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Original Article

Significant Role of Pyocyanin and Exotoxin A in the Pathogenesis of *Pseudomonas aeruginosa* Isolated from Hospitalized Patients

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Abstract

Aim: Due to the importance of exotoxin A and pyocyanin in the pathogenicity of this bacterium, we decided to evaluate the prevalence of genes encoding these virulence factors in clinical isolates of *P. aeruginosa*.

Materials and methods: In this study, 100 clinical isolates were collected and identified by conventional biochemical tests. The ability to produce pyocyanin was determined by culture in a specific liquid medium (GSNB) at 37°C. The DNAs of the bacteria were extracted by the SDS method and a PCR test was performed to identify the *exoA*, *phzA1B1C1D1E1F1G1*, *phzM*, and *phzS* genes.

Results: In this study, 90 isolates were pyocyanin-producing in phenotypic test. Also, 96%, 98%, 92%, and 96% of the isolates carried *exoA*, *phzM*, *phzS*, and *phzA1B1C1D1E1F1G1* genes, respectively. Interestingly, 2 isolates lacking the *exoA* gene did not have the *phzM* and *phzS* genes at the same time, but the other 2 isolates carrying all three gene loci required for pyocyanin synthesis. Also, among the strains that lacked the ability to produce pyocyanin, 2 isolates concurrently lacked the *phzS* and *phzA1B1C1D1E1F1G1* genes and 2 isolates simultaneously lacked the *phzM* and *phzS* genes, while the 2 isolates did not carry only the *phzS* gene. The other 2 isolates did not contain only *phzA1B1C1D1E1F1G1* operon.

Conclusion: The presence of the *phzA1B1C1D1E1F1G1* operon, along with the *phzM* and *phzS* genes, plays a significant role in the production of pyocyanin pigment. However, the high prevalence of *exoA* gene in this study indicates the importance of this factor for vaccine designing.

Keywords

exoA, Pseudomonas aeruginosa, *phzM*, *phzS*, *phzA1B1C1D1E1F1G1*

INTRODUCTION

Pseudomonas aeruginosa is an important cause of health care-related infections and high mortality, especially in patients with underlying diseases.[1] Due to the production of various virulence factors and antibiotic resistance, this organism is classified as a critical pathogen by the World Health Organization.[1-3] *P. aeruginosa* is distinguished by the production of various pigments, with more than 90% of the strains producing pyocyanin, which corresponds to

a slower growth rate.[4] Also, the exotoxin A (ETA), related to the bacterial pathogenesis in burn and wound infections, corneal ulcers, and keratitis, could be produced by more than 90% of the *P. aeruginosa* isolates.[4]

Water-soluble pyocyanin (phenazine) is expressed only by *P. aeruginosa*.[5] This phenazine damages several enzymes and DNA due to increased reactive oxygen species (ROS), leading to cell lysis, severity of disease, and decreased lung function.[6] In addition, it slows the respiratory cilia movement, disrupts the function of epithelial cells, increases the secretion of respiratory mucus and colonization of bacteria, and increases the inflammation.[6] Phenazine production in *P. aeruginosa* is controlled by the QS system which stimulates the operon *phzA1B1C1D1E1F1G1* to produce phenazine-1-carboxylic acid (PCA), which is converted to 5-methylphenazine-1-carboxylic acid betaine by *phzM* and then to pyocyanin by *phzS* gene product.[7,8]

On the other hand, the most toxic virulence factor of *P. aeruginosa* is exotoxin A, an ADP-ribosyltransferase.[9] ETA inhibits the host protein synthesis and IL-18 secretion, reduces the production of TNF-α, IL-6, IL-8, and IL-10, and induces the apoptosis of the host cell.[3,9,10]

P. aeruginosa is one of the most important causes of nosocomial and community-acquired infections with high morbidity and mortality. The high pathogenicity of this organism is due to the abundance of virulence factors, significant metabolic flexibility, and the adaptation to various harsh conditions.[1] Identification of the specific virulence factors in the pathogenesis of this bacterium can help design a vaccine and specific infection control measures in each region.

AIM

In this study, we aimed to investigate the prevalence of genes encoding exotoxin A (*exoA*) and pyocyanin (*phzA1B1C1D1E1F1G1*, *phzM*, and *phzS*) in *P. aeruginosa* clinical isolates.

