Detection of Biofilm Formation in *Pseudomonas aeruginosa* Isolates from Clinical Specimens

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**ABSTRACT**

The ability of a microorganism to develop biofilm is considered as a marker of clinically relevant infection. Infections caused by *P. aeruginosa* are difficult to treat as the majority of isolates exhibit high level of innate resistance to many antibiotics and tendency to form biofilms. The study was undertaken to investigate biofilm-forming capabilities of *P. aeruginosa* isolated from clinical specimens by two main different methods tissue culture plate (TCP) method, and congo red agar (CRA) method. Further, to investigate the antimicrobial resistance profile among biofilm producing isolates. A total of 300 specimens were collected from patients. Ninety six isolates of *P. aeruginosa* were obtained from various clinical samples. We applied Vitek-2 automated system as a panel of antimicrobial agents. TCP method and CRA method assay was chosen to detect the biofilm formation. Out of the 96 isolates, the results by TCP method and CRA method were 84(87.5%) and 76(79.1%) isolates respectively. TCP method was the most sensitive method for detection of biofilm production. TCP method detected 78 as strong, 6 as moderate and 12 as weak or non-biofilm producers. By CRA method, 65, 11 and 20 of isolates were strong, moderate and weak or non-biofilm producers, respectively. The antibiotic resistance pattern of *P. aeruginosa* was found higher in biofilm producers than in biofilm non-producers. TCP method was considered as the gold standard and reliable method for detection of biofilm formation. We conclude that there has been a positive association between drug resistance and biofilm formation of *P. aeruginosa*.

1. **INTRODUCTION**

*Pseudomonas aeruginosa* (*P.aeruginosa*) is a remarkably one of the most adaptive prevalent nosocomial pathogens. They have been implicated in serious and life-threatening infections (Bo Fu et al., 2017). Infections caused by *P. aeruginosa* associated with a higher death rate particularly in clinical settings (Karthic and Gopinath, 2016). They are frequently responsible for various acute and chronic opportunistic infections (Heydari and Eftekhar, 2015). Biofilm formation is another important characteristic of *P.aeruginosa* contributes to the chronicity of infections as they reduce susceptibility to antimicrobial agents and consequently decreased therapeutic options (Bo Fu et al., 2017). It is now commonly accepted that biofilm formation is the most common mode of growth of bacteria (Mathur et al., 2006). Biofilm is defined as an assemblage of microbial cells that is associated (not removed by gentle rinsing) with a surface and covered by an exopolysaccharide matrix (slime) (Bose et al., 2009, Stepanovic’et al., 2007). Various changes occur during their transition from planktonic to a surface attached community. In response to certain environmental signals,
and exhibit an altered phenotype with respect to growth rate and gene transcription (Bose et al., 2009, Hassan et al., 2011). These phenotypic differences are manifested in various ways, depending on the species of bacteria. According to a publication by the National Institutes of Health, more than 60% of all infections are related to biofilms (Bose et al., 2009). Microorganisms growing in a biofilm are generally well protected against environmental stresses, antibiotics, desiccation, disinfectants as well as the host immune system and consequently are notoriously difficult to eradicate (Steenackers et al., 2012). Biofilm formation is main medical significance as they reduce the sensitivity to the antimicrobial agents. Further, the vicinity of cells within a biofilm can facilitate a plasmid exchange and promote the distribution of antimicrobial resistance (Niveditha et al., 2012). There are various number of tests are available to detect biofilm production. These methods include the TCP, TM, CRA, bioluminescent assay and light or fluorescence microscopic (Mathur et al., 2006). Nevertheless, the TCP (microtiter plate) method remains among the most frequently used assays for investigation of biofilm, and a number of modifications have been developed for the in vitro cultivation and quantification of bacterial biofilms (Stepanovic’ et al., 2007). Under laboratory conditions, this form of biofilm is characterized by the expression of cellulose and curli fimbriae, two major extracellular matrix components in Salmonella spp. and E. coli (Monteiro et al., 2011, Uhlich et al., 2006). The in vitro production of curli fimbriae and cellulose is usually manifested as a distinct colony morphology on congo red agar plates. Both components are capable of interacting with the dye Congo Red, which results in the production of redar (red, dry and rough) colonies of typical dark purple colour, with rough and dry surface and undulate margins (Milanov et al., 2015, Monteiro et al., 2011). The study was undertaken to investigate in vitro biofilm-forming capabilities of P. aeruginosa isolated from clinical specimens by two main different methods tissue culture plate (TCP) method and congo red agar (CRA) method and to compare these methods for biofilm detection. In addition to investigate the antimicrobial resistance profile among biofilm producing isolates.

