

## Investigation of the Relationship between the Presence of Chromosomal and Plasmid-Encoded AmpC Genes and Type of Clinical Specimen in *Pseudomonas Aeruginosa*

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J Babol Univ Med Sci; 20(3); Mar 2018; PP: 36-43

Received: Sep 16<sup>th</sup> 2017, Revised: Dec 17<sup>th</sup> 2017, Accepted: Dec 24<sup>th</sup> 2017.

### ABSTRACT

**BACKGROUND AND OBJECTIVE:** Different clinical specimens play a decisive role in the type and nature of drug resistance in pathogenic organisms. Occasionally, the presence of certain antibiotic resistance genes is associated with the type of clinical specimen. The aim of this study was to determine the relationship between the presence of chromosomal and plasmid-encoded AmpC genes and type of clinical specimen in *Pseudomonas aeruginosa*.

**METHODS:** In this descriptive and experimental study, 114 isolates of *Pseudomonas aeruginosa*, and clinical specimens including blood, urine, wound secretion, burn injuries were collected from teaching hospitals in Hamadan. The presence of chromosomal and plasmid-encoded AmpC genes was evaluated using multiplex PCR technique.

**FINDINGS:** The plasmid-encoded AmpC genes were observed more than chromosomal genes in *Pseudomonas aeruginosa* isolates. The FOX gene with a value of 29 (37.66%) ( $p \leq 0.037$ ) and DHA gene with a value of 5 (6.4%) ( $p \leq 0.015$ ) in plasmid-encoded AmpC genes, while FOX gene with a value of 39 (48.75%) ( $p \leq 0.001$ ) and MOX gene with a value of 2 (7.36%) in chromosomal AmpC genes had the highest and lowest frequency, respectively.

**CONCLUSION:** The results of the study showed that the presence of chromosomal and plasmid-encoded AmpC genes may have various frequencies according to the type of clinical specimen.

**KEYWORDS:** *Pseudomonas Aeruginosa*, Drug Resistance, Ambler Classification of B-Lactamases, Plasmid, Chromosome.

### Please cite this article as follows:

Tahmasebi H, Yousef Alikhani M, Dehbashi S, Arabestani MR. Investigation of the Relationship between the Presence of Chromosomal and Plasmid-Encoded AmpC Genes and Type of Clinical Specimen in *Pseudomonas Aeruginosa*. J Babol Univ Med Sci. 2018; 20(3):36-43.

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## Introduction

Any pathogenic agent that enters the human body should be placed in its target tissue to create pathogenicity. Pathogenic bacteria are not excluded from this, and each one can have different levels of damage and infection according to their contaminating tissues and their inhabitants (1, 2). For the treatment of bacterial infections, depending on the amount of infection and the involved organ, the type of antibiotics and dosage levels are always different (3). Hence, different clinical samples always have a determining role in the type and way of resistance of pathogenic organisms to treatment (1).

In many bacterial diseases, the amount of administered drug is determined according to the target tissue, and this leads to treatment with the lowest dose in open spots that have a faster access to the source of the infection (2). Bacteria that are more prevalent in terms of presence in public and hospital spaces and actually cause hospital-associated infection make the process easier. *P. aeruginosa* is one of these bacteria that has the ability to reside in different places of the human body (4).

*P. aeruginosa* is the most important human pathogen and the second most common cause of burn infections. It is also found in hospitals and in humid areas (5, 6). The existence of strains with multiple drug resistance in this bacterium is a major problem in the treatment of bacteria in important hospital areas such as burn unit and intensive care unit (7). Beta-lactamases are a heterogeneous group of bacterial enzymes (2) classified according to various criteria such as the hydrolysis spectrum of enzyme, the sensitivity to beta-lactamase inhibitors, the genetic location of the enzyme (plasmid, chromosome, integrin) and amino acid sequence of the protein (8). There are several methods for classifying beta-lactamases, among which the Ambler classification and the Bush classification have become more widely used. Based on Amber classification, group C beta-lactamases, also known as *AmpC*, are one of the most important resistance agents against beta-lactam antibiotics (8).

