Original Article

Molecular Analysis of lasA, lasB, and toxA Genes in Clinical Isolates of *Pseudomonas Aeruginosa* by the PCR Technique

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Abstract

Background: Pseudomonas aeruginosa *(P. aeruginosa)* is a type of opportunistic bacteria, which may infect almost all tissues, immunocompromised people, and cause hospital-acquired illnesses as well. It has a variety of virulence variables that might attribute to its pathogenesis. The toxA (exotoxin A), lasA (protease) and lasB (elastase) genes are among these virulence variants. **Methods:** In present study, 115 clinical samples were collected from patients. Conventional morphological and biochemical assays were used to identify the isolates of *P. aeruginosa*. PCR technique was performed for detection of lasA, lasB, and toxA genes in clinical specimens of *P. aeruginosa*. **Results:** Of all the samples, *P. aeruginosa* isolates were identified in 25 clinical samples and their distribution was as follows: 8 (40%) wounds, 5 (7.14%) urine, 3 (100%) blood, 3 (37.5%) sputum, 2 (28.57%) CSF, 2 (50%) pus, and 2 (66.7%) renal abscess. Results of molecular analysis indicated the presence of virulence genes lasA, lasB, toxA in 14 (56%) of *P. aeruginosa* isolates while both lasA and lasB genes were detected in 15 (60%) isolates. While 16 (64%) of isolates harbored lasB and toxA genes. All these virulence genes were mostly found in pus, sputum, blood, and wound samples.

Keywords: *P. aeruginosa,* virulence variants, PCR.

Introduction

Gram-negative bacterium *P. aeruginosa* results in a wide range of illnesses, including illnesses of the urinary system, bloodstream infections, and pneumonia. This bacterium has inherent mechanism of resistance to various antibacterial drugs such as tetracycline, beta-lactams, trimethoprim/sulfamethoxazole, and chloramphenicol. In addition, through mobile genetic elements, *P. aeruginosa* may develop other drug resistance mechanisms, which also complicate the treatment of the infections it induces.[1,2]

The increase in the prevalence of *P. aeruginosa* species resistant to such antibacterial drugs has increased the risk of hospitalization, total cost as well as rate of death.[3] Its ability to infect the host is related to its role in regulating virulence genes which respond to external stimuli.[4,5] Pathogenesis of *P. aeruginosa* is complex and depends on the production of several cell-associated genetic variants, bacterial toxins and enzymes.[6] Exotoxins are either passively or actively released from the cell through various protein secretory mechanisms such as I, II, III, IV, and V.[7,8]

P. aeruginosa carries several genes including toxA, lasA, and lasB which comprise type II secretory mechanisms.[9] Exotoxin A, which is expressed via toxA gene is a toxin that suppresses protein production.[10] Throughout this mechanism, the nicotinamide adenine dinucleotide (NAD) and adenosine-5'-diphosphate-ribosyl (ADP-ribose) are transmitted to the multicellular organisms' elongation factor-2 that inhibits the activity of this factor and eventually suppresses protein production.[11]

LasA (protease) and lasB (elastase) have potent elastolytic function and are able to repress a broad variety of biological tissues; whilst the lasA has a limited elastolytic action, yet it may increase the expression of the lasB gene.[7] Elastase B, produced via the lasB gene, is a zinc metalloprotease which shows elastolytic action on lung tissue and destroys proteins like collagen and elastin.[12] Moreover, it also degrades proteins of immune system such as cytokines.[13]

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Such toxins have been considered to enhance the bacteria's proliferation at the infection sites. In addition, the bacteria invade the human immune system, and suppress DNA synthesis, resulting in destruction of host tissue and other damages.[6,8]

The significance of these potential virulence factors is determined by the location and type of the illness. The proteolytic enzymes (proteases); have a significant effect in corneal lesion, burn infections, and are linked to persistent lung infection.[14] Detection of *P. aeruginosa* from environmental and clinical samples by microbiological procedures is reliable, however, it typically takes many days to complete. It is important to use molecular analysis to rapidly diagnose the isolates that are causing hospital-acquired infections before making final decisions on the patients' treatment. Multiplication of sequences distinct to a certain organism can be done through PCR which helps to rapidly determine the microbial species.[15]

The aim of the current study is to identify, through PCR technique, the incidence of virulence genes lasA, lasB, toxA, in *P. aeruginosa* clinical specimens collected from various diseases of humans admitted in hospital in Kirkuk city.