2. MATERIALS AND METHODS

2.1. Specimen collection

The study was conducted at Rizgyar Teaching Hospital and West Erbil emergency in Erbil City, Iraqi Kurdistan Region, during the first 6 months of 2017. A total of 300 specimens were collected from patients admitted to the hospitals. 96 clinical isolates of P. aeruginosa were isolated from various clinical samples including wounds and burns 40 (41.6%), ear discharge 17 (17.7%), blood cultures 15 (15.6%), urine 13 (13.5%), sputum 6 (6.25%), and abdominal fluid 5 (5.2%).

2.2. Bacterial identification

The Vitek-2 automated system by using Gram-Negative cards according to the manufacturer’s instructions (GNI-20and 22) (bioMérieux, USA) (Vitek Systems Version: 05.04) was used for diagnosis to the species level. The isolated bacteria were stored in trypticas soy broth (TSB) with 40% glycerol at −70°C until used.

2.3. Detection of biofilm formation

All bacterial isolates were tested by the following two methods for detection of biofilm formation (tissue culture plate method and congo red agar method):

2.4. Tissue culture plate method (TCP)

Tissue culture plate (TCP) assay described by Christensen et al. (1995) is considered the gold-standard method for biofilm detection. Isolates from fresh agar plates were inoculated in 10 mL of TSB with 1% glucose. Broths were incubated at 37°C for 24 h. The cultures were then diluted 1:100 with fresh medium. Individual wells of sterile 96 well flat bottom tissue culture plates were filled with 200 µL of the diluted cultures. Only sterile broths were served as blank to check sterility and non-specific binding of media. Similarly, control
organisms were also diluted and incubated. All three controls and blanks were put in the tissue culture plates. The culture plates were incubated at 37°C for 24 h. After incubation, to remove free-floating bacteria, gentle tapping of the plates were done. The wells were washed with 0.2 mL of phosphate buffer saline (pH 7.2) four times then were exposed to air-dry. The wells were then stained with 200 μL of 0.1% crystal violet for 30 minutes at room temperature. The wells were washed with distilled water to remove the unbounded dye, allowed to dry. The adhered stain was solubilized by addition 200μl of 95% ethanol. Optical densities (OD) of stained adherent biofilm were obtained by using micro ELISA auto reader at wavelength 630 nm. The experiment was performed in triplicate and repeated three times. Average of OD values of sterile medium were calculated and subtracted from all test values. ODs below 0.120 was considered as non-biofilm producers, 0.120 - 0.240 as moderate biofilm producers and more than 0.240 as strong biofilm producers(Bose et al., 2009, Hassan et al., 2011, Mathur et al., 2006).

2.5. Congo Red Agar method (CRA)
Freeman et al. (1989) had described a simple qualitative method to detect biofilm production by using CRA medium; which requires the use of a specially prepared solid medium -brain heart infusion broth (BHI) supplemented with 5% sucrose and Congo red. The medium was composed of BHI (Oxoid, UK) 37 g /L, sucrose (50 g /L), agar no.1 (Oxoid, UK) 10 g /L and congo red indicator (Oxoid, UK) 0.8 g /L. Congo red stain was prepared as concentrated aqueous solution and autoclaved at 121°C for 15 minutes. Then it was added to autoclaved Brain heart infusion agar with sucrose at 55 °C. Plates were inoculated with test organism and incubated at 37°C for 24 to 48 hours aerobically. Biofilm production was indicated by black colonies with a dry crystalline consistency. Brownish or reddish growth was considered as non biofilm producing organisms. The experiment was performed in triplicate and repeated three times (Hassan et al., 2011, Mathur et al., 2006, Bose et al., 2011).

2.6. Antimicrobial susceptibility
In vitro susceptibility testing of all collected isolates to a wide range of antimicrobials (ampicillin, cefepim, cefotaxime, cefoxitine, ceftazidime, cefuroxime, gentamycin, imipenem, meropenem, norfloxacin, ciprofloxacin, tobramycin, and colistin) was determined by Vitek-2 automated system which usually uses different Antimicrobial Susceptibility Test cards (AST-cards) according to the expected pathogens. The related cards were inoculated and incubated in the machine according to the manufacturer’s instructions (Lima et al., 2017).

