ORIGINAL ARTICLE

Genetic Relationship between Virulence Genes and *arcDABC* Operon Genes of Arginine Deaminase Pathway in *Pseudomonas aeruginosa* isolated from Burn Patients

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Key words:
Pseudomonas aeruginosa, burns, antibiotics resistance, virulence genes, arcDABC operon

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ABSTRACT

Background: *Pseudomonas aeruginosa possesses a diverse of virulence genes located on chromosome, which cause infections in burn patients. It has the ability to grow under any condition of limited oxygen or its absence, because it possesses the arcDABC operon, which encodes enzymes of the arginine deaminase pathway, thus giving it ability to resist antibiotics.* ***Objectives:*** *The aim of our current research is knowing the relationship between the effect of the arcDABC genes of P. aeruginosa and virulence genes in causing infection and determine its antibiotic resistance and its genetic relationship with arcDABC genes of ADI pathway using qRT-PCR technique.* ***Methodology:*** *Twenty-five samples were obtained from patients attending and hospitalized at the Burns Hospital in Diwaniyah City / Iraq from the period January to June 2024, was isolated and identified P. aeruginosa by the standard bacteriological methods and by 16S rRNA diagnostic gene by PCR technique. The antibiotic sensitivity test was done by Kirby-Baures disc diffusion sensitivity test.* ***Result:*** *Fifteen bacterial isolates of P. aeruginosa were obtained at a rate of 60% and they possess 16S rRNA gene at a rate of 53.3%. The antibiotic resistance rate was (53.3% -73.3%). All isolates were sensitive to colistin. P. aeruginosa possess virulence genes at a rate (100%).* ***Conclusion:*** *P. aeruginosa isolated from burn infections showed high resistance to many antibiotics and high sensitivity to colistin, which is an effective antibiotic. The gene expression was high in the pathogenic strains and there is a relationship between the virulence genes and arc* of *DABC* genes.

INTRODUCTION

pseudomonas aeruginosa continues to be one of the most virulent opportunistic pathogens[1]. It is considered to be the main deriving reason of mortality and morbidity in immunocompromised patients admitted to the hospitals[2]. The bacteria are Gram-negative, non-fermentative, aerobic, rod-shaped, and living in different environments[3]. Many types of infections are acquired from the hospitals, e.g. burns, wounds, conjunctivitis, lung infections, and middle and external ear infections[4]. The ideal habitat for these opportunistic microbes, whether external or internal, is burns and wounds infections, which result from exposed body surfaces, immunocompromised status, and prolonged hospitalization during surgical operations[5]. The genes encoded by *P. aeruginosa* are responsible for its multidrug resistance and virulence which are an international problem and require urgent interest and intervention. However, there are few studies examining the virulence genes responsible for the pathogenicity of the bacteria and its resistance to multiple antibiotics. *P. aeruginosa* was isolated from clinical samples in 11countries with low income[6,7]. The genes responsible for the virulence and pathogenicity are, *toxA, exos, exoU, oprL, plcN, nanl, oprl, lasA, lasB, plcH, exoY*, and *oprD*[8]. The *toxA* gene encodes the function of exotoxin A[9]. *P. aeruginosa's* outer membrane proteins (OMPs), oprL and oprl, are crucial for how bacteria interact with their surroundings and are also a major contributor to antibiotic resistance[8]. The large genome of *P. aeruginosa* contains virulence genes that are expressed in the occurrence of many diseases that are difficult to treat[10]. The ADI pathway is widespread in bacteria that use it as a primary energy source, this pathway will provide some of the energy needed by *P. aeruginosa* under anaerobic growth conditions due to lack of O_2 or end electron acceptors[11]. Expression of the functional genes (*arcA, arcB, and arcC*) in *P. aeruginosa* is precedent by the *arcD* gene, which encoded the arginine-ornithine anti-carrier[12]. The aim of our work was to identify the relation between the effect of the *arc* of *DABC* genes of *P. aeruginosa* and its virulence genes.

METHODOLOGY

Bacterial isolates:

The samples that were subjected to the current study were 25 samples collected from patients who visited and

were admitted to the burn's hospital in Diwaniyah City/Iraq during the period from January to June 2024. The samples were obtained from all ages and both sexes and then transferred to the laboratory using culture media for primary isolation, blood agar and MacConkey agar. Identification of *P. aeruginosa* was done by morphological and cultured characteristics then sub-cultured on cetrimide agar[13]. Also, diagnosis of the isolates was done by the diagnostic gene *16S rRNA* using PCR technique.