Materials and Methods

Clinical Specimens

One hundred fifteen samples of sputum, blood, urine, cerebrospinal fluid (CSF), pus, renal abscess, and wound infections were obtained from patients (68 females, and 47 males) hospitalized in Azadi teaching hospital, Kirkuk, Iraq, from December 2020 to August 2021. Using sterile transport medium, the clinical specimens were transported to the microbiological laboratory at the College of Medicine, University of Kirkuk, Iraq.

Identification of P. aeruginosa Isolates

The specimens were cultured on MacConkey agar medium, and maintained at 37°C for 24-hours. Conventional morphological identification based on gram- stain, biochemical assays; oxidase, citrate utilization, sugar indole, iron, and nitrate reduction tests were used to identify *P. aeruginosa* isolates.[16]

DNA Extraction

The extraction of the bacterial DNA was done by using the DNA extraction kit (Jena Bioscience GmbH, Germany) as instructed by the manufacturer. The concentration of extracted DNA was then determined using UV spectrophotometric absorbance at 260 nm. Furthermore, samples were analyzed through on 1% agarose gel to screen for genomic DNA before being stored at -20°C for future molecular investigations.[17]

Detection of the Virulence Genes

The virulence genes in *P. aeruginosa* isolates i.e. elastase (lasA), elastase (lasB), and exotoxin A (toxA) were identified through PCR. Primers used in this study were supplied by Macrogen Company, Korea in a lyophilized form (Table 1).

Table 1. The primers utilized in present study.

Primer	Primer sequence (5`-3`)	Annealing Temperature (o C)	Product Size (bp)	Reference
lasA-F	GCAGCACAAAAGATCCC	55	1075	[18]
lasA-R	GAAATGCAGGTGCGGTC			
lasB-F	GGAATGAACGAAGCGTTCTCCGAC	60	284	[19]
lasB-R	TTGGCGTCGACGAACACCTCG			
toxA-F	CTGCGCGGGTCTATGTGCC	55	270	[19]
toxA-R	GATGCTGGACGGGTCGAG			

PCR Components

The PCR kit used in this study was provided by Promega, USA. The PCR amplification was done in a 20µl volume which comprised 10µl master mix, 1µl of forward and reverse primer each, 6µl nuclease-free water, and 2µl of template DNA.

PCR Program

Initial incubation was done at 95°C for 5min followed by 30 cycles of reaction having the steps of denaturation (95°C/30 sec), annealing (55, 56, 60°C/30 sec), and elongation (72°C/1 min). Thereafter, the last step of extension was set at 72°C for a min. Finally, the amplified products were held at 10°C for 10 minutes.

PCR amplification was proceeded by the gel electrophoresis to verify the existence of PCR products and for this purpose, the agarose gel (1.5%) stained with ethidium bromide was used. Optimal product size was confirmed by comparing it with DNA ladder of 100-1500 bp size.

Statistical Analysis

The ANOVA test was used to determine the incidence of virulence genes according to the source of *P. aeruginosa* isolates. The P value less than 0.05 was considered statistically significant.

