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Detection of Immunological Response of Exotoxins Producing *Pseudomonas aeruginosa*

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ABSTRACT

Pseudomonas aeruginosa is responsible for increasing the mortality rate of burn patients, especially those strains that produce toxins. Therefore, this study aimed to evaluate the immune response to exotoxins produced by isolates that were collected from burn patients at the Burns Center in Najaf City of Iraq for the period from October 1st to December 1st, 2023. A total of 48/ 178 (27%) P. aeruginosa test isolates identified, 23 and 25 of which were female and male, respectively. The age groups of inpatients ranged from 5 to 55 years. Using molecular techniques, the polygenic pattern of exotoxin-encoding genes was predominant, especially the pattern (exoT+exoS+exoY) in 7 (58.4%) isolates, while no single gene was identified. Immunologically, the concentration of Interferon-Gamma (IFN-γ) was measured, and it was found higher in the inpatient group contrasted to the healthy control group. Moreover, it was found higher in the patient group (300.233 and 298.212) pg/ml in the 10- and 20-year age groups, respectively. While the level of IFN-γ in the inpatient group and the healthy control group was (310.123 and 52.222) pg/ml in females, which was higher than its concentration in males.

Keywords: Pseudomonas aeruginosa, Exotoxins, Burn, Iraq, PCR, ELISA, T3SS, IFN-γ.

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INTRODUCTION

Pseudomonas aeruginosa is a frequent opportunistic bacterium seen in burn wounds. Its responsible for more than 50% of all chronic and acute nosocomial infections [1]. *P. aeruginosa* causes mortality at rates that range from 20% to 60%, which is rather substantial [2]. The pathogenicity of the bacteria is dependent on a vast variety of variables that are linked with cells and those that are found outside of cells. Numerous infections can be brought on by *P. aeruginosa*, which infiltrates the body and its immune system to cause infections that are almost hard to cure. Pathogenicity can sometimes be precipitated by virulence factors such as secretion systems, biofilm formation, proteases, exotoxin A, type III secretion systems (T3SS), and flagellum [3, 4].

Another well-known feature of *P. aeruginosa* is its capacity to release toxins from the T3SS which may have a significant impact on cellular virulence factors and pathogenicity. Most studies have suggested that the T3SS constitutes the most common and distinct virulence factor in *P. aeruginosa*. Scientists predict that toxin-producing-*P. aeruginosa* will make infections more difficult to treat, manage, and control [5]. Several virulence factors in *P. aeruginosa* work against the host and may harm human tissues directly

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or boost the bacterium's level of competition. *P. aeruginosa* exhibits several pathogenic features that allow it to remain in the host and cause recalcitrant and chronic infections even in hostile conditions, making it the most common pathogen [6, 7]. The immune system combats microbes and their multiple virulence characteristics using several mechanisms, including the production of cytokines like Interferons type γ (IFN-γ), which plays an important function in the human immunological response to several pathogens, but its role in *P. aeruginosa* is not clearly defined.

It has been shown that exogenous IFN-γ administration via adenoviral vectors prior to bacterial challenge with *P. aeruginosa* enhances bacterial clearance by increasing the production of increasing Major Histocompatibility Complexes (MHC)-II molecules expression and Tumor Necrosis Factor (TNF)-α in macrophages associated with overexpression of IFN-γ [8]. Several molecular studies have linked the production of exotoxins by *P. aeruginosa* to immune responses, but local studies are lacking to our knowledge. Therefore, this investigation aimed to reveal the potential of a association between immune response and exotoxin production, due to the lack of studies on this issue, and to investigate the possibility of a role for these factors in stimulating and increasing the virulence characteristics of strains acquired in hospitals, especially those strains producing exotoxins in patients with wound infections in Najaf City.

MATERIALS AND METHODS

Patients and Specimens Collection

Swabs were collected from infected burn wounds from inpatients with burns at Najaf Burn Center from October 1st to December 1st, 2023, after obtaining permission from the patients and adopting the regulations of the Research Ethics Committee of Al-Furat Al-Awsat Technical University. The isolates that had green fluorescent colonies under UV light, were oxidase positive, and able to grow at 42°C were confirmed as *P. aeruginosa*. To determine the immune response of both the inpatient and control groups were subjected to measuring the concentrations of IFN-γ using the enzymes-linked immunosorbent assay (ELISA) system, and it was confirmed that both groups were identical in number, age group and gender [9].

