World Journal of
Experimental Biosciences

Volume 11, Number 1: 11 - 15
ISSN: 2313-3937
2023

Research article

Comparison Study between Clinical and Environmental Isolates of *Pseudomonas aeruginosa* in terms of Pro-inflammatory Cytokines Production in Mice Lungs

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ABSTRACT

Pseudomonas aeruginosa is one of the most widespread bacteria. It causes infectious diseases and at the same time is found in soil and water. It stimulates the pro-inflammatory immune response. The study aims to compare the role of *P. aeruginosa* isolated from infected burned wounds and that isolated from fresh water in terms of stimulating the pro-inflammatory response of the respiratory tract. In the current study, P. aeruginosa isolates were isolated from infected burned wound swabs (PAC) and from freshwater (PAE). Laboratory mice were stimulated by giving them intranasal (i.n.) standard inoculum of pathogenic and environmental isolates of *P. aeruginosa* (PAC and PAE respectively). The levels of interleukin (IL-) 1 and tumor necrosis factor (TNF)-alpha (α) were measured in the homogenized mice lungs. The study demonstrated that both isolates (PAC and PAE) stimulated the respiratory system to secrete pro-inflammatory cytokines (IL-1β and TNF-α). Both isolates significantly ($P<0.05$) increased the level of IL-1β and TNF-α at the first-hour post-administration, and the highest level was observed at the fourth hour after administration with PAC and PAE. After that, the level of cytokines decreased dramatically over time. At 48 hours after administration, it was found that a significant increase of IL-1β and TNF-α was seen only in the lungs of the mice that were given the pathogenic isolates. It can be concluded that both isolates of P. aeruginosa (PAC and PAE) stimulate respiratory system cells to produce pro-inflammatory cytokines, but PAC stimulates mice lungs to produce IL-1β and TNF-α in a higher level and for a longer period as compared with stimulation of mice lungs with PAE.

Keywords: Clinical isolates, Environmental isolates, Interleukin 1, *Pseudomonas aeruginosa,* Tumor necrosis factor.

Citation: Mohammed HA, Alshalah LAM. (2023) Comparison study between clinical and environmental isolates of *Pseudomonas aeruginosa* in terms of pro-inflammatory cytokines production in mice lungs. *World J Exp Biosci* **11**: 11-15. https://doi.org/10.65329/wjeb.v11.01.003 .

Received February 6, 202; Accepted March 22, 2023; Published: March 30, 2023.

1. INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium known for its ability to thrive in various environments, including soil, water, and moist surfaces. It is also a significant opportunistic human pathogen associated with a wide range of infections [1]. Clinical Isolates of *P. aeruginosa* are a leading cause of nosocomial (hospital-acquired) infections, particularly in immunocompromised individuals and those with underlying health conditions. Clinical isolates are associated with diseases

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such as pneumonia, urinary tract infections, wound infections, and sepsis [2]. They possess virulence factors like exotoxins, proteases, and biofilm-forming capabilities, which enable them to colonize and infect host tissues effectively. Clinical isolates of *P. aeruginosa* often exhibit resistance to multiple antibiotics, making them challenging to treat. This resistance is often due to the selective pressure imposed by antibiotic use in clinical settings [3].

Environmental isolates of *P. aeruginosa* have applications in bioremediation. They are known for their ability to break down and degrade various organic compounds, including hydrocarbons and pollutants [4]. Their metabolic versatility makes them valuable for cleaning up contaminated sites. In the environment, *P. aeruginosa* is a part of the natural microbial community. It plays a role in nutrient cycling and is involved in interactions with plants and other organisms [5]. The environment serves as a reservoir for genetic diversity in *P. aeruginosa*. This diversity can influence the genetic traits found in clinical isolates. For insta-nce, antibiotic-resistance genes can be exchanged between environmental and clinical strains. Environmental isolates, when not properly managed, can become a source of clinical infections [6]. Contaminated water sources, soil, or medical equipment can introduce *P. aeruginosa* into clinical settings. While *P. aeruginosa*'s ability to degrade pollutants is beneficial for bioremediation, the release of these pollutants into the environment poses environmental challenges. These pollutants can contaminate water bodies and soil, impacting ecosystems [4].

