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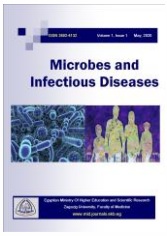

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Original article

LasB, *ExoS* and *Nan1* genes as potential predictors of site-specific *Pseudomonas aeruginosa* pathogenicity in nosocomial isolates

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ABSTRACT

Background: *Pseudomonas aeruginosa* is an opportunistic pathogen that represents a global public health threat. Immunocompromised hosts and cystic fibrosis patients are primarily susceptible to high morbidity and mortality due to pulmonary tract colonization with mucoid *Pseudomonas aeruginosa*. Differentially expressed virulence factors govern *Pseudomonas aeruginosa* pathogenicity in different sites of infection. Therefore, the detection of site-specific virulence genes in *Pseudomonas aeruginosa* isolates may potentially predict the level of *Pseudomonas aeruginosa* pathogenicity and the outcome in infected patients. **Objectives:** This study aims to phenotypically and genotypically characterize nosocomial *Pseudomonas aeruginosa* isolates from different clinical specimens to determine the frequency of *LasB*, *ExoS* and *Nan1* virulence genes in the collected isolates relative to the site and severity of *Pseudomonas aeruginosa* infection using conventional polymerase chain reaction. **Methods:** The study was carried out on 30 *Pseudomonas aeruginosa* isolates collected from hospitalized patients who have been diagnosed with respiratory tract, bloodstream or burn infection in Tanta University Hospitals. *Pseudomonas aeruginosa* isolates were phenotypically characterized using standard microbiology techniques and genotypically characterized using conventional PCR for the detection of *LasB*, *ExoS* and *Nan1* virulence genes. **Results:** Both *Nan1* and *ExoS* genes were detected in *Pseudomonas aeruginosa* isolates from 30% of respiratory and burn infections but they were undetectable in all isolates from bloodstream infections. While, *LasB* gene was detected in all types of clinical specimens (100% of respiratory specimens, 90% of pus and 90% of blood specimens). A significant correlation was found between the site, severity of infection and the presence of multiple virulence genes. **Conclusion:** Our study reveals site and severity specific detection of certain *Pseudomonas aeruginosa* virulence genes which may suggest site and severity specific pathogenesis of certain *Pseudomonas aeruginosa* strains. Our data also imply that precision targeting of specific virulence factors detected in certain infection sites and/or severity should improve therapeutic outcomes of *Pseudomonas aeruginosa* infections.

Introduction

Pseudomonas aeruginosa (P. aeruginosa) is a ubiquitously distributed opportunistic pathogen that is capable of infecting nearly all tissues. Pulmonary tract colonization with mucoid *P. aeruginosa* is a major cause of morbidity and

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mortality in immunocompromised hosts and in patients with cystic fibrosis (CF). Additionally, *P. aeruginosa* hospital-acquired infections mainly affect critical care patients, in particular, patients on assisted ventilation and burn patients [1, 2].

Various virulence factors govern *P. aeruginosa* pathogenicity, and their expression is tightly regulated via quorum sensing such as *Exotoxin A, Exoenzyme S, Elastase and sialidase* [3]. Protein biosynthesis in infected host cells is inhibited by *Exotoxin A* while a *zinc metalloprotease* called *LasB* displays an elastolytic activity on infected lung tissue [4-8].

Pseudomonas aeruginosa generally displays cytotoxic or invasion phenotype in the infected host which have been correlated with the presence of *ExoU* gene encoding *Exotoxin U* or *ExoS* gene encoding *Exotoxin S* [9]. *Nan1* gene encodes a distinct virulence factor named *sialidase* which plays an important role in adhering to the respiratory tract epithelium and has been suggested to play a role in cystic fibrosis pulmonary disease and to be more prevalent in pulmonary isolates than in burn isolates [7-11].

Pseudomonas aeruginosa owns a diversity of virulence factors that can intensify the bacterial pathogenicity and consequently the infection severity. The expression of virulence genes can provide bacteria with an evolutionary advantage across various habitats and enhance their adaptation to specific ecological niches. Additionally, multiple studies have demonstrated differential expression of certain *P. aeruginosa* genes in various infection types. Therefore, there is a potential to improve clinical outcomes in patients with *P. aeruginosa* infections through identifying those virulent strains and targeting them [12-16].