*AmpC* beta-lactamases appeared and were studied in the late 1970s. These enzymes hydrolyze broad-spectrum cephalosporins such as ceftazidime, ceftriaxone, cefepime, and monobactams, such as aztreonam and cephamycins, but are not controlled by conventional inhibitors such as clavulanate (9). *AmpC* enzymes, which are often inducible by beta-lactam, are

coded by chromosomal genes. Plasma-mediated *AmpC* enzymes can be transmitted to organisms that lack this enzyme and produce resistance similar to that of beta-lactam (9). In a study by Chiquet et al., it was found that there is a direct relationship between the presence of certain bacteria in keratitis and resistance to some broad-spectrum antibiotics (10).

In addition, through a study on *P. aeruginosa*, Jansen et al. showed a significant difference in antibiotic resistance patterns between isolates of cystic fibrosis and other clinical samples (11). However, it is likely that there is a relationship between the various clinical samples that have been isolated from *P. aeruginosa* strains and the amount and type of antibiotic resistance (12).

This may increase to a degree that causes the appearance of different strains in a person infected by a shared species of bacteria. Therefore, the aim of this study was to determine the relationship between the presence of chromosomal and plasmid-encoded *AmpC* genes and the clinical samples in *P. aeruginosa*, so that in addition to examining the relationship between the type of clinical sample and the presence of some chromosomal-plasmid genes encoding beta-lactam enzymes, this relationship could be approached and used for better and more definitive treatment.

## Methods

**Collection, identification and isolation of *P. aeruginosa*:** This descriptive and experimental study was approved by the Ethics Committee of Hamadan University of Medical Sciences with the code of ethics IR.UMSHA.REC.1395.402, and was conducted through random convenience sampling. Overall, 114 isolates of *P. aeruginosa* were isolated from a total of 288 clinical samples during a 9-month period from June 2016 to March 2017. Samples with bacterial source were included in the study. For initial screening, the collected samples were cultured on Cetrimide Agar (Merck, Germany) and incubated at 42 °C. Finally, isolates were determined in terms of genus and species (13) using different biochemical tests (oxidase, catalase, indole, methyl red and culture on the identifier media).

**Determination of the phenotype of *AmpC*-producing strains by disc diffusion method:** To investigate the presence of *AmpC*, cefoxitin (30 µg) and cefpodoxime (10 µg) (Mast, England) discs and *AmpC* inhibiting compounds were used. In all cases,

*Klebsiella pneumoniae* ATCC700603 was used as a positive control and *P. aeruginosa* ATCC27853 was used as a negative control (14).

**DNA extraction and bacterial plasmid:** Plasmid and DNA were extracted using an extraction kit. After isolation, the isolates were cultured on a Mueller-Hinton agar (Merck, Germany). Then, several colonies from each cultured isolate were inoculated in 5 ml LB Broth medium (sigma Aldrich, USA) and were incubated at  $35 \pm 2$  °C for 24 hours. Other steps of DNA and plasmid extraction were performed using CinnaGen extraction kit (Iran).

**Preparation of primers and performing PCR tests:** To perform a PCR reaction for each sample, 25 µl of Master Mix RED (Ampliqon Company, Germany) was

prepared with one lambda of the extracted DNA and 1 µl of each Primer Mix (Table 1) and to achieve a final volume of 50 µl, distilled water was added. ICycler thermocycler (BioRad, USA) was used for amplification of the desired genes with a thermal shock setting of 94 °C for 30 seconds and 35 cycles for 94 °C for 30 seconds, annealing temperature of 61 °C for 30 seconds and 72 °C for 30 seconds.

The final lengthening was done at 72 °C for 10 minutes. Finally, the products were examined using 2.5% agarose gel.

**Data analysis:** The results from the determination of antibiotic resistance by phenotypic method were analyzed using SPSS software version 16 and x2 statistical test and  $p \leq 0.05$  was considered significant.

