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BIOFILM FORMATION AND METALLO β-LACTAMASE PRODUCTION IN CLINICAL AND ENVIRONMENTAL ISOLATES OF *PSEUDOMONAS AERUGINOSA*

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ABSTRACT : In recent years, the worldwide emergence of multidrug-resistant isolates of *Pseudomonas aeruginosa* has been observed. This opportunistic pathogen produces mechanisms of resistance to several antibiotics. The resistance to antibiotic in *P. aeruginosa* isolates has been associated with bacterial biofilm formation and production of metalobetalactmase. Present study undertook to assess antibiotic susceptibility, metalobetalactamase and biofilm formation in clinical and environmental isolates of *P. aeruginosa* that resistant to antibiotics. A total of 586 samples were collected from ICU unit in Hilla Teaching Hospital. *Pseudomonas aeruginosa* isolates were recovered using cetrimide agar base. Kirby-Bauer disc diffusion technique was used for antimicrobial susceptibility testing. Combined disc diffusion technique was used for the detection of MBL production, while we use 96 well flat plate bottoms for detection of biofilm formation. In this study, the most isolates of *P. aeruginosa* were obtained from burns 30(62.5%). In environmental samples, the most isolates were obtained from tools 20 (42.4%). Biofilm formation was observed in clinical sample of the 48 test isolate only 8(16.7%) strong positive in which that formed biofilm weakly positive 6(12.5%). While in the environmental sample of the 48 test isolate only 4(8.3%)strong positive and formed biofilm and 8(16.7%) weakly positive. The result showed that the majority of *P. aeruginosa* isolates were found in burns followed by wounds, urine and sputum samples.While, tools followed by beds, and floors have the major isolates in the environmental samples. The most *P. aeruginosa* isolates displayed multidrug resistance to tested antibiotics as well as a variable activity in biofilmformation as weak positive and strong positive results.

Key words : *Pseudomonas aeruginosa*, antibiotic susceptibility, metallo â-lactamase, biofilm formation.

INTRODUCTION

Pseudomonas aeruginosa is associated with an ever-widening spectrum of infections. Some infections occur predominantly in the community, in healthcare settings especially in the intensive care unit (ICU). Infections may be associated with significant morbidity, mortality and antimicrobial resistance (Kerr *et al*, 2009). The *P. aeruginosa* is the main cause of mortality in cases of bacteremia and the second most common bacterium causing sepsis in the ICU. In addition, it has been implicated in urinary tract infections, burn wounds, ventilator associated pneumonia and multiorgan system failure (Gad *et al*, 2007).

Infections caused by *P. aeruginosa* are difficult to treat, because it has innate resistance to several antibiotics (Heydari and Eftekhar, 2015). Multi-drug resistant *P. aeruginosa* isolates are by definition resistant to at least three antibiotics from the following classes: carbapenems (β-lactams), aminoglycosides and fluoroquinolones. Therefore, the treatment of pseudomonal infections now represents a significant challenge to clinicians and health authorities (Zhao and Hu, 2010). *Pseudomonas aeruginosa* resistance to antibiotic are due to the outer membrane permeability, active efflux pump system, alteration of the penicillin binding proteins and hydrolyzing enzymes. Acquired metalobetalactamase (MBL) have emerged recently as one of the most important mechanism, it was able to hydrolyze almost all the β-lactams as well as carbapenems (Jena *et al*, 2015). Emergence of MBL producing *P. aeruginosa* in intensive care units (ICUs), due to high usage of broad spectrum antibiotics in ICUs. This results in eradication of competitive flora and subsequent selection of multidrug-resistant strains (Kali *et al*, 2013). It was first reported from Japan in 1991, described various parts of the world, including Asia, Europe, Australia, South America and North America. In some countries, *P. aeruginosa* possessing MBLs constitute highly percentage of all nosocomial isolates (Manoharan *et al*, 2010).

Biofilms are dense bacterial communities attached to a solid surface and surrounded by an exopolysaccharide

matrix. Persistent infections caused by bacterial biofilms have been associated with a number of medical conditions. One of the most important features of bacterial biofilms is their resistance to antimicrobial agentsand components of the host immune system and consequently produces chronic and difficult infections (Drenkard, 2003). The present study aimed to isolate *P. aeruginosa* from clinical and environmental samples at Hilla teaching hospitals by using *Pseudomonas* chromogenic agar and cetrimide agar base as a selective media to determine antibiotic susceptibility patterns and biofilm formation.

