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ORIGINAL ARTICLE

# Coordination of *las* regulated virulence factors with Multidrug-Resistant and extensively drug-resistant in superbug strains of *P. aeruginosa*

**Sanaz Dehbashi[1] · Mohammad Reza Pourmand[2] · Mohammad Yousef Alikhani[1] · Sara Soleimani Asl[3] · Mohammad Reza Arabestani[1,4]**

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## Abstract

Successful pathogenicity often resulted from a complicated association between virulence and antibiotic resistance in *Pseudomonas aeruginosa* infections. Therefore, the current study aimed to investigate the relationship between the *las* system and antibiotic resistance. Seventy-three (73) *P. aeruginosa* isolates were collected from burn wounds (26.02%), blood cultures (30.13%), catheters (12.32%), and urine culture (31.50%). Among the 73 collected isolates, 22 isolates were considered as multi-drug resistant (MDR) and 11 isolates as extensively-drug resistant (XDR). Furthermore, phenazines and LasA protease were detected among 21.91% and 32.87% of isolates, respectively. Quantitative real-time PCR assessment of *KPC*, *MBL,* and *lasI/R* indicated that resistance and virulence factors are more expressed in XDR strains than MDR strains. Also, the expression level of *KPC* and *MBL* reduced in non-biofilm forming strains. However, increased expression levels of *lasI*, *lasR,* and the *KPC* genes were observed in LasA and LasB protease producing strains. Interestingly, 16 known sequence types (including ST108, ST260, ST217) and three novel STs (ST2452, ST2427, and ST2542) were characterized among the collected isolates, which are related to the virulence and resistance. In MDR-XDR strains, a strong correlation between *lasI/R* and the variants of antibiotic resistance genes was found. In conclusion, the pathogenicity of *P. aeruginosa* may increase the prevalence of antibiotic-resistant strains.

**Keywords** *P. aeruginosa* · Antibiotic resistance · Quorum sensing · Virulence factors

✉ Mohammad Reza Arabestani
mohammad.arabestani@gmail.com

Sanaz Dehbashi
s.dehbashi@edu.umsha.ac.ir

Mohammad Reza Pourmand
mpourmand@tums.ac.ir

Mohammad Yousef Alikhani
alikhani@umsha.ac.ir

Sara Soleimani Asl
s.soleimaniasl@umsha.ac.ir

[1] Department of Microbiology, Faculty of Medicine, Hamadan University of Medical Sciences, Hamadan, Iran

[2] Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

[3] Department of Anatomy, Faculty of Medicine, Hamadan University of Medical Sciences, Hamadan, Iran

[4] Nutrition Health Research Center, Hamadan University of Medical Sciences, Hamadan, Iran

## Abbreviations

| | |
|---|---|
| MDR | Multidrug-resistant |
| XDR | Extensively drug-resistant |
| ESBL | Extended-spectrum β-lactamase |
| MBL | Metallo β-lactamase |
| QS | Quorum sensing |
| CF | Cystic fibrosis |
| MEGA5 | Molecular evolutionary genetics analysis version 5 |

## Introduction

*Pseudomonas aeruginosa* possesses an arsenal of virulence factors and a variety of antibiotic resistance mechanisms to cause disease successfully. To regulate the pathogenicity in response to cell density, *P. aeruginosa* develops three different quorum sensing (QS) systems, including Las, Rhl, and PQS [1]. QS causes organisms to act in synchrony for controlling a variety of functions such as bioluminescence,

biofilm formation, virulence factor production, and antibiotic resistance [2]. The systems mentioned above dependently regulate QS- related processes in the organism and are auto-regulated to commence regulatory cascades [3].

The Las is a two-component system which includes a sensor-*lasI*, and a response regulator- *lasR*. In response to the growth phase and accumulation of autoinducer peptides (AIPs), such as acyl-homoserine lactones (AHL), *LasI* expresses, which results in *LasR* activation [4]. *LasR,* as a global regulator, controls the production of siderophores, metalloprotease elastase (LasB), metalloprotease (LasA), phenazines, and exotoxin A [5]. Moreover, pyocyanin as a critical virulence factor of *P. aeruginosa* is regulated and secreted under the control of QS system. This factor is a blue redox-active phenazine, which acts through reactive oxygen species (ROS) production, and plays different roles in disturbing critical organs, including respiratory, cardiovascular, urogenital, and central nervous system (CNS) [6, 7].

QS plays a vital role in the biofilm formation by microorganisms, particularly in chronic infections, and consequently increases antibiotic resistance [8]. Intrinsic or acquired resistance to antibiotics have been evolved through decades and resulted in multi-drug- or extensively drug-resistant (MDR and XDR) strains of *P. aeruginosa* [9]. Due to high energy-consuming processes of antibiotic resistance, microorganisms neatly fine-tune the resistance and the virulence. During *P. aeruginosa* infections, QS plays a notable role to control the resistance and the virulence. In order to illustrate, efflux pumps upregulate in exposure to C4HSL (an AIP from Rhl QS system), and as a consequence, antibiotic resistance increases [10]. However, the relationship between virulence and resistance is still unclear, and contradictions are not resolved.

Although resistance to different antibiotics can affect the fitness costs of the organism and results in attenuation of the production of virulence factors [11, 12], some studies demonstrated a contradiction. It has been mentioned that aztreonam-resistance leads to hypervirulent strains of *P. aeruginosa* in cystic fibrosis (CF) patients [13]. Therefore, the present study aimed to determine the relationship between presence and expression of *las* regulated virulence factors and resistance genes in MDR and XDR *P. aeruginosa* strains.

## Material and methods

### Study design and sample collection

In this descriptive-analytic study, which was performed during six months (April 2015 till August 2016), 73 different clinical specimens, including burn wounds, blood cultures, catheters, and UC (urine culture) samples, were collected from educational Hospitals of Hamadan, Iran. The isolates were characterized and identified by oxidase test (Himedia, India), triple sugar iron (TSI) agar (Merck, Germany), oxidation- fermentation (OF) media (Merck, Germany), and MacConkey agar (Merck, Germany). The oxidase-positive colonies which had a grapelike odor, grown on TSI agar with an alkaline/no change reaction, and consumed glucose only in the aerobic condition in OF media, considered as *P. aeruginosa* isolates [14].

### Antimicrobial agents susceptibility testing

The collected isolates were investigated using antibiotic susceptibility tests for different antibiotic categories including Amikacin (30 µg), Doripenem(10 µg), Meropenem(10 µg), Imipenem(10 µg), Cefoxitin(30 µg), Cefpodoxime(30 µg), Cefotaxime(30 µg), Ceftazidime(30 µg), Ceftriaxone (30 µg), Ciprofloxacin (5 µg), Piperacillin-Tazobactam (100/10 µg), Piperacillin (100 µg),Ticarcillin (75 µg) and Aztreonam (30 µg). The antibiotic susceptibility testing was done based on CLSI 2016. Resistance to at least one antibiotic in more than three antimicrobial categories was considered as MDR. Also, resistance to at least one agent in more than 6 antibiotic families was regarded as XDR. *P. aeruginosa* ATCC 27,853 was used as the reference strain in each assay. All antibiotic disks were purchased from Mast, UK.

