistance detection (table 3).

Table 3: Relation between Carba NP test results and presence of MβL genes

***P. aeruginosa* C.I.s (N= 62)**			**Z test (p value)**
MβL genes	**Carba- NP test**		
	+ve 40(64.5%)	**-ve 22 (35.5%)**	
1- *IMP* gene +ve (21: 33.8%)	16(76.2%)	5(23.8%)	3.09(0.002)
2- *VIM* gene +ve (15: 24.2%)	11(73.4%)	4(26.6%)	2.20 (0.03)
3- *IMP & VIM* genes +ve (10:16.2%)	10(100.0%)	0(0.0%)	4.02 (<0.001)
4- *IMP & VIM* genes -ve (16:25.8%)	3(18.8%)	13(81.2%)	3.18 (0.001)
Carbapenem resistance	MβL genes		
	+ve 46 (74.2%)	**-ve** 16 (25.8%)	Total 62 (100%)
Carba NP +ve	37 (80.4%)	1 (18.7%)	40 (64.5%)
Carba NP -ve	9 (19.6%)	13 (81.3%)	22 (35.5%)
Sensitivity	80.4%		
Specificity	81.3%		
Positive predictive value and	92.5%		
Accuracy	85.5%		

In the current work, there was non significant relation between biofilm formation by *P. aeruginosa* C.I.s and carbapenemase activity or presence of MβL genes (*P= 0.51*) (table 4).

Table 4: Association between biofilm formation and carbapenem susceptibility

Carbapenem susceptibility	Biofilm formation by TCP		Total N = 62	Z test (p value)
	non producer 18(29.1%)	producer 44(70.9%)		
Carba NP test				
- CRPA	10(55.5%)	30(68.2%)	40 (64.5%)	0.66 (0.51)
- Carbapenem sensitive *p. aeruginosa*	8(44.5%)	14(31.8%)	22 (35.5%)	0.66 (0.51)
MβL genes				
- *IMP* +ve	5(27.8%)	16(36.4%)	21 (33.8%)	0.35 (0.72)
- *VIM* +ve	4(22.2%)	11(25%)	15 (24.2%)	0.09 (0.93)
- *IMP & VIM* +ve	3(16.7%)	7(15.9%)	10 (16.2%)	0.30 (0.76)
- *IMP & bla VIM* -ve	6(33.3%)	10(22.7%)	16 (25.8%)	0.55 (0.58)

DISCUSSION

Due to an increasing number of non curable, continual *P.aeruginosa* infections; so we have to develop new advances that manage this problem. Biofilm formation is a survival approach for *P. aeruginosa* to adjust them self to their living environment. Under the biofilm protection, microbial cells become tolerant and resistant to antibiotics and immune responses, that make the clinical treatment of biofilm infections more difficult[15].

In our study, a total of 62 *P. aeruginosa* C.I.s were recovered from different clinical specimens where the highest percentage was from catheter specimens (27.5%); and the least from peritoneal fluid specimens (3.2%). Our results matched with those by Khorvash et al[2] where urine (30.5%), catheter (29.08%), and blood (25%) specimens represented the highest percentage of *P. aeruginosa* C.I.s followed by tracheal aspirate (22.9%), wound (8.3%), and peritoneal fluid (9%). Regarding Fattouh et al[16]results, the highest percentage of *P. aeruginosa* isolates were from cystic fibrosis patients (20%); followed by those with infected orthopedic implants (15%) or ventilator-associated pneumonia (VAP) (15%); then UTI ones (14%); those with surgical site infection (10%); infected diabetic foot (10%); patients with COPD (10%) and lastly (6%) from patients with infected burn.

For *P. aeruginosa* C.I.s biofilm formation, herein, the majority (70.9%) were biofilm producers by TCP method that matched with Lima et al[4]results where 75% were biofilm producers. But is higher than that by Fattouh et al[16] and Hassan et al[17] where 58% were biofilm producers. Stepanovic et al[18] and Gurung et al[19]described that only 33% were biofilm producers. With respect to isolation site, in our work tracheal aspirate C.I.s represented the highest tendency (85.7%) for biofilm formation. While Gurung et al[19] observed that *P. aeruginosa* isolated from sterile fluid showed 100 % biofilm production and the respiratory isolates showed least tendency (27.58%).

Carbapenem resistance among *Pseudomonas* spp. is a concern, some reports have suggested recent increases in the prevalence of carbapenem resistant *P. aeruginosa* (CRPA)[20].