Antibiotic sensitivity test:

The antimicrobial sensitivity of *P. aeruginosa* isolates was tested by Kirby-Bauer disc diffusion sensitivity testing based on standards guide Clinical and Laboratory Standard Institute (CLSI)[14], utilizing Mueller-Hinton Agar. The antibiotic discs (Oxoid, England) were: Meropenem (MER; 10 mg), Ceftazidime (CAZ; 30 mg), Cefepime (FEP; 30 mg), Piperacillin (PRL; 30 mg). Colistin (CL; 10 mg), Ciprofloxacin (CIP; 10 mg), Levofloxacin (LEV; 5 mg), Amikacin (AMK; 30 mg), Gentamicin (CN; 10 mg), Imipenem (IMI; 10 mg).

Bacterial genomic preparation:

DNA was extracted from bacteria under study using a special genomic DNA extraction kit provided by Presto ™Mini gDNA Bacteria Kit. DNA concentration was determined using a Nanodrop spectrophotometer at absorbance of 260/280 nμ.

Detection of virulence genes by PCR:

PCR was performed to detect (*toxA*, *oprL* and *oprI*) in *P. aeruginosa* isolates. All primers used in this technique were designed by using NCBI-GenBank sequence and Primer3 plus by Scientific Researcher.Co. Ltd from Iraq, as shown in table 1.

Table 1: Primers of some virulence genes

PCR Primer	Nitrogenous base sequence (5'-3')		Amplification size	NCBI code
16S rRNA	F	TTGGATGTGAAATCCCCGGG	719 bp	FJ972538.1
	R	CAGACTGCGATCCGGACTAC		
toxA gene	F	GTGCTGCACTACTCCATGGT	539bp	JX026663.1
	R	TCCCAGGTATCGTCGAGGTT		
OprL gene	F	TTACCTGAACTGACGGTCGC	416bp	EU286532.1
	R	GCCCAGAGCCATGTTGTACT		
oprI gene	F	ATTCTCTGCTCTGGCTCTGG	234bp	JX040480.1
	R	TACTTGCGGCTGGCTTTTTC		

The master PCR reaction mixture for all genes was prepared using (GoTaq® Green PCR master kit) and this master mixture was made according to the protocol: - 5 µl of 5-50ng DNA template, 2 µl of Forward (10 pmol), 2 µl of Reveres (10 pmol), 12.5 µl of GoTaq & Green PCE master and 3.5 µl of PCR water and the total volume was 25 µl. Then the PCR cycle conditions according to manufacturer's instructions was performed for all genes using convention PCR thermocycler (Bio-Rad. USA) as follows: The initial denaturation was performed at 95°C for 5 min, then 35 cycles of denaturation were also performed at 95°C but at 30s, the next step was annealing at 58°C also for 30s, then extension at 72°C for 1min, the final extension step was imposed at 72°C for 5 min. The PCR product was detected by electrophoresis done on a 2% agar gel and imaged by UV light under conditions of 100 V and 80 mA current for a full hour[14].

Real time PCR primers:

Real-time PCR primers for gene expression of arcDABC operon in *P. aeruginosa* isolates under study were designed by NCBI-Genbank sequences and Primer3 plus from Scientific Reserecher. Co. Ltd from Iraq as shown in table 2.

Table 2: qPCR primers for *arcDABC* genes

qPCR Primer	Nitrogenous base sequence (5'-3')		Amplification size	NCBI code
arcD gene	F	TTCATCAACACCGTCACCAC	89 bp	NC_002516.2
	R	AAGATGTCCAGCTTGAAGGC		
arcA gene	F	AACTGCGCAAAGTGATGGTC	100bp	NC_002516.2
	R	TTCACCCAGATCACGTCGTC		
arcB gene	F	GCTTTCAACATGCACAACCG	143 bp	NC_002516.2
	R	ATGTTCTTGCGCTTCAGGTG		
arcC gene	F	AAAGGCACCATCGTCATCTG	109bp	NC_002516.2
	R	AGCAGAGGTCCTTGTCGATC		
rpoS gene	F	ACTCCAAAAGCCACCACTTC	89bp	LN811443.1
	R	TTGAGATACAGCTGCGTTGC		

Real-Time (RT-qPCR):

The RT-qPCR technique was used for measurement relative gene expression analysis of *acrDABC* operon genes that is normalized by housekeeping *rpoS* gene in *P. aeruginosa* isolates. The method was implemented according to Rattanachak *et al.*[15-16], and included the following steps:

RNA extraction:

The total RNA was extracted from *P. aeruginosa* by (easy-BLUEM Total RNA Extraction Kit) based on the manufacturer's protocol.