### Phenotypic characterization of pyocyanin production

In order to examine pyocyanin production, the chloroform and HCl (Merck, Germany) extraction method based on El Fouly et al. was used [15]. The $OD_{520nm}$ of samples was multiplied to 17.072 to determine the concentration of pyocyanin in µg/mL.

### Phenotypic characterization of pyoverdine production

Strains were inoculated to RPMI1640 (Invitrogen, USA) and incubated at 37 °C by shaking 100 rpm overnight. The $OD_{600nm}$ of the cultures was measured. Then the cultures were centrifuged at 200 g for 30 min. The supernatants were collected and filtered by 0.22 µm Millipore filters (Merck, Germany). Using a spectrophotometer, the $OD_{405nm}$ of supernatants was measured, and then Relative Pyoverdine Production (RPP) was calculated by the following formula: RPP: $OD_{405}/OD_{600}$.

### Biofilm formation

To examine the capacity of isolates to produce biofilm, the Crystal violet assay was done according to O'Toole et al.

study [16]. To define the biofilm production of the isolates, $OD_{cut\ off}$ was calculated using a formula as below: $OD_{cut\ off}$: $OD_{avg}$ of negative control + 3*SD of ODs of the negative control. If OD of tests were less than $OD_{cut\ off}$, the isolates were a non-biofilm producer. The isolates interpreted as weak biofilm producers if the OD lays between $OD_{cut\ off}$ and 2*ODcut off. And strong biofilm formers showed an OD more than $4*OD_{cut\ off}$.

## Phenotypic characterization of LasA and LasB enzymatic activity

LasA enzymatic activity was measured according to Oldak et al. [17]. Briefly, an overnight culture of *Staphylococcus aureus* ATCC25923 was centrifuged and re-suspended in PBS buffer, and the $OD_{600nm}$ was adjusted to 0.8. Also, overnight cultures of *P. aeruginosa* strains were centrifuged and re-suspended in the CDMC solution. The CDMC was (pH 7.4) consisted of glucose (30 mM), NaCl (8 mM), $K_2HPO_4$ (60 mM), $KH_2PO_4$ (35 mM), $ZnCl_2$ (0.025 mM), $(NH_4)_2SO_2$ (15 mM), L-glutamine (7 mM), $CaCl_2$ (0.05 mM), $FeCl_3$ (0.017 mM), $C_6H_5Na_3O_7$ (35 mM), $MgCl_2$ (1.4 mM), thiamine (0.15 mM), DL-arginine (0.22 mM), uracil (0.2 mM), and nicotinic acid (0.1 mM). This solution stimulates LasA Staphylolytic activity. Then, 100 µL of CDMC was added to 900 µL of Staphylococcal suspension, and the decrease in absorbance of the solution was spectrophotometrically monitored at $OD_{595nm.}$ To determine the protease activity of LasB, 1% skim milk medium was used. Isolates were cultured on the plates, incubated overnight at 37 °C, and the clear zone around colonies was monitored the next day.

## Genomic DNA

Strains were inoculated into LB broth (Merck, Germany), and then incubated at 37 °C. In order to extract genomic DNA and plasmid, the Qiagen extraction kit (Germany) was applied using manufacturer instructions.

## Virulence factor production and resistance genes detection

PCR method was applied to determine virulence factor production for *lasB, lasA, apr, plcH, phzI, phzM*. Also, resistance genes, including carbapenemase, ESBLs, MBLs, and AmpC families, were detected using primers, according to Fazeli and et al. study [18, 19]. Multiplex PCR was done in a 50 µL volume reaction containing 2 µL DNA, 1 µL of each primer (5 pmol $L^{-1}$) and 20 µL of master mix (Amplicon, Denmark) reached 50 µL by adding deionized water. The PCR program consisted of an initial denaturation at 96 °C for 10 min, 30 cycles of 1 min denaturation at 96 °C, annealing 58 °C for 1 min, an extension for 1 min at 72 °C, and a final extension at 72 °C for 10 min.

## Sequencing

PCR products were purified and sequenced by Bioneer Co., Korea mediated by Pishgam Co., Iran. The data were analyzed using the Chromas software and compared to the microbial genome using the Basic Local Alignment Search Tool (BLAST) to confirm the sequence authenticity.

## Sample selection for gene expression

All strains of MDR and XDR *P. aeruginosa* were selected to evaluate gene expression. The selected isolates demonstrated variation in Las-regulated virulence production, antibiotic susceptibility patterns, and demographic characteristics.

## RNA extraction and qRT-PCR

The strains were inoculated into LB broth (Merck, Germany), and then incubated at 37 °C. RNA was extracted, and cDNA synthesis was performed using the GeneAll RNA extraction kit and GeneAll cDNA synthesis kit (GeneAll, Korea) according to the manufacturer instructions. Quantitative RT-PCR was used to determine the expression of *lasR*, *lasI, amp*, *mexR. KPC*, *MBL* genes using a syber green master mix (Amplicon, Denmark) and *aroC* was applied as the reference gene. The primers of Lima et al. [20], Quale et al. [21], Zaman et al. [22], and Geyer et al. [23], studies were used for q-RT PCR. To qualify the qPCR test, the standard validation tests for assessing the sensitivity and Melt curve analysis for determining the specificity of primers were performed. To determine the amplification efficiency (EFF %), 10 dilutions of cDNA were prepared, and the most efficient dilution was determined according to the $10^{-slope}$ equation. The most suitable amount in this equation is less than 2. To calculate the expression level, the $2^{-\Delta\Delta CT}$ equation was used based on the Pfaffl study [24]. To normalize expression levels, *P. aeruginosa* PAO1 was used as a reference strain.

## Multilocus sequence typing (MLST)

MLST was performed using the scheme described by Vernez et al., [25]. The primers used to detect seven housekeeping genes (*acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, and *trpE*). For each of the seven MLST loci, 50 µL PCR reactions were performed in 96-well plates. The PCR mixture contained 39.75 µL of molecular grade water, 5 µL of 10 × PCR buffer (Qiagen, UK), 1 µL of a 10 µM concentration of each forward and reverse primer, 1 µL of 10 mM deoxynucleoside triphosphate (dNTP) mix (Invitrogen, UK), 0.25 µL of HotStart Taq DNA polymerase (Qiagen, UK) and 2 µL of *P.*

*aeruginosa*gDNA. The MLST PCR was performed using a Bio-Rad C1001 thermocycler (California, United States) and thermocycling conditions were; 35 cycles at 94 °C for 15 s, 50 °C for 1 min, 72 °C for 1 min. This was followed by a hold step of 72 °C for 7 min.

### Statistical analysis

All statistical analyses of phenotypic data were carried out in SPSS software (version 16, Chicago, IL, USA), using One-Way and Two-Way analysis of variance (ANOVA) for individual comparisons and Tukey's for multiple comparisons. A p-value of less than 0.05 was reported as statistically significant (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). The statistical significance was defined as $P < 0.05$. Gene expression analysis was performed using REST® 2009 (Qiagen, Germany) software. For MLST analysis, strain information that includes strain designation, subspecies, serovar, year of isolation, host, continent, and the source was stored in a MEGA 6 (version 6.0,https://www.megasoftware.net). The nucleotide sequences of gene fragments were also stored in the same database.

