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Original Article

Evaluation of Antibiotic Resistance Pattern, Alginate and Biofilm Production in Clinical Isolates of *Pseudomonas aeruginosa*

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Abstract

Background: *Pseudomonas aeruginosa* is one of the most common opportunistic bacteria causing nosocomial infections, which has significant resistance to antimicrobial agents. This bacterium is a biofilm and alginate producer. Biofilm increases the bacterial resistance to antibiotics and the immune system. Therefore, the present study was conducted to investigate the biofilm formation, alginate production and antimicrobial resistance patterns in the clinical isolates of *P. aeruginosa*.

Methods: One hundred isolates of *P. aeruginosa* were collected during the study period (from Dec 2017 to Jul 2018) from different clinical samples of the patients admitted to Milad and Pars Hospitals at Tehran, Iran. Isolates were identified and confirmed by phenotypic and genotypic methods. Antimicrobial susceptibility was specified by the disk diffusion method. Biofilm formation and alginate production were measured by microtiter plate and carbazole assay, respectively.

Results: Sixteen isolates were resistant to all the 12 studied antibiotics. Moreover, 31 isolates were Multidrug-Resistant (MDR). The highest resistance rate was related to ofloxacin (36 isolates) and the least resistance was related to piperacillin-tazobactam (21 isolates). All the isolates could produce the biofilm and alginate. The number of isolates producing strong, medium and weak biofilms was equal to 34, 52, and 14, respectively. Alginate production was more than 400 µg/ml in 39 isolates, 250-400 µg/ml in 51 isolates and less than 250 µg/ml in 10 isolates.

Conclusion: High prevalence of MDR, biofilm formation, and alginate production were observed among the clinical isolates of *P. aeruginosa*. The results also showed a significant relationship between the amount of alginate production and the level of biofilm formation.

Keywords: Alginate; Antibiotic resistance; Biofilm; *Pseudomonas aeruginosa*

Introduction

Pseudomonas aeruginosa, as a non-fermenter gram-negative bacterium can survive under various environmental conditions. It is an opportunistic pathogen that can colonize in healthy individuals and moist sites in the hospitals (1). This bacterium causes a wide range of infections, particularly in immunocompromised, burn, and cystic fibrosis patients. Other infections caused by this bacterium include wound infections, corneal infections, urinary tract infections, respiratory infections, and

septicemia (1, 2). *P. aeruginosa* is intrinsically resistant to many antibiotics and can develop resistance to others limiting treatment options. Several mechanisms are conferring the bacterial resistance to biocides. Antibiotic overuse has been shown to lead to an increase in the resistance of this bacterium and the emergence of Multidrug-Resistant (MDR) isolates (3).

P. aeruginosa is able to produce the biofilm. Biofilms comprise any syntrophic consortium of microorganisms in which cells stick to each other on a surface coated by a matrix of Extracellular Polymeric Substances (EPS) (4). It is also recognized as a major cause of chronic infections due to its ability to form biofilm. Biofilm formation increases the bacterial resistance to environmental stresses and also protects it against antimicrobial agents and immune system (5). An extracellular exopolysaccharide called alginate is one of the most important structures produced during biofilm formation. Overexpression of alginate protects the bacterium against phagocytosis by white blood cells (6) and can enhance the attachment of mucoid strains to the lung epithelial cells thus, preventing pulmonary clearance mechanisms (7). The *P. aeruginosa* is a major cause of nosocomial infections and is also resistant to antibiotic treatment, this study was conducted to evaluate the antibiotic resistance pattern and the amount of alginate and biofilm production in the clinical isolates of *P. aeruginosa*.

Materials and Methods

Bacterial Isolates

One hundred isolates of *P. aeruginosa* were collected during the study period (from Dec 2017 to Jul 2018) from different clinical samples of the patients admitted to Milad and Pars Hospitals at Tehran, Iran. Standard strains of *P. aeruginosa* ATCC 27853, *P. aeruginosa* PAO1 and *P. aeruginosa* 8821M were used as controls. Bacteria were stored at -70 °C in the nutrient broth medium (Merck, Germany) containing 15% glycerol. Nutrient agar medium (Merck, Germany) was used for bacterial growth (8).