However, limited evidence is reported, particularly from Egypt, on the tissue-specific detection of *LasB*, *ExoS* and *Nan1* in *P. aeruginosa* isolates from distinct infection sites. In an attempt to address this gap, the current study aims to phenotypically and genotypically characterize nosocomial isolates of *P. aeruginosa* from different clinical specimens to determine the frequency of *LasB, ExoS and Nan1* virulence gene detection in distinct infection sites which may predict infection severity and outcome.

Material and methods

Study design and ethical approval statement

The present cross-sectional study was performed on 30 nosocomial isolates of *P. aeruginosa* from patients admitted to Internal Medicine Department, ICU and Burn Unit at Tanta University Hospitals, Tanta, Egypt. Isolates were collected from ten respiratory tract, ten burn, and ten bloodstream infections. Written informed consent was obtained from all participants in this study. The study protocol has been approved by the Ethical Committee Review Board, Faculty of Medicine, Tanta University (approval code "33417/10/19").

To be included in the study, enrolled cases had to fulfill the following requirements: a) manifesting infection symptoms and signs after at least 48hrs of hospital admission; b) having one or more risk factors for *P. aeruginosa* infection such as lowered immunity, cystic fibrosis, productive cough, particularly greenish sputum in patients with respiratory tract infection; c) having burn infection with characteristically greenish pus; d) admitted to the ICU, particularly with requirement for assisted ventilation; e) any signs of bacteremia and /or septicemia such as fever, increased heart rate, and low blood pressure. Demographic and clinical data were collected from all patients including age, sex, date of admission, underlying diseases, symptoms, onset, course and duration of illness and course of treatment. Cases were classified according to disease severity into mild, moderate and severe based on clinical, laboratory and radiological parameters and guidelines [17,18,19].

Specimen collection

Samples were collected under complete aseptic precautions as follows:

Respiratory samples

Samples were collected from inpatients early in the morning after overnight accumulation before drinking or eating. Collection was done in semi-setting position by directing the patients to take deep inspiration, hold for 30 sec and then spit hard cough in sterile screw capped containers (≥ 5 ml). Oropharyngeal or endotracheal suctioning was done for patients on assisted ventilation. Collected samples were kept at room temperature for less than 2 hours [20, 21].

Burn samples

Samples were collected from inpatients with sterile cotton swabs in sterile containers under complete aseptic conditions. Swabs were introduced

into the depth of the lesion and rolled the swab to collect pus or exudation from the burn wound. Obtained swabs kept at room temperature for less than 2 hours [22, 23].

Blood samples

Patients' skin was disinfected using alcoholic iodine tincture to prevent any skin flora from contaminating the specimen. Five to ten ml of blood was withdrawn. Each blood sample was immediately added to a blood culture bottle containing broth after sterilizing the top of bottle by using alcohol pad to avoid contamination [24, 25].

All collected samples were transported as soon as possible to Medical Microbiology and Immunology Department, Faculty of Medicine, Tanta University where they were subjected to microbiological isolation and identification.

Phenotypic identification

Specimens were subjected to the phenotypic identification protocol of *Pseudomonas aeruginosa* including wet unstained smear, direct Gram stained film (Oxoid, UK), culture on nutrient, blood and MacConkey's agar plates (Oxoid, UK) by streak plate method then all plates were incubated aerobically at 37°C for 24 hrs. Blood culture bottles were reported negative after 10 days of incubation at 37°C with monitoring of potential growth every 48hrs by subculture on nutrient, blood and MacConkey's agar plates. Biochemical reactions (Oxoid, UK) were used for identification of the isolated *P. aeruginosa* by sugar fermentation, triple sugar iron (TSI) agar, oxidase test strips, glucose oxidation test, motility indole ornithine (MIO) and lysine decarboxylation (LDC) tests [26, 27].

Molecular detection of *LasB, ExoS and Nan1* genes

Detection of *LasB*, *ExoS* and *Nan1* genes in different *P. aeruginosa* clinical isolates was done using conventional PCR. Each gene was tested in a separate PCR reaction. DNA was extracted from different isolates by DNA extraction kit (QIAamp DNA mini kits QIAGEN) followed by amplification of the target genes by conventional PCR (PCR Biosystems, USA). Detection of the amplified genes was done by horizontal agarose gel electrophoresis. Specific primers used for each gene and expected amplicon sizes are shown in **Table 1**. Additionally, DNA extraction, conventional PCR and agarose gel electrophoresis were also done for a positive control *P. aeruginosa PAO1 strain (ATCC 47085)* where the three virulence genes (*Las B, Exo S* and *Nan)* are present, and negative control was also used [28-30].

