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Detection of New Delhi Metallo-Beta-Lactamase-1 (*bla*$_{NDM-1}$) in Carbapenem-Resistant *Pseudomonas aeruginosa* Isolated from Clinical Samples in Wasit Hospitals

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Abstract

Background *Pseudomonas aeruginosa* (*P. aeruginosa*) infections are clinical problem, it is a difficult to treat because of high resistant to many antibiotics (Multi-drug resistant) and a high risk of emergence of resistance during therapy. Carbapenems are therapeutic choice against infections caused by Gram-negative bacilli including strains of *P. aeruginosa*. New Delhi metallo-β-lactamase-1 (*bla*$_{NDM-1}$) gene, an acquired class B carbapenemase. Dissemination predominantly involves transfer of the *bla*$_{NDM-1}$ gene among promiscuous plasmids and clonal outbreaks. Bacteria with NDM-1 are typically resistant to nearly all antibiotics.

Objective To detect *bla*$_{NDM-1}$ in the isolates of *P. aeruginosa*, which were recovered from various clinical samples from hospitalized patients in Wasit hospitals.

Methods This cross-sectional study involved 200 clinical samples were collected from three major hospitals in Wasit province. Samples were inoculated in Mackonkey and blood agar for primary isolation and then biochemical tests were used to confirm diagnosis of *P. aeruginosa*. The susceptibility test for 14 types of antibacterial drugs were tested by using disk diffusion method. Chromosomal and plasmid DNA were extracted by using special methods.

Results Out of 36 carbapenems resistant *P. aeruginosa* (CRPA) isolates, there were 18 isolates (50%) positive for *bla*$_{NDM-1}$ gene.

Conclusion Rate of occurrence of *bla*$_{NDM-1}$producers is highest among carbapenem-resistant *P. aeruginosa* isolated from clinical samples in Wasit hospitals. Therefore, its recognizable proof in clinical bacterial diseases will be suspected in any carbapenem resistance *P. aeruginosa*.

Keywords *P. aeruginosa*; carbapenems; metallo-β-lactamase; *bla*$_{NDM-1}$

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List of abbreviations: BHI = Brain-heart infusion, bla-NDM1 = New Delhi metallo-β-lactamase-1, CRPA = Carbapenem- resistant *Pseudomonas aeruginosa*, ESBLs = Extended-spectrum beta lactamases, MDRs = multi-drug resistance

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is an aerobic Gram-negative rod-shaped. It is widely distributed in nature and can adapt to many environments, it can be isolated from nearly any conceivable source within hospitals [(1)]. It is an important cause of both community and hospital-acquired infections. Infections with these bacteria have been associated with high mortality and morbidity when compared with other bacterial pathogens [(2)]. *P. aeruginosa* infections are clinical problem, it is a difficult to treat because of high resistant to many

antibiotics (Multi-drug resistant) and a high risk of emergence of resistance during therapy [3].

Beta-lactam as antibacterial agent are broadly used to treat diseases caused by Gram-negative Pathogens However, the adequacy of these medications is lessened impressively because of the presence of extended-spectrum beta lactamases (ESBLs) and the consequent emergence of multi-drug resistant (MDRs) strains [3].

Carbapenems are a group of β-lactam antibiotics with a broad spectrum of antibacterial activity. Their structure makes them highly resistant for most β-lactamases [4]. They include meropenem and imipenem, which are among the few therapeutic options still available for treating infections caused by *P. aeruginosa* [5]. Carbapenems are considered to be antimicrobial agents of choice and are frequently used for the treatment of hard-to-manage *P. aeruginosa* infections. However, carbapenem resistance in *P. aeruginosa* has been reported to increase steadily over the years across the world, but the relative contribution of different carbapenems resistance mechanisms is not well established [6,7]. bla_{NDM-1} is an enzyme that cleaves the amide bond of β-lactam ring and provides resistance against major classes β-lactam antibiotics [8]. New Delhi Metallo-β-lactamase-1 gene (bla_{NDM-1}) codes for NDM-1 [2]. An association with other resistance mechanisms makes majority of *P. aeruginosa* with blaNDM-1 gene extensively resistant to antibiotics.