Bacterial Identification Test

Isolates were cultured on the nutrient agar and were incubated overnight at 37 °C. After 24 h, the *P. aeruginosa* strains were identified by standard microbiological tests. All the isolates were checked for the presence of *oprL* gene to approve bacterial isolates as *P. aeruginosa* using the specific primers of *oprL* (5'ATGGAAATGCTGAAATTCGGC3' and 5'CTTCTTCAGCTCGACGCGACG3'). Product length of amplicon was equal to 504bp. PCR amplification for *oprL* genes was set with the following conditions: Initial denaturation for 5 min at 95 °C and 30 cycles consisted of denaturation for 30 sec at 95 °C, annealing for 30 sec at 57 °C, extension for 1 min at 72 °C, and final extension for 10 min at 72 °C. The final volume of each reaction was equal to 25 µl. *P. aeruginosa* PAO1 was considered as positive control in this assay (9).

Antimicrobial Susceptibility Tests

Antimicrobial susceptibility test was performed on the isolates by disk diffusion method based on the Clinical and Laboratory Standards Institute (CLSI) guideline. The tested antibiotic disks (MastGroup Ltd., UK.) for *P. aeruginosa* isolates were piperacillin (PRL, 100 µg), piperacillin-tazobactam (PTZ, 100/10 µg), ceftazidime (CAZ, 30 µg), aztreonam (ATM, 30 µg), imipenem (IMI, 10 µg), doripenem (DOR, 10 µg), gentamicin (GM, 10 µg), tobramycin (TN, 10 µg), amikacin (AK, 30 µg), ciprofloxacin (CIP, 5 µg), norfloxacin (NOR, 5 µg), and ofloxacin (OFX, 5 µg). *P. aeruginosa* ATCC 27853 was used as the reference strain for antibiotic susceptibility testing (10). MDR isolates were defined if they were non-susceptible to at least one agent in three or more antimicrobial categories (11).

Biofilm Formation Assay

Microtiter plate assay was used for investigation of biofilm formation in *P. aeruginosa* isolates (12). The isolates were cultured in Muller-Hinton Broth (MHB) at 37 ºC and after 24 h, the cultures were diluted at a ratio of 1:100 in a fresh MHB medium and 200 µl of them was inoculated onto each three wells of sterile 96-well flat-bottomed polystyrene plate (SPL, South Korea). Control wells contained

fresh MHB media. The plates were covered and incubated aerobically for 24 h at 37 °C. Then, each well was washed three times with 250 µl of the Phosphate-Buffered Saline (PBS) buffer. The wells were dried in an inverted position at room temperature. The remaining attached bacteria were fixed with 250 ml of 99% methanol, and after 15 min, the plates were emptied and left to dry. Then, the plates were stained for 5 min with 200 µl of 1% crystal violet. Stain was rinsed off and the wells were washed by water to remove extra dye. In the end, 200 µl of 33% acetic acid was added and the optical density (OD) of each well was measured at 570 nm by a microplate reader (Elx808, BioTek, USA). The mean OD of the three wells for each isolate was recorded as ODt, and the mean OD of the three wells for control was recorded as ODc. The biofilm formation levels were classified based on the OD as shown in Table 1.

Table 1: Interpretation of result of OD in detect level of biofilm formation (12)

OD	*Results*
$OD_t \leq OD_c$	Non-biofilm
$OD_c < OD_t < 2x\ OD_c$	Weak biofilm
$2x\ ODc < OD_t < 4xOD_c$	Moderate biofilm
$OD_t \geq 4xOD_c$	Strong biofilm

Alginate Production Assay

Knutson's method (13) was used with a few modifications to investigate the amount of alginate production by *P. aeruginosa* isolate. Suspension with the turbidity of 0.5 McFarland standard was prepared from overnight culture of *P. aeruginosa* isolate and 100 µl of it was added to 4 ml of fresh MHB medium. This culture was placed in the incubator at 37 ºC and after 48 h, 70 µl of it was mixed with 600 µl of borate-sulfuric acid solution (24.74 gr of H_3BO_3 was dissolved in 45 ml of 4 M KOH, and was diluted in 100 ml of distilled water) and 20 µl of 0.2% carbazole in ice bath using the vortex. This suspension was incubated for 30 min at 55 ºC and then, OD was measured at 540 nm by the microplate reader (Elx808, BioTek, USA). Upper OD value indicates higher alginate production.