Molecular Detection of Type 3 Secretion System

In this study, the T3SS-encoding genes were detected using primers and polymerase chain reactions (PCR) technique where the PCR thermal-program was set up with 36 cycles after a 3-minute initial denaturation step at 94 °C. Then, 40 seconds of 94 °C were used to prepare the second denaturation step, and 40 seconds of 56 °C were used for the annealing step. The extension step lasted 60 seconds at 72 °C. An extension step lasting 5 minutes marked the end of the thermal process at 72 °C [10] **[Table 1].**

Table 1. Primers that were used to detect T3SS-encoding genes.

Gene	Oligonucleotide sequence		Size (bp)	Reference
exoT	F	5'AATCGCCGTCCAACTGCATGCG3'	153	10
	R	5'TGTTCGCCGAGGTACTGCTC3'		
exoS	F	5'GCGAGGTCAGCAGAGTATCG3'	118	
	R	5'TTCGGCGTCACTGTGGATGC3'		
exoY	F	5'CGGATTCTATGGCAGGGAGG3'	289	
	R	5'GCCCTTGATGCACTCGACCA3'		

Collection of Blood Samples

Three ml of fresh blood was collected by cleaning the skin of the arm area with a cotton swab soaked in 70% dilute alcohol. The blood was drawn from the indicated vein and placed in a test tube free of coagulants. The blood sample was left at room temperature to clot for 60 minutes, and wooden sticks were used to delicately remove the blood clot. Finally, the blood samples underwent a 10-minute centrifugation at 3 thousand revolutions per minute. The sera were then transported to other test-tube for separation and the serum was stored in the deep freezer [11].

Determination of IFN-γ Concentration

Principle of ELISA Assay

Invitro immune response was evaluated by using the ELISA assay that was performed according to the manufacturing company. The micro-ELISA plate provided with an antibody specific to IFN-γ of human. The micro-ELISA was properly filled with serum samples. At a wavelength of (450) nm, the optical density (OD) was detected by using a spectrophotometer. The OD of the samples was compared to the standard curve in order to detect the levels of IFN-γ.

Statistically Analysis

All data of the current study were analyzed statistically and mathematically using the Statistical Packages for the Social-Science (V-20).

RESULTS

Isolation and Detection of *Pseudomonas aeruginosa*

Out of 178 samples, 48 (27%) *P. aeruginosa* isolates were detected, including 23 (48%) females and 25 (52%) males.

Determination of T3SS-Encoding Genes

Using PCR technique, 1 (8.3%), 1 (8.3%), 3 (25%), and 7 (58.4%) of *P. aeruginosa* isolates were identified as carrying the genes with patterns: (*exoY*), (*exoT*+*exoY*), (*exoT*+*exoS*) and (*exoT*+ *exoS*+*exoY*), respectively. the polygenic pattern of exotoxin-encoding genes was predominant, especially the pattern (*exoT*+*exoS*+*exoY*), while no single gene was identified **(Table 2).**

Table 2. Presence of exotoxin-coding genes in the isolates under study.

Genes Type	No. of genes, N= 12/ 48 (25%) n (%)
exoT	0 (0)
exoY	1 (8.3)
exoT* + *exoY	1 (8.3)
exoT* + *exoS	3 (25)
exoT* + *exoS* + *exoY	7 (58.4)

Detection of Interferon Gamm level

The immune system is highly dependent on IFN-γ, which is a unique, effective, and efficient type II interferon. The current study included 48 resident patients and the same number of individuals in the control group. The inpatient and healthy control groups were matched in (number of age groups and sex of contributors). In this study, the concentration of IFN-γ was measured using the ELISA system. It was also noted that the levels of IFN-γ increased significantly in the inpatient group compared to the healthy control group. The highest concentration of IFN-γ was in the (300.233, 298.212, and 278.265) pg/ml inpatient group in the age groups 10-, 20-, and

50-60, respectively. While the lowest concentration of IFN-γ was in the age groups 30- and 40-, respectively. All the results of the immune response were summarized in **Figure (1).** Moreover, the levels of IFN-γ in the inpatient and control groups were (310.123 and 52.222) pg/ml in females, which was higher than that in males, which was (287.322 and 40.312) pg/ml, respectively **(Figure 2).** According to **Table (3),** both the arithmetic means, and the statistical standard-deviations were computed at a significance level of $P<0.05$. The mean concentrations of IFN-γ in the inpatients group was 250.937, whereas in the healthy control groups it was 44.166.

