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Profiling virulence genes in ceftazidime-resistant *Pseudomonas aeruginosa*: A comprehensive study across five hospitals in Iraq

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Abstract: This study addresses the crucial problem of antibiotic-resistant *Pseudomonas aeruginosa*, a major pathogen in severe skin infections that is becoming increasingly resistant to antibiotics worldwide. The research was conducted in Iraq and aimed to isolate and identify *P. aeruginosa* from wound and burn patients, with a particular focus on identifying isolates that are resistant to ceftazidime. Between January and May 2022, a total of 283 samples were collected from five hospitals, leading to the identification of 117 *P. aeruginosa* isolates. Notably, 35.04% of these isolates were found to be resistant to ceftazidime. The study's crucial findings include the identification of the most common resistance genes, pel_F and bla_{GES}, in the resistant isolates using conventional PCR and real-time PCR. Additionally, the researchers performed a detailed sequence analysis of the pel_F genes in SE36 isolates that showed a high prevalence of virulence genes. This analysis was conducted using bioinformatics tools. These insights are critical for understanding the resistance patterns of *P. aeruginosa* in Iraq and developing more effective treatment strategies against this increasingly resistant pathogen.

Keywords: Burn; Conventional PCR; *Pseudomonas aeruginosa*; Resistance genes; Wound

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INTRODUCTION

Pseudomonas aeruginosa, one of the most opportunistic rod-shaped non-spore-forming, straight or slightly curved Gram-negative bacteria (0.5-1.0 µm by 1.5-5.0 µm), is resistant to many antibiotics due to its genetic ability to adapt to various resistance mechanisms and therefore poses a great danger to human health (Moradali *et al*., 2017). Over time, the bacterium has become a serious source of nosocomial infections due to its extensive virulence factors and is one of the most important pathogens commonly seen in intensive care units (ICUs); therefore, it causes serious respiratory and urinary tract infections as well as sepsis and bacteremia (Moradali *et al*., 2017; Diggle and Whiteley *et al*, 2020).

The tendency to produce biofilms due to genetic background and environmental factors enables *P. aeruginosa* to thrive on unfavorable contaminated surfaces in healthcare facilities, thus becoming an important source of infection (Singh *et al*., 2013; Bagge *et al*., 2004; Kaiser *et al*., 2017; Wei and Ma *et al*, 2013). Despite causing serious infections, multidrug-resistant *P. aeruginosa* (MDR-PA) prolongs hospital stay and increases care costs (Kaiser *et al*., 2017). Biofilm produced by bacterial clusters by integrating with extracellular polymeric compounds protects the bacteria against the human immune system and antibacterial agents, so that the bacteria are repeatedly colonized and thus resist antibiotics, making treatment difficult (Wei and Ma, 2013).

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The ability to adapt to changing environments, to have a wide variety of metabolic mechanisms and to develop resistance to antibiotics has made the bacterium a model organism in this regard (Bagge *et al*., 2004). It can protect itself from antibiotics by limiting the permeability of the outer membrane, changing antibiotic targets, forming biofilms, producing drug efflux pumps and beta-lactamases and using some other mechanisms (Wei and Ma *et al*, 2013; Reygaer *et al* t, 2018; Goudarzi *et al*., 2019). Beta-lactamases are subdivided into extended-spectrum beta-lactamases (ESBLs), metallo-beta-lactamases (MBLs), or AmpC-beta-lactamases (Cesur and Demiroz *et al*, 2013). Cephalosporin group antibiotics are used in the treatment of multidrug-resistant isolates such as *P. aeruginosa*, but recently the frequency of cephalosporin-resistant isolates has increased in many parts of the world and 88.3% of them developed resistance to cephalosporin, aminoglycosides and fluoroquinolones (Bagge *et al*., 2004; Wei and Ma *et al*, 2013; Singh *et al*., 2013).

Recently, the prevalence of *P. aeruginosa* strains with ESBL and MBL genes has increased further, thus making antimicrobial treatments more difficult. MDR-PA infections pose a significant threat to the burned and wounded patient population, especially in countries such as Iraq, where health conditions are poor and hospital facilities are inadequate. The poor operating room conditions in hospitals, the irregular use of antibiotics by patients without consulting a doctor and the easy access to all kinds of antibiotics increase the antibiotic resistance of infectious agent bacteria throughout Iraq (Al-Jumaili and Ahmed *et al*, 2024), causing the rapid spread of resistant

strains such as *P. aeruginosa*. Therefore, in this study, resistance gene profiles and antibiotic susceptibility patterns of clinical *P. aeruginosa* isolates were investigated to better understand the local epidemiology of multidrug-resistant strains and to guide effective treatment strategies.

