In vitro biofilm formation and antimicrobial resistance pattern in Pseudomonas aeruginosa recovered from infected burn wounds in Erbil city

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ABSTRACT

A biofilm is a population of cells enclosed in an extracellular polysaccharides matrix, which make them grow to biotic and abiotic surfaces. Pseudomonas aeruginosa is one of the most important biofilm-forming pathogens found to be responsible for infections on various host tissues, especially burns and surgical wounds. Biofilm producing enables their existence in an aggressive environment of host immune system and antimicrobial agents. The main aim of this study is to evaluate the prevalence of biofilm producers among P. aeruginosa and to find out their antibiotic susceptibility pattern with special reference to extended-spectrum beta-lactamase (ESBLs) from patients with wound and burn infections. All clinical isolates of P. aeruginosa were screened for extended-spectrum beta-lactamase production and biofilm formation. This study included 90 isolates of P. aeruginosa from the wound and burn specimens. About 47 out of 90 isolates were ESBLs producers, and 28 showed to be biofilm-forming pathogens. In conclusion the biofilm producing pathogenic P. aeruginosa showed high resistance rate to almost all the frequently used antibiotics. Prominently extended-spectrum beta-lactamase phenotype was not strongly associated with biofilm formation in P. aeruginosa.

1. INTRODUCTION

The worldwide appearance of drug-resistant bacterial strains in the healthcare setting and community continues to be a problem and toughly compromises the selection of suitable treatments and is therefore associated with significant morbidity, particularly in the burn wards and intensive care units (ICU) that cause infections with a high mortality rate, antibiotic costs, and a hospital stay (Kumar et al., 2012; Zafer et al., 2014). Pseudomonas aeruginosa is widely known as an opportunistic human pathogen, frequently complicated in infections of immunocompromised patients, causes various acute and chronic hospital-acquired infection such as bacteremia, pneumonia, and urinary tract infections (Jiang et al., 2006). Infections caused by P. aeruginosa are difficult to treat as the majority of isolates exhibit intrinsically resistant to a wide range of antibiotics and disinfectants including anti-Pseudomonal antimicrobial classes (Hill et al., 2005;
Tavajjohi et al., 2011). Resistance in \( P. \) aeruginosa may be mediated via several distinct mechanisms, including mutation mediated resistance mechanisms that leading to the inactivation of the carbapenem porin OprD, target site outer membrane modification and efflux pumps encoded gene (Salimi and Eftekhar, 2013) or overexpression of the naturally occurring cephalosporinase AmpC and acquired beta-lactamases such as extended-spectrum \( \beta \)-lactamases (ESBL) (Umadevi et al., 2011) Such enzymes are most commonly found in other gram-negative bacteria and have been recently detected in \( P. \) aeruginosa at low frequency (Jiang et al., 2006). ESBLs are typically beta-lactamases that hydrolyze penicillins, cephalosporins, and aztreonam and are encoded by mobile genes (Tavajjohi et al., 2011). The rise in the worldwide spread of resistance towards antibiotics mostly in hospital area among \( P. \) aeruginosa strains are observed (Aryanezhad et al., 2016) especially, on indwelling devices in hospital environments including sinks, drains, respirators, and disinfectant solutions on which these organisms grow as a biofilm, subsequently it can be transmitted rapidly among hospitalized burn patients (Qi et al., 2016; Shaikh et al., 2015).

Biofilm is a complex community of microorganisms embedded in self-produced extracellular polymeric matrices consisting of polysaccharide, and protein. (Norouzi et al., 2010) which hold the cells of the biofilm community together in order to resist phagocytosis and other defense mechanism of the host (Di Domenico et al., 2017; El-Khashaab et al., 2016). Biofilm growth is associated with an increased level of resistance as well as with quorum-sensing-regulated mechanisms, all of the noteworthy resistance mechanisms such as extended spectrum \( \beta \)-lactamase, up-regulated efflux pumps and mutations in antibiotic target molecules in bacteria also contribute to the existence of biofilms (Norouzi et al., 2010). More studies found that biofilm formation is one of a common strategy for bacterial survival in hard environmental conditions (Matsukawa and Greenberg, 2004). According to National Institutes of Health, “more than 60% of all microbial infections are caused by biofilms.” \( P. \) aeruginosa responsible for serious outbreaks of multi-drug resistant diseases due to the uncontrolled usage of antibiotics (El-Khashaab et al., 2016). Bacterial biofilms are a major cause of recurrent or chronic infections and may impair cutaneous wound healing and reduce topical antibacterial efficiency in treating infected skin wounds (Di Domenico et al., 2017). The resistance of biofilm against a component of the host immune system as well as their capability to acquire resistance to different antibiotics appears to be a risk factor for persistent infections and makes infections due to \( P. \) aeruginosa hard to eradicate (Kumar et al., 2012).