Statistical analysis

Data were analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp). The Kolmogorov-Smirnov test was used to verify the normality of distribution. Chi-square test was used for categorical variables and to compare between different groups. A p-value less than 0.05 was considered statistically significant.

Results

Demographic criteria of enrolled study participants

The basic information of enrolled study participants is demonstrated in **Table 2**. We have collected nosocomial isolates of *P. aeruginosa* from 30 patients (63.3% males; mean age 53 years). No significant difference was found between the frequency of *P. aeruginosa* detection in either respiratory, pus or blood samples in relation to patient's gender or age.

Differential frequency of *Pseudomonas aeruginosa Nan1, LasB* and *ExoS* genes in distinct clinical isolates

Data in **Table 3** demonstrate that *P. aeruginosa LasB* gene is the most consistently detected gene across different types of clinical specimens. Both *Nan1* and *ExoS* genes were present in 30% of respiratory and pus specimens and absent in all blood specimens, while *LasB* gene was present in 100% of respiratory specimens, 90% of pus and 90% of blood specimens **(Table 3)**.

Relationship between number of detected virulence genes and the site of *Pseudomonas aeruginosa* infection

To examine the relationship between the number of detected virulence genes and the site of *P. aeruginosa* infection, collected clinical specimens were divided into three groups based on the number of detectable virulence genes. Specifically, group I consisted of specimens with all three detectable virulence genes, group II included those with two, group III comprised specimens with one, and group IV contained isolates with no detected virulence genes. Notably, group I was found in 20% of respiratory specimens but was absent in pus or blood. Group II was present in 50% of pus specimens and 20% in respiratory specimens, but not in blood. Group III was the most common, occurring in 60% of respiratory, 50% of pus, and

90% of blood samples. Interestingly, group IV was only identified in 10% of blood samples **(Table 4)**.

Differential *P. aeruginosa* infection severity based on the number of detected virulence genes

The data in **Table 5** examine the relationship between the severity of *P. aeruginosa* infection and the number of detected virulence genes. The data are categorized into cases with a single virulence gene (n=20) and cases with more than one virulence gene (n=9). The findings reveal a significant correlation: all cases with more than one virulence gene (100%) were associated with severe infections, while cases with a single virulence gene (100%) were associated with moderate infection severity **(Table 5)**.

Table 1. Primer sequence for PCR amplification of *ExoS, LasB and Nan1* genes

Gene ID	Primer sequence	Amplicon size in bps	GC Content%
Elastase B (Las B)	F: GGAATGAACGAAGCGTTCTC R: GGTCCAGTAGTAGCGGTTGG	300	64.3
Exoenzyme S (Exo S)	F: CTTGAAGGGACT CGACAAGG R: TTCAGGTCCGCGTAGTGAAT	504	64
Neuraminidase1 (Nan 1)	F: AGGATGAATACTT ATTTTGAT R: TCACTAAATCCATCTCTGACCCGATA	1316	48.4

Table 2. Distribution of *P. aeruginosa isolates* according to patients' demographics

Demographic criteria		Type of specimen						Test of significance	P-value
		Respiratory specimens (N=10)		Pus (N=10)		Blood (N=10)			
		N	%	N	%	N	%		
Gender	Male	6	60.0	4	40.0	9	90.0	□□□ □□□□□	^{MC}p = 0.088
	Female	4	40.0	6	60.0	1	10.0		
Age (years)	Range	38.0 – 81.0		39.0 – 61.0		39.0 – 56.0		F = 0.941	0.403
	Mean ± SD.	53.20 ± 12.80		48.10 ± 7.39		49.0 ± 4.22			
	Median (IQR)	51.50 (44.0–59.0)		46.50 (42.0–52.0)		50.0 (48.0–50.0)			

Abbreviations: N (number of samples; IQR (interquartile range); χ^2 (Chi square test); MC (Monte Carlo); F (F statistic for ANOVA**).** Asterisks (*) represent a statistically significant difference at $p \leq 0.05$.

