Research article

Molecular detection and expression of virulence factor encoding genes of *Pseudomonas aeruginosa* isolated from clinical samples

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ABSTRACT

Introduction and Aim: *Pseudomonas aeruginosa* virulence factors genes are a growing concern as they are involved not only in its pathogenicity, but also cause bacterial resistance to multiple classes of antibiotics. Laboratory identification of clinical isolates carrying the virulence genes would be critical in limiting the bacteria's spread and reducing its pathogenicity. The purpose of this study was to investigate a simple and inexpensive real-time PCR test to find the level of expression of *Pseudomonas aeruginosa* virulence genes before and after treatment with specific concentration of *Lactobacillus acidophilus* cell-free supernatants (CFSs).

Materials and Methods: Between December 2021 and June 2022, 350 clinical samples collected from Baghdad hospitals, Iraq, were tested for the presence of *P. aeruginosa*. The *P. aeruginosa* isolated were tested for their antimicrobial susceptibility using the Kirby-Bauer disk diffusion method. *P. aeruginosa* virulence genes were detected by using the reverse transcription-PCR method. The expression levels of these genes before and after treatment with *Lactobacillus acidophilus* cell-free supernatants were measured by real-time PCR.

Results: Out of 350 samples tested, 60 isolates were positive for the presence of *P. aeruginosa*. Antibiotic susceptibility tests revealed a high level of antibiotic resistance, while genetic techniques identified the presence of several virulence genes that exhibited variable expression under the influence of *Lactobacillus acidophilus* supernatants.

Conclusion: The study findings showed that L. acidophilus supernatants had an effect on reducing the expression of certain virulence genes of P. aeruginosa, implying that L. acidophilus could be used as an option in treating *P. aeruginosa* infection.

Keywords: *Pseudomonas aeruginosa;* MIC; virulence factor genes; gene expression; real time- PCR; *Lactobacillus acidophilus*.

INTRODUCTION

Pseudomonas aeruginosa are aerobic, motile, non-spore forming gram-negative rods that can easily grow at temperatures of 40–41°C, *P. aeruginosa* occur commonly in soil, water, plants, humans, animals, and hospitals (1). The bacteria are significant opportunistic pathogens responsible for nosocomial infections in immunocompromised individuals and the high mortality rates in burn centers (2). This pathogen is also implicated in Infections of the bladder, eye, blood, surgical wounds, and cystic fibrosis (3).

The pathogenicity of *P. aeruginosa* has been largely attributed to its virulence factors and genetic adaptability that enable it to live in a variety of environments (4). Antibiotic resistance in bacterial populations has nearly reached a crisis level in nosocomial healthcare (5). *P. aeruginosa* is unique in that they have a variety of virulence factors that enable them to colonize their host organism's body and cause disease-causing infections (6). Virulent factors enable them to infect and invade the host organism, by destroying the immune response in the host and forming a barrier against antibiotics, where it plays an important pathogenic role in colonization, survival of bacteria, and invasive tissues (7). These bacteria's pathogenicity is a result of their virulence components, which include pili, flagella, proteases, lipases, elastase, and numerous toxins, such as pyocin, hydrogen cyanide, toxins of the type III secretion system, and exotoxin A (8).

Most microorganisms used as probiotics are bifidobacteria and lactic acid bacteria (LAB). Due to their potential benefits as probiotics, LAB *lactobacilli* species are the most frequently used group of microorganisms. Numerous pathogenic bacteria are known to be inhibited by the antagonistic activities of these bacteria (9). It has been established that *Lactobacillus* spp. plays a function in preventing and treating certain infections.Commensally residing *Lactobacillus* bacteria in the human body has been shown to exert its positive effect due to its ability to secrete antibacterial compounds such as lactic acid and hydrogen peroxide (10).