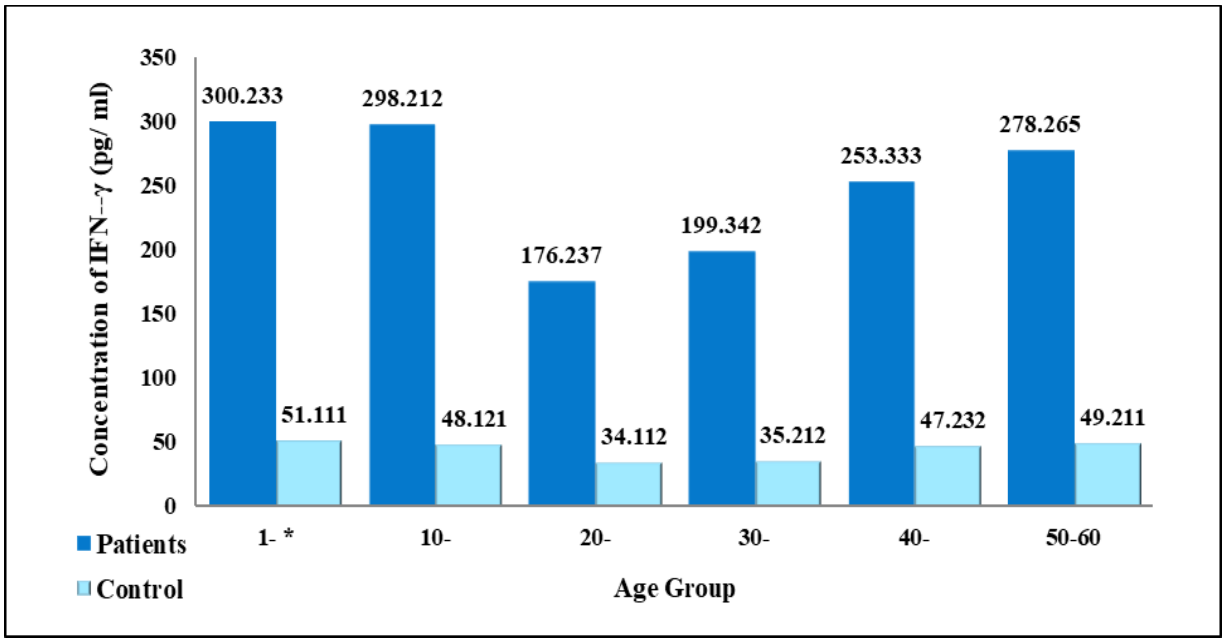

Figure 1. IFN-γ concentration levels according to age in the control and patient groups.

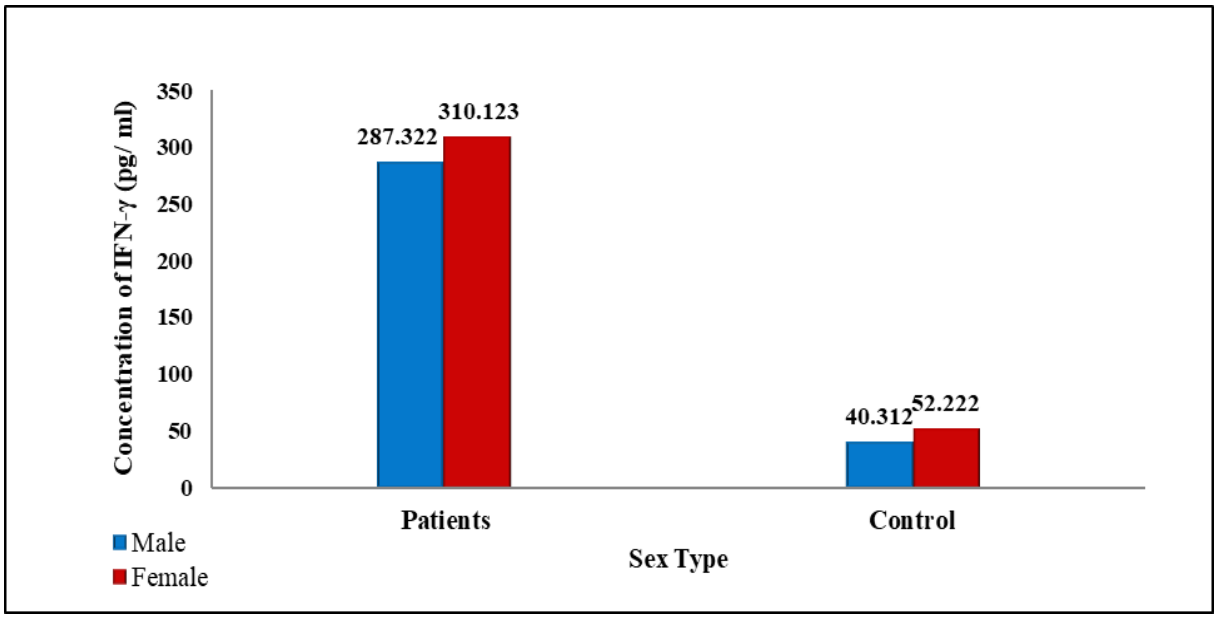

Figure 2. IFN-γ concentration levels according to sex type in the inpatient and control healthy groups.

