Original Article

Association of the Exotoxin A and Exoenzyme S with Antimicrobial Resistance in *Pseudomonas Aeruginosa* Strains

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Abstract

Introduction: *Pseudomonas aeruginosa* is a gram-negative and opportunistic bacterium that causes opportunistic infections in immunosuppressed patients. The main purpose of this study was to investigate the association between exotoxin A and exoenzyme S production with antibiotic resistance patterns.

Methods: The toxA and exoS genes were detected in 102 clinical isolates by PCR. Antibiotic susceptibility tests were performed by disk diffusion method (Kirby Bauer). The Chi-square and Fisher's test were used for evaluation of the association between "toxins and infections source" and "toxins and antibiotic resistance" respectively.

Results: Frequency of toxA+ and exoS+ strains was 81% and 61%, respectively. The association between drug resistance and toxA+ genotype was significant for all antibiotics tested ($P < 0.05$) except aztreonam; however, no significant association was observed between drug resistance and exoS gene ($P > 0.05$).

Conclusions: High frequency of toxA+ resistant strains isolated from inpatients and significant association between the toxin and drug resistance in more antibiotics, reinforces possible role of exotoxin A as an extracellular protein in the regulation of drug resistance genes. The results may be further verified by Southern blot analysis of toxA and exoS gene expression and elucidation of the mechanism of antibiotic resistance.

Keywords: Antibiotic resistance, exoenzyme S, exotoxin A, Pseudomonas aeruginosa

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Introduction

Pseudomonas aeruginosa is an opportunistic Gram-negative bacterium that has become a major cause of nosocomial infections, especially in patients with severe burns, immunocompromised patients, and individuals with HIV infection.[1] There are six classes of secretary systems (T1SS to T6SS), which operate in Gram-negative bacteria. These Secretary systems are involved with secretion of different virulence factors. T2SS and T3SS systems secrete the most important virulence factors in *P. aeruginosa*.[2] Exotoxin A is considered to be a major virulence factor of *P. aeruginosa*.[3] It is secreted into bacterial extracellular space in stationary phase of growth under iron-limiting circumstances, environmental temperature changes, and presence of specific factors like the amino acid glutamine.[4–6] The exotoxin is an ADP-ribosyltransferase which inactivate elongation factor-2 and thereby halts protein synthesis and eventually leads to cell death.[3]

Exoenzym S is a major cytotoxin required for colonization, invasion and bacterial dissemination during infection, which is secreted by the type III secretion system in *Pseudomonas aeruginosa*.[7] The toxin has two active domains, comprises a C-terminal ADP ribosyltransferase domain and an N-terminal Rho GTPase-activating protein (GAP) domain.[3] The exoenzyme is secreted in stationary phase of growth under environmental changes such as depletion of calcium or host cell contact.[8] Together GAPRho and ADP ribosyltransferase change the host cell cytoskeletal function and results in impaired cell migration and adhesion. Furthermore, by preventing the action of phagocytes and disrupting epithelial and endothelial cells and their apoptosis,[9] they can prevent wound healing.[10] *ExoS* is considered to be a major virulence factor involved in burns and chronic pulmonary infections.[11] Regulation of growth, production of many virulence factors and resistance to different antimicrobial agents is controlled by Quorum Sensing (QS). Expression of virulence genes and multi drug resistance (MDR) trait can be influenced by efflux pumps.[12] Multi drug resistance in *P. aeruginosa* can lead to serious outcomes such as amputation or death.[13] The bacterium intrinsic and acquired resistance is due to various factors, including efflux pumps, reduced cell wall permeability, acquisition of resistant plasmids, expression of various enzymes, and biofilm formation.[1]

Pseudomonas aeruginosa is resistant against three major classes of antibiotics, including: beta-lactams, aminoglycosides, and fluoroquinolones.[14] According to previous studies, the resistance is due to four main mechanisms, including: low-permeability membrane,[6,15,16] efflux pumps,[17–19] enzymatic modification[20,21] and resistance due to mutations.[22] Strains which possess genes encoding virulence factors are more likely to be exposed to high levels of antibiotics which will in turn be translated to high level antibiotic resistance.[23] Among the extracellular toxins, exotoxin A has a major role in bacterial virulence; although it seems to have a lesser

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impact than the type III secretion toxins.[3]

The aims of this study were to isolate *toxA*+ and *exoS*+ strains from blood, urine, wounds and trachea specimens; examine possible associations between antibiotic resistance and exotoxin A and exoenzyme S production; as well as compare antibiotic resistance patterns in strains possessing the *toxA* and *exoS* genes. In this study, analysis of the *toxA* and *exoS* genes expression and determination of the mechanism of antibiotic resistance will be verified using Southern blotting.