Probiotics are viewed as very safe, non-pharmaceutical solutions to treat and prevent a variety of diseases, including UTIs. Probiotics are defined as "live microorganisms which, when administered in adequate amounts confer a health benefit on the host" due to presence of members of the genus *Lactobacillus* which

are considered and recognized as safe (11). Hence, the purpose of this work was to examine a simple real-time PCR assay to determine the level of expression of *P. aeruginosa* virulence genes before and after treatment with specific amounts of *Lactobacillus acidophilus* cell-free supernatants (CFSs).

MATERIALS AND METHODS

Collection and identification of bacterial isolates

The clinical samples were taken directly from patients in different Baghdad hospitals (Al-Yarmouk Hospital, Baghdad Hospital, Al-Kadumia Medical City, and Al-Karama Hospital) between December 2021 and June 2022. These samples included wounds, burns, sputum, urine, and ear swabs. in sterile conditions for the isolation of *P. aeruginosa*, samples were collected and cultivated in suitable media, and the detection of smelly scent, hemolytic activity, sugar fermentation, and other biochemical features (12). To isolate *Lactobacillus* spp. were taken using sterile swab sticks. These sterile swabs were then put into sterile screw cap tubes filled with MRS broth and transported under refrigerated conditions to the lab. In the laboratory the sample was cultured on MRS agar (13).

The identification of *P. aeruginosa* and *lactobacillus spp*. in this study was based on colony morphology, microscopic investigations and specific biochemical assays. An automated Vitek2 system was used to identify *P. aeruginosa* and *lactobacillus spp*., which is a new technique for identifying bacteria in clinical samples (14).

Antimicrobial susceptibility test

P. aeruginosa isolates were tested for their antimicrobial susceptibility using the Kirby-Bauer disk diffusion method. Briefly, the isolate was first grown in 5 ml Mueller-Hinton broth to 0.5 McFarland turbidity (15). A loopful of the culture grown was transferred onto Mueller-Hinton agar plate and spread plated. Using sterile forceps, the antibiotic disk was placed on the inoculated plate, followed by overnight incubation at 37° C. The results were interpreted as per Clinical and Laboratory Standards Institute guidelines (CLSI) (16). The strains were considered as multidrug drug resistance (MDR) if they were susceptible to three or more antibiotics.

Amplification of *P. aeruginosa* 16SrRNA and virulence genes

The 16SrRNA gene and selected virulence genes (*toxA, phzI, phzII, exoS, exoT*) in *P. aeruginosa* genomic DNA was investigated by PCR. The primers employed to amplify the respective genes are designed in this study according to NCBI, listed out in Table 1. PCR was carried out in a thermal cycler (Thermo Fisher Scientific, USA) using the following thermocycling conditions: 1 cycle of initial denaturation at 94ºC for 5min, followed by 38x cycles of all (first denaturation at 94ºC for 30 sec, second annealing at 57ºC for 45 sec, and extension at 72ºC for 45 sec.) and the final extension at 72ºC for 7 min. The PCR amplified products were stained with ethidium bromide and the band size determined by gel electrophoresis.

Preparation of *lactobacillus acidophilus* supernatant

L. acidophilus strain was inoculated to MRS broth and incubated overnight at 37°C. After growth, the culture was centrifuged at 5000 rpm for 30 minutes, and the supernatant filter sterilized using 0.22 m pore size filter paper to obtain a crude cell-free supernatant (CFS) (17). The *L. acidophilus* CSF was tested for its minimum inhibitory concentration (MIC) for *P. aeruginosa* by broth dilution method **(**13).

Determination of minimum inhibitory concentration (MIC) and sub-MIC of *P. aeruginosa*

The MIC and sub-MIC of *P. aeruginosa* under effect of CFSs was detected by the broth dilution method (13). The dilution concentrations used are 10^{-1},10^{-2}, 10^{-3}, 10^{-4} ,$10^{-5}$and 10^{-6} (13).

