



## Typing of *fliC* Gene in *Pseudomonas aeruginosa* Metallo-Beta-Lactamase Producer Strains Isolated from Clinical Specimens

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#### Authors

Moosavian M.<sup>1</sup> PhD,  
Moradzadeh M.\* MSc,  
Ghadri H.<sup>2</sup> MSc

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\*Infectious & Tropical Diseases Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran  
<sup>1</sup>Microbiology Department, Medical Sciences Faculty and "Infectious & Tropical Diseases Research Center, Health Research Institute", Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran  
<sup>2</sup>Microbiology Department, Medical Sciences Faculty, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

#### Correspondence

Address: Infectious & Tropical Diseases Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Golestan Avenue, Ahvaz, Iran. Postal Code: 1579461357  
Phone: +98 (61) 13369539  
Fax: +98 (61) 33332036  
mi.1366@yahoo.com

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### ABSTRACT

**Aims** Carbapenem resistant *Pseudomonas aeruginosa* resulting from metallo- $\beta$ -lactamases (MBLs) has been reported to be an important cause of nosocomial infection and is a serious therapeutic problem worldwide. The aim of the present study was to determine the *fliC* (flagellin) typing and their prevalence rate in *P. aeruginosa* producing MBL isolated from clinical specimens in Ahvaz, Iran.

**Materials & Methods** In the present experimental study, isolates were related to the previous study collected from hospitalized patients in Golestan and Imam Khomeini, in Ahvaz, Iran, during 9 months in 2012. Strains were identified using microscopic and biochemical tests. Then, the susceptibility antibiotic tests were performed on all isolates. Imipenem (IMP) and IMP+EDTA (IMP/IMP+EDTA) combined disk phenotypic test was performed for detection of MBL producing strains that were resistant to IMP. Finally, PCR was performed to detect *fliC* genes in IMP resistant strains.

**Findings** Out of 100 examined isolates, 47 isolates were resistant to IMP. Among 47 imipenem resistant strains, 41 strains were MBL producers. Eighty-three percent of the strains contained *fliC* gene that 48 isolates had type A and 32 isolates had type B.

**Conclusion** Eighty-three percent of the specimens have flagellin (*fliC*) gene, which out of them, 48 strains of *P. aeruginosa* (60.0%) have type A flagellin and 32 strains (40.0%) have type B. Twenty-four of the 41 strains of MBL producer (60.0%) have type A and 16 strains (40.0%) have type B and only one strains lacks the flagellin gene, so the flagella plays a significant role in the bacterial virulence.

**Keywords** *Pseudomonas aeruginosa*; Metallo Beta-Lactamases; *fliC*; Flagellin; PCR

### CITATION LINKS

[1] *Pseudomonas aeruginosa*: New insights into pathogenesis and host ... [2] Antibiotic resistance patterns of *Pseudomonas aeruginosa* in a tertiary care hospital in Central ... [3] Prognostic factors for nosocomial bacteraemia outcome: A prospective study in a Greek ... [4] Identification of *Pseudomonas aeruginosa* ... [5] Preparation of *Pseudomonas aeruginosa* alginate-flagellin ... [6] Bacterial flagellins: Mediators of pathogenicity and host immune ... [7] Flagellin gene and protein variation amongst clinical isolates ... [8] Sequence polymorphism in the glycosylation ... [9] Flagellar preparations from *Pseudomonas* ... [10] *Pseudomonas* immunotherapy: A historical ... [11] Evaluation of flagella and flagellin of *Pseudomonas* ... [12] Imipenem-resistant *Pseudomonas aeruginosa* ... [13] Detection of *Pseudomonas aeruginosa* producing metallo-beta-lactamases ... [14] Current challenges in antimicrobial chemotherapy ... [15] The mechanisms of resistance to  $\beta$ -lactam ... [16] Detection of metallo- $\beta$ -lactamases-encoding ... [17] Carbapenemase detection using the  $\beta$ -CARBA test ... [18] Updated functional classification of ... [19] Evaluation of different methods for detection ... [20] Isolation of *Pseudomonas aeruginosa* strains ... [21] Clinical and Laboratory Standards ... [22] Molecular detection of metallo- $\beta$ -lactamase ... [23] Morbidity and survival probability in burn patients ... [24] SARI Study Group. Detection and characterization ... [25] Identification and characterization of metallo- $\beta$ -lactamases ... [26] Antibiotic resistance pattern and evaluation of ... [27] Prevalence and clonal dissemination of ... [28] Phenotypic and molecular characterization of antimicrobial ... [29] Characteristics of metallo- $\beta$ -lactamase-producing ... [30] Molecular identification and detection of virulence ... [31] The epidemiology, pathogenesis and treatment ... [32] Passive immunisation against *Pseudomonas* ... [33] The role played by contaminated surfaces in the transmission ... [34] A double-blind randomized placebo-controlled phase III study of ... [35] Cloning, expression, purification ... [36] Cloning and comparison of *fliC* ... [37] Glycosylation of b-Type flagellin of *Pseudomonas* ... [38] *Pseudomonas aeruginosa* flagellin as an adjuvant: Superiority ...