P. aeruginosa stimulates the pro-inflammatory cytokines via their appendages proteins or their products. The ability of this bacteria to produce pro-inflammatory cytokines such as Interleukin (IL)-1 beta (β) and tumor necrosis factor (TNF)- alpha (α) in vivo after exposure to bacterial structural proteins or their products. The mechanism of pro-inflammatory cytokines production happens via Pattern Recognition Receptors (PRRs) when *P. aeruginosa* infects the host, its molecular components, such as lipopolysaccharides (LPS) and flagellin, are recognized by host PRRs like Toll-like receptors (TLRs) and NOD-like receptors (NLRs) [7]. This recognition triggers a signaling cascade that leads to the activation of pro-inflammatory pathways and occurs via the activation of nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways. These pathways play a crucial role in the production of pro-inflammatory cytokines [8]. This response is critical for the early defense against the pathogen but needs to be carefully regulated to avoid excessive inflammation and tissue damage [9]. Understanding the mechanisms by which *P. aeruginosa* stimulates pro-inflammatory cytokine production is essential for developing therapeutic strategies to modulate the immune response during infections caused by this bacterium.

Understanding the distinctions and interactions between clinical and environmental isolates of *P. aeruginosa* is crucial for managing infections, studying its pathogenicity, and addressing environmental issues. Additionally, proper infection control measures in clinical settings and responsible management of environmental pollutants are essential to mitigate problems associated with this versatile bacterium. The differentiation between clinical isolates of *P. aeruginosa* (isolated from infected patients) and environmental isolates (found in non-clinical settings) is important for several reasons, including understanding its pathogenesis and addressing environmental problems [10].

The study that focused on the comparison between the *P. aeruginosa* isolated from clinical cases and that isolated from environmental samples is scanty in the literature. This is why, the present study aims to study the comparative between the clinical and environmental isolates of *P. aeruginosa* in terms of their ability to stimulate the host body to produce the pro-inflammatory cytokines in vivo.

2. MATERIALS AND METHODS

2.1. Bacterial Isolation and Identification

The clinical swab samples were collected from patients suffering from infected burn wounds. The freshwater samples were collected in a sterile container. The samples were transferred to the lab for microbiological examination. The samples were immediately cultured onto MacConkey agar, and the non-lactose fermenting colonies were re-cultured onto Cetrimide agar. The VITEK 2 DensiCheck instrument, fluorescence system (bioMe´rieux) was used to identify the pre-examined isolates of *P. aeruginosa* after following the instructions of the manufacturing company [11]. In the further experiment, two isolates of *P. aeruginosa*, a clinical isolate of *P. aeruginosa* (PAC) and an environmental isolate (PAE) were used in the mice experiments (*in vivo* study). The bacterial isolates were stored for the short term by culturing onto nutrient agar slant (stored at 4 °C) and for the long term, the bacterial isolates were cultured into nutrient broth containing 15% glycerol (stored at -20 °C) for a year.

2.2. Preparation of Bacterial Suspension

The bacterial isolates of *P. aeruginosa* (PAC) were grown in Luria Bertini (LB) broth (Hi-media, India) for 18 h at 37°C. The bacterial cells were washed three times with sterile phosphate buffer saline (PBS, 0.1 M, pH 7.2) (10000 rpm, 15 min). The pellets of bacteria were re-suspended with PBS (0.1M, pH, 7.2) and then the bacterial numbers were prepared at the final number of 10^8 colony form unite (c.f.u.)/milliliter (ml).