MATERIALS AND METHODS

Samples, isolation and identification of bacteria

A total of 100 non-duplicated *P. aeruginosa* were included in this descriptive analytical study. The isolates were collected from 5 therapeutic and educational hospitals (Imam Khomeini, BuAli Sina, Razi, Zare, and Fatemeh Zahra) of Mazandaran, North Iran, during 2020 to 2021. The bacterial isolates were collected from urine, sputum, catheter, wound, stool, blood, and eye secretion. *P. aeruginosa* clinical isolates were identified using the standard microbiological and biochemical tests. The tests were as follows: observation of the gram-negative bacilli in Gram staining, positive oxidase test, growth on cetrimide agar and MacConkey agar (as non-fermenter) (Condalab, Spain), non-fermentation reaction on triple sugar iron agar (Condalab), utilization of the citrate as a carbon source (Condalab), positive motility, positive glucose and negative lactose oxidation/fermentation test (Condalab), colony morphology and odor, pigment production in Mueller Hinton agar (Condalab), and growth at 42°C.[11] These tests were done two times. First, the bacteria were identified in the hospital laboratory and then the final identification was carried out in the microbiology laboratory of the medical school. The bacteria were then frozen at −20°C in trypticase soy broth (TSB) containing 10% glycerol (Merck, Germany) until used. The isolates were stored for two weeks and then used for the following tests.

Evaluation of the ability of clinical isolates to produce pyocyanin pigment

To enhance the production of pyocyanin, the bacteria were cultured in 5 ml of glycerol supplemented nutrient broth (GSNB).[12] Then, the samples were incubated for 96 hours in a 150-rpm shaker incubator at 37°C, and the green-blue dye production was considered as a positive result for pyocyanin production.[13] We used the PAO1 *P. aeruginosa* and *Acinetobacter baumannii* ATCC 19606 as the positive and negative control strains, respectively.

Bacterial DNA extraction

The bacterial genomic DNAs were extracted by alkaline lysis method.[14] We used 0.5 g of SDS (Sigma, Germany) and 0.4 g of NaOH (Sigma) in 200 µl of sterile distilled water as an extraction buffer. Then, 4-6 colonies of the bacteria were dissolved in 20 µl of this solution. Next, the microtubes were placed at 95°C for 10 minutes and then were centrifuged for 3 minutes at 13000 g. Finally, 180 µl of sterile distilled water was added to the microtubes containing the extracted DNAs. After assessing the quality of DNAs by determining the optical density (OD) using a NanoDrop (ND1000, USA) and electrophoresis on 1.5% agarose gel (Wizbiosolutions, South Korea), the extracted DNAs were frozen at -20°C until used. The extracted DNAs were used for polymerase chain reaction after two weeks.

Polymerase chain reaction for detection of pyocyanin and exotoxin A encoding genes

The presence of pyocyanin and exotoxin A encoding genes was detected using specific primers **(Table 1)**. The PCR reactions were carried out in a final volume of 15 µl containing 7.5 µl of Master Mix (Ampliqon, Denmark), 300 ng of the extracted DNA for *exoA*, *phzM*, and *phzS*, and 600 ng for *phzA1B1C1D1E1F1G1*, 5 pmol of each primer for *exoA*, *phzM*, and *phzS*, and 10 pmol for *phzA1B1C1D1E1F1G1*, and added distilled water to the final volume. The Thermal Cycler (BioRad, USA) running conditions in 34 cycles are shown in **Table 1**. Then, the PCR products were observed

Table 1. Primers used for detection of pyocyanin and exotoxin A encoding genes by PCR

Target genes	Primer sequences (5‘ to 3‘)	PCR product size (bp)	Denaturation	Annealing	Extension	No. in References
			temperature (°C) and time (sec)			
exoA	GGTAACCAGCTCAGCCACAT TGATGTCCAGGTCATGCTTC	352	94°C for 20 sec	53°C for 20 sec	72°C for 25 sec	[15]
phzM	AACTCCTCGCCGTAGAAC ATAATTCGAATCTTGCTGCT	313	94°C for 20 sec	53°C for 20 sec	72°C for 25 sec	[16]
phzS	TGCGCTACATCGACCAGAG CGGGTACTGCAGGATCAACT	664	94°C for 20 sec	63°C for 20 sec	72°C for 25 sec	[16]
phzA1B1C1D1E1F1G1	CCGTCGAGAAGTACATGAAT CATAGTTCACCCCTTCCAG	448	94°C for 20 sec	53°C for 20 sec	72°C for 25 sec	[16]

by electrophoresis on 1% agarose gel (Wizbiosolutiotions, South Korea) containing 1% safe stain (SinaClon, Iran).