The present study was undertaken to find the prevalence of ESBL production in burn and wound isolates of \( P. \) aeruginosa and to estimate isolates forming biofilms. Furthermore, I have evaluated the correlation between biofilm formation and ESBL production among the isolates along with I studied the association of biofilm producer with an antibiotic-resistant pattern of the isolates.

2. MATERIALS AND METHODS

2.1. Specimens collection

A total of 90 nonduplicate \( P. \) aeruginosa isolates were obtained from wounds swabs submitted for bacteriological testing from hospitalized burn patients admitted to the West Emergency Hospital Erbil City, Iraqi Kurdistan in the period from January 2016 to June 2017.
The study was approved by the Ethics Committee and permission was obtained from all patients participating in the study, with regard to the sampling site. All the collected specimens were quickly sent to the Microbiology laboratory to be processed (Rafiee et al., 2014).

2.2. Bacterial identification

Identification of P. aeruginosa were done on the basis of colony morphology on MacConkey’s agar, motility, pigment production, oxidase reaction, for diagnosis to the species level Vitek II automated system (bioMérieux Marcy l’Etoile, France) (Vitek Systems Version: 06.01) was used with the ID-GNB card for identification of Gram-negative bacilli. The identified bacteria were stored in Trypticase Soy Broth (TSB) containing 15% glycerol at -20°C until used (Chen et al., 2015; Jiang et al., 2006).

2.3. Antimicrobial susceptibility

A panel of antimicrobial agents (cefepime, cefotaxime, cefoxitin, ceftazidime, cefuroxime, gentamycin, imipenem, meropenem, norfloxacin, ciprofloxacin, tobramycin, trimethoprim, trimethoprim-sulfamethoxazole, and colistin) was determined by Vitek II automated system. All bacterial species that showed resistant to any of third-generation cephalosporin was tested for ESBL production (Chen et al., 2015).

2.4. Detection of ESBLs production by the phenotypic method

ESBL production was screened out by the Phenotypic Confirmatory Combination Disc Diffusion Test (Rafiee et al., 2014). A 0.5 MacFarland’s suspension of each isolate was spread on a Muller – Hinton agar (MHA) plate then ceftazidime (30μg) and cefotaxime discs (30μg) with or without clavulanic acid (10μg) were placed aseptically on the agar plate as recommended by the CLSI (CLSI, 2017). A distance of about 20mm was kept between the two discs and the cultures were incubated at 37 °C for 24hrs. The observation of a ≥ 5mm increase in the zone diameter for the antimicrobial agent which was tested in combination with clavulanic acid, versus its zone diameter when tested alone, interpreted as ESBL production.

2.5. Biofilm induction in Vitro

Micro-titer plate assay was chosen to detect the biofilm formation, as previously reported (Hassan et al., 2011; Heydari and Eftekhar, 2015). All isolates were grown overnight in tubes containing 5 ml Trypticase soy broth (TSB) (Merck, Germany) at 37°C. The growths were then suspended into fresh medium in 1:100 dilutions. 200μL of diluted cultures were inoculated into 96 well polystyrene microtiter plates (Costar/USA) and incubated at 37°C for 24 hours without shaking. The edge of the plate was covered with parafilm to avoid evaporation during incubation. The broth was then removed by washing the wells three times with 200μL of PBS pH 7.2 then were exposed to air-dry. The wells were then stained with 200μL of 0.1% crystal violet for 30 minutes at room temperature. The plates were washed with distilled water to remove the unbounded dye, allowed to dry. The adhered stain was solubilized by addition 200 L of 95% ethanol. The optical density (OD) of the dissolved crystal violet was measured at 630nm using ELISA reader. 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 (Hassan et al., 2011). Each test was repeated on three different days and the results were reported as the mean of the obtained values.

3. Results
Of 90 *Pseudomonas aeruginosa* isolated from the wound and burn samples, 28(31%) were biofilm producers and 62(69%) were non-biofilm producers, and 47(52%) were seen to be ESBL producers, while 43(48%) were non-ESBL producers. See Figure 1.

