

Role and Function of KPC and MBL Enzymes in Increasing the Pathogenicity of *Pseudomonas Aeruginosa* Isolated from Burn Wounds

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ABSTRACT

BACKGROUND AND OBJECTIVE: *Pseudomonas aeruginosa* is one of the main causes of hospital infections. Pathogenic factors in this bacterium may play a role in the resistance to carbapenem and beta-lactam. The purpose of this study was to evaluate the role and function of KPC and MBL enzymes in increasing the pathogenicity of *Pseudomonas aeruginosa* isolated from burn wounds.

METHODS: In this cross-sectional study, 63 isolates of *Pseudomonas aeruginosa* from burn wounds of different patients were isolated using biochemical tests such as fermentation of sugars in the OF medium, oxidase test, and so on. Determination of resistance pattern and strains with metalloβ-lactamase and carbapenemase was done by disc diffusion method. The oprD gene was used for molecular confirmation of isolates. PCR method was used to detect pathogenicity genes.

FINDINGS: Out of 63 isolates of *Pseudomonas aeruginosa* isolated from burn patients, 10 isolates (15.83%) had KPC enzyme and 13 isolates (20.63%) had MBL enzymes. Doripenem, Ertapenem and meropenem were the most frequent. Also, the *lasB* gene was observed in 43 isolates (68.25%), *plcN* gene in 41 isolates (65.07%), *lasA* gene in 20 isolates (31.74%), *apr* in 60 isolates (95.23%), *phzI* gene in 53 isolates (84.12%), the *phzII* gene in 38 isolates (60.31%), *phzH* gene in 30 isolates (47.61%) and *plcH* gene in 56 isolates (88.88%).

CONCLUSION: The results of this study showed that the production of Carbapenemase and MBL enzymes increased the pathogenicity of *Pseudomonas aeruginosa* isolated from burn wounds.

KEY WORDS: Antibiotic Resistance, *Pseudomonas Aeruginosa*, Virulence Factors, Carbapenem Antibiotics.

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Introduction

Pseudomonas aeruginosa is one of the main causes of hospital infections and causes infectious diseases in people with immune deficiency (1). When using catheters, skin damage such as rupture or burns, *Pseudomonas aeruginosa* binds to the mucous membrane and, after fixing its position, it spreads to other tissues and causes systemic illness (2, 3). One of the most important pathogenic factors in *Pseudomonas aeruginosa* is biofilm. The alginate produced by *Pseudomonas aeruginosa* is the main source of biofilm production. In this bacterium, chromosensing is controlled by two las and rhl systems (5, 4). Las system contains the lasR and lasI transcription activator genes, and the rhl system contains the rhlI and rhlR genes. LasI gene regulates elastase, exotoxin A and alkaline protease production (4).

Two types of phospholipase (PLC) are produced by *Pseudomonas aeruginosa*; high molecular weight phospholipase, which is hemolytic (PLC-H), while low molecular weight is non-hemolytic phospholipase (PLC-N), which leads to the decomposition of sulfidrylcholine, which leads to release of diacylglycerol and choline (6). In addition to these factors, the presence of phenysin and extracellular polysaccharides can also play a significant role in the pathogenicity of *Pseudomonas aeruginosa*. In this regard, two opron phzA1 and phzA2 play a major role in controlling and regulating the production of phenysin in this bacterium (7). Beta-lactamase enzymes, which cause resistance to various β -lactamate groups, are classified into four groups A to D, according to structural classification (8). The beta-lactams of groups A and C are the most common and, as in class D, they contain serine in its active site. Class B beta-lactamases include metallo-beta-lactamases and their coenzyme is zinc (9). Carbapenems in group A of amber are used to treat serious infections in hospitals (10). Compared with penicillins, cephalosporins or beta-lactams containing beta-lactamase inhibitors have a wide range of antimicrobial activity, including gram-positive bacteria (e.g., imipenem, dipropenem) and gram negative (e.g., meropenem, ortpanem) (11, 12).

