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ORIGINAL ARTICLE

Study of Biofilm Relationship with ESBL and MBL Positive *Pseudomonas aeruginosa* Isolates from Hospital and Environmental setting in Tabuk, KSA

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ABSTRACT
Pseudomonas aeruginosa (P. aeruginosa) are increasingly found among the patients suffering from some sort of underlying diseases like cystic fibrosis. It has emerged as a model organism to investigate the formation of bacterial biofilm. Therefore, the present study aims to focus on the biofilm production and antibiotic resistance pattern of P. aeruginosa from the clinical and environmental settings. High resistance to Imipenem was found among the clinical isolates, as compared to environmental isolates. Based on the study findings, there were significant differences in positive biofilm between clinical and environmental isolates of P. aeruginosa, with clinical isolates showing high degree resistance to the antibiotics.
Keywords; *Antibiotic Resistance, Clinical Isolates, Environmental Isolates, Negative Biofilm, Positive Biofilm, Pseudomonas aeruginosa*

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INTRODUCTION

Pseudomonas aeruginosa (*P. aeruginosa*) is involved in diverse and severe opportunistic infection as it is commonly known hospital pathogens among immunocompromised patients [1]. The complications of *P. aeruginosa* result due to increase in multidrug-resistance (MDR) strains, which is a global issue [2,3]. In chronic infections, there is increased involvement of *P. aeruginosa* because of its capability of producing biofilm. It is known that biofilm comprises of microbial cells within extracellular DNA, exopolysaccharides, and extracellular matrix composed of proteins [4]. It provides protective life-style to the bacteria, making the treatment of antimicrobial compounds challenging, as well as costly [4].

There are three distinct exopolysaccharides comprising biofilm components of *P. aeruginosa* that include; polysaccharide synthesis locus (Psl), Pel, and Alginate [5]. The clinical isolates of *P. aeruginosa* originating from the lungs of patients of cystic fibrosis mainly produce alginate [5]. Alginate plays significant role in protecting biofilm and maintaining structural stability as it consists of α-l-guluronic acid and β-d-mannuronic acid [6]. AlgACD operon is responsible for controlling the synthesis of alginate in *P. aeruginosa*. The making of GDP-mannuronic acid from GDPmannose is catalyzed by AlgD, which is encoded by algD [7]. algD is a GDP-mannose dehydrogenase, which is responsible for mediating the transcription of Alg proteins and controlling alginate biosynthesis. It also has significant impact on the polymerization, alginate synthesis, and production of precursor GDP- mannuronic acid [5].Two different exopolysaccharides are produced by the isolates of *P. aeruginosa* obtained from different environments. Psl is made up of pentasaccharide repetitions that include; d-glucose, l-rhamnose, and d-mannose, and known as a neutral polysaccharide. Psl renders cell to surface as well as cell to cell interaction during the formation of biofilm that plays significant role to initiate its formation and offer further protection [8]. There are 15 co-transcribed genes in Psl operon that need to be synthesized. Ps1D gene encodes Ps1D protein in periplasm/outer membrane. This gene is responsible for the formation of biofilm, based on export of bioflm-relevant exopolysaccharide [9]. Pellicle formation is defined as formation of

polymer/cell layer at the air–liquid interface of a *P. aeruginosa*. The pel (pellicle) operon is responsible for controlling the formation of this layer. Partially acetylated glucosamine and galactosamine sugars make up Pel that is a cellulose-sensitive exopolysaccharide [10]. Seven genes ranging from pelA to pelG comprises the pel operon. It is known that UDP-glucose is used as a donor by PelF to act as a soluble glycosyltransferase for the biosynthesis of Pel exopolysaccharide [5].