Table 1: Primers used in this study

Gene	Primer	Sequence 5' to 3'	Size (bp)
16SrRNA	Forward Reverse	AGGGCCATGATGACTTGACG TCGTGTCGTGAGATGTTGGG	143
Virulence genes			
Tox A	Forward Reverse	AGCCCTCGAACATCAAGGTG CCTGACGAAGAAGGTGGCAT	137
Exo T	Forward Reverse	AAATCGCCGTCCAACTGCAT GTTCGCCTAGGTACTGCTCC	153
Exo S	Forward Reverse	CGAAATCACCGACCAGTTGC GCTGTCTGCCCAGGTACTTT	158
PhzI	Forward Reverse	TCAGCTTAGCAATCCCGCAT TCGCGAAGACTTTCAGCGT	153
PhzII	Forward Reverse	ATGAGAAAGACCGCCGTGAG GACGACGAAACGAGGCTGAA	151

Real-time assay (qRT-PCR) for *P. aeruginosa* genes

Six antibiotic-resistant *P. aeruginosa* isolates which showed the presence of virulence genes were chosen for further investigation into their expression levels before and after probiotic treatment using the qRT-PCR assay. RNA was extracted from each of the six *P.*

aeruginosa isolates using the manufacturer's protocol for TRIzol™ (Invitrogen, USA). The RNA concentration was determined using a Quantus fluorometer (Promega, USA). The total RNA obtained was reverse transcribed to cDNA using a protoscript cDNA synthesis kit (NEB®, UK) and stored at -80 °C until use. Real-time assay was carried out using a 20 µl volume of the DNA in a QUBIT® Real-time PCR System (Thermofisher®, USA). The qPCR reaction run was set as follows: 1 cycle of initial denaturation at 95ºC for 60sec, followed by 40-45 cycles of denaturation (95ºC for 15 sec) and extension (60ºC for 30sec), and a melt curve set at 60-95ºC for 40 min. The results obtained were analyzed using the qPCRsoft software (18,19).

Gene expression analysis

To evaluate the transcript levels in various samples at various CFSs concentrations, the CT of the target gene was adjusted to be equal to the CT of the internal control gene. The fold of expression difference between isolates was calculated based on the concentration of CFSs, with the low concentration of CFSs serving as a calibrator and the high concentration as a test group:
The results obtained were evaluated using the Ct and Livac formulae.
ΔCt A = CtGol A – CtRef A
ΔCt B = CtGol B – CtRef B
ΔΔCt = ΔCt A - ΔCt B
Normalized Ct expression Formula= 2-(ΔΔ Ct)

RESULTS

Isolation of *P. aeruginosa*

In this study, *P. aeruginosa* was detected in 60 (17%) of the 350 clinical samples analysed.

Antibiotics susceptibility test

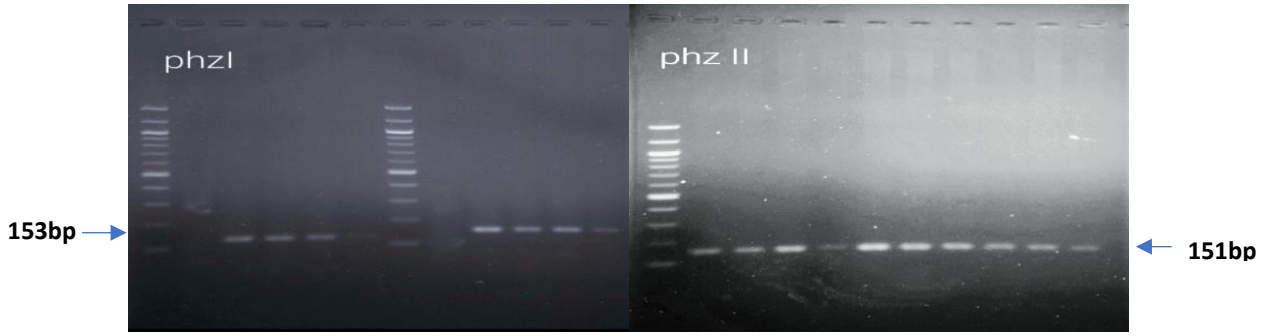
Table 2 shows the antibiotic susceptibility pattern of *P. aeruginosa* isolates against 15 different antibiotics. As seen from Table 4, *P. aeruginosa* isolates exhibited high resistance to the antibiotics tetracycline (90%), Aztreonam (86.7%), erythromycin (85%), Ceftriaxone (66.7%), Ceftazidime (65%). On the other hand, the *P. aeruginosa* isolates were highly sensitive to the antibiotics Imipenem 85%, Ciprofloxacin (80%), Levofloxacin Meropenem (78.3%) and (66.7%; Table 2).