2.3. Animals

BALB/c mice 6-8 weeks old, weighing 20-25 gm were procured from Central Animal House, AL-Nahrain University, Baghdad, Iraq. Animals were kept in clean polypropylene cages and fed on a standard antibiotic-free diet

2.4. Experiment

In the present study, three test groups of mice were used. Test group A (n: 15 mice), mice administrated intranasal (i.n.) 50 µl PBS (0.1 M, pH 7.2) containing $5x10^6$ c.f.u/ml clinical isolate of *P. aeruginosa*. In test group B (n: 15 mice), mice were administrated (i.n.) 50 µl PBS (0.1 M, pH 7.2) containing $5x10^6$ c.f.u/ml of an environmental isolate of *P. aeruginosa*. The control group consisted of 15 mice administrated i.n. with 50 µl PBS. Three animals were sacrificed at different time intervals (1, 4, 24, 48, and 72 h) post-bacterial administration. The lungs from respective groups were sampled to determine the viable bacterial count and pro-inflammatory cytokines.

2.5. Levels of IL-1β and TNF-α

The mice's lungs of different groups of mice were collected (post-killing by cervical dislocation) at different time intervals (1, 4, 24, and 48h), weighted, and homogenized in 2 ml of lysis buffer containing 0.5% Triton X-100, 150 mM NaCl, 15 mM Tris, and 1 mM MgCl (pH 7.4), using a tissue homogenizer and then centrifuged at 3000 ×g, for 10 min, 300 µl of the supernatants of homogenizes of mice lungs were stored at -20 °C. The enzyme linkage immunosorbent assay (ELISA) was used to measure the mice's

pro-inflammatory cytokines. The mouse TNF-α ELISA kit and mouse IL-1β ELISA kit (KOMA BIOTECH INC) in a wavelength of 450 nm were used. The manufacturer's instructions of companies were followed [13].

2.6. Bacteria Count in Mice Lungs

The lungs of mice groups that were exposed to PAC and PAE were collected at different time intervals (4, 24, 48, and 72 h). The mice were killed by cervical dislocation. The Lungs were sectioned into several pieces. One piece of lung was placed in separate sterile tubes and weighted. Lung tissue was homogenized in PBS (0.1 M, pH 7.2) in a corning glass homogenizer. Serial dilutions of homogenized lung tissue in PBS (0.1 M, pH 7.2) were made and plated on nutrient agar plates. The plates were incubated at 37°C for 18 h and the bacterial counts were counted [13].

2.7. Statistical Analysis

The Origin 8 software was used to do all operations of statistical analysis. The data were expressed as means ± SE. The differences were evaluated using a student t-test and one-way ANOVA. The P values less than 0.05 were considered to be statistically significant.

3. RESULT

3.1. Isolation and Identification of *P. aeruginosa*

The clinical isolate of *P. aeruginosa* (PAC) was isolated from the swabs of infected burn wounds, while the environmental *P. aeruginosa* (PAE) was isolated from freshwater. The biochemical methods and VITEK technology were used to identify the isolates.

3.2. Levels of IL-1β in mice lung

In the present study, ELISA was used to measure the levels of IL-1β in lung homogenates of three groups (test group A, test group B, and control group) at different time intervals (1, 4, 24, 48 h) post-instillation intranasally (i.n.) with a standard inoculum of bacterial suspension. The significant elevation of IL-1β was observed as early as 1 h post-instillation in the test groups (as compared with the control group) and the levels of IL-1β increased dramatically with time. The maximum levels of IL-1β were observed at 4 h post-instillation. After this time point, the levels of IL-1β decreased dramatically with time and the decrease was slow in the lung homogenized of mice instilled i.n. with PAC. While the decrease was sharp in the case of the mice that instilled i.n. with a standard inoculum of PAE at 48 h post instillation. At the time point 48h, the significant elevation of IL-1β was still observed only in the case of mice that were instilled with PAC as compared with the control group. The current study proved that a clinical isolate of *P. aeruginosa* stimulates the production of IL-1β at a high level and for a long time, while the elevation of IL-1β in the case of mice instilled with PAE was limited and for not long time (**Fig. 1**). In both case the clinical isolates and environmental isolates of *P. aeruginous* could stimulate the mice lungs to produce IL-1β.