In our study, CRPA represented 64.5% by Carba NP test. That was observed in 100% of peritoneal fluid isolates followed by cathter isolates (70.6%), urine (69.9%), wound (63.6%), tracheal aspirate (57.1%) and finally blood isolates. Walters et al[20] mentioned that respiratory specimens (44.0%) including Includes sputum, tracheal aspirate and bronchoalveolar lavage represented the the most frequent sources of CRPA followed by blood (47.5%), urine (40.7%), peritoneal fluid (4.8%) and wound (12.8%).

In the present study we used PCR for detection of quorum sensing genes (*Rh1I* and *LasI*) as indicators for biofilm formation ability where both *RhlI* and *LasI* quorum sensing genes were present in 27.4% of *P. aeruginosa* C.I.s. *Rh1I* gene was present in (67.7%) of isolates and *LasI* gene was present in (48.4%) of isolates. The *RhlI* gene or *LasI* gene presented alone in 38.7% and 20.9% of C.I.s respectively that is higher than results documented by Fattouh et al[16] that (4%) of isolates contain both *LasI* and *Rh1I* genes, *Rh1I* gene was present in (43%) of isolates and *LasI* gene was present in (74%) of isolates, (2%) of isolates contain *Rh1I* gene only and (9%) of them found to contain *LasI* gene only.

In our study, the genes coding for carbapenem resistance were distributed as follow, both *IMP* and *VIM* genes were present in 16.1%, *IMP* or *VIM* gene presented alone in 33.9% and 24.2 % of the C.I.s respectively. That is nearly similar to Khorvash et al[2] results where *IMP* and *VIM* genes were detected in 31.3% and 14.6% of the isolates, respectively and Yousefi et al[21] results that 17.31% of CRPA had *VIM* gene. While Kazeminezhad et al [22]documented that 31.5% and 10.5% of the isolates had respectively *VIM* and *IMP* gene alone .

Here in, both *IMP* and *VIM* genes and mostly present in wound isolates (27.3%) that differs from results EL Maraghy et al[23] study that the *IMP* gene was

not expressed in any strain and the *VIM* gene was present at the highest level in blood specimens.

Regarding biofilm quantification analyses, 81.9%, 13.6%, 4.5% of *P. aeruginosa* C.I.s were strongly, moderately, weakly adherent and 29.1% were non adherent that higher than that by Lima et al[4]where 10%, 25%, 40% were strongly, moderately and weakly adherent, and 25% were non-adherent and that by Perez et al[24] who recorded that 4% of *P. aeruginosa* C.I.s were moderately adherent and 96% were weakly adherent. Fattouh et al[16] study recorded that 17%, 19% and 22% of *P. aeruginosa* C.I.s were strongly, moderately and weakly adherent respectively.

In the current work, there was strong association between the presence of QS genes and biofilm quantification where 82.4% of the isolates that had both *RhlI* and *LasI* genes showed strong biofilm adherence, while all isolates that had not any QS genes couldn't form biofilm this is opposite to that by Fattouh et al[16] where, two isolates were negative for all QS genes but they were biofilm producers by TCP. They explained that by presence of other virulence factors which may not be controlled by QS.

The TCP method showed 81.5% sensitivity, 100% specificity,100% positive predictive value, 44.4% negative predictive value and 83.9% accuracy for biofilm detection in comparison with PCR method that nearly matched with Fattouh et al[16] reported 70% sensitivity, 90% specificity, 96.6% positive predictive value and 42.9% negative predicative value of this method.

In our study, the isolates that had *IMP* gene, *VIM* gene and those had both *IMP* and *VIM* genes showed carbapenemase activity by Carba NP test in the following rates, 76.2%, 73.4% and 100%. The Carba NP test had 80.4% sensitivity, 81.3% specificity, 92.5% positive predictive value and 85.5% accuracy in relation to PCR results for detection of carbapenem resistance that is matched with Dortet et al[6] results where specificity and sensitivity of the Carba NP test 100% and 94.4%, respectively while Elanany et al[25] mentioned that Carba NP test had 40% sensitivity, 100% specificity, 100% PPV and 96.9% NPV for detection of carbapenem resistance.

In the current work, there was non significant relation between biofilm formation by *P. aeruginosa* C.I.s and carbapenem resistance. This was in accordance with Lima et al[4] results that the biofilm production was independent of the susceptibility profile of the bacteria. And explained this by that, biofilm production may be related to the failure of empirical therapy, as the biofilm reduces the access of antimicrobials, preventing them from eliminating the bacteria present in the biofilm.

CONCLUSIONS

There was a high tendency for biofilm formation by *P. aeruginosa* C.I.s. The presence of QS genes increased the biofilm quantification level. Carba NP test is novel test for carbapenem resistance detetion. And non significant relation between biofilm formation and carbapenem resistance among *P. aeruginosa* C.I.s.

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Conflicts of interest:

- The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.
- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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