MATERIALS AND METHODS

All chemicals including ABIOpure TM Total DNA (ABIOpure, USA), Crystal violet stain 1% (Promega (USA), GoTaq® qPCR Master Mix and Quantifier dsDNA System (Promega, USA), Absolute ethanol (Bioneer, Korea), Kovacs reagent (Vac and sealant, Iraq), Agarose (Bio Basic INC, Canada), Gram stain: crystal viole iodine, Aceton, and safranin (Institute of sera and vaccines, Iraq), Methyl red (Fluka, Switzerland), Normal Saline (Schuchard, German), Primers (Macrogen, Korea), AP20E Test system and E- TEST (Bio mexieux, France), Ethanol and Glycerol (BDH, England), Nuclease-Free Water (Promega, USA), N,N,N,N (tetramethyl-p-phenylenediamine dihydrochloride) (Difco, England),Agar-agar (Biolife, Italy) whilst other materials were purchased from Oxoid, England: Blood agar base, Brain-Heart infusion broth, Methyl red-Voges- Proskauer broth, Brain-Heart infusion agar, Cetrimide agar, Kligler-Iron agar, MacConkey agar, Gelatin medium, Muller-Hinton agar, Nutrient agar, Urea agar, Indol agar and HiFluoro Pseudomonas Agar Base. Instruments and major apparatus need in this study include Autoclave (Hirayama, Japan), Distillator (G.F.L Germany), Balance (Kevn, Jordan), Compound Light Microscope (Olympus, Japan), Vitek2 (Biomerieux, France), Cooler Incubator (Binder, England), Waterbath (Gallenkamp, England), Electric Oven (Binder, England), Hot Plate with Magnetic Stirrer (Biocote, UK), Quantus Fluorometer (Promega, USA), Refrigerator (Fiocchetti, Italy), Centrifuge (Fisher Scientific, USA), Mic Qpcr Cycler (BioMolecular System, Australia), Vortex (Biocote, UK).

Sample collection and P. aeruginosa isolation

Sample collection was conducted using a random sampling method. A total of 283 samples were collected from patients aged between 1 to 70 years, admitted to various hospitals, including Kirkuk Education, Mosul General, Baghdad Karama, AL Ramadi General Education and AL Ramadi Maternity and Children Teaching Hospitals, from January to May 2022. The objective was to isolate *P. aeruginosa* from patients with wounds and burns on various body parts.

Bacterial Identification

Initially, swabs collected from the patients were inoculated on MacConkey and blood agar. Subsequently, colonies that emerged were transferred to Cetrimide agar, a selective medium used specifically for the detection of *P. aeruginosa*. Identification of all isolates was meticulously carried out based on cultural characteristics, biochemical testing, and Gram staining. Additionally, cellular and aggregation patterns of the isolates were examined under a microscope using Gram staining.

For further identification, oxidase, catalase, and Kligler iron urease-positive isolates were analyzed using the Analytical Profile Index 20 Non-Enterobacteriaceae (API 20NE; bioMérieux, France) diagnostic kit. This involved adding 0.25 ml of saline and bacterial colonies to each of the 20 wells on the strip-shaped kit, followed by incubation at 37°C for 24 hours. The reactions in each well were observed, and the identification number for *P. aeruginosa* was determined by comparing it with the reference value. For future studies, all isolates were preserved at -80°C in Brain Heart Broth containing 50% glycerol.

Antibiotic susceptibility testing

The susceptibility of *P. aeruginosa* isolates to Ceftazidime was determined using two standardized techniques: the Disc Diffusion Method and the E Test (Minimum Inhibitory Concentration - MIC).

For the Disc Diffusion Method, paper discs impregnated with Ceftazidime were placed on inoculated Mueller-Hinton agar plates exhibiting intensive *P. aeruginosa* growth. Following 24 hours of incubation, the diameter of the inhibition zone around each disc was measured in millimeters, and bacterial susceptibility was calculated as a percentage based on established zone diameter breakpoints.

Concurrently, the E Test was performed to ascertain the quantitative MIC value. A standard E-test strip, measuring 5 mm in width and 60 mm in length, was placed on the inoculated agar plate to establish a continuous antimicrobial concentration gradient. The Ceftazidime concentration range used for the strips was 0.16 to 256 mcg/mL. After overnight incubation, the MIC value was determined by identifying the intersection point of the symmetrical inhibition ellipse with the test strip. The resulting MIC values were then used to classify the isolates as sensitive or resistant.

Molecular studies

Extraction of genomic DNA

DNA extraction was performed from each isolate in accordance with the Alliance Bio ABIO pure extraction protocol for use in molecular studies. For this, 1 ml of bacterial samples was incubated overnight and centrifuged at 13000 rpm for 2 minutes. A mixture of 100 µl of nuclease-free water and 100 µl of lysozyme solution was added to the pellet and vortexed. The sample was incubated at 37°C for 30 minutes. After centrifugation as above, 20 µl of Proteinase K (20 mg/ml) and 200 µl of buffer were added to digest the proteins and lyse cells, vortexed vigorously and then incubated at 56°C for 30

minutes and for further lysis, samples additionally incubated for another 30 minutes at 70°C.

Quantitation of DNA

Calculating DNA concentration accurately and precisely is critical for the efficiency of molecular studies. In this study, a Quantus Fluorometer was used to measure the concentration of the extracted DNA. For this, 1 µl of DNA was mixed with 199 µl of diluted Quantity Fluor dye, and after 5 minutes of incubation at room temperature, DNA concentrations were determined (15ng/µl to 24ng/µl).

Conventional PCR and real-time PCR

Virulence factor gene profiles of *P. aeruginosa* isolates were determined by both conventional and real time, and the performance of both methods was compared. The PCR mixture consisted of 5µl master mix for each sample, primer (0.5 Mm F and 0.5 Mm R), 1 ng/µl of DNA template, and 3 µl nuclease-free water up to a final volume of 10 µl. PCR amplification was performed in a thermal cycler, with an initial denaturation step of 95°C for 5 min, followed by cycles between 35-60 depending on the genes. The characteristics of the primers of the genes screened during the study are given in Table 1.

