Identification of *exoS, exoU* genes in *Pseudomonas aeruginosa*

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ABSTRACT

The bacterium *Pseudomonas aeruginosa* is a common cause of infections, and infection with antibiotic-resistant strains can be dangerous. The aim of the present study is to investigate the presence of the *exoS* and *exoU* genes and the formation of biofilms in clinically isolated *P.aeruginosa* strains. A total of 126 samples were isolated, and *P.aeruginosa* was identified using biochemical tests. Bacterial genomic DNA was extracted, and the presence of the *exoS* and *exoU* genes were detected by PCR. Biofilms were formed by culturing *P.aeruginosa*on glass slides in rich medium. The *exoU* (76%) *exoS* (68%) genes were detected in of strains isolated from burn (burns caused by heat) patients, respectively. Among the 113 strains isolated from patients with burn infections. 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*; *exoU*; *exoS*; burn; biofilm

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative, aerobic, non-fermentative, rod-shaped and motile bacterium that is ubiquitous and very versatile. *P.aeruginosa* secretes a variety of pigments, including pyocyanin (blue-green), fluorescein (yellow-green), fluorescent, pyoverdin, and pyorubin (red-brown). *P.aeruginosa* does not produce spore and non-acid-fast. This bacterium uses a unipolar fellagelum for motility [1-3]. Many strains of *P.aeruginosa* were initially isolated based on their ability to produce pyocyanin, their typical colony structure when grown on agar media, and their distinctive grapelike odor, which is due to the production of aminoacetophenone [4-6]. Because these bacteria can grow on most surfaces, including medical devices such as catheters, they are an important cause of nosocomial infections [7]. *P.aeruginosa* tends to form biofilms, which are complex bacterial communities that adhere to a variety of surfaces, including metals, plastics, and medical implant materials, and tissues. Growth in biofilms promotes bacterial survival; once a biofilm is formed, it is extremely difficult to destroy [8, 9]. *P.aeruginosa* are an important cause of corneal ulcers worldwide, with *P.aeruginosa* infection resulting in severe corneal opacity, ocular pain and visual impairment. One of the main causes of encomia infections, including pneumonia, urinary tract infections, surgical wound infections and bloodstream in fictions. Understanding bacterial virulence and, more generally, the host-pathogen relationship at the cellular and molecular level is essential to identify new targets and develop new strategies to fight infection [11]. *P.aeruginosa* harbors at least one or more *exoS, exoT, exoU* and *exoY* genes that translated into protein products related to type III secretion systems (TTSS). *ExoS* and *exoT* are bio functional enzymes that 75% amino acid identity and encode Gtpase-activating protein (GAP) and ADP-ribosyl transferase (ADP-RT) activities. *ExoY* is Adenylate Cyclase [11-13]. In addition to its ability to grow in biofilms, *P.aeruginosa* possesses other important virulence factors. *Exos* transfer the ADP-ribose moiety from NAD^+ to many eukaryotic cellular proteins. *P.aeruginosa exoS* is an adenosine diphosphate ribosyl transferase that is distinct from Pseudomonas toxin A [14, 15]. *ExoU* has phospholipase/lysophospholipase activity and disrupts eukaryotic cell membranes after translocation into the cell by type III secretion systems [16, 17]. The aim of present study was to characterize the presence of the *exoS* and *exoU* 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.

MATERIALS AND METHODS

A total of 126 strains of *P.aeruginosa* bacteria were collected from burnt (Burns caused by heat) infections of the patients hospitalized respectively in Motahary Hospital (Burn center) in Tehran. The present study was performed with a convenience sample that included newborns recruited from 2011 through 2013. Bacteria were isolated 72 women and 54 men patients, the patients were aged between 40 and 62 years.

Bacterial strains and growth condition

Cultured on certified 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 detecting motility, Indolic and H_2S production. Others 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 detecting *P.aeruginosa*. All strains were stored in LB and 20% glycerol at -20°C was added. Single colonies were grown in 50 ml LB medium at 37°C for 24 hours with shaking. After incubation, 1.5 of the medium was transferred to a new test tube and centrifugation was carried out at 12000 rpm for 1min at 4°C. Supernatants were discarded. When precipitate of bacteria were low, this process was repeated 2 or 3 times. The chromosomal genome bacterium was extracted through DNA purification kit (MBST DNA Extraction). Cells were stored at -20°C for further experiments within a week.