Patients and Methods

Clinical samples

For a period of six months in 2012, based on Modal Instance Sampling, a total of 102 isolates were recovered from blood (14 isolates), wound (58 isolates), urine (19 isolates) and trachea (11 isolates) infections. Samples were collected from outpatients (13 isolates) and inpatients (89 isolates) in Tehran hospitals. The samples were collected in tubes containing 2 mL LB broth (Merck, Germany) and incubated aerobically at 37°C for 24 hours.

Diagnostic tests

For bacterial identification different tests were employed, including: Gram stain, oxidase (Roshd Co. of Iran), growth at 42°C in cetrimide agar (Merck, Germany), and glucose fermentation. Glycerol (20%) was added to each microtube containing bacterial suspension and stored at -80°C.[24]

Antibiotic susceptibility testing

According to CLSI guidelines (M100-S22, 2012),[25] six different types of antibiotics; Ceftazidime (Caz. 30 µg), Imipenem (Imp. 10 µg), Aztreonam (Azt. 30 µg), Piperacillin (Pip. 100 µg), Gentamicin (Gen. 10 µg), and Ciprofloxacin (Cip. 5 µg), as well as a non-recommended antibiotic (Cefotaxime (30 µg)) were tested. The tests were performed by disk diffusion method (Kirby Bauer) on Mueller Hinton agar medium (Hungary Micromedia) in accordance with 0.5 McFarland turbidity standard.

Molecular detection of toxA and exoS genes

Following extraction of bacterial DNA by a commercial kit (Fermentans Co.), polymerase chain reactions (PCR) were used for detection of toxA and exoS genes. The complete genome sequence of *P. aeruginosa* PAO1, deduced from NCBI (National Centre for Biotechnology Information), was utilized to design PCR primers. Gene Runner software was used for designing upstream and downstream primers (Table 1). The primer sequences were synthesized by GenFanAvaran Co., Tehran, Iran.

PCR assays were performed in a total volume of 50 µL. Reagents were added to 0.5 mL microtubes in the following order: 5 µL of 10X standard buffer, 1 µL of 200 µM each dNTP, 2 µL of 0.5 µM primer mix, 1 µL of 0.5 mM $MgCl_2$, 2 µL of template DNA, and 0.25 µL of 1.25 units of Taq DNA polymerase. Fifty µL of mineral oil were added onto each tube and placed in a thermocycler (Bioneer XP, Japan).

Reaction mixtures were subjected to 35 amplification cycles of the following incubations: Hot- start PCR at 94ºC for 4 min (1 cycle), denaturation at 94ºC for 1 min, annealing at 62ºC for 1 min, extension at 72ºC for 1 min and final extension at 72ºC for 5 min (1 cycle).[26] *Pseudomona aeruginosa* strain ATCC 15692 (containing both the *toxA* and the exoS genes) was used for positive control and distilled water as a negative control. Two µLs of the amplification products was analysed by 0.8% agarose gel electrophoresis. Gels were stained with Ethidium Bromide and visualized under UV (Figure 1 and 2). The amplicons were purified from the gels (Fermentans Kit) and sent for sequencing to Macrogen Co. Korea. The gene sequence chromatograms were viewed and edited using Chromas Lite software version 2.1.1. Finally, the output of the gene sequence was transferred to the ClustalW software in FASTA format.

Statistical analysis

The Chi-square and Fisher's test were used for evaluation of the association between "toxins and infections source" and "toxins and antibiotic resistance" respectively.