DNase I remediation:

The method was performed to remove the genomic DNA contamination from extracted RNA samples using DNase I enzyme treatment. A specific reaction mix, including total RNA, DNase I enzyme, buffer, and DEPC water, was prepared and incubated at 37°C for 30 min. Then, 1µl stop reaction was added and incubated at 65C° for 10 minutes for activation of DNase enzyme action.

cDNA manufacturing:

cDNA combination was performed using DNase-treated total RNA, which was mixed with random hexamer primers and DEPC water. This mixture was added to AccuPower® Rocket ScriptTM RT PreMix kit strip tubes containing the necessary reagents for cDNA synthesis, including reverse transcriptase. After brief centrifugation, the tubes were incubated under 42°C for 1 hour to allow cDNA synthesis, followed by a 5-minute heat inactivation step at 95°C.

qPCR master mix for target genes and housekeeping gene:

qPCR master mix for *arcDABC* target genes and housekeeping gene was prepared according to the Standard qPCR master mix protocol manufacturers.

qPCR Thermocycler conditions:

These conditions were done according to qPCR kit instructions and primers annealing calculation by using Therma Scientific^{Tm} Calculator for primers annealing calculation as follows: an initial denaturation was applied at 95°C for 10 min, then 40 cycles of denaturation took place at 95°C for 20 sec., an annealing\ Extension detection (scan) at 60°C and finally melting step at 65-95°C.

Data analysis of qPCR

The expression analysis (fold change) was carried out using the Livak method[17] and the following equations were used to collect the qPCR data results for the target gene and the gene of interest: CT (target gene, test) minus CT (HKG gene, test) equals ΔCT (test).

CT (target gene, control) minus CT (HKG gene, control) = ΔCT (Control).

ΔΔCT is equal to ΔCT (Test) minus ΔCT (Control).

2^{-CT}ΔΔCT is the fold change (target / HKG).

Statistical analysis:

Implemented by IBM SPSS Statistics (Chi-square at ($P < 0.01$) and LSD in one way ANOVA method at ($P< 0.05$).

RESULTS

Bacterial isolates and antimicrobial susceptibility test:

P. aeruginosa isolates were identified by morphological and cultural characteristics. In our current research, 15 isolates of *P. aeruginosa* were diagnosed from burn samples from 25 patients in the burn's hospital. Regarding the antibiotic resistance test of the bacterial as shown in table 3, the highest drug resistance was to cefepime and levofloxacin (73.3%), while for meropenem, imipenem, ceftazidime, piperacillin, ciprofloxacin, amikacin and gentamicin it was (53.3%). All bacterial isolates were sensitive to colistin (100%).

Table 3: Percentage of *P. aeruginosa* sensitive and resistant to antibiotics

No.	Antibiotics	Sensitive No. %	Resistant No. %
1.	Meropenem	7 (46.6%)	8 (53.3%)
2.	Ceftazidime	7 (46.6%)	8 (53.3%)
3.	Cefepime	4 (26.6%)	11 (73.3%)
4.	Piperacillin	7 (46.6%)	8 (53.3%)
5.	Colistin	15 (100%)	0 (0%)
6.	Ciprofloxacin	7 (46.6%)	8 (53.3%)
7.	Levofloxacin	4 (26.6%)	11 (73.3%)
8.	Amikacin	7 (46.6%)	8 (53.3%)
9.	Gentamicin	7 (46.6%)	8 (53.3%)
10.	Imipenem	7 (46.6%)	8 (53.3%)

Detection of virulence genes:

The current study showed that 8 isolates out of 15 strains (53.3%) of *P. aeruginosa* contain the *16S rRNA* gene, which represents the diagnostic gene of the bacteria using the PCR technique, as shown in Fig. 1.