MATERIALS AND METHODS

Bacterial isolates

The study included 586 samples were taken different hospitals in Hilla city during period from October 2018 to January 2019 and only 96 samples was diagnosed as *P. aeruginosa*. The isolates were collected from clinical and environmental samples. A total numbers of clinical samples isolates from different infections such as burn (30), wound (12), UTI (4) and sputum (2) isolates while environmental isolates (48) were collected from the different sites of hospitals including tools mask, sacker, surgical instruments (20), hospital floors (10), beds (10) and other place of hospital (8) isolates. These specimens collected using transport swabs and immediately inoculated into pseudomonas chromogenic agar and cetrimide agar base (selective media for *P. aeruginosa*), then incubated at 37°C for 24 hours. The standard laboratory diagnosis procedures can be depended on the diagnostic steps mentionedby (MaCfaddin, 2000). The grown colonies on the culture media with characterized diffusible pigments were selected for further analysis.

Antimicrobial susceptibility test

Antimicrobial susceptibility testing was performed on Mueller-Hinton agar (MHA) plates by Kirby-Bauer disc diffusion method, according to Clinical Laboratory Standards Institute (CLSI) guidelines. The following antibiotics were tested by disk diffusion method: Aminoglycosides (Gentamicin, tobramycin, amikacin), Monobactams (Aztreonam), Anti pseudomonal carbapenems (Impenem, Meropenem), Anti pseudomonal cephalosporin's (ceftazidime, cefepime, ceftriaxone, cefotaxime) Anti pseudomonal fluoroquinolones (ciprofloxacin, levofloxacin), Anti pseudomonal penicillin's â-lactamase inhibitors (Ticarcillin-clavulanic acid, anti-cell membrane synthesis (colstin, polymyxin) (Mast Diagnostic, UK). The *in vitro* susceptibilityof96*P. aeruginosa* isolates to 16 antimicrobial agents was determined via diffusion method according to Clinical and Laboratory Standards Institute Instructions (CLSI, 2016). The bacterial isolates were cultured in a nutrient broth (NB, Conda, Spain) and then sub-cultured on nutrient agar (NA) for 24 h at 37°C. Thereafter, turbidity was adjusted in 0.85% sterile normal saline solution to 0.5 McFarland's standard (10^8 CFU/mL) and then spread on MHA with a sterile cotton swab. Antibiotic discs were placed onto MHA, gently pressed down to ensure complete contact with the agar inoculated with bacteria and then incubated for 24 h at 37°C. The tests were performed in triplicate, and the antibacterial activity was expressed as mean of the inhibition zone diameter in millimeters (mm).

Biofilm assay

Biofilm formation was determined by the microtiter plate assay, as previously reported by Heydari and Eftekhar (2015). Briefly, 200 µL of a 1:100 dilution of each overnight bacterial culture in trypticase soy broth (TSB) (Conda, Spain) was inoculated into four wells of a 96-well flat bottomed polystyrene plate. Following incubation at 37°C for 22-24 h, the cultures were removed and the wells were washed twice with 200 µl of phosphate buffered saline (PBS, pH = 7.4) and dried at room temperature. Biofilms were stained with 0.1% safranin (CDH, India) solution in water for 15 minutes and the plates were washed in distilled water and dried at room temperature. The optical density (OD) of the biofilms was measured at 492 nm using an ELISA reader (BioTeck, USA). Biofilm formation was considered negative at ODs below 0.12, weakly positive at ODs 0.12-0.24 and strong positive at ODs > 0.24. Each test was repeated on three different days and the results were reported as the mean of the obtained values.

RESULTS

Bacterial isolation

Out of the 587 samples, only 96 isolate of *Pseudomonas aeruginosa* isolated from clinical and environments (48 isolates each). In clinical isolate, the most isolates were obtained from burns 30(62.51%), wound 12(25), urine 4(8.33%), sputum 2(4.16%). In environmental samples, the most isolates were obtained from tools 20(42.40%), beds 10(20.73%), floor 10(20.73%), others 8(16.14%) (Table 1).

