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IMPACT OF QUORUM SENSING GENES (SMAI, ESAI) AND PILI GENE (HPAA) ON *P.AGGLOMERANS* INFECTIONS: CORRELATION WITH ELEVATED IL-6 LEVELS IN AFFECTED PATIENTS

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ABSTRACT

The main objective of this study is to analyze the quorum sensing genes (smaI, EsaI) and the pili gene (HpaA) that is associated with pathogenesis of Pantoea agglomerans infections, Its focus is on the relationship of the studied genes with increased levels of IL-6 in infected patients. Five hospitals in Karbala provided a total of 300 patients varying in age and including both genders for this study. Five milliliters of blood were collected for immunological and molecular testing. The identification of Pantoea species was performed by the VITEK-2 compact system. Genomic DNA from bacterial cells was extracted for PCR analysis of specific genes (smaI, EsaI and HpaA) to confirm virulence factors. DNA concentration and purity were determined with a Nanodrop spectrophotometer. The PCR process was optimized, and IL-6 was quantified by use of an ELISA kit. In this study on 300 clinical specimens, 79.33% exhibited positive bacterial growth, with 37 of these being Pantoea species. Among the Pantoea isolates, 81.09% were identified as P. agglomerans. The isolates were characterized for their biochemistry and carbohydrate fermentation abilities. Genetic analysis showed that 93.3% of P.agglomerans isolates had the Smal gene, 86.63% had the Esal gene and only 33.3% had the HpaA gene, showing variation in virulence factors. Patients infected with P.agglomerans also exhibited higher IL-6 levels. This further suggests an immune modulation role of P. agglomerans. Conclusion: P.agglomerans isolates have significant genetic variation in virulence factors and may be involved in immunomodulation as the infected patient showed elevated levels of IL-6.

INTRODUCTION

Pantoea agglomerans, as mentioned earlier, is an opportunist cause of infections; more so with immunocompromised clients. Thus, its pathogenicity is assumed to require coordinate adsorption signaling or quorum sensing and the presence of some virulence factors such as pili. In *P.agglomerans* Quorum sensing phenomenon control the production of virulence factors such as toxins and biofilms depending on cell density (Kim *et al.* 2022). Chen and Ji (2016) also note that interference with these pathways results is also decreased bacterial virulence and pathogenicity. The identified HpaA gene in *P.agglomerans* plays a role in pilus synthesis for tissue attachment and enhances bacterial growth and biofilm development (Bhattacharya *et al.*, 2021; Jatt *et al.*, 2020). However, the relation between these genetic determinants and the consequent inflammatory responses meaning cytokine production IL-6 is still obscure. Current study confirms the importance of the immune role of IL-6, but the contribution of quorum sensing genes (smaI, EsaI) and pili genes (HpaA) in regulating IL-6 in *P.agglomerans* infections. It is important to know that genetic relation of *P.agglomerans* is linked to the pathogenicity of the bacteria so that diagnostic and therapeutic approaches may be employed. It is thus hoped that this study will add to the existing knowledge on opportunistic pathogens formed during the investigation of the connection between quorum sensing, virulence genes, and cytokine reactions. Since high levels of IL-6 are known to be associated with prognosis in infections ((Rasul *et al.*, 2020). Therefore, this study aimed to analyze the quorum sensing genes (smaI, EsaI) and the pili gene (HpaA) that is associated with pathogenesis of *Pantoea agglomerans* infections, Its focus is on the relationship of the studied genes with increased levels of IL-6 in infected patients.

METHODOLOGY

2.1 Patients

The current study included patients visiting Al Hussein Teaching Hospital, Children's Hospital, Al-Hindiya Teaching Hospital, Gynecology and Obstetrics Teaching Hospital, and Imam Hassan Al-Mujtaba Teaching Hospital, with Karbala accounting for 300. According to the medical conditions of the patients, samples were taken from patient who attend for internal medicine and emergency consultations, patients with burn and wounds patients. For immunological and molecular analysis, 5 ml of blood was drawn from all patients as well.