The hospitalized patients need to be concerned about multidrug resistant strains infections of *P. aeruginosa*. It has emerged as a problematic human pathogen because it causes severe infection such as meningitis, urinary tract infections, urinary tract infections, and septicemia. The outstanding survival properties, antibiotic resistance, and increased virulence of *P. aeruginosa* is explained based on its increased potential of producing biofilm [11,12]. The antimicrobial therapy against bacteria is retarded because of the development of biofilm that act as a barrier resulting in treatment failure and incapability of immune system to recognize micro-organisms [13]. This intensifies the need of evaluating the ability to produce biofilm of multi drug resistant *P. aeruginosa* genotypically and phenotypically from clinical samples. Moreover, it is important to identify isolates that enhance understanding about organism pathogenesis, considering the increasing potential of biofilm towards antimicrobial resistance resulting in persistent infections by *P. aeruginosa*. In the similar context, the present study aims to evaluate the Biofilm Relationship with ESBL and MBL Positive *P. aeruginosa* Isolates from Clinical/Hospital and Environmental Origin in Tabuk.

MATERIAL AND METHODS

The bacterial isolates (*P. aeruginosa*) were obtained from University affiliated hospitals and also from different soil and food substances in Tabuk during December 2017 to May 2019. Standard biochemical and microbiological methods were performed to identify the isolates of *P. aeruginosa*. These methods include; oxidase and catalase tests, pigment production in agar, and reactions in triple sugar iron (TSI) agar at 42°C [2]. Disk diffusion agar method was used for determining the susceptibility of *P. aeruginosa* isolates, based on the recommendations by the Clinical and Laboratory Standards Institute (CLSI) [5]. The susceptibility testing was controlled using Escherichia coli ATCC 25922. It has been shown that the isolated of multidrug-resistant *P. aeruginosa* shows resistance toward one antimicrobial agent categorized into three or more antimicrobial agents [14].

Antimicrobial Sensitivity Testing

The antimicrobial sensitivity testing was done by the Kirby-Bauer disk diffusion method using Mueller-Hinton agar. This method was recommended by the Clinical and Laboratory Standards Institute. The selection of antibiotic panel for each group of isolates was done according to the Clinical and Laboratory Standards Institute (Table 1). All the discs were obtained from Hi-Media labs, India. Moreover, the guidelines of manufacturer were followed for deciding interpretative criteria for each antimicrobial test.

Table 1: Selection of antibiotic panel for each group of isolates.

Isolates	Unit
Amikacin	30 µg
Ceftazidime	30 µg
Cefepime	30 µg
Levofloxacin	5 µg
Tobramycin	10 µg
Amoxicillin	10 µg
Cefodoxime	30 µg
Cefepime	30 µg
Cefoxitin	30 µg
Cefixime	10 µg
Ofloxacin	5 µg
Cefotaxime	30 µg
Aztreonam	30 µg

Detection of Extended Spectrum β lactamases (ESBL)

Initially, the screening of *P. aeruginosa* for ESBL production by disc diffusion method. This method utilized cefepime, piperacillin, cephotaxime, and cefoparazone. It was later confirmed by cephalosporin/clavulanate combination disk test (disk potential test) using cefepime, cefepime+clavulanic acid, cefoparazone, cefoparazone+sulbactam, piperacillin, and piperacillin+ tazobactam and cephotaxime, cephotaxime+clavulanic acid. The control strains used in this study were *E. coli* ATCC 25922 (non ESBL-producer), *K. pneumoniae* 700603 (ESBL-producer).

Metallo-beta-lactamase (MBL) detection

A: Imipenem -EDTA Combined Disc Synergy Test (CDST-Imipenem)

The detection of Metallo-beta-lactamase was performed through EDTA-impregnated imipenem disc [15]. The test organism was inoculated for Mueller–Hinton agar plate (opacity adjusted to 0.5 McFarland opacity standards). 186.1g of disodium EDTA 2H2O in 1000 ml of distilled water was used for preparing an EDTA (0.5m) and the pH 8.0 was adjusted by using NaOH and sterilization by autoclaving. Two 10-µg imipenem discs were placed on the plate, and 5 µl of EDTA solution was added to one 10-µg imipenem discs. Recordings were obtained for increase in the zone diameter ≥7 mm around the imipenem-EDTA disc to that of imipenem alone as an MBL-positive strain after 16–18 hours of incubation at 35°C.

