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Virulence Genes and Antibiotic Resistance Profile of *Pseudomonas aeruginosa* Isolates in Northwest of Iran

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***Pseudomonas aeruginosa* (*P. aeruginosa*) is one of the common pathogen that causes serious infections in hospitalized patients throughout the world. It has been reported that the clinical isolates of *P. aeruginosa* are difficult to treat because of their virulence factors and antibiotics resistances. The aim of present study was to determine whether a correlation exists between the prevalence of virulence factors including *lasB* , *lasA* , *PopB*, *toxA* and antibiotic resistance in *P. aeruginosa* isolated from different wards of hospitalized patients in Northwest of Iran. In this study, 150 isolates of *P. aeruginosa* were collected from the wound, UTI, LRT, sputum, burn and blood stream infections. The prevalence of *toxA, lasA*, *lasB* and *PopB* genes was determined by PCR. Antimicrobial susceptibility testing was performed using the kirby-bauer method. Prevalence of the isolates encoding exotoxin A was 87.33 %, *lasA* was 30 %, *lsaB* was 46.66 % and *PopB* was 28.66%. Prevalence of *lasB* gene was significantly higher in isolates from blood and respiratory tract infection in comparing with isolates from wound infections. High resistance levels to Gatifloxacin (81.33 %), Piperacillin (71.33 %), gentamicin (69.33%) and Ciprofloxacin (64%) were observed. Colistin and Polymyxin B were the most effective antibiotics. findings of the present study showed type II secretion toxin, *toxA*, *lasA* and *lasB* were predominant in *P. aeruginosa* infections from our region. Prevalence of the *PopB* gene was significantly lower than other previous studies. The high antibiotic resistances against antimicrobial agents were observed except for colistin and Polymyxin B which shows priority needs for developing antibiotic stewardship in our regional hospitals.**

Key words: *Pseudomonas aeruginosa*, infection, secretion toxin, resistance, virulence, Iran.

Pseudomonas aeruginosa is a gram negative pathogen that causes opportunistic infections in human [1, 2]. In normal hosts *P. aeruginosa* rarely leads to disease, However , it is responsible for UTI, respiratory tract infection, bacteremia, pneumonia, post-operative infections and blood stream infection in hospitalized patients and immunosuppressed hosts [2-6]. *P. aeruginosa* is associated with high morbidity and mortality and it has been reported as acute infection in over than 70% cases [7, 8] . It has been reported that isolates of *P. aeruginosa* in Iran were resistance to almost all conventional antibiotics and recently has been shown that in the USA the prevalence of fluoroquinolones resistance *P . aeruginosa* is more than 60% in hospitals [3, 9-11]. Numerous studies have been indicated that human infections with this organism is life threatening since treatment of clinical isolates are difficult because of their resistance to antibiotics [12, 13].

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The ability of *P. aeruginosa* in causing of infections in hosts is related to its ability in regulating of virulence genes in response to environmental conditions [14, 15]. Although increasing multidrug resistance complicates treatment of this bacterial infection, the pathogenesis of *P. aeruginosa* is multifactorial and depends on the production of various secreted and cell-associated virulence genes including exotoxins and enzymes [16]. Exotoxins are passively secreted from the cell or actively secreted via different types of protein secretion systems including types I, II , II I , V, and IV [1, 17, 18] . *P. aeruginosa* has one or more of the genes *toxA, lasA, lasB,* and *popB* which are constituted of the type II (*toxA, lasA, lasB)* and type II I (*popB*) secretion systems [8, 18]. The *exotoxin A*, is an ADP- ribosyltransferase toxin that inhibits protein synthesis [19, 20]. *lasA* (protease) and *lasB* (elastease) [18, 21] have a strong elastolytic activity capable of inactivating a wide range of biological tissues ,whereas *lasA* possesses a low level of elastolytic activity, but can enhance the activity of *lasB* [12] and *popB* gene is able to induce necrosis of phagocytes that is essential for the translocation of effector proteins to host cell cytoplasm [2, 22]. These toxins are thought to promote the organism, diffusion in the site of infection and the organism invade to host immune system and inhibits DNA synthesis, so leads to host cell damage and death [16, 20]. Despite the high incidence of *P. aeruginosa* infections in hospitalized patients and increasing antibiotic resistance, there is no comprehensive investigation about the prevalence of virulence factors and antibiotic resistance of *P. aeruginosa* isolated from human clinical samples in northwest of Iran. Therefore, the present study aimed to determine whether exists correlation between the prevalence of virulence factors, encoding elastease *(lasB)* , protease *(lasA)* , *PopB, toxA* and antibiotic resistance in *P. aeruginosa* isolated from different clinical specimens in hospitalized patients.