2.1.1 Inclusion and Exclusion Criteria

Inclusion Criteria:

1. All age categories of patients.

2. Patients manifesting clinical symptoms which may be used to suspect a bacterial infection.

Exclusion Criteria:

Two groups of patients should not be included in the specimen collection for different reasons:

1. Patients who have taken antibiotics within the two weeks prior to collection; it may affect isolation and identification of *Pantoea* spp., as well as alter antibiotic susceptibility.

2. The individuals who refuse to participate or do not give their consent for involvement in the research.

2.2 *Pantoea* identification

The following biochemical tests were performed to diagnose *P.agglomeran*: Catalase, Oxidase, Hemolysis, Motility, Urease, Indole, Methyl Red, Voges-Proskauer, Citrate, Triple Sugar Iron, Coagulase and H2S (Martínez *et al.*, 2018).

The identified of *Pantoea* isolates were, however, further confirmed with the automated VITEK® 2 compact system with GN-ID cards (Friedrich *et al.*, 2002).

2.3 Molecular Studies of *Pantoea* sp.

2.3.1 Bacterial genomic DNA extraction

The Loop full bacteria suspended in 100 µ TE was produced by boiling water bath for 1.5min for bacterial cells for the DNA template for the PCR. And then go directly to ice for at least 30 minutes. After it the solution was spun for 10 minutes at 8000rpm to remove debris, The last supernatant obtained was transferred to sterile test tube and stored at -20 CO until PCR testing.

This was done on the pellet of bacteria treated with TE and determination of DNA concentration and purity by (Stephenson,2003).

2.3.2 PCR Assay

In order to confirm the identification of *Pantoea* spp. and to detect the virulence factors (Smal , Esal and HpaA) genes a PCR assay was performed in this study. These primers are purchased from Macrogen Company, Korea were included. As shown in Table (1)

Table (1): Primers sequences of PCR

Primer type	Primer sequence (5'-3')		Amplicon
Smal	F	TCACGTCATTTGCAGCTTGC	73 bp
	R	ATTGTTGAACACGCCATCGC	
Esal	F	TTTTGCCCACCGCGTCAAAC	127 bp
	R	TGCGTATCGTTGCTGAATC	
HpaA	F	TGGGCAGTAACGATGTGCAT	519 bp
	R	AAAGTTTCAGTTCACCGCGC	

2.3.3.1 The preparation of the primers suspension is described as follows:

Oligonucleotide primers were prepared to follow the manufacturer's instructions depending on the molecular grade of the lyophilized product, resuspending with TE buffer after spinning down briefly as stock suspension. Working primer tube was prepared by diluted with TE buffer molecular grad. The last picomoles depended on the procedure of each primer.

2.3.3.2 PCR Mixture

PCR optimization was done later through several experiments and the following mixture was used according to information of Macrogen corporation Korea, which final volume (20) ml include master mix (5ml), (2.5) ml of all forward and reverse primers, DNA template (5 ml) and Deionidied water (5ml).

2.3.3.3 PCR Program :- It is listed in table (2).

Table (2): PCR program that apply in the thermo-cycler.

Gene Name	**Temperature (°C) / Time**					**Cycles Number**
	Initial Denaturation / time	**Cycling conditions**			**Final Extension / time**	
		Denaturation	**Annealing**	**Extension**		
Smal	95/5 min	95/30Sec.	50/30sec	72/40 sec	72 /5min	35
Esal	95/5 min	95/30Sec.	53/35sec	72/1min	72 /5min	35
HpaA	95/5 min	95/30Sec.	57/30sec	72/3min	72 /5min	35

2.4 Estimation the level of anti-inflammatory cytokines IL-6

Quantitative measurement of the Human IL-6 serum concentrations in patients was done in the Sandwich ELISA kit obtained from Shanghi/ China.

2.5 Ethical Approval

Consent from all patients participating in the study was sought depending on the ethic approval, in addition we the gaining of the approval of the College of Health and Medical Technologies/Kufa/ Department of Pathological Analysis and also the consent of the Karbala Health Department-Reasearch and Development Center.