Imipenem and meropenem have a better effect on *Pseudomonas aeruginosa*. carbapenems (imipenem-meropenem) are used for treatment of Beta-lactam-resistant strains which resistant to beta-lactams. However, in recent years resistance to carbapenems has been reported in many cases. This resistance is due to decreased penetration of the drug and the production of carbapenems hydrolyzing enzymes. These enzymes

have a wide range of substrates and are able to hydrolyze all beta-lactams, except monobactam (aztreonam) (13). The genes encoding these enzymes are located on integrons and can be integrated into the plasmid or chromosome. Therefore, they are also capable of transferring to susceptible strains of *Pseudomonas aeruginosa* (14). In some studies, the relationship between antibiotic resistance and the presence of *Pseudomonas aeruginosa* has been addressed, but the fact that the strains of Carbapenemase and Metallobetalactamase differ in terms of pathogenicity pattern has not been studied (15).

The aim of this study was to determine the role and function of Klebsiella pneumoniae Carbapenemase and Metallobetalactamase enzymes in increasing the pathogenicity of *Pseudomonas aeruginosa* isolated from burn wounds, in order to obtain a suitable pattern for pathogenicity and the occurrence of metallo-beta-lactamase and carbapenem resistance in *Pseudomonas aeruginosa*.

Methods

Isolation, identification and culture of bacteria: In this cross-sectional study, after approval by the Ethics Committee of Hamedan University of Medical Sciences with the code 249.1395IR.UMSHA.REC, samples of burn wounds from selected hospitals of Hamadan during 9 months from Bahman 95 to Azar 96 were collected. The conditions for the entry of samples to this study were considered as being hospitalized in the burn wards and having severe skin infections and the exclusion factor was also considered for those who did not have burn infections. Sampling method was considered easy, fast, accessible and random. Isolates from burn wounds were cultured on a medium of Merck, Germany, and the colonies were cultured on specific media of *Pseudomonas aeruginosa* after purification to determine the genus and species. The oprD gene was used for molecular confirmation of isolated isolates (16).

Antibiotic resistance phenotypic pattern study: Determine the susceptibility of clinical isolates to 5 different antibiotics including Cefoxitin (30 μ g), imipenem (10 μ g), meropenem (10 μ g), orporen (10 μ g), doripenem (30 μ g) and aztreonam (30 μ g) (Mast, UK) using the Kirby-Bauer Disk Diffusion method. To minimize contamination, discs were placed on the plate surface by the Disc Dispenser (Mast, UK).

After 24 hours of incubation at 35 °C, the diameter of the non-growth holes was evaluated using the latest

version of the Clinical and Laboratory Standards Institute (CLSI) (17).

Determination of *Pseudomonas aeruginosa* strains with KPC enzyme: Houch modified test was used to determine the isolates with Kabapnemase enzyme. In this method, all steps were carried out according to Kouhsari et al. method. The standard strain of *Pseudomonas aeruginosa* ATCC 27853 was used as a negative control and the *Klebsiella pneumonia* strain ATCC 700603 was used as a positive control (6).

Determination of *Pseudomonas aeruginosa* strains with MBL enzyme: Disc diffusion method was used to detect metalloβ-lactamase strains. In this way, the Imipnem disk was placed adjacent to an IMP-EDTA disk. According to Panchal et al., if the diameter of non-growth halo of the IMP-EDTA is greater than 7 mm in relation to the IMP disc, the MBL is positive and, if less than 7 mm, MBL is considered negative (13).

Genomic extraction using boiling method: Boiling was used to extract DNA. All stages of work were optimized according to Shahbazi et al. (18).

Preparation of primers and PCR: Primers used after dilution with 10 picomolar concentrations were used to

prepare a PCR mixture. The final volume of the PCR reaction was 25 µL, which included: 1 µL of DNA template, 1 µL of each primer with 10 µM concentration and 12 µL of Master-mix (Ampliqon, Germany). The BioRad C1001 thermocycler (US) was used to amplify the genes. For all genes the initial denaturation temperature and final elongation temperature were considered, 96 °C and 72 °C for 5 minutes, respectively, (Table 1).