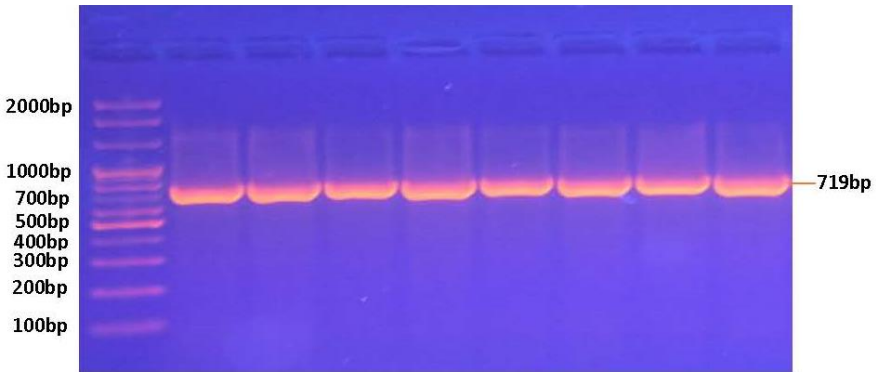

Fig. 1: Agarose gel electrophoresis showing *16S rRNA* gene in *P. aeruginosa* isolates. Marker (M) (100-2000) bp. Lanes (1-8) Positive for *16S rRNA* (719) bp.

Genetic investigation of some virulence factors, where the bacterial isolates were mostly resistant to antibiotics showed that they possessed the virulence genes t*oxA, oprL, oprI at a rate* 100% as shown in Fig. 2- Fig. 4.

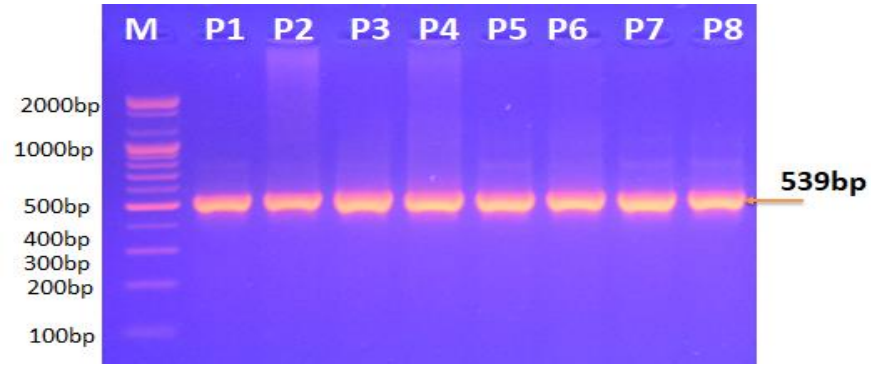

Fig. 2: Agarose gel electrophoresis showing *toxA* gene in *P. aeruginosa* isolates. Marker (M) (100-2000) bp. Lanes (1-8) Positive for *toxA* gene (539) bp.

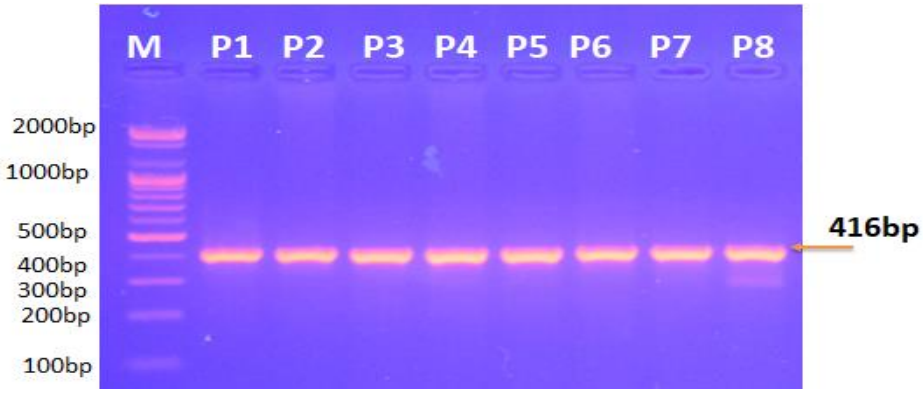

Fig. 3: Agarose gel electrophoresis showing *oprL* gene in *P. aeruginosa* isolates Marker (M) (100-2000) bp. Lanes (1-8) Positive for *oprL* gene (416) bp.