Statistical analysis

The data were entered into SPSS v. 22, and the desired results were statistically analyzed using Pearson's chi-square test. However, *P*-values <0.05 were considered statistically significant.

RESULTS

Samples and clinical isolates

One hundred non-repetitive *P. aeruginosa* isolates were collected in the present study. The isolates were obtained from non-repetitive patients hospitalized in Imam Khomeini Hospital (n=40), Razi Hospital (n=22), Bu-Ali Sina Hospital (n=17), Zare Hospital (n=11), and Fatemeh Al-Zahra Hospital (n=10) in Northern Iran. However, 60 isolates were obtained from men (mean age, 44.76 years) and the rest were obtained from women (mean age, 47.85 years). Considering the clinical specimens, the isolates were obtained from respiratory samples (n=37), urine (n=26), wounds (n=20), catheters (n=8), blood (n=5), stool (n=2), and ocular discharge (n=2). In terms of hospital wards, the isolates were collected from an intensive care unit (ICU) (n=53), an emergency unit (n=13), a burn unit (n=6), an operating room and surgery unit (n=6), a cardiac care unit (CCU) (n=5), a pediatric unit (n=5), an internal unit (n=4), men (n=3), women (n=2), neurology (n=2), and oncology (n=1).

Ability to produce pyocyanin pigment by clinical isolates of *P. aeruginosa*

Out of 100 clinical isolates studied, 90 isolates had the ability to produce pyocyanin pigment in phenotypic testing. The production of pyocyanin improved with increasing incubation time, so that after 96 hours, we observed more color intensity. The results for pigment production are shown in **Fig. 1**. The ability of pyocyanin production in different clinical isolates varied in this study, while some strains produced more color intensity.

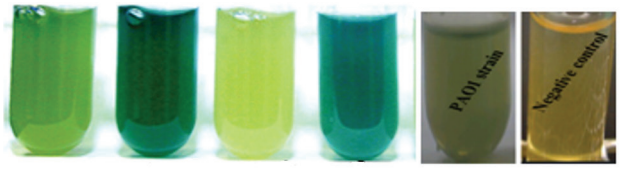

Figure 1. Pyocyanin producing clinical isolates of *Pseudomonas aeruginosa* after 96 hours.

Molecular identification of virulence genes

In the present study, 98%, 92%, 96%, and 96% of *P. aeruginosa* clinical isolates carried the *phzM*, *phzS*, *phzA1B1C1D1E1F1G1*, and *exoA* genes, respectively. **Fig. 2** shows the

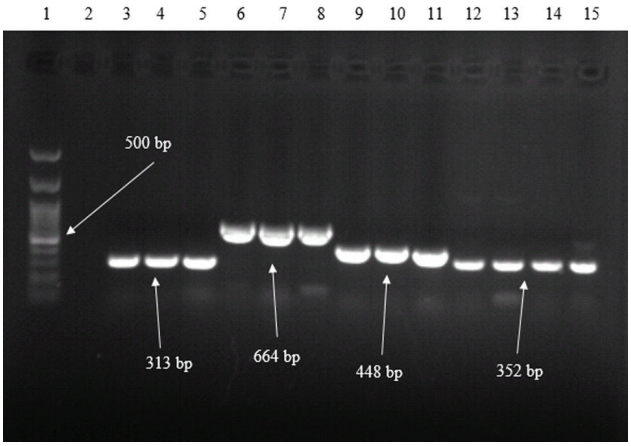

Figure 2. PCR product electrophoresis results for *phzM*, *phzS*, *phzA1B1C1D1E1F1G1*, and *exoA* genes. Line 1: DNA ladder 100 bp plus; line 2: negative control (Master Mix without DNA); lines 3, 4, and 5: clinical isolates carrying *phzM* gene; lines 6, 7, and 8: clinical isolates carrying *phzS* gene; lines 9, 10, and 11: clinical isolates carrying the *phzA1B1C1D1E1F1G1* gene; lines 12, 13, and 14: clinical isolates carrying the *exoA* gene in this study.

PCR results of studied genes identification.