Sequencing

After the PCR products were separated on a 2% agarose gel, the bands stained with Redsafe were visualized under ultraviolet light at 302 nm wavelength. gene extraction from the gel was performed according to the method reported by (Vogelstein and Gillespie*,* 1979). The genetic sequencing was carried out by the Biotechnology Laboratory at the National Hardware Center (Microgene-Korea), utilizing the DNA Sequencer 3730XL device. Homology analysis was performed using the Basic Local Alignment Search Tool (BLAST; Altschul *et al*. 1990), along with the BioEdit program (Hall, 1999).

Statistical analysis

The information collected during the research underwent a thorough statistical analysis using Analysis of Variance (ANOVA), which is a powerful method for comparing means across several groups. We used the Statistical Analysis System (SAS, 2004) for this analysis, which is well-known for its ability to handle complex datasets accurately.

Duncan's Multiple Range Test was used to investigate the differences observed in the study (Duncan, 1955). This test is particularly effective in identifying significant differences. The Duncan's test, applied at a significance level of $P<0.05$, allowed us to discern whether the observed differences in MIC values were statistically significant. This threshold ($P<0.05$) indicates that there is less than a 5% probability that the observed differences occurred by chance, thus providing a high confidence level in the results (SAS Institute Inc., 2004; Field, 2018).

RESULTS

In the study, a total of 283 samples were taken from patients hospitalized in hospitals from different regions in Iraq, as indicated in Table 2. These samples were used to diagnose *P. aeruginosa, and* the most positive results were detected at Kirkuk Training Hospital. While 31 of a total of 117 *P. aeruginosa* isolates were obtained from Kirkuk Training Hospital, 26, 24, 18 and 18 were isolated from Karama Hospital, AL-Ramadi General Teaching Hospital, Maternity and Children's Teaching Hospital, and Mosul General Hospital, respectively, and the highest incidence of *P. aeruginosa* is in Karama Hospital. Of the 283 swap samples, 107 (37.94%) were taken from the burned areas of the patients' skin and 176 (62.4%) of them were taken from the wounds. Although more samples were taken from the wound, a higher rate of *P. aeruginosa* (44.8% vs. 39.2%) was isolated from the burned body areas. Of these 117 *P. aeruginosa* isolates, 41 were resistant to ceftazidime and the majority (27 to 14) were from wounds. The isolates were pale in color, non-lactose fermenting, with irregular borders and fruity odor on MacConkey agar medium and were morphologically compatible with *P. aeruginosa* colony. These bacteria then grew into colonies producing water-soluble greenish blue colored pyocyanin and yellowish green and bright pyoverdin on Muller-Hinton agar. Colonies were confirmed to be *P. aeruginosa* by additional physical and biochemical testing.

Cells obtained after Gram staining were gram-negative, rod-shaped and non-spore forming type under the microscope. The bacteria tested positive with both oxidase and catalase, converted hydrogen peroxide into water and oxygen gas, metabolized citrate, no H_2S gas was formed and the surface of the medium was alkaline. Contaminated samples were sieved with cetrimide agar. In addition to all these, urease, indole and methyl red tests were negative. The VITEK 2 system (BioMérieux) was used to confirm the identity and ascertain the antibiotic susceptibility patterns of 117 *P. aeruginosa* isolates, and API 20E was used to validate the validity of the biochemical tests. Of these, 41 exhibited ceftazidime resistance, comprising 14 isolates from burn specimens and 27 isolates from wound specimens.

The Ceftazidime susceptibility of the *P. aeruginosa* isolates was determined using the Disc Diffusion Method and the E-test (MIC). A total of 41 clinical isolates were found to be resistant to Ceftazidime, based on the breakpoint interpretation of the high MIC values derived from the E Test, which used strips ranging from 0.16 to 256 mcg/mL. Ceftazidime is a primary treatment for *P. aeruginosa* infections in wounds and burns in Iraq. Our resistance analysis indicated that isolates recovered from

