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 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 Khomaini 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 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 37ºC in LB broth. (2) Bacterial suspensions were provided for each isolates (MacFar-land 0.5 standard, including, 1.5×108 CFU⁄mL). (3) 190 µl LB broth was added to each polyvinylchloride 96-well microtiter plate wells, and (4) 10 µl bacterial suspension added, (5) and incubated in 37ºC 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 µl 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≤ Ac=no biofilm formation, Ac < A≤ (2×Ac) = weak biofilm formation, (2 ×Ac) < A≤ (4 ×Ac) = moderate biofilm formation and (4×Ac) < 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 µg/ml), ticarcillin (MIC=256 µg/ml), carbenicillin (MIC=32 µg/ml), piperacillin (MIC=8 µg/ml), amikacin (MIC=256 µg/ml) and ciprofloxacin (MIC=16 µg/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 µg/ml) and chlorohexidin (MIC=0.125 µg/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, pipericillin, nafcillin, cloxicillin, 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 beast antimicrobial agent against biofilm formation in *P. aeruginosa*.

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