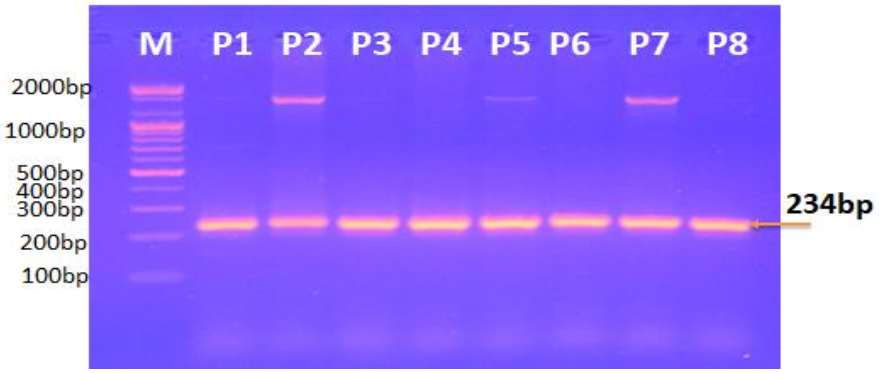

Fig. 4: Agarose gel electrophoresis showing *oprI* gene in *P. aeruginosa* isolates. Marker (M) (100-2000) bp. Lanes (1-8) Positive for *oprI* gene (234) bp.

Gene Expression Analysis of the arcDABC Operon in *P. aeruginosa*:

Table 4: Fold change of gene expression of arcDABC Operon in pathogenic strain isolates & control strain.

Target	Sample	Control	Mean Cq	Expression	Expression SD	Corrected Expression SD	Cq SD	P-Value
HKG-rpoS	P.A		27.86	N/A	N/A	N/A	0.25982	N/A
arcC	P.A		26.60	1.13012	0.21755	0.21755	0.09807	N/A
arcB	P.A		26.40	1.31392	0.32462	0.32462	0.24401	N/A
arcA	P.A		26.28	2.58899	1.05303	1.05303	0.52613	N/A
arcD	P.A		24.08	3.27310	1.02407	1.02407	0.36911	N/A
HKG-rpoS	Control	C	27.84	N/A	N/A	N/A	0.00000	N/A
arcD	Control	C	25.77	1.00000	0.00000	0.00000	0.00000	N/A
arcC	Control	C	26.76	1.00000	0.38480	0.38480	0.55514	N/A
arcB	Control	C	26.77	1.00000	0.23417	0.23417	0.33784	N/A
arcA	Control	C	27.64	1.00000	0.00000	0.00000	0.00000	N/A

The RT-qPCR analysis revealed the following findings: *arcA* expression: The pathogenic (P.A) strain showed a 2.58-fold more in *arcA* expression comparative to the control strain. *arcB* expression: The (P.A) showed a 1.31-fold more in *arcB* expression comparative to the control. *arcC* expression: The (P.A) demonstrated a 1.13-fold more in *arcC* expression relative to the control. *arcD* expression: The (P.A) displayed a 3.27-fold more in *arcD* expression comparative to the control. The reference gene *rpoS* showed no significant differences in expression between the two strains, as shown in table 4.

The gene expression data was further analyzed using various visualization techniques, including bar charts, box-and-whisker plots, and scatter plots. These analyses confirmed the upregulation of the *arcDABC* genes in the (P.A) strain compared to the control as shown in fig. 5 which shows the amplification curves for the *arc* genes (*arcA*, *arcB*, *arcC*, *arcD*) in pathogenic *P. aeruginosa* was exhibited earlier with steeper increase in fluorescence compared to the control isolate.

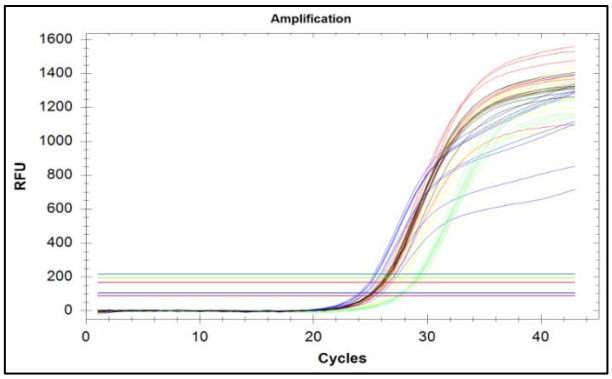

Fig. 5: shows the RT-qPCR amplification plots for the analysis gene expression in pathogenic and a control.