Among all the isolates we studied, 4 isolates just did not have the *phzS* gene and 2 isolates did not contain *phzS* and *phzA1B1C1D1E1F1G1* genes at the same time. Also, 2 isolates did not carry the *phzA1B1C1D1E1F1G1* gene, but contained other studied genes. Moreover, 2 isolates showed negative result for *exoA* gene, while 2 isolates did not have simultaneously the *phzM*, *phzS*, and *exoA* genes. Importantly, all isolates that lacked one or more genes involved in the production of pyocyanin pigment did not produce any pigment in the phenotypic test. Out of 10 isolates that did not contain at least one of the virulence genes studied, 6 isolates were collected from the fecal samples of the patients in ICU, the remaining 3 urinary isolates were obtained from a pediatric ward (2 isolates) and emergency department (one isolate). Also, the remaining 3 isolates were obtained from ICU (wound sample), oncology (stool specimen), and surgery (catheter).

Besides, **Table 2** shows the frequency of virulence genes studied in *P. aeruginosa* clinical isolates considering the sample type. Accordingly, the isolates collected from blood, fecal, and ocular specimens carried all of the virulence genes, whereas among wound isolates, only one of them lacked the *phzS* gene, but the other isolates had all genes. Although a small number of urinary and respiratory isolates lacked some of the investigated genes, these genes were observed in the majority of isolates, and no statistically significant relationship was found between the presence of genes and the type of clinical sample.

Also, according to **Table 3**, which shows the frequency of virulence genes studied in terms of hospital ward, all isolates related to CCU, burn, internal, men, women, oncology, and neurology carried all 4 genes studied, but some isolates from other hospital wards lacked the genes studied.

Table 2. Number (%) of *Pseudomonas aeruginosa* clinical isolates containing the studied virulence genes based on clinical sample type

Samples		Isolates carrying the relevant genes based on the clinical sample type n (%)						
Genes	**PCR result**	**Urine (n=26)**	**Respiratory (n=37)**	**Wound (n=20)**	**Catheter (n=8)**	**Blood (n=5)**	**Stool (n=2)**	**Eye (n=2)**
phzM	Positive	26 (100)	36 (97.29)	20 (100)	7 (87.5)	5 (100)	2 (100)	2 (100)
	Negative	-	1 (2.7)	-	1 (12.5)	-	-	-
phzS	Positive	25 (96.15)	32 (86.48)	19 (95)	7 (87.5)	5 (100)	2 (100)	2 (100)
	Negative	1 (3.84)	5 (13.51)	1 (5)	1 (12.5)	-	-	-
phzA1B1C1D1E1F1G1	Positive	24 (92.3)	35 (94.59)	20 (100)	8 (100)	5 (100)	2 (100)	2 (100)
	Negative	2 (7.69)	2 (5.4)	-	-	-	-	-
exoA	Positive	26 (100)	34 (91.89)	20 (100)	7 (87.5)	5 (100)	2 (100)	2 (100)
	Negative	-	3 (8.1)	-	1 (12.5)	-	-	-

Table 3. Number (%) of *Pseudomonas aeruginosa* clinical isolates carrying virulence genes based on hospital ward

Hospital wards		Isolates carrying the relevant genes considering the hospital wards n (%)										
Genes	**PCR result**	**ICU (n=35)**	**Emergency (n=13)**	**Burn (n=6)**	**Surgery (n=6)**	**CCU (n=5)**	**Pediatric (n=5)**	**Internal (n=4)**	**Men (n=3)**	**Women (n=2)**	**Neurology (n=2)**	**Oncology (n=1)**
phzM	Positive	52 (98.11)	13 (100)	6 (100)	5 (83.33)	5 (100)	5 (100)	4 (100)	3 (100)	2 (100)	2 (100)	1 (100)
	Negative	1 (1.88)	-	-	1 (16.66)	-	-	-	-	-	-	-
phzS	Positive	47 (88.67)	13 (100)	6 (100)	5 (83.33)	5 (100)	4 (80)	4 (100)	3 (100)	2 (100)	2 (100)	1 (100)
	Negative	6 (11.32)	-	-	1 (16.66)	-	1 (20)	-	-	-	-	-
phzA1B1C1D1E1F1G1	Positive	51 (96.22)	12 (92.3)	6 (100)	6 (100)	5 (100)	4 (80)	4 (100)	3 (100)	2 (100)	2 (100)	1 (100)
	Negative	2 (3.77)	1 (7.69)	-	-	-	1 (20)	-	-	-	-	-
exoA	Positive	51 (96.22)	13 (100)	6 (100)	5 (83.33)	5 (100)	5 (100)	4 (100)	3 (100)	2 (100)	2 (100)	-
	Negative	2 (3.77)	-	-	1 (16.66)	-	-	-	-	-	-	1 (100)

ICU: intensive care unit; CCU: cardiac care unit

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However, there was no statistically significant relationship between the frequency of the studied genes and the hospital ward from which the clinical samples were collected.

