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Original Research Article

The prevalence of some *Pseudomonas* virulence genes related to biofilm formation and alginate production among clinical isolates

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ABSTRACT

Chronic infections caused by *Pseudomonas aeruginosa* (*P. aeruginosa*) isolates are mainly related to resistance to antimicrobials and the production of certain virulence factors. The purpose of this study was twofold: to investigate the prevalence of virulence genes and to study the relationship between biofilm formation/alginate production/antibiotic resistance and the presence of genes associated with biofilm, alginate, flagella and exotoxin A in clinical isolates of *P. aeruginosa*.

Microtiter plate biofilm assay and Carbazole method were used to examine the biofilm formation and alginate production ability of the isolates, respectively. The genes, *ppyR*, *pslA*, *pelA* (biofilm formation), *algD*, *algU*, *algL* (alginate production), *fliC* (flagella) and *exoA* (exotoxin A) were detected by PCR.

Biofilm formation as well as alginate production ability was found in 47.1% of the clinical isolates. Based on PCR data, the frequency distribution of the genes in the clinical isolates was as follows: *ppyR* (99%), *pslA* (83.7%), *pelA* (45.2%), *algU* (90.4%), *algL* (73.1%), *algD* (87.5%), *exoA* (84.6%) and *fliC* (70.2%). Biofilm formation ability of the isolates was significantly correlated with the presence of the genes, *pelA* and *fliC* (statistically significant). In addition, 58.65% of the isolates were resistant to three or more different classes of antibiotics.

Introduction

The persistence of prolonged infections caused by biofilm-forming and multi-drug-resistance (MDR) *Pseudomonas aeruginosa* isolates has created serious problems in hospitals. Increased length of stay and mortality rates in the hospitals are frequently reported among the individuals contracting chronic infections (Chastre and Trouillet, 2000; Shigemura et al., 2006; Wang et al., 2009). Immunocompromised patients

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with respiratory infections, urinary tract infections or incurable wounds are the main victims of this biofilm-forming bacterium. Based on previous studies, biofilm has been found to cause prolonged treatment of chronic infections due to its ability to combat the immune response of the host and thrive in antibiotic therapy (Jeff, 2009; Chen and Wen, 2011).

The *pslA* and *pelA* genes play an important role in formation of carbohydrate-rich structure of biofilm matrix. Therefore, the mutations in *pslA* and *pelA* genes cause deficiency in the biofilm formation ability. In addition, *psl* operon was repressed following inactivation of the *ppyR* gene (putative transmembrane protein), and biofilm formation was decreased. According to several studies, the production of alginate and formation of flagella increases the virulence of bacteria (Feldman et al., 1998; Leid et al., 2005; Cotton et al., 2009).

Alginate is a linear unbranched polymer composed of 1–4 linked saccharides β-D mannuronic acid (M) and a C-5 epimer of α-L-guluronic acid (G) (McIntyre-Smith et al., 2010). The three genes, *algD*, *algU* and *algL*, are involved in the production of alginate, which encode GDP-mannose dehydrogenase and the transcription of the operon is increased by sigma factor AlgU (Hershberger et al., 1995; Franklin et al., 2011). *algU* contributes to the expression of the *ppyR* gene (Bazire et al., 2010). AlgL (alginate lyase) was shown to be an enzymatic and structural protein, which participates as a component of the alginate transport scaffold (Jain and Ohman, 2005). Alginate production decreases macrophage phagocytosis and complement activation (Pier et al., 2001; Leid et al., 2005; Ramsey and Wozniak, 2005). It was also shown in a series of studies that overproduction of alginate might in fact create a resistance to some types of antibiotics (Hentzer et al., 2001).

The *fliC* gene is one of the key genes in flagella production due to its role in encoding the subunit protein, flagellin (FliC) (Feldman et al., 1998; Wolfgang et al., 2004). The flagellar complex makes a contribution in chemotaxis, random motility and gain of essential nutrients through attachment to surfaces and mediates human infections (Shapiro, 1995; Feldman et al., 1998). Exotoxin A (encoded by *exoA*) is an extracellular ADP-ribosyl transferase enzyme with three distinct functional domains. As a result of the catalytic domain, ADP-ribosylation of eukaryotic elongation factor-2 (eEF-2) occurs, and protein synthesis is inhibited (Beattie et al., 1996; Yates and Merrill, 2001). Consequently, the number of polymorphonuclear leukocytes (PMNLs) decreases and the mortality rate of the patients infected with ExoA producing clinical isolates significantly increased (Slack and Nichols, 1981; Baltimore et al., 1987).