Electrophoresis on agarose gel 5.1%: 5 µL of the final product of PCR in 0.1% agarose gel was electrophoresed in X5/0 buffer. The 100 bp Fermantaz ladder (American Thermofisher) was used to identify the target bands. In this study, the standard strains of *Pseudomonas aeruginosa* 27853ATCC and 14425ATCC were used as positive control and the strain of *Pseudomonas aeruginosa* ATCC 15692 was used as negative control.

Data analysis: Results from determination of antibiotic resistance using phenotypic method by WHOnet software version 5.5. Data were analyzed by SPSS software version 16 and by χ^2 test. $P < 0.05$ was considered significant.

Table1. List of primers and temperature settings used to amplify the *Pseudomonas aeruginosa* genes isolated from burn wounds

Genes	Nucleotide sequence	Length of sequences (bp)	Temperature setting	References
<i>lasB</i>	F:GGAATGAACGAAGCGTTCTCCGAC R: TGGCGTCGACGAACACCTCG	284	35 (1 min 96 °C, 50 seconds 58 °C, 1 min 72°C)	(19)
<i>lasA</i>	F: GCAGCACAAAAGATCCC R: GAAATGCAGGTGCGGTC	1075	25 (1 min 96 °C, 50 seconds 57 °C, 1 min 72 °C)	(19)
<i>plcH</i>	F: GCACGTGGTCATCCTGATGC R: TCCGTAGGCGTCGACGTAC	608	35 (1 min 96 °C, 55 seconds 57 °C, 1 min 72 °C)	(19)
<i>plcN</i>	F: TCCGTTATCGCAACCAGCCCTACG R: TCGCTGTCGAGCAGGTGCAAC	481	35 (1 min 96 °C, 1 min 57 ° C, 1 min 72 °C)	(19)
<i>apr</i>	F: TGTCCAGCAATTCTCTTGC R: CGTTTTCCACGGTGACC	1017	25 (1 min 96 °C, 45 seconds 59 °C, 1 min 72 °C)	(19)
<i>phzII</i>	F: GCCAAGGTTTGTGTGTCGG R: CGCATTGACGATATGGAAC	1036	25 (1 min 96 °C, 50 seconds 56 °C, 1 min 72 °C)	(19)
<i>phzI</i>	F: CATCAGCTTAGCAATCCC R: CGGAGAACTTTCCCTC	392	35 (1 min 96 °C, 1 min 58 °C, 1 min 72 °C)	(19)
<i>phzH</i>	F: GGGTTGGGTGGATTACAC R: CTCACCTGGGTGTTGAAG	1752	25 (1 min 96 °C, 50 seconds 59 °C, 1 min 72 °C)	(19)

Results

Results of isolation of *Pseudomonas aeruginosa* isolates from burn wounds: Of 250 isolated isolates, 63 isolates (25.2%) were obtained as *Pseudomonas aeruginosa* from burn wounds.

Antibiotic Resistance Pattern Results: Of 63 isolates of *Pseudomonas aeruginosa* isolated from burn patients, antibiotic-resistant of Imipenem strains had the lowest frequency and the antibiotic-resistant of

dorypnem, ortpanem and meropenem had the highest frequency (Fig 1).

Results of *Pseudomonas aeruginosa* isolated from carbapenemase and metallobetalactamase enzymes:

Of 63 isolates of *Pseudomonas aeruginosa* isolated from burn patients, 10 isolates (15.83%) had KPC enzyme and 13 isolates (20.63%) had MBL (Fig 2,3)

The results of proliferation of *Pseudomonas aeruginosa* genes from burn isolates: The distribution pattern of pathogenic genes was that the LasB gene was

observed in 43 isolates (68.25%), *plcN* gene in 41 isolates (65.07%), *lasA* gene In the 20 isolates (31.74%), the *gen* gene in 60 isolates (95.23%), the *phzI* gene in 53 isolates (84.12%), the *phzII* gene was isolated in 38 isolates (60.31%), isolated genes (30 *phzH*) 47.61%) and the *plcH* gene was observed in 56 isolates (88.88%) (Fig 4).The results of the study showed that there is a significant relationship between the presence of KPC enzymes and MBL enzymes and pathogenicity genes (Table 2, 3).