Table 3. Comparison of mean IFN-γ concentration between patient and control samples.

Group Type	IFN-γ		Confidence Level	Margin of Error	SE	*P.* Value
	Mean (μ)	±SD (σ)				
Patient	250.937	52.26	95%, 1.960σx̄	250.937 ±38.179 (±15.21%)	21.33	<0.0*
Control	44.166	7.483	95%, 1.960σx̄	44.1665 ±5.466 (±12.38%)	3.054	
SD, Standard deviation; SE, Standard error; *P.* value, probability.						

DISCUSSION

In the current study, the test isolates expressed the production of exotoxins encoded by genes encoding the T3SS system such as the *exoY*, *exoT*, and *exoS* genes, which are known to contribute to the invasion and cytotoxicity process [12]. The secretion system allows the injection of proteins into eukaryotic cells. ExoT, ExoY, ExoU and ExoS were identified as effector proteins, especially ExoU and ExoS, which are highly toxic and responsible for host cell death, severity of infection and accelerated early death [13]. Virulence factors, notably the T3SS, which contributes to the process of invasion and cytotoxicity, are some of the most important variables relevant to the virulent *P. aeruginosa* infection [12]. The secretion system permits effector proteins to be injected into eukaryotic cells. ExoT, Y, U, and S were identified as effector proteins. Exotoxins U and S contribute to *P. aeruginosa* pathogenicity, especially the highly cytotoxic ExoU that is responsible for the phenotype that leads to the death of host cells and is considered a factor responsible for the severity of infection and an accelerating factor for premature death [13].

There are several studies that found that the ExoU and ExoS toxin were common [14, 15], in contrast to the current study that documented that multiple *exoS*, *exoT*, and *exoY* genes were more common than single genes, which may be evidence that multiple exotoxins genes may be transmitted together, linked together, or perhaps have a synergistic association.

The present study recorded significant differences in IFN-γ concentration levels in the age groups from 10 to 30 years. The reason for the high levels of IFN-γ may be the immune system response and mobilization of immune capabilities to combat infections caused by *P. aeruginosa* in immunocompromised age groups such as the elderly and children. In this study, significant statistical differences were also observed in IFN-γ levels between the patient groups and the control group, while no significant differences were observed in the levels of IFN-γ between males and females. Therefore, the current study did not record a relationship between IFN-γ production and sex at the level of the immune responses, indicating that the immune response is almost parallel and not affected by the sex of the participant, whether the participant was a patient or healthy. Interestingly, isolates characterized by the presence of exotoxins collected from patients who showed high levels of IFN-γ may indicate the fact that there is a relationship between immune stimulation and toxic encoding.

This fact was inferred from the molecular profile of each isolate and the statistical results. These results can be confirmed by future extended studies and by increasing the study sample size. Several studies have suggested that endogenous expression of IFN-γ is not beneficial to host defenses against *P. aeruginosa*, but that increased concentrations of IFN-γ may enhance the host's ability to clear the bacteria. The reasons for these discrepant results are not clear and further studies are

needed before any definitive conclusion can be reached about the beneficial or detrimental effect of this cytokine [16]. It is known in scientific fields that IFN-γ may coordinate a variety of defense mechanisms to boost immune response during infection. It can demonstrate its immunomodulatory effect by altering cellular proliferation, promoting antimicrobial activities, increasing leukocyte trafficking, presentation and processing, improving antigens, and apoptosis [17].

IFN-γ contributes to bacterial resistance, clearance, and elimination and defending the body through several mechanisms. It is a major driver of the interaction between alveolar macrophages and natural killer cells, which leads to the stimulation of host defense and bacterial eradication. The role of IFN-γ in stimulating antibacterial immunity has not been previously recognized because IFN-γ is a central effector of cellular immunity, and it also coordinates a wide range of antibacterial functions by enhancing antigen recognition, increasing the Reactive-Nitrogen Intermediates production, antigen presenting cells, and stimulating antimicrobial responses [18, 19].

CONCLUSIONS

Unfortunately, it can be concluded that *P. aeruginosa* is endemic in the burn center environment and that there is an immunological relationship between the exotoxin production and the high levels of IFN-γ, indicating the presence of an immune response to the virulence factors possessed by this bacterium, which qualifies it to survive, colonize and increase mortality rates among burn patients. Regarding the molecular aspect, it indicates the fact that multiple genes were more dominant than single genes, indicating the acquisition of virulence factors in package. This issue is worrisome and requires further research and molecular studies.

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