Alginate Standard Curve

Stock solution of the alginate with a concentration of 10 mg/ml was prepared by dissolving 50 mg of alginic acid (Sigma-Aldrich, USA) in 5 ml of distilled water to plot the standard curve for alginate. Ten dilutions with concentrations between 100-1000 µg/ml were prepared from this stock solution. Alginate production assay was done for all the prepared concentrations, based on Knutson's method (13) and alginate standard curve was plotted by obtained OD values with respect to concentration.

Statistical Analysis

All the experiments were conducted as triplicates. Chi-Square test was used to investigate the relationship between the tested factors. SPSS software ver. 25 (Chicago, IL, USA) was used for data analysis and *P*-value of less than 0.05 was considered as statistically significant.

Results

Collected Isolates

One hundred *P. aeruginosa* isolates were isolated from different clinical samples in this study. The isolates were collected from the 31 sputum samples (31%), 39 urine samples (39%), nine wound samples (9%), four blood samples (4%), and five tracheal samples (5%), and 12 isolates were prepared from other samples (unknown origin) (12%). Among which, 50 isolates (50%) were obtained from the inpatients and 50 isolates were collected (50%) from the outpatients. Polymerase Chain Reaction (PCR) technique was used to identify the *oprL* gene after identification of the isolates by phenotypic tests to confirm the isolates as *P. aeruginosa* (Fig. 1).

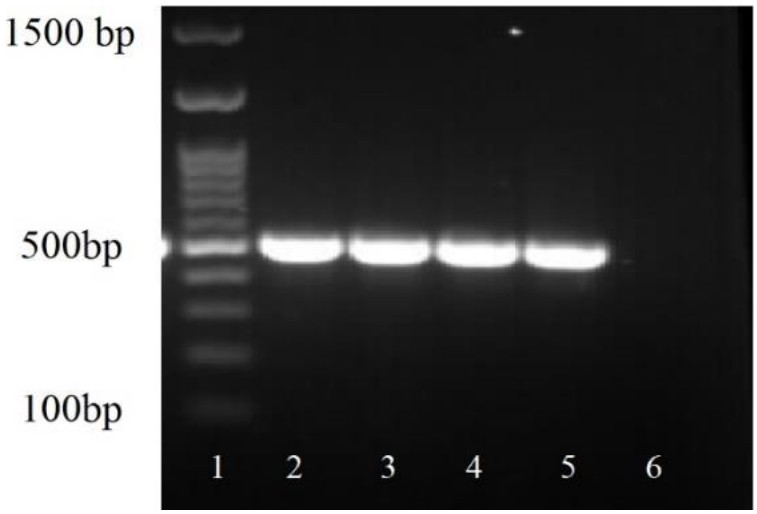

Fig. 1: Electrophoresis snapshots for PCR of *oprL* gene (504 bp). 1: Ladder – 2, 3, 4: clinical isolates of *P. aeruginosa* – 5: Positive control (*P. aeruginosa* PAO1) – 6: Negative control.

Results of Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of the clinical isolates of *P. aeruginosa* to 12 antibiotics was evaluated by the disk diffusion method (Table 2). Intermediate resistance was also considered as a low resistance level. Isolates exhibited the highest antibiotic resistance to ofloxacin (36%) and the lowest antibiotic resistance to piperacillin/tazobactam (21%).

Table 2: Antibiotic resistance result of *P. aeruginosa* isolates

	Antibiotics											
Antimicrobial susceptibility	PRL	PTZ	CAZ	ATM	IMI	DOR	GM	TN	AK	CIP	NOR	OFX
Susceptible	73	79	72	72	73	72	71	71	73	71	73	64
Resistant	27	21	28	27	27	28	29	29	27	29	27	36

The antibiotic resistance pattern of the isolates was also determined (Table 3). Overall, 21 different resistance patterns were identified. Most isolates (62 isolates) showed resistance pattern 1 and were sensitive to all the studied antibiotics. Moreover, 16 isolates (pattern 21) were resistant to all the studied antibiotics. Out of 62 susceptible isolates, 26 isolates (41.9%) belonged to the inpatients and 36 isolates (58.1%) were obtained from the outpatients. Besides, out of 16 resistant isolates, ten isolates (62.5%) belonged to the inpatients and six isolates (37.5%) were from the outpatients.