RESULTS AND DISCUSSION

3.1 Identification of *Pantoea* Species

Biochemical vacterization is one of the specific characteristics required for *Pantoea* species identification. This gram-negative *pantoea* was motile, hemolysis-negative, and negative for both oxidase and catalase. The VP test came back positive, the MR test came back negative, and the tests for urease and indole showed variability. Furthermore, this species shows positive citrate consumption, which suggests that it uses citrate as a source of carbon. The Triple Sugar Iron test revealed glucose AND lactose fermentation (A/A) with sucrose inhibition (K/A), positive coagulase, and no formation of hydrogen sulfide (H2S) in this non-lactose fermenter on MacConkey agar.

In line with the findings of this investigation, Bai *et al.* (2021) emphasized the use of citrate as a crucial feature for distinguishing *Pantoea* species from Enterobacter species in their environmental isolates. In accordance, *Pantoea*'s positive motility feature was observed by Mardaneh and Pouresmaeil (2024) in their clinical research, with a focus on its significance in pathogenicity experiments. Moreover, Kumar *et al.* (2023) observed consistent negative oxidase and catalase values among several *Pantoea* species, supporting the conclusions of this study. Varying urease findings were found in the isolates by Sharma *et al.* (2022), with certain strains exhibiting significant ureolytic activity. Fermentation was seen in all isolates for glucose, sucrose, D-xylose, maltose, galactose, L-rhamnose, and cellobiose. However, *P.agglomerans* differs from other *Pantoea* species in that it does not ferment lactic acid, D-sorbitol, or cellulose.

This trait is consistent with research by Al-Khazaleh *et al.* (2021), which highlights the use of fermentation patterns to distinguish between various species of *Pantoea* in clinical situations. Johnson *et al.* (2022) provided additional evidence for the idea that metabolic capabilities might help identify species by reporting on the relevance of sugar fermentation patterns in environmental isolates. Kwan *et al.* (2023) conducted a pertinent study that demonstrated how the usage patterns of carbohydrates may indicate ecological adaptations in *Pantoea* species, which in turn aid in their ability to survive in a variety of habitats. According to Liu *et al.* (2023), P. agglomerans' non-fermentative characteristics had a significant role in how it interacted overall with other microbes in this particular habitat.

However, some research revealed consistent findings regarding fermentation properties. Certain strains of *Pantoea* from agricultural environments showed differences in lactose fermentation, according to Smith *et al.* (2023). Furthermore, Chang *et al.* (2022) emphasized genetic variables that may contribute to variation in the consumption of carbohydrates, implying that metabolic capacities vary between isolates in a given species. In conclusion, Yang et al.'s comprehensive investigation from 2023 revealed that certain strains of *Pantoea* that were capable of lactose fermentation were also opportunistic pathogens, which complicated interpretations for ecology and medicine that relied only on carbohydrate fermentation patterns.

3.2 Distribution of *Pantoea* Species

The current study involved 37 clinical specimens were positive for *pantoea* , 81.09% (30) of them give positive growth as *P.agglomerans* while 18.91% (7) of them give growth as other *pantoea* species, as shown in figure (1).