## Results

### Isolate characterization and antimicrobial resistance profiles

Totally, 73 isolates of *P. aeruginosa* were isolated from 19 (26.02%) burn wounds, 22 (30.13%), blood cultures, 9 (12.32%) catheters, and 23 (31.50%) urine culture samples in Hamadan University hospitals. Also, according to the disk diffusion method, resistance to cefoxitin, ciprofloxacin, and cefotaxime reported in 79.45%, 75.34% and 97.26% of isolates. Moreover, more than 49% of the isolates were resistant to cefpodoxime (67.12%) and ceftazidime (49.31%). Besides, 39.73% of the isolates were non-susceptible to ceftriaxone, 36.97% to imipenem, 31.50% to doripenem, 30.13% to piperacillin, and 28.76% to meropenem. Resistance to other antibiotics (except for ticarcillin and amikacin) was observed in more than 20% of the isolates (Table 1).

### Pyocyanin and pyoverdine Production

Out of 73 *P. aeruginosa* isolates, 16 isolates (21.91%) possess phenazines, and pyocyanin was detected in 22 isolates (30.13%) (Table 1).

### Biofilm formation

Out of 73 *P. aeruginosa* isolates, 17 (21.91%) isolates were strong biofilm producers, 1 (21.91%) isolates moderately formed biofilm, and 55 (75.34%) isolates were a weak or non-biofilm producer.

### LasA and LasB enzymatic activity

Among 73 *P. aeruginosa* isolates, LasA and LasB proteases were detected in 24 (32.87) isolates, and 22 (30.13) isolates, respectively (Table 2).

### Distribution of virulence factor genes

Out of 73 *P. aeruginosa* isolates, 24 (36.87%) isolates carried *lasA* gene, 20 (27.39%) isolates carried *lasB* gene. Also, the frequency of *apr* and *plcH* were 26.02% (19 isolates) and 21.91% (16 isolates), respectively. Moreover, *phzI* and *phzM* were detected in 21 (28.76%) and 23 (31.50%) isolates, respectively (Table2) (Fig. 1).

### Distribution of antibiotic resistance genes

Among 73 *P. aeruginosa* isolates, 12 (16.43%) isolates carried the *KPC* gene, 10 (13.69%) isolates carried the *IMP* gene. *VIM*, *FOX*, and *MOX* genes were detected in 10 (13.69%) isolates, 55 (75.34%), and 48 (65.75%) isolates, respectively. 11 (15.06%) isolates carried the *SIM* gene, 9 (12.32%) isolates carried the *GIM* gene. Moreover, 29 (39.72%) and 27 (36.98%) isolates were identified to carry *TEM* and *SHV* plasmid genes, respectively (Table1) (Fig. 1).

### Gene sequencing

All PCR products were assigned to the microbial genome using the Basic Local Alignment Search Tool (BLAST) and showed the same DNA sequences, which confirmed all PCR assay results.

### Resistance and Virulence genes functionality evaluation:

According to Fig. 2a, the activity of antibiotic resistance and virulence factors genes in XDR strains is more than MDR strains. In non-MDR-XDR strains, the activity of virulence factor genes was increased, and the activity of antibiotic resistance genes was decreased. In Fig. 2b, the expression of the virulence factor genes was higher in the biofilm producing strains. However, the expression of antibiotic resistance genes in weak/non-biofilm producer strains was reduced.

Strains with strong biofilm showed increased expression of virulence factor and antibiotic resistance genes. Also, *KPC* and *MBL* genes were associated with reduced expression in non-biofilm forming strains. Due to the distribution of LasA and LasB protease enzymes in different strains (Tables 1 and 2), the increased frequency of these

**Table 1** Prevalence of phenotypic characteristics of virulence factor and antibiotic resistance genes in *P. aeruginosa*