PCR-based genotyping assays

The oligonucleotide primers used in this study are given in table1. To design these primers first nucleotide sequence of genes were blasted in NCBI site. Then, the primers were made by Roobin Teb Gostar Company showed in Table 1. The genome was electrophoresed on agarose gel 1% in voltage 50 Volt for 2 hours. PCR reaction with final 25µl concentration was done. Each reaction included 2.5 µl buffer of PCR, 1µl Deoxy nucleotide three phosphate (dNTP), 1µl of each primers, 1µl extracted genome, 0.5µl of Taq DNA Polymerase enzyme and 18µl twice distilled water were prepared. The *exoU* gene was amplified from p. aeruginosa by use of primers:
FW: 5'- GCTAAGGCTTGGCGGAATA-3'
(up-stream and amplification siza: 204 bp)
RV: 5'-AGATCACACCCAGCGGTAAC-3'
(downstream and amplification siza: 204 bp), and the *exoS* gene was amplified from p. aeruginosa by use of primers:
FW: 5'-ATGTCAGCGGGATATCGAAC-3' (up-stream and amplification siza: 230 bp), and RV: 5'-CAGGCGTACATCCTGTTCCT-3'
(downstream and amplification siza: 230 bp),
PCR (for *exoU*) was performed by use of *pfu* proofreading polymerase (strata gene), according to the following protocol: 94°C for 10 min, then 25 cycles at 94°C for 30″, 56°C for 45″ and 72°C for min 45″, then a final extension at 72°C for 10 min. Also, PCR (for *exoS*) was performed by use of pfu proofreading polymerase (strata gene), according to the following protocol: 94°C for 10 min, then 25 cycles at 94°C for 30″, 58°C for 45″ and 72°C for min 45″, then a final extension at 72°C for 10 min. To investigate the reaction product, 5µl of production was transferred on 1% agarose gel to Electrophores. They were stained with Ethidium Bromide and photographed. 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 have resentment equivalent to 0.5 mac far land, about 200 µl was removed and added to 20 ml BHI broth in a tube. Then it was poured into a sterile plate with one glass slide and they were 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 slowly were shaken in distilled water until BHI broth was washed on surface slides. Slides were dried at room temperature for at least 30 min. Then, the slides were stained with Safranin or without staining were observed under light and phase contrast microscope.

RESULTS

126 strains of *P.aeruginosa* were isolated from 215 burn injury patients hospitalized. From all of strains in this study 82 (71%) *P.aeruginosa* after cultivation on certified agar medium were produced green-blue pigments. Bacteria colonies were dispersed. For detection of *exoS* and the *exoU* PCR reaction was done and the following results were obtained. PCR results of *exoU* gene (204 bp) expression is shown in Figure 1. Also, PCR results of *exoS* gene (230 bp) expression is shown in Figure 2.

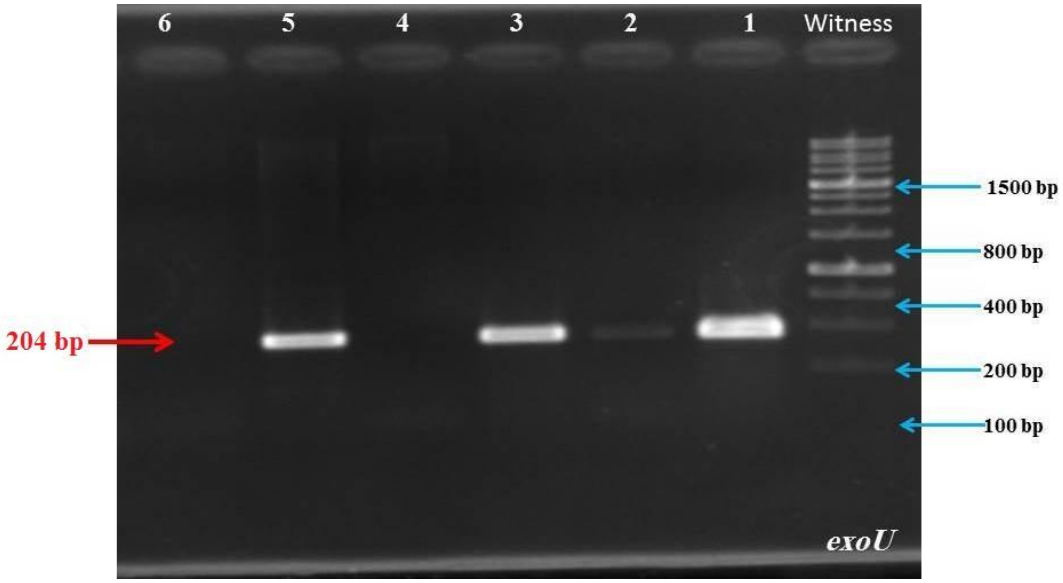

Figure1. Gel Electrophoresis of PCR products following amplification with specific primers for *exoU* gene (204bp).