On the other hand, **Table 4** shows the isolates carrying virulence genes based on medical centers, from which all isolates collected from Zare Hospital (burn center) and Fatemeh Al-Zahra Hospital (heart center) had all the genes studied. However, the prevalence of these genes were slightly different in other medical centers, and there was no statistically significant relationship between the frequency of virulence genes studied and the hospital which the clinical samples were collected.

DISCUSSION

Pseudomonas aeruginosa is a serious opportunistic pathogen and an infectious agent in a diverse group of organisms that is largely related to the ability of this bacterium to produce a variety of pathogens.[17] The expression of many virulence genes in *P. aeruginosa* is controlled by quorum sensing, which affects the transcription of hundreds of downstream genes.[17,18] The production of a green color phenazine called pyocyanin (PCN) by this bacterium is also linked to several QS systems and is often used as a marker to assess QS behavior.[17] PCN is a potent bacterial pigment that has five different derivatives produced by *P. aeruginosa* and has an inhibitory effect on pathogenic microbes.[19] PCN secretion has been detected in sputum, ear secretions, wounds, and urine of patients following chronic infection by *P. aeruginosa*.[20]

The biosynthesis of this phenazine begins with the induction of phenazine-1-carboxylic acid (PCA) production by the action of enzymes encoded by the conserved operon *phzA1B1C1D1E1F1G1*.[21] The PCA produced can be converted to phenazine-1-carboxamide by *phzH* and to 1-hydroxyphenazine by *phzS*. Also, the *phzM* gene product can convert PCA to 5-methylphenazine-1-carboxylic acid betaine, whereas it can be converted to PCN by *phzS*.[21,22] Inactivation of *phzM* and *phzS* genes in *P. aeruginosa* prevents the production of pyocyanin pigment by this bacterium.[7] However, we concluded in our study that the absence of any of the three genes *phzM*, *phzS*, and *phzA1B1C1D1E1F1G1* operon could lead to a lack of pyocyanin production, so that among the 100 clinical isolates studied in the present study, 10 isolates lacked the ability to produce pyocyanin. In the genetic study of these strains, we observed that all isolates, which individually or in combination did not have genes related to the production of pyocyanin pigment, did not produce any pigment in the phenotypic test.

An Iranian study conducted on 80 *P. aeruginosa* isolates collected from burn wounds, soil, and plants showed that 3 of the 48 clinical strains were unable to produce pyocyanin on cetrimide and Müller Hinton agar, while two strains lacked both phenazine-modifying genes (*phzM* and *phzS*) and the other strain lacked the *phzM* gene. Also, all 32 environmental isolates had the studied genes (*phzA1B1C1D1E1F1G1*, *phzM*, and *phzS*), and out of 48 burn wound isolates, only 2 isolates did not produce pyocyanin. On the other hand, the isolates without *phzM* gene had yellow pigment and the one without *phzS* gene also had reddish brown pigment.[16] In our study, 10 isolates were unable to produce pyocyanin, four of which lacked the phzS gene. Of these four isolates, three produced brown pigment and one produced reddish brown pigment on Müller-Hinton agar. In such isolates, the absence of the *phzS* gene prevents the production of 1-hydroxyphenazine and then pyocyanin. We also observed that the strains lacking the *phzS* and *phzA1B1C1D1E1F1G1* genes produced the yellow pigment, while the strains lacking the *phzS* and *phzM* genes produced the brown pigment. However, 2 isolates without the *phzA1B1C1D1E1F1G1* operon produced the black pigment. So, absence of any of these three genes lead to a lack of pyocyanin production, which was consistent with previous studies.[7,16,17,23] In another study published in 2020 in Brazil, a total of 54 clinical isolates of *P. aeruginosa* collected from the ICUs were evaluated, while 22 (78.5%), 12 (85.7%), and 7 (58.3%) isolates collected from ICU of

Table 4. Number (%) of *Pseudomonas aeruginosa* clinical isolates carrying virulence genes considering the hospitals of sample collection