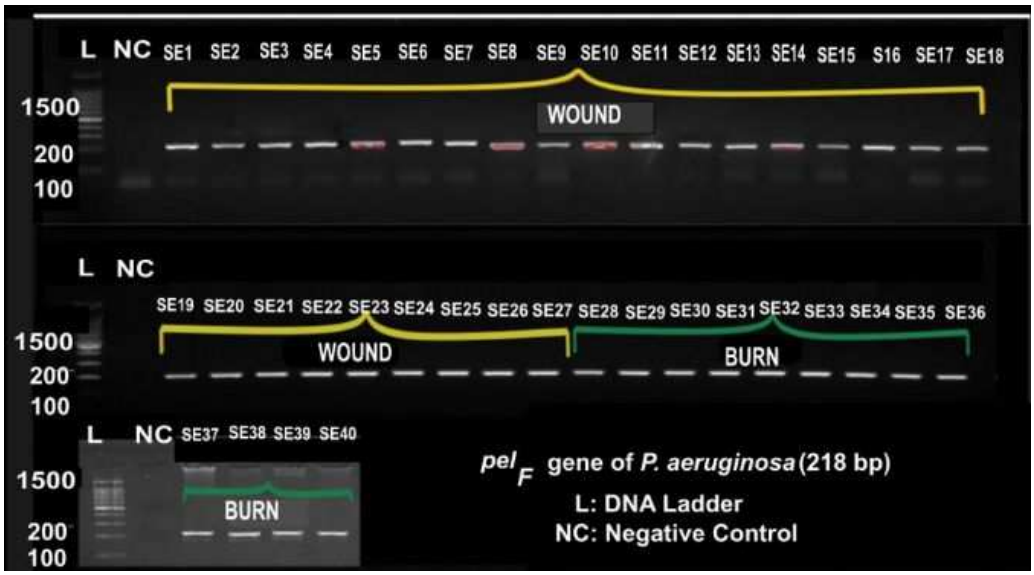


Fig. 1: Agarose gel electrophoresis of the pel_F gene of *P. aeruginosa* isolates obtained from wound and burn

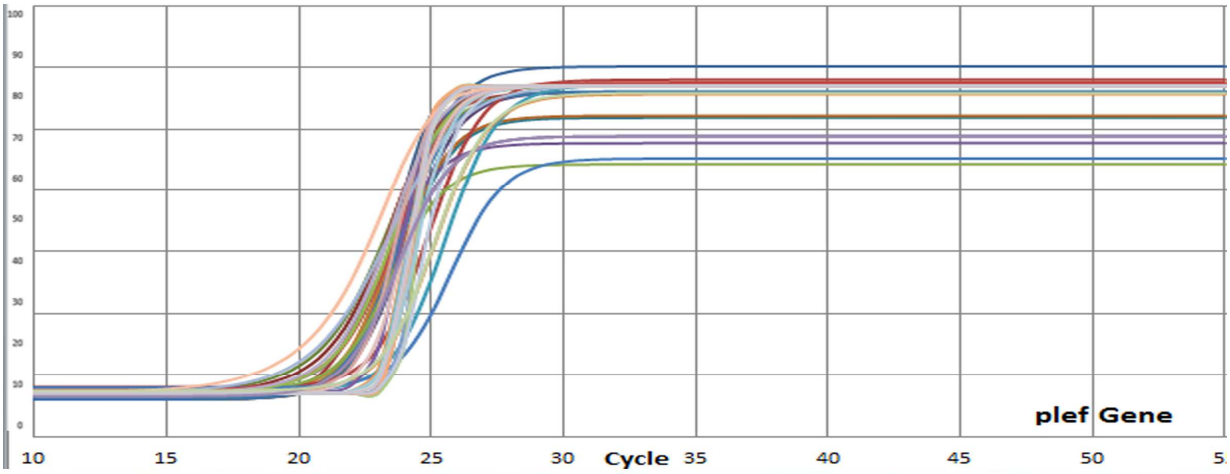
wound samples were notably more resistant to Ceftazidime than those from burn samples (Table 2). Furthermore, a statistically significant difference in susceptibility (mean MIC values) was observed among the hospitals for the wound samples, but no such difference was noted for the burn samples (Table 3).

When the positive samples in the conventional PCR were subjected to the real-time PCR, the gene pel_F, bla_{GES}, and mex_A did not show any difference between conventional PCR and real-time PCR. The results were positive for exo_S, exo_A, mex_B, bla_{IMP}, bla_{AmpC} and bla_{AmpD} genes from 9 different isolates by conventional PCR, but real-time PCR did not confirm this result. Furthermore, pel_F and bla_{GES} genes are the most common virulence genes, While the pel_F gene was observed in all the ceftazidime-resistant isolates taken from the wound and burn units of all hospitals (Fig. 1), The real-time quantitative PCR (qPCR) plot and table (Fig. 2) illustrate the expression profile of the ple_F Gene, which encodes biofilm formation, in 41 *P. aeruginosa* isolates (27 from wounds and 14 from burns). The numerical data provide the quantification cycle (Cq) and reaction efficiency for the wounds and burns groups, supporting the finding that the ple_F genes were present across all isolates. the bla_{GES} gene was not found only in the burn units of AL Ramadi General Training Hospital and Ramadi Maternity and Children's Teaching Hospital (Table 4).

The presence of bla_{GES}, bla_{AmpC}, bla_{AmpD}, bla_{Imp} mex_B, mex_A, exo_S, exo_A and pel_F genes in these 41 resistant *P. aeruginosa* isolates was screened by both conventional PCR and real time PCR (Table 4). The pel_F gene was found in all isolates from hospital wound and burn samples. The bla_{GES} gene was absent in the isolates only from the burn units of 2 hospitals (H2: AL Ramadi General Teaching Hospital, H3: Ramadi Maternity and Children's Teaching Hospital). The mex_A gene was not found in the wound and burn units of the Ramadi Maternity and Children's Training Hospital and the burn units of the Kirkuk Training Hospital (Table 4). The bla_{AmpD} gene was absent only in the burn unit of Al Karama General Hospital but was present in all others. The exo_A, mex_B and bla_{Imp} genes were absent only in the burn units of the AL Ramadi General Teaching hospital. As demonstrated in the accompanying images for each gene, the percentage of isolates harbouring each gene ranged from 100% for the pel_F gene to 43% for the bla_{AmpC} gene. The pel_F gene of SE36 isolates from burn units of Mosul State Hospital was sequenced and compared with the gene bank, nucleotide changes were observed in 4 different places, while insertion was determined in one place and deletion in the other. The associated protein sequence showed amino acid substitution at 9 different positions (Fig. 3).