Results

Total 115 clinical samples were collected which are as follows: urine (n=70, 60.68%), pus (n=4, 3.47%), sputum (n=8, 6.95%), renal abscess (n=3, 2.60%), blood (n=3, 2.60%), wounds (n=20, 17.39%), and CSF (n=7, 6.08%). Using conventional microbiologic tests, *P. aeruginosa* was identified in 25 (21.73%) of all clinical specimens. Their distribution is as follows: 8 (40%) wounds, 5 (7.14%) urine, 3 (100%) blood, 3 (37.5%) sputum, 2 (28.57%) CSF, 2 (50%)

pus, and 2 (66.7%) renal abscess (Table-2).

Table 2. Prevalence of *P. aeruginosa* in clinical samples.

Clinical samples	Total samples		Positive Isolates	
	No.	(%)	No.	(%)
Urine	70	60.68	5	7.14
Wounds	20	17.39	8	40
Sputum	8	6.95	3	37.5
CSF	7	6.08	2	28.57
Pus	4	3.47	2	50
Blood culture	3	2.60	3	100
Renal abscess	3	2.60	2	66.7

Molecular analysis was used to screen the virulence genes (lasA, lasB, toxA) in all *P. aeruginosa* clinical isolates. When PCR products of these genes were subjected to gel electrophoresis, the results showed that 15 (60%) of the isolates carried lasA gene (Figure 1), (68%) harbored lasB gene (Figure 2) while 16 (64%) of the isolates had toxA gene (Figure 3). In total, all the three genes were detected in 14 (56%) isolates while both lasA and lasB genes were found in 15 (60%). Whereas, 16 (64%) isolates carried lasB and toxA genes.

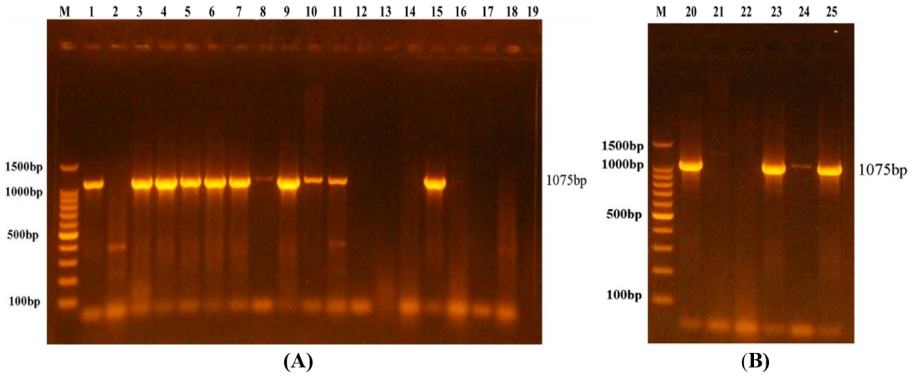

Figure 1. (A, B): Agarose gel showing PCR product of the virulence gene lasA (1075 pb) in *P. aeruginosa* clinical isolates. DNA marker size (100-1500bp), Lane;1, 3-11, 15, 20, 23-25 represent positive isolates of *P. aeruginosa* for lasA gene.

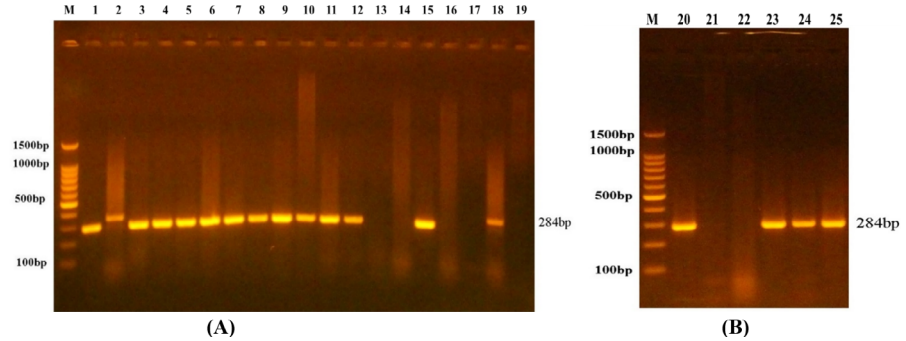

Figure 2. (A, B): Agarose gel showing the PCR product of the virulence gene lasB (280 pb) in *P. aeruginosa* clinical isolates. DNA marker size (100-1500 bp), Lane;1, 3-12, 15, 18, 20, 23-25 represent positive isolates of *P. aeruginosa* for lasB gene.