![Figure 1: Biofilm and ESBL among *P. aeruginosa* isolates](image)

| Antibiotics | Biofilm producers | Biofilm nonproducers | Total resistant |
|-------------|-------------------|----------------------|-----------------|
|             | *n* = 28(31.1%)   | *n* = 62(68.9%)      |                 |
| Amoxicillin | 28(100%)          | 62(100%)             | 90(100%)        |
| Cefotaxime  | 28(100%)          | 62(100%)             | 90(100%)        |
| Ceftazidime | 21(75%)           | 39(63%)              | 60(67%)         |
| Cefepime    | 26(93%)           | 44(71%)              | 70(78%)         |
| Ciprofloxacin | 17(61%)     | 35(56%)              | 52(58%)         |
| Tobramycin  | 18(64%)           | 40(65%)              | 58(64%)         |
| Gentamicin  | 20(71%)           | 36(58%)              | 56(62%)         |
| Meropenem   | 22(79%)           | 25(40%)              | 47(52%)         |
| Imipenem    | 21(75%)           | 33(53%)              | 54(60%)         |
| Colistin    | 0                 | 0                    | 0               |

Out of 47 ESBLs producing *Pseudomonas aeruginosa*, only 13(28%) were biofilm producers and 34(72%) were weak/non biofilm producers, whereas, among 43 ESBL non-producing *P. aeruginosa*, 15(35%) showed biofilm forming isolates, and 28(65%) were non biofilm forming. (See table 1).

**Table 1: Biofilm and ESBL distribution of isolates**

| ESBL producers No. = 47 (52%) |
|-------------------------------|
| Biofilm producers | Biofilm nonproducers |
| 13 (28%)        | 34(72%)              |

| Non-ESBL producers No. = 43(48%) |
|-----------------|
| Biofilm producers | Biofilm nonproducers |
| 15(35%)          | 28(65%)              |

Out of 90 *P. aeruginosa* isolates, the highest number showed to be non-susceptible to amoxicillin and cefotaxime followed by, cefepime, ceftazidime, tobramycin, gentamicin, imipenem and meropenem, all isolates were susceptible to colistin. Biofilm producer displayed the highest resistance to cefotaxime 28(100%) cefepime 26(93%) followed by meropenem 22(79%), imipenem, and ceftazidime 21(75%). The non biofilm producers after colistin showed the lowest resistance to meropenem 25(40%) followed by imipenem 33(53%) ciprofloxacin 35(56%) gentamicin 36(58%).

**Table 2: Antibiotic resistance pattern of *P. aeruginosa* among biofilm producers and nonproducers**

The study displayed that, out of a total of 90 *P. aeruginosa* isolates, only 28(31%) formed a biofilm, of which 8(9%) were strong and 20(22%) showed to be moderate producers. The results illustrate the low percentage of
biofilm in ESBL producing isolates. Of the 47 ESBL producers, 5(11%) were strong biofilm producers and 34(72%) were weak or non biofilm producers as in table 3.

Table 3: Biofilm formation by 90 isolates of P. aeruginosa.
The association between the potential to form various degrees of biofilms by P. aeruginosa and ESBL production has also been shown in Figure 2.

| ESBL production | Biofilm formation in each group |
|-----------------|---------------------------------|
|                 | Weak or none | Moderate | Strong |
| Positive (n = 47)| > 0.24       | 0.12-0.24 | < 0.12 |
| Negative (n = 43)| 28(65%)     | 12(28%)    | 3(7%)  |
| Total (n = 90)  | 62(69%)      | 20(22%)    | 8(9%)  |