Table 3. Frequency of detection of *Pseudomonas aeruginosa* virulence genes in distinct infection sites

Virulence Gene	Type of specimen						Total (N=30)		χ^2	^{MC}p	P-value
	Respiratory specimens (N=10)		Pus (N=10)		Blood (N=10)						
	No.	%	No.	%	No.	%	No.	%			
Nan1											
Present	3	30.0	3	30.0	0	0.0	6	20.0	3.921	0.197	$^{FE}p_1$=1.000 $^{FE}p_2$=0.211 $^{FE}p_3$=0.211
Absent	7	70.0	7	70.0	10	100.0	24	80.0			
ExoS											
Present	3	30.0	3	30.0	0	0.0	6	20.0	3.921	0.197	$^{FE}p_1$=1.000 $^{FE}p_2$=0.211 $^{FE}p_3$=0.211
Absent	7	70.0	7	70.0	10	100.0	24	80.0			
LasB											
Present	10	100.00	9	90.0	9	90.0	28	93.3	1.312	1.000	$^{FE}p_1$=1.000 $^{FE}p_2$=1.000 $^{FE}p_3$=1.000
Absent	0	0.0	1	10.0	1	10.0	2	6.7			
$^{FE}p_4$	0.003*		0.020*		<0.001*						
$^{FE}p_5$	1.000		1.000		1.000						
$^{FE}p_6$	0.003*		0.020*		<0.001*						

Abbreviations: χ^2 (Chi square test); MC (Monte Carlo); FE (Fisher Exact); p_1: p value for comparing the frequencies of virulence genes between respiratory and pus specimens; p_2: p value for comparing the frequencies of virulence genes between respiratory and blood specimens; p_3: p value for comparing the frequencies of virulence genes between pus and blood; p_4: p value for comparing the frequency of *Nan1* and *LasB* across all specimen types*;* p_5: p value for comparing the frequency of *Nan1* and *ExoS* across all specimen types*;* p_6: p value for comparing the frequency of *LasB* and *ExoS in* all specimen types*;* Asterisks (*) represent a statistically significant difference at $p \leq 0.05$.

Table 4. Relationship between number of detected virulence genes and the site of infection

Virulence gene groups	Type of specimen						χ^2	^{MC}p
	Respiratory specimen (N=10)		Pus (N=10)		Blood (N=10)			
	No.	%	No.	%	No.	%		
Group I	2	20.0	0	0.0	0	0.0	10.660	0.025*
Group II	2	20.0	5	50.0	0	0.0		
Group III	6	60.0	5	50.0	9	90.0		
Group IV	0	0.0	0	0.0	1	10.0		
Significance level between gene groups	$^{MC}p_1$=0.201, $^{MC}p_2$=0.089, $^{MC}p_3$=0.033*							

Abbreviations: χ^2 (Chi square test); MC (Monte Carlo); p_1 (p value for comparing between respiratory and pus specimens); p_2 (p value for comparing between respiratory and blood specimens); p_3 (p value for comparing between pus and blood); Asterisks (*) represent a statistically significant difference at $p \leq 0.05$; Group I: Presence of *Nan1, LasB and ExoS;* Group II: Presence of any two virulence gens; Group III: Presence of only one virulence gens; Group IV: *Nan1, LasB and ExoS* are not detected.

Table 5. Relationship between *P. aeruginosa* infection severity and number of detected virulence genes

Infection severity	**Cases with > 1 virulence gene (n=9)**	**Cases with single virulence gene (n=20)**	χ^2	^{FE}p
Severe	9 (100%)	0 (0%)	29.0*	<0.001*
Moderate	0 (0%)	20 (100%)		

Abbreviations: χ^2 (Chi square test); FE (Fisher Exact); p value for comparing *P. aeruginosa* infection severity between cases with 2-3 genes and cases with single gene; Asterisks (*) represent a statistically significant difference at $p \leq 0.05$

Figure 1. Agarose gel electrophoresis *shows ExoS* gene in two *Pseudomonas aeruginosa* isolates (lanes 1 and 2) detected at 504 bp using DNA size marker (lane 1). The black line indicates noncontiguous lanes from a single horizontal electrophoresis.