**Table 1. List of primers used for amplification of chromosomal and plasmid genes of *AmpC* – producing *P. aeruginosa* strains**

The gene	Primer name	Nucleotide sequence	Ampliqon length (bp)	Reference
MOX	MOXMF	GCTGCTCAAGGAGCACAGGAT	520	(15)
	MOXMR	CACATTGACATAGGTGTGGTGC		
CIT	CITMF	TGGCCAGAACTGACAGGCAAA	462	(15)
	CITMR	TTTCTCCTG AACGTGGCTGGC		
DHA	DHAMF	AACTTTCACAGGTGTGCTGGGT	405	(15)
	DHAMR	CCGTACGCATACTGGCTTTGC		
EBC	EBCMF	TCGGTAAAGCCGATGTTGCGG	302	(15)
	EBCMR	CTTCCACTGCGGCTGCCAGTT		
FOX	FOXMF	AACATGGGGTATCAGGGAGATG	190	(15)
	FOXMR	CAAAGCGCGTAACCGGATTGG		
ACC	ACCMF	AACAGCCTCAGCAGCCGGTTA	346	(24)
	ACCMR	TTCGCCGCAATCATCCCTAGC		
<i>AmpC1</i>	Pre <i>AmpC</i> -PA1	ATGCAGCCAACGACAAAGG	1243	(15)
	Post <i>AmpC</i> -PA2	CGCCCTCGCGAGCGCGCTTC		
<i>AmpC2</i>	<i>AmpC</i> -PA-A	CTTCCACACTGCTGTTCGCC	1063	(16)
	<i>AmpC</i> -PA-B	TTGGCCAGGATCACCAGTCC		

## Results

**Distribution of *AmpC*-containing isolates:** After primary phenotypic screening test in this study, of 114 *P. aeruginosa* isolates, 97 isolates (85.08%) were considered to contain *AmpC* enzyme using phenotypic method. Of 97 *AmpC* – containing isolates of *P. aeruginosa*, 80 isolates (82.42%) carried chromosomal genes and 77 isolates (79.38%) carried plasmid genes.

**Distribution of clinical samples in *AmpC*-containing isolates:** In this study, of 80 chromosomal *AmpC* – containing isolates, 22 isolates (27.5%) were obtained from blood, 10 isolates (12.5%) from urine, 16 isolates (20%) from the wound secretions, 19 isolates (23.75%) from burn wounds, 9 isolates

(11.25%) from catheter and 4 isolates (5%) were obtained from cerebrospinal fluid. Moreover, of the 77 plasmid *AmpC* – containing isolates, 12 isolates (10.52%) were obtained from blood, 23 isolates (28.75%) from urine, 8 isolates (10%) from ulcers, 27 isolates (33.75%) from burn wound, 2 isolates (2.25%) from the catheter and 5 isolates (6.25%) were obtained from cerebrospinal fluid. Most of the chromosomal and plasmid *AmpC* strains were isolated from ICU and pediatric sections.

In terms of the type of clinical sample, most *AmpC* positive strains were obtained from urine and culture samples, and the least of them were obtained from the wound secretions. These values included both plasmid

and chromosomal groups. In addition, according to the obtained samples, most strains of chromosomal and plasmid-encoded *AmpC* genes were isolated from female patients.

Of the 80 chromosomal *AmpC*-containing isolates, 59 isolates (73.75%) were isolated from female patients and 21 isolates (25.26%) were isolated from male patients. There was a significant correlation

between the presence of chromosomal *AmpC* genes and different clinical samples (Table 2). Of the 77 plasmid *AmpC* – containing isolates, 41 isolates (53.24%) were isolated from men and 36 isolates (46.53%) were isolated from women, and there was a significant relationship between the presence of plasmid *AmpC* genes and different clinical samples (Tables 2, 3).

**Table 2. Frequency of chromosomal *AmpC* – producing *P. aeruginosa* isolates based on genotypic tests in clinical samples**

<i>P. aeruginosa</i> (n=80)								
Isolated section	Cerebrospinal fluid culture	Culture of catheter contamination	Burn wound	Blood cultures	Secretions of the wound	urine culture	Total	P-value
CIT	0	0	3	5	0	0	8	≤ 0.004
DHA	0	0	0	0	1	3	4	≤ 0.001
EBC	2	1	8	3	2	0	16	≤ 0.009
FOX	0	2	8	13	10	6	39	≤ 0.001
ACC	2	3	0	0	3	1	9	≤ 0.018
MOX	0	2	0	1	0	0	2	≤ 0.015

**Table 3. Frequency of plasmid *AmpC* – producing *P. aeruginosa* isolates based on genotypic tests in clinical samples**