DISCUSSION

Having a relatively larger genome compared to other bacteria, encoding regulatory enzymes that provide metabolism, transport and efflux of organic compounds, the existence of intrinsic, acquired and adaptive antibiotic resistance mechanisms allows *P. aeruginosa* to develop versatile metabolic activities against changing environmental conditions, causing it to become a multi-drug resistant pathogen all over the world. Although the bacterium affects healthy people to a lesser extent, it causes high morbidity and mortality, especially in cystic fibrosis patients and immunocompromised individuals; recent studies point to the effect of multidrug-resistant strains on surgical wounds and burns (Dantas and Moretti-Branchini 2003).



Wounds						Burns					
Well	Color	Sample Name	Cq	Efficiency	Result	Well	Color	Sample Name	Cq	Efficiency	Result
1		SE1	17.6	0.82		28		SE28	17.6	0.75	
2		SE2	28.2	0.56		29		SE29	18.2	0.78	
3		SE3	33.2	0.87		30		SE30	33.2	0.79	
4		SE4	19.4	0.92		31		SE31	18.1	0.88	
5		SE5	29.8	0.88		32		SE32	19.0	0.89	
6		SE6	21.3	0.75		33		SE33	21.3	0.90	
7		SE7	25.8	0.78		34		SE34	22.8	0.82	
8		SE8	27.4	0.79		35		SE35	27.4	0.56	
9		SE9	23.6	0.88		36		SE36	23.6	0.87	
10		SE10	24.5	0.89		37		SE37	24.5	0.92	
11		SE11	26.8	0.79		38		SE38	26.8	0.88	
12		SE12	22.5	0.91		39		SE39	22.5	0.93	
13		SE13	28.3	0.92		40		SE40	28.3	0.75	
14		SE14	19.8	0.99		41		SE41	19.8	0.86	
15		SE15	44.6	0.82							
16		SE16	32.1	0.56							
17		SE17	23.7	0.87							
18		SE18	20.3	0.92							
19		SE19	18.3	0.88							
20		SE20	24.5	0.93							
21		SE21	26.8	0.75							
22		SE22	22.5	0.75							
23		SE23	28.3	0.78							
24		SE24	19.8	0.79							
25		SE25	44.6	0.88							
26		SE26	20.1	0.89							
27		SE27	23.7	0.77							

Fig. 2: Real-time PCR results of pel_F gene of *P. aeruginosa* isolates isolated from wound and burn samples.

These bacteria, considered a member of the life-threatening "ESKAPE bugs" known for their increasing prevalence of drug resistance and virulence, have been listed by the World Health Organization as a "priority pathogen" with the highest antibiotic resistance and critically needed to develop new antibiotics (Santajit *et al*., 2015; Wongsawat *et al*, 2017).

In this study, *P. aeruginosa* isolates were determined from different hospitals in Iraq, where sanitation conditions are inadequate and intensive and irregular antibiotic use is common and the virulence genes of these ceftazidime-resistant isolates were screened by conventional PCR and real-time PCR. In addition, the pel_F gene of the SE36 isolate, which was determined to carry all the virulence factors screened, was sequenced and the results analyzed using bioinformatics tools. Out of a total of 283 samples taken from the 5 different hospitals mentioned above, 117 were *P. aeruginosa* and 41 (35.04%) of them were resistant to ceftazidime. The results obtained from the Antibiotic Susceptibility Test (AST) and E Test indicated that *P. aeruginosa* specimens from wound units at each hospital were more resistant to ceftazidime than those from the burned, a finding further