Detection of *P. aeruginosa* virulence genes

PCR for the virulence genes *toxA* (137bp), *phzI* (153bp), *phzII* (151bp), *exoS* (158 bp) and *exoT* (153bp) revealed that except for 4 strains of *P. aeruginosa* which was negative for the *phzI* gene, the remaining were all positive for all the virulence genes tested (Fig.1).

Table 2: *P. aeruginosa* antibiotic sensitivity test from clinical sample isolates

Antibiotics	**Conc. µg/disc**	**Code**	***P. aeruginosa* isolate (n=60)**		
			Resistant	**Intermediate**	**Sensitive**
			No. (%)	**No. (%)**	**No. (%)**
Amikacin	30	AK	23 (38.3%)	13(21.7%)	24(40%)
Aztreonam	30	AZT	52(86.7%)	1(1.6%)	7(11.7%)
Cefepime	30	FEP	38(63.3%)	0	22(36.7%)
Ceftazidime	30	CAZ	39(65%)	3(5%)	18(30%)
Ceftriaxone	30	CRO	40(66.7%)	4(6.7%)	16(26.6%)
Ciprofloxacin	5	CIP	12(20%)	0	48(80%)
Erythromycin	15	E	51(85%)	0	9(15%)
Gentamicin	10	CN	33(55%)	2(3.3%)	25(41.7%)
Imipenem	10	IMP	7(11.7%)	2(3.3%)	51(85%)
Levofloxacin	5	LEV	17(28.3%)	3(5%)	40(66.7%)
Meropenem	10	MEM	11(18.4%)	2(3.3%)	47(78.3%)
Norfloxacin	10	NOR	26(43.3%)	8(13.4%)	26(43.3%)
Piperacillin	100	PIP	10(16.7%)	18(30%)	32(53.3%)
Tetracycline	30	TE	54(90%)	0	6(10%)
Tobramycin	10	TOB	30(50%)	6(10%)	24(40%)