B:Imipenem -EDTA Double disc synergy test (DDST- Imipenem)

A Imipenem (10ug) disc was placed 15 mm centre to centre from a blank sterile disc containing 10ul of 0.5M EDTA (750ug). Inoculated plates were incubated for 16-18 hours at 37ºC. If enhancement in zone of inhibition between Imipenem and EDTA disc which was considered as positive for MBL production by DDST method [16].

Biofilm Production

The biofilm production assay was adopted from Zubair et al. [17]. Biofilm formation was examined by the quantitative determination of biofilm formation in 96-well flat bottom plates. Briefly, fresh bacterial suspensions were prepared in TSB from overnight cultures and adjusted to OD600 of 0.1 (~ 107 CFU/mL). in the next step, 100 µL aliquots of bacterial suspension were inoculated into individual wells of a 96-well flat-bottomed polystyrene plate and incubated at 37°C for 48h. Following overnight incubation, plates were gently washed with 1X phosphate buffered saline (PBS; pH 7.4) and stained with 100µL of 0.1% Crystal Violet (Sigma-Aldrich, St. Louis, MO) for 30 min at room temperature. Excess crystal violet was removed by washing, and biofilm was quantified by measuring the corresponding OD590nm of the supernatant following the solubilization of CV in 95% ethanol. For each clinical strain tested, biofilm assays were performed in triplicate and the mean biofilm absorbance value was determined. Biofilm formed were classified as weak (OD590 0.1 to ≤0.400), moderate (OD590 > 0.400) and strong (OD590 > 0.800) according to the method described by Stepanovic *et al*. [18]. For this study, moderate and strong were clubbed as positive isolates and weak non-biofilm were clubbed as negative for biofilm production.

Data Analysis

The relationship between categorical variables, including biofilm characteristics and antimicrobial resistance was performed using chi square test using SPSS software.

RESULTS

Standard microbiological and biochemical methods were used to test 28 hospital and 30 environmental isolates. The resistance pattern of different antibiotics on *P. aeruginosa* from hospital and environmental settings has been shown in Table 2. The biofilm activity was higher in hospital isolates [24(85.7%)] compared with environmental isolates [19(63.3%)]. For instance, significant results have been obtained for cefodoxime antibiotic among the hospital isolates (p-value=0.000) as 100% *P. aeruginosa* were present on the film in *hospital* isolates (Table 2). Figure 1 shows the comparative graphic representation of classification of biofilm activity as strong, moderate, weak and negative among the *P. aeruginosa* from hospital and environmental isolates**.**

Table 2: Antibiotic resistance pattern and biofilm producers among resistant isolates.

Resistance pattern	Hospital Isolates n(%)			Environmental isolates n(%)		
	Total	**Biofilm positive**	**p-value**	**Total**	**Biofilm positive**	**p-value**
Amikacin	20	18(75.0%)	0.306	9	6(31.6%)	0.804
Ceftazidime	14	11(45.8%)	0.280	4	3(15.8%)	0.603
Cefepime	18	16(66.7%)	0.520	11	7(36.8%)	0.979
Levofloxacin	20	18(75.0%)	0.306	12	9(47.4%)	0.279
Tobramycin	18	16(66.7%)	0.520	8	5(27.8%)	0.976
Amoxicillin	20	18(75.0%)	0.306	8	6(31.6%)	0.424
Cefodoxime	26	24(85.5%)	0.000	16	10(55.6%)	0.958
Cefepime	18	16(66.7%)	0.520	11	7(36.8%)	0.979
Cefoxitin	25	23(95.8%)	0.006	10	7(36.8%)	0.592
Cefixime	18	16(66.7%)	0.520	8	5(26.3%)	0.854
Ofloxacin	17	15(62.5%)	0.636	9	6(31.6%)	0.804
Cefotaxime	20	18(75.0%)	0.306	2	1(5.3%)	0.685
Aztreonam	21	17(70.8%)	0.212	10	5(27.8%)	0.331