MATERIALS AND METHODS

Isolation and identification of *pseudomonas aeruginosa*

One hundred and fifty clinical isolates of *P. aeruginosa* were collected from samples including wound, respiratory tract infection, urinary tract infections, bloodstream infection and sputum of patients admitted to the Imam Reza, Shahid Madani, and Sina hospitals in Tabriz (Northwest of Iran) during September 2013 till July 2014. The isolates were confirmed as *P. aeruginosa* by colony morphology, motility, pigment production, growth at 42ºC and 4ºC, Gram staining and conventional biochemical tests.

Antibiotic susceptibility tests

Antimicrobial susceptibility of the isolates against 11 antibiotics was performed by Kirby- Bauer disk diffusion method on Muller-Hinton agar according to CLSI guideline [23]. The susceptibility and resistance of *P. aeruginosa* to the following antibiotic disks were tested: Amikacin, Cefepime, Ceftazidime, Tobramycin, Gentamicin, Imipenem, Colistin, Ciprofloxacin, Piperacillin, Gatifloxacin, Polymyxin B. The interpretation of sensitivity was done according to CLSI breakpoint. *P .aeruginosa* (ATCC 27853) used for the quality control.

DNA extraction

DNA extraction was done according to Tissue buffer boiling method. First, 20µl of Tissue Buffer (0.25% SDS + 0.05M NaoH) was mixed with single colony of bacterial isolate and the mixture was incubated for 10 minutes in 95°C, after incubation mixture centrifuged for 1minute in 13000g and finally 180µl of MilliQ water was added and extracted DNA freezed in -20°C for long time storage[24, 25]

PCR for type II and type III secretion systems

The virulence genes including *lasA, lasB, toxA* and *PopB* were amplified by PCR method and using the specific primers shown in Table 1**.** Each PCR reaction was done in a total volume of 20µl as follows: 2 µl of template DNA, 0.6 µl MgCl2, 0.4 µl of each primer, 0.2 µl dNTP , 2µl of 10 x PCR buffer , 0.5 µl of *Taq DNA polymerase* (5U/µl) (SinaClon, Tehran, Iran) and 13.9 µl of molecular grade water. The PCR condition carried out as the following steps: initial denaturation step at 94 ºC for 10 min followed by 35 cycles repetitions of 40 s at 94 ºC (denaturation), 50 s at 57 to 68 ºC (annealing) and 55 s at 72 ºC(extension) with a final extension at 72 º C for 10 min. PCR products were analyzed by electrophoresis in 1 % of agarose gels for 70 – 80

min at 100 V. Finally stained with ethidium bromide (0.5 mg / ml) and detected by UV transllumination.

Statistical Methods

The prevalence of virulence gene with respect to the site of infection was compared by chi-square test. The correlation between the prevalence of virulence gene and antibiotic resistance patterns were tested by the t-test.