3. RESULTS
A total of 300 specimens were included in the study. P. aeruginosa was present in 96 of various clinical specimens. Biofilm production by CRA method and TCP methods was seen in 76(79.1%) and 84(87.5%) isolates respectively. 12(12.5) were negative by TCP, while 20(20.8) were negative by CRA method. Table1

Table1. The percentage of biofilm formation among Pseudomonas aeruginosa isolates (n=96)

| Biofilm producers No. (%) | Biofilm non producers No. (%) |
|--------------------------|-------------------------------|
| 84(87.5)                 | 12(12.5)                      |

As shown in Figure 1 among 96 P. aeruginosa isolates, in TCP method detected 78 as strong, 6 as moderate and 12 as weak or non-biofilm producers. Different results were observed by the CRA method, the number of strong biofilm producers were 65, moderate were 11 and weak or non-biofilm producers were 20.
Figure 1. Screening of P. aeruginosa isolates for detection of biofilm formation by tissue culture plate and congo red agar methods

Out of 13 antimicrobes tested, all ninety six isolates were found to be 100% sensitive to colistin. They revealed a high resistance rate for ampicillin, cefotaxime and cefuroxime (100%). Biofilm producer among P. aeruginosa displayed the highest resistance to cefoxitin 81(96%) followed by cefepim 65(77%), norfloxacin 62(73%), tobramycin 54(64%), gentamicin 53(63%), imipenem 51(60%), ciprofloxacin 48(57%), meropenem 44(52%), and ceftazidime 56(47%). The non biofilm producers after colistin showed lowest resistance to meropenem 6(50%) followed by imipenem, ciprofloxacin and gentamicin 7(58%), ceftazidime and tobramycin 8(66%), cefepim and norfloxacin 9(75%) and cefoxitin 11(91%). The antibiotic resistance pattern of P. aeruginosa was found higher in biofilm producers than in biofilm non-producers. Table 2

Table 2. Antimicrobial- resistance pattern of P. aeruginosa among biofilm producers and non producers

| Antimicrobial | Biofilm Producers No. (%) | Biofilm Non producers No. (%) | Total resistant No. (%) |
|---------------|----------------------------|-------------------------------|------------------------|
| Ampicillin    | 84(100%)                   | 12(100%)                      | 96(100%)               |
| Cefotaxime    | 84(100%)                   | 12(100%)                      | 96(100%)               |
| Cefuroxime    | 84(100%)                   | 12(100%)                      | 96(100%)               |
| Cefoxitin     | 81(96%)                    | 11(91%)                       | 92(95%)                |
| Cefepim       | 65(77%)                    | 9(75%)                        | 74(77%)                |
| Ceftazidime   | 56(47%)                    | 8(66%)                        | 64(66%)                |
| Ciprofloxacin | 48(57%)                    | 7(58%)                        | 55(57%)                |
| Tobramycin    | 54(64%)                    | 8(66%)                        | 62(64%)                |
| Gentamicin    | 53(63%)                    | 7(58%)                        | 60(62%)                |
| Meropenem     | 44(52%)                    | 6(50%)                        | 50(52%)                |
| Imipenem      | 51(60%)                    | 7(58%)                        | 58(60%)                |
| Norfloxacin   | 62(73%)                    | 9(75%)                        | 71(74%)                |
| Colistin      | 0                          | 0                             | 0                      |