| Characteristics | Number (%) of isolates | | | $p^a$ | Number (%) of isolates | | | | | | | | | $p^a$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Total | MDR[b] | XDR[c] | | *KPC* | *IMP* | *VIM* | *FOX* | *MOX* | *SIM* | *GIM* | *TEM* | *SHV* | |
| | n = 73 | n = 21 | n = 11 | | n = 12 | n = 10 | n = 10 | n = 55 | n = 48 | n = 11 | n = 9 | n = 29 | n = 27 | |
| *Biofilm production* | | | | | | | | | | | | | | |
| Low and non | 55 (75.34) | 5 (23.80) | 0 (0) | 0.836 | 0 (0) | 1 (10) | 2 (20) | 37 (64.91) | 31 (64.58) | 0 (0) | 0 (0) | 12 (41.37) | 10 (37.37) | 0.152 |
| Moderate | 1 (1.36) | 0 (0) | 1 (9.09) | 0.050 | 1 (8.33) | 1 (10) | 1 (10) | 1 (1.81) | 0 (0) | 1 (9.09) | 0 (0) | 0 (0) | 0 (0) | 0.006 |
| High | 17 (23.28) | 16 (76.19) | 10 (90.90) | 0.012 | 11 (91.66) | 8 (80) | 7 (70) | 17 (30.90) | 17 (35.41) | 10 (90.90) | 9 (100) | 17 (58.62) | 17 (62.96) | 0.066 |
| *Proteases* | | | | | | | | | | | | | | |
| LasA Protease Negative | 49 (67.12) | 3 (14.28) | 1 (9.09) | 0.152 | 3 (25) | 2 (20) | 3 (30) | 33 (60) | 34 (70.83) | 1 (9.09) | 0 (0) | 10 (34.48) | 7 (25.92) | 0.152 |
| LasA Protease Positive | 24 (32.87) | 18 (85.71) | 10 (90.90) | 0.006 | 9 (75) | 8 (80) | 7 (70) | 22 (40) | 14 (29.17) | 10 (90.90) | 9 (100) | 19 (65.51) | 20 (74.07) | 0.006 |
| LasB Protease Negative | 51 (69.86) | 1 (4.76) | 2 (18.18) | 0.066 | 2 (16.66) | 2 (20) | 1 (10) | 33 (60) | 29 (60.41) | 0 (0) | 0 (0) | 8 (27.58) | 5 (18.51) | 0.066 |
| LasB Protease Positive | 22 (30.13) | 20 (95.23) | 9 (81.81) | 0.031 | 10 (83.33) | 8 (20) | 9 (90) | 22 (40) | 19 (39.58) | 11 (100) | 9 (100) | 21 (72.41) | 22 (81.48) | 0.031 |
| *Phenazine pigments* | | | | | | | | | | | | | | |
| Negative | 57 (78.08) | 6 (28.57) | 0 (0) | 0.413 | 1 (8.33) | 0 (0) | 0 (0) | 41 (74.54) | 32 (66.66) | 0 (0) | 0 (0) | 14 (48.27) | 13 (48.14) | 0.010 |
| Positive | 16 (21.91) | 15 (71.42) | 11 (100) | 0.012 | 11 (91.66) | 10 (100) | 10 (100) | 14 (25.45) | 16 (33.33) | 11 (100) | 9 (100) | 15 (51.72) | 14 (51.85) | 0.036 |
| *Pyocyanin production* | | | | | | | | | | | | | | |
| Negative | 51 (69.86) | 2 (2.73) | 1 (9.09) | 0.413 | 2 (75.34) | 0 (0) | 0 (0) | 36 (65.45) | 28 (58.33) | 0 (0) | 0 (0) | 11 (35.71) | 7 (25.92) | 0.152 |
| Positive | 22 (30.13) | 19 (90.47) | 10 (90.90) | 0.047 | 10 (75.34) | 10 (100) | 10 (100) | 19 (35.54) | 20 (41.66) | 11 (100) | 9 (100) | 18 (62.08) | 20 (74.07) | 0.025 |
| *Resistance* | | | | | | | | | | | | | | |
| Cefoxitin | 58 (79.45) | 20 (95.23) | 11 (100) | 0.026 | 12 (100) | 10 (100) | 10 (100) | 55 (100) | 48 (100) | 9 (81.81) | 2 (22.22) | 19 (65.51) | 17 (62.96) | 0.006 |
| Cefpodoxime | 49 (67.12) | 21 (100) | 11 (100) | 0.050 | 12 (100) | 10 (100) | 10 (100) | 49 (89.90) | 48 (100) | 11 (100) | 9 (100) | 29 (100) | 27 (100) | 0.066 |
| Cefotaxime | 71 (97.26) | 21 (100) | 11 (100) | 0.055 | 12 (100) | 10 (100) | 10 (100) | 55 (100) | 48 (100) | 11 (100) | 9 (100) | 29 (100) | 27 (100) | 0.031 |
| Ceftazidime | 36 (49.31) | 21 (100) | 11 (100) | 0.055 | | | | | | | | | | 0.152 |
| Ciprofloxacin | 55 (75.34) | 21 (100) | 11 (100) | 0.025 | 12 (100) | 10 (100) | 10 (100) | 55 (100) | 48 (100) | 11 (100) | 9 (100) | 29 (100) | 27 (100) | 0.006 |
| Aztreonam | 13 (17.80) | 11 (40.74) | 11 (100) | 0.025 | 5 (41.66) | 4 (40) | 10 (100) | 13 (23.63) | 13 (27.08) | 11 (100) | 4 (44.44) | 9 (31.03) | 7 (25.92) | 0.066 |
| Ceftriaxone | 29 (39.73) | 20 (95.23) | 11 (100) | 0.019 | 12 (100) | 10 (100) | 10 (100) | 29 (52.72) | 29 (60.41) | 11 (100) | 9 (100) | 29 (100) | 27 (100) | 0.031 |
| Amikacin | 16 (21.91) | 11 (40.74) | 11 (100) | 0.022 | 6 (50) | 6 (60) | 7 (70) | 16 (29.09) | 16 (33.33) | 11 (100) | 9 (100) | 13 (44.82) | 21 (77.77) | 0.152 |
| Piperacillin | 22 (30.13) | 16 (76.19) | 11 (100) | 0.044 | 9 (75) | 8 (75.34) | 4 (60) | 22 (40) | 22 (45.83) | 7 (63.63) | 4 (44.44) | 11 (37.93) | 19 (70.37) | 0.006 |
| Doripenem | 23 (31.50) | 13 (61.90) | 11 (100) | 0.062 | 12 (100) | 10 (100) | 10 (100) | 21 (38.18) | 19 (39.58) | 11 (100) | 9 (100) | 19 (65.51) | 22 (81.48) | 0.066 |
| Meropenem | 21 (28.76) | 12 (57.14) | 11 (100) | 0.012 | 12 (100) | 10 (100) | 10 (100) | 19 (34.54) | 17 (35.41) | 11 (100) | 9 (100) | 20 (75.86) | 19 (70.37) | 0.031 |
| Imipenem | 27 (36.97) | 16 (76.19) | 11 (100) | 0.013 | 12 (100) | 10 (100) | 10 (100) | 25 (45.45) | 21 (43.75) | 11 (100) | 9 (100) | 13 (85.41) | 24 (88.88) | 0.152 |
| Ticarcielin | 13 (17.80) | 11 (40.74) | 11 (100) | 0.013 | 7 (58.33) | 5 (50) | 4 (60) | 13 (23.63) | 13 (27.08) | 5 (45.45) | 5 (55.55) | 13 (44.82) | 13 (48.14) | 0.006 |
| Piperacilin-Tazobactam | 19 (26.02) | 11 (40.74) | 11 (100) | 0.636 | 5 (41.66) | 5 (50) | 2 (20) | 19 (34.54) | 19 (39.58) | 6 (54.54) | 3 (33.33) | 7 (24.13) | 10 (37.07) | 0.066 |