Samples		Isolates carrying the relevant genes in different hospitals n (%)				
Genes	**PCR result**	**Imam (n=40)**	**Razi (n=22)**	**BuAli Sina (n=17)**	**Zare (n=11)**	**Fatemeh Al-Zahra (n=10)**
phzM	Positive	38 (95)	22 (100)	17 (100)	11 (100)	10 (100)
	Negative	2 (5)	-	-	-	-
phzS	Positive	36 (90)	19 (86.36)	16 (94.11)	11 (100)	10 (100)
	Negative	4 (10)	3 (13.63)	1 (5.88)	-	-
phzA1B1C1D1E1F1G1	Positive	40 (100)	20 (90.9)	15 (88.23)	11 (100)	10 (100)
	Negative	-	2 (9.09)	2 (11.76)	-	-
exoA	Positive	36 (90)	22 (100)	17 (100)	11 (100)	10 (100)
	Negative	4 (10)	-	-	-	-

adults, children, and infants contained *phzM* gene, and 20 (71.4%), 12 (85.7%), and 5 (41.6%) of the isolates carried the *phzS* gene, respectively. On the other hand, 18 (78.2%) and 8 (57.2%) isolates collected from respiratory and blood samples carried the *phzM* gene, respectively, while 17 (73.9%) and 6 (42.8%) isolates carried the *phzS* gene, respectively. Also, all isolates from rectal swab, eye, stomach, urethral secretions, and urine samples carried both the *phzM* and *phzS* genes. Besides, of 3 surgical wound isolates, 2 carried both genes and out of the 6 catheter isolates, 5 and 4 isolates carried the *phzM* and *phzS* genes, respectively.[24] Compared to the above study, in which 75.92% and 68.51% of the total isolates carried the *phzM* and *phzS* genes, respectively, 98.11% and 88.67% of the ICU isolates in our study carried the *phzM* and *phzS* genes, respectively, which indicates a higher presence of these genes in our study. Another study in Iran showed that the frequency of *phzS* gene was 27.9% and 31.2% of the isolates had 8 virulence genes simultaneously.[25] In our study, 90% of the isolates had all the genes studied simultaneously, and among the remaining 10 isolates, most of them carried at least 2 virulence genes simultaneously. In addition, the frequency of *phzI* (*phzA1B1C1D1E1F1G1*) operon in our study was 96%, which is consistent with the study of Haghi et al.[25] These isolates may belong to the PA7 group (group 3) of *P. aeruginosa* that cannot be identified by PCR method due to their high genetic variability.[26]

The pathogenesis of *P. aeruginosa* is due to the production of several cellular and extracellular virulence factors, whereas exotoxin A is a secreted extracellular enzyme encoded by the *toxA* gene that causes cell death, severe tissue damage, and necrosis in host cells.[27] According to the literature, it has been found that 56.7% of *P. aeruginosa* isolates in bacteremia cases are able to produce exotoxins and isolates collected from acute infections express more virulence factors than chronic infections.[9] In a study by Rodrigues et al.[24] in Brazil, the *toxA* gene was detected in 49 (90.74%) isolates, which was almost consistent with our research. Meanwhile, 89.2%, 92.8%, 91.6%, 91.3%, 92.8%, 83.3%, 100%, 66.6%, 100%, 100%, and 100% of the isolates collected from adults, children, and neonate ICUs, and respiratory, blood, catheter, rectal swab, surgical wound, ocular secretion, gastric secretion, and urethral secretion samples carried the *toxA* gene, respectively.[24] Also, similarly to our study, 95.7% of the isolates collected in a study conducted in southern Iran, carried the *toxA* gene.[28] This data shows the approximate similarity of the strains in terms of production of this virulence factor in the North and South of Iran, although the presence of this gene in our study was not significantly related to the type of clinical specimens. Also, in another study conducted in northwestern Iran, as in our study, the *toxA* virulence gene was identified in 97.8% of *P. aeruginosa* isolates.[25]