3.3. Levels of TNF-α in mice lung

In the current study, indirect ELISA method was used to measure the levels of TNF-α in lung homogenates of three groups (test group A, test group B, and control group) of mice at different time intervals (1, 4, 24, 48 h) post-instillation intranasally (i.n.) with a standard inoculum of bacterial suspension ($2*10^6$ c.f.u./50 µl). The significant elevation of IL-1β was observed as early as 1 h post-instillation in the mice lung homogenizes of test groups A and test group B as compared with the control group. The levels of TNF-α were increased dramatically with time.

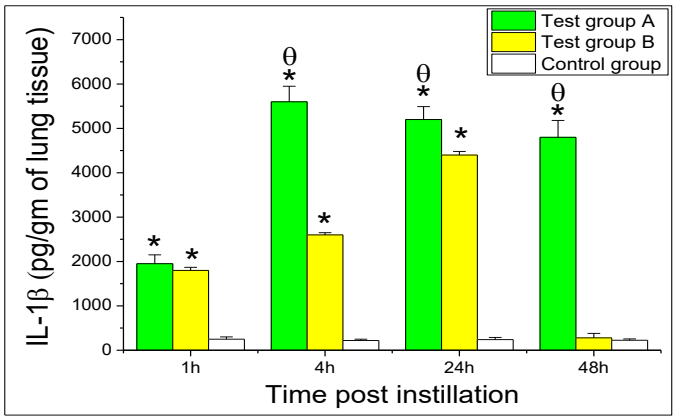

Fig. 1. Time-course of changes in IL-1β levels of homogenized lungs obtained from different groups of mice [test group A, mice instilled (i.n.) with clinical isolate *P. aeruginosa* (PAC); test group B, mice instilled (i.n.) with environmental isolate *P. aeruginosa* (PAE); control group, mice instilled (i.n.) with sterile PBS (0.1 M, pH 7.2). θ, $p < 0.05$ vs control group, *, $P < 0.05$ vs test group B.

The highest increase of TNF-α in the mice lungs was observed by 4 h post instillation i.n. in both mice groups. After this time point the levels of TNF-α decreased with time, the lowest levels of TNF-α were at the time point of 48 h. At the time point of 48 h, the level of TNF-α in the mice lungs of group A (mice instilled with PAC) was still higher than the level of TNF-α in the mice lungs of the control group. The levels of TNF-α in the mice lungs of group A were higher than the levels of TNF-α in the mice lungs of test group B (mice instilled i.n. with PAE) at the time points of 4, 24, 48 hours ($P<0.05$). The interest finding that the level of TNF-α in the mice lungs of group B was similar to the level of TNF-α in the mice lungs of control group (Fig.2). The current study proved that a clinical isolate of *P. aeruginosa* stimulates the production of TNF-α at a high level and for a long time, while the elevation of TNF-α in case of mice that instilled with PAE was limited and for not long time (**Fig. 2**). In both cases the clinical isolates and environmental isolates of *P. aeruginous* could stimulate the mice lungs to produce TNF-α, but in different quantity and the variation was in a time-dependent manner.