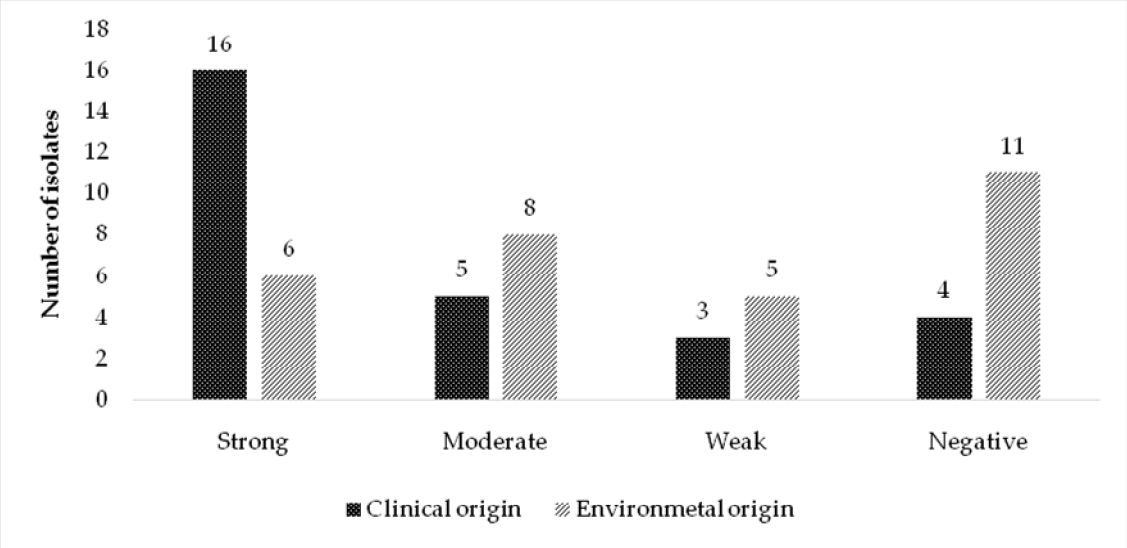

Figure 1: Classification of biofilm activity as strong, moderate, weak and negative among the *P. aeruginosa* from Hospital (clinical) and environmental isolates.

The Minimum Inhibitory Concentration (MIC) is considered as the lowest concentration of an antimicrobial agent, which is bacteriostatic preventing visible growth of bacteria. Table 3 shows results for MIC of *P. aeruginosa* strain for Imipenem. It has shown categorized hospital and environmental isolates as sensitive, intermediate, and resistant. The MIC was performed to select the real imipenem resistant isolates for further analysis.

Table 3: Minimum inhibitory concentration analysis of *P. aeruginosa* strain for Imipenem

MIC- (µg/ml)	Sensitive					Intermediate	Resistant						
	0	0.5	1	2	4	8	16	32	64	128	256	512	1024
Hospital isolates													
Biofilm positive n = 24	0	1	2	1	1	3	3	3	4	4	1	1	0
Biofilm negative n = 4	0	1	1	2	0	0	0	0	0	0	0	0	0
Environmental isolates													
Biofilm positive n= 19	0	1	3	4	2	3	3	2	1	0	0	0	0
Biofilm negative n = 11	0	1	3	2	2	2	1	0	0	0	0	0	0

The imipenem-resistant strains were selected for MBL production and the results are shown in Table 4. It was observed that *P. aeruginosa* of hospital origin show majority MBL-positive by CDST method, then by both DDST and CDST method (28.5%) and DDST method (25%). While in case of *P. aeruginosa* of environment origin, only shows MBL positive by CDST method (100%) (Figure 2).

Table 4: Number of isolates for MBL positivity [Data in n(%)]

	Total	Only by DDST method	Only by CDST method	By both CDST and DDST methods	P value
Hospital Isolates n=28	16(57.1)	3 (25%)	9 (46.4%)	4(28.5%)	0.654
Environmental Isolates n=30	6 (20.0)	0	6 (100)	-	0.831