## Introduction

*Pseudomonas aeruginosa* is an originally environmental bacterium that is considered as an opportunistic pathogen which infects hospitalized and immunocompromised patients. The Carbapenem-resistant *P. aeruginosa* causes serious infections, such as nosocomial pneumonia which based on the reports is increasing in the hospitalized patients [1]. *Pseudomonas* infections have been reported often in burns, urinary tract infections and lung diseases, such as cystic fibrosis [2]. *P. aeruginosa* uses many virulence factors include exotoxins, polysaccharides, pyocyanin, lipase, protease, phospholipase, rhamnolipid and flagella [3]. *P. aeruginosa* has polar flagella that composed of protein units (flagellin), which encoded by the *fliC* gene. Flagella are responsible for the pathogenicity, chemotaxis, and colonization of bacteria to the host cells [4].

Pathogenic bacteria that have flagella can infect host cells [5], which in *P. aeruginosa* the appropriate status of the connection and the inflammatory response is acquired by Toll-Like Receptor 5 (TLR5) [6]. The flagella of *P. aeruginosa* can be divided into two serotypes. Serotype A (A<sub>1</sub>, A<sub>2</sub>) and serotype B [7]. Type A is a heterogeneous molecule that has a changeable molecular weight ranging from 45 to 50KDa, while the type B flagellin is a heterologous molecule that has 53KDa molecular weight [8]. *P. aeruginosa* expresses only one of these two types of flagellin. Sixty percent of the strains have type A and B [9, 10]. *FliC* flagellin has an important role in stimulating of the immune response, with the emergence of multi-resistant strains, is evidence of the importance of alternative methods of antibiotic therapy such as recombinant vaccines. So flagella can be regarded as a vaccine candidate [11].

Due to extensive metabolic and genetic capabilities, such as relative impermeability of the outer membrane of bacteria, efflux pump, chromosomal and plasmid enzymes degrading antibiotics, the bacteria are one of the most resistant microorganisms to the drug. Resistant to carbapenems is often associated with the production of Metallo-Beta-Lactamases (MBL). Of these, nosocomially acquired *P. aeruginosa* remains the major cause of mortality, particularly because of the emergence of multidrug-resistant strains [12]. Beta-lactam antibiotics are the most effective antibiotics against *P. aeruginosa* which among them, imipenem (IMP) as a carbapenem is a selective antibiotic used against these organisms. The occurrence of carbapenem-resistant *P. aeruginosa* strains is due to decrease in antibiotics absorption due to lack of an outer membrane porin, as oprD, exclusion from the cell by efflux pump, decrease in outer membrane permeability and production of MBL [13-15].

MBLs are in class B of the Ambler classification and group 3 of the Bush classification and requires to divalent cations (e.g. zinc metal) as a cofactor for its enzymatic activity [16, 17]. In 1995, based on substrate and inhibitor type, beta-lactamases were classified into four classes. Group 1 is cephalosporinases that are not well inhibited by clavulanic acid. Group 2 is beta-lactamases that hydrolyze penicillins, cephalosporins, and carbapenems and almost are weakly inhibited by all molecules that contain beta-lactam. Group 4 is beta-lactamases that their features are not fully understood currently [18]. Nowadays, the emergence of antibiotic-resistant *P. aeruginosa* and beta-lactamase-producing strains are considered as one of the challenges for treating patients with burns [12].

There are different ways to identify MBLs. Among these methods, it can point out to Pulsed-Field Gel Electrophoresis (PFGE), Modified Hodge Test (MHT), Double Disk Synergy (DDST); E-test Metallo-Beta-Lactamase (Etest MBL) noted [19].