The aim of this study was to evaluate the prevalence of virulence genes including *algD*, *algL*, *algU*, *pelA*, *pslA*, *ppyR*, *fliC* and *exoA* using PCR in multidrug resistant clinical isolates of *P. aeruginosa* and their relationship with ability of biofilm formation and alginate production.

Materials and methods

Bacterial isolates and culture conditions

A total of 104 *P. aeruginosa* were isolated from the clinical specimens (urine, respiratory tract, wound, pleural effusion, ear, blood, bronchial wash, trachea, BAL, sputum, chest tube, bone, pharynx). The specimens were collected from one of the educational hospitals in northern Tehran, Iran between 2010 and 2012. The *P. aeruginosa* isolates were identified by colony morphology, gram staining and standard biochemical tests (production of pigments, oxidase, nitrate reduction, growth at 42 °C and hydrolysis of acetamide) (Winn and Koneman, 2006; Engelkirk and Duben-Engelkirk, 2008). The isolates were grown in Luria–Bertani (LB) broth at 37 °C overnight. Then, the overnight broth culture was serially diluted with autoclaved distilled water (equivalent to a 0.5 McFarland turbidity standard). We used *P. aeruginosa* PAO1 as a positive control for the biofilm formation and alginate production assays. *P. aeruginosa* ATCC 27853 was used for quality control in the antibiotic susceptibility tests.

Quantitative biofilm assay

Microtiter plate biofilm assay described by Merritt et al. (2005) was used, with some modifications. Briefly, 100 μl of each diluted culture was transferred into individual wells of sterile, 96-well microtiter plate, containing 100 μl of the appropriate Mueller Hinton Broth (MHB) with 0.5% glucose, and grown at 37 °C overnight. After incubation, the planktonic bacteria were removed by rapidly shaking and submerging into the tray containing tap water. 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 rinsed twice with distilled water and the plate was allowed to air-dry. 200 μl of 95% ethanol was added to solubilize the stained biofilm and incubated for 10–15 min at room temperature. 125 μl of each well was transferred into a new microtiter plate. Finally, the optical density (OD) of each sample was measured at 570 nm by a spectrophotometer (Smart Spec Plus Spectrophotometer Bio-RAD, USA). The experiment was repeated three times and the mean value of the optical densities (OD) was calculated. Based on Wakimoto model (Wakimoto et al., 2004), the isolates with the optical density higher than 0.2 were considered as biofilm-forming isolates.

Alginate production assay

In order to determine the alginate production level, the Carbazole method described by May and Chakrabarty (1994) was performed. Briefly, the clinical samples were cultured in Luria–Bertani (LB) broth with rapid aeration at 37 °C overnight and then centrifuged for 30 min at 13,700 × *g*. To precipitate alginate, the supernatant was combined with 95% ethanol (−70 °C) and then centrifuged for 15 min at 13,700 × *g*. 70 μl of alginate solution was mixed with 600 μl of borate–sulfuric acid (24.74 g of H_3BO_3 in 45 ml of 4 M KOH that was diluted in 100 ml distilled water) and 20 μl of the 0.1% (w/v) Carbazole (Sigma, Germany). In order to develop the color reaction the mixture was heated at 55 °C for 30 min. Finally, the optical density was measured at 530 nm. The standard curve of alginate concentration (Sigma, Germany) was prepared and the amount of alginate produced was measured per mg cell dry weight. The experiment was repeated three times and the mean value for OD was calculated.