Results

Among 102 strains, 79.4% of the isolates possessed *toxA* gene and 59.8% contained *exoS* gene. *ToxA*+ isolates were predominantly from the wound, blood and trachea specimens. The Fisher's exact test analysis showed a significant association between possession of *toxA* gene and infection sources ($P < 0.05$). However, there was no significant association between the presence of *exoS* gene and infection sources ($P > 0.05$). In hospitalized patients, the highest frequency in both *toxA*+ and *exoS*+ strains was observed in wound samples. However, in outpatient the highest frequency was observed in urine isolates (Table 2).

Sequence alignment of the replicons in comparison with GenBank database revealed 97.49% similarity to *P. aeruginosa* strain ATCC 25619 exotoxin A and 97.11% to *P. aeruginosa exoS* gene (ORF1 of strain PAO1); thus the identity of isolates were confirmed.

The frequency of drug resistance in *toxA*+, *toxA*-, *exoS*+ and *exoS*- strains isolated from the multiple infection sources were shown in Table 3. In a total of 14 strains isolated from blood samples, twelve strains were possessed *toxA* gene. Six *toxA*+ strains isolated from blood samples were resistant to piperacillin and aztreonam; whereas, only four strains were resistant to Gentamicin and Ciprofloxacin. Five tested strains were also resistant to Cefotaxime, Imipenem and Ceftazidime. For *toxA*- strains, only one strain was resistant to all three antibiotics (Cefotaxime, imipenem, and ciprofloxacin). Another strain was sensitive to

Table 1. Primer sequences used in PCR assays for detection of *toxA* and *exoS* genes in *P. aeruginosa* strains

Type and size of gene	sequence type	Sequences (5 '- 3')	starting index	Sequence length	GC%	Tm
toxA, 710 bp	Forward primer	CACAGGCAACGACGAGGC	1137	18	66.7	66.2
	Reverse primer	CCTTGTCGGGGATGCTGG	1846	18	52.4	67.9
exoS, 367 bp	Forward primer	CGGTAGAGAGCGAGGTCAGC	1768	20	65	66.4
	Reverse primer	GAGGTGGAGAGATAGCGTCG	2134	21	61.9	67.9

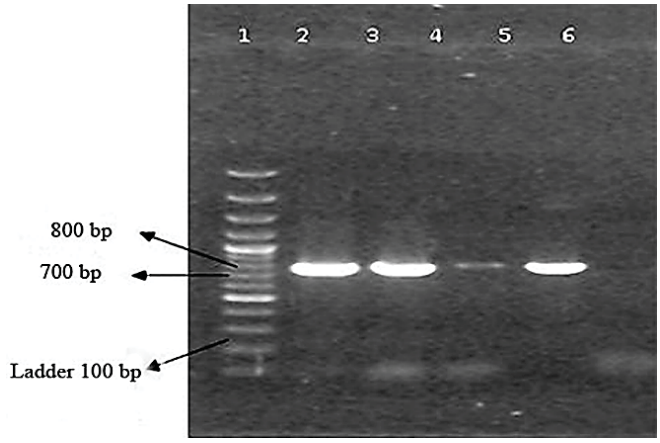

Figure 1. Electrophoresis of PCR products- detection of *toxA* gene using designed primer for *P. aeruginosa* in clinical strains. Lane 1) 100bp DNA marker; Lane 2) *P. aeruginosa* (strain ATCC® 15692™ /PAO1) as a positive control. Lane 3–5) Clinical isolates with *toxA* gene. Lane 6) Distilled water as a negative control. *ToxA*+ strains were observed in the 750bp band