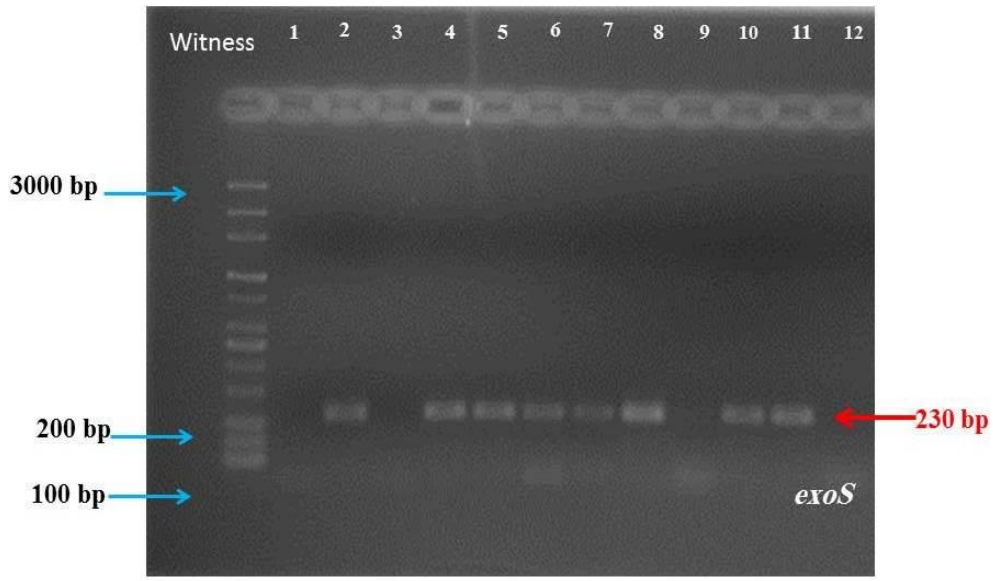

Figure 2. After Electrophores an observation studied *exoS* gene (230bp) on a 1% agarose gel.

Also, all strains were isolated from burn infection patients formed biofilms. Five days of biofilm structure, with vertical growth and the formation of water channels. The image of the *P.aeruginosa* biofilm after 6, 24, 48, and 72 hour after the painting were photographed [Figure 3]. Prevalence of *exoU* and *exoS*genes in patients with burn infections caused by heat, shown in Figure 5.Four different modes (*exoU*+ & *exoS*+, *exoU*+ & *exoS*-, *exoU*- & *exoS*+, *exoU*- & *exoS*-) were examined. In 54% of patients in both *exoU* and *exoS* gene expression have been, In contrast, 10% of burn infections caused by bacteria, the expression of both *exoU* and *exoS* genes are not shown. 22% of infections have shown only *exoU* gene expression, In contrast, *exoS* genes have been shown to burn 14% of the infections.