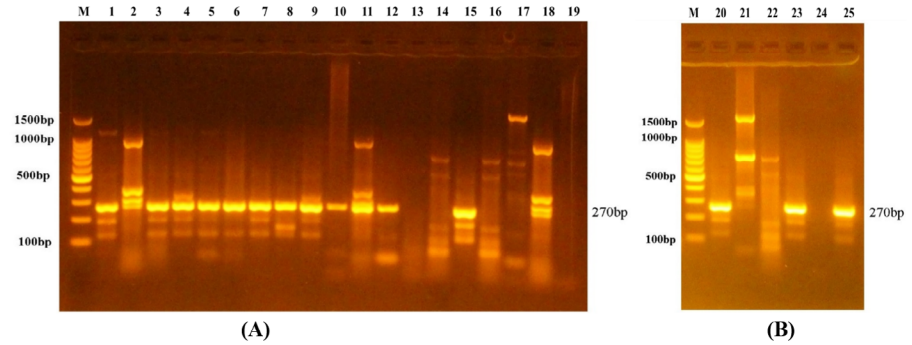

Figure 3. (A, B): Agarose gel showing PCR product of the virulence gene toxA (270 pb) in *P. aeruginosa* clinical isolates. DNA marker size (100-1500bp), Lane;1, 3-12, 15, 18, 20, 23, 25 represent positive isolates of *P. aeruginosa* for toxA gene.

Regarding the frequency of virulence genes in the clinical isolates of *P.seudomonas aeruginosa*, the results of the present study showed that the frequency of lasA gene was substantially greater in the isolates from sputum, pus, blood, and wound samples than in the samples from urine, CSF, and renal abscess (P<0.008). Similar results were obtained with lasB gene as their frequency was also significantly higher in specimens from sputum, pus, blood, and wounds than from CSF, urine, and renal abscess (P<0.01). While in case of toxA gene, their frequency was significantly hogher in blood, sputum, and wound samples than in urine, CSF, pus, and renal abscess samples (P<0.012). Results are shown in Table- 3.

Table 3. Frequency of the genes (lasA, lasB, toxA) in *P. aeruginosa* strains from various clinical samples.

Clinical samples	Virulence genes		
	lasA No. (%)	lasB No. (%)	toxA No. (%)
Urine	1(20)	2 (40)	2(40)
Wounds	5(62.5)	6(75)	6(75)
Sputum	3(100)	3(100)	3(100)
CSF	1(50)	1(50)	1(50)
Pus	2(100)	2(100)	1(50)
Blood	2(66.7)	2(66.7)	3(100)
Renal abscess	1(50)	1(50)	1(50)
P value	0.008	0.01	0.012

Discussion

One of the sources of nosocomial and acquired infections is *Pseudomonas aeruginosa* and is commonly associated with elevated mortality rate.[18,19] The results of current study indicated the presence of *P. aeruginosa* in 25 out of 115 different clinical isolates with the following distribution: 8 (40%) in wounds, 5 in (7.14%) urine, 3 each in blood (100%) and sputum (37.5%) and 2 each in CSF (28.57%), pus (50%) and renal abscess (66.7%). This bacterium is the most prevalent nosocomial pathogen and can result in various diseases including endocarditis, pneumonia, and inflammation in urinary system.[20] In addition, many other organs may also be affected by the bacterium such as; nervous system, skeletal muscles, skin, eyes, and ears.[21-24]