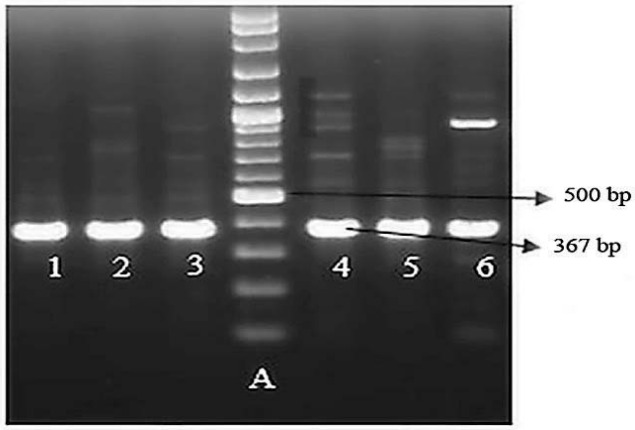

Figure 2. Electrophoresis of PCR products- detection of *exoS* gene using designed primer for *P. aeruginosa* clinical strains. Lane A: 100bp DNA marker. Lane 1) *P. aeruginosa* (strain ATCC® 15692™ /PAO1) as a positive control; Lane 2–6) Clinical isolates with *exoS* gene. *ExoS*+ strains were observed in the 367 bp band.

Table 2. Frequency distribution of strains with *toxA* and *exoS* genes in clinical samples

Gene name		Inpatient				Outpatient			
		Wound	Blood	Trachea	Urine	Wound	Blood	Trachea	Urine
toxA	Positive	51	9	8	4	2	3	0	4
	Negative	5	1	3	8	0	1	0	3
	Total	**56**	**10**	**11**	**12**	**2**	**4**	**0**	**7**
exoS	Positive	35	4	9	4	1	3	0	5
	Negative	21	6	2	8	1	1	0	2
	Total	**56**	**10**	**11**	**12**	**2**	**4**	**0**	**7**

Table 3. Frequency of resistant and susceptible strains with *toxA* and *exoS* genes and the Fisher exact test to determine the association between drug resistance and *toxA* and exoS genes

Source Name	Genes	Ceftazidime		Cefotaxime		Gentamicin		Piperacillin		Imipenem		Aztreonam		Ciprofloxacin	
		S	R	S	R	S	R	S	R	S	R	S	R	S	R
Blood	toxA+	7	5	7	5	8	4	6	6	7	5	6	6	8	4
	toxA-	2	0	1	1	2	0	2	0	1	1	2	0	1	1
Trachea	tozA+	5	3	3	5	4	4	5	3	4	4	4	4	5	3
	toxA-	2	1	1	2	2	1	2	1	3	0	1	2	2	1
urine	tozA+	6	2	4	4	5	3	7	1	6	2	3	5	5	3
	toxA-	9	2	9	2	8	3	8	3	10	1	9	2	10	1
Wound	tozA+	12	41	12	41	17	36	15	38	13	40	18	35	13	40
	toxA-	2	3	1	4	2	3	2	3	2	3	1	4	2	3
Total	**tozA+**	**30**	**51**	**26**	**55**	**34**	**47**	**33**	**48**	**30**	**51**	**31**	**50**	**31**	**50**
	toxA-	**15**	**6**	**12**	**9**	**14**	**7**	**14**	**7**	**16**	**5**	**13**	**8**	**15**	**6**
***P*-value**		0.006		0.044		0.050		0.049		0.003		0.082		0.013	
Blood	exoS+	6	1	5	2	7	0	5	2	5	2	3	4	5	2
	exoS-	3	4	3	4	3	4	3	4	3	4	5	2	4	3
Trachea	exoS+	6	3	4	5	5	4	6	3	5	4	5	4	6	3
	exoS-	1	1	0	2	1	1	1	1	2	0	0	2	1	1
Urine	exoS+	7	2	5	4	5	4	8	1	7	2	4	5	6	3
	exoS-	8	2	8	2	8	2	7	3	9	1	8	2	9	1
Wound	exoS+	10	26	9	27	14	22	11	25	9	27	11	25	10	26
	exoS-	4	18	4	18	5	17	6	16	6	16	8	14	5	17
Total	**exoS+**	**29**	**32**	**23**	**38**	**31**	**30**	**30**	**31**	**26**	**35**	**23**	**38**	**27**	**34**
	exoS-	**16**	**25**	**15**	**26**	**17**	**24**	**17**	**24**	**20**	**21**	**21**	**20**	**19**	**22**
***P*-value**		0.423		1.000		0.420		0.544		0.551		0.222		0.498	

S: Susceptible; R: Resistant

all antibiotics. In the isolates, frequency of resistant strains possessed exos+ was less than sensitive strains except for aztreonam. No resistance was observed to gentamicin in the *exoS*+ Strains.