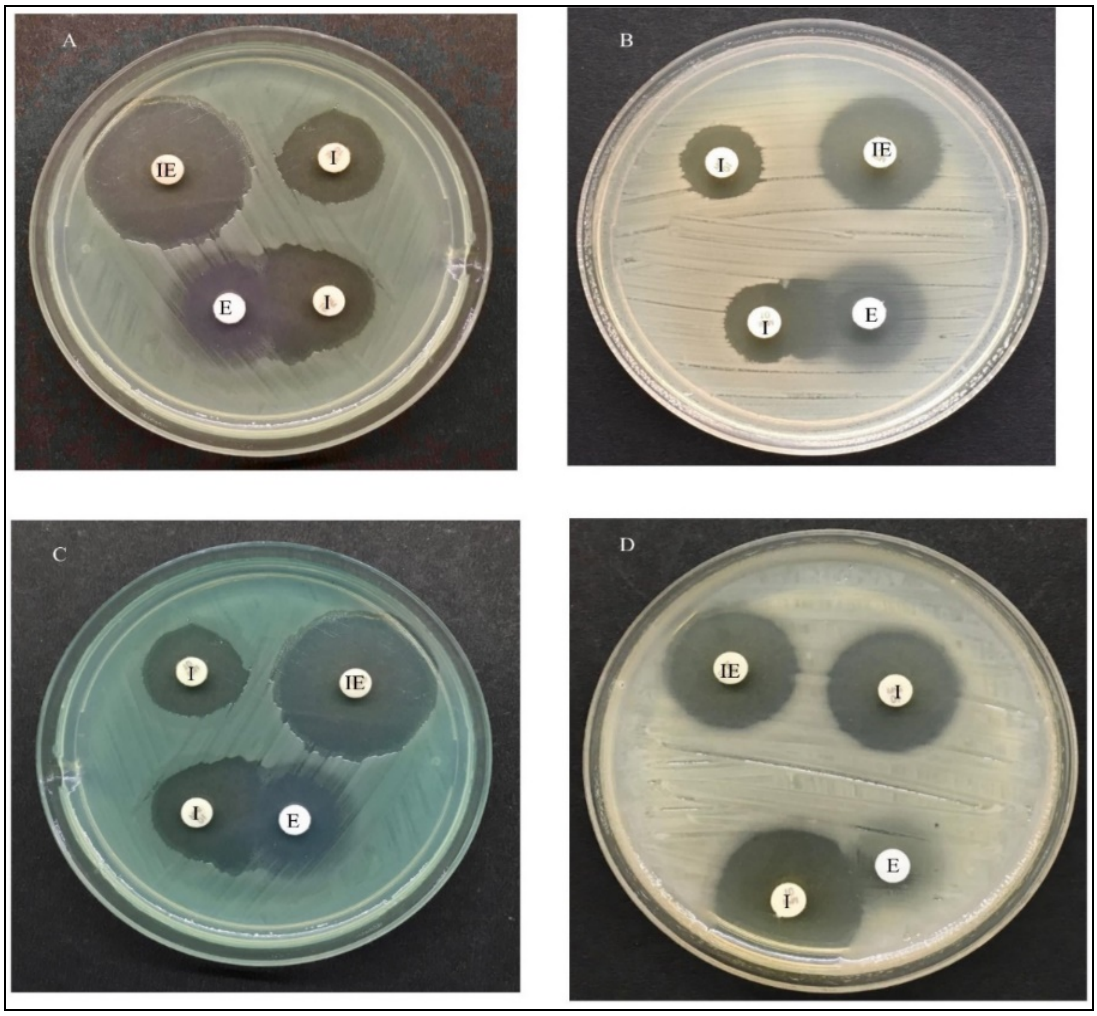

Figure 2. CDST and DDST test [(A: Pseudomonas sp from environmental origin showing MBL positive by CDST and negative DDST) (B: Pseudomonas sp from environmental origin showing MBL negative by both CDST and DDST), (C: Pseudomonas sp from hospital origin showing MBL positive by CDST and negative DDST), (D: Pseudomonas sp from hospital origin showing MBL negative by CDST and positive DDST)]. Legends: I=10µg/ml imipenem disk, IE= 10µg/ml imipenem disk + 5 µL of EDTA solution, E=10ul of 0.5M EDTA (750ug) disk. [Clinical=Hospital]

Table 5 has compared the biofilm positivity in relation to MBL production (CDST and DDST). The results clearly show that *P. aeruginosa* from the hospital origin, majority (43%) of them were biofilm producers compared to environmental origin (one isolate only).Figure 3 showed comparative results of ESBL screening and confirmatory analysis of *P. aeruginosa* from the Hospital origin and environmental origin. In both the screening and confirmatory analysis of ESBL, the overall positivity was higher (85.7% in screening and 68.7% in confirmatory) shown by *P. aeruginosa* from hospital origin compared with the environment origin (56.6% in screening and 25.8% in confirmatory).

