Journal of Genetic and Environmental Resources Conservation, 2017, 5(1):28-32.
www.jgerc.com Print ISSN: 2306-8663, Online ISSN: 2306 – 8280

Correlation between biofilm, protease production and antibiotic resistance in clinical bacterial isolates

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Abstract

Six clinical bacterial isolates (*Enterobacter* sp., *Serratia marscense, Pseudomonas aeruginosa, Salmonella* sp., *Staphylococcus aureus and Micrococcus* sp.*)* isolated from blood of pulmonary patients in Ibn Al-Baladi hospital, in Baghdad- Iraq, were included in this study to determine their biofilm formation capability and other virulence properties (protease production and antibiotic resistance). Biofilm formation was down by; Congo red agar method (CRA) and tube method. Four isolates (*Pseudomonas aeruginosa, Serratia* sp., *Enterobacter spp.* and *Staphylococcus aureus*) were positive for biofilm production, while two weakly adherent isolates (*Salmonella* sp. *and Micrococcus* sp.) considered as negative or non-biofilm producers. Most of the positive isolates showed thick blue ring at the liquid-air interface in tube method and black colonies with a dry crystalline consistency indicated biofilm production in CRA method. Cotrimoxazol, Lincomycin, Tetracyclin and Vancomycin found to be more effective against all of the isolates, while Amikacin found effective only for *Enterobacter* sp. and *Staphylococcus aureus*, the rest kinds of antibiotic tested were non-effective for all bacterial isolates. All bacterial isolates showed a high protease activity (defined as a clear circle zone by degrading milk proteins). The results showed there are significant correlation between production of biofilm, protease and resistant to antibiotic from clinical bacterial isolates tested in this study. *Pseudomonas aeruginosa, Serratia* sp.*, Enterobacter* sp. and *Staphylococcus aureus* were produce strong biofilm and good protease producers, also they resisted to 4 kinds of antibiotic (Cotrimoxazol, Lincomycin, Tetracyclin and Vancomycin), in addition to *Enterobacter* sp. and *Staphylococcus aureus* which resisted to Amikacin too. Understanding the correlation between antibiotic resistance and biofilms, protease production and their role in serious infections of clinical isolates will help in development of new therapy for pulmonary infections.

Keywords: Clinical bacterial isolates, Biofilm formation, Protease production, Antibiotic resistance.

Introduction

Bacterial biofilms are complex communities of microorganisms embedded in a self-produced matrix, in addition, adhering to inert or living surfaces (Rijavec *et al*., 2008). Biofilms are architecturally complex assemblies of microorganisms that form on biotic or abiotic surfaces or at interfaces. Biofilms exhibit altered phenotypes with respect to growth rate and gene transcription (Taj *et al*., 2012). Biofilm production considered as a marker of clinically relevant infection and persistence of bacterial biofilms in the human body is a major cause of recurrent or chronic infections (Murugan and John, 2011). A role for bacterial biofilms in pathogenesis well established for a number of infections and opportunistic pathogens (Lassaro, *et al*., 2009). Biofilm mediates interaction between bacteria and host tissue through adhesion, which is pre-requisite for invasion and tissue-specific colonization (Ghanbarpour and Salehi, 2010). Biofilms are not only resistant to antibiotics but also to a variety of disinfectants, which emphasizes that their characterization is an important aspect of infection control. Biofilm also facilitates coexistence of otherwise competing bacterial species (Mathur *et al*., 2006).