According to studies, there are various results in different geographic regions in terms of the prevalence of *P. aeruginosa* producing MBLs. Antibiotic resistant of *P. aeruginosa* and flagella are a major factor in bacterium virulence.

The aim of the present study was to determine the *fliC* (flagellin) typing and their prevalence rate in *P. aeruginosa* producing MBL isolated from clinical specimens in Ahvaz, Iran.

## Materials and Methods

In the present experimental study, isolates were related to the previous study and collected from hospitalized patients in Golestan and Imam Khomeini hospitals, in Ahvaz, Iran. These bacteria had been isolated from different clinical specimens such as urine, wound, blood, and trachea and so on. A total of 100 clinical isolates from *P. aeruginosa* were studied.

After transporting the samples to the microbiology laboratory in Medical School, the colonies were again inoculated to MacConkey agar medium (Merck; Germany) and pure isolates were identified as *P. aeruginosa* based on gram staining characteristics and biochemical tests such as oxidase, catalase, oxidative-fermentative test, growth on media such as Triple Sugar Iron (TSI; Merck; Germany), Sulfide, Indole, Motility (SIM; Merck; Germany), cetrimide agar (Merck; Germany) and growth at 42°C [8]. Then, *P. aeruginosa* isolates were preserved in Trypticase Soy Broth media (TSB; Merck; Germany) containing 20% glycerol [20].

**Susceptibility test:** The susceptibility patterns of isolates to different antibiotics were examined using disk diffusion method (Kirby-Bauer) on Muller-Hinton agar plates (Merck; Germany)

according to the guidelines of Clinical and Laboratory Standard Institute (CLSI) [20]. The antimicrobial disks were included: Imipenem (10µg), meropenem (10µg), ceftazidime (30µg), carbenicillin (100µg), tobramycin (10µg), amikacin (30µg), ticarcillin (75µg), gentamicin (10µg), cefotaxime (30µg), and 30µg ceftizoxime (MAST; UK). *P. aeruginosa* ATCC27853 was used as a control strain for susceptibility tests [21].

**MBL phenotypic test:** Combination disk diffusion test (CDDT) was used for phenotypic detection of MBLs producing *P. aeruginosa* strains. In brief, 930µg of EDTA plus 10µg of imipenem were placed on the Muller Hinton agar (Merck; Germany) plates which were inoculated with *P. aeruginosa*. After 18 to 24h of incubation at 37°C, an organism was considered MBL positive, if growth inhibition zone was increased 7mm or more in comparison to IMP disk alone [21].

**MBL Molecular test:** The existence of encoding genes of *blaIMP-1*, *blaVIM-2*, and *blaSPM-11* metallo-beta-lactamases between imipenem-resistant *P. aeruginosa* strains was evaluated by polymerase chain reaction (PCR) [22].

**Extraction of DNA:** DNA was extracted from *P. aeruginosa* colonies using a simple boiling method. A few colonies of an overnight culture of *P. aeruginosa* isolates were suspended in 500µl of TE buffer (10mM Tris, 0.5mM EDTA) by vortex. The suspension was heated in a boiling bath at 95°C for 10min. After centrifugation at 14000×g for 4min, the supernatant was used as a source of template for amplification [22].

**Molecular analysis:** In order to reproduce and identify the *fliC* gene, a specific primer pair was used in order to identify different types of flagellin (A, B) in *P. aeruginosa* (Table 1).

**Table 1)** Sequence and conditions of PCR Primers [7]

Primers of <i>fliC</i> gene	Sequence	The size of the PCR product (bp)
Forward primer	5'GGCAGCTGGTTNGCCTG3'	A type: 1160
Reverse primer	5'GTCAACTTATCGCGTTTGCT3'	B type: 1460

In order to assess the specificity of primers, the desired sequences were blasted in the NCBI site and the amount of overlap and cross-reactivity with other organisms were examined. In this study, the standard strains of *P. aeruginosa* 8821 (type A) and PAO1 (type B) were used as a positive control and distilled water as a negative control [22].

## Findings

Out of 100 clinical isolates, 42 (42.0%) were isolated from urine (Table 2).