Non-susceptibility to at least one antibiotic in three or more different antibiotic families was considered as MDR. In this study, 31 isolates (31%) were identified as MDR. Thirty-eight % (38%) of the isolates (19 of 50 isolates) isolated from the inpatients were MDR, while MDR level in the isolates from outpatients was equal to 24% (12 of 50). Therefore, MDR was higher in the isolates from the inpatients, but this difference was not statistically significant (P=0.19).

Table 3: Resistance Patterns of *P. aeruginosa* isolates

Groups	Number of strains	Resistant	MDR	Biofilm	Alginate
1	62	-	-	9W, 35M, 18S	6A, 31B, 25C
2	2	IMI	-	1S, 1M	B,C
3	1	OFX	-	M	B
4	1	ATM, OFX	-	M	B
5	1	CAZ, ATM, OFX	-	W	B
6	1	IMI, DOR, OFX	-	M	C
7	1	CAZ, ATM, OFX	+	S	B
8	1	PRL, CAZ, ATM, OFX	+	S	C
9	1	PRL, CAZ, ATM, DOR, OFX	+	M	A
10	2	GM, TN, AK, CIP, OFX	-	1M, 1S	1B, 1C
11	1	PRL, CAZ, IMI, DOR, GM, TN, CIP, NOR, OFX	+	S	A
12	1	PRL, CAZ, ATM, GM, TN, AK, CIP, NOR, OFX	+	S	C
13	1	CAZ, IMI, DOR, GM, TN, AK, CIP, NOR, OFX	+	W	A
14	1	PRL, PTZ, ATM, DOR, GM, TN, AK, CIP, NOR, OFX	+	M	B
15	1	PRL, PTZ, CAZ, IMI, DOR, GM, TN, CIP, NOR, OFX	+	S	B
16	1	CAZ, ATM, IMI, DOR, GM, TN, AK, CIP, NOR, OFX	+	S	B
17	1	PRL, CAZ, IMI, DOR, GM, TN, AK, CIP, NOR, OFX	+	S	C
18	1	PRL, CAZ, ATM, IMI, DOR, GM, TN, AK, CIP, NOR, OFX	+	W	B
19	2	PRL, PTZ, ATM, IMI, DOR, GM, TN, AK, CIP, NOR, OFX	+	2M	2B
20	1	PRL, PTZ, CAZ, ATM, DOR, GM, TN, AK, CIP, NOR, OFX	+	S	C
21	16	PRL, PTZ, CAZ, ATM, IMI, DOR, GM, TN, AK, CIP, NOR, OFX	+	2W, 8M, 6S	1A, 9B, 6C

Biofilm: W: Weak, M: Moderate, S: Strong. Alginate: A: <250 μg/ml, B: 250-400 μg/ml, C: >400 μg/ml.

Results of Biofilm Formation Assay

Biofilm formation for 100 *P. aeruginosa* isolates was also evaluated. All the isolates were able to form the biofilm, of which 34 (34%), 52 (52%), and 14 (14%) isolates formed strong, intermediate, and weak biofilms, respectively. Therefore, among all the isolates, the intermediate biofilm formation was higher than the production of strong and weak biofilms. The standard strain of *P. aeruginosa* PAO1 was also a strong biofilm-forming strain. Among 31 MDR isolates, 14 isolates (45%) formed strong biofilm, 12 isolates (38%) formed intermediate biofilm, and five isolates (16%) formed weak biofilm. Therefore, among the MDR

isolates, the percentage of strong biofilm formation was higher than that of intermediate and weak biofilms. Evaluation of biofilm formation in the isolates from outpatients and inpatients revealed that 40% (20 out of 50) of isolates from the inpatients were strong biofilm –forming strains, whereas the amount of strong biofilm formation in the isolates from outpatients was equal to 28% (14 out of 50). However, this difference was not statistically significant (*P*=0.35). Biofilm formation was not significantly associated with resistance to individual antibiotics and multiple drug resistance (*P*>0.05).

Results of Alginate Production Assay

Alginate production of 100 *P. aeruginosa* isolates was also evaluated. Standard alginate curve was initially plotted based on OD values for concentration to obtain the value of produced alginate (Fig. 2). Since the curve was linearly constant up to a concentration of 1000 µg/ml; it was applicable and valid in the concentration range of this study. Among these 100 clinical isolates of *P. aeruginosa*, alginate production level in ten isolates was <250 µg/ml, in 51 isolates was between 250-400 µg/ml, and in 39 isolates was >400 µg/ml. Alginate production level in *P. aeruginosa* 8821M was equal to 450 µg/ml. Among 31 MDR isolates, four isolates (13%) produced <250 µg/ml, 17 isolates (55%) produced 250-400 µg/ml, and ten isolates (32%) produced >400 µg/ml of alginate.