Detection of the genes by PCR

For detection of the genes, *ppyR*, *pslA*, *pelA*, *algU*, *algL*, *algD* (Fig. 1), *exoA* (Fig. 2) and *fliC* (Fig. 3) in the clinical samples of *P. aeruginosa*, pairs of specific primers were designed for each gene which are listed in Table 1, except for *exoA* (Imani Fooladi et al., 2011) and *fliC* (Imani Fooladi et al., 2009). The specificity of the primers was checked by using the primer BLAST program through the server hosted by the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nigh.gov/BLAST) and synthesized by CinaClon Company of Tehran, Iran. *P. aeruginosa* PAO1 was used as a positive control to confirm the amplification of *ppyR*, *pslA*, *pelA*, *algU*, *algL*, *algD*, *fliC* and *exoA* gene products.

The PCR mixture contained 2.5 μl of 10× PCR buffer (200 mM Tris/HCl, 500 mM KCl), 1 μl of 2.5 mM $MgCl_2$, 1 μl of each dNTP (2.5 mM), 1 μl of each primer (1 ρm/μl), 1 μl of Ampli Tag DNA polymerase (1u/μl) and 300 ng of each template DNA which were extracted by Bioneer DNA extraction kit (Cat. No.: K-3032).

The DNA was amplified using the following protocol: 95 °C for 5 min (for *fliC* gene: 94 °C for 4 min and for *exoA* gene: 94 °C for 3 min), 35 cycles of 95 °C for 1 min (for *fliC* gene: 94 °C for 1 min and for *exoA* gene 94 °C for 5 min), 60 °C for 1 min (for *fliC* gene: 56.2 °C for 1 min) and 72 °C for 1 min, and 72 °C for 5 min (for *fliC* gene 72 °C for 10 min). The PCR products were separated in a 1.5% agarose gel for 1 h at 100 V, stained with ethidium bromide and detected by a UV transillumination (Figs. 1–3). The bands were cut and the eluted PCR products were sequenced by CinaClon in order to confirm the expected full length gene as listed in Table 1.

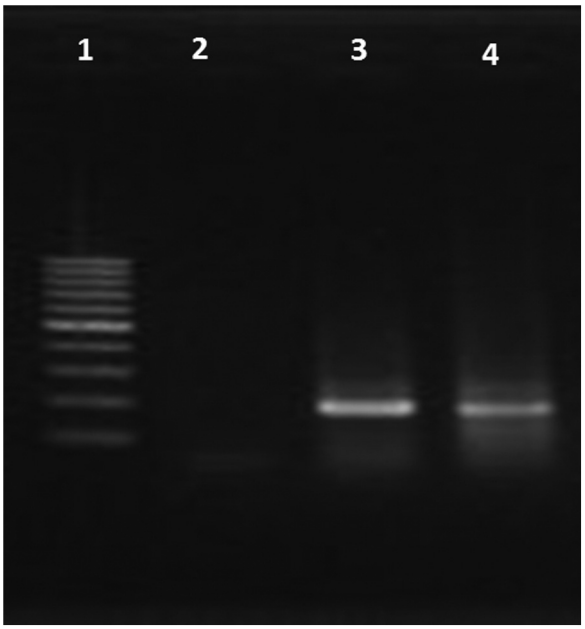

Fig. 2 – Agarose gel electrophoresis (1.5%) of PCR products of *exoA* gene. Lane 1: 1 kb ladder, **Lane 2**: negative control, **Lane 3**: positive control (190 bp) and **Lane 4**: clinical sample (190 bp). *P. aeruginosa* PAO1 was used as positive control.

Antibiotic susceptibility test

The disk diffusion method was used to evaluate the antibiotic susceptibilities of *P. aeruginosa* clinical isolates based on the