**Table 2)** Frequency of examined clinical specimens

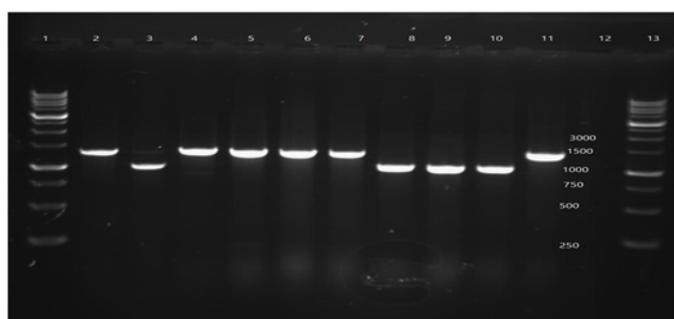
Sample type	Number	Female	Male
Urine	42	25	17
Scars	18	10	8
Chip	20	12	8
Eye discharge	5	1	4
Ear discharge	5	3	2
Suppurative discharge	5	4	1
Blood	5	4	1

Forty-seven isolates (47.0%) were resistant to imipenem (Table 3). Among of 47 imipenem resistant isolates, 41 strains (87.0%) were MBL producer. All of the MBL producing isolates were resistance to the examined antibiotic disks.

Twenty-six (55.3%) MBL-producing isolates contained *blaIMP-1* follow by two isolates (4.2%) contained *blaVIM-2* that isolated from urinary tract infection (n=1) and blood culture (n=1), whereas none of them were positive for *blaSPM-1* gene.

**Table 3)** Antimicrobial resistance pattern of *P. aeruginosa* strains

Antibiotics	Sensitive	Intermediate	Resistant
Imipenem (10µg)	47.0%	6.0%	47.0%
Meropenem (10µg)	49.0%	3.0%	48.0%
Gentamicin (10µg)	44.0%	2.0%	54.0%
Carbenicillin(100µg)	33.0%	0.0%	67.0%
Cefotaxime (30µg)	27.0%	3.0%	70.0%
Ticarcillin (75µg)	32.0%	0.0%	68.0%
Tobramycin (10µg)	34.0%	0.0%	66.0%
Ceftazidime (30µg)	37.0%	0.0%	63.0%
Amikacin (30µg)	42.0%	9.0%	49.0%
Ceftizoxime (30µg)	25.0%	3.0%	72.0%



**Figure1)** PCR of *fliC* gene

Lanes 1 and 13: DNA ladder (1kbp); Lane 2: Positive control of type B; Lane 3: Positive control of type A  
Lanes 4, 5, 6, 7 and 11: Clinical isolates of type B; Lanes 8, 9 and 10: Clinical isolates of type A; Lane 12: Negative control

Out of 100 *P. aeruginosa* strains 83.0% of the specimens had flagellin (*fliC*) gene, which out of them, 48 strains (60.0%) had type A flagellin (52.0% were female and 48.0% were male) and 32 strains (40.0%) had type B (53.0% were female and 47.0% were male).

Twenty-four of the 41 strains of MBL producer (60.0%) had type A and 16 strains (40.0%) had type B. Out of 41 positive MBL producers only one strain lacks the flagellin gene (Figure1).

## Discussion

The aim of the present study was to determine the *fliC* (flagellin) typing and their prevalence rate in *P. aeruginosa* producing MBL isolated from clinical specimens in Ahvaz, Iran.

*P. aeruginosa* is one of the most common opportunistic pathogen in nosocomial infections such as severe burns and cystic fibrosis patients [23]. In severe and acute such as septicemia and bacteremia, these bacteria can cause death of patients, so early diagnosis and appropriate treatments are essential. MBLs are considered as one of the mechanisms involved in resistance to pharmaceutical compounds such as carbapenem, including imipenem and meropenem that are among the antimicrobial antibiotics in the treatment of *P. aeruginosa*, MBLs have been identified from clinical isolates in different parts of the world and with increasing prevalence in the past years and strains producing these enzymes are responsible for nosocomial infections [24]. A study was conducted in Japan showed that mortality rates due to infection by *P. aeruginosa* producing MBL are more than the amount happened with negative MBL strains [13]. A study conducted by Doosti *et al.* in Zanjan hospital in 2013 showed that among 70 strains of *P. aeruginosa* isolated by phenotypic methods, out of 44 isolates resistant to imipenem, 36 isolates (81.8%) are MBL-producer [25].