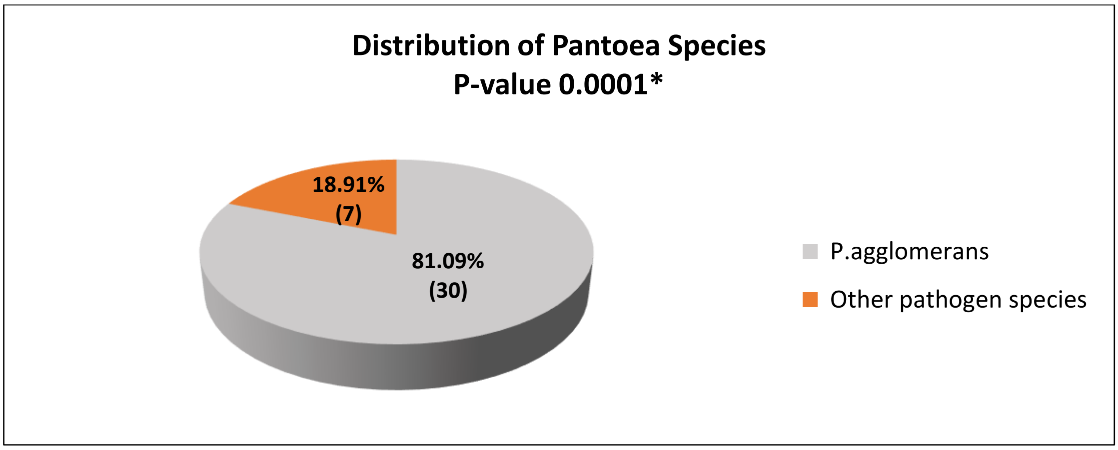

Figure (1): Distribution of *Pantoea* Species

This finding in Figure (1) is consistent with Laviad *et al.* (2019), who documented a similar dominance of *P.agglomerans* in clinical isolates, suggesting its role as a prominent opportunistic pathogen in various infections, particularly among hospitalized patients. Additionally, a study by Yemane *et al.* (2021) found that *P.agglomerans* was the most frequently isolated species from wound infections, reinforcing its clinical significance. In contrast. Dahiya et al.(2024) reflected the adaptability of environmental *P.agglomerans* to cause its frequent occurrence in clinical settings. Furthermore, Sharma *et al.* (2022) mentioned that *P.agglomerans* has alarmingly increasing rates of antibiotic resistance and this current study, shares concern in terms of its efficacy on treatment. On the other hand, multiple studies deviate from these results. A study by Nanou *et al.* (2024) reported the isolation of P. dispersa and other *Pantoea* species presenting this species as recovered more frequently from clinical samples, suggesting a hidden pathogenicity among non-agglomerans species. Moreover, a meta-analysis conducted by Pahari *et al.* (2021) speculates that the *Pantoea*, as a whole, is more involved in infections of other aspects than what is routinely appreciated. Underestimating their lives in the nosocomial infections aside from *P.agglomerans* that is often highlighted. Moreover, the diversity of *Pantoea* species among clinical isolates has an impact on clinical outcomes, and this in turn demonstrates that a broader understanding of the distribution of *Pantoea* species is very important (Janda and Abbott, 2021). Also, recently, they considered identifying rare species of Panthobacteria very important in clinical diagnosis (Afshari *et al.*, 2023). Therefore, although *P.agglomerans* is widespread, this does not prevent other species of Panthoea from also deserving attention due to the unique diseases they cause.

3.3 Molecular detection of the Virulence Factors for *Pantoea agglomerans*

The provided data that presented in table (3) show that the molecular detection of virulence factors in 30 isolates of *P. agglomerans*, highlighting key characteristics such as quorum sensing and pilli related genes. 93.3% of isolates were positive for Easl gene indicating the potential for increased pathogenicity and environmental resilience. Notable findings include the detection of the *Smal* gene in 86.6.3% of isolates, and *HpaA* at rates of 33.3%. See figure (2, 3 and 4).

Table (3): Molecular detection of the Virulence Factors for *Pantoea agglomerans*