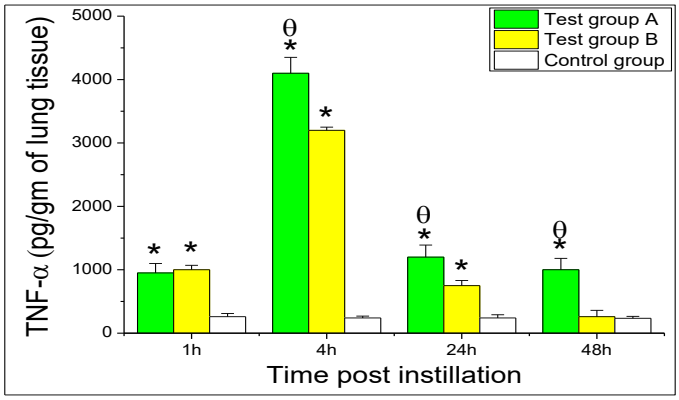

Fig. 1. Time-course of changes in TNF-α levels of homogenized lungs obtained from different groups of mice [test group A, mice instilled (i.n.) with clinical isolate *P. aeruginosa* (PAC); test group B, mice instilled (i.n.) with environmental isolate *P. aeruginosa* (PAE); control group, mice instilled (i.n.) with sterile PBS (0.1 M, pH 7.2). θ, $p < 0.05$ vs control group, *, $P < 0.05$ vs test group B.

3.4. Lung bacterial load

In the present study, the standard inoculums (2*10^6 c.f.u.) of a clinical isolate of *P. aeruginosa* (PAC) and environmental isolates of *P. aeruginosa* were administrated (i.n.) to test groups A and test group B respectively. The bacterial counts were measured at different times post-instillation. The bacterial count (number) was measured in terms of c.f.u. /gm of lung tissue. The results show that the number of PAC was higher than the number of PAE at all-time points especially at the time points 24 h and 48 h ($P<0.05$). The PAC persisted in the lung of mice up to 72 h while the PAE presence in lung tissue up to 48 h post instillation. The present study proved that the ability of clinical isolate of *P. aeruginosa* to persist in the mice lung was higher than the presence of *P. aeruginosa* that was isolated from freshwater.

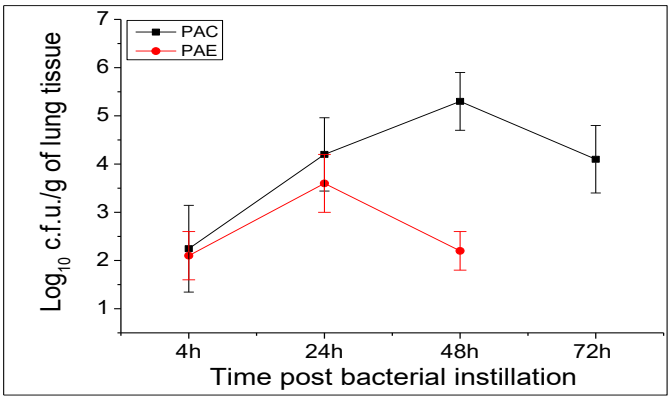

Fig. 3. Lung bacterial burden in test groups A and B of mice. Test group A, mice instilled (i.n.) with 50 µl (2 x 10^6 c.f.u.) of clinical isolate of *P. aeruginosa* (PAC); test group B, mice Instilled (i.n.) with 50 µl (2 x 10^6 c.f.u.) of environmental isolates of *P. aeruginosa* (PAE6).

4. DISCUSSION

P. aeruginosa induces a pro-inflammatory immune response. In serious infections with *P. aeruginosa* forms biofilms that escape treatment with common antibiotics and get rid of the host immune responses [13]. The biofilms stimulate a hyper-inflammatory state, resulting in collateral damage to the host tissue [14]. The infections with *P. aeruginosa* that are resistant to a wide spectrum of antibiotics lead to increased expression of pro-inflammatory cytokines such as IL-6, IL-8, IL-10, IL-1β, and TNF-α [15]. The elevation of expression of these pro-inflammatory cytokines relates to disease severity and is associated with the activation of immune signaling molecules [16]. Furthermore, the colonization of highly resistant *P. aeruginosa* as compared to commensal strains results in local and systemic pro-inflammatory responses, indicating the role of *P. aeruginosa* in stimulating pro-inflammatory immune responses [17]. However, there is no previous study highlighting the comparison between clinical and environmental isolates of *P. aeruginosa* in terms of stimulating IL-1β and TNF-α in the animal model (*in vivo*).