[a]P-value

[b]Multiple drug resistance

[c]Extensively drug-resistant

**Table 2** Prevalence of Phenotypic characteristics of virulence factor and virulence genes in *P. aeruginosa*

| Characteristics | Number (%) of isolates | | | $p^a$ | Number (%) of isolates | | | | | | $p^a$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Total | MDR | XDR | | *lasA* | *lasB* | *apr* | *plcH* | *phzI* | *phzM* | |
| | n = 73 | n = 21 | n = 11 | | n = 24 | n = 20 | n = 19 | n = 16 | n = 21 | n = 23 | |
| *Biofilm production* | | | | | | | | | | | |
| Low and non | 55 (75.34) | 5 (23.80) | 0 (0) | 0.836 | 7 (29.16) | 2 (10) | 5 (26.31) | 6 (37.5) | 3 (14.28) | 6 (26.08) | 0.015 |
| Moderate | 1 (1.36) | 0 (0) | 1 (9.09) | 0.050 | 0 (0) | 1 (5) | 2 (10.52) | 1 (6.25) | 1 (4.76) | 1 (4.34) | 0.025 |
| High | 17 (23.28) | 16 (76.19) | 10 (90.90) | 0.012 | 17(70.83) | 17(85) | 12 (63.15) | 9 (56.25) | 17 (80.95) | 16 (69.56) | 0.049 |
| *Proteases* | | | | | | | | | | | |
| LasA Protease Negative | 49 (67.12) | 3 (14.28) | 1 (9.09) | 0.152 | 0 (0) | 4(20) | 4(21.05) | 11(68.75) | 17(80.95) | 15(65.21) | 0.001 |
| LasA Protease Positive | 24 (32.87) | 18 (85.71) | 10 (90.90) | 0.006 | 24(100) | 16(80) | 15(78.94) | 5(31.25) | 4(19.04) | 8(34.78) | 0.035 |
| LasB Protease Negative | 51 (69.86) | 1 (4.76) | 2 (18.18) | 0.066 | 5(20.83) | 1(5) | 6(31.57) | 9(56.25) | 14(66.66) | 17(73.91) | 0.001 |
| LasB Protease Positive | 22 (30.13) | 20 (95.23) | 9 (81.81) | 0.031 | 17(70.83) | 19(95) | 13(68.42) | 7(43.75) | 7(33.33) | 6(26.08) | 0.024 |
| *Phenazine pigments* | | | | | | | | | | | |
| Negative | 57 (78.08) | 6 (28.57) | 0 (0) | 0.413 | 20(83.33) | 17(85) | 3 (15.78) | 0 (0) | 5 (23.80 | 7 (30.44) | 0.057 |
| Positive | 16 (21.91) | 15 (71.42) | 11 (100) | 0.012 | 4(16.66) | 5(25) | 16(84.21) | 16(100) | 16(76.20) | 16(69.56) | 0.001 |
| *Pyocyanin production* | | | | | | | | | | | |
| Negative | 51 (69.86) | 2 (2.73) | 1 (9.09) | 0.413 | 19(79.61) | 14(70) | 2(10.5) | 0 (0) | 0 (0) | 2 (8.69) | 0.091 |
| Positive | 22 (30.13) | 19 (90.47) | 10 (90.90) | 0.047 | 5(20.83) | 6(30) | 17(89.47) | 16(100) | 21(100) | 21(95.45) | 0.005 |
| *Resistance* | | | | | | | | | | | |
| Cefoxitin | 58 (79.45) | 20 (95.23) | 11 (100) | 0.026 | 20(83.33) | 19(95) | 15(78.94) | 13(81.25) | 16(76.19) | 20(86.95) | 0.007 |
| Cefpodoxime | 49 (67.12) | 21 (100) | 11 (100) | 0.050 | 17(70.83) | 17(85) | 13(68.42) | 9(56.25) | 17(80.95) | 18(78.26) | 0.001 |
| Cefotaxime | 71 (97.26) | 21 (100) | 11 (100) | 0.055 | 11(45.83) | 11(55) | 6(31.57) | 8(50) | 6(28.57) | 11(47.82) | 0.031 |
| Ceftazidime | 36 (49.31) | 21 (100) | 11 (100) | 0.055 | 13(54.16) | 7(35) | 11(57.89) | 13(81.25) | 14(66.66) | 13(56.52) | 0.057 |
| Ciprofloxacin | 55 (75.34) | 21 (100) | 11 (100) | 0.025 | 18(64.28) | 17(85) | 14(73.68) | 13(81.25) | 17(80.95) | 21(91.30) | 0.007 |
| Aztreonam | 13 (17.80) | 11 (40.74) | 11 (100) | 0.025 | 5(20.83) | 9(45) | 3(15.78) | 3(18.75) | 7(33.33) | 9(39.13) | 0.019 |
| Ceftriaxone | 29 (39.73) | 20 (95.23) | 11 (100) | 0.019 | 21(87.5) | 18(90) | 14(73.68) | 11(68.75) | 17(80.95) | 21(91.30) | 0.046 |
| Amikacin | 16 (21.91) | 11 (40.74) | 11 (100) | 0.022 | 9(37.5) | 11(55) | 12(63.15) | 7(43.75) | 11(52.38) | 9(39.13) | 0.013 |
| Piperacillin | 22 (30.13) | 16 (76.19) | 11 (100) | 0.044 | 17(70.83) | 14(70) | 14(73.68) | 7(43.75) | 16(76.19) | 17(73.91) | 0.005 |
| Doripenem | 23 (31.50) | 13 (61.90) | 11 (100) | 0.062 | 14(58.33) | 7(35) | 7(36.84) | 7(81.25) | 13(61.90) | 14(60.86) | 0.075 |
| Meropenem | 21 (28.76) | 12 (57.14) | 11 (100) | 0.012 | 16(66.66) | 9(45) | 5(26.31) | 4(25) | 10(47.61) | 12(52.17) | 0.021 |
| Imipenem | 27 (36.97) | 16 (76.19) | 11 (100) | 0.013 | 10(41.66) | 5(25) | 11(57.89) | 9(56.25) | 15(71.42) | 16(69.56) | 0.036 |
| Ticarcielin | 13 (17.80) | 11 (40.74) | 11 (100) | 0.013 | 4(16.66) | 3(15) | 6(31.57) | 6(37.5) | 9(42.85) | 8(34.78) | 0.057 |
| Piperacilin-Tazobactam | 19 (26.02) | 11 (40.74) | 11 (100) | 0.636 | 9(37.5) | 5(25) | 4(21.05) | 5(31.25) | 10(47.61) | 11(47.82) | 0.018 |

[a]P-value

[b]Multiple drug resistance

[c]Extensively drug-resistant

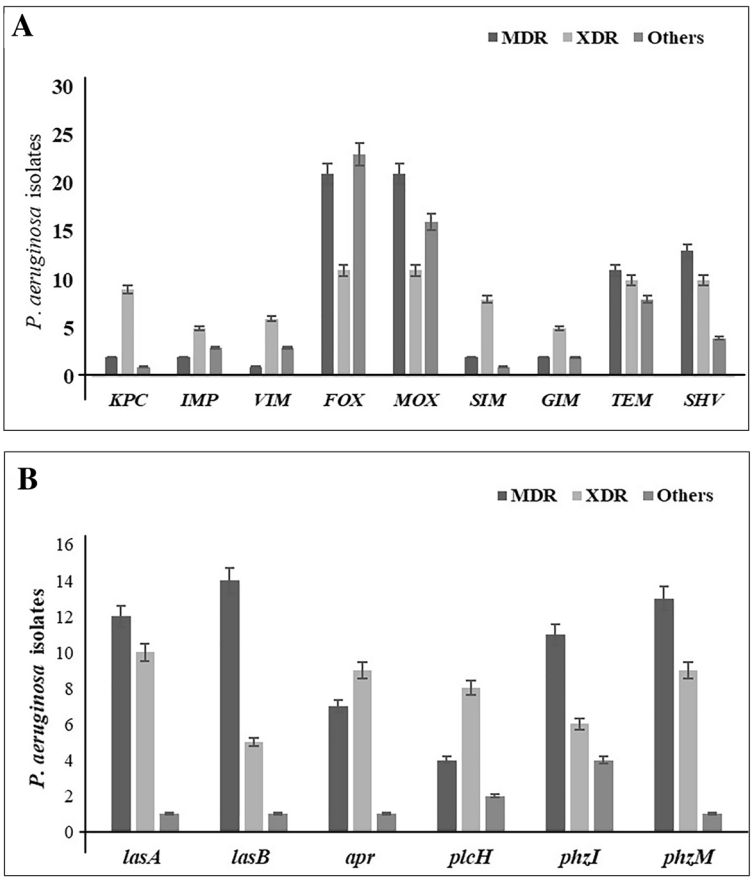


**Fig. 1** Prevalence of antibiotic resistance (**a**) and virulence factor (**b**) genes in MDR, XDR and other strains of *P. aeruginosa*

enzymes was associated with increased expression of the virulence factor and antibiotic resistance genes.

The frequency of phenazine and pyocyanin in MDR and XDR strains was higher than sensitive strains. Moreover, the expression of virulence factor and antibiotic resistance genes in XDR and MDR strains comparing to sensitive strains was extremely high. Of course, the expression of the *MBL* and *ampC* genes differed from other genes and did not follow the distribution of these enzymes.

