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Presence of *exo*U and *exo*S Genes in *Pseudomonas aeruginosa* Isolated from Urinary Tract Infections

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Background: *Pseudomonas aeruginosa* is considered an opportunistic pathogen; several reports indicate that the organism can also cause infections in healthy hosts. Four effector proteins have been described in *P. aeruginosa*: *exo*U, *exo*S, *exo*T, and *exo*Y. These genes that are translated into protein products related to type III secretion systems.
Materials and Methods: A total of 134 samples were isolated, and *P. aeruginosa* was identified using biochemical tests. Bacterial genomic DNA was extracted, and the presence of the *exo*S and *exo*U genes were detected by PCR. Biofilms were formed by culturing *P. aeruginosa*on glass slides in rich medium.
Results: The *exo*U (73%), *exo*S (62%) genes were detected from infections caused by *P. aeruginosa* in urinary tract infection patients. Among the 119 strains isolated from patients with urinary tract infections.
Conclusion: An improved understanding of virulence genes and biofilm formation in *P.aeruginosa* may facilitate the future development of novel vaccines and drug treatments.

Keywords: Pseudomonas aeruginosa, *exo*U, *exo*S, Urinary tract infection, Biofilm

1. Background

Pseudomonas aeruginosa is a gram-negative, aerobic, oxidase-positive, non-fermentative, motile, rod-shaped bacterium that inhabits a wide range of environments, such as water, soil, the rhizosphere, and animals. It is also known as a frequent opportunistic pathogen in both animals and human (1-3). The *P. aeruginosa* is considered an opportunistic pathogen; several reports indicate that the organism can also cause infections in healthy hosts. Furthermore, evidence has suggested that there are no major differences in virulence between clinical and environmental (4). Corneal infection with *P. aeruginosa* is a major cause of visual impairment and blindness worldwide (5-8). The *P. aeruginosa* bacterial keratitis is a major cause of corneal ulcers in the developing countries. Predisposing factors include contact lens wearing in the industrialized countries, whereas trauma to the ocular surface is the most common risk factor in developing countries (9, 10).

Urinary tract infections (UTIs) are the other most common community-acquired bacterial infections (11, 12). The diagnosis of UTI is based on semi-quantitative urine culture, used as reference standard that provides both quantification as well as identification of the uropathogen (13-15). In complicated UTIs the gram-negative proteobacterium *P. aeruginosa* plays a major role (16, 17). *P. aeruginosa* is an important cause of the most problematic in multidrug-resistant form of gram-negative pathogens. Many strains of *P. aeruginosa* are resistant to all commonly used antimicrobial agents, so there is a critical need for novel approaches to suppress the growth of this pathogen in the bladder. In the numerous patients who have a legitimate requirement for long-term urinary catheter use, no strategy is truly effective at preventing bladder invasion by urinary pathogens (18). In general, *P. aeruginosa* can be regarded as a successful environmental bacterial genus with the human body as one of its habitats. Because of its high intrinsic antibiotic resistance and its ability to develop new resistances during antibiotic treatment, infections with *P. aeruginosa* are difficult to eradicate (19). Their multidrug resistant strains are normally intractable in any infectious episode, including urinary tract infection, suppurating wounds, or bloodstream infections, thereby causing a fear of the onset of various ailments in people of all age groups (20). To promote severe illness, *P. aeruginosa* uses a type III secretion system to inject toxic effector proteins into the cytoplasm of eukaryotic cells. To date, four effector proteins have been described in *P. aeruginosa*: *exo*U, *exo*S, *exo*T and *exo*Y (21, 22). These genes that are translated into protein products are related to type III secretion systems (TTSS). These products have been demonstrated to show a cytotoxic effect in vitro (23). Furthermore, in clinical studies, the presence of these toxins is associated with a dissatisfactory clinical outcome among patients with *P. aeruginosa* infection (24). The *Exo*U is a 74 kDa, hydrophilic, and slightly acidic protein with a PI of 5.9. The *Exo*U is an important virulence factor of *P. aeruginosa*, and causes rapid cell death during in vitro infections (25, 26). A recent study with a mutant with a specific deletion in the *exo*S structural gene failed to detect a contribution of *exo*S to virulence in a model (21). Genes encoding the cytotoxins *exo*S are present as variable traits and are mutually exclusive in most strains (27).