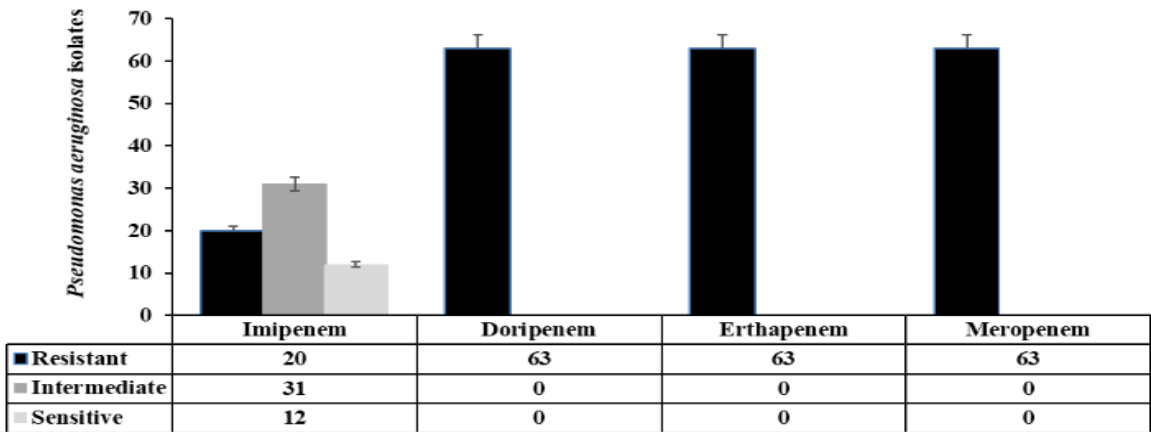


Figure 1. Antimicrobial resistance pattern in *Pseudomonas aeruginosa* isolated from burn wounds

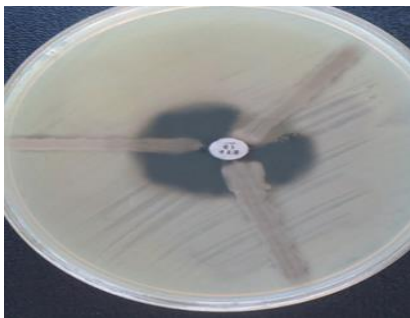


Figure 2. *Pseudomonas aeruginosa* strains with KPC enzyme

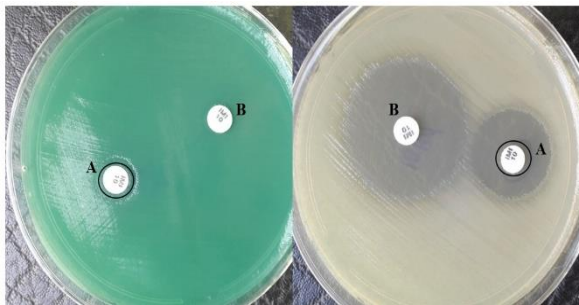


Figure 3. *Pseudomonas aeruginosa* strains with MBL enzyme (right) and without MBL enzyme (left), A: IMP disk. B: IMP + EDTA disk