RESULTS

The resistant properties of *P. aeruginosa* to the 11 antimicrobial disks tested is shown in Table 2 . Bacterial strains exhibited the highest level of resistance to Gatifloxacin (81.33%) and Piperacillin (71.33%). The most effective antibiotics against *P. aeruginosa* were colistin (8%) and Polymyxin B (28 %)**.** The resistance patterns in respect to site of infections was different in *P . aeruginosa* isolates according to Table 3. The distribution of type II and type III secretion-toxin encoding gene varied in respect to the infection localization in patients are shown in Table 4 and Fig 1. The highest spred of the *lasB* was detected among *P. aeruginosa* from blood (76.93 %) and respiratory tract infection(76.19%), which was significantly higher than that in isolates from in-patients with wound infections (44%) , (P = 0.004 and P = 0.005, respectively). Our results showed that, isolates of respiratory tract infection had the highest frequency of virulence genes .Moreover, the frequencies of *PopB* gene among the isolates of blood infection was higher than other isolates. The occurrence of virulence genes in all isolates of the present study were as follows: *toxA* (87.33 %) *lsaB* (46.66 %) , *lasA* (30 %) , *PopB* (28.66%) . Coexistence of *lasA*, *lasB* and *PopB* was seen in 19.33 % of the isolates, while 7.33% of the isolates had concomitant existence of *toxA, lasA, lasB* and *PopB***.** Just in 25 (16.66 %) of isolates, *lasA*+, *lasB*+ was found. No significant association between MDR resistance and prevalence of virulence gene carriage was observed (P = 0.409).

DISCUSSION

P. aeruginosa isolates exhibit high incidence of resistance to antimicrobial agents and are frequency multidrug resistance [26, 27]. According to the results of the present study, there is a high frequency (>60%) of resistance against at least four or more antibiotics, which is in agreement with the previous reports from Iran

Table 1. Primer s used for the amplification of different virulence genes among Pseudomonas aeruginosa isolates.

Target Gene	Product size bp	Cycle	Annealing	Primer Sequence (5'> 3')	Refrens
toxA	396	35	68	F- GACAACGCCCTCAGCATCACCAGC R--CGCTGGCCCATTCGCTCCAGCGCT	[22]
lasA	1,075	30	57	F- GCAGCACAAAAGATCCC R- GAAATGCAGGTGCGGTC	[23]
lasB	1,220	30	50	F- ACAGGTAGAACGCACGGTTG R- GATCGACGTGTCCAAACTCC	[23]
PopB	1,200	40	55	F- TTTGGATCCATGAATCCGATAACGCTT R-TTTGAATTCTCAGATCGCTGCCGGTCG	[8]

Table 2. Prevalence o f toxA, lasA, lasB and PopB among *P. aeruginosa* obtained from various sources. NO %

virulence-genes	Woundn =50	Urinen =53	Bloodn =13	Respiratoryn =21	Burnn =5	Sputumn =5	Csfn =3
toxA	37(74)	43(81.13)	10(76.93)	18(85.71)	5(100)	4(80)	2(66.66)
lasA	18(36)	15(30.18)	7(53.84)	8(38.09)	2(40)	1(20)	1(33.33)
lasB	20(44)	18(34.61)	10(76.93)	16(76.19)	2(40)	2(40)	0
PopB	13(29.16)	13(25)	5(38.96)	6(28.57)	1(20)	1(20)	1(33.33)

and neighbor regions [3, 16, 27-29]. In a same study from Malaysia, Idris *et al*. reported that the majority of the isolates were resistant to at least two of the beta-lactam group of antibiotics (CAZ, FEP and TZP), while the number of isolates resistant against the fluoroquinolones and aminoglycoside groups (CIP and GEN), were low (<11%) [30]. *Rossolini* et al. indicated that the fluoroquinolones retain significantly better activity in the Asia Pacific region compared with North America [31] . In the contrary, Our results showed that the isolates were resistance(> 64 %) against the fluoroquinolones and aminoglycoside groups (CIP and GEN), while low numbers of isolates were resistant (<28 %) against the two beta-lactam groups. 64% of isolates in the present study were resistance against ciprofloxacin, which was higher than Latin America with 26.8% and European countries with 10–32% resistance [9]. Probably, overuse of the fluoroquinolones and aminoglycoside groups as the first line of therapy regimen resulted in selective proliferation of isolates resistant to these antibiotics. According to data from the largest multicentre surveillance study [32] Amikacin,

Piperacillin–tazobactam and carbapenems remain the most active drugs worldwide, while ticarcillin and aztreonam had the lowest activities,

Table 3. Antimicrobial Resistance Properties in *Pseudomonas aeruginosa* Isolated From Clinical Infections. n=150 , NO % ,

