

The association of biofilm formation and sub-minimal inhibitory concentrations of antimicrobial agents

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Abstract

Introduction: Although bacteria producing biofilm are more resistance to antimicrobial agents, biofilm formation can be stimulated by sub-minimal inhibitory concentrations (sub-MICs) of some antimicrobial agents. Therefore, we designed present study to investigate the *in vitro* efficacy of several antibiotics (including ceftazidime, piperacillin, ticarcillin, carbenicillin, aztreonam, meropenem, gentamicin, amikacin and ciprofloxacin) and biocides (including savlon, benzalkonium chloride and chlorohexidin) on biofilm formation in *Pseudomonas aeruginosa* (*P. aeruginosa*) isolates.

Materials and methods: A total of 10 uropathogenic *P. aeruginosa* isolates were collected from Mostafa Khomani Hospital in Ilam. The isolates were evaluated for MIC, biofilm formation ability and finally the effect of different concentration of antimicrobial agents on the biofilm formation.

Results: Our finding demonstrated that all antimicrobial agents except gentamicin, aztreonam, and savlon were able to induce biofilm formation at sub-MICs. Moreover, savlon was the best agents for encountering biofilm formation in *P. aeruginosa*.

Conclusion: Some antimicrobial agents are able to induce biofilm formation at sub-MICs. Biofilm formation inducement depended on antimicrobial agents, strains, and matrix composition.

Keywords: *Pseudomonas aeruginosa*, Biofilm, Biofilm formation inducement

Introduction

The low requirement nutrition cause *P. aeruginosa* isolated from many places such as lakes, soils and plants. *Pseudomonas aeruginosa* is known as human opportunistic pathogens, which able to infect animals, plants, and insects. It is also considered as a major threat for infection in the immunocompromised individuals and cystic fibrosis patients (1-3). These isolates have high tend to biofilm formation on the tissues or medical devices (4, 5). A bacterial biofilm is known as bacterial community enclosed in a self-

produced polymeric matrix that adherent to an inert or living surface (6). Although it is clear that the bacteria producing biofilm are more resistance to antimicrobial agents, its formation can be stimulated by sub-MICs of some antimicrobial agents (7). Notably, biofilm formation inducement is known as a major health issue, because bacteria commonly expose to sub-MICs of antimicrobial agents (8). Therefore, we investigated *in vitro* effect of several antibiotics (e.g. ceftazidime, piperacillin, ticarcillin,

carbenicillin, aztreonam, meropenem, gentamicin, amikacin, ciprofloxacin) and biocides (e.g. savlon, benzalkonium chloride and chlorohexidin) on the biofilm formation of *P. aeruginosa* isolated from clinical samples.

Materials and methods

Bacterial identification and culture conditions: The isolates were identified as standard protocols. Briefly, they cultured on MacConkey agar media and examined by microscope after Gram staining. Then, according to Bergey's Manual of Systematic Bacteriology, the activities of oxidase and catalase, MR-VP test, gelatin hydrolysis and starch hydrolysis, indole production, motility and citrate utilization were tested (9).

MIC determination: Briefly, planktonic susceptibility testing of *P. aeruginosa* was applied by the reference broth microdilution assay according to CLSI protocol with 96-well polystyrene flat-bottom microtitre plate and adjusted Mueller–Hinton broth. MIC for each antimicrobial agent was determined using ten dilutions. It was performed as triplicate and MICs were reported after 24 h of incubation in 35°C by microplate reader RT-2100C at 492 nm (10).

Biofilm formation assay: For biofilm formation; (1) strains were incubated overnight at 37°C in LB broth. (2) Bacterial suspensions were provided for each isolates (MacFar-land 0.5 standard, including, 1.5×10^8 CFU/mL). (3) 190 μ l LB broth was added to each polyvinylchloride 96-well microtiter plate wells, and (4) 10 μ l bacterial suspension added, (5) and incubated in 37°C for overnight. Biofilm assay was performed as triplicate, and LB broth used as negative control (10).