Bacterial biofilms ubiquitous in natural, medical, and engineering environments, because of their increased tolerance to antimicrobial treatment, biofilms formed by pathogenic bacteria can pose serious problems to human health, such as cystic fibrosis pneumonia, prostatitis, and periodontitis (Tirumalai and Prakash, 2012). In natural niches, bacteria grow in polymicrobial communities where competition or cooperation between the community members is important for bacterial survival in limited

resources and space. As a survival strategy, many bacteria are able to form biofilms and some bacteria produce biofilm-inhibiting molecules against other species (Rendueles and Ghigo, 2012). Biofilm formation is a well-known pathogenic mechanism in device related infections in hospitals (Stewart and Costerton, 2001). Moreover, the environmental survival of some nosocomial pathogens may facilitate by biofilm formation on abiotic surfaces (Srinivasa *et al*., 2008). The present work was undertaken to evaluate the correlation of biofilm formation with protease production and antibiotic resistance of six clinical bacterial isolates isolated from pulmonary infections.

Materials and Methods

Bacterial isolates and culture media: Six clinical bacterial isolates (4 Gram-negative isolates; *Enterobacter* sp., *Serratia marscense, Pseudomonas aeruginosa, and Salmonella),* and (2 Gram-positive isolates; *Staphylococcus aureus and Micrococcus* sp.*)* isolated from blood of pulmonary patients in Ibn Al-Baladi hospital, Baghdad, Iraq. Isolates cultured on Brain Heart infusion broth (BHI) provided from Oxoid (England) at 37°C for 24-48hr. Bacterial isolates maintained at 4°C on nutrient agar slants.

Antibiotic susceptibility testing: All the clinical isolates of bacteria tested for antibiotic resistance by well diffusion agar method on Mueller Hinton agar. The following antibiotics with the disc strength in parentheses were used: Tetracycline (Tet, 30µg), Cotrimoxazol (STX, 30µg), Ciprofloxacin (CIP, 5µg), Norfloxacin (NOR, 10µg) Lincomycin (L, 15µg), Amikacin (AK, 30µg), Netillin (NET,30 µg), Vancomycin (VA, 30µg). Antimicrobial break points and interpretation take from the CLSI standards (CLSI, 2011).

Biofilm formation: Biofilm formation down by two methods:

1-Congo red agar method (CRA): This method down according to (1) briefly, Congo Red Agar medium prepared with: (37g/L) Brain Heart Infusion broth, (50g/L) sucrose, (10g/L) agar, and (0.8g/L) Congo red stain (Donlan, 2001).

2-Tube method: The isolates cultured in trypticase soya broth, and incubated at 37°C for 24hr., then tubes drain, and add 10 ml of safranin stain (0.1%) to each of it, and leave it for 1 min. after that tubes drain and up dawn. The scoring for tube method was doing according to the results of the control strains. Biofilm formation considered positive when a visible film lined the wall and the bottom of the tube (Afreenish *et al*., 2011).

Screening of protease by using Skim milk agar: The isolates were sub cultured on Trypticase Soya Agar (TSA), and incubated at 37°C for 24 hr. Then they cultured on skim milk agar plates (containing 5 g of nonfat dry milk and 0.5g of Bacto-agar (Difco) in 50 mL of distilled water) and incubated at 37°C for 24hr. Protease production tested by observing hydrolysis of casein when grown on milk agar medium (Mansour *et al*., 2014).

Results and Discussion

The overall resistance to antibiotic observed among all the clinical isolates including for eight antibiotics tested, given in (Table 1). Cotrimoxazol, Lincomycin, Tetracyclin and Vancomycin found to be more effective against all of the isolates, while Amikacin found effective only for *Enterobacter* sp. The rest kinds of antibiotic tested were non-effective for all bacterial isolates.

Clinical bacterial isolates (including species tested in this study) present a global medical challenge. They are opportunistic pathogens and are particularly successful at colonizing and persisting in the hospital environment. They are able to resist desiccation and survive on inanimate surfaces for years (Navon-Venezia *et al.*, 2005). They are growing rapidly because of the emergence of multi-drug-resistant strains, some of which are pan-resistant to antimicrobial agents. They also among the most common causes of device-related nosocomial infection that results when the organism is able to resist physical and chemical disinfection, often by forming a biofilm (Srinivasa *et al.*, 2008).