Another study showed that out of 212 clinical specimens, 100 strains were resistant to imipenem, which of these 75 strains were MBL producers [26]. In 2015, a study conducted by AKA *et al.* showed that out of 60 *P. aeruginosa* isolates using DDST, 29 isolates (48.3%) were positive MBL producers [27]. In the present study, out of 100 *P. aeruginosa* strains, 47 strains were resistant to imipenem and of these, 41 isolates (87.0%) by combination disk method were MBL producers that this is indicative of more prevalence of MBL producing strains in the region. In a study conducted in 2012, out of 108 *P. aeruginosa* isolates, 40 isolates (37.0%) were imipenem resistant that 20 isolates of them (50.0%) were MBL producers [28]. Another study in Korea in 2014 showed that out of 329 *Pseudomonas* isolates from 23 hospitals, 100 isolates were resistant to

imipenem, which of these, 21 isolates were MBL producers [29]. This study showed that increasing the number of antibiotic resistance of *P. aeruginosa* isolates to imipenem is due to production of MBL enzymes.

A bacterial infection is established by virulence factors [30]. Extensive studies are conducted for identifying the structure of flagella and flagellin. Flagella in *P. aeruginosa* are divided into two types: A and B and is known as a vaccine candidate [31-33]. The bivalent vaccine (A, B) of flagella has been tested in patients with cystic fibrosis and satisfactory results have yielded [34].

Since these two types of flagella play an important role in the intensity of pathogenicity, in this study, have tried to identify and type the *fliC* gene of both types of flagella using a pair of specific primer and determine how many percentage of strains have flagella.

In the present study, 83.0% of the specimens had *fliC* gene that was consistent with the study conducted by Goudarzi *et al.* in which 85.0% of the isolates have *fliC* gene, and indicates that the flagella plays an important role in the bacterial pathogenesis [35].

In the present study, the results of PCR showed that the sequence of strain M8821 (type A) is smaller than the sequence of strain PAO1 (type B) that these results are consistent with *fliC* sequences in the GenBank of NCBI. Furthermore, Brimer *et al.* duplicated several strains of the type A, and after cloning them in vector T, compared these sequences with the strain PAO1 (type B). The results of their study showed that the sequence of the type A is shorter than the sequence of type B, which is consistent with the results of the present study [36]. In a study of Verma *et al.* on the flagella, it became clear that type A is smaller than type B, which is consistent with result of the present study [37].

Fast detection of *P. aeruginosa* can be very important in the treatment process and since flagella play a considerable role in colonization and disease, identification of *P. aeruginosa* through flagella by molecular methods can be a quick method to detect bacteria.

Generally, using this method can distinguish the flagella *P. aeruginosa* with flagella from strains without flagella and determine their frequencies in each region. In addition, this method can be used alongside other methods for typing of flagellin, and the data obtained can be used in epidemiological studies and the preparation of vaccine, because, as noted, flagella in *Pseudomonas* can be a good candidate in the preparation of vaccines [38]. The rapid distinction between *P. aeruginosa* strains with flagella and without flagella by PCR method can be a good alternative method for serological methods in flagella typing.

In this study, the results of the antibiogram showed that the prevalence of the MBL-producing *P. aeruginosa* in the patients in Ahvaz is high. Therefore, the identification of *P. aeruginosa* resistant to Carbapenems which is able to produce the MBL was essential. In addition, the majority of the positive MBL strains were also positive in terms of the presence of *fliC* gene; so it can be concluded that the presence of flagella plays a considerable role in the bacterial virulence. The limitations of the present study include the lack of access to all of the samples collected from the previous study, the frequency of the present sample was low, in addition to identified different types of MBL, must use more gene such as *GIM*; *SIM* genes and the suggestions is a study is to be done in the future that does not have these limitations. In conclusion, the results showed that the majority of the positive MBL strains were also positive in terms of the presence of *fliC* gene, so that recognizing, *fliC* gene could be a suitable and reliable genetic region for epidemiologic study.

## Conclusion

Eighty-three percent of the specimens have flagellin (*fliC*) gene, which out of them, 48 strains of *P. aeruginosa* (60.0%) have type A flagellin and 32 strains (40.0%) have type B. Twenty-four of the 41 strains of MBL producer (60.0%) have type A and 16 strains (40.0%) have type B and only one strains lacks the flagellin gene, so the flagella plays a significant role in the bacterial virulence.

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**Conflicts of Interests:** There is no conflict of interests.

**Authors' Contribution:** Moosavian M. (First author), Methodologist/ Original researcher or Assistant/ Statistical analyst/ Discussion author (10%); Moradzadeh M. (Second author), Introduction author/ Methodologist/ Original researcher or Assistant/ Statistical analyst/ Discussion author (80%); Ghadri H. (Third author), Statistical analyst/ Discussion author (10%).

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