2. Objectives

The purpose of this study is to examine the relationship between genes (*exo*S and *exo*U genes), biofilm formation, and create disease. The present study was to characterize the

presence of the *exo*S and *exo*U genes in clinically isolated *P. aeruginos*a strains. An improved understanding of these virulence factors is important for the future development of vaccines, because *P. aeruginos*a is an opportunistic bacterium that is resistant to common antibiotics

3. Materials and Methods

A total of 134 strains of *P. aeruginosa* bacteria were collected from UTIs of 325 patients hospitalized in Baqiyatallah hospital in Tehran. Bacteria were isolated from 85 female and 49 male patients; the patients were aged between 36 and 71 years.

3.1. Bacterial strains and growth condition

Samples were cultured on cetremide agar for 24 hours. Gram negative bacillus was recognized by gram staining, then catalase and oxidase tests were done. If these tests were positive, bacteria were recognized by SIM for detection of motility, indol and H_2S production. Other biochemical tests including triple sugar iron (TSI), methyl red-Voges-Proskauer (MR-VP), oxidase fermentation (OF), urease broth, citrate tests, lysine and ornithine decarboxylase and growth in 42°C were done for detection of *P.aeruginosa*. All strains were stored in Luria Bertani (LB) containing 20% glycerol at -20°C. Single colonies were grown in 50 ml LB medium at 37 °C for 24 hours with shaking. After incubation, 1.5 ml of medium was transferred to a new test tube and centrifugation was carried out at 12000rpm for 1min at 4°C. Supernatants were discarded and when precipitate of bacteria was low, this process was repeated 2 or 3 times. The chromosomal bacterial genome was extracted through DNA purification kit (MBST Inc., Iran) and stored at -20°C for further experiments.

3.2. PCR-based genotyping assays

The oligonucleotide primers used in this study are presented in Table 1. To design these primers first nucleotide sequence of genes were blasted in NCBI site. PCR amplification was performed in a total volume of 25µL in 0.5mL tubes containing 1µg of the extracted DNA sample, 1µM of each four primers, 2mM $MgCl_2$, 200µM deoxynucleoside triphosphates, 2.5µL of 10X PCR buffer (10mM Tris-HCl, 1.5mM $MgCl_2$, 50mM KCl [pH 8.3]) and 1 unit of Taq DNA polymerase (Roche Applied Science, Germany). All oligonucleotide primers were synthesized by CinaGene (CinaGene Co, Tehran, Iran). Amplification was carried out by using the Mastercycler Gradient Thermal Cycler (Eppendorf, Germany) with the programs which have been shown in Table 2. PCR (for exoU) was performed by use of pfu proofreading polymerase (Stratagene, United States), according to the following protocol: 94°C for 10 min, then 25 cycles at 94°C for 30s, 56°C for 45s and 72°C for 45s, then a final extension at 72°C for 10 min. Also, PCR (for exoS) was performed by use of pfu proofreading polymerase (Stratagene, United States), according to the following protocol: 94°C for 10 min, then 25 cycles at 94°C for 30s, 58°C for 45s and 72°C for 45s, followed by a final extension at 72°C for 10 min. To investigate the reaction product, 5µl of each product was transferred on 1% agarose gel to Electrophores. They were stained with Ethidium Bromide and photographed.

Table 1. Primers used for detection of *exo*U and *exo*S genes and lengths of the PCR products.

Target gene	Primer sequences (5'-3')	Amplification size (bp)
***exo*U**	FW: GCTAAGGCTTGGCGGAATA RV: AGATCACACCCAGCGGTAAC	204 bp
***exo*S**	FW: ATGTCAGCGGGATATCGAAC RV: CAGGCGTACATCCTGTTCCT	230 bp

Table 2. PCR program for amplification of *exo*U and *exo*A genes.

Gene	Denaturation		Annealing		Extension		Cycles
***exo*U**	Initial: 94°C	10 min					1
	Cycle: 94°C	30″	56°C	45″	Cycle:72°C	45″	30
					Final: 72°C	10 min	1
***exo*S**	Initial: 94°C	10 min					1
	Cycle: 94°C	30″	58°C	45″	Cycle:72°C	40″	30
					Final: 72°C	10 min	1

3.3. Biofilms

In the present study, biofilms were formed on glass slides. First, the slides were sterilized at 121˚C. From a fresh culture of bacteria (18-24 hours) that has represented equivalent to 0.5 Macfarland, about 200µL was removed and added to 20mL BHI broth in a tube. Then it was poured into a sterile plate with one glass slide and stored at 37°C for 6, 24, 48, 72 and 120 hours. After a period of time set, slides were removed slowly with sterile forceps and were gently shaken in distilled water until BHI broth was washed on surface slides. Slides were then dried at room temperature for at least 30min. Then, the slides were stained with safranin or without staining were observed under light and phase contrast microscope.

