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Comparison of biofilm formation and antibiotic resistance pattern of *Pseudomonas aeruginosa* in human and environmental isolates

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Pseudomonas aeruginosa is an opportunistic human pathogen especially in patients with underlying diseases such as cyctic fibrosis and has been established as a model organism to study bacterial biofilm formation. The aim of this study was to compare the biofilm formation and antibiotic resistance in human and environmental *P. aeruginosa* isolates. Numbers of positive samples for *algD* and *algU* genes in human samples were 98% and the positive samples for *algD* and *algU* genes in the environmental samples were 80% and 70%, respectively. Ability to create biofilms by the human and environmental samples were 70% and 28%, respectively. The incidences of various antibiotic resistance genes in human samples including bla_{TEM} and bla_{SHV} were 92% and 16%, respectively but antibiotic resistance genes in environmental samples including bla_{TEM} and bla_{SHV} were 20% and 6%, respectively. High resistance to gentamicin (74%) and meropenem (70%), were found in the human samples, were as in the environmental samples high level of resistance were observed to ceftazidime (30%), gentamicin and meropenem (28%). According to findings of this study, differences in genes involve in biofilm synthesis between human and environmental isolates are highly significant and the environmental isolates of *P. aeruginosa* stile are sensitive to most antibiotics because they lacks the antibiotic resistance genes. But after transfer to human and isolation from diseased people have been taken the antibiotic resistance genes that would be resistant to many antibiotics.

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1. Introduction

Pseudomonas aeruginosa is a gram negative bacilli that is found all around us and is widely found in soil and water [1,2]. *P. aeruginosa* is an opportunistic human pathogen especially in patients with underlying diseases such as cyctic fibrosis [3]. *P. aeruginosa* infections principally affect the patients in intensive care units, chronic illnesses and burn patients [4].

Biofilms are microbial society encased in extracellular polymeric substances (EPS) [5]. Biofilm formation symbolizes a protective mode of growth that allows microorganisms to survive in hostile environments [6]. Biofilm is responsible for persistent in chronic infections, due to their inherent resistance to antimicrobial agents. A better understanding of the genetic and molecular mechanisms of biofilm formation may provide strategies for the control of chronic infections and problems related to biofilm formation [7].

Three exopolysaccharides have been identified *in P. aeruginosa* including: alginate, Psl and Pel [9,11]. Each of these exopolysaccharides has been found to be involved in biofilm formation [11]. Alginates are a linear unbranched polymer composed of 1–4 linked saccharides b-D mannuronic acid (M) and a C-5 epimer of a-L-guluronic acid (G) [14]. The three genes, *algU*, *algL* and, *algD* are involved in the production of alginate [15,16].

Alginate is mainly produced by *P. aeruginosa* clinical isolates from the lungs of patients [10] and plays important roles in structural protection and stability of biofilms. It is necessary for nutrient and water retention in biofilms [13]. Alginate has been shown to contribute to reduce susceptibility of biofilms to antibiotic treatment and human antibacterial defense mechanisms [8,12]. Alginate production reduce complement activation and macrophage phagocytosis [18–21]. As shown in a series of studies the excessive productions of alginate in fact create a resistance to some types of antibiotics [22]. Alginate characteristics caused precedence over

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other factors in the biofilm structure and selected for studies.

There are three basic mechanisms by which organisms resist the action of antimicrobial agents: restricted uptake and efflux, drug inactivation and changes in targets, all three are affected in antibiotic resistance in *Pseudomonas* [49]. Drug susceptible strains of *P. aeruginosa* have serious defenses. *P. aeruginosa* has an inducible AmpC β-lactamase and is intrinsically resistant to those β-lactams that induce this enzyme and are hydrolyzed by it [48].

This high antibiotic resistance of *P. aeruginosa* was against commonly especially β-lactams and used antibiotics, including cephalosporins, monobactams, carbapenems, and penicillin, mainly encoded by several antibiotic resistance genes, including bla_{TEM}, bla_{SHV}, bla_{OXA}, $bla_{CTX\text{-}M}$, bla_{DHA} and bla_{VEB} [31]. The aim of this study was to compare the biofilm formation and antibiotic resistance in human and environmental *P. aeruginosa* isolates.