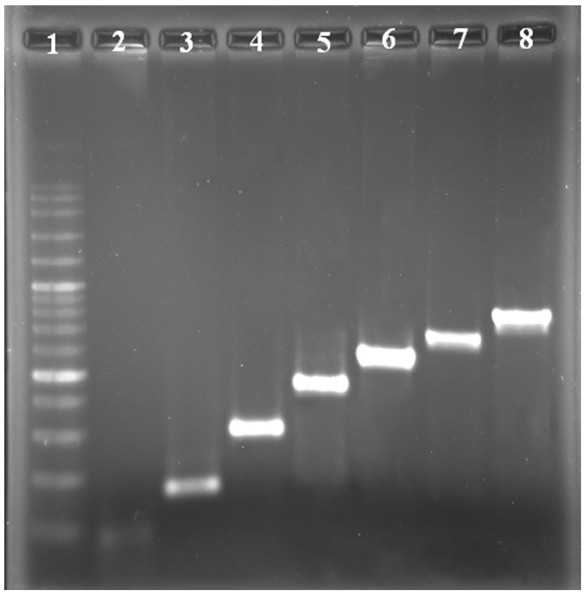

Fig. 1 – Agarose gel electrophoresis (1.5%) of PCR products. Lane 1: 1 kb ladder, **Lane 2:** negative control, **Lane 3:** *ppyR* gene (160 bp), **Lane 4:** *algU* gene (292 bp), **Lane 5:** *algL* gene (432 bp), **Lane 6:** *algD* (550 bp), **Lane 7:** *pslA* gene (656 bp) and **Lane 8:** *pelA* (786 bp). *P. aeruginosa* PAO1 was used as positive control.

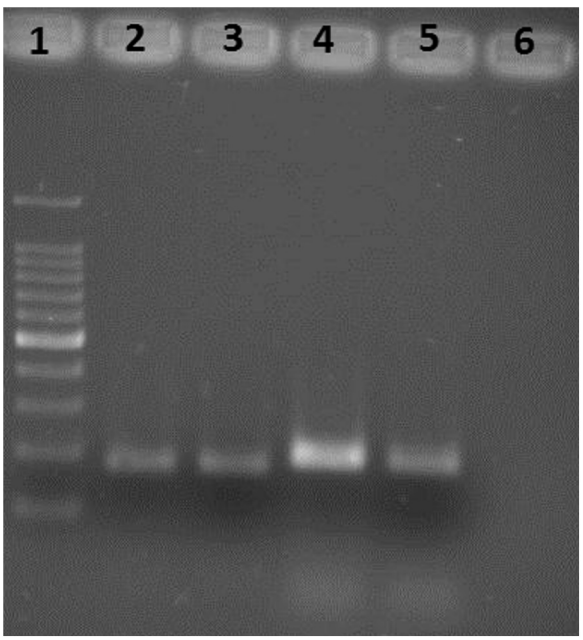

Fig. 3 – Agarose gel electrophoresis (1.5%) of the PCR products of *fliC* gene. Lane 1: 1 kb ladder, **Lane 2**: positive control (180 bp), **Lane 3**: clinical sample (180 bp), **Lane 4**: clinical sample (180 bp), **Lane 5**: clinical sample (180 bp) and **Lane 6**: negative control. *P. aeruginosa* PAO1 was used as positive control.

Table 1 – Primers used for the PCR amplification of virulent factors.

Target gene	Primer sequence (5′–3′)	Product size (bp)	Annealing temperature (°C)	Reference
ppyR	F: 5′-CGTGATCGCCGCCTATTTCC-3′ R: 5′-ACAGCAGACCTCCCAACCG-3′	160	60	Original
algU	F: 5′-CGATGTGACCGCAGAGGATG-3′ R: 5′-TCAGGCTTCTCGCAACAAAGG-3′	292	60	Original
algL	F: 5′-CCGCTCGCAGATCAAGGACATC-3′ R: 5′-TCGCTCACCGCCCAGTCG-3′	432	60	Original
algD	F: 5′-AGAAGTCCGAACGCCACACC-3′ R: 5′-CGCATCAACGAACCGAGCATC-3′	550	60	Original
pslA	F: 5′-TCCCTACCTCAGCAGCAAGC-3′ R: 5′-TGTTGTAGCCGTAGCGTTTCTG-3′	656	60	Original
pelA	F: 5′-CATACCTTCAGCCATCCGTTCTTC-3′ R: 5′-CGCATTCGCCGCACTCAG-3′	786	60	Original
fliC	F: 5′-TGAACGTGGCTACCAAGAACG -3 R: 5′-TCTGCAGTTGCTTCACTTCGC -3	180	56.2	Imani Fooladi et al. (2009)
exoA	F: 5′-TGCTGCACTACTCCATGGTC-3′ R: 5′-ATCGGTACCAGCCAGTTCAG-3′	190	60	Imani Fooladi et al. (2011)

zone of inhibition (diameter interpreted by CLSI (Cockerill, 2013). The diluted cultures were spared on Mueller Hinton agar plates and incubated at 37 °C. The zone of inhibition was measured after cultures had been incubated overnight. The standard antibiotic discs (MAST, England) for antibiotic susceptibility tests included amikacin (30 μg), ceftazidime (30 μg), ceftriaxone (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), norfloxacin (10 μg), tetracycline (25 μg) and trimethoprim–sulfamethoxazole (1.25/23.75 μg).