The goal of this research was to detect the presence of NDM-1 producers in clinical *P. aeruginosa* isolates producer between clinical P. aeruginosa isolates in Wasit hospitals.

Methods

Clinical isolates

Over six months from November 2016 to April 2017, different samples including (burn swab, ear swab, urine, sputum and wound swab) from two hundred patients admitted to (Al-Zahraa Teaching Hospital, Al-Karama Teaching Hospital and Al-Kut Hospital for Gynecology, Obstetrics and Pediatrics) in Wasit province were enrolled in this study. In the case of swab samples, two swabs were taken from each patient, while sputum and urine were divided directly into two parts, the first one was prepared for wet smear preparation (Gram stain), and the other was used for culturing on different culture media for further isolation and characterization of the causative agents. The isolated bacteria were identified by standard laboratory methods and API20E system (BioMerieux), *P. aeruginosa* isolates in Brain-Heart Infusion (BHI) broth containing 15%, and the tubes were stored in deep freezing at -20 °C [9].

Antimicrobial susceptibility testing

Resistance patterns of the *P. aeruginosa* isolates to different antibiotics was determined using disk diffusion test (Kirby-Bauer) on Muller Hinton agar media [10], the antibiotic discs used in this study was Levofloxacin (5 µg), Meropenem (10 µg), Imipenem(10 µg), Aztreonam (30 µg), Ceftazidime (30 µg), Amikacin (30 µg), Gentamicin (30 µg), Ciprofloxacin (10 µg), Piperacillin (10 µg), Colistin sulphate (25 µg). The standard isolates from central public health laboratory *E. Coli* ATCC25922 used as a negative control. When the incubation was completed temperature and time, the resulting zones of inhibition were measured and compared with the break points standard value of Clinical Laboratory Standards Institute CLSI (2016) [10]. The minimum inhibitory was determined by Vitik2-System (VITEK MS, bioMérieux, Nürtingen, Germany), and standard agar dilution method [11] according to the CLSI (2016) [10].

Phenotypic detection of metallo-β-lactamases (MBL)

All imipenem and meropenem-resistant isolates were examined for MBL production using the IMP-EDTA double disk synergy test as described by [12], furthermore, Modified Hodge test (MHT) was used for detection of carbapenemases production *P. aeruginosa*

isolates according to CLSI guidelines using 10 µg meropenem susceptibility disk, which was placed in the center of the test area. P. aeruginosa was streaked in a straight line from the edge of the disk to the edge of the plate. The plate was incubated overnight at 37 °C in ambient air for 16-24 hours. After 24 hours, MHT positive test showed a clover leaf-like [13].

DNA Extraction and polymerase chain reaction (PCR) amplification

In this study, both plasmid DNA and chromosomal DNA were extracted, plasmid DNA was extracted according to (14), while chromosomal DNA was extracted by Genomic DNA Mini Kit (Genaid) according to company instructions. All carbapenem-resistant isolates were screened by standard PCR conventional using specific primers for *bla*$_{NDM-1}$ gene as shown in table (1). PCR reaction tubes were transferred into thermal cycler (Agilent, USA) that was programmed as following: initial denaturation for 5 mints at 95 °C, (the conditions for each cycle were: 30 sec. at 94 Ċ, 30 sec. at 60 °C and 30 sec. at 72 °C), and final extension at 72 °C for 5 mints. Amplified products were electrophoresed on 1.5% agarose for 90 mints at 5 V/cm.

Table 1. Sequences of primer that used in the detection bla$_{NDM-1}$ gene

Gene		Nucleotide sequences (5'→3')	Products size bp	References
bla$_{NDM-1}$	F	GGG CAG TCG CTT CCA ACG GT	475	(15)
	R	GTA GTG CTC AGT GTC GGC AT		

Statistical Analysis

Statistical analysis was performed with Graph Pad Prism version 6 software, percentages were used for the comparison between samples of the study. Data analysis was done using Chi-square for the comparison of categorical data.

Results

A total of two hundred samples were enrolled in this study which include, burn swabs (n=105, 52.50%), ear swabs (n=19, 9.50%), wound swab (n=36, 18.00%), sputum from patients with lower respiratory tract infection (n=7, 3.50%) The patient's ages ranged from one to older than 61 years (Table 2).