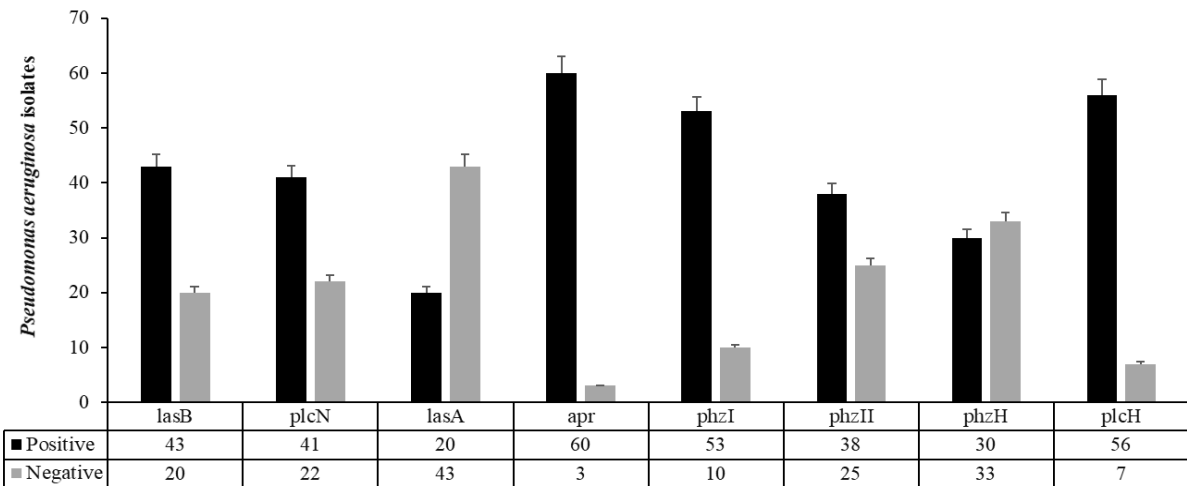


Figure 4. Frequency of pathogenic genes in *Pseudomonas aeruginosa* isolates

Table2. Investigation of statistical relationship between the studied variables in *Pseudomonas aeruginosa* isolates carrying KPC enzyme isolated from burn wounds

Variabls	<i>Pseudomonas aeruginosa</i> with KPC enzyme	<i>Pseudomonas aeruginosa</i> without KPC enzyme	Level	P-value
<i>lasA</i>	9	11	A*	≤0.003
	1	42	B*	
<i>lasB</i>	10	0	A	≤0.001
	0	53	B	
<i>plcH</i>	8	2	A	≤0.008
	2	51	B	
<i>plcN</i>	10	0	A	≤0.001
	0	53	B	
<i>apr</i>	8	2	A	≤0.008
	2	51	B	
<i>phzII</i>	9	1	A	≤0.005
	1	52	B	
<i>phzI</i>	9	1	A	≤0.003
	1	52	B	
<i>phzH</i>	10	0	A	≤0.001
	0	53	B	

A: Detect presence. B: Not recognizing presence

Table3. Investigation of statistical relationship between the studied variables in *Pseudomonas aeruginosa* isolates carrying MBL enzymes isolated from burn wounds (not referenced)

Variabls	<i>Pseudomonas aeruginosa</i> with MBL enzyme	<i>Pseudomonas aeruginosa</i> without MBL enzyme	Level	P-value
<i>lasA</i>	12	1	A*	≤0.007
	1	49	B*	
<i>lasB</i>	13	0	A	≤0.005
	0	50	B	
<i>plcH</i>	13	0	A	≤0.005
	0	50	B	
<i>plcN</i>	11	2	A	≤0.007
	2	48	B	
<i>apr</i>	39	11	A	≤0.006
	10	3	B	
<i>phzII</i>	9	2	A	≤0.009
	4	48	B	
<i>phzI</i>	10	3	A	≤0.006
	3	47	B	
<i>phzH</i>	11	2	A	≤0.007
	2	48	B	

Discussion

The results of this study showed that the frequency of Carbapenem antibiotics in *Pseudomonas aeruginosa* isolated from burns was very high, but the strains with MBL and KPC enzymes were very low. However, the isolated isolates were resistant to most antibiotics in

other classes, such as cefpodoxime and ceftriaxone, with a resistance of 100%. In the study of Akhavan-Tafti et al. the frequency of metallo-β-lactamase enzymes in *Pseudomonas aeruginosa* isolates from burn wounds was 29.5% (20). The study by Golshani et al. showed that 18% of *Pseudomonas aeruginosa*

isolates had MBL gene (21). In the study of Malek Mohamad et al., it was found that the highest frequency is related to the morphine antibiotic. Khorvash et al. also showed that in clinical isolates of *Pseudomonas aeruginosa*, more than 60% of isolates were resistant to carbapenem antibiotics (22, 23). In studies by Sobouti et al. and Liakopoulos et al., the prevalence of *Pseudomonas aeruginosa* strains producing KPC and MBL was more than ours (24, 25).