Biofilm formation measurement: After incubation, (1) microplate was washed with distilled water. (2) The wells stained with 0.1% crystal violet and left at room temperature for 10 min (3) and washed with distilled water for three times. (4) 200

μ l 95% ethanol was added and OD was measured at 570 nm with ELISA reader. These OD values considered as bacteria adhering index to surface and biofilm formation. For biofilm formation quantitative analysis, average absorbance of the control wells (Ac) subtracted from the A570 nm of all test wells. To all strains, averages and standard deviations (SD) calculated and they classified as follows: $A \leq A_c$ = no biofilm formation, $A_c < A \leq (2 \times A_c)$ = weak biofilm formation, $(2 \times A_c) < A \leq (4 \times A_c)$ = moderate biofilm formation and $(4 \times A_c) < A$ = strong biofilm formation (10).

Determination effects of antimicrobial agents on the biofilm formation:

Different concentration of antibiotics and biocides were applied to measure biofilm formation by microtitre plates as mentioned above. Drug-free medium applied as control negative for each concentration.

Statistical analysis

All data entered to SPSS 16.0 software. Thereafter, the data was analysed by this software.

Results

Our finding demonstrated that all antibiotics (except meropenem, gentamicin and aztreonam) including ceftazidime (MIC=8 μ g/ml), ticarcillin (MIC=256 μ g/ml), carbenicillin (MIC=32 μ g/ml), piperacillin (MIC=8 μ g/ml), amikacin (MIC=256 μ g/ml) and ciprofloxacin (MIC=16 μ g/ml) induced biofilm formation at sub-MICs. We also observed that all antibiotics, except gentamicin and aztreonam, decreased biofilm formation by increasing concentration. Benzalkonium chloride (MIC=0.007 μ g/ml) and chlorohexidin (MIC=0.125 μ g/ml) also induced biofilm formation at sub-MICs, while savlon severely decreased biofilm formation.

Discussion

Pseudomonas aeruginosa cause diverse infections, especially in the immunocompromised patients (11). *Pseudomonas aeruginosa* biofilms leads to increasing bacterial survival in the unfavorable environment and risk of persistent infections (12-14). These isolates are the main opportunistic human pathogens cause hospital-acquired infections such as respiratory system, urinary tract, gastrointestinal infections as well as keratitis, otitis media, folliculitis, endocarditis and bacteremia (15, 16).

The antibiotics in the present study belonged to three groups, including β -lactams (ceftazidime, piperacillin, ticarcillin, carbenicillin, aztreonam, and meropenem), aminoglycosides (amikacin, gentamicin), and quinolones (ciprofloxacin). We observed that all antibiotics and biocides induced biofilm formation except gentamicin, aztreonam, and savlon. Previously, it has been also demonstrated that several drugs caused biofilm formation (17-22). Mandy *et al.* (2014) investigated sub-MIC effects of β -lactam antibiotics (e.g. penicillin, amoxicillin, ampicillin, piperacillin, nafcillin, cloxacillin, ceftriaxone and cefazolin) and six non- β -lactam antibiotics (e.g. trimethoprim/sulfamethoxazole, linezolid, vancomycin, daptomycin, tigecycline, and rifampicin) on the biofilm formation in methicillin-resistant *Staphylococcus aureus* (MRSA). They observed that all eight β -lactam antibiotics induced biofilm formation at sub-MIC (23). Bleich *et al.* (2015) reported that although antibiotics are chemical tools for elicit biofilm formation, they also have potential to stimulate biofilm formation (24). This phenomenon probably is related to (i) a defensive response against sub-

MICs, (ii) bacterial cooperation or (iii) actual ecological and evolutionary role of antimicrobial drugs in the nature (25). The diffusion gradients also cause that cells buried in the deep of biofilm exposed to sub-MICs of antimicrobial agents (8). In addition, this effect observed by many drugs, chemicals or biological molecules and physical stressors (26). Biofilm formation is also increased antibiotic resistance (29, 30). We recommended that other novel mechanisms related to biofilm formation should be investigated in future studies (31).