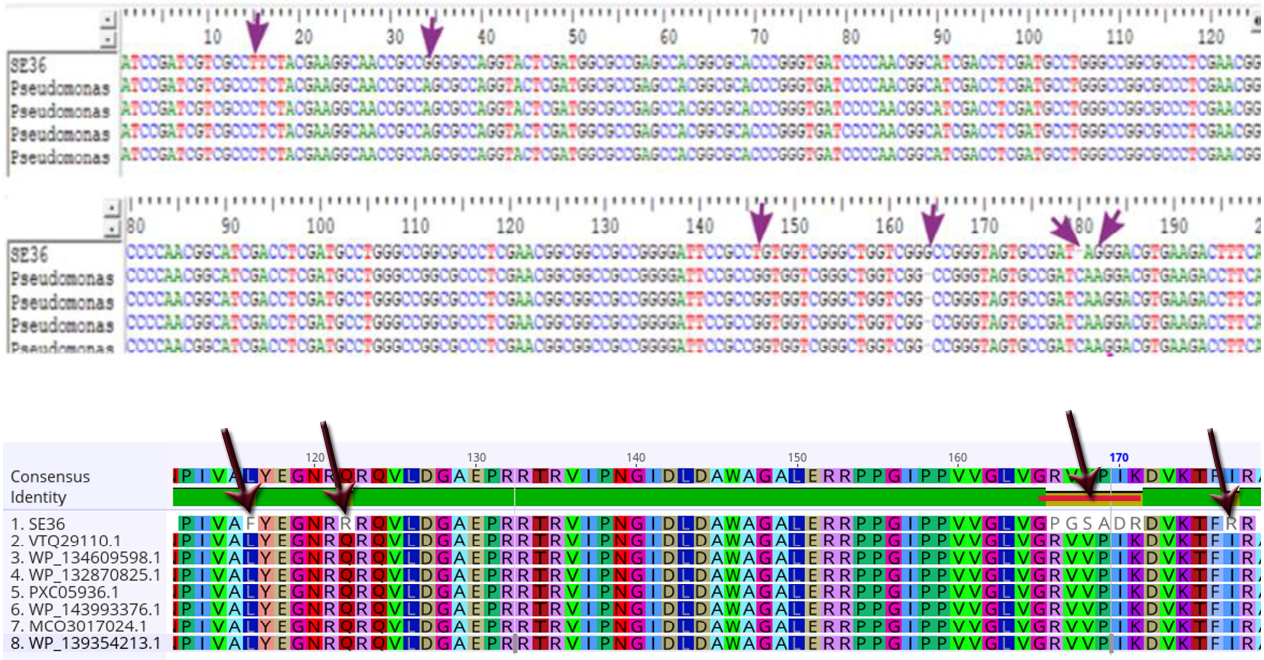


Fig. 3: Comparison of *P. aeruginosa* SE 36 isolate *pel*$_F$ gene sequences with gene bank and novel mutations occurring in the *Pel*$_F$ Protein (small arrows indicates point mutation in nucleotides 15, 34, 146, 164, 179, 180, 182 and 192 of the gene; arrows with dashed lines indicate novel missense mutations at 9 different positions of the protein).

Table 1: Characteristics of the primers used during the study.

Gene	Primer (5'-3')	Size (bp)	Annealing Temp (°C)	Reference
exoS	F: GCG AGG TCA GCA GAG TAT CG R: TTC GGC GTC ACT GTG GAT	504	56.7	Horna *et al*., 2019
exoA	F: CTTCTTCAGCTCGACGCGACG R: GAC AACGCCCTCAGCATCACCAGC	396	64.1	Xu *et al*., 2004
pelF	F: GTGGCTTCAGTGCTTGTAGGTA R: TGCTTGATTGAGTTGTTGCCG	250	54.8	Nathaniel *et al*., 2017
blaGES	F: ATGCGCTTCATTCACGCAC R: CTATTTGTCCGTGCTCAGG	390	54.5	Wang *et al*., 2005
blaIMP	F: TTGACACTCCATTTACTGCTA R: TCATTTGTTAATTCAGATGCATA	587	56.2	Goudarzi *et al*., 2019
blaAmpC	F: TGGGGTCGAACCAATCTCTA R: ACGTCGAGGTGGGTCTGTT	303	54.8	Berrazeg *et al*., 2015
blaAmpD	F: TCGCTGCTGGTTATCCACAA R: ACCTTACCGGTGCCGAACT	255	69.8	Tsutsumi *et al*., 2013
mexA	F: AGACGGTGACCCTGAATACC R: GTC GGC CTC GTA GGTGG	276	56.6	Abet *et al*., 2021
mexB	F: CAACGCGCAGTTCAACGG R: GTACGCCCTGGTCCTCGTC	360	54.3	Middlemiss *et al*., 2004

supported by the significantly higher mean MIC values recorded for wound isolates (Table 3). This difference is notable since burn infections are generally considered more serious than chronic wound infections. *P. aeruginosa* specimens from wound units at each hospital were more resistant than those from the burned.

However, burn infections are more serious than chronic wound infections because *P. aeruginosa* from burned body parts reaches other parts of the body in a shorter time by systemic route and causes death (Cobos-Trigueros *et al*., 2015). Although bacteria live longer in chronic wounds, they cause less mortality. This difference

Table 2: *P. aeruginosa* growth resistance in selected Iraq hospitals.

Hospital	Total of isolate (N)		Total of *Ps.*		No. resist. (R, Ceftazidime)	
	Wound	Burn	Wound	Burn	Wound	Burn
H1	25	19	15	11	4	3
	44(15.6%)		26(22.2%)		7(17.0%)	
H2	33	25	14	10	7	2
	58(20.5%)		24(20.5%)		9(21.95%)	
H3	41	22	12	6	3	3
	63(22.34%)		18(15.3%)		6(14.63%)	
H4	30	17	10	8	6	4
	47(16.6%)		18(15.3%)		10(24.39%)	
H5	46	24	18	13	7	2
	70(24.8%)		31(26.49%)		9(21.95%)	
Total	176(62.4%)	107(37.94%)	69(39.2%)	48(44.8%)	27(39.1%)	14(29.0%)
	283		117 (41.48)%		41(35.0)%	

H1: Al Karama General Hospital, Baghdad;
H2: AL Ramadi General Teaching Hospital, Ramadi;
H3: Ramadi Maternity and Children's Teaching Hospital, Ramadi;
H4: Mosul General Hospital, Mosul;
H5: Kirkuk Teaching Hospital, Kirkuk;
N: Total number of isolates;
R: Resistant isolates.