The box plot as shown in fig. 6 representation demonstrates significant upregulation of genes *arcA*, *arcB*, *arcC*, and *arcD* in the pathogenic strain of *P. aeruginosa* compared to the control strain.

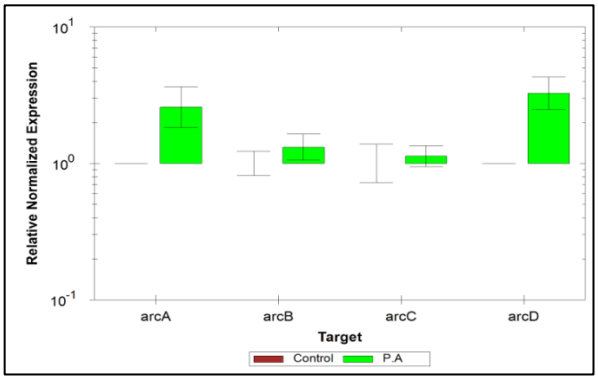

Fig. 6: The box plot shows the relationship between *arc* genes pathogenic strain and control strain.

The scatter plot as shown in fig. 7 compares the normalized expression levels between the (P.A) and control strains of *P. aeruginosa*, the x-axis represents the normalized expression in the control strain, while the y-axis shows the corresponding normalized expression in the (P.A) strain, the data points are clustered around a diagonal line. The clustergram genes as shown in fig. 8 explained expression fold change of *arcA, arc C, arcB and arcD* gene in Pathogenic strains and control Strain, the color scale is used to visualize the expression levels of different genes across pathogenic strains and the control strain.

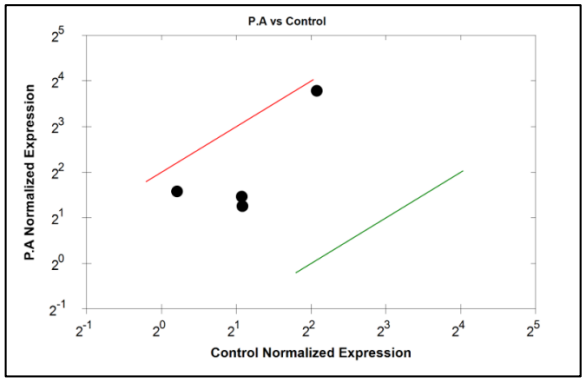

Fig. 7: This scatter plot compares the normalized expression levels between the Pathogenic and control.

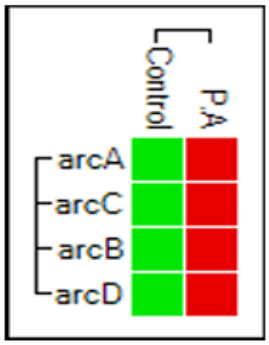

Fig. 8: Glustergram data gene fold change of *arc* genes in pathogenic & control

The image shows a melt curve analysis which is a common technique used RT-qPCR analysis to evaluate the specificity of amplified DNA products. The melt curve depicts the change in fluorescence (RFU) on the y-axis as a function of temperature (°C) on the x-axis as shown in fig. 9.

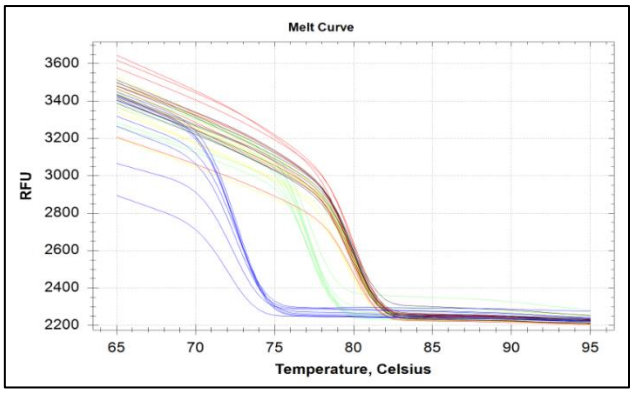

Fig. 9: The image shows a melt curve analysis of *arcDABC* genes *p. aeruginosa* isolates and control strain.