Antibiotic susceptibility

Antimicrobial susceptibility testing was carried out following the method outlined by the CLSI (2016). Sixteen antibiotics were tested for antibacterial activity by Kirby-Bauer Disc diffusion method. In clinical samples, the result showed that the resistance were 100% for (Amoxicillin-clavulanic acid, Ceftazidime, Cefepime, Ceftriaxone,

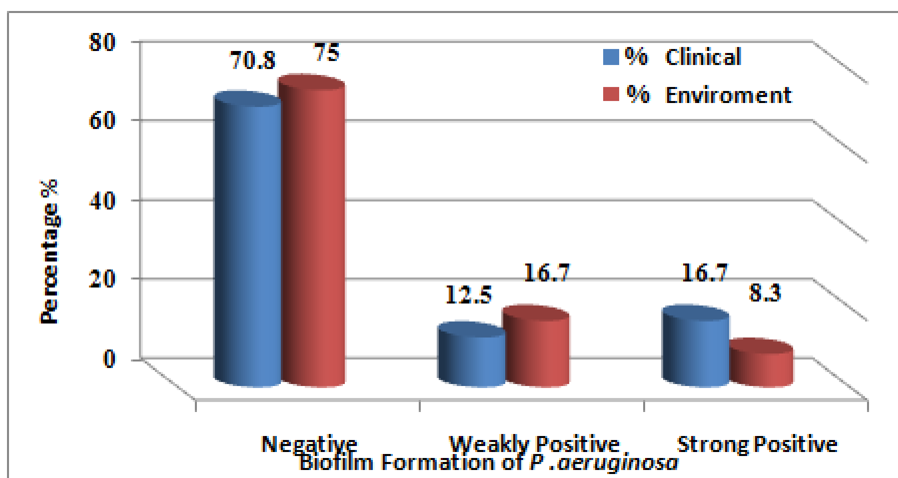

Fig. 1 : Biofilm formation of *P. aeruginosa* among clinical and environmental samples.

Table 1 : Numbers and percentage of *P. aeruginosa* isolates from clinical and environmental samples

Source of samples	Samples	No (%)
Clinical	Burn	30(62.51%)
	Wound	12(25%)
	Urine	4(8.33%)
	Sputum	2(4.16%)
	Total	48(100%)
Environmental	Tools	20(42.40%)
	Beds	10(20.73%)
	Floor	10(20.73%)
	Others	8(16.14%)
	Total	48(100%)

Cefotaxime, Meropenem, Ticarcillin-clavulanic acid, Aztreonam, Gentamicin, Tobramycin) and intermediate for Imipenem 16(33.33%, amikacin 2(4.16%), ciprofloxacin 2(4.16%), levofloxacin 2 (4.16%) and sensitive for colstin 45(93.75%), polymyxin B 47(97.91%).

In the clinical samples, the result showed that the resistance were 100% for (Amoxicillin / clavulanic acid, Ceftazidime, Cefepime, Ceftriaxone, Cefotaxime, Meropenem, Ticarcillin-Clavulanic acid, Aztreonam, Gentamicin, Tobramycin) and intermediate for Imipenem 16(33.33%) , amikacin 2(4.16%), ciprofloxacin 2(4.16%), levofloxacin 2(4.16%) and sensitive for colstin 45(93.75%), polymyxin B 47(97.91%).

While in the environmental samples the result showed that the resistance were 100% for (Amoxicillin-clavulanic

Table 2 : Antibiotic susceptibility of 48 clinical isolates.

No	Antibiotic	Sensitive isolates		Resistance isolates	
		No	%	No	%
1	Amoxicillin / clavulanic acid	0.0	0.0	48	100
2	Ceftazidime	0.0	0.0	48	100
3	Cefepime	0.0	0.0	48	100
4	Ceftriaxone	0.0	0.0	48	100
5	Cefotaxime	0.0	0.0	48	100
6	Imipenem	16	33.33	32	66.67
7	Meropenem	0.0	0.0	48	100
8	Ticarcillin-clavulanic acid	0.0	0.0	48	100
9	Aztreonam	0.0	0.0	48	100
10	Gentamicin	0.0	0.0	48	100
11	Tobramycin	0.0	0.0	48	100
12	Amikacin	2	4.16	46	95.83
13	Ciprofloxacin	2	4.16	46	95.83
14	Levofloxacin	2	4.16	46	95.83
15	Colistin	45	93.75	3	6.25
16	Polymyxin B	47	97.91	1	2.08

Table 3 : Antibiotic Susceptibility of 48 environmental isolates.