Among tracheal isolates, eight strains were possessed *toxA* gene that was highly resistant to cefotaxime (five strains). Amongst the *toxA*+ isolates, the lowest resistance levels were observed towards ceftazidime, piperacillin and ciprofloxacin (three isolates); and for other drugs, resistance level was equal to four isolates. Regarding the *toxA*- samples, resistance to cefotaxime and aztreonam was remarkable. In these strains, no resistance was observed to imipenem. In exos+ strains, the highest resistance was observed to cefotaxime, gentamicin, imipenem and aztreonam. In this isolates only two strains were resistant to all drugs.

Among urine isolates, eight strains were possessed *toxA* gene. The four isolates, which possessed the *toxA* gene and recovered from UTI cases, had higher resistance. These isolates had very little resistance to piperacillin but displayed high resistance levels towards cefotaxime and aztreonam. The least resistance rate amongst toxA- strains was seen towards imipenem and ciprofloxacin and higher levels noted to gentamicin and piperacillin. In isolates with *exoS*+ trait, a high level of resistance to Cefotaxime, gentamicin and aztreonam was observed. They had the lowest resistance to piperacillin (one isolates), imipenem and ceftazidime (two isolates). In exoS- strains, resistance to all drugs was at the lowest amount for all the strains.

In a total of 58 strains isolated from wound samples, 53 strains were possessed *toxA* gene. Amongst those recovered from blood infections, there was no significant difference between resistance rate to Cefotaxime and towards those antibiotics recommended by CLSI; although the resistance level was slightly higher in the antibiotic.In the *toxA*- strains, resistance to all antibiotics was remarkable. Although in both types of exoS+ and exoS- isolates, the frequency of resistant strains was higher than susceptible strains, but the trend of increasing resistance in exoS+ strains was remarkable.

For six antibiotics tested, the Fisher exact test showed a significant association between drug resistance and *toxA* gene ($P < 0.05$). Only for aztreonam, the Fisher test was not significant. However, no significant association was observed between drug resistance and possession of *exoS* gene ($P > 0.05$, Table 3).

Discussion

The distribution of strains with *toxA*+ gene was variable in different infection sources. Wound infection bacteria had the highest frequency of *toxA* gene (91.4%). This result is consistent with those of R.I. Badr, et al. (2008), Aslani, et al. (2014), Cotar, et al. (2013), and Sadeghifard, et al. (2012) who reported the frequency of *toxA* gene in wound infection isolates to be 89%[4], 90%,[27] 100%,[9] and 80%,[28] respectively. Hashemipour, et al. in Iran (2010) reported the frequency of *toxA* gene in isolates obtained from tracheal infection to be 77%;[29] in this study this frequency was 72.7%. Sharma, et al. (2004) reported the frequency of this gene in the urinary infections isolates to be 11%;[30] in this study the frequency of this gene was reported as 42.1%.

These findings revealed that detection of toxA gene in strains isolated from clinical specimens can be a criterion for diagnosis of infections caused by *P. aeruginosa*.[27]

The frequency of *exoS* gene in our isolates is shown in Table 2. According to this table, the frequency of genes in strains isolated from wounds, blood and tracheal infections were 62.1%, 50%, and 81%, respectively. Feltman, et al. (2001) reported the frequency of genes in strains isolated from wounds, blood and tracheal infections to be 60%, 60% and 75%, respectively.[31] Thus, these results are consistent with our findings. Cotar, et al. (2013) reported the frequency of this gene for wound isolates to be 61.90%.[9] Aslani, et al. in Iran (2012) reported it to be 62%.[27] Mitov, et al. (2010) reported its frequency in the wound isolates 62%, blood isolates 87.5%, lower respiratory tract isolates 55.6%, and in urinary tract infections isolates 66%,[15] (in this study for urine samples 47.4%). Feltman, et al. (2001) reported the frequency of this gene in urine isolates to be 70%.[31] These findings showed that detection of exoS gene in strains isolated from clinical specimens couldn't be a criterion for the diagnosis of infections caused by *P. aeruginosa*. These results are consistent with the finding of Mitov, et al.[15]

Pseudomonas aeruginosa is resistant against many antibiotics due to possession of a number of different mechanisms including; low-permeability membrane, efflux pumps, and a variety of beta-lactamases.[1] Indirect epidemiological evidence and some case studies have raised the possibility of an association between some of *P. aeruginosa* virulence factors and antibiotic resistance.[32] In this study, using the Fisher's test analysis the possible dependency of antibiotic resistant to the presence of two pseudomonas virulence related genes, *toxA* and *exoS*, was investigated.