2. Material and methods

2.1. Identification of P. aeruginosa isolates

All human (27samples of respiratory infections, 13samples of urinary tract infections and 10samples from burns patients) and environmental (32 samples from soil and 18 samples of surface and springs water) samples were inoculated on Pseudomonas Cetrimide Agar (PCA) (Merck, Germany) (Table 1). The identification of *P. aeruginosa* isolates were carried out by using standard microbiological and biochemical tests such as colony characteristics, motility, oxidase reaction, citrate utilization, sugar fermentation and H_2S production. All isolates were stored in Trypticase Soy broth with 15% glycerol at −70 °C for future experiments.

2.2. DNA extraction and detection of biofilm factors and antibiotic resistance genes

DNA was extracted from each *P. aeruginosa* isolate by DNA extraction kit (MBST, Zistmolecule, Iran). The detection of *16s rRNA*, *algU*, *algD*, bla_{TEM}, and bla_{SHV} genes was performed by PCR using specific primers (Table 2). PCR amplifications performed with an automated thermal cycler (Technet tc512, England) in the PCR cycling based on conditions have been shown in Table 1. *P. aeruginosa* and *E. coli* were used as positive and negative control, respectively (Tehran University Treasure gene Department of Microbiology).

Amplification reactions were prepared in a total volume of 25 µl (24 µl of PCR master mix plus 1 µl of template DNA) including 5 ng of genomic DNA, 2.0 U of *Taq* DNA polymerase, 10 mM dNTP mix at a final concentration of 0.2 mM, 50 mM $MgCl_2$ at a final concentration of 1.5 mM, 1 µM of each primer, and 1X PCR buffer (final concentration). Gel electrophoresis of PCR products were performed for 1 h in a 1.5% agarose gel at 90 V and stained with ethidium bromide solution and finally visualized in gel documentation system (Kiagen, Iran).

2.3. Quantitative biofilm assay

In order to evaluate the phenotype ability to create biofilms in *P. aeruginosa* used the method of Merritt*et al.* and Wakimoto model [23,24]. Briefly, 100 µl of each diluted culture was relocate into individual wells of polystyrene microtiter plate. The plates were incubated in a static condition at 37 °C for 24 h. Then, the medium in each well was carefully removed and washed with the hygienic phosphate buffer saline to remove loosely attached bacteria. All wells were stained with 125 µl of 0.2% (w/v) crystal violet solution for 10 min at room temperature. The excess stain was washed twice with distilled water and the plate was allowed to air dried. 200 µl of 95% ethanol was added to solubilize the stain and incubated for 10–15 min at room temperature. Then, 125 µl of the crystal violet/ethanol solution was transferred from each well to a separate well in an optically clear flat-bottom 96-well plate. The optical density was measured at a wavelength of 570 nm, using a microplate reader (BioTek ELx 808 instrument, USA). The isolates with the optical density higher than 0.2 were considered as biofilm forming isolates.

2.4. Antibiotic susceptibility test

Antimicrobial susceptibility test was performed on Mueller-Hinton agar by the standard disk diffusion method recommended by Clinical and Laboratory Standards Institute (CLSI) [50]. The antibiotics used against the test bacteria were as follows: amikacin (30 µg); eftazidime (10 µg); aztreonam (30 µg); piperacillin (25 µg); gentamicin (10 µg); imipenem (5 µg); ofloxacin (30 µg); ciprofloxacin (5 µg); tobramaycin (5 µg); meropenem (300 µg); cefepime (10 u); piperacillin + tazobactin (10 u). The diameter of inhibition zone was measured in millimeters and isolates were scored as sensitive or resistant by comparing results with values recommended on standard charts [50].

2.5. Statistical analysis

Statistical analysis was performed using the computer software program SPSS version 18 for descriptive statistics including frequencies, cross-tabulation of microbiological data. A *P* value of less than 0.05 was considered as statistically significant.

3. Results

3.1. Identification of P. aeruginosa isolates

493 environmental samples and 152 human samples have been tested, using standard microbiological and biochemical methods then 52 and 55 samples were confirmed, respectively. Samples were confirmed using PCR amplification of *16s rRNA* gene. Finally 50 isolates of both human and environmental samples were identified and selected for further investigations. The PCR results for detection of *16s rRNA* genes are shown in Fig. 1.

3.2. Detection of algU and algD genes in the different isolates

The PCR results of *algU* and *algD* genes were showed in Figs. 2 and 3. The numbers of positive samples for *algD* and *algU* genes in human samples were 98%, but the numbers of positive samples

Table 1
Origin of human samples.

Sampling site	Sample Type	Number of samples	Number of positive samples	Male	Female
Respiratory	Phlegm	280	27	18	9
Urinary	Urine	117	13	3	7
Burns	Swab and Necrotic tissue	96	10	5	8

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