P. aeruginosa has several virulence variables that are related to its pathogenesis such as exotoxins, proteases, various enzymes, secretory systems, flagella, pigments, and pili.[25] These virulence factors aid bacteria in attaching and colonizing their host by interfering with immune responses and producing a barrier against antibiotics. Cell-associated or secreted virulence variants are located on plasmid or on chromosome.[26] The current work focused on the molecular characterization of virulence genes (lasA, lasB, and toxA) of the *P. aeruginosa* isolates in various clinical samples. The results indicated the presence of lasA, lasB, and toxA genes in 60%, 68%, and 64% of *P. aeruginosa* clinical isolates respectively. All these genes were observed in 14 (56%) isolates, while lasA and lasB genes were found in 15 (60%) isolates. Moreover, lasB and toxA genes were found in 16 (64%) of the *P. aeruginosa* samples. Studies have shown the presence of lasB gene in *P. aeruginosa* isolates from samples of blood, sputum, cerebral fluid, wound, urine, and bronchial wash.[27,28] Another study depicted that most of the *P. aeruginosa* isolates were from the urinary tract, wounds and trachea.[28] Furthermore, a study found a high incidence of the lasB gene i.e. 100% in blood samples from Egyptian clinics.[29] Similarly, in India, isolates from UTIs showed a high incidence of toxA (100%) and lasB (75%) genes.[30] While in another study, toxA gene has been found in patients with chest infections.[15]

The virulence of *P. aeruginosa* is multifactorial and is related to its cellular components such as exotoxin A, elastase, and protease. It has been demonstrated that lasA and lasB are released proteases that have a significant role in this bacterium's invasion.[31] Exotoxin A (toxA) is anti-phagocytic, and cytotoxic to the cell.[10] These virulence variables are crucial in the pathogenicity of *P. aeruginosa* causing illnesses of respiratory system, urinary tract, wounds, bloodstream infections etc.[32]

Secretion systems types II (T2SS) is formed by three proteases released by *P. aeruginosa* i.e. 2 elastase enzymes (lasA, lasB), and an exotoxin (toxA). Elastases have a strong elastolytic activity and work synergistically, primarily in the lungs and blood vessels, whereas toxA suppresses protein production in the host resulting in cell destruction.[33] These enzymes are involved in breakdown of both collagen and non-collagen proteins in the host. Elastin causes damage to the host's physical barrier and promotes the spread of infection. Besides, immunoglobulins, complement proteins, and α-1 antitrypsin are all involved in the probable pathogenic processes. Proteases may improve the organism's adhesion by destroying the tissue. In addition, they increase nutrient availability by digesting host proteins, and facilitate the bacterium to invade. These have been reported to be prevalent in many areas worldwide.[34-36]

LasB is one of *P. aeruginosa*'s most significant proteases which increases lasB's elastolytic ability.[37,38] *P. aeruginosa* invasion is significantly reduced when the lasB gene is mutated. The lasB gene's presence in clinical and environmental samples indicates that this gene is necessary for *P. aeruginosa* viability in a variety of environments.[39] The majority of these virulence genes are controlled by a gene regulatory two-component mechanism and the quorum sensing, both are required in the growth and survival of bacteria within the host.[40,41] The transcription of virulence genes in a specific infection is critical to the ability of *P. aeruginosa* isolates to develop and keep spreading the infection.[42]

Regarding prevalence of virulence genes (lasA, lasB, toxA) in various clinical isolates of *P. aeruginosa*, the current study's findings revealed that their frequency was substantially greater in sputum, pus, blood, and wound samples than in urine, CSF, renal abscess isolates. High

association between virulence genes and infection origins can contribute to the treatment of these pathogens in the population.[43] The variations in data distribution of the virulence genes in different communities increase the possibility that certain species of *P. aeruginosa* are best adapted to specific infectious sources. Therefore, the transcription of these genes varies depending on the site as well as the severity of the infection.[44]

Conclusion

The findings of this study revealed significant prevalence of virulence genes (lasA, lasB, toxA) in *P. aeruginosa* isolates from different clinical samples.

Declarations

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