The frequency of resistant *toxA*+ strains isolated from the total infections was higher than *toxA*- and *exoS*+ strains. This indicates a possible role for exotoxin A in drug resistance. The relatively high sensitivity of *toxA*+ strains isolated from blood stream to gentamicin and ciprofloxacin suggests that these strains have low-level resistance to aminoglycosides and fluoroquinolones. Flamm, et al. (2004) and Kollef, et al. (2005) showed these two antibiotics were effective against 75% of their isolates; therefore suggested them as best medicines against pseudomonas infections.[33,34]

Increased frequency of resistant *toxA*+ strains than resistant *toxA*- isolates in tracheal infections is indicative of a possible role for exotoxin A as a virulence factor, which also induces drug resistance. In accordance with the findings of Kulich, et al. that have demonstrated involvement of exoenzyme S in respiratory infections,[11] higher levels of resistance were observed in the *exoS*+ strains than the *exoS*- strains.

Lower frequency of *P. aeruginosa* strains isolated from urine samples indicates a weak role for this bacterium in urinary tract infections. Similarly, Sumithra, et al. (2014) reported very week involvement of *P. aeruginosa* in UTI cases.[35] Despite the reduced frequency of resistant strains isolated from urinary tract infections, an increased frequency of the resistant *toxA*+ strains points to a probable role for exotoxin A in resistance mechanism. Lack of association between drug resistance and exotoxin S, rejects the role of the toxin in antibiotic resistant bacteria. However, the frequency rate for the resistant strains possessing both genes with those containing solely the *toxA*+ gene, confirms the possible role of *toxA* and non-interference of *exoS* in drug resistance.

The highest frequency of resistant *toxA*+ strains was observed in isolates obtained from wound infection. Comparison of resistant *toxA*+ and *toxA*- strains (Table 3) indicate that exotoxin A, especially in the wound infections, plays a role in inducing resistance. In other words, drug resistance is due to resistance factors and exotoxin A has a contributory role. Badr, et al. (2008) reported that all of their 38 *toxA*+ isolates were associated with delayed wound healing; whereas, their *toxA*- isolates were associated with

a normal wound healing process.[4] The increased frequency of resistant strains with *toxA* gene and the reduced incidence of resistant strains possessing both toxin genes in wound isolates, indicate a possible role for exotoxin A in increasing antibiotic resistance. This finding is consistent with those of Mitov, et al.[15]

Prolonged hospitalization and use of the broad-spectrum antimicrobial agents were shown to be the main cause of increased drug resistance in hospitalized patients.[36] The results obtained in this study, showed high levels of multi-drug-resistance in *toxA*+ strains among hospitalized patients. In The *toxA*- strains isolated from hospitalized and outpatients, was observed an irregular trend of resistance. Goldsworthy (2008), has shown that production of exotoxin A increased 1839-fold when *P. aeruginosa* and methicillin resistant *Staph. aureus* (MRSA) bacteria were grown together in a mixed biofilm.[37] This finding and the significant level of Fisher's test in the present study points to a possible role for exotoxin A in increasing bacterial resistance in the hospital environment. On the contrary, in spite of decreasing resistance rates in the exoS+ strains in hospitalized patients, it is not indicative of a possible role for exoenzyme S in increasing drug resistance rates.

This study revealed, strains of P. aeruginosa that have the ability to produce exotoxin-A show high levels of resistance to drugs that are generally used against this pathogen. The increase in multi resistant strains possessing toxA gene can represent a considerable genetic switch between exotoxin A activity and resistance to antibiotics. If these results are further substantiated, exotoxin-A could potentially be used to produce vaccines against P. aeruginosa.

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