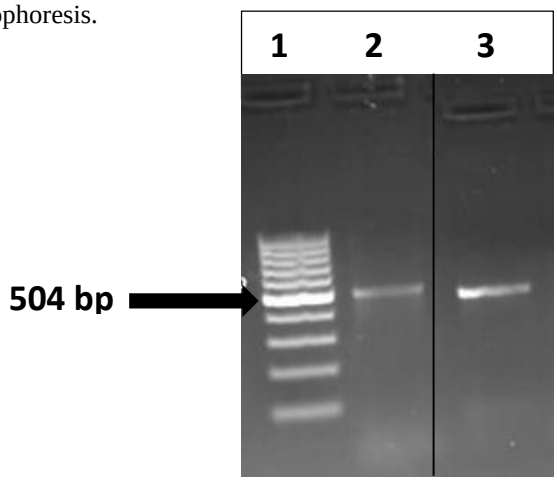


Figure 2. Agarose gel electrophoresis shows LasB gene in seven *Pseudomonas aeruginosa* isolates detected at 300 bp (lanes 3 -8). Lane 2 is a positive control of virulent *P. aeruginosa* strain (PAO1) using DNA size marker (lane 1). The black line indicates noncontiguous lanes from a single horizontal electrophoresis.

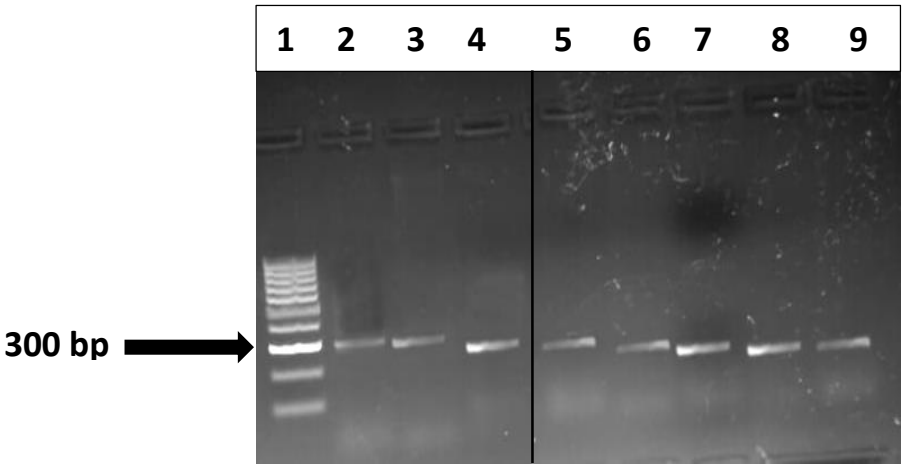
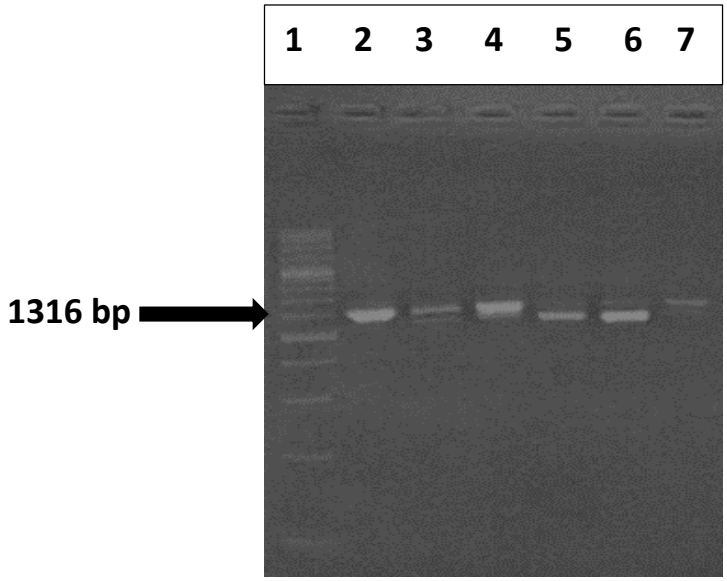


Figure 3. Agarose gel electrophoresis shows Nan1 gene in five *Pseudomonas aeruginosa* isolates detected at 1316 bp (lanes 3-7). Lane 2 is a positive control of virulent *P. aeruginosa* strain (PAO1) using DNA size marker (lane 1).



Discussion

Pseudomonas aeruginosa is an opportunistic pathogen that is considered a major health hazard, especially in immunocompromised patients. It is mostly implicated in nosocomial infections, particularly affecting ICU patients and those having catheterization, burn, and/or chronic illnesses. *Pseudomonas aeruginosa* possesses a variety of virulence genes that may contribute to its pathogenicity such as *Nan1, ExoS, LasB* and other genes [31].