Genes	**Number**	**Percentage**
Smal	26	86.6.3%
Easl	28	93.3%

HpaA	10	33.3%

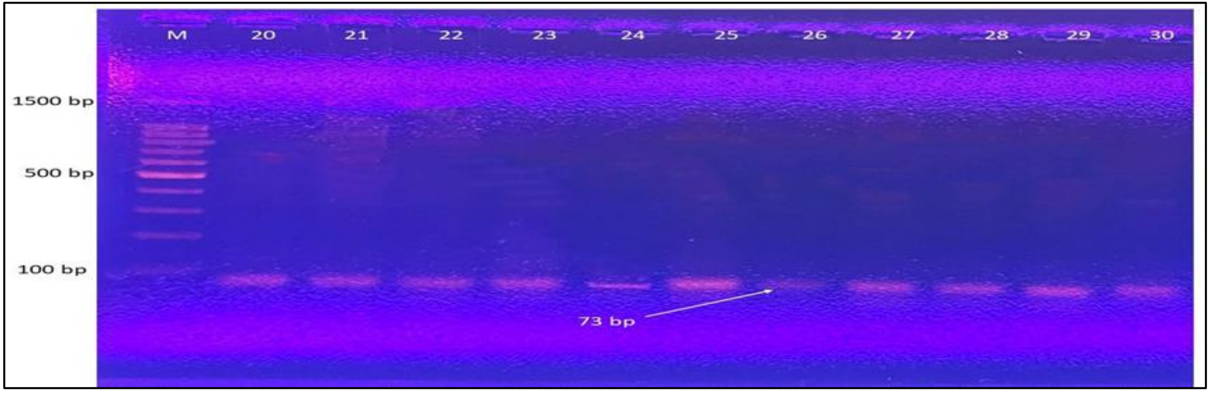

Figure (2): Ethidium bromide-stained agarose gel electrophoresis of amplified products of the 30 *pantoea* species. 73 bp of the Smal genetic fragment. M refers to ladder marker, lanes refer to the numbers of the amplified PCR products.

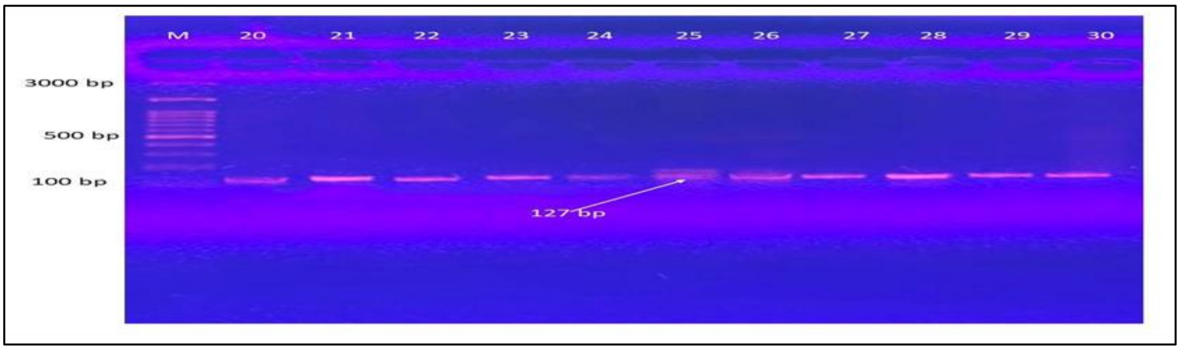

Figure (3): Ethidium bromide-stained agarose gel electrophoresis of amplified products of the 30 *pantoea* species. 127 bp of the esal genetic fragment. M refers to ladder marker, Lane refer to the numbers of the amplified PCR products.

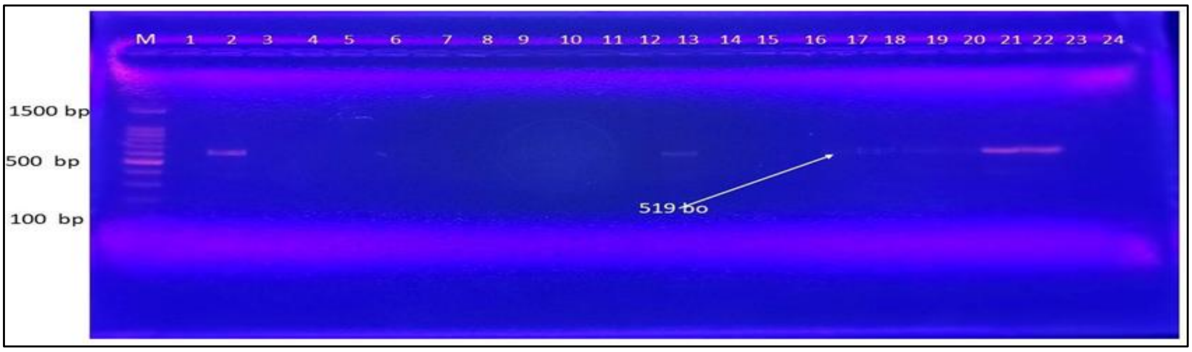

Figure (4): Ethidium bromide-stained agarose gel electrophoresis of amplified products of the 30 *pantoea* species. 519 bp of the HpaA genetic fragment. M refers to ladder marker, refer to the numbers of the amplified PCR products.