Conclusion

It is clear that bacteria during treatment period or in the environment exposed to various concentrations of antimicrobial drugs, which can cause many clinically problems by biofilm formation inducement. Moreover, widespread antimicrobial drugs abuse in agriculture exposed microorganisms to low drugs concentrations that cause biofilm formation inducement and transfer resistance factors by horizontal transfer mobile genetic elements (7, 8). Current study can be useful to investigate clinical isolates for optimizing antibiotic chemotherapy in clinical settings as well as increasing people awareness about hazards of widespread antimicrobial abuse (26-28). Moreover, we observed that savlon was the best antimicrobial agent against biofilm formation in *P. aeruginosa*.

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References

1. Daniel JH, Ju-Fang M, James GE, Timothy RM, Urs AO, Susan EHW, *et al.* Quorum sensing in *Pseudomonas*

aeruginosa controls expression of catalase and superoxide dismutase genes and mediates biofilm

- susceptibility to hydrogen peroxide. *Mol Microbiol.* 1999; 34(5):1082-93.
2. Sudha A, Chugani MW, Kimberly ML, David DA, Colin Manoil, Greenberg EP. QscR, a modulator of quorum-sensing signal synthesis and virulence in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A.* 2001; 98 (5):2752–7.
 3. Susan LM, Barbara HI, Everett CP. The *Pseudomonas* Quinolone Signal Regulates rhl Quorum Sensing in *Pseudomonas aeruginosa*. *J Bacteriol.* 2000;182 (10):2702–8.
 4. Hwang SJ, Michael O. Molecular Basis of In Vivo Biofilm Formation by Bacterial Pathogens. *Chem Biol.* 2012;19(12):1503-13.
 5. Lokender K, Sanjay C, Kusum H. Zingerone inhibit biofilm formation and improve antibiofilm efficacy of ciprofloxacin against *Pseudomonas aeruginosa* PAO1. *Fitoterapia.* 2013;90:73-8.
 6. Aleksandra T, Grzegorz F, Mariusz G, Joanna N. Innovative strategies to overcome biofilm resistance. *Biomed Res Int.* 2012;2013:1-13.
 7. Kaplan JB, Izano EA, Gopal P, Karwacki MT, Kim S, Bose JL, et al. Low levels of β -lactam antibiotics induce extracellular DNA release and biofilm formation in *Staphylococcus aureus*. *MBio.* 2012;3(4):e00198-12.
 8. Kaplan JB. Antibiotic-induced biofilm formation. *Int J Artif Organs.* 2011;34(9):737-51.
 9. Raja CE, Anbazhagan K, Selvam GS. Isolation and characterization of a metal-resistant *Pseudomonas aeruginosa* strain. *World J Microb Biot.* 2006;22(6):577-85.
 10. Hemati S, Jalilian FA, Pakzad I, Taherikalani M, Maleki A, Karimi S, et al. The correlation between the presence of quorum sensing, toxin-antitoxin system genes and MIC values with ability of biofilm formation in clinical isolates of *Pseudomonas aeruginosa*. *Iran J Microbiol.* 2014;6(3):133-9.
 11. Andera DT. Biofilms: The T3SS translocon in biofilm formation. *Nat Rev Microbiol.* 2015;13(1):2-3.
 12. Chalabaev S, Chauhan A, Novikov A, Iyer P, Szczesny M, Beloin C, et al. Biofilms formed by Gram-negative bacteria undergo increased lipid a palmitoylation, enhancing *in vivo* survival. *MBio.* 2014;5(4):e01116-14.
 13. Wiens JR, Vasil AI, Schurr MJ, Vasil ML. Iron-regulated expression of alginate production, mucoid phenotype, and biofilm formation by *Pseudomonas aeruginosa*. *MBIO.* 2014;5(1):e01010-13.
 14. Ta CA, Freundorfer M, Mah TF, Otárola-Rojas M, Garcia M, Sanchez-Vindas P, et al. Inhibition of bacterial quorum sensing and biofilm formation by extracts of neotropical rainforest plants. *Planta Medica.* 2014;80(4):343-50.
 15. Nathwani D, Raman G, Sulham K, Gavaghan M, Menon V. Clinical and economic consequences of hospital-acquired resistant and multidrug-resistant *Pseudomonas aeruginosa* infections: a systematic review and meta-analysis. *Antimicrob Resist Infect Control.* 2014;3(1): 2-16.
 16. Meadows JA, Wargo MJ. Catabolism of host-derived compounds during extracellular bacterial infections. *J Cell Biol.* 2014;115(2):217-23.
 17. Dorota KW, Ewelina M, Beata FB, Daria L, Alicja G, Pawe S and et al. Antibiotics promoting oxidative stress inhibit formation of *Escherichia coli* biofilm via indole signalling. *Res Microbiol.* 2010;161(10):847-53.
 18. Virginia A, Ana IB, Inés A. Resistance to ciprofloxacin by enhancement of antioxidant defenses in biofilm and planktonic *Proteus mirabilis*. *Biochem Biophys Res Commun.* 2010;393(1):84-8.
 19. Kamel C, Tarek Z, Yosra S, Kacem M, Amina B. XTT assay for evaluating the effect of alcohols, hydrogen peroxide and benzalkonium chloride on biofilm