Antibiotic	Wound	Burn	Urine	Sputum	Respiratory	Csf	Blood
AMK	70	80	42.30	60	57.14	100	69.23
CEP	64	40	46.15	80	38.09	100	76.92
TOP	64	80	48.07	80	76.19	100	69.23
CAZ	86	60	55.76	100	71.42	100	69.23
GEM	76	80	44.23	80	71.42	100	69.23
IMP	56	80	25	80	66.66	100	69.23
COL	12	0	5.76	80	19.047	0	0
CIP	68	40	48.07	0	66.66	100	61.53
PRL	70	60	52.92	80	66.66	100	69.23
GAT	74	60	72.07	100	90.47	100	69.23
PB	34	0	26.92	0	23.80	0	30.76

Abbreviations :
AMK= amikacin (30 ¼g/disk); CEP = ciprofloxacin (5 ¼g/disk); TOB = tobramycin (30 ¼g/disk); CAZ = ceftazidime(30 ¼g/disk); GEM = gentamycin(30 ¼g/disk); IMP = imipenem (30 ¼g/disk); COL = colistin (10 ¼g/disk); CIP = ciprofloxacin (5 ¼g/disk); PRL =Piperacilin(100 µ) ; GAT = Gaticilin ; PB = Polymyxin B (300 U/disk)

Table 4. Antimicrobial susceptibility testing results of 150 isolates of P. aeruginosa collected from 3 hospitals.n=150 ,NO %

Antibiotic	Resistance N0%
Amikacin (30µ)	150
Cefipime(30µ)	66
Ceftazidime(30µ)	66.68
Tobramycin(30µ)	63.33
Gentamycin (30µ)	69.33
Imipenem(30µ)	54.66
Colistin (10µ)	8
Ciprofluxacin(5µ)	64
Piperacilin(100 µ)	71.33
Gaticilin	81.33
Polymyxin B (300 U/disk)	28

but findings of present study indicated that isolates had high resistance against amikacin and piperacillin (> 60 %). In a study by Japoni et al in Tehran, Majority of the *isolates* were resistant to more than 5 antibiotics, which was in agreement with resistance in our isolates [29].

Virulence of *P. aeruginosa* is clearly multi-factorial and has been attributed to cell associated factors , including the exoenzymes or secretory virulence factors like protease, elastease and exotoxin A [9]. It has been shown that *lasA* and *lasB*, were the Proteases secreted that play a major role in the invasion of *P. aeruginosa* [33]. exotoxin A (*toxA*) has an antiphagocytic and cytotoxic effect on host [19] *P.*

aeruginosa isolates are able to induce the necrosis presumably, via the pore-forming activity of the "translocation" proteins *PopB* [22]. these factors play an important role in pathogenesis of *P. aeruginosa* induced infections like respiratory tract infections, burn wound infections , urinary tract infection and blood stream infections [9] . findings of present study showed that *toxA* (87.33 %) , *lasA* (30 %), *lasB* (46.66 %) and *PopB* (28.66 %) were the most commonly detected virulence factors in our isolates of *P. aeroginosa*. Wolska and Szweda. reported that the frequency of *lasB* and *toxA* virulence factors in clinical isolates of *P. aeruginosa* were 76.9% and 76.95%, respectively [34]. Endimiani et al. founded that 100% of *P. aeruginosa* isolates from their clinical infections