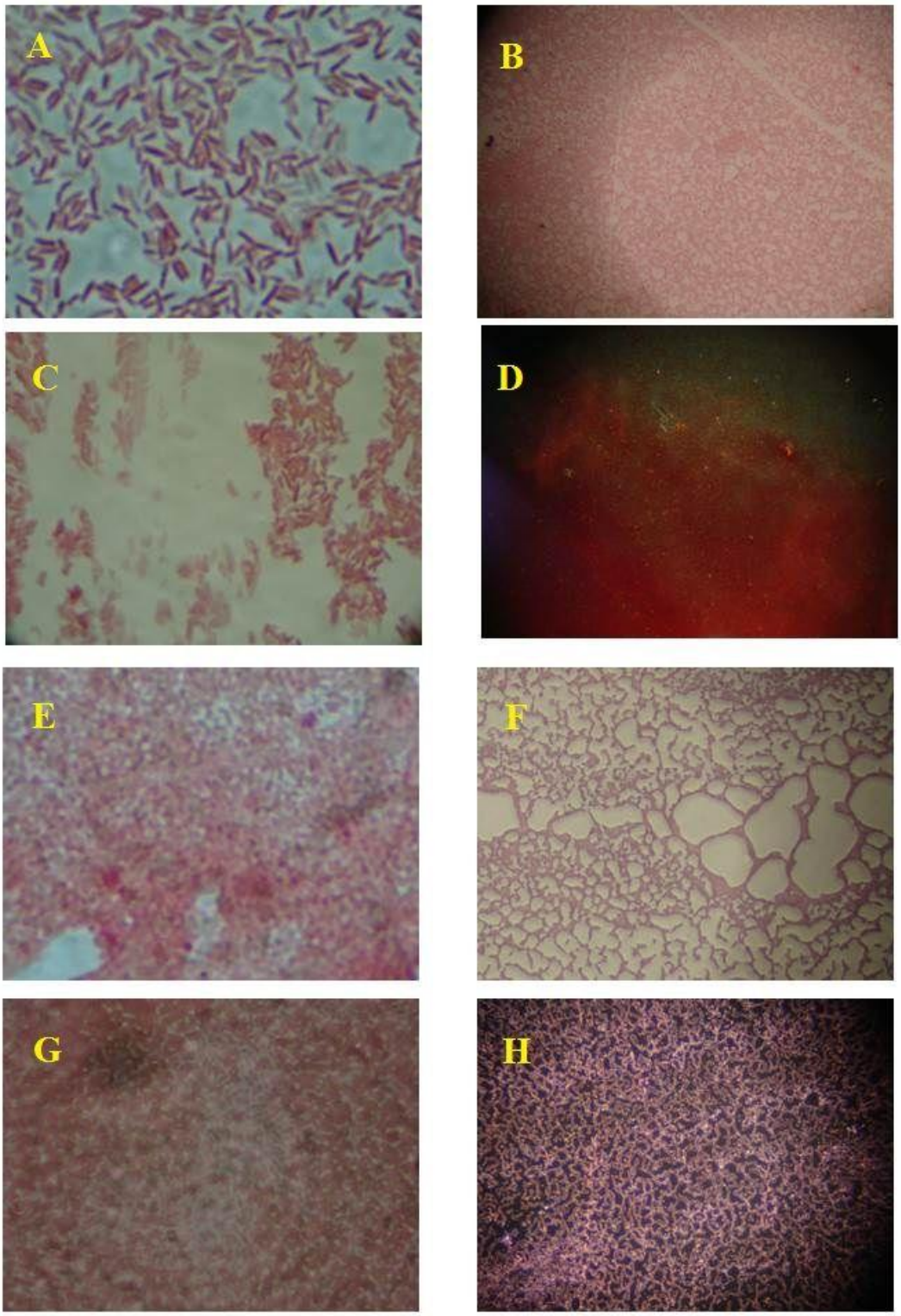

Figure 3. Biofilms formed by *P.aeruginosa* at different times,
A: 6- hour biofilms by optical microscope. **B:** 6- hour biofilms by phase microscopy.
C: 24- hour biofilms by optical microscope. **D:** 24- hour biofilms by phase microscopy.
E: 48- hour biofilms by optical microscope. **F:** 48- hour biofilms by phase microscopy.
G: 72- hour biofilms by optical microscope. **H:** 72- hour biofilms by phase microscopy

