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Original Article

The Prevalence of Virulence Factors in Human and Environmental Isolates of *Pseudomonas aeruginosa*

Sayyad Gholami, Mohammad Tabatabaei*

Faculty of Veterinary Medicine, Shiraz University, Shiraz, Iran

***Corresponding author:**
Mohammad Tabatabaei,
Address: School of Veterinary Medicine, Shiraz University, Shiraz, Iran.
Email:
Tabatabaie@shirazu.ac.ir

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Abstract

Background: *Pseudomonas aeruginosa* as a gram-negative bacterium can be detected all around us, as it is generally distributed in different environments and can act as a probable cause of human diseases especially in immunocompromised patients. This bacterium also is a leading cause of nosocomial infections in burn intensive care units. The aim of this study was to evaluate the presence of some virulence genes in *P. aeruginosa* isolated from different sources.

Methods: *Pseudomonas aeruginosa* isolates were evaluated for the presence of virulence factors including *toxA, phzM, lasB, exoU, exoS* genes by polymerase chain reaction (PCR).

Results: According to our findings, the prevalence of *toxA, phzM, lasB, exoU,* and *exoS* virulence genes in the isolates originated from human sources was 86%, 16%, 94%, 100%, and 78%, respectively. While, the above-mentioned virulence factors were present in 46%, 4%, 68%, 64%, and 62% of environmental samples, respectively.

Conclusions: Findings of this study indicated that the presence of virulence genes in human isolates was greater than their presence in environmental isolates. Hence, it seems this difference may have caused infections in humans.

Keywords: *Pseudomonas aeruginosa*, Virulence genes, Environmental isolates, Polymerase chain reaction

Background

Pseudomonas aeruginosa, as a gram-negative bacterium, has a wide environmental distribution. It can occasionally cause human diseases especially in patients with hidden diseases such as cystic fibrosis (CF) (1). This bacterium also is one of the most significant causes of nosocomial infections in burn intensive care units (2).

Pseudomonas aeruginosa harbors different virulence genes including *elastase, sialidase, exoenzyme S* (*exoS*), *exotoxin A* (*ETA*), and *exoenzyme U* (*exoU*), which are strongly checked by cell-to-cell signaling systems (3). ExoS and ETA cause a defect in elongation factor 2 (EF2) that in turn contributes to the protein biosynthesis (4). These enzymes are secreted by a type III secretion system (T3SS) and exhibit an ADP-ribosyltransferase activity (5,6). ExoS has a potent phospholipase and GTPase activity that causes the rapid lysis of host cells (7,8). ExoU is encoded by *exoU* gene and has been shown to be over 100 times more toxic than ExoS (9). *P. aeruginosa* strains lack the *exoU* gene, thereby limiting the toxicity to the lungs (10,11). On the other hand, ExoU has been implicated as an agent associated with mortality in pneumonia and septic shock (12,13). A relation between *exoU* and invasive diseases has been proposed that causes bloodstream infections (14).

Zinc metalloprotease does an elastolytic activity on the lung tissue. Accordingly, pulmonary elastase can be effective on different basic materials such as components of connective tissue including laminin, elastin, fibronectin, and collagen (15).

Pseudomonas aeruginosa secretes the precursor of 3 phenazine compounds including pyocyanin, 1-hydroxyphenazine, and phenazine-1-carboxamide (16,17). Phenazines induce intracellular oxidative stress through the intracellular redox cycling of oxygen and reducing the agents through the production of superoxide and hydrogen peroxide (17). Phenazines also promote the growth and survival of *P. aeruginosa* in the lung tissue of CF patients (17).

Objectives

This study aimed to evaluate the presence of different virulence factors including *toxA, phzM, lasB, exoU,* and *exoS* genes in *P. aeruginosa* strains isolated from different human and environmental sources by polymerase chain reaction (PCR) method.