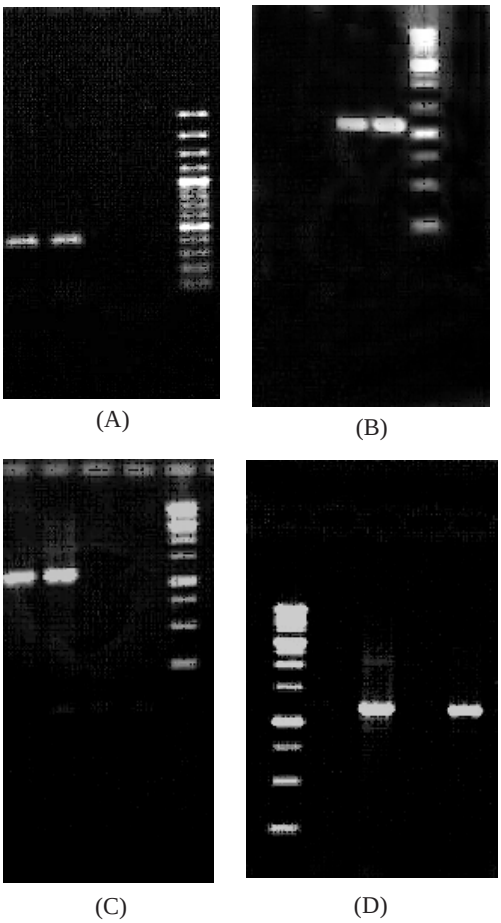

Fig. 1. Gel electrophoresis of PCR products of the virulence genes among Pseudomonas aeruginosa isolates. (A) Amplification of the toxA gene ,(B) amplification of the PopB gene , (C) amplification of the lasA gene and (D) amplification of the lasB gene.M= Marker

were positive for *toxA* and *lasB* , while over 80 % of the studied isolates from clinical infections of our investigation contained *toxA* gene and over 40 % contained *lasB* gene [9]. In present study the prevalence rate of *lasA* was 30% , which was similar with othe same studies in Iran [3, 9] . Moreover, The prevalence of *lasB* among our clinical isolates was significantly lower ($P<0.05$) than that in Bulgaria non CF *P. aeruginosa* (cystic fibrosis *P. aeruginosa*) isolates [23]. Feltman et al and Alonso et al. reported the *PopB* gene as a marker for the presence of the large chromosomal locus encoding the type III secretion machinery proteins (psc-pcr-exs-pop genes) [8]. They also noted that each of seven examined environmental isolate contained the *pscJ* gene, which is also located in the large type III secretion gene cluster. Dacheux et al. demonstrated that the *exsA* gene member of the *PopB* gene cluster, was present in 28 (97%) of 29 examined CF *P. aeruginosa* isolates [8, 35, 36]. Although no study about the prevalence of the *PopB* gene and presence of the chromosomal locus (psc-pcr-exs-pop genes) were conducted in our country, In the current study, 28.66 % of isolates carried *PopB* genes, which is higher than previous studies [35, 36] .

lasA, and *lasB* activities were observed in multidrug- resistant (MDR) *P. aeruginosa* clinical isolates, but no significant difference was observed in the production of proteases between MDR and multidrug-susceptible *P. aeruginosa* [37]. Zhenzhen Sun et al. indicated that produced amounts of extracellular enzymes decreased and lower *lasB* activity was also observed in all of the ²-lactam–resistant isolates [12]. Our data showed that, lower activity of *lasA* and *lasB* was observed in all of the ²-lactam–resistant isolates, which is in agreement with previous studies [12]. Also, our results indicated co presence of *toxA* and fluoroquinolones resistance and ²-lactam–resistant among the large number of isolates, but no statistically significant difference was found. In this study there was no correlation between the fluoroquinolones resistance, MDR resistance and frequencies of virulence genes ($P>0.05$).

In conclusion, findings of the present study showed type II secretion toxin, *toxA*, *lasA* and *lasB* were predominant in *P. aeruginosa* infections from our region. Prevalence of the *PopB* gene was significantly lower than other previous

studies. The high antibiotic resistance against antimicrobial agents were observed except colistin and Polymyxin B which, Prescription of this antibiotic can be effective for the treatment of human infections due to *P. aeruginosa* in our region. Also high resistance against gentamicin and ciprofloxacin indicate that irregular high prescription of these antibiotics in our region concluded to this high resistance. Findings of present study shows priority needs for developing antibiotic stewardship in our hospital sets, importance of virulence genes related to type II (*toxA, lasA, lasB)* and type III (*popB*) secretion systems on clinical isolates and Further studies with large sample size and expression rate analysis are required for finding out the actual role of this gene in different clinical infection caused by *P. aeruginosa*.

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