Various studies have indicated that *P. aeruginosa* exhibits variable expression of certain genes across different types of infections. Nonetheless, scarce data exist, especially from Egypt, on how virulence genes like *LasB*, *ExoS*, and *Nan1* are detected in *P. aeruginosa* strains from distinct clinical sources. This study aimed to bridge this knowledge gap by phenotypic and genetic analysis of hospital-acquired *P. aeruginosa* samples to examine the prevalence of these virulence genes in various infections, which could help predict their severity and potential outcome [12-16].

Our analysis of nosocomial *P. aeruginosa* isolates revealed that *LasB* gene was most frequently detected across all clinical specimens, implying its pivotal role in the pathogenesis and adaptability of the bacterium. In contrast, *Nan1* and *ExoS* genes showed a more selective presence, being identified in respiratory and pus samples but not in blood, suggesting a potential tissue-specific role in infection severity. Moreover, the presence of multiple virulence genes was strongly associated with increased infection severity, indicating that a cumulative effect of these genes may contribute to the pathogenicity and outcome of *P. aeruginosa* infections.

In examining the role of *Nan1* gene in site-specific pathogenesis of *P. aeruginosa*, our data further elucidate this by showing a selective distribution of *Nan1* gene, predominantly in respiratory and pus specimens, and its association with severe infection outcomes when present in conjunction with other virulence genes, as evidenced by our significant findings. This profile aligns with other studies that highlighted a prominent role for *Nan1* gene in pulmonary infections, likely due to its contribution to bacterial adherence and invasion of respiratory epithelium mediated by the encoded sialidase [11, 14]. On the other hand, other studies have observed a ubiquitous presence of *Nan1* in various clinical specimens [32]. Further studies with a bigger sample size are needed to confirm the role of *Nan1* in site-specific pathogenesis.

Importantly, the detection of the *ExoS* gene in 30% of respiratory and pus specimens, and its absence in all blood specimens, may be attributable to invasive phenotypes of *P. aeruginosa* isolated from tissue specimens. This correlates with the

expression of the *ExoS* gene, which encodes cytotoxic factors, consistent with findings from other studies [29]. The low prevalence of the *ExoS* gene among isolates from bloodstream infections suggests its role is potentially more significant in burn and pulmonary tract infections.

In agreement with other reports, our results showed also that *LasB* gene was significantly present more than *Nan1* and *ExoS* genes in all specimen types. Our data indicate the potential importance of *LasB* gene for bacterial survival in addition to its role in ubiquitous *P. aeruginosa* pathogenesis [10, 29, 33].

Importantly, our analysis showed that *Nan1, ExoS, and LasB* virulence genes are unevenly present across various clinical samples. *LasB* was prevalent in blood samples, possibly due to its role in the bacterial survival [34]. Meanwhile, respiratory and pus samples showed a combination of genes, including *Nan1* and *ExoS*, which are linked to the severity of the infections. Specifically, multiple genes were associated with severe infections, while single genes were found in moderate cases. This pattern suggests that the number of virulence genes could reflect the seriousness of the infection, thereby influencing treatment approaches that focus on inhibition of virulence gene expression, particularly in severe cases.

In conclusion, the variation in gene distribution among specimens suggests that different *P. aeruginosa* strains may be uniquely adapted to particular niches within infection sites, which in turn could impact the severity of infection. A significant correlation was found between the severity of infection and the presence of multiple virulence genes, emphasizing the potential for developing targeted inhibitors as novel treatments for severe *P. aeruginosa* infections resistant to conventional antibiotics [35-42]. This research marks a significant step in Egypt towards understanding the relationship between *P. aeruginosa* virulence, infection site and severity, paving the way for future studies to further decipher these relationships and develop life-saving therapies against this highly antibiotic-resistant pathogen.

Still, there are some limitations to this study as we could not investigate the presence and association of other important virulence genes to the site and severity of infection as no single study would be able to test the presence of all virulence determinants especially in a highly virulent pathogen like *P. aeuroginosa*. Also, further studies with a bigger sample size are needed to confirm the role of each virulence gene in site-specific pathogenesis.

Declarations

Ethics approval and consent to participate

This study complies with all relevant ethical regulations. The experimental protocols were constructed in accordance with the International Ethical Guidelines for Biomedical Research Involving Human Subjects and were approved by The Ethical Committee Review Board, Faculty of Medicine, Tanta University, Tanta, Egypt (approval code "33417/10/19").

Consent for publication

Not applicable

Availability of data and material

The data generated in the present study are available from the corresponding authors upon request.

Competing interests

The authors declare no competing interests.

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