Statistical methods

The distributions of the above-mentioned genes with respect to the amount of biofilm formation, alginate production and the origin of the isolates, were evaluated using Chi-square and Fisher's tests at the significance level $2\alpha = 0.05$.

Results

We analyzed 104 clinical isolates which were obtained from different sites of infection. 27.9% of the isolates were obtained from wounds, 23% from bronchoalveolar lavage (BAL), 19.2% from urine, 9.6% from sputum and 20.3% from other sources (blood, pleural effusion, ear, bronchial wash, trachea, chest tube, bone and pharynx).

Biofilm formation and alginate production in the clinical isolates

90.4%, 87.5% and 73.1% of the isolates had the *algU*, *algD* and *algL* genes, respectively (Table 2). *ppyR*, *pslA* and *pelA*, genes responsible for biofilm formation, were detected in 99%, 83.7%, and 45.2% of the isolates, respectively (Table 2).

In the current study, 50.9% of the clinical isolates were found to be biofilm-forming. In addition, 89.4% of the isolates had the ability to produce alginate, and 47.1% of them had the combined ability of biofilm formation and alginate production. In the latter group, 41.3%, 45.8% and 60% of the isolates were obtained from wounds, BAL and urine (Table 3).

The importance of alginate production as the main compound of biofilm structure was evaluated, but the differences were not significant. Although, the lack of biofilm was shown in *pslA*-, *pelA*- and *ppyR*-positive *P. aeruginosa* isolates, the significant correlation between prevalence of *pelA* gene and the biofilm forming capacity was detected (statistically significant) (Fig. 4). Furthermore, a correlation between the prevalence of *algD* (unlike *algU* and *algL* genes) and capacity

Table 2 – Distribution (%) of virulence genes in *P. aeruginosa* isolates with respect to their origin.

Gene	Infection sites												Total (n = 104)
	Wound (n = 29)	BAL (n = 24)	Urine (n = 20)	Sputum (n = 10)	Blood (n = 5)	Pleural effusion (n = 4)	Ear (n = 3)	Bronchial wash (n = 3)	Trachea (n = 2)	Chest tube (n = 2)	Bone (n = 1)	Pharynx (n = 1)	
fliC	86	62.5	65	70	60	75	66.6	0	100	100	100	0	70.2
exoA	93	79	85	80	60	100	100	66.6	100	100	100	0	84.6
ppyR	96.5	100	100	100	100	100	100	100	100	100	100	100	99
pslA	89.6	75	95	60	80	100	66.6	100	50	100	100	100	83.7
pelA	51.7	37.5	45	40	60	50	33	33	50	50	0	100	45.2
algU	100	91.6	80	100	60	100	66.6	66.6	100	100	100	100	90.4
algL	82.7	87.5	75	60	60	50	33	66.6	50	0	0	100	73.1
algD	93.1	91.6	85	80	60	100	66.6	66.6	100	100	100	100	87.5

Table 3 – The prevalence (%) of biofilm formation and alginate production in *P. aeruginosa* isolates with respect to their origin.

	Infection sites												Total (n = 104)
	Wound (n = 29)	BAL (n = 24)	Urine (n = 20)	Sputum (n n = 10)	Blood (n = 5)	Pleural effusion (n = 4)	Ear (n = 3)	Bronchial wash (n = 3)	Trachea (n = 2)	Chest tube (n = 2)	Bone (n = 1)	Pharynx (n = 1)	
Biofilm[a]	44.8	50	60	40	60	75	33	66.6	50	0	100	100	50.9
Alginate[b]	82.7	91.6	100	100	80	75	100	100	0	100	100	100	89.4
Biofilm and alginate	41.3	45.8	60	40	40	75	33	66.6	0	0	100	100	47.1

[a] Optical density (OD) > 0.2.
[b] μg of alginate per mg of dry cell.

of alginate production was found statistically significant (Fig. 5).