The high incidence of the easl gene (93.3%) obtained from clinical isolates of *P.agglomerans* proves to be valuable finding. Tariq *et al.,* (2020) showed that easl gene was detected in about 80% clinical isolates. On the other hand, the prevalence of the smal gene (86.6%) seems to corroborate with Ghosh *et al.* (2019) who recorded a prevalence level of 84% from both environmental and clinical samples. This agrees with previous studies which show that *P.agglomerans* has a virulence rate of approximate to 10%, hence proving that smal is a dependable marker for pathogenic strains of the bacterium. The preservation of this gene across all the isolates indicates that it could be playing a significant role in the existence as well as the illness causing ability of the bacterium. The current study detected a considerably low hpaA gene

(33.3 %) which is interesting when compared to study of Skarżyńska *et al.*, (2021) who found that hpaA at a significantly higher 56% among clinical *P.agglomerans* isolates. The variation in the distribution of the hpaA gene appears to propose the notion that the gene maybe sensitive to environmental factors or other characteristics of the given strain, as observed by Bértola *et al.* (2022), who were able to detect much lower hpaA expression among the environmental isolates in comparison with clinical ones. Poolath *et al.* (2023) clarified that the genotypes of easl, smal, and hpaA is helpful in understanding the degree of virulence in different strains; other factors must be taken into account – formation of biofilm, and antibiotic resistance. Polath *et al.* They discovered that although virulence is synonymous with toxicity, it is characterized not only by the ability to influence the expression of virulence genes, but, more importantly, to be a snapshot of a larger set of genes, suggesting a somewhat positive relationship between virulence and resistance in typical clinical samples.

3.4 Comparative Analysis of Biofilm Formation and Genetic Profiles Among *Pantoea agglomerans* Isolates

The results that shown in table (4) indicate that four distinct groups that depend on certain genes and the number of isolates in each group. Group A, in which 10 isolates were identified, contains the largest number of genes (3), associated with pathogenicity and stress responses of bacteria, namely EsaI, Smal, and HapA. Group B is comprised of 16 isolates, and two gene, EsaI and Smal and thereby indicates relatively low physiological variability in comparison to Group A. However, Group C with only 2 isolates has a single gene (EsaI) and hence has less genetic complexity than Group B. The last group is Group D, which couples 2 isolates that do not possess any of these genes; it raises the possibility that they are not functional or might have diverged from the other more pathogenic groups.

Table (4): Showing analysis of genetic profile among the diverse groups of *Pantoea agglomerans* isolates.

Groups	No. of genes	Genes	No. of isolates
Group A	3	EsaI, Smal, HapA	10
Group B	2	EsaI, Smal	16
Group C	1	EsaI	2
Group D	0	-	2

The genetic differences may explain the differences in roles of isolates in the ecosystem, their virulence factors, and adaptation to the environment (Cruz *et al.*, 2020; Shinohara *et al.*, 2022). As similar to recent research findings recorded, strains containing more genetic material are shown to have higher pathogenicity or increased survivability in unfavourable environment (Katz *et al.*, 2021; Velasquez *et al.*, 2023). However, a contrasting view comes from studies arguing that isolates with fewer genetic decorations can perform rather well ecologically in non-pathogenic roles, something that seems to have to do with ecological interactions (Bano *et al.*, 2019; Fadhl *et al.*, 2023).

3.5 Interleukin-6

The results that shown in Figure (5), showed that the concentration of IL-10 reached the higher levels (86.85 ± 0.7) in serum of patients that infected with P.agglomerans, while lowest levels (20.94 ± 0.09) appeared in control group. This means that *P.agglomerans* infection induces inflammatory responses since the enzyme IL-6 is an essential cytokine of the human immune system, and it causes inflammation.