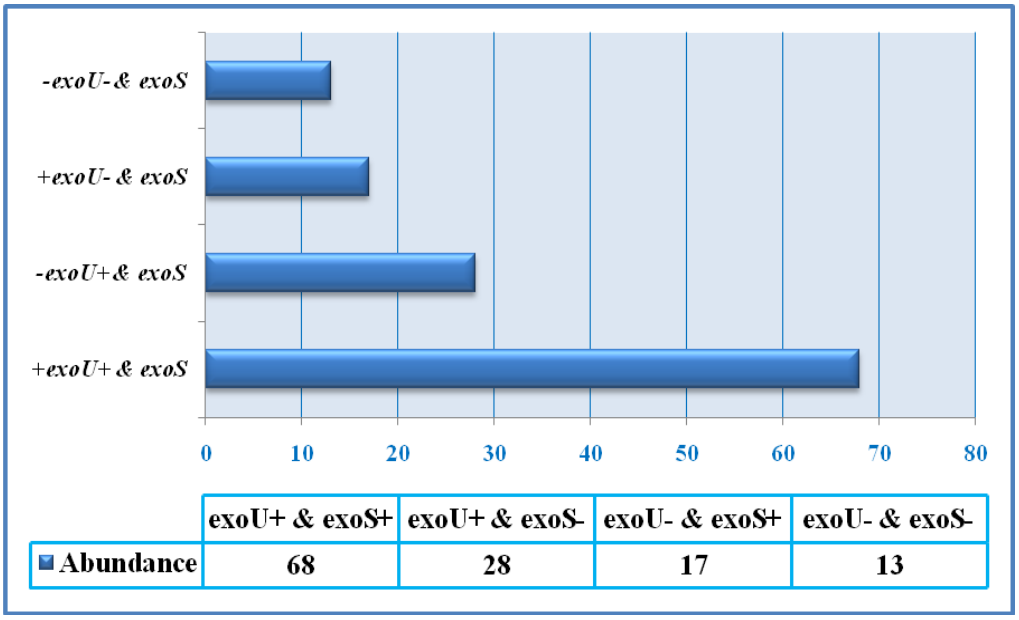

Figure4.prevalence of genes *exoS*, *exoU* infections in patients with burns

DISCUSSION

In Iran and most country of the developing, *P.aeruginosa* is the major risk factor underlying bacterium infection. We mentioned in the previous section *P.aeruginosa* was the most common nosocomial infection in the Motahary Hospital. In a study conducted by Guggenheim et al in 2009 [18], Mohammadzadeh et al in 2013 [19], Yah et al in 2006 [20], and Agnihotri et al in 2004 [21], which was done on burn wound samples, all of them reported that *P.aeruginosa* as the most common bacteria isolated from burn infections and it was consistent with the present study. In this study frequency of *exoU* gene in *P.aeruginosa* isolated from burn patients in Motahary Hospital was 76% a head bands of this gene. In a study done by Mitove et al on 202 samples in 2010 reported 30.2 % frequency of this gene [22]. The result of the present study was similar to their study of frequency of *exoU* gene. The frequency of *exoU* gene in *P.aeruginosa* from burnt patients was 76%. In a studied by Bradbury et al in 2010 was conducted in Australia on 184 clinical and environmental samples, 18% of the patients had *exoU* gene. The difference may be due to the differences of isolated strains [23].

In addition, the frequency of *exoS* gene isolated from burn infection was 68%. In a studied by Mitov et al conducted in 2010, on 202 clinical samples, the frequency of the *exoS* gene was reported 62.4% [22]. The result of their study was similar to the present study. Since most of the samples of burnt patients had *exoU* gene. It indicates that, this gene has an important role in bacterial infection, but it is not essential because some of the samples had not this gene. In another study done by Idrisetalin 2012, on 44 clinical samples [24], Win Stanley et al in 2005 of 63 clinical samples [25], they reported the frequency of *exoS* gene 54.54%, 38% respectively. The results of their study were different from our study, this difference may be due to the dissimilarity strains of bacterium or the disparity years. Our findings verify an association between secretion of *exoU* and high levels of cytotoxicity. These results indicate that *exoU* is a predominant cytotoxin of *P.aeruginosa*.

Despite reports of *exoS*-associated cytotoxic activity, *exoS*-secreting isolates were no more cytotoxic than non-secreting isolates, under the conditions of our assays. *Exos* mediated cytotoxicity has been reported to require longer coincubation times than *exoU*-mediated cytotoxicity [26]. *P.aeruginosa* develops resistance to the fluroquinolones primarily from acquiring target site mutations or overexpression of multidrug efflux pumps from the resistance-nodulation-division (RND) family. Confirming our previously published findings, Poole et al showed that the fluoroquinolone-resistant subpopulation is predominated by *exoU*+ strains in a large sample of respiratory isolates, despite the higher prevalence of *exoS*+ strains overall. This may be related to our observation that *exoU*+ strains

more readily acquire multiple target site mutations and at a lower Minimal Inhibitory Concentration (MIC) than *exoS*+ strains [27].
In addition, all of the *P.aeruginosa* isolated from burn and urinary patients formed biofilms. The samples which were isolated from patients with urinary tract infections formed poor biofilms in comparison to burn patients. Since *exoU* gene involved in biofilm formation and even the frequency of this genes were low in urinary tract infections, consequently, the quantity of biofilm formation in these patients was not high.
Hence, one reason for the increase of mortality among burn patient is the speed and the quality of biofilm formation in these patients. The study done by Choy et al in 2008, reported that clinical samples of *P.aeruginosa* could form biofilms, and the samples having *exoU* gene formed better biofilms. The results of our study were similar to theirs.

The study done by Zaborina et al on clinical samples in 2006, indicated that *exoU* gene involved in biofilm formation in isolated cases. The results of the present study were consistent with them [28, 29].

CONCLUSION

The results of this study showed, *P.aeruginosa* bacteria isolated from burn patients established better biofilms. That is perhaps one reason for the high mortality in burn patients. Indeed, it is suggested to find ways to prevent *exoU* gene activity to prevent biofilm formation, especially in burn patients with high mortality.
It is likely for *exoS* gene to play an important role in the infections caused by *P.aeruginosa*. Due to the advantages of molecular methods in diagnosis of opportunistic pathogenic bacteria, it could be convenient and swift technique to prevent the progress of infections and mortality among these patients.

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