Detection of fliC and exoA genes in the clinical isolates

70.2% of the isolates were positive for the *fliC* gene (Table 2). The variations in the distribution of the *fliC* gene among biofilm-forming *P. aeruginosa* isolates, were significant with respect to the extent of biofilm formation (Fig. 4). However, the presence of *fliC* gene was not correlated with the amount of alginate production or the origin of the isolates.

93.1%, 85% and 79% of the clinical isolates obtained from wounds, urine and BAL were positive for the presence of *exoA* gene, respectively (Table 2). Furthermore, based on the statistical analysis, the variation in the distribution of the *exoA* gene was not significantly correlated with the amount of biofilm formation, alginate production or the origin of the clinical isolates (statistically significant).

Prevalence of multidrug resistance in the clinical isolates

Table 4 illustrates the prevalence of resistance among the clinical isolates. The lowest resistance, 13.46%, was found in norfloxacin. Multidrug resistance (defined as resistance to 3 or more antibiotic classes) was detected in 58.65% of the isolates.

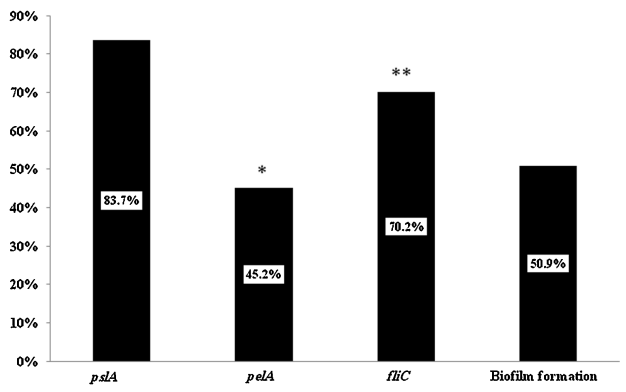

Fig. 4 – Detection frequency (%) of *pelA*, *fliC* and *pslA* genes associated with biofilm formation. *Statistically significant relation between *pelA* distribution and biofilm formation **Statistically significant relation between *fliC* distribution and biofilm formation.

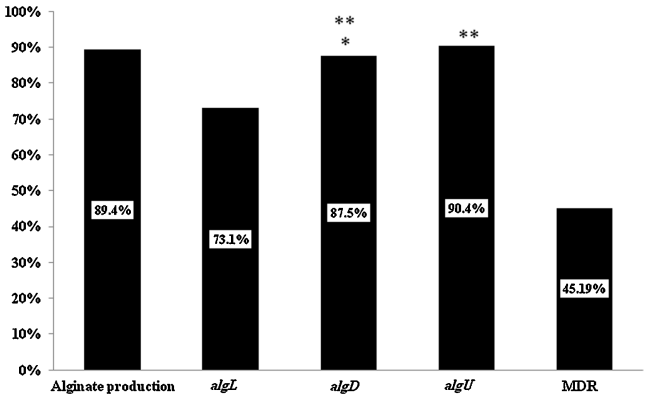

Fig. 5 – Detection frequency (%) of *algL*, *algD* and *algU* genes associated with alginate production and multidrug resistance (MDR) to trimethoprim–sulfamethoxazole, tetracycline and ceftriaxone. *Statistically significant association between *algD* distribution and alginate production. **Statistically significant relation between the *algD* and *algU* distribution and the MDR property.

Table 4 – The frequency of antibiotic resistance among the *P. aeruginosa* clinical isolates.

Antibiotics	Number (%) of resistance isolates
AMK	18 (17.3)
GM	24 (23.07)
CAZ	35 (33.65)
CP	19 (18.26)
NOR	14 (13.46)
AMK-CAZ-CP	
AMK-CAZ-NOR	4 (2.88)
GM-CAZ-CP	
GM-CAZ-NOR	
SXT	98 (94.23)
TE	98 (94.23)
CRO	70 (67.3)
SXT-TE-CRO	47 (45.19)

AMK, amikacin; GM, gentamicin; CAZ, ceftazidime; CP, ciprofloxacin; NOR, norfloxacin; SXT, trimethoprim–sulfamethoxazole; TE, tetracycline; CRO, ceftriaxone.