Table 5: Comparison of biofilm activity in relation to MBL detection (CDST & DDST) methods among clinical and environmental isolates.

***P. aeruginosa* from Hospital setting**		**MBL positive by**	***P. aeruginosa* from environment setting**	
Biofilm negative	**Biofilm positive**		**Biofilm positive**	**Biofilm negative**
3	6	**CDST**	1	5
-	3	**DDST**	-	-
1	3	**both methods**	-	-

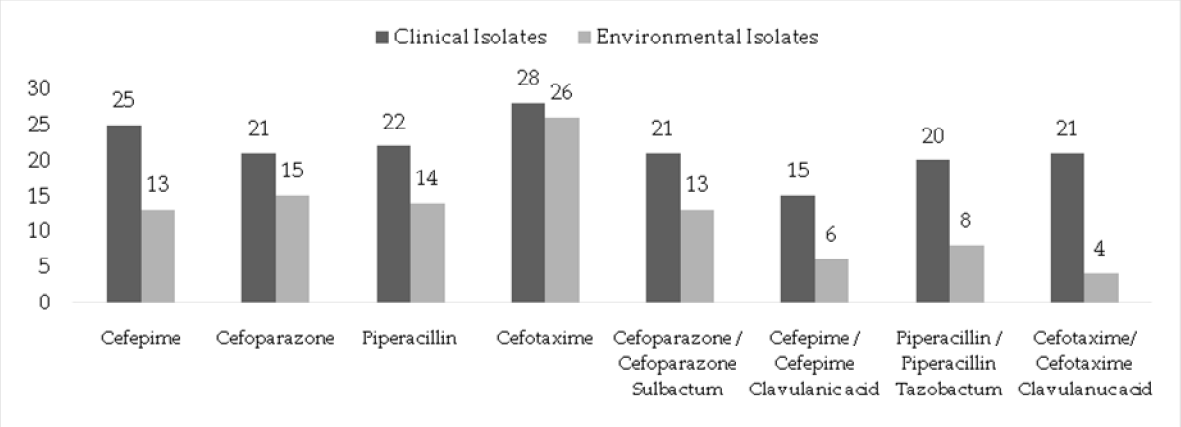

Figure 3: ESBL (screening and confirmatory) results details of Clinical Isolates and Environmental isolates of *P. aeruginosa* (Hospital Isolates n=28; Environmental isolates n=30; data represents in chart was in numbers). [Clinical=Hospital]

Table 6 showed the biofilm positivity among ESBL positive isolates. Majority of the *P. aeruginosa* (hospital origin) isolates were ESBL positive also shows high biofilm activity compared with environmental origin with the same antibiotic.

Table 6: Biofilm positivity among ESBL positive isolates.

	Hospital Isolates N=28		Environmental isolates N=30		P value
	Total	**Biofilm positive**	**Total**	**Biofilm positive**	
Cefoparazone / CefoparazoneSulbactum	21	16 (76%)	13	8 (61.5%)	0.345
Cefepime / Cefepime Clavulanic acid	15	11 (73.3%)	6	2 (33.3%)	0.456
Piperacillin / Piperacillin Tazobactum	20	14 (70.0%)	8	3 (37.5%)	0.121
Cefotaxime/ CefotaximeClavulanuc acid	21	4(19.0%)	15	1 (25.0%)	0.202