In the current study, a group of laboratory mice was given i.n. a standard inoculum of *P. aeruginosa* isolated from infected burn wounds, and another group was given a standard inoculum of *P. aeruginosa* isolated from freshwater. The results showed that both isolates stimulated the pro-inflammatory response in terms of IL-1β and TNF-α, but the stimulation was more severe and prolonged when the pathogenic bacteria were given to the mice. The results showed that giving pathogenic bacteria will stimulate the harmful inflammatory response because it was for a long period, while bacteria isolated from the environment stimulate a moderate pro-inflammatory immune response because the stimulation occurred for a short period and at a moderate level. This represents the first study to provide a comparison between clinical and environmental isolates of *P. aeruginosa* in terms of their stimulation of pro-inflammatory immune responses.

P. aeruginosa is a formidable pathogen that can instigate various infections, especially in individuals with compromised immune systems or underlying health conditions. It initiates the production of pro-inflammatory cytokines through the activation of PRRs. When *P. aeruginosa* invades the host, its molecular constituents, such as lipopolysaccharides (LPS) and flagellin, are identified by host PRRs like Toll-like receptors (TLRs) and NOD-like receptors (NLRs) [18, 19]. This identification event sets off a signaling cascade that ultimately leads to the activation of pro-inflammatory pathways. The activation of PRRs also triggers the activation of nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways [20]. These pathways are pivotal in the synthesis of pro-inflammatory cytokines. The activation of NF-κB and MAPK pathways brings about the transcription of pro-inflammatory genes [20]. Consequently, immune cells such as macrophages and dendritic cells are stimulated to produce pro-inflammatory cytokines [21]. Notable pro-inflammatory cytokines induced by *P. aeruginosa* infection encompass TNF-α, IL-1β, and interleukin-6 IL-6. Pro-inflammatory cytokines serve as signaling molecules that prompt the recruitment of immune cells to the site of infection. Neutrophils, specifically, are critical in the defense against *P. aeruginosa* as they aid in the eradication of the infection and combatting the pathogen [22]. While pro-inflammatory cytokines play a pivotal role in the host's defense against *P. aeruginosa*, an excessive and prolonged pro-inflammatory response may result in inflammation and damage to tissues [23]. This is especially pertinent in cases of chronic infections and conditions such as cystic fibrosis, where *P. aeruginosa* infections can persist [24]. Furthermore, the pro-inflammatory response caused by *P. aeruginosa* infection also contributes to the activetion of the adaptive immune system, involving T cells and B cells [25]. This facilitates the development of a more specific and targeted immune response.

The current study needs to know the histological effect of the respiratory tract pro-inflammatory immune response produced as a result of given *P. aeruginosa* i.n. isolated from infected burn wounds and the damage that may result from this stimulation and compared that with the histological effect of stimulation of pro-inflammatory immune response of respiratory tract of mice's lungs stimulated with *P. aeruginosa* isolated from freshwater. This will contribute to knowing the possibility of using environmental isolates to stimulate the safe pro-inflammatory response.

5. CONCLUSION

The current study demonstrated that clinical isolates and environmental isolates of *P. aeruginosa* can stimulate the pro-inflammatory response by increasing the production of IL-1β and TNF-α in the lungs of mice, but the ability of isolates isolated from infected burns wounds is greater in stimulating the pro-inflammatory immune response compared to the environmental isolate isolated from Freshwater.

Acknowledgments

We would like to thank the staff of the branch of the microbiological laboratory at, the College of Dentistry, University of Babylon, Babylon, Iraq for their valuable assistance.

Funding information

This work received no specific grant from any funding agency.

Conflict of interest

The authors declare that they have no conflict of interests.

Ethical Approval

This review was approved by the Ministry of Health, Baghdad, Iraq (No 351, 2022).

Author contributions

Huda Abbas Mohammed. Conceptualization; Data curation; Investigation; Methodology; Roles/Writing - original draft; Visualization; Writing - review & editing.

Lubna Abd Muttalib Alshalah. Formal analysis; Project administration; Resources; Software; Supervision; Validation.

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