No	Antibiotic	Sensitive isolates		Resistance isolates	
		No	%	No	%
1	Amoxicillin/clavulanic acid	0	0	48	100
2	Ceftazidime	0	0	48	100
3	Cefepime	0	0	48	100
4	Ceftriaxone	0	0	48	100
5	Cefotaxime	0	0	48	100
6	Imipenem	7	14.58	41	85.41
7	Meropenem	5	10.41	43	89.58
8	Ticarcillin-clavulanic acid	0	0	48	100
9	Aztreonam	2	4.16	46	95.84
10	Gentamicin	1	2.08	47	97.92
11	Tobramycin	0	0	48	100
12	Amikacin	3	6.2	45	93.75
13	Ciprofloxacin	2	4.16	46	95.84
14	Levofloxacin	1	2.08	47	97.91
15	Colistin	45	93.75	3	6.2
16	Polymyxin b	46	95.84	2	4.16

acid, Ceftazidime, Cefepime, Ceftriaxone, Cefotaxime, Ticarcillin-clavulanic acid, Tobramycin) and intermediate for imipenem 7 (14.58%), Neropenem 5(10.41%), amikacin 3(6.2%), ciprofloxacin 2(4.16%), aztreonam 2(4.16%), Gentamicin 1 (2.08%), Levofloxacin 1(2.08%) and sensitive to colstin 45(93.75%), polymyxin B 46(95.84%).

Biofilm formation

In clinical samples, out of 48 isolates only 8(16.7%) were strong positive in which that formed biofilm, weakly positive 6(12.5%) and negative 34 (70.8%). In the environmental samples, out of 48 isolates only 4(8.3%) were strong positive and formed biofilm and 8(16.7%) weakly positive and 36(75%) are negative.

DISCUSSION

The present studyrevealed high rate of *P. aeruginosa* in clinical samples found in burns patients due to that significant colonization of *P. aeruginosa* in injured tissues that may induce a state of immunosuppression leading to patients complications.

This result agreement with the other result obtained by Pal *et al* (2017), he found that the high percentage of *P. aeruginosa* concentrated in burn patient and agreement with Nikokar *et al* (2013) he found that the high frequency rate of *P. aeruginosa* found in burn centers might be due to the prolonged hospital stay and intensive use of antibiotics.

While in the environmental samples (environment could be a main reservoir for *P. aeruginosa* infections in hospital). The high rate of *P. aeruginosa* isolates found in tools. This result was agreement with other result obtained by Tajeddin *et al* (2015) they said that the patients' oxygen masks, ventilators and bed linens were mostly contaminated with *P. aeruginosa*. In other study, Ana Lucia *et al* (2015), they said that the light of varied sources of bacterial transmission and infection, pieces of equipment that are not used in invasive procedures and which are collectively and repeatedly handled by the team providing care to critical patients in hospitals are potential reservoirs of pathogenic agents, which may survive or persist on their surfaces for months, besides being a continuous source of transmission if regular disinfection is not performed in these pieces of equipment. Because it has multidrug resistance activity to antibiotics used in present study, might be due to rapid genetic variability of *P. aeruginosa* as well as, it exhibits innate resistance to a wide range of antibiotics. This result was agreement with Streeter and Katouli (2016) they showed that *P. aeruginosa* has MDR due to intrinsically resistant to various antibiotics and the result agreement with Ranjan *et al* (2014) they show that *P. aeruginosa* showed very high resistance to various antibiotics. In present study a variable activity in biofilm formation this result agreement with the result obtained by Halstead *et al* (2015) they said that isolate of *P. aeruginosa* exhibited variable biofilm production. Steven *et al* (2015), they mentioned that; Biofilms are of great importance in infection control and healthcare-associated infections owing to their inherent tolerance and 'resistance' to antimicrobial therapies. It has been shown to develop on medical device surfaces, and dispersal of single and clustered cells implies a significant risk of microbial dissemination within the host and increased risk of infections.

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