Methods

Identification of *Pseudomonas aeruginosa* Isolates

In this study, a total of 100 *P. aeruginosa* isolates, which had been prepared and recognized for a previous published

article, were used (18). All the human samples (27, 13, and 10 samples isolated from respiratory and urinary tract infections and burns, respectively) and environmental samples (32 and 18 samples from soil and surface and spring waters, respectively) collected from February to September 2016 were cultured on Pseudomonas Cetrimide Agar (PCA) (Merck, Germany). *P. aeruginosa* isolates were identified according to the general bacteriological and biochemical characteristics. Then isolated bacteria were inoculated into TSB containing 25% glycerol and stored at -70°C.

DNA Purification and PCR Conditions

From overnight cultures of *P. aeruginosa* isolates on blood agar plates, a few colonies were picked up and chromosomal DNA was extracted based on the manufacturers' instructions (Cinnagen, Iran). Quantity and quality of extracted DNA were checked using Nanodrop (10000 V 3.52). Then *16S rRNA*, *toxA, phzM, lasB, exoU,* and *exoS* genes were amplified by PCR method using different primer sets (Table 1). Amplification was done by an automated thermal cycler (Technet tc512, England) according to PCR cycling procedure (Table 1).

For preparation of a 25 µL PCR reaction mixture, 2.5 µL PCR buffer, 1.5 µL $MgCl_2$, 1.5 µL deoxyribonucleotide triphosphate mixture, 1.5 µL forward and reverse primers, 0.5 U *Taq* DNA polymerase, 14.5 µL DDH_2O, and 2 µL extracted genomic DNA were mixed. Then PCR procedure was followed as: initial denaturation at 96°C for 5 minutes, 30 cycles of PCR amplification including denaturation at 94°C for 40 seconds, annealing (according to temperatures determined in Table 1), and extension at 72°C for 40 seconds, and a final extension at 72°C for 7 minutes. The cycling procedure was repeated in a Block assembly 96G thermocycler (Analytic Jena, Germany). For prevention of contamination with extraneous nucleic acids, the extraction of genomic DNA, preparation of reaction mixture, and PCR amplification were done in different isolated areas.

Agarose gel electrophoresis was employed for the evaluation of the PCR products. To this end, PCR amplicons were assessed by mixing the PCR products and loading dye (8 and 2 µL, respectively) in wells of 1.5% agarose gel (Cinnagen, Iran) including safe stain immersed in 0.5×TBE buffer with a voltage difference of 5 v/cm. Moreover, PCR products were detected using a 100 bp DNA ladder (Cinnagen, Iran). Finally, PCR amplicons were visualized in and images were recorded by a GelDoc UV gel documentation system (AlphaEase; Alpha Innotech, Genetic Technologies, Inc. Miami).

Statistical Analysis

SPSS software version 18.0 was employed for analysis of descriptive statistics including frequencies, and cross-tabulation of microbiological data. A *P* value less than 0.05 was considered statistically significant.

Results

Identification of *Pseudomonas aeruginosa* Isolates

According to general bacteriological and physiological characteristics, 52 and 55 isolates of *P. aeruginosa* were confirmed, respectively, from different human and environmental samples. The PCR results for amplification of *16s rRNA* genes are shown in Figure 1.

Detection of Virulence Genes

The most and the lest frequent virulence genes in human isolates were *exoU* (100%) and *phzM* (16%), but in environmental samples were *lasB* (68%) and *phzM* (4%), respectively (Table 2). The results of amplification of some virulence genes in *P. aeruginosa* isolates are shown in Figures 2-6.

Discussion

In pneumonia associated with ventilator devices, *P. aeruginosa* can be the main cause of infection with high mortality rate (4). In the Europe and the United States, it acts as a general cause of infection in the urinary tract (22,23).