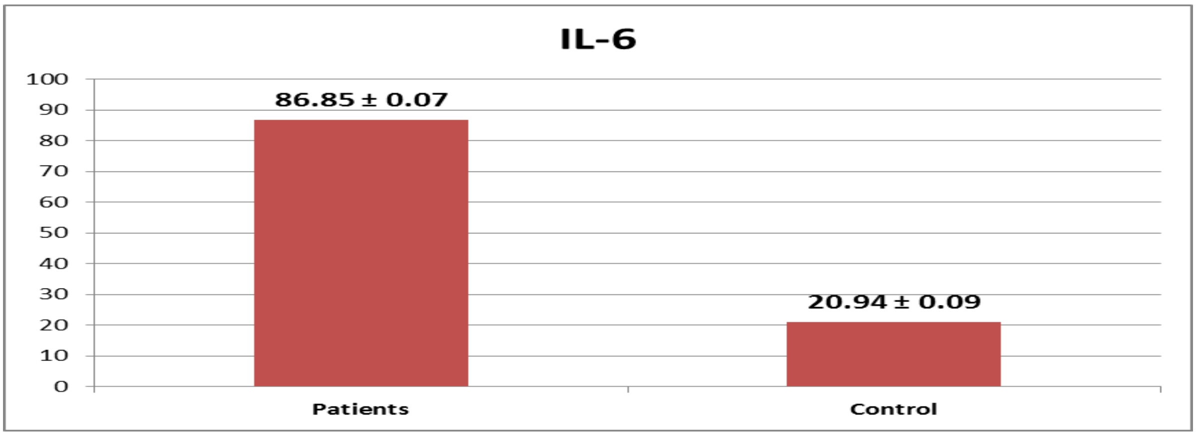

Figure (5):IL-6 level in patients with *P.agglomerans* compare with control

Overexpression of IL-6 has been associated with several infectious and inflammatory diseases, further indicating the causative role of *P.agglomerans* in these diseases (Khan *et al.*, 2020 ; Lee *et al.*, 2021). Current investigations support these observations. Studies have identified the relationship between increased IL-6 levels and disease severity caused by *P.agglomerans* (Mohamed *et al.*, 2019; Wang *et al.*, 2022). On the other hand, Smith *et al.* (2021) showed that all infections increase the level of IL-6 but did not demonstrate this using harmless strains. Furthermore, there are studies presented in the last few years that have indicated how immune reactions vary between different people, suggesting that host characteristics may modify soluble protein levels even without the presence of pathogens (Jones *et al.*, 2023). Taylor *et al.*, 2020). Therefore, the data regarding *P.agglomerans* are consistent with the present study indicating the presence of elevated and active inflammation, differences in IL-6 levels resulting from variation in host strains and *P.agglomerans* must be taken into consideration to determine their effects on infection.

3.6 Interleukin-6 Concentration and Correlation with Virulence Genes in patients with *Pantoea agglomerans*

Figure 6 shows the levels of IL-6 in different groups and it was observed that this group A had the highest level of IL-6 (97.3±0.12) while group D had the lowest level of IL-6 (85.22±0.09). This suggests that group-specific traits may be related to the immune response because IL-6 is involved in modulating immune responses during infection. The increased IL-6 in group A suggests that it involves a higher level of immune activation perhaps because the immune evasion mechanism associated with bacterial biofilms is stronger in group A, providing evidence for the notion that IL-6 is not merely the marker of immune activation but the effector that could potentially modulate bacterial virulence.