While the melt peak plot displays the derivative of the fluorescence with respect to temp. (-dF/dT) on y-axis, plotted against the temp. (°C) on x-axis as shown in fig. 10.

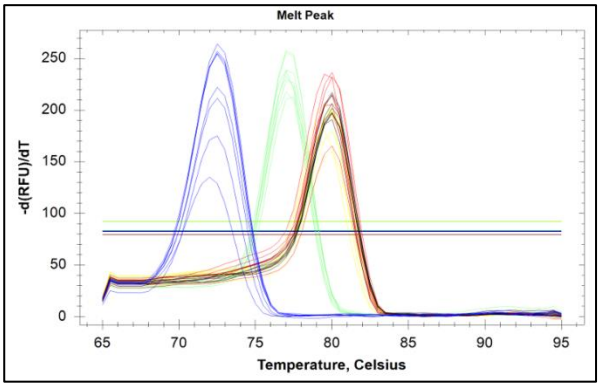

Fig. 10: The image shows a melt peak analysis of *arc* genes *P. aeruginosa* isolates and control strain.

DISCUSSION

P. aeruginosa is an opportunistic pathogen with the ability to infect all tissues, and has the potential to infect immunocompromised individuals as well as being responsible for hospital-acquired infections[18]. Because these *P. aeruginosa* have a variety of virulence factors and antimicrobial resistance, they can survive in a variety of environments[19]. In this study, *P. aeruginosa* isolated from burn patients were identified based on their phenotypic and genetic characteristics by *16SrRNA* gene and virulence factors (*toxA* , *oprL* and *oprI*) genes. In our current study, 15 isolates (60%) of *P. aeruginosa*

were obtained from a total of 25 samples of burn resistant patients. Most isolates were resistant to antimicrobials. This is consistent with the findings of the study conducted on Algerian burn patients, which was (62%)[20]. While another study[21] in Iraq revealed a high rate of burn infection (97.6%) but studies from Morocco[22] and Egypt[23] showed lower incidence rates of drug resistance (15.1% and 19.8%) respectively. Also, another study showed most of the *P. aeruginosa* isolates (76.06%) (54/71) were identified as multidrug resistant based on the results of antimicrobial susceptibility testing[24] .All isolates in their study were completely sensitive to colistin, which agree with the current study. This variability is attributed to the misuse of antibiotics, different methods of infection control that are tiring for hospital management, sanitary conditions, and regional climate of the region.

P. aeruginosa possesses a large number of virulence factors that have an impact on the infection events e.g. exotoxin A, exotoxin S, elastase and sialidase are which controlled by cell-to-cell signaling regulation[18]. Our results are consistent with previous studies of the *toxA* virulence gene, where they reported that the percentage of the *toxA* gene was (100%) in the bacterial isolates from burn patients in Iraq[25], also consistent with another study conducted on burn patients in Cairo, and the percentage of the *toxA* gene was (100%) in ten *P. aeruginosa* isolates[26], and the study conducted in Nigeria[27] on 10 *P. aeruginosa* isolates, and found *toxA* gene in (100%) of the isolates, but Neamah[28] in Iraq found 13 *P. aeruginosa* isolates containing the *toxA* gene at a rate of (92.8%). On the other hand, our study does not agree with the study of Polse *et al.*[24] where the rate of the presence of the *toxA* gene was (64.81%) from 35 isolates. The outer membrane proteins of *P. aeruginosa*, known as lipoproteins L and I, are what give the bacterium its resistance to antimicrobials and disinfectants.

Our study is also consistent with a study conducted on the *oprL* and *oprI* genes isolated from burn patients, which was present in (100%) of the isolates[26], and also found the *oprL* gene in (100%) of the isolates, while the same study reported the *oprl* gene in (74.5%)[27]. Ghazaei[29] found the percentage of bacterial isolates possessing the *oprL* gene were (80.76%).While our current study does not agree with Neamah[28] where genes *oprL* and *oprI* were present in (50%) and (42.8%), respectively. Previous studies were also consistent with our current study, which showed a relationship between virulence genes and antibiotic resistances. Laila *et al.*[30] found *P. aeruginosa* isolates more prevalent in the burns studied, with a higher incidence in males than females and the incidence of drug-resistant *P. aeruginosa* was increased in hospitals, and the virulence gene EXO-A was considered an important factor for burns infected with *P. aeruginosa*.