Pseudomonas aeruginosa is responsible for pulmonary infections, urinary tract infections (UTIs) (24), CF (25), infections particularly in burns patients, bed ulcers, and

Table 1. Oligonucleotide Primers and Preferred PCR Conditions (16, 20, 21)

Genes	5′ to 3′ Sequences	Annealing	Amplicon size (bp)
16s rRNA	F: GGGGGATCTTCGGACCTCA R: TCCTTAGAGCTGCCACCCG	58 °C, 30 s	956
phzM	F: ATGGAGAGCGGGATCGACAG R: ATGCGGGTTTCCATCGGCAG	54°C, 1 min	875
lasB	F: GGAATGAACGAAGCGTTCTCCGAC R: TGGCGTCGACGAACACCTCG	55°C, 1 min	284
exoU	F: CCAACACATTAGCAGCGAGA R: TGGGAGTACATTGAGCAGCA	58°C, 30 s	94
exoS	F: CGTCGTGTTCAAGCAGATGGTGCTG R: CCGAACCGCTTCACCAGGC	55°C, 1 min	444
toxA	F: GACAACGCCCTCAGCATCACCAGC R: CGCTGGCCCATTCGCTCCAGCGCT	68°C, 1 min	369

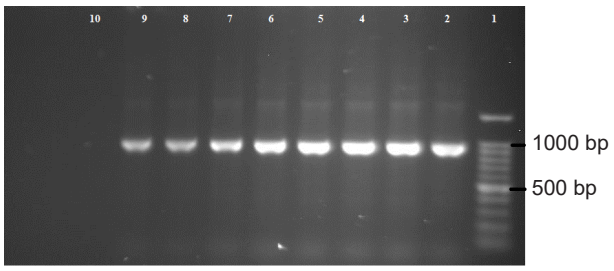

Figure 1. Electrophoresis of PCR Products for *16S rRNA* Gene of *Pseudomonas aeruginosa* Isolates in Agarose Gel. Lane 1: 100 bp DNA molecular weight marker; Lanes 2-8: Positive samples of *16S rRNA* gene; Lanes 9 and 10: Positive and negative controls, respectively.

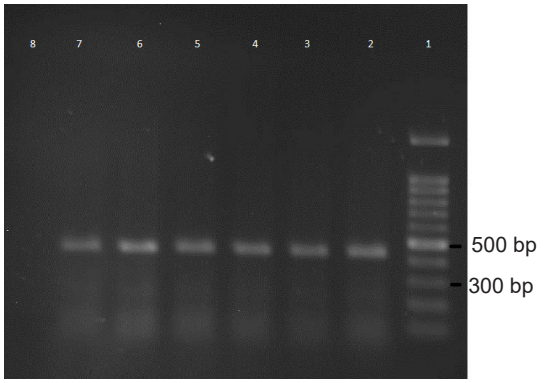

Figure 2. Electrophoresis of PCR Products for *exoS* Gene of *Pseudomonas aeruginosa* Isolates in Agarose Gel. Lane 1: 100 bp DNA molecular weight marker; Lanes 2-6: Isolates positive for *exoS* gene; Lanes 7 and 8: Positive and negative controls, respectively.

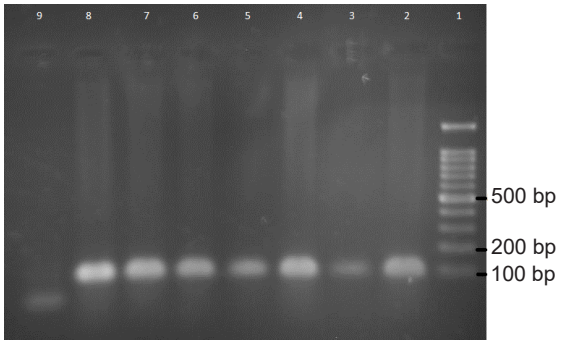

Figure 3. Electrophoresis of PCR Products for *exoU* Gene of *Pseudomonas aeruginosa* Isolates in Agarose Gel. Lane 1: 100 bp DNA molecular weight marker; Lanes 2-7: Isolates positive for *exoU* gene; Lanes 8 and 9: Positive and negative controls, respectively.

infections in immunocompromised patients such as cancer or AIDS (26). Based on reports from different studies for the identification of *P. aeruginosa*, molecular procedures were regarded well than general bacteriological methods (27). The strains of *P. aeruginosa* can be identified by phenotypic characteristics with the inclusion of bacteriological and physiological traits and some professional procedures such as *16s rRNA* sequencing for the confirmation of species type.