Qualitative tube and congo red methods of biofilm screening as in Figure (1) showed 4 isolates (*Pseudomonas aeruginosa, Serratia* sp., *Enterobacter* sp. and *Staphylococcus aureus*) positive for biofilm production. Two weakly adherent isolates (*Salmonella* sp. *and Micrococcus* sp.) considered as negative or non-biofilm producers. Both the methods for biofilm detection thus showed similar results. Most of the positive isolates showed thick blue ring at the liquid-air interface in tube method and black colonies with a dry crystalline consistency indicated biofilm production in CRA method.

During their evolution, bacteria have been able to develop successful strategies for survival, which include attachment to surfaces and the development of protective biofilms where bacteria behave very differently to the free-floating types (Joo-Hyeon *et al.*, 2012).

Table (1): Antibiotic resistant to bacterial isolates

Bacterial isolates	STX	CIP	NOR	L	AK	TE	NET	VA
Pseudomonas aeruginosa	R	S	S	R	S	R	S	R
Salmonella sp.	R	S	S	R	S	R	S	R
Serratia sp.	R	S	S	R	S	R	S	R
Enterobacter sp.	R	S	S	R	R	R	S	R
Micrococcus sp.	R	S	S	R	S	R	S	R
Staphylococcus aureus	R	S	S	R	R	R	S	R

STX: Cotrimoxazol (30µg), CIP: Ciprofloxacin (5µg), NOR: Norfloxacin (10µg), L: Lincomycin (15 µg), AK: Amikacin (30µg), TE: Tetracyclin (30µg), NET: Netillin (30µg), VA: Vancomycin (30µg).

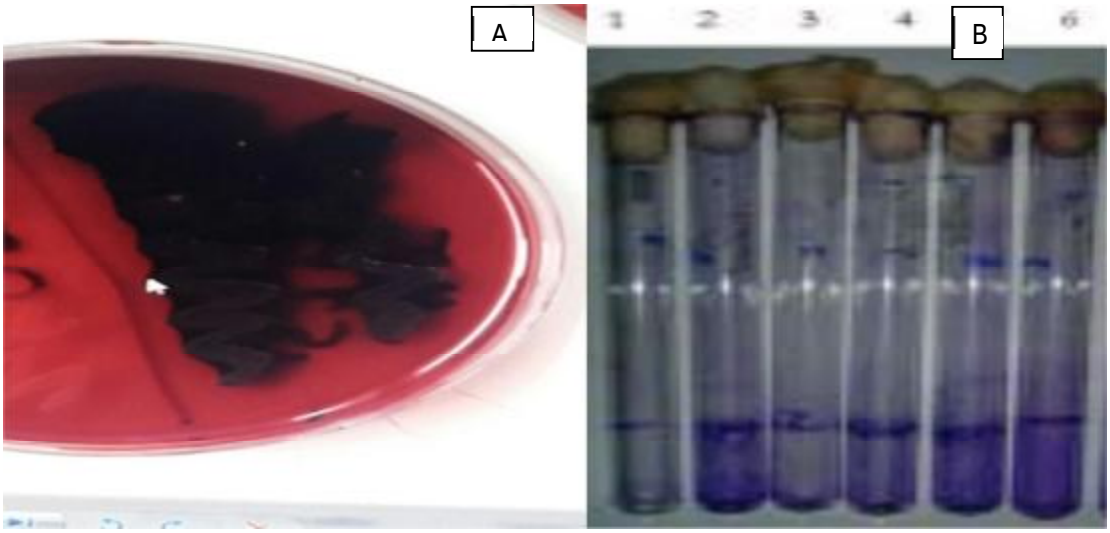

Figure (1): Biofilm formation of bacteria on: A- Congo red agar plate, B-Tube method.

These successful strategies make it difficult to control biofilm growth, with a biofilm providing bacteria with a 10- to 1,000-fold increase in antibiotic resistance compared to free ones. Some mechanisms of resistance appear to be intrinsic to growth in a biofilm; with the inhibited diffusion through the matrix, reduced metabolism by nutrient limitation, formation of dormant persisters, and increased production of oxidative stress all appearing to impact on the development of a protective environment within the biofilm; in addition to extrinsic factors such as -induced gene expression by antibiotics in biofilm cells (Boles, BR. and Horswill, AR. 2011).