Table 2. Distribution of *Pseudomonas aeruginosa* isolates according to age groups

Age groups	*P. aeruginosa*	Others bacteria	Negative	Total
1-10 yr	11	10	2	23
11-20 yr	10	9	2	21
21-30 yr	20	12	4	36
31-40 yr	27	13	2	42
41-50 yr	11	14	3	28
51-60 yr	9	7	4	20
≥61 yr	15	13	2	30
Total	**103**	**78**	**19**	**200**

One hundred and eighty-one bacterial species were isolated from these samples with the percentage of *P. aeruginosa* (n=103, 51.50%) followed by *E. coli* (n=28, 14.00%) and the lowest percentage were *K. pneumoniae* (n=2, 1.00%). There is non-significant association between Pseudomonas infections and age groups as shown in table (3).

Table 3. Distribution of *Pseudomonas aeruginosa* and other bacteria according to the growth

Type of Bacteria	Samples	%
Negative	19	9.50%
Klebsiella pneumoniae	2	1.00%
Staphylococcus epidermidis	4	2.00%
Acinetobacter baumanni	7	3.50%
Pseudomonas putida	7	3.50%
Streptococcus pyogenes	12	6.00%
Staphylococcus aureus	18	9.00%
Escherichia coli	28	14.00%
Pseudomonas aeruginosa	103	51.50%
Total	200	100%

Antimicrobial susceptibility test

The results of antibiotic susceptibility test for isolated *P. aeruginosa* indicated different antibiotic profiles as shown in table (4). In total, 55.5% (n=103) resistance to the third-generation ceftazidime 57.28% of the isolates exhibited resistance to the fourth generation cefepime. While the resistance to monobactams, aztreonam was 51.46%. The highest resistance percentage was found against gentamicin (91.26%). According to the results of the fluoroquinolones susceptibility testing, 83.50% and 60.19% of the isolates were resistant to ciprofloxacin and levofloxacin, respectively (Table 4).

Table 4. Susceptibility patterns of *Pseudomonas aeruginosa* to different antibiotics

Antibiotic	Sensitive (S)		Intermediate (IR)		Resistant (R)	
	No. of isolates	%	No. of isolates	%	No. of isolates	%
Imipenem	67	65.05	0	0	36	34.95
Meropenem	67	65.05	0	0	36	34.95
Ciprofloxacin	12	11.65	5	4.85	86	83.50
Levofloxacin	38	36.89	3	2.91	62	60.19
Amikacin	9	8.74	6	5.83	88	85.44
Gentamicin	5	4.85	4	3.88	94	91.26
Cefepime	42	40.77	6	5.83	55	53.40
Ceftazidime	39	37.86	5	4.85	59	57.28
Aztreonam	40	38.83	10	9.71	53	51.46
Piperacillin	17	16.50	16	15.53	70	67.96
Piperacillin/Tazobactam	50	48.54	8	7.77	45	43.69
Colistin	102	98.03	0	0	1	0.97
Ticarcillin/Clavulanic acid	47	45.63	5	4.85	51	49.51
Ticarcillin	40	38.83	7	6.80	56	54.37

Phenotypic detection of MBLs

From 36 *P. aeruginosa* carbapenem-resistant isolates, MHT revealed 16 (44.44%) were positive showing their ability to produce carbapenemases, moreover, double disc synergy indicates that in 32 (88.89%) isolates, MBLs were produced. Those isolates, which were found MBL positive by Double disc synergy test and were also found to be MBL positive MHT.

PCR screening for NDM-1 encoding gene

PCR using specific primers for NDM-1 was performed on all the IMP-resistant isolates for generation of specific amplification band with certain molecular weight that were 475 bp fragment which represented blaNDM-1 gene. The results showed MBL gene *bla*$_{NDM-1}$ (475 bp) was detected in 18 (50.00%) of the carbapenem-resistant isolates on plasmid DNA, while MBL gene *bla*$_{NDM-1}$ not found on chromosomal DNA (Figure 1).