Zarei et al. evaluated *P. aeruginosa* isolates obtained from ICU clinical specimens, ICU environmental samples, and beetle exterior specimens, and found that the *exoA* gene was found in 57.5%, 72.5%, and 20% of them, respectively.[29] Also, in a study published in Iraq in 2017, 100% of the *P. aeruginosa* isolates collected from ICU clinical specimens and hospital environmental samples carried the *exoA* gene.[30] However, the 4% increase in the presence of the *exoA* gene compared to our study could be due to the fact that they also used hospital environmental samples, although their clinical samples confirmed the presence of 100% of this gene, too. Given that their clinical specimens were collected from the ICUs, these results indicate the higher prevalence of virulence genes in clinical isolates collected from immunocompromised patients. In other studies conducted in Pakistan, Bangladesh, and Poland, 33.33%, 55.56%, and 88.7% of *P. aeruginosa* clinical isolates, respectively, carried the gene encoding exotoxin A.[15,27,31] Due to the fact that the expression of bacterial virulence factors are different in various conditions, this can be due to the differences in bacterial clones and the high diversity of clinical strains in these studies. Interestingly, in most of the studies on the frequency of *P. aeruginosa* virulence genes, we observed a high presence of these factors in samples such as feces, wounds, and blood, which may be related to the severe conditions in these samples.

Limitations

One of the most important limitations in this study was the lack of financial resources and the small sample size for a prevalence study.

CONCLUSIONS

The high prevalence of genes encoding exotoxin A and pyocyanin in clinical isolates of *Pseudomonas aeruginosa* in Northern Iran indicates the significant role of these virulence factors in bacterial pathogenicity. Meanwhile, no significant difference has been observed in the prevalence of these genes in the isolates collected from different hospitals and different clinical samples. These results indicate that probably the source of the isolates is similar and they are spreading in the region. Therefore, maybe in the future, we can take an important step in controlling the severity of pathogenicity caused by this organism by designing compounds that can be used against these bacterial virulence factors.

Ethics

Ethical approval statement and consent to participate

We received the clinical samples without names from the laboratories of the hospitals affiliated to the Mazandaran University of Medical Sciences. This study was conducted

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in accordance with the Declaration of Helsinki; however, written informed consent form was provided by the patients or their close relatives, and any classifying information of each sample was kept secret. This study was approved by the Iran National Committee for Ethics in Biomedical Research with the national ethical code IR.MAZUMS.REC.1398.075.

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Consent for publication

The participant has agreed to the submission of this article. Also, this manuscript has neither been published nor is currently under consideration and is approved by all coauthors.

Conflict of interests

The authors declare no conflict of interest.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Author contributions

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Значимая роль пиоцианина и экзотоксина А в патогенезе *Pseudomonas aeruginosa*, выделенного от госпитализированных пациентов

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Резюме

Цель: Ввиду важности экзотоксина А и пиоцианина в патогенности этой бактерии мы решили оценить распространённость генов, кодирующих эти факторы вирулентности, в клинических изолятах *P. aeruginosa*.

Материалы и методы: В ходе исследования было собрано и идентифицировано с помощью традиционных биохимических тестов 100 клинических изолятов. Способность продуцировать пиоцианин определяли путём культивирования на специальной жидкой среде (GSNB) при 37°C. ДНК бактерий экстрагировали методом SDS и проводили PCR -тест для идентификации генов *exoA*, *phzA1B1C1D1E1F1G1*, *phzM* и *phzS*.

Результаты: В этом исследовании по фенотипическому тесту 90 изолятов продуцировали пиоцианин. Кроме того, 96%, 98%, 92% и 96% изолятов несли гены e*xoA, phzM, phzS* и *phzA1B1C1D1E1F1G1* соответственно. Интересно, что у двух изолятов, лишённых гена *exoA*, одновременно не было генов *phzM* и *phzS*, а у двух других изолятов были обнаружены все три генных локуса, необходимые для синтеза пиоцианина. Также среди штаммов, лишённых способности продуцировать пиоцианин, у 2 изолятов одновременно отсутствовали гены *phzS* и *phzA1B1C1D1E1F1G1*, а у 2 изолятов одновременно отсутствовали гены *phzM* и *phzS*, при этом 2 изолята не несли только ген *phzS*. Остальные два изолята не содержали только оперона *phzA1B1C1D1E1F1G1*.

Заключение: Наличие оперона *phzA1B1C1D1E1F1G1*, а также генов *phzM* и *phzS* играет существенную роль в продукции пигмента пиоцианина. Однако высокая распространённость гена exoA в этом исследовании указывает на важность этого фактора для разработки вакцин.

Ключевые слова

exoA, Pseudomonas aeruginosa, *phzM*, *phzS*, *phzA1B1C1D1E1F1G1*