In the present work, we analyzed the expression of the *arcDABC* genes of a pathogenic strain and compared it to a control strain to better understand the role of this operon in the bacterium's pathogenesis. Gene expression was implemented by (RT-qPCR), RNA extracted for (P.A) strain and a control strain of *P. aeruginosa* and cDNA was synthesized. Primers targeting the *arcA, arcB, arcC, arcD,* and the reference gene *rpoS* were used to quantify the relative expression of the arcDABC operon, our current research revealed that the arginine-ornithine antiporter-encoding *arcD* gene's expression increased more than that of other genes. These findings were consistent with a previous study that demonstrated that in *P. aeruginosa*, the expression of functional genes (*arcA*, *arcB*, and *arcC*) is preceded by *arcD*[12]. In our study, we provide clear evidence of the increased expression of *arcA*, *arcB,* arcC and *arcD* genes compared with control strains. Hydroquinine treatment directly affected the mRNA articulation of the circular segment operon, also has been shown the ADI-related characteristics, such (arcD) with the three (arcA), (arcB), and (arcC)[31]. Since clinical strains have virulence factors like adhesion, invasion, and biofilm formation, they should theoretically be able to adapt to more problematic situations than control strains from a variety of sources, such as blood, discharge, and sputum[32].The present study also showed that the amplification curves *arcA, arcB, arcC, arcD* genes in pathogenic *P. aeruginosa* showed an earlier and sharper increase in fluorescence compared to the control isolate, indicating higher expression levels of these genes in the bacteria that caused burn infection. In contrast, the reference gene *rpoS* showed relatively similar amplification patterns between the pathogenic and control, also showed the mean relative expression levels of all target genes were significantly above in P.A strain, with little overlap between the two groups as shown by the box plot, significant upregulation of *arcA*, *arcB*, *arcC* and *arcD* in the P.A strain of compared to the control strain. Furthermore, the P.A strain shows less variation in expression levels, as shown by the smaller interquartile ranges of the box plot. The study of Gamper *et al.*[33] outlined how the relative abundance of corresponding arc transcripts roughly correlates with the expression levels of the *arcDABC* gene products, with *arcA* being the highest and *arcB-arcC-arcD* being the lowest. Our study represents the distinct peaks in the graph and represents the specific melting temperatures of the amplified DNA fragments, where the double-stranded DNA dissociates in to single strands. This explains the presence of a single sharp peak for each target *arcDABC*, indicating that the primers specifically enhanced the desired sequences without any non-specified amplification or primer dimer formation. Thus, melting peak analysis help in confirming the reliability and specificity of qPCR. Furthermore, our

study generally indicated the prevalence of some virulence genes in all clinical isolates from burn patients, as well as the emergence of multi-antibiotic resistance, which provides support for the (ADI) pathway in the pathogenic *P. aeruginosa*, which in turn encodes the genes (*arcD, arcA, arcB, and arcC*) specific to the arcDABC operon, enables bacteria to grow under oxygen-limited conditions and constitutes a burden on public health worldwide.

CONCLUSION

Most of the *P. aeruginosa* strains isolated from burn infections showed high resistance to antibiotics and showed sensitivity to the antibiotic colistin which is considered an effective antibiotic against *P. aeruginosa*. All the bacterial isolates resistant to antibiotics possessed the virulence genes *toxA*, *oprL*, *oprI*, which enable them to cause burn infections. The result s study showed that the highest expression rate of the genes in the pathogenic bacterial strains were (*arcD, arcA, arcB, arcC*) on (ADI Pathway), while the reference *rpoS* gene showed non-significant differences in expression between the pathogenic and the control strains.

Ethical approval:

The current study was approved by the Research Ethics Committee of the College of Biotechnology/University of Al-Qadisiyah (No.1106 in 20/5/2024), after obtaining the verbal consent of adult patients who were exposed to burns in order to take swabs from the burn sites under sterile conditions.

Conflict of Interests: There are no conflicts of interest in the research that was submitted. This research paper has not been published in any other journal or even received acceptance for publication from any other journal.

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