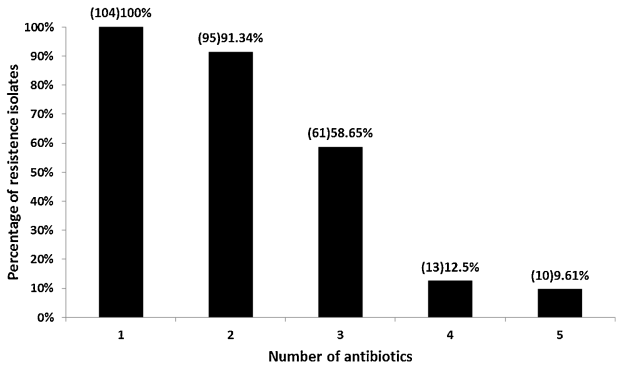

Fig. 6 – Resistance of *P. aeruginosa* clinical isolates to one, two or more classes of antibiotics. MDR strains were detected in 58.65%, 12.5% and 9.61% of isolates (aminoglycosides, cephems, fluoroquinolones, folate pathway inhibitors and tetracyclines).

Additionally, among the MDR isolates, the highest prevalence of resistance was related to trimethoprim–sulfamethoxazole, tetracycline and ceftriaxone (45.19%), but the lowest resistance was observed against aminoglycosides (amikacin or gentamicin), cephems (ceftazidime) and fluoroquinolones (ciprofloxacin or norfloxacin) (2.88%). 12.5% and 9.61% of MDR clinical isolates were resistant to four and five antibiotics, respectively (Fig. 6). Moreover, only resistance to trimethoprim–sulfamethoxazole, tetracycline and ceftriaxone among the MDR isolates was significantly correlated to the prevalence of *algD* and *algU* genes (Fig. 5, statistically significant).

Discussion

The crucial role of biofilm formation, alginate, exotoxin A and flagella in the establishment and persistence of infections have been highlighted in previous studies (Gallant et al., 2000; Fux et al., 2005; Patankar et al., 2013). In this study, the relationship between the prevalence of virulence genes and the capacity of biofilm formation, alginate production and antibiotic resistance were compared with one another in the clinical isolates of *P. aeruginosa*.

In two separate studies performed by Prince et al. (2008) and Oncel et al. (2010), the ability to form biofilm was found in 28.6% and 65.2% of clinical isolates of chronic rhinosinusitis patients, respectively. Based on Kádár et al. (2010) findings, 23.3% of *P. aeruginosa* isolates from clinical samples were positive for biofilm formation. Coban et al. (2009) assessed *P. aeruginosa* isolates from patients with cystic fibrosis and showed 33.3% of the isolates had biofilm formation capabilities. However, Hou et al. (2012) reported no biofilm production in *P. aeruginosa* isolates from patients with ocular infections. In the current study, the prevalence of biofilm formation was found in 50.1% of *P. aeruginosa* clinical isolates. The differences in the prevalence of biofilm forming isolates between our results and previous studies may be due to the variation in the sites of clinical isolates.

To the best of our knowledge, there is no report on the prevalence of alginate production in clinical isolates. In this study, alginate production was detected in 89.4% of the clinical isolates. The real role of AlgL in alginate production is still controversial. In one model, AlgL degraded alginates, and high level of alginate was toxic to the cell when the *algL* gene was absent in the isolates (Bakkevig et al., 2005). In another model, AlgL was shown to contain a dual structural and enzymatic function (Jain and Ohman, 2005). In the current study, unlike *algD* gene, alginate production was observed in both *algL* positive and *algL* negative isolates. In other words, regardless of the presence of *algL* gene, alginate was produced. Therefore, as shown by Bakkevig et al. (2005), *algL* may participate in the regulation of alginate production.

Moreover, alginate production ability and the frequency of alginate genes (*algU*, *algL* and *algD*) was not significantly different between the biofilm forming and non-biofilm forming isolates. Therefore, our study does not confirm the essential role of alginate in the biofilm structure, which corresponds with the findings of McIntyre-Smith et al. (2010).