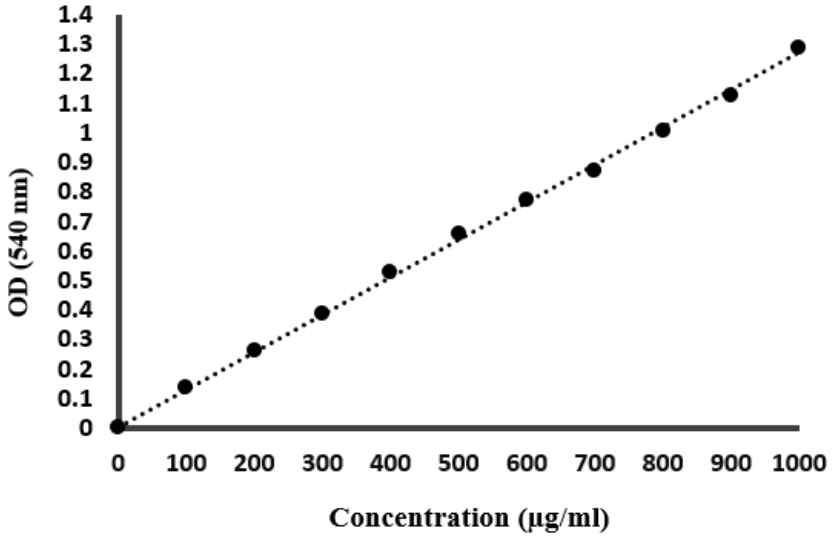

Fig. 2: Standard curve related to the alginate production

Analysis of alginate production in the isolates from outpatients and inpatients showed no significant difference between the two groups (*P*>0.05). About 42% (21 of 50) of isolates from the inpatients produced >400 µg/ml of alginate, while 36% (18 out of 50) of isolates from the outpatients produced >400 µg/ml of alginate.

Alginate production was not significantly associated with resistance to individual antibiotics and multiple drug resistance (*P*>0.05).

The Relationship between Biofilm Formation and Alginate Production

A significant relationship was observed between alginate production of >400 µg/ml and strong biofilm formation in this study. In other words, alginate production of >400 µg/ml was significantly higher among strong biofilm-forming isolates than intermediate biofilm-forming isolates (*P*=0.047). Alginate production in 53% of strong biofilm-forming isolates (18 of 34 isolates) was >400

μg/ml, while 30.7% of intermediate biofilm-forming isolates (16 of 52 isolates) produced >400 μg/ml of alginate.

Discussion

P. aeruginosa is an opportunistic bacterium that produces different virulence factors including biofilm and alginate. Moreover, this bacterium is resistant to most antibiotics (14). In this study, 100 clinical isolates of *P. aeruginosa* were evaluated for biofilm and alginate production and antibiotic resistance. All the isolates were capable of biofilm and alginate production. Moreover, these isolates had resistance against many antibiotics and MDR isolates were considerable.

Many studies have been conducted on the formation of biofilms in the *P.aeruginosa*. Frequency of biofilm formation and antibiotic resistance in *P. aeruginosa* isolates was investigated in Bandar Abbas (Hormozgan Province, Iran). In their study, 75 isolates of *P. aeruginosa* were isolated from different samples such as ulcer, urine, sputum, blood, and cerebrospinal fluid (CSF) samples. The rate of biofilm formation was determined by microtiter plate method. 98.6% of isolates were capable of biofilm formation, among which 60% had strong biofilm, 34.3% had moderate biofilm, and 4.3% had weak biofilm (1). In our study, 100% of the isolates contained biofilm, which is consistent with another study in which 98% of the isolates produced biofilm. Biofilm formation in urine isolates was higher than in other samples. In our study, however, strong biofilm formation was observed in the isolates obtained from sputum samples.