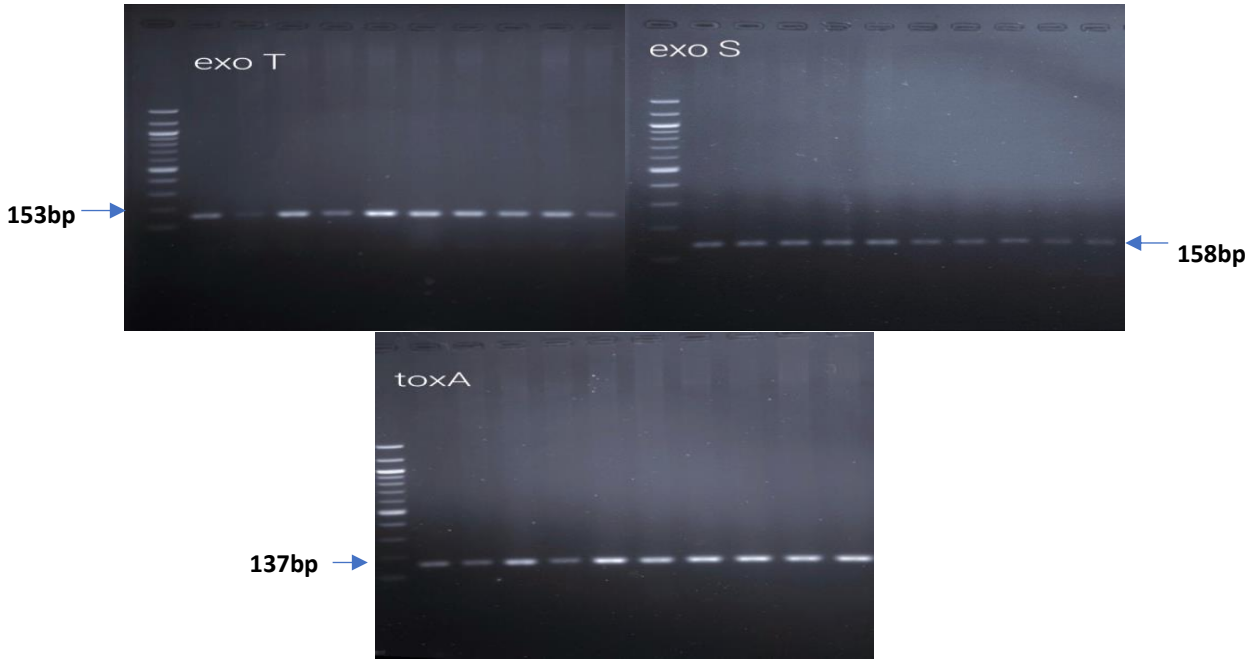


Fig. 1: PCR results for the detection of virulence genes in *P. aeruginosa* isolates

Table 3: The MIC values for *P. aeruginosa*

Isolate	*L. acidophilus* CFSs concentrations					
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
P9	-	+	+	+	+	+
P17	-	-	+	+	+	+
P32	-	-	+	+	+	+
P38	-	+	+	+	+	+
P55	-	-	+	+	+	+
P59	-	-	+	+	+	+

(+) *mean inhibition of P. Aeruginosa* growth, (-) mean, *P. aeruginosa* growth.

Determination of minimal inhibitory concentration (MIC) for CFSs

In order to determine the minimum inhibitory dose for *P.aeruginosa* in this study, six antibiotic-resistant isolates harboring all of the virulence genes tested were chosen. *P.aeruginosa* isolates grown in the presence of varying concentrations of *L.acidophilus* cell free supernatants for a period of 24h. The growth inhibition of *P. aeruginosa* at different concentrations is given in Table 3.

Gene expression studies

Studies for the expression of the virulence *genes toxA, phzI, phzII, exoS,* and *exoT* of *P. aeruginosa* showed the levels of expression varied due to CFSs stress (Table 4). As shown in Table 4, varying alterations were seen for gene expression between isolates, specifically in the genes *phzII, exoS, & exoT*. Notably, a drop in gene expression was observed in the *toxA* gene, but the *phzI* gene exhibited a minor rise in gene expression across several samples.

Based on the findings of the present study, a reduction in the expression of the *phzI* gene was observed in isolates P6 and p38, with a decrease of 33.3%. Similarly, the gene *phzII* exhibited a decrease in gene expression by 50% in isolates P9, P17, and P38. Furthermore, the *exoS* gene displayed a decrease in isolates P9, P17, and P38, amounting to 50%. Likewise, the *exoT* gene exhibited a 50% decrease in isolates P17, P32, and P38.

Table 4: Expression of *P.* aeruginosa virulence genes under CFSs stress

Genes	Expression	*P. aeruginosa* isolate					
		P9	**P17**	**P32**	**P38**	**P55**	**P59**
phzI	Expression Fold	0.87	2.14	4.92	0.0384	6.964	4.28
	Reduction	1.14	-	-	26	-	-
	Induction	-	1.14	3.92	_	5.964	3.28
phzII	Expression Fold	0.329	0.625	6.49	0.0625	4.287	1.231
	Reduction	3.03	1.626	-	16	-	-
	Induction	-	-	5.49	_	3.287	0.231
exoS	Expression Fold	0.68	0.267	3.73	0.0544	1.624	5.65
	Reduction	1.47	3.84	-	18.38	-	-