4. Results

A total of 134 strains of *P. aeruginosa* were isolated from 325 hospitalized patients with UTI. From all of the strains in this study 119 *P. aeruginosa* (88%) produced green-blue pigments after cultivation on cetrimide agar medium. Bacterial colonies were dispersed. For detection of *exo*S and the *exo*U genes PCR reaction was done and the following results were obtained. PCR results of *exo*U gene (204 bp) are shown in Figure 1. Also, PCR results of *exo*S gene (230bp) are shown in Figure 2.

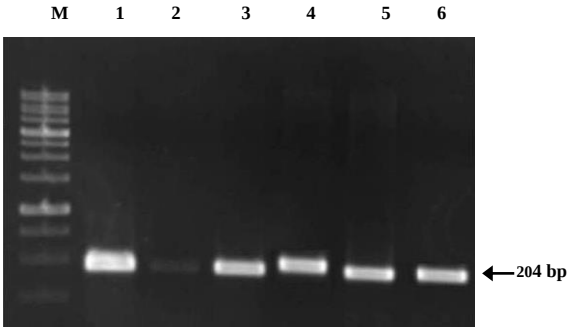


Figure 1. Gel electrophoresis of PCR products following amplifycation with specific primers for *exo*U gene (204 bp). M: DNA size marker.

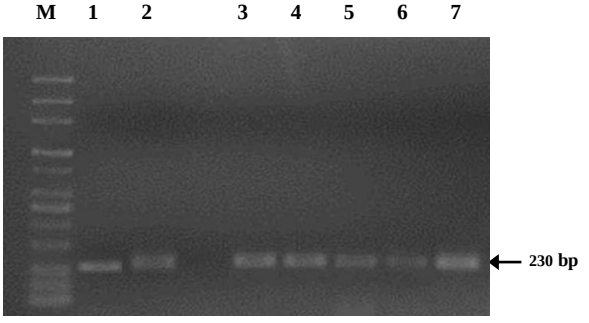


Figure 2. After Electrophores an observation studied *exo*S gene (230bp) on agarose gel.

Moreover, all strains isolated from UTI patients formed biofilms. After five days biofilm structure showed vertical growth and the formation of water channels. The image of the *P. aeruginosa* biofilm after 5 days was photographed (Figure3). Prevalence of *exo*U and *exo*S genes in patients with urinary tract infections caused by *P. aeruginosa* is shown in Figure4. Four different modes including: *exo*U^{+}*exo*S^{+}, *exo*U^{+}*exo*S^{-} or *exo*U^{-}*exo*S^{+}, *exo*U^{-}*exo*S^{-} were observed. In contrast, 11% of UTIs caused by *exo*U^{-}*exo*S^{-} bacteria. In contrast, *exo*S genes were seen in UTIs in 16% of the infections. Statistical analysis of these two genes is shown in Figure 4.

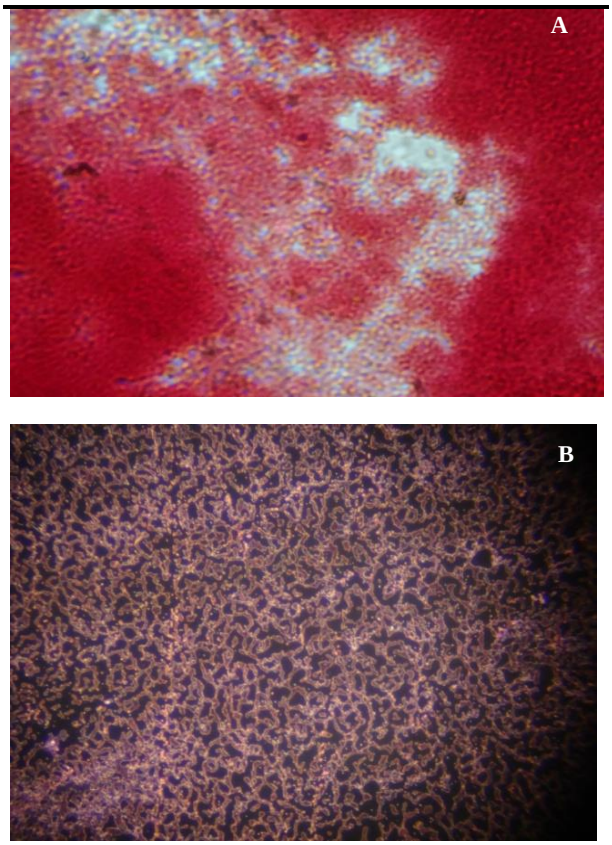
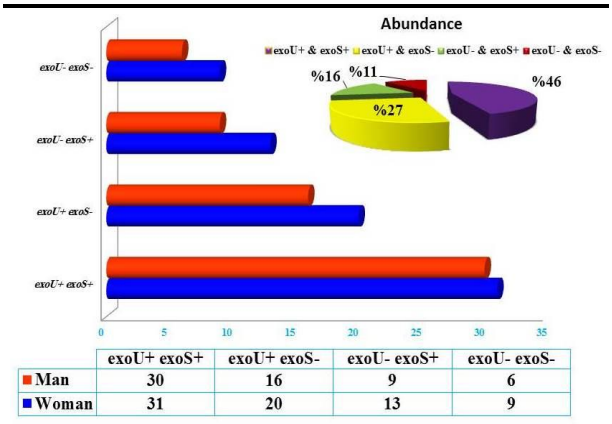