### Phylogenetic analysis of the MLST sequences

MLST was performed on all 73 isolates with reference strains. MLST analysis identified 16 known sequence types (ST108, ST260, ST217) and found 3 sequence types (ST2452, ST2427, and ST2542) to be novel (Fig. 3). The phylogenetic tree shows that ST108, ST217, ST260, ST274, ST501, ST1075, and ST3335 were highly resistant and pathogenic isolates (Fig. 3). Several close clusters were identified. Typing by MLST further illustrated the large diversity found within the strains, as isolates with similar β-lactamase genes had different STs.

### Statistical analysis

The frequency of antibiotic resistance genes and virulence factor genes had a significant correlation with the antibiotic resistance pattern ($p \leq 0.05$). Also, the frequency of antibiotic resistance genes and virulence factors were significantly correlated with biofilm expression ($p \leq 0.05$) (Table 3).

Data analysis by Wilcoxon signed-rank test showed that the activity of QS genes had an inductive effect on each other. Also, the results clearly showed that there was a significant relationship between *ampC* and *lasR* gene expressions. On the other hand, a weak correlation was observed between the activity of *KPC* and *lasR* genes in the isolates (Fig. 2) (Table 3).

## Discussion

*Pseudomonas aeruginosa,* as an opportunistic pathogen, is capable of causing a wide range of infections, particularly in patients with a compromised immune system due to long term hospitalization or other damages [18]. Prevalence of *P. aeruginosa* resistant to at least one of the

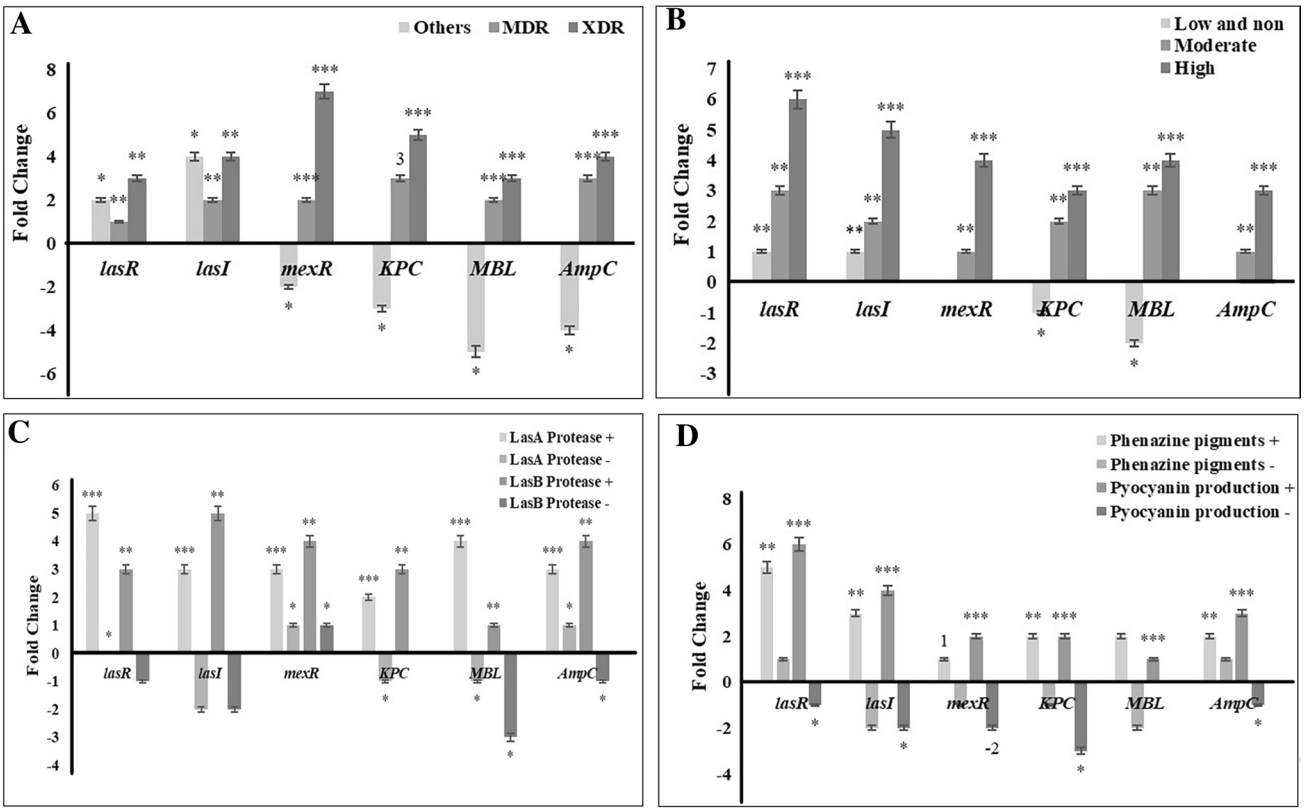


**Fig. 2** Virulence and antibiotic resistance gene expression average's in MDR and XDR strains. **a** Gene expression of virulence and antibiotic resistance genes in MDR and XDR strains. **b** Gene expression of virulence and antibiotic resistance genes in biofilm positive and biofilm negative strains. **c** Gene expression of virulence and antibiotic resistance genes in strains with/without lasA and lasB enzymes. **d** Gene expression of virulence and antibiotic resistance genes in strains with/without phenazine and pyocyanin enzymes. Bars represent means ± SD of the results of three independent experiments. Asterisks indicate significant differences in gene expression levels between (*P < 0.05; **P < 0.01; ***P < 0.001)

studied antibiotics was 70%, higher than reported by Dou et al. [26], in their study investigating antibiotic resistance among *P. aeruginosa*, also during the seven-year period, resistant strains of this bacterium have increased. In our study, the most frequent resistance was detected against cefoxitin (79.45%), cefotaxime (97.26%), and ciprofloxacin (75.34%) (approximately 70% of isolates). This prevalence rate was comparatively higher than the reports of Acar et al. [27], Liews et al. [28], and less than Choudhary et al. [29] studies.

During a study in Turkey, it was found that 55% of bacteria isolated from the hospital during 2007–2017 were resistant to many beta-lactamase antibiotics [27]. In this case, the prevalence of ESBL, MBL, AmpC, and KPC-producing *P. aeruginosa* was around 38.35%, 13.69%, 70.54%, and 16.43%, respectively, relatively lower than the figures reported by Ghasemian et al. [30] and Choudhary et al. [29]. One of the most important reasons for these differences is the type of weather in the regions, the pattern of antibiotic use, various mutations in bacteria, and the health of the communities. Although some issues, including the emergence of new antibiotics and the inter-relationship of bacteria, have contributed to the appearance of such a resistance.

There are many reports that show an association between antimicrobial resistance patterns and resistance genes and virulence factor genes in *P. aeruginosa*. Our results showed that there is a significant relationship between antibiotic resistance and pathogenesis. Nearly all MDR and XDR strains strongly formed biofilm (more than 95%), and about 80% of the MDR and XDR strains of *P. aeruginosa* produced phenazine, pyocyanin and protease enzymes. Hwang et al. [31] and Tahmasebi et al. [32] showed a significant correlation between antibiotic resistance and pathogenicity in different strains of *P. aeruginosa*.