<i>P. aeruginosa</i> (n=77)								
Isolated section	Cerebrospinal fluid culture	Culture of catheter contamination	Burn wound	Blood cultures	Secretions of the wound	urine culture	Total	P-value
CIT	0	0	4	1	1	2	8	≤ 0.005
DHA	0	0	1	0	1	3	4	≤ 0.015
EBC	0	0	4	4	2	0	10	≤ 0.007
FOX	0	0	8	6	1	14	29	≤ 0.037
ACC	1	0	6	0	3	1	11	≤ 0.023
MOX	0	2	5	1	0	3	14	≤ 0.041

**Frequency of chromosomal and plasmid *AmpC* gene families:** Compared to the chromosomal genes, plasmid *AmpC* genes were observed more in the *P. aeruginosa* isolates. Of 77 plasmid *AmpC* – producing *P. aeruginosa* isolates, 29 isolates (37.66%) carried *FOX* plasmid gene, 10 isolates (12.95%) carried *EBC* plasmid gene, 11 isolates (14.82%) carried *ACC* plasmid gene, 5 isolates (6.4%) carried *DHA* plasmid gene, 8 isolates (10.38%) carried *CIT* plasmid gene and 14 isolates (18.18%) carried *MOX* plasmid gene. In addition, of 80 chromosomal *AmpC* – producing *P. aeruginosa* isolates, 39 isolates (48.75%) carried *FOX* chromosomal gene, 16 isolates (20%) carried *EBC* chromosomal genes, 9 isolates (23.57%) carried *ACC* chromosomal genes, 4 isolates (27.5%) carried *DHA* chromosomal gene, 8 isolates (4.21%) carried *CIT* chromosomal genes and 2 isolates (7.36%) carried

*MOX* chromosome gene. Most samples with *FOX* gene were obtained from ICU and pediatric sections (Fig 1,2).

**The results of statistical analysis:** Statistical analysis of the variables in this study showed that there is a significant relationship between the type of clinical sample and the distribution of resistance agents to *AmpC* beta-lactamases.

**Relationship between the presence of chromosomal genes encoding the *AmpC* enzyme and the clinical sample type:**

Considering the significance level of  $P \leq 0.05$ , there was a significant correlation between the presence of chromosomal *AmpC* genes and different clinical samples (Table 2).

**Relationship between the presence of *AmpC*-encoding plasmid genes and clinical sample type:**

Considering the significance level of  $p \leq 0.05$ , there was a significant relationship between the presence of *AmpC* plasmid genes and different clinical samples (Table 3).

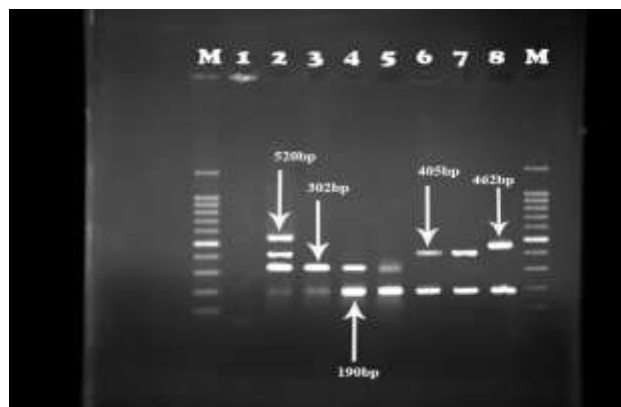


Figure 1. Results of amplification of *AmpC* genes. *MOX* gene with a length of 520 bp, *CIT* gene with a length of 462 bp, *DHA* gene with a length of 405 bp, *EBC* gene with a length of 302 bp, *FOX* gene with a length of 190 bp and *ACC* gene with a length of 346 bp. Well 1: negative control. Wells 2 to 8: positive samples in terms of the presence of genes. Well M: 100 bp Marker