Protease determination in the bacterial isolates: The protease activity in the supernatants of all bacterial species measured using milk agar plates as protease activity plays an important role in the disassembly of bacterial biofilms (Paraje, 2011). All bacterial isolates showed a high protease activity (defined as a clear circle zone by degrading milk proteins) (Figure 2).

SecDF is an accessory factor of the conserved Sec protein translocation machinery, and belongs to the resistance-nodulation-cell division (RND) family of multidrug exporters. SecDF has shown in *Escherichia coli* and *Bacillus subtilis* to be involved in the export of proteins (Quiblier *et al.*, 2011).

Table (2) showed the correlation between production of biofilm and protease from clinical bacterial isolates tested in this study. It was obvious that all isolates were good producers to protease enzyme, while only *Pseudomonas aeruginosa, Serratia* sp., *Enterobacter* sp. and *Staphylococcus aureus* were produce biofilm, and as mentioned in Table (1) all of these isolates also exhibit resistance to 4 kinds of antibiotic (Cotrimoxazol, Lincomycin, Tetracyclin and Vancomycin), in addition *Enterobacter* sp. and *Staphylococcus aureus* resisted to another antibiotic (Amikacin).

Figure (2): Protease determination on agar of bacterial isolates

One of the most important aspects of bacterial biofilm formation is the increased resistance of the constituent microbes to antibiotics and other stressors. The structural natures of the biofilms, and the characteristics of the sessile cells, are produce resistance towards the antimicrobial agents, leading to a protected environment against adverse conditions and the host´s defenses (Joo-Hyeon *et al.*, 2012). Bjarnsholt *et al*. (2015) demonstrated an important correlation between the antibiotic susceptibilities of biofilms In vitro using the efficacy of antibiotic treatment in vivo. Clinical bacteria acquired multi-drug resistance is one of the major sources of illness and death, thereby increasing healthcare cost (Akond *et al.*, 2009). Antibiotic susceptibility pattern of the isolates revealed multi-antibiotic resistance in the isolates but resistance to third generation antibiotics were comparatively lower. Most of the strong biofilm formers with virulence traits were more antibiotic resistant than other isolates. (Golia *et al*., 2012) Suggested that is screening of biofilm could consider as virulence marker in drug resistant *E. coli* isolates. (Fakruddin *et al.*, 2015) Remarked that biofilm production in *E. coli* may promote colonization and lead to increased rate of infections like UTI and such infections may be difficult to treat as they exhibit multi drug resistance.

Conclusion

The results of this study showed significant correlation between production of biofilm and protease, and resistance to multiple kinds of antibiotic from six clinical bacterial isolates tested. *Pseudomonas aeruginosa, Serratia* sp., *Enterobacter* sp. and *Staphylococcus aureus* were produce strong biofilm, and good protease producers, also they resisted to 4 kinds of antibiotic (Cotrimoxazol, Lincomycin, Tetracyclin and Vancomycin), in addition, *Enterobacter* sp., and *Staphylococcus aureus* which resisted to Amikacin too. A greater understanding of biofilms formation, protease production and antibiotic resistance and their role in serious infections of clinical isolates will help in development of new and more effective treatment for pulmonary infections.

Acknowledgment

The corresponding author would like to thank Al-Mustansiriyah University (www.uomustansiriyah.edu.iq), Baghdad, Iraq for its support in the present work.

Table (2): production of biofilm and protease

Bacterial isolates	Biofilm	protease
Pseudomonas aeruginosa	+	+
Salmonella sp.	-	+
Serratia sp.	+	+
Enterobacter sp.	+	+
Micrococcus sp.	-	+
Staphylococcus aureus	+	+

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