Our findings also indicated a direct relationship between phenazine production and *lasI/R* expression. *lasI/R* was expressed in 11 isolates, which produced phenazine, protease, and siderophores. According to Abd El-Aziz et al. [33], *lasI/R* system plays a key role in the regulation and production of phenazine. There was a converse relationship in one isolate. It might be due to unidentified mutations in *lasI/R*. Proteases including *lasA*, *lasB,* and *aprA*

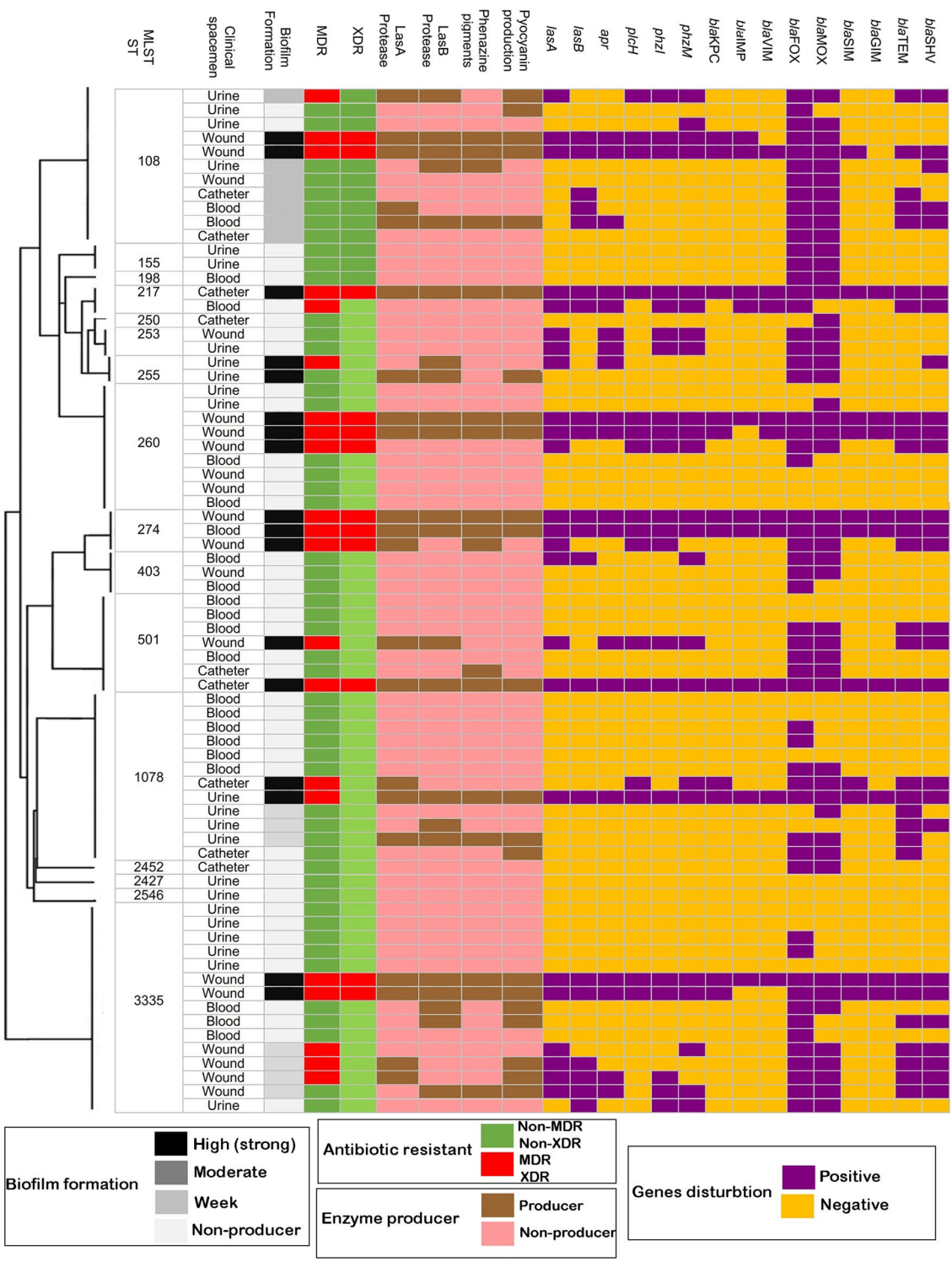


**Fig. 3** Dendrogram of 73 strains of colistin-resistant *P. aeruginosa*

are positively regulated by *lasI/R* QS system [5, 34], and as our result showed so, the proteases were detected in 10 isolates out of 11 MDR-XDR isolates. Furthermore, There was not an established association between *MBL* and *lasI/R* expression in our study except for MDR-XDR isolates, which were consistent with Goncalves et al., study [35]. In contrast to Al Dawodeyah et al., and Lee et al. studies, there was a high expression level of *KPC* in 10 isolates in