	Induction	-	-	2.73	-	0.624	4.65
exoT	Expression Fold	3.03	0.535	0.535	0.0291	5.656	6.06
	Reduction	-	1.86	1.86	34.3	-	-
	Induction	2.03	-	-	-	4.6565	5.5
tox A	Expression Fold	0.42	0.61	1.24	0.211	0.615	1.41
	Reduction	2.38	1.63	-	4.739	1.62	-
	Induction	-	-	0.24	-	-	0.41

The *toxA* gene showed the highest level of expression in isolates P9, P17, P32, and P55. The isolates P9, P17, and P38 exhibited the highest rates (66.7%) of susceptibility to the decrease in virulence gene expression, whereas isolate P55 had the lowest percentage (16.6%) of susceptibility to the drop in gene expression. Isolate P59 exhibited no significant reduction in gene expression (Table 4).

DISCUSSION

The presence of *Pseudomonas aeruginosa* within a community can be attributed to the heightened prevalence of individuals with impaired immune systems, which can be caused by the contamination of hospital environments. Additionally, patients who have prolonged hospital stays are particularly susceptible to *P. aeruginosa* infections. This finding is consistent with the research conducted by Okafor *et al.*, (17).

The bacteria *P. aeruginosa* has demonstrated resistance to a wide range of commonly used antibiotics inside hospital settings. Furthermore, these bacteria possess virulence genes that confer the ability to withstand adverse environmental conditions, resist biological interventions, and facilitate long-term colonization and survival. Based on the results of this investigation, it was observed that 78.3% (47 out of 60) of the *P. aeruginosa* isolates exhibited multidrug resistance. A microorganism that exhibits resistance to a minimum of three distinct classes of antibiotics is commonly known as multi-drug resistant (MDR). This discovery contradicts the findings of previous research conducted by (20), which reported that 44.4% of the isolates exhibited multidrug resistance (MDR). A study conducted in Yemen (21) yielded similar findings, indicating that approximately 65.2% of the isolates exhibited multidrug resistance (MDR). Hence, the objective of this investigation was to explore an alternate therapeutic approach for combating *P. aeruginosa* infections.

In this study, few of the *P. aeruginosa* isolates harboring the virulence genes showed a decrease in the expression of the genes, indicating the effectiveness of the *L. acidophilus* cell free supernatants (CFSs) on the virulence genes. Given the significance of inhibiting the growth of *P. aeruginosa*, it is imperative to devise inhibitors that selectively target this pathogen. There exist studies that explore the impact of probiotics on the expression of virulence genes in *Pseudomonas* spp, specifically in relation to gene expression. For instance, a study conducted by (22) demonstrated that salicylic acid and trans-cinnamaldehyde significantly decrease the expression of quorum sensing regulatory virulence genes in *P. aeruginosa*, even at sub-inhibitory levels, without exerting a bactericidal effect. Moreover, this treatment successfully down-regulated both the *las* and *rhl* quorum sensing system genes. Furthermore, a research conducted by (23, 24) investigated the impact of the monosaccharide D-mannose on the expression of the neuraminidase gene in *P. aeruginosa* isolates. The inclusion of D-mannose resulted in a reduction in the rate of neuraminidase activity, indicating that D-mannose has an inhibitory effect on the expression of the *nan1* gene. Given its role as a competitive neuraminidase inhibitor, this sugar exhibits potential for application in the development of novel antibacterial drugs. There are many reports observing alternatives for antimicrobial agents such as plant extracts, oil, pigments, and nanoparticles were applied against pathogenic bacteria (25, 26). Ongoing scientific investigations aim to determine optimal concentrations of probiotics that can effectively down regulate the gene expression of virulence genes in *P. aeruginosa*. Consequently, this approach holds promise for diminishing the efficacy of virulence factors, mitigating pathogenicity, and potentially eradicating them altogether.

CONCLUSION

The study concluded that *L. acidophilus* supernatants reduced the expression levels of virulence factors found in *P. aeruginosa*. The study also suggests the probable use of *L. acidophilus* supernatants as an alternative in treating infections due to this pathogen.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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