4. DISCUSSION

The major problem attributed with infections formed by biofilm producer bacteria is abundance of resistance to various antibiotics (Karthic and Gopinath, 2016). Production of an extracellular matrix is the hallmarks of a mature biofilm acts as a barrier for any antibiotics and increases resistance to these
antibiotics (Heydari and Eftekhar, 2015). In current study we tested 96 clinical isolates of 
P. aeruginosa for their ability to form biofilm by two phenotypic methods. TCP method could detect 84(87.5%) of 96 P. aeruginosa as biofilm producers compared to the CRA method 76(79.1%). Table 1 Based on the current results, there was a high prevalence of biofilm production in our isolates. Also Bakir and Ali (2016) from Erbil (Iraq) reported that out of 34 Gram-negative bacteria including P. aeruginosa isolates were 25 (73.5%) isolates produce biofilm by TCP method but in CRA method 20 (58.8%) produce biofilm. Another finding obtained by Ahmed (2013) from Erbil who documented that out of (73) Gram-negative bacteria isolates in TCP method 28 (38.4%) isolates were produce biofilm as (strong and moderate) which were lower than present results. Furthermore, Lima et al. (2017) tested TCP to detect biofilm formation among P. aeruginosa isolates. According to their results, 75% of the isolates exhibited biofilm formation. Devaraj and Sajjan (2015) described maximum biofilm production in P. aeruginosa (100%) amongst Gram negative bacilli. TCP method was the most effective method and detected biofilm production in 93% of Gram negative bacilli. Study conducted by Rewatkar and Wadher (2013), employed both tube and congo red agar methods, he reported in his study that (54/60) P. aeruginosa isolates detected by CRA. In a study by Abdallah et al. (2011) from Cairo have reported that the percentage of biofilm production by P. aeruginosa was (50%) from urinary tract infection. Samant and Pai (2012) recommend the TCP method for biofilm detection in their study; they found a very high incidence of biofilm production in staphylococcal isolates (89%). From this study TCP method gave significant result 81.2% strong biofilm production as compared to the CRA method (67.7%). Figure 1 It was found that the TCP could differentiate between strong, moderate, and weak biofilm producers as compared to CRA. It is also reported as gold standard by other researchers (Lima et al., 2017, Samant and Pai, 2012, Karthic and Gopinath, 2016, Bakir and Ali, 2016). Hence TCP method was considered as standard method for additional interpretation of results. In consistent finding have been obtained by Bakir and Ali (2016) they presented that for biofilm production by TCP method as (moderate and strong) by P. aeruginosa was (81.8%) and CRA (72.7%). Study performed by Karthic and Gopinath (2016) noted that out of 20 clinical isolates of P. aeruginosa isolated from different clinical specimens, 35% and 25% as moderate and strong biofilm producers respectively, which was incomparable with our results. Indeed, P. aeruginosa possesses a variety of resistance mechanisms, which allow its survival under the action of antimicrobial or biocides, as well as make it an important nosocomial infection threat (de Almeida Silva et al., 2017). It is evident from Table 2; there was a high frequency of resistance against all the frequently used antimicrobial agents. Biofilm producing P. aeruginosa isolates showed markedly high-level antimicrobial resistant to many groups of antibiotics as compared to biofilm non-producing isolates. This finding is important because therapy of patients with pseudomonal infections becomes more difficult when the isolate is biofilm producer, as biofilm is known to block the distribution of antibiotics. This observation is supported by various other researchers (Bakir and Ali, 2016, Karthic and Gopinath, 2016, Rewatkar and Wadher, 2013). The results in current study highlights the existence of association between antibiotic resistance and biofilm formation. Similar data have been recorded in the literature, Karthic and Gopinath (2016) showing high levels of biofilm in resistance isolates. This incidence is possibly attributed to gene transfer mechanisms within the biofilm environments, which is often acquired by transfer of genetic information from one organism to another as well as delayed diffusion of antibiotics inside the bacterial cell (Sahal and Bilkay, 2015). The highest resistance overall was observed against 3rd and 4th generation cephalosporins which is similar to Devaraj and Sajjan (2015) study which showed maximum resistance to penicillin (100%) and cephalixin (100%). This finding might be due to the inappropriate use
of antibiotics in this situation. According to our observation meropenem and ciprofloxacin were observed to be less frequently resistant, the present investigation is similar to the report of Karthic and Gopinath (2016). Both Bakir and Ali (2016) and Rewatkar and Wadher (2013) confirmed that all their isolates were sensitive to colistin which is in correlation with our results that showed none of the isolates were resistant to colistin, meanwhile colistin is effective antibiotic against biofilm-forming bacteria.

5. CONCLUSION

This study documents a high incidence of biofilm productions were demonstrated among P. aeruginosa isolates. TCP method was considered as effective test for detection of biofilm formation and was also able to verify biofilm production by the P. aeruginosa. Based on our findings we recommended TCP method as a screening method. Importantly, our P. aeruginosa isolates were observed to be resistant to most commercially used antimicrobials. This indicated a higher propensity among the clinical isolates of P. aeruginosa to form biofilm and there were a positive correlation exists between biofilm formation and antibiotic resistance.

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