Mitov et al. (2010) found that 92.5% of their clinical isolates were positive for *algD* gene. Wolska and Szweda (2009) investigated *P. aeruginosa* clinical isolates for *algD* and *exoA* genes, and found them in 93.55% and 88.7% of isolates, respectively. However, based on Valéria Zaranza et al. (2013) 39% of the isolates were positive for alginate production. In our study, 87.5% and 84.6% of the isolates carried *algD* and *exoA* genes, respectively.

99% of clinical isolates were positive for *ppyR* gene. The role of PpyR in enhancing biofilm formation (via *psl* operon) and elastase activity (as a virulence factor) was shown in previous studies (Attila et al., 2008). Therefore it may be possible for *ppyR* gene to be as a conserved gene because of its high prevalence and activity in virulence gene expression among clinical isolates.

Based on statistical analysis, the differences in the distribution of all the eight genes among the clinical isolates were not significantly correlated with the origin of the strains. This corresponds with Wolska and Szweda (2009). Therefore, the production of virulence genes may be possible in different infection sites.

psl and *pel* are two main loci for biofilm formation. Colvin et al. (2011) showed the occurrence of biofilm formation in *psl* mutation isolates, because the lack of Psl was compensated with the high transcription of *pelA* gene. Similarly, in our study, unlike *psl* gene, the difference in the distribution of *pelA* gene was significant with respect to biofilm formation (Fig. 4). Therefore, *pelA* gene was considered as a biofilm-formation marker for *P. aeruginosa* isolates.

Based on the findings of Feldman et al. (1998), flagella play an important role in establishing pulmonary infections. Furthermore, O'Toole and Kolter (1998) demonstrated the role of flagella in biofilm formation by using *P. aeruginosa* PA14 isolates. In the current study, the significant relationship between biofilm formation and prevalence of *fliC* gene in the clinical isolates confirms the essential role of flagella in biofilm formation (Fig. 4).

Moradian et al. (2012) found that 77.3% of their clinical isolates were resistant to ceftriaxone. Ghazi et al. (2012) investigated antibiotic resistance pattern in clinical isolates of *P. aeruginosa* and showed that 96.5% of the isolates were

resistant to trimethoprim–sulfamethoxazole. Akingbade et al. (2012) reported that 70% of *P. aeruginosa* clinical isolates were resistant to tetracycline. Similarity, our data showed high resistance rate to trimethoprim–sulfamethoxazole, tetracycline and ceftriaxone, 94.2%, 94.2% and 67.3%, respectively. The increased rate of MDR *P. aeruginosa* isolates can cause limitations in antibiotic therapy as a final strategy for the treatment of infections. Therefore, it is important to investigate the prevalence of MDR *P. aeruginosa* isolates. In Iran, Yousefi et al. (2010) and Nikokar et al. (2013) found that 30.1% and 45.3% of the isolates were multi-drug resistant, respectively. In Georgia, Burjanadze et al. (2007) revealed that 31.5% of *P. aeruginosa* isolates were MDR but in the current study, 58.65% the prevalence of MDR isolates were 58.65% (Fig. 6).

In addition, there is no relationship between the capacity of alginate production and the prevalence of gentamicin resistance isolates. Therefore, our findings is in agreement with Diaz et al. (2011) which showed, the changes in alginate production have no significant effect on susceptibility to gentamicin. Based on previous findings the cell wall-inhibitory antibiotics can induce alginate production operon via AlgU (Wood et al., 2006). In our study, a significant correlation was found between the prevalence of *algD* and *algU* genes and multiple-drug resistance to three different classes of antibiotics, trimethoprim–sulfamethoxazole, tetracycline and ceftriaxone (Fig. 5). Therefore it may be possible to consider them as two candidate genes which have some roles in the mechanisms of antibiotic resistance.

In conclusion, the high frequency of colonization and toxicity in the clinical isolates may increase the pathogenicity of *P. aeruginosa* isolates due to their adhesion and protective properties caused by biofilm and antibiotic resistance. Consequently, it may be possible to promote the stability of infections. This study offers new data on the correlation between biofilm formation and alginate production with respect to the presence of virulence genes. Further experimentation using a variety of clinical isolates from different regions is required to fully determine the depth of our analysis.

Conflict of interest

None.

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