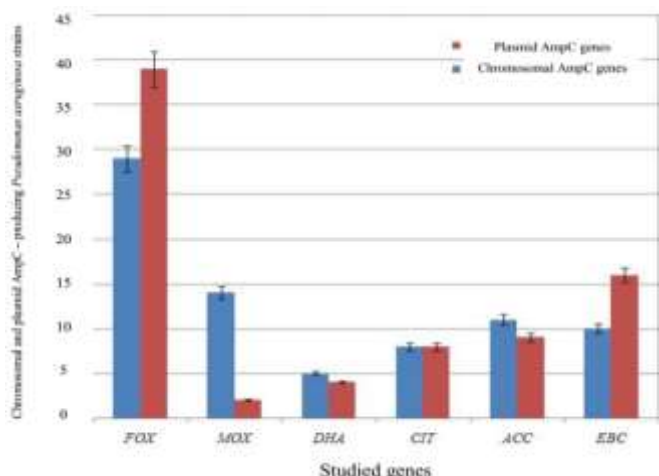


Figure 2. Frequency of chromosomal and plasmid *AmpC* gene families

## Discussion

Due to its specific characteristics, *P. aeruginosa* can infect various organs. In some cases, the presence and activity of bacteria in different organs may also affect the type and extent of resistance (17). In this study, of 114 isolates of *P. aeruginosa*, 97 isolates were detected using *AmpC*-producing phenotypic methods. However, the result was different from the results obtained from the molecular methods. In a

study by Lin et al., it was found that phenotypic methods to detect *AmpC*-producing strains were not susceptible (18). Due to its specific characteristics, *P. aeruginosa* can infect various organs. In some cases, the presence and activity of bacteria in different organs may also affect the type and range of its resistance (18, 19). Furthermore, a study by Garrec et al. showed that phenotypic methods are most often associated with errors in diagnosis and reporting (17), which may be related to the experimenter, the quality of the materials used for test, the environmental conditions of the test, such as thermal conditions and pH, incubation conditions of the samples, as well as equipment used for testing, all of which can influence the results of the phenotypic methods. *P. aeruginosa* has a high resistance to many antimicrobial and antiseptic agents, such as ammonium compounds, hexachlorophene, soaps and iodine solutions (20).

Therefore, it should be anticipated that isolates collected from parts of the body show more resistance to treatment than antiseptic agents such as iodine, alcohol, and other substances. The results of this study showed that the samples obtained from burn wounds and secretions had the highest genetic distribution. Catheter-related infections and urinary tract infections also had a significant distribution of *AmpC* genes. In a study by Cornut et al., it was found that samples that have been exposed to infections from open surfaces are more resistant to antibiotics than treatment with other bacterial infections (19). *P. aeruginosa* accounts for 11 to 13% of nosocomial infections, especially in patients with cystic fibrosis, people with burns or immunodeficiency, and people using ventilator devices. Sepsis caused by this bacteria is a serious complication of burn infections (22).

This was consistent with the results obtained from burns and wounds, with most of the chromosomal and plasmid *AmpC* gene families being found in infections of the wounds. Nevertheless, in the case of resistances to plasmid-encoded beta-lactam antibiotics, the reduction in the permeability of the organism to the drug should not be overlooked, which is accompanied by changes in the specific receptor, drug desire and a slight change in one or more components of the cellular coating and the loss of active transmission capacity of drug from the cell membrane (23). However, the less interfering factors in the living environment of the bacterium, the easier it is for the organism to adjust its resistance pattern. In the present study, the isolates of *P. aeruginosa* plasmid-mediated

*AmpC* isolated from the blood sample were less frequent than isolates with chromosomal genes. Among the differences observed in the clinical trials, there is inconsistency in terms of the presence of plasmid and chromosomal genes in the obtained bacteria. In the study of Rafiee et al. on a wide range of bacteria isolated from clinical specimens, it was found that there is a relationship between the type of clinical sample and the distribution of plasmid and chromosomal genes. In this 25-year study, the active presence of hospital bacteria and the transmission of genes in different clinical samples was investigated (24, 25).

The results of this study indicated that the presence of chromosomal and plasmid-encoded *AmpC* genes can vary according to the type of clinical sample. Distribution of plasmid and chromosomal *P. aeruginosa* strains in different clinical samples showed that even the source of infection and tissue involved by the bacteria could also affect the genetic pattern of the

organism. Hence, some isolates are resistant to the treatment and antibiotics and change the route of treatment. Hence, by identifying these relationships, the pathway for treatment of *P. aeruginosa*-dependent infections can be controlled. On the other hand, due to the cross-sectional nature of the study and its limitations, it cannot be used at wider levels or with a decline in the range of probabilities, and therefore, further studies are suggested.

**Conflict of Interest:** No conflicts of interest.

### Acknowledgment

Hereby, we express our deepest sense of gratitude and indebtedness to Deputy of Research and Technology of Hamadan University of Medical Sciences for their financial support and Microbiology Laboratory Personnel.



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