Table 3: Mean Minimum Inhibitory Concentration (MIC) Values (µg/Ml ± Standard Error) for Ceftazidime against *P. aeruginosa* Isolates from Wound and Burn Samples in Selected Iraqi Hospitals

Hospital	Wound (Mean ± SE)	Burn (Mean ± SE)
H1	1.76 ± 0.55	4.75 ± 2.02
H2	1.76 ± 1.54	12.0 ± 2.30
H3	8.00 ± 2.11	10.8 ± 2.49
H4	10.7 ± 2.24	11.6 ± 2.11
H5	21.0 ± 5.00	13.2 ± 7.16

H1: Al Karama General Hospital, Baghdad
H2: AL Ramadi General Teaching Hospital, Ramadi
H3: Ramadi Maternity and Children's Teaching Hospital, Ramadi
H4: Mosul General Hospital, Mosul
H5: Kirkuk Teaching Hospital, Kirkuk

in burns and wounds may be due to different responses of the patient's immune system and the host's subsequent failure to heal, as well as the fact that antimicrobial agents used during long-term treatment of chronic wounds affect bacterial gene expression at different rates (Codjoe and Donkor *et al*, 2017). The isolation rate of *P. aeruginosa* was reported as 32.8% and 30.00% in Egypt and Saudi Arabia, whereas in the current study, this rate was 41.49% in Iraq and varied between 28.57% and 59.09% in 5 different hospitals. While the isolation rates obtained at different times in Saudi Arabia are similar (El-Ageery *et al*, 2016), there are great differences in the rates stated in Iraq (Al-Kaisse *et al*., 2014).

This situation necessitates a review of the studies conducted in Iraq and the hospital conditions in terms of this pathogen. There is evidence that antibiotic resistance will vary based on geographical differences and hospital conditions. A large-scale study in Turkey showed the rate of ceftazidime-resistant strains was 26.80% in the last 10 years (Akkaya Isik *et al*., 2021), while this rate was 34.05% in this study. Health conditions are different in different countries, and the higher rate observed in Iraq may be attributed to various factors such as less stringent antibiotic use protocols, differences in infection control measures, and local epidemiological factors.

Table 4: Presence of virulence factor genes with conventional PCR and real time -PCR

Genes	Conventional PCR																			
	Wound										Burns									
	H1		H2		H3		H4		H5		H1		H2		H3		H4		H5	
	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N
pel$_F$	4	0	7	0	3	0	6	0	7	0	3	0	2	0	3	0	4	0	2	0
bla$_{GES}$	4	0	7	0	3	0	6	0	7	0	3	0	1	1	2	1	4	0	2	0
exo$_S$	2	2	6	1	3	0	4	2	7	0	3	0	1	1	2	1	4	0	1	1
exo$_A$	2	2	5	2	2	1	4	2	5	2	2	1	0	2	3	0	4	0	1	1
mex$_B$	3	1	5	2	2	1	2	4	5	2	2	1	0	2	3	0	3	1	2	0
bla$_{IMP}$	4	0	2	5	2	1	5	1	4	3	3	0	0	2	2	1	2	2	1	1
bla$_{AmpC}$	1	3	4	3	1	2	2	4	4	3	1	2	1	1	2	1	4	0	1	1
bla$_{AmpD}$	2	2	2	5	1	2	2	4	3	4	0	3	2	0	2	1	1	3	1	1
mex$_A$	3	1	4	3	0	3	5	1	4	3	1	2	1	1	0	3	3	1	0	2
Total	25	11	42	21	17	10	36	18	46	17	18	9	8	10	19	8	29	7	11	7
Genes	Real Time- PCR																			
	Wound										Burns									
	H1		H2		H3		H4		H5		H1		H2		H3		H4		H5	
	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N
pel$_F$	4	0	7	0	3	0	6	0	7	0	3	0	2	0	3	0	4	0	2	0
bla$_{GES}$	4	0	7	0	3	0	6	0	7	0	3	0	1	1	2	1	4	0	2	0
exo$_S$	1	3	6	1	3	0	4	2	7	0	2	1	1	1	1	2	4	0	1	1
exo$_A$	2	2	5	2	2	1	3	3	5	2	2	1	0	2	3	0	4	0	1	1
mex$_B$	3	1	5	2	2	1	2	4	3	4	2	1	0	2	3	0	3	1	2	0
bla$_{IMP}$	4	0	2	5	2	1	5	1	3	4	3	0	0	2	2	1	2	2	1	1
bla$_{AmpC}$	1	3	3	4	1	2	2	4	4	3	1	2	1	1	2	1	4	0	1	1
bla$_{AmpD}$	2	2	2	5	1	2	2	4	3	4	0	3	2	0	1	2	1	3	1	1
mex$_A$	3	1	4	3	0	3	5	1	4	3	1	2	1	1	0	3	3	1	0	2
Total	25	11	42	21	17	10	36	19	44	20	18	9	8	10	19	8	29	7	11	7

H1: Al Karama General Hospitals in Baghdad, H2: AL Ramadi General Teaching Hospital, H3: Ramadi Maternity and Children's teaching hospital, H4: Mosul General Hospital and H5: Kirkuk Teaching Hospital; P: Positive, N: Negative.

Health conditions are different in different countries and the rates of resistant strains have increased over time around the world. Prolongation of hospital stays in intensive care units and surgical departments, non-compliance with antibiotic use protocols and limited opportunities to take adequate precautions in hospitals may also be the cause of increasing rates of antibiotic resistance. According to the report of the European Center for Disease Prevention and Control (ECDC), MDR-resistant *P. aeruginosa* infections are relatively low in European Union countries and show a decreasing trend between 2014 and 2017. Again, in the same report, the increasing resistance in Eastern European and Middle Eastern countries is emphasized and its relationship with the level of development of the countries, strict infection control measures and antibiotic use protocols is stated (Evans *et al*, 2014).