DISCUSSION

The importance of microbial biofilm can be measured with a number of different assays such as XTT and crystal violet methods. The biofilm detection can be performed visually automatically with multi-well plate readers or by experienced personnel in vitro. The former system is influenced by the experience and sensitivity of the analyst, while the prior converges continuous data of absorbance that must be explained. A single threshold must be selected for discriminating the positive over the threshold from the negatives in the case of a yes-or-not output. In other cases, it has been selected for dividing the range of values in quartiles, which lead to a more expressed portrayal of the strains based on their competence in producing biomass, if CV was used or to be metabolically active based on the XTT assay. *P. aeruginosa* is an essential nosocomial pathogen, supported with a myriad of resistance mechanisms that may cause pan-drug resistance or multidrug resistance. The most causative agents are MBLs and ESBLs.

Many infections such as urinary tract, bed ulcers, burns, pediatric patients, and adult AIDS are basically caused by a pathogen *P. aeruginosa* that is usually acquired from hospitals [19]. Previous studies reflect that *P. aeruginosa* is affiliated with a high rate of morbidity and mortality ranging between 20 and 60% of these infections [20]. In terms of preference, the Molecular methods play an important role in identifying *P. aeruginosa*, as compared to the phenotypic methods [21]. It is possible to perform phenotype methods in combination to identify *P. aeruginosa*; whereas, the molecular method follows use of gene sequencing for confirming the type of species.

In the present study, hospital isolates were more resistant as compared to the environmental isolates based on the characteristics of culture. Considering the hospital isolates, majority of the antibiotics showed biofilm positive, specifically for Cefodoxime antibiotic. Previous studies have exhibited similar occurrence rates for hospital isolates in different regions of the world [22-24]. A similar study by Shi et al. [25] examined 201 environmental samples of environmental isolates that showed positive results for *P. aeruginosa* (56%). There is increased resistance among the biofilms by killing wide range of antimicrobial agents. *P. aeruginosa* secretes exopolysaccharide alginate among the individuals suffering from respiratory tract infection, chronic obstructive disease, and cystic fibrosis [26]. The present study has

showed significant difference in positive biofilms among the hospital isolates, as compared to the environmental isolates. Among the hospital isolates of *P. aeruginosa*, the present study showed that 75% were resistant to amikacin, 45.8% to ceftazidime, 66.7% to cefepime, 75% to levofloxacin, 66.7% to tobramycin, 75% to amoxicillin, 66.7% to cefepime, 95.8% to cefoxitin, 66.7% to cefixime, 62.5% to ofloxacin, 75% to cefotaxime, and 70.8% to Aztreonam. However, the percentages of resistance to different antibiotics among the environmental isolates was found to be 31.6% to amikacin, 15.8% to ceftazidime, 36.8% to cefepime, 47.4% to levofloxacin, 27.8% to tobramycin, 31.6% to amoxicillin, 55.6% to cefodoxime, 36.8% to cefepime, 36.8% to cefoxitin, 26.3% to cefixime, 31.6% to ofloxacin, 5.3% to cefotaxime, and 27.8% to aztreonam. The findings of current study have indicated elevated resistance to commonly used antibiotics. Piperacilin and Piperacillin/tazobactam alone proved to be influential antibiotics. The second most effective antibiotic group was carbapenemes, which account for 10.2% and 12.5% resistance for meropenem and imipenem, respectively.