This discrepancy can be attributed to the sample size. Because 2 factor of limitation of sample and time are defects of descriptive, cross-sectional and non-epidemiological studies. By expanding the time series in different seasons and taking samples in different years, this error can be greatly reduced. In addition, the pattern of antibiotic use and strains differentiation with respect to specific patterns in that area provide conditions that bacteria can transfeer resistance more quickly. Of course, it should not be overlooked that the prevalence of MBL and KPC strains in *Pseudomonas aeruginosa* is followed by a different pattern in Asia. In such, the abundance of these strains is very low. While most reports indicate a high resistance to pncm in *Pseudomonas aeruginosa* isolates. In the study of Hong et al., it was found that the highest resistance to *Pseudomonas* isolates was found in Korea, China and Taiwan, with the highest resistance to Imipenem, Doripenem and Meropenem (26).

In this study, the genes of biofilm, phenazine and external enamel layers of cell wall were the most frequent. In Tutunchi et al., which was performed on *pseudomonas* isolates, it was found that 96.5% of isolates carries the *phzI* gene, 93.1% of the isolates carry the *phzII* gene and 27.2% of the isolates also carry *phzH* (27). Also, studies by Meskini et al., Radlinski et al. on clinical isolates of *Pseudomonas aeruginosa* showed that the frequency of quorum-sensing and biofilm genes play a very important role in pathogenicity and resistance to certain antibiotics, this view is consistent with the two studies mentioned. In this study, all pathogens were highly prevalent, so that 15% of the studied isolates had the genes of the pathogenic agents and had a 100% resistance to the penem group antibiotic. One of the most important cases studied in this study was the presence of some variables in changing the pattern of pathogenicity or resistance of bacteria. One of the most important issues was the presence of several

bacteria in the wound and the activity of these two bacteria against each other can change the pathogenicity of the other, Like the *Pseudomonas aeruginosa* bacterium, which, in conjunction with *Staphylococcus aureus* bacteria in wound specimens, extensively changes pathogenicity and antibiotic resistance (28, 29).

The results of this study showed that there is a significant relationship between the activity of KPC and MBL enzymes and the pathogenicity of *Pseudomonas aeruginosa* isolates isolated from burn wounds, and the presence of these enzymes may cause bacterial pathogenesis. However, there are several other factors involved in the abundance of pathogenic genes in *Pseudomonas aeruginosa*. Gupta et al. showed that the presence of some pathogenic factor such as biofilms, quorum sensing and host immune deficiency can increase the risk of this bacterium and also make the bacteria more resistant to the treatment. The results of this study also showed that the producing genes of pyli, phenazine, biofilm generators and enamel layers along with some control genes such as *apr* indicated that the regulation and control of pathogenic genes according to the presence or absence of some environmental factors can undergo many changes that were clearly seen in isolates isolated from burn wounds (30).

The analysis of this study showed that there is a significant relationship between the presence of antibiotic resistance enzymes and the pathogenicity of *Pseudomonas aeruginosa* isolated from burn wounds. Because *Pseudomonas aeruginosa* produces a wide range of pathogenic and resistant enzymes such as Carbapenemase and Metallobetalactamase, the severity of the pathogenicity of this bacterium varies with respect to the target tissue and resistance and pathogenicity is higher in surface tissues such as skin. On the other hand, the results of this study showed that the pathogenicity of *Pseudomonas aeruginosa* also increases the activity and resistance of Carbapenemase and metallobetalactamase enzymes.

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