- formation of *Staphylococcus epidermidis*. *Microb Pathog*. 2011;50(1):1-5.
20. Amit K, Yen-Peng T. Effect of sub-inhibitory antibacterial stress on bacterial surface properties and biofilm formation. *Colloids Surf B Biointerfaces*. 2013;111:747-54.
 21. Huang CY, Hsieh SP, Kuo PA, Jane WN, Tu J, Wang YN, et al. Impact of disinfectant and nutrient concentration on growth and biofilm formation for a *Pseudomonas* strain and the mixed cultures from a fine papermachine system. *Int Biodeterior Biodegradation*. 2009; 63:998–1007.
 22. Nasim K, Mahboobeh A, Seyed Kamran K. Effect of sub-lethal photodynamic inactivation on the antibiotic susceptibility and biofilm formation of clinical *Staphylococcus aureus* isolates. *Photodiagnosis Photodyn Ther*. 2013;10(4):368-73.
 23. Ng M, Epstein SB, Callahan MT, Piotrowski BO, Simon GL, Roberts AD, et al. Induction of MRSA biofilm by low-dose β -lactam antibiotics: Specificity, prevalence and dose-response Effects. *Dose Response*. 2014;12(1):152-61.
 24. Bleich R, Watrous JD, Dorrestein PC, Bowers AA, Shank EA. Thiopeptide antibiotics stimulate biofilm formation in *Bacillus subtilis*. *Proc Natl Acad Sci U S A*. 2015;112(10):3086-91.
 25. Linares JF, Gustafsson I, Baquero F, Martinez JL. Antibiotics as intermicrobial signaling agents instead of weapons. *Proc Natl Acad Sci U S A*. 2006;103(51):19484-9.
 26. Kaplan JB, Izano EA, Gopal P, Karwacki MT, Kim S, Bose JL, et al. Low Levels of β -Lactam Antibiotics Induce Extracellular DNA Release and Biofilm Formation in *Staphylococcus aureus*. *Mbio*. 2012; 3(4):e00198-12.
 27. Maillard JY. Bacterial resistance to biocides in the healthcare environment: should it be of genuine concern? *J Hosp Infect*. 2007;65(2):60–72.
 28. Leung CY, Chan YC, Samaranyake LP, Seneviratne CJ. Biocide resistance of *Candida* and *Escherichia coli* biofilms is associated with higher antioxidative capacities. *J Hosp Infect*. 2012;81(2):79-86.
 29. Ghafourian S, Sadeghifard N, Soheili S, Sekawi Z. Extended spectrum beta-lactamases: Definition, classification and epidemiology. *Curr Issues Mol Biol*. 2014;17(1):11-22.
 30. Mohebi R, Ghafourian S, Sekawi Z, Neela V, Raftari M, Aboualigalehdari E, et al. Extended-spectrum beta-lactamases producing *Klebsiella* species isolated from several major hospitals in Iran. *Eur J Inflamm*. 2012;10(3):269-78.
 31. Ghafourian S, Raftari M, Sadeghifard N, Sekawi Z. Toxin-antitoxin systems: Classification, biological function and application in biotechnology. *Curr Issues Mol Biol*. 2014;16(1):9-14.