**Table 3** Relationship between the biofilm formation, genotypic virulence factors and genotypic antibiotic resistance

| Antibiotics | Biofilm | Genotypic virulence factors ($p^a$) | | | | | | | | | Genotypic Antibiotic resistance | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *lasA* | *laSB* | *apr* | *plcH* | *phzI* | *phzM* | *KPC* | *IMP* | *VIM* | *Fox* | *Mox* | *SIM* | *GIM* | *ampC* | *TEM* | *SHV* |
| Cefoxitin | 0.015 | 0.041 | 0.065 | 0.003 | 0.001 | 0.004 | 0.033 | 0.015 | 0.033 | 0.022 | 0.001 | 0.005 | 0.010 | 0.045 | 0.047 | 0.044 | 0.025 |
| Cefpodoxime | 0.004 | 0.003 | 0.080 | 0.033 | 0.033 | 0.029 | 0.074 | 0.045 | 0.048 | 0.008 | 0.003 | 0.002 | 0.020 | 0.011 | 0.015 | 0.012 | 0.015 |
| Cefotaxime | 0.017 | 0.069 | 0.01 | 0.040 | 0.032 | 0.021 | 0.097 | 0.015 | 0.015 | 0.018 | 0.002 | 0.004 | 0.015 | 0.030 | 0.019 | 0.022 | 0.005 |
| Ceftazidime | 0.009 | 0.024 | 0.007 | 0.020 | 0.046 | 0.051 | 0.064 | 0.049 | 0.05 | 0.023 | 0.03 | 0.03 | 0.019 | 0.017 | 0.025 | 0.006 | 0.009 |
| Ciprofloxacin | 0.004 | 0.005 | 0.305 | 0.083 | 0.117 | 0.047 | 0.043 | 0.038 | 0.047 | 0.005 | 0.05 | 0.05 | 0.045 | 0.021 | 0.02 | 0.017 | 0.008 |
| Aztreonam | 0.009 | 0.033 | 0.597 | 0.020 | 0.076 | 0.051 | 0.142 | 0.022 | 0.022 | 0.027 | 0.019 | 0.01 | 0.040 | 0.019 | 0.017 | 0.016 | 0.002 |
| Ceftriaxone | 0.022 | 0.002 | 0.824 | 0.081 | 0.018 | 0.047 | 0.006 | 0.035 | 0.039 | 0.004 | 0.004 | 0.007 | 0.020 | 0.044 | 0.001 | 0.038 | 0.005 |
| Amikacin | 0.060 | 0.079 | 0.099 | 0.064 | 0.072 | 0.065 | 0.091 | 0.045 | 0.016 | 0.009 | 0.037 | 0.065 | 0.058 | 0.029 | 0.007 | 0.05 | 0.035 |
| Piperacillin | 0.014 | 0.009 | 0.416 | 0.420 | 0.046 | 0.602 | 0.004 | 0.005 | 0.028 | 0.016 | 0.011 | 0.043 | 0.077 | 0.001 | 0.007 | 0.01 | 0.019 |
| Doripenem | 0.005 | 0.016 | 0.214 | 0.002 | 0.008 | 0.013 | 0.080 | 0.002 | 0.009 | 0.014 | 0.047 | 0.004 | 0.005 | 0.305 | 0.083 | 0.018 | 0.042 |
| Meropenem | 0.065 | 0.008 | 0.029 | 0.061 | 0.055 | 0.008 | 0.001 | 0.028 | 0.001 | 0.029 | 0.045 | 0.009 | 0.033 | 0.597 | 0.020 | 0.05 | 0.024 |
| Imipenem | 0.003 | 0.007 | 0.801 | 0.086 | 0.003 | 0.858 | 0.900 | 0.015 | 0.008 | 0.001 | 0.002 | 0.040 | 0.027 | 0.001 | 0.026 | 0.043 | 0.018 |
| Ticarcielin | 0.062 | 0.027 | 0.801 | 0.180 | 0.603 | 0.365 | 0.900 | 0.065 | 0.065 | 0.801 | 0.063 | 0.072 | 0.020 | 0.041 | 0.080 | 0.091 | 0.095 |
| Piperacilin-Tazobactam | 0.041 | 0.088 | 0.488 | 0.073 | 0.067 | 0.782 | 0.309 | 0.048 | 0.048 | 0.088 | 0.058 | 0.080 | 0.043 | 0.060 | 0.052 | 0.090 | 0.073 |
| MDR | 0.05 | 0.02 | 0.007 | 0.02 | 0.07 | 0.05 | 0.042 | 0.025 | 0.045 | 0.04 | 0.019 | 0.041 | 0.024 | 0.019 | 0.037 | 0.05 | 0.002 |
| XDR | 0.02 | 0.008 | 0.009 | 0.001 | 0.005 | 0.008 | 0.001 | 0.048 | 0.041 | 0.009 | 0.005 | 0.009 | 0.043 | 0.007 | 0.030 | 0.05 | 0.044 |

accordance with *lasI/R* expression. These results may indicate that the KPC enzymes are responsible for carbapenem resistance in MBLs [36, 37].

Our results confirm a relationship between *mexR* expression and MDR-XDR strains and virulence enzyme. In addition, the expression of antibiotic resistance and virulence factors in XDR strains is higher than MDR strains. In non-MDR and non-XDR strains, the activity of virulence factor genes was increased, and the activity of antibiotic resistance genes was decreased. Although, it seems that more factors might influence the association, such as environmental elements. Efflux pump families as functional and metabolic pumps significantly act in drug resistance of *P. aeruginosa*. Resistance- nodulation- division (RND) family is commonly regulated via *mexR*, *nalC,* or *nalD*.

The initial regulator of RND efflux pumps-*mexR* can determine efflux function as a negative regulator, and Cabot et al. reported that the *mexR* mutant indicates a significant reduction in susceptibility to β-lactams and fluoroquinolones [11, 38]. As our findings demonstrated, the *mexR* expression level was increased in 11 isolates in accordance with *lasR/I* and, as a consequence, *kpc* expression raised in the XDR and MDR strains.

Therefore, QS systems could impressively interconnect virulence and efflux pump functions [39, 40]. Poole [41] and Al Dawodeyah et al. [36] reported that there was a significant relationship between the presence of beta-lactamase enzymes and pathogenicity of bacteria so that the MBL and AmpC possessing strains of *P. aeruginosa* were more pathogenic than antibiotic susceptible strains. In line with previously mentioned studies, there was a direct relationship between *kpc* and *lasI/R,* and also the expression of efflux pump genes was decreased.

In the present study, we have done MLST for 73 *P. aeruginosa* and found predominantly high-risk clones of ST108, ST217, ST260, ST274, ST501, ST1075, and ST3335 of MDR-XDR positive *P. aeruginosa* among different isolates (Fig. 3). We have also confirmed a high frequency of ST108 and ST3335 in virulent strains. Similar findings have been reported from Estonia more than 50% of beta-lactamase-producing *P. aeruginosa* belong to ST108 and ST260 [42]. Furthermore, the study also testified ST108, ST147, ST250, and ST260 in ESBL, NDM, and KPC-producing *P. aeruginosa* isolated from Poland and Germany [43, 44]. However, Vatansever et al. [45] demonstrated that ST235 is a high-risk *P. aeruginosa* producing clone, but to our knowledge, ST235 has not been identified in NDM positive *P. aeruginosa* so far.

The presence of sequence types belonging to ST260, ST274, and ST3335 beta-lactamase-producing isolates is also worrisome as their expansion has been reported previously from other countries [46, 47]. Identification of high-risk clone ST260, ST274, and ST3335 isolates in association with *ESBL*, *MBL*, and *ampC* genes is significant. ST260, ST274, and ST3335 have been associated with the successful dissemination of biofilm formation and virulence factors with high prevalence worldwide. *P. aeruginosa* ST274 encoding biofilm forming genes, which have been reported to cause serious infections in patients from Spain [47].

In order to define a comprehensive and accurate relationship between antibiotic and virulence, further studies are necessary to investigate a considerable number of isolates from different sources, various antibiotic resistance patterns, and virulence factors. In this regard, host-related factors should be taken into account. The low number of isolates and less diverse patterns of antibiotic and virulence factors could be acknowledged as some limitations of this research.

## Conclusion

The correlation of virulence factors with antibiotic resistance is a complex topic which has yet to be explained. However, based on our results, there is a clear association between QS-regulated virulence and antibiotic resistance. Furthermore, in *P. aeruginosa*, QS tends to be able to regulate antibiotic tolerance and pathogenicity. In addition, the expression of genes involved in the pathogenesis of *P. aeruginosa* showed that MDR and XDR strains are more virulent than antibiotic-sensitive strains. Also, according to phylogenetic tree, dangerous strains were identified in ST108, ST260, and ST274, which indicates the high distribution of dangerous STs in Iran.

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**Author contributions** SD performed microbiological and molecular tests and wrote the manuscript. MRA supervised all of the stages of designing the study, conducting the research, and writing the manuscript. MRP, MYA and SSA play a role in Project Administration.

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**Data Availability** The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Compliance with ethical standards

**Conflicts of interest** All authors declare that they have no conflict of interest.

**Ethics approval** This study was approved by the Ethics Committee of Hamadan University of Medical Sciences (Code No: IR.UMSHA.REC.1395402.) about the consent to participate is not applicable.

**Consent for publication** Not Applicable.

**Research involving Human Participants and/or Animals** In this study, there isn't any research involving Human participant or animals.

**Informed consent** Not Applicable.

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