Therefore, in this study, 9 different virulence factor genes (*bla*$_{GES}$, *bla*$_{AmpC}$, *bla*$_{AmpD}$, *bla*$_{IMP}$, *mex*$_B$, *mex*$_A$, *exo*$_S$, *exo*$_A$ and *pel*$_F$) of ceftazidime-resistant *P. aeruginosa* isolates, which pose a problem for the world and Iraq, were screened with conventional PCR and real-time PCR and the current situation for 5 Iraqi hospitals was analyzed. The findings revealed the presence of *pel*$_F$ genes encoding biofilm formation in all 41 *P. aeruginosa* isolates (14 burns, 27 wounds).

The prevalence of this gene in some other studies reveals that biofilm formation is very common in *P. aeruginosa* and is an important weapon of the bacterium in preventing its antimicrobial effect (Nathaniel *et al*., 2017; Bacalso *et al*., 2011).

Again, in the present study, the *bla*$_{GES}$ gene was also found to be quite common in both wound and burn infections, confirming another study conducted in South Africa (Nathaniel *et al*., 2017; Bacalso *et al*., 2011).

In the current study, the frequencies of *bla*$_{AmpC}$ and *bla*$_{AmpD}$ genes were similar in wound and burn infections (44.4%). In comparison, the prevalence of *bla*$_{AmpC}$ was lower in France, reported as 21.3% in hospital isolates (Cavallo *et al*., 2002), whereas it was higher in Portugal at 68.6% (Rafiee *et al*., 2014). Co-existence of *bla*$_{AmpC}$ and *bla*$_{AmpD}$ was observed in all hospitals in the current study except Al Karama General Hospital. Notably, inactivation of *ampD* leads to overexpression of *ampC* in *P. aeruginosa*, potentially enhancing resistance (Tsutsumi *et al*., 2013).

In the current study, the *exoA* gene related to exotoxin production was observed in wound (66.6%) and burn (71.4%) isolates, whereas a lower prevalence (13%) of *exoU*-positive *P. aeruginosa* isolates among multidrug-resistant strains has been reported (Peña *et al*., 2015). This highlights the necessity of characterizing the virulence factors of *P. aeruginosa* populations in different geographical regions. Additionally, the frequency of the exo_S gene in our study was similar to that reported in *P. aeruginosa* isolates from Iran (Elahi *et al*., 2024), suggesting that certain virulence determinants may be conserved across distinct locations. Since efflux pumps (mex_A and mex_B) facilitate the transport of necessary substances during biofilm formation, the coexistence of virulence factors (e.g., exo_A and exo_S) alongside these resistance mechanisms further complicates treatment strategies and contributes to multidrug resistance. The presence of biofilm formation in almost all the isolates from all hospitals during our studies makes their co-existence with *mex* genes more important. In this respect, while the mex_A gene was determined as 59.2% and 35.7% in isolates from wound and burn, these rates were 70.3% and 71.4% for the mex_B gene (Edward *et al*., 2024). Considering that the pel_F gene is seen at a rate of 100% in isolates of wound and burn infection, it increases the interest in resistance when it is considered together with the presence of a high rate of mex_B gene. The bla_{IMP} gene related to beta-lactam resistance was also present in 62.96% and 64.28% of wound and burn units (Ali *et al*., 2024).

CONCLUSION

This study represents a significant advancement in both microbiology and clinical practice. Primarily, it offers critical insights into the antibiotic resistance profiles of *P. aeruginosa* isolates responsible for skin infections in Iraq. This information is invaluable for clinicians, aiding them in making more informed decisions regarding antibiotic selection. Furthermore, identifying and analyzing specific resistance genes, such as bla_{GES}, illuminate the molecular mechanisms driving antibiotic resistance. This knowledge is crucial for researchers delving into resistance pathways and instrumental in guiding the development of targeted interventions against these genes.

Additionally, the study's emphasis on local epidemiology provides a deeper understanding of the regional challenges in combating antibiotic resistance. This localized focus enables the formulation of more customized strategies to address this global health issue effectively. Moreover, the investigation into virulence genes and the bioinformatic analysis of pel_F genes in virulent isolates enrich our comprehension of the relationship between virulence factors and resistance mechanisms. This aspect of the study opens new avenues for innovative approaches in managing infections caused by *P. aeruginosa*.

Overall, this research makes pivotal contributions by delivering essential data that can directly influence clinical management and public health policies. These strategies aim to mitigate the effects of antibiotic resistance, particularly in the context of severe skin infections caused by *P. aeruginosa* in Iraqi healthcare settings.

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Authors' contributions

SAB designed the study, performed the experiments, collected the data, performed the molecular analysis, and conducted the statistical evaluation. SOGA contributed to the analyses and experimental procedures. SAB and AA wrote the manuscript and AA also provided supervision. All authors have read and approved the final manuscript.

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Data availability statement

All data generated or analyzed during this study are included in this published article. The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethical approval

This study was approved by the Ethics Committee of Al-Ramadi General Hospital, Iraq (Approval Number: ARGH-2022-0147). All procedures involving human participants were conducted in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments. Written informed consent was obtained from all participants or from their legal guardians in the case of minors.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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