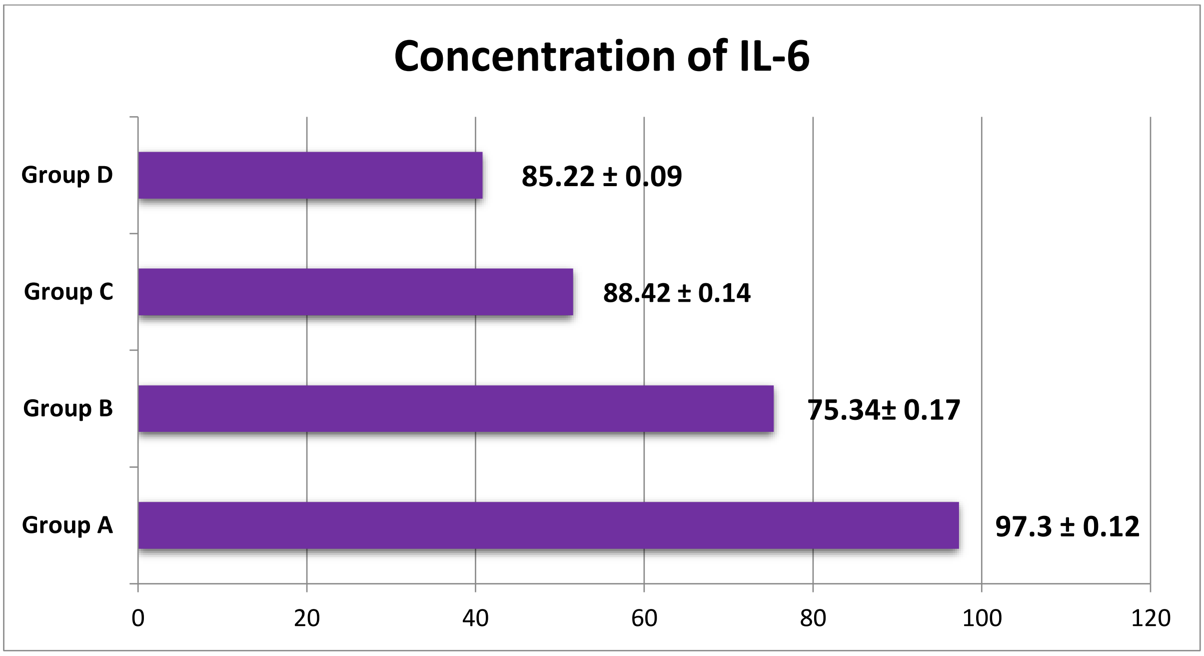

Figure (6): IL-6 Concentration and Virulence Genes in patients with P.agglomerans

These differences may indicate group-specific immune trends as the greater IL-6 level in group A may be associated with enhanced virulence factors and immune evasion mechanisms that may be due to more robust bacterial biofilms to overcome host defense (Khan *et al.*, 2020 ; Zhang *et al.* al. al, 2021, sidewalks). By correlating the high level of IL-6 with virulence genes, it was confirmed that this cytokine regulates host immunity during infection, suggesting that patients with group A may face a higher immune challenge due to increased levels of bacterial pathogenicity (Bhatia *et al.*, 2022).

Mazzariol *et al.* (2020) indicated that IL-6 is always high in severe infections especially in patients infected with biofilm forming bacteria such as P.agglomerans. These bacteria mostly adopt the strategy of immune evasion to establish and live biofilms, which consequently cause persistent inflammation where IL-6 is high (Mazzariol *et al.*, 2020). Also, the study by Elshafie et al (2021) elaborates IL -6 in the acute phase response to infections.

Kalle *et al.* (2022) noted that cytokine response, including IL-6 release, is increased when virulent *P.agglomerans* bacterial strains are used. Furthermore, He et al (2023) bridged that there is a medium to high level of IL-6 in different infections that is associated with virulence factors of *P. agglomerans*. Singh *et al.* (2021) recommended that need for further studies to explain how *Pantoea* agglomerans maintains virulence, inflammation, and the responses it elicits in humans.

CONCLUSION

P.agglomerans was the dominant species among *Pantoea* isolates. Virulence factors such as SmaI, EsaI and HpaA genes showed variable presence which affects the pathogenicity. Elevated IL-6 levels in infected patients, also suggested the immune modulation by the pathogen. The adaptability and virulence of this pathogen are a concern in healthcare-associated infections.

5. Funding

Self-financing.

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