A similar study by Choy et al. [27] showed that 65% of the hospital isolates could form positive biofilm. This was supported by another study that worked on 93 clinical isolates and majority of them (78%) secreted biofilm [28]. *P. aeruginosa* comprises of different types of b-lactamases and aminoglycoside-modifying enzymes that are commonly found in Southeast Asia, Europe, and Turkey. A total of 102 strains of *P. aeruginosa* were isolated by Bazire et al. [29] from different clinical sources. It is further revealed that antibiotics need to be transferred to cell wall for reaching the target and treat the infections of *P. aeruginosa* [30]. Protein synthesis through binding with 30S ribosomal subunit is not allowed in the presence of aminoglycosides that includes gentamicin, amikacin, and tobramycin [30]. The structure of chromosome within the cell is hold by ciprofloxacin that is connected to subunit A of DNA gyrase. The assembling of transpeptidases and peptidoglycan is restricted by b-lactams that include imipenem, aztreonam, piperacillin, ceftazidime, and meropenem [30]. This entire mechanism takes place on the outer side of the cytoplasmic membrane. A similar study testing the isolates of *P. aeruginosa* showed that 29.4% of the isolates from the canines exhibited resistance to gentamicin; while, other antibiotics showed sensitivity [31]. Another study by Bonfigli et al. [32] showed 9.1% resistance to meropenem, 13.4% to ceftazidime, 10.6% to amikacin, 19.3% to imipenem, 12% to piperacillin, and 31.9% to ciprofloxacin. Another study conducted in United Kingdom showed that 19.1%, 14.2%, 11.5%, 15.8%, 7.1%, and 29.5% of the isolates remain untouched to gentamicin, imipenem, ceftazidime, piperacillin amikacin, and ciprofloxacin respectively [33]. Moreover, meropenem and imipenem resistance of 66% and 74% was recorded by Akhavan et al. [34] in hospital-isolates of *P. aeruginosa*. Resistance of 81% to amikacin, 84% to tobramaycin, and 88% to gentamicin was shown among the isolates obtained from burn patients [35].

P. aeruginosa was majorly undertaken as non-MDR that articulated biofilm and those conducted bio-film related genes. This may induce a misunderstanding that biofilm production in the first place is not linked with antibiotic resistance. It is essential that all isolates were subjected to antimicrobial susceptibility testing as planktonic cells and not in biofilm form in this study. Therefore, multiple mechanisms of biofilm and those conducted biofilm-related genes were majorly undertaken as non-MDR based on the findings.

Surprisingly, it was witnessed that Cefoparazone and Cefotaxime were significantly higher in ESBL-positive EC isolates as compared to that of ESBL-negative isolates. This finding is in-line with the investigations on other infectious types. Similar observations were reported in Subramanian et al [36] and Neupane et al [37] in patients with UTI that ESBL-EC strains more often articulated biofilm as compared to that of non-ESBL-EC isolates. The reason of the higher competence of ESBL-EC strains to establish biofilm was not apparent. During bacterial chromosomal gene rearrangements, activation of several stress response genes and expression of specific virulence genes need the ESBL plasmids that might be the underlying mechanism. Enhanced biofilm formation capacity, among these studies, in ESBL-plasmid carrying ST131 and ST648 strains that produce survival and virulent-related extracellular matrix elements. An in-depth comprehension of the contribution of transcription factor csgD might be solution to biofilm formation and motility capacity in pandemic ESBL-producing EC lineages. Early screening of *P. aeruginosa* isolates to detect ESBL and MBL-production should be emphasized. Therefore, routine testing of the isolates of *P. aeruginosa* for sensitivity to ceftazidime, cefotaxime and carbapenems may represent a cost-effective way for screening of ESBLs and MBLs. Our study has introduced an easy and cost-effective inhibitor potentiated disk diffusion (IPD) method for MBL detection. Thus, double disk synergy test and combined disk synergy test (CDST) / inhibitor-potentiated disk diffusion method (IPD) can easily be used to confirm the ESBL and MBL phenotypically. The emergence of these β-lactamases along with MDR genes in *P. aeruginosa* may adversely muddle the clinical management of such patients. High frequency of these enzymes urges the infection control teams of hospitals to design some preventive measures to stop the dissemination of these resistant strains.

CONCLUSION

The findings of present study show that difference in sampling methods and geographical area result in difference of occurrence of positive biofilm and resistance patterns of antibiotics. As compared to the environmental isolates, the frequency of biofilm formation and antibiotic resistance pattern of *P. aeruginosa* among the hospital isolates was higher. The main reasons for these differences might be incomplete duration of antibiotics, improper use of detergents/disinfectant, use of medications, high use of drugs, and upsurge of mutagens bacteria in today industrial life. This study might be limited by the lack of clinical information of the patients from whom bacteria were isolated. In addition, this study has indicated that determination of expression levels of biofilm-related genes via quantitative real-time PCR may assist in evaluating the role of each subsequent gene in biofilm production.

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