According to the results of this study, 36.18% of human samples and 10.54% of environmental samples contained *P. aeruginosa* based on routine bacteriological methods. Moreover, by PCR amplification of *16S rRNA* gene, 90.9% of human isolates and 96.15% of environmental isolates were confirmed as *P. aeruginosa.*

The frequency of *P. aeruginosa* isolated from human sources in current research was similar to the reports from India (29.6%) (28), Georgia (31.5%) (29), Norway, Sweden (25.8% to 45.9%) (30), Turkey (16.4%) (31), and Brazil (37.3%) (32). In the study of Shi et al on 201 environmental samples, 56% of them were positive for presence of *P. aeruginosa* (19).

Different pathogenic traits of *P. aeruginosa* has helped

Table 2. The Comparative Analysis of Virulence Genes in *Pseudomonas aeruginosa* Isolates

Genes	Human Isolates (%)	Environmental Isolates (%)	*P* Value
exoS	78%	62%	< 0.05
exoU	100%	64%	< 0.05
lasB	94%	68%	< 0.05
phzM	16%	4%	< 0.05
toxA	86%	46%	< 0.05

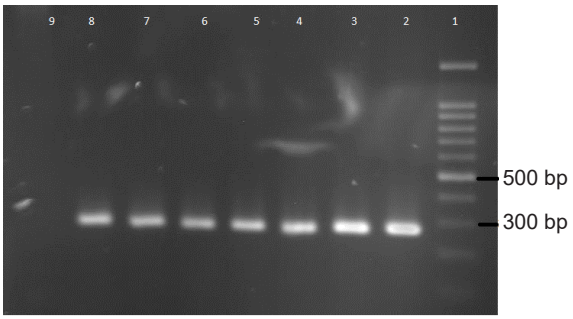

Figure 4. Electrophoresis of PCR Products for *lasB* Gene of *Pseudomonas aeruginosa* Isolates in Agarose Gel. Lane 1: 100 bp DNA molecular weight marker; Lanes 2-7: Isolates positive for *lasB* gene; Lanes 8 and 9: Positive and negative controls, respectively.

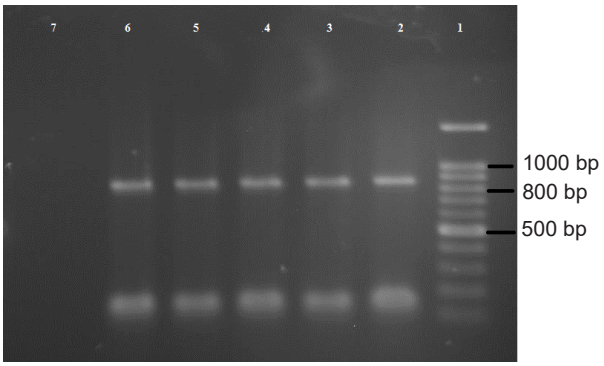

Figure 5. Electrophoresis of PCR Products for *phzM* Gene of *Pseudomonas aeruginosa* Isolates in Agarose Gel. Lane 1: 100 bp DNA molecular weight marker; Lanes 2-5: Isolates positive for *phzM* gene; Lanes 6 and 7: Positive and negative controls, respectively.

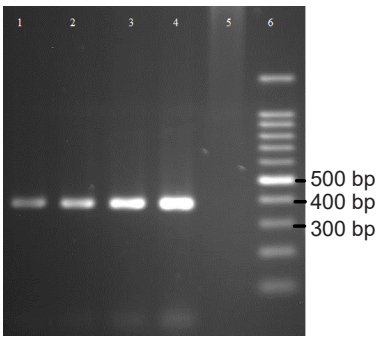

Figure 6. Electrophoresis of PCR Products for *toxA* Gene of *Pseudomonas aeruginosa* Isolates in Agarose Gel. Lanes 1-3: Isolates positive for *toxA* gene; Lanes 4 and 5: Positive and negative controls, respectively; Lane 6: 100 bp DNA molecular weight marker.