Resistance to tetracycline (32.82%) and ofloxacin (30%) was reported and among which, 24.3% of the isolates showed MDR (1). These results were consistent with our studies, and in our study, the highest antibiotic resistance was observed against the ofloxacin (36%). According to the definition of MDR, there must be at least one antibiotic resistance from the three antibiotic groups. Since there were no antibiotics in the study group, it would be possible to increase the number of MDR strains if all antibiotics were available also, if the isolated strains are from a prevalent strain (especially in hospitalized patients). A MDR strain may spread among the patients (especially inpatients). In this case, the number of MDR strains will decrease. Further studies on genotyping should be carried out to solve this problem. This may be an important factor while comparing several studies. This analysis can be considered for other researches as well. In other words, it is necessary to study all the antibiotics referred to the CLSI of different antibiotics to have accurate statistics of MDR, Extensively Drug-Resistant (XDR), and Totally Drug Resistant (TDR) strains. The strains identified by this non-MDR study method may yield to identification of different MDR strains by increasing the number of antibiotics.

In the study of Pournajaf et al., overall 143 *Pseudomonas* isolates were tested for drug resistance and biofilm formation and among which, 8.4% of isolates had MDR (15). In our study, the number of isolates with drug resistance was equal to 31% which is inconsistent with the results of the mentioned research. This may be due to the variety in the studied samples in our study and the only sample with cystic fibrosis in mentioned study. Moreover, about 87% of the isolates produced biofilm, of which 69% had strong biofilm, 11% had moderate biofilm, and 7% had weak biofilm, representing high biofilm production. This research is consistent with our study. The biofilm formation and antimicrobial were measured susceptibility of *P. aeruginosa* in the patients with cystic fibrosis before and after antibiotic use and found that biofilm interferes with resistance to some antibiotics (16). However, in our study, no association was observed between biofilm productions in the strains with multiple antibiotic patterns.

The strains were able to form biofilm among which, 60% had strong biofilm, 34.3% had moderate biofilm, and 4.3% had weak biofilm. In this study, most isolates formed strong biofilms, whereas in our study, the intermediate biofilms were mostly formed. In this study, the rate of formation of strong biofilm in the isolates from urine samples was higher than other samples, which is in line with our research. In another study, 78.3% of the isolates were biofilm-forming. Among

them, 54.5% of the isolates co-produced the alginate and biofilm (15). This was lower than the specific results of our study, which may be due to the differences in the biofilm measurement in the two studies.

Many studies have been performed on alginate production in *P. aeruginosa*. For example, 88.8% of the isolates were alginate-producing, which is consistent with our study, which may be due to the use of carbazole method in both studies. In contrast to that study, our study classifies alginate production.

Among 50 samples, 92% (46 strains) of them were alginate positive. Most alginate positive samples were from urine samples. The abundance of alginate in the samples is in line with the amount of alginate observed in our study. Alginate was produced by 100% in the samples from burn patients, which is true in our study. Alginate plays a more important role in urinary tract infection (17).

Many studies have been performed on antibiotic resistance in *P. aeruginosa*. For instance, the highest antibiotic resistance was against the ofloxacin (36%) and the lowest antibiotic resistance was against the piperacillin-tazobactam (21%). Among all the isolates, 62 isolates were resistant to all the used antibiotics and eight isolates were resistant to all the used antibiotics. Moreover, 24.3% of the isolates had MDR, which is similar to our study. Among the 31 MDR isolates, 14 were strong biofilm-forming isolates, 12 were medium biofilm-forming isolates, and five were weak biofilm-forming isolates.

Out of 115 isolates, 94 (81.7%) isolates had MDR patterns, which is completely inconsistent with our research (18).

High presence of alginate coding gene (81.7%) was reported in *P. aeruginosa* isolates with MDR patterns (18). However, in our study, there was no significant relationship between the increase in drug resistance and the increase in alginate production.

The highest resistance was observed to the ceftazidime and showed that all the isolates were resistant to it. These results are inconsistent with our study, which may be due to the differences in the sampling such that, only the corneal eye samples were evaluated in their study while six types of samples were assessed in our study (19).

Conclusion

Biofilm and alginate production can play important role in pathogenesis. The number of MDR strains is also increasing and the resistance pattern of these bacteria is constantly changing, so there is an urgent need to control these cases and its corresponding results should be applied in the infection control systems and evaluation of therapeutic protocols. Molecular epidemiological studies are also required for genotyping.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interest.

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