Figure 2: Number and percent of isolates forming biofilm and ESBLs

4. DISCUSSION

P. aeruginosa is accomplished by causing acute and chronic infections mostly due to its potential to form biofilms (Matsukawa and Greenberg, 2004). The burn was the most common clinical sample from which P. aeruginosa were isolated (Heydari and Eftekhar, 2015). Wound and burn infections caused by P. aeruginosa can cause mortality. This is consistent with the idea that wounds and burned patients may not have a skin barrier and a steady defense system (Akers et al., 2014; Zaranza et al., 2013). Biofilms-producing bacterial infections are difficult to eliminate by using antimicrobial agents. The biofilm may be involved in the defense mechanism of the bacteria against antimicrobials (Tadepalli et al., 2016). The biofilm producing isolates have been initiated to be involved in nosocomial infections and it has established resistance to a wide range of antimicrobial agents in burn centers (Kumar et al., 2012). The ESBL screening results established a high incidence of ESBLs in P. aeruginosa isolates examined in wounds and burns infection. Out of a total of 90 P. aeruginosa isolated in our study 47 isolates (52%) were ESBL producers and only 43(48%) non-ESBL producers. Whereas, a study by (Rani et al., n.d.) phenotypically revealed that (37.28%) of P. aeruginosa isolated from burn patients were ESBL positive, wherein a study done by (Goel et al.,
2013) from India, (42.3%) isolates were found to be positive for ESBL production. All β-lactamase genes in *P. aeruginosa* characterize a serious therapeutic challenge, modification in antimicrobial policy is essential for the treatment of patients (Ingti and Maurya, 2017). The existent study determined the association between biofilm and ESBL producer in burns isolates. The results showed 28(31%) of *P. aeruginosa* from wounds and burns isolates were biofilm produced. A study in the same field showed around 43.5% of *P. aeruginosa* in burn isolates were moderate to strong biofilms (Heydari and Eftekhar, 2015). Whereas another study conducted by (El-Khashaab et al., 2016) showed 25.7% *P. aeruginosa* were strong biofilm producer. This study showed the higher incidence of resistance among biofilm producers rather than non biofilm producer. Supporting our result in a study conducted by (Norouzi et al., 2010) perceived that, the higher percentage of resistance towards different antimicrobial agent predominantly among biofilm producing gram-negative microorganisms. *Pseudomonas aeruginosa* is a bacterium seems to play a significant role in the remarkable ability of the bacteria to persist and extend in the environment of the hospital. (F. Al Marjani and Khadam, 2016). The ability of *P. aeruginosa* to spread in the environment may be due to its ability to biofilm formation on surfaces. Biofilm formation is also a mechanism of pathogenesis in infections related to the device and provides a source of repeated transmission by prolonging survival on abiotic stuff (Zaranza et al., 2013). According to the current results, there was a high frequency (> 60%) of resistance against all the frequently used antimicrobial agents, except for colistin. These outcomes had shown a severe antimicrobial resistance among *P. aeruginosa* in burn infection which might be due to the inappropriate use of antibiotics in this situation. In the present study, also I have observed a high prevalence 54(60%) of isolates showed resistance to imipenem and 47(52%) to meropenem, which is an “alarming sign” since carbapenems are the last resort of the drug. Furthermore, a study by (Kalaivani et al., 2013) also showed 36% of *P. aeruginosa*, were resistant to imipenem and 80% to meropenem. Another report from Iraq by (Al Marjani and Khadam, 2016) confirmed that all their isolates were sensitive to colistin. The present study showed the relationship between biofilm formation and antibiotic resistance, exposed that the biofilm producer isolates considerably more resistant as compared to biofilm nonproducers, possibly due to gene transfer mechanisms within the biofilm environments are more possible (Sahal and Bilkay, 2015), and delayed diffusion of antibiotics inside the bacterial cell (Vahdani et al., 2012). Reports revealed that fluoroquinolones are effective antibiotics against biofilm-forming bacteria which is in correlation with the existent results that showed low resistance to ciprofloxacin 52(58%), meanwhile, ciprofloxacin is one of
the most prescribed antibiotic in burn centers in Iran (Moazami-Goudarzi and Eftekhar, 2013). The results in current study highlight the existence of an association between antibiotic resistance and biofilm formation, several reports confirmed resistant strains realize high levels of biofilm in resistance isolates in spite of producing weak biofilms (Nucleo et al., 2010; Qi et al., 2016). In the existing study, a nonsignificant correlation was found between the degree of biofilm formation and ESBL production in P. aeruginosa. In another study, biofilm formation correlated with MBL production in burn isolates of P. aeruginosa but not to ESBL (Singhai et al., 2013). Comparable results have been shown for other non lactose fermenting bacteria, where the possibility to form biofilm was significantly higher in β-lactamase (MBL and AmpC) - producing strains (Heydari and Eftekhar, 2015; Sahal and Bilkay, 2015; Singhai et al., 2013). Although another result did not support a direct connection between biofilm formation and β-lactamase production (Nucleo et al., 2010). In contrast a study by (Singhai et al., 2013) showed that extended spectrum beta-lactamase producing isolates particularly blaCTX-M had a better aptitude to produce biofilm than non biofilm producing isolates. Furthermore, a study exhibited that ESBL (but not MBL or AmpC) inhibited biofilm production by impairing the twitching motility, which plays an important role in manifesting as a perturbation of structures involved in bacterial adhesion that is required to induce biofilm formation (Gallant et al., 2005). Additional explorations would progress our understanding of these processes and afford understandings in the therapeutics and prevention of P. aeruginosa infections.

5. CONCLUSION

The current study verified the high prevalence of extended spectrum β-lactamases among P. aeruginosa isolates in burns infection. I conclude that biofilm forming and ESBL producing P. aeruginosa showed a high resistance rate to almost all the commercially used antibiotics. As there is no any literature found concerning the association between biofilm production and ESBL producing P. aeruginosa in Erbil city.

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