it to establish in different niches and cause infection. Adhesions, enzymes, and toxins are these virulence factors. *P. aeruginosa* exotoxins mainly consist of exotoxin A, which is an important ADP ribosyltransferase toxin (8). In our study, 86% of human isolates of *P. aeruginosa* and 46% of environmental isolates had *toxA* gene. The prevalence of this gene was significantly different between the isolates from two sources ($P < 0.05$). The study of Qin et al. displayed that 93.7% of *P. aeruginosa* isolated from CF patients contained *toxA* gene (27). Lavenir et al also reported that 55 (95%) out of 59 isolates of *P. aeruginosa* from various sources harbored *toxA* gene (33). In addition, Shi et al. evaluated 201 isolates of *P. aeruginosa* from drinking water, soil, and river, and found that 93% of them had *toxA* gene (19). Difference in the frequency of the *toxA* gene in this study and that in previous research in environmental samples is attributed to the difference in the resources from which the samples were taken.

The maximal phospholipase activity of ExoS causes the rapid lysis of the host cells (7). ExoS has ADP ribosyltransferase (ADPR) activity, along with its role as a GTPase activating protein (8). In this study, 78% of human isolates and 62% of environmental isolates had *exoS* gene; the difference between two sources in representing the *exoS* gene was statistically significant ($P < 0.05$). Feltman et al demonstrated that 60% of environmental and human isolates of *P. aeruginosa* had *exoS* (34). Lomholt et al studied the isolates of *P. aeruginosa* originated from various samples and showed that all of them (100%) had *exoS* gene (35). In another study, Lanotte et al revealed that 64.7% of isolates were positive for *exoS* gene (36). These differences in the frequency of *exoS* gene could be related to the difference in the sources from which the bacteria were isolated.

ExoS and ExoU greatly contribute to pathogenesis and cause great concerns (10). Nonetheless, these proteins are not usually secreted by the same strain (10,37). In our study, 100% of human isolates and 64% of environmental isolates harbored *exoU* gene, with a statistically significant difference ($P < 0.05$). Strateva et al reported that the prevalence of *exoU* virulence gene in the samples isolated from nosocomial infections was 45% (38). In the study conducted by Shi et al, 9% of 201 environmental isolates had *exoU* gene (19). The difference in the results is tagged to the differences in the sampling and geographical area.

The production of different proteases such as alkaline protease LasA and LasB, and membrane protease and protease IV by *P. aeruginosa* has been reported in various studies (39,40). In our study, 94% of human isolates and 68% of environmental isolates had *lasB* gene, and the difference between the sources in presenting this virulence gene was statistically significant ($P < 0.05$). In the study conducted by Shi et al, 80% of 201 environmental isolates had *lasB* gene (19). When Fazeli and Momtaz evaluated 102 human isolates of *P. aeruginosa,* 18 strains (17.6%) contained *lasB* gene (20). Difference between the results of our study and those of others could be linked to differences in the sampling field.

Human pathogenic *P. aeruginosa* along with other pseudomonads produce some important and biologically attractive pigmented secondary metabolites named pyocyanins. Thus, produced phenazines and pyocyanins by *P. aeruginosa* and other pseudomonads can act as virulence factors (41). In our study, 16% of human isolates and 4% of environmental isolates were positive for *phzM* gene, which was a significant difference ($P < 0.05$). The study of Shi et al on 201 environmental isolates of *P. aeruginosa* demonstrated that 84% of them harbored *phzM* gene (19). While, the study of Fazeli and Momtaz showed the prevalence of *phzM* gene as 36.2% in human isolates of *P. aeruginosa* (20).

Conclusions

Results of our study showed that *P. aeruginosa* isolated from environmental samples harbored less frequency of virulence factors compared to the human isolates. These dissimilarities may be because of the presence of mutagenic factors in bacteria, improper use of disinfectants and detergents, unsuitable use of medication in human infections, and unrestrained use of drugs in animals and poultry in today's industrial life.

Ethical Approval

Not applicable.

Conflict of Interest Disclosures

None.

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