Figure 3. biofilm formation by *P. aeruginosa* with (A) optical microscope and (B) opposite phase microscopy (After 5 days).



	exoU+ exoS+	exoU+ exoS-	exoU- exoS+	exoU- exoS-
Man	30	16	9	6
Woman	31	20	13	9

Figure 4. Prevalence of genes *exo*S, *exo*U infections in UTI patients.

5. Discussion

Several factors are involved in causing diseases including the induced genes encoded regulators, transporters, biosynthetic enzymes and other proteins of unknown function. The majority of genes differentially expressed in biofilms grown under different conditions are related to pathogenesis in human (28). The overall observed gene regulation principle has indicated strong treatment limitation under pathogenic conditions (29). Urinary tract infections occur in both hospital and in community (30). *P. aeruginosa* is an ubiquitous environmental bacterium responsible for a variety of infection in human as well as urinary tract infections (31). *P. aeruginosa* utilizes the TTSS to deliver effector toxins (*exo*S, *exo*U, *exo*Y, and *exo*T) directly into host cells, which can cause rapid cell necrosis or can modulate the actin cytoskeleton, allowing the pathogen to invadethe host cells and evade phagocytosis depending on the disease site or patient background. The genes encoding the cytotoxins *exo*U and *exo*S are present as variable traits and are mutually exclusive in most strains (32, 33).

The relative virulence associated with each of these effector proteins are important since clinical isolates of *P. aeruginosa* commonly fall into one of the following four phenotypic categories: Patients with both gene expressions is an infection caused by *P. aeruginosa* (exoU^{+} and *exo*S^{+}). Statistical analysis of both genes is shown in Figure 4.

Those strains that ware incapable of type III secretion and therefore do not secrete *exo*U, *exo*S, *exo*T may be the least virulent. Both *exo*S and *exo*T have been implicated in the induction of apoptosis in vitro (34, 35). The role of *exo*S and *exo*T in *P. aeruginosa* keratitis is almost entirely due to the ADA ribosyltransferase activities that appear to have non-redundant roles in bacterial survival in neutrophils, and in the induction of neutrophil apoptosis. To date, only fully enzymatic and catalytically inactive exoU proteins have been examined for phospholipase activity (21, 25). These results indicate that *exo*U is a predominant cytotoxin of *P. aeruginosa* (36, 37). Expression of virulence factors in *P. aeruginosa* is under comprehensive regulation, and in vitro expression does not necessarily reflect expression during infection processes one notable exception was the isolates from urinary tract infections, all of which had the *exo*S gene but lacked *exo*U (38, 39). Increased *exo*S activity in urinary tract isolates was recently demonstrated which, combined with our findings, indicates that this exo enzyme may be important in the pathogenesis of urinary tract infections caused by *P. aeruginosa*. The *exo*U production was recently shown to be associated with increased virulence in a murine model of acute pneumonia and systemic spread in accordance with the hypothesis that cytotoxicity plays a role in dissemination of *P. aeruginosa* (40-42).

6. Conclusion

Based on the results of this study, it is suggested to find ways to prevent *exo*U gene activity in order to prevent biofilm formation, especially in burn patients with high mortality. It is likely for *exo*S gene to play an important role in the infections caused by *P. aeruginosa*. Due to the advantages of molecular methods in the diagnosis of opportunistic pathogenic bacteria, it could be convenient and swift technique to prevent the progress of infections and mortality among these patients.

Conflict of Interests

The authors declare they have no conflict of interests.

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Authors' Contributions

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Leila Firouzi-Dalvand, Jamileh Nowroozi and Mehdi Pooladi designed the study, dathcred and analyzed the data and wrote the paper; Mansooreh Hooshiyar and Abbas Akhvan-Sepahi contributed to study desigh. Mehdi Pooladi contributed to samples collection and indentation.

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