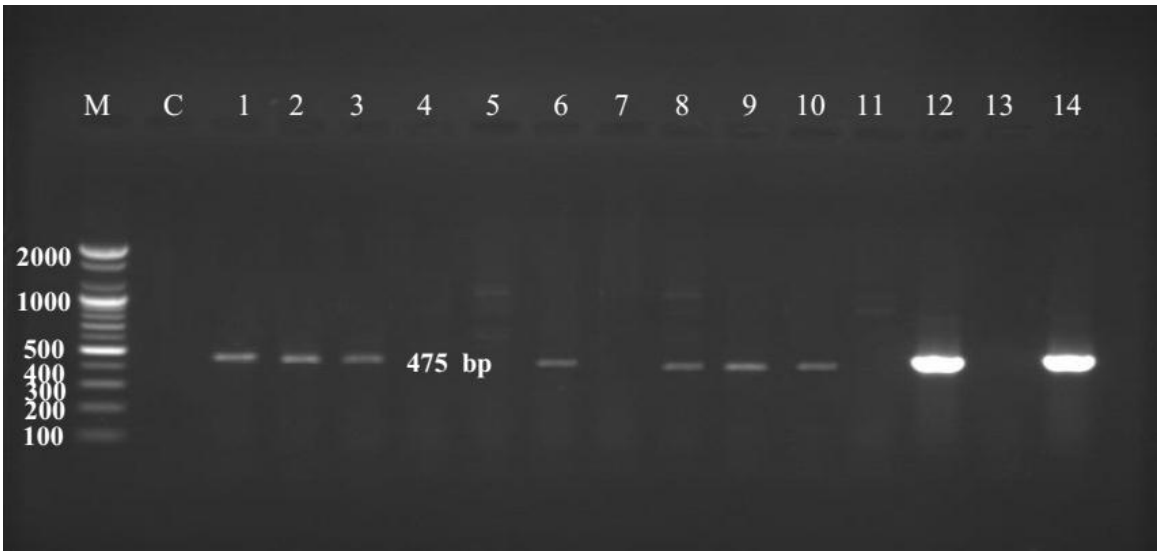

Figure 1. Gel electrophoresis of amplified plasmid DNA for detection of MBL bla-NDM1 gene (475bp) using PCR with specific primers; 1,5% Agarose for 90 minutes at 70 V\cm. Lane M: Marker DNA ladder Size (100bp), Lane C: Negative control and Lanes (1-14) positive for *bla*$_{NDM-1}$ (475 bp) except (4,5,7,11,13)

Discussion

The current emergence of *P. aeruginosa* carbapenem-resistant represents a major threat to the clinical approach because it exhibits intrinsically decreased susceptibility to a range of antimicrobials and possesses a great ability to develop resistance to multiple classes of agents [16]. Among two hundred samples were enrolled in this study, the mean age of the patients were 36.61 years. Results of current study revealed that, there is non-significant association between Pseudomonas infections and age. It is noteworthy to mention that result was disagreed with a study conducted by Magliano et al. [17] who was reported the high rate of *P. aeruginosa* infection among age group (≥ 60 years). In the present study, *P. aeruginosa* has been the predominant bacterial isolated among study group followed by *E. coli* (14%) and the lowest percentage were *K. pneumoniae* (1%). These findings are compatible with study conducted in Egypt by Gad et al. 2007 [18], the present study is incompatible with a study in Baghdad by Al-Huraishi [19] who found that *Acinetobacter baumannii* (31%) is even more common than *P. aeruginosa* (12%), and study conducted in Baghdad by Al-Kadhmi in 2016 [20], who reported that *S. aureus* (30%) was the most common agents, then *P. aeruginosa* (14.6%) this difference in results can be attributed to sample difference and kind of test

used in isolation and diagnosis of different bacterial species.

Carbapenems are a class of β-lactam antibiotics with good antimicrobial activity against *P. aeruginosa* but the arises and spread of acquired carbapenem-resistance in this species have challenged the success of therapeutic and control efforts [21]. Result in current study showed there was no different found in activity of imipenem and meropenem to *P. aeruginosa* (both of them have the same percentage 34.95% resistant, respectively), which disagree with Gupta et al. 2006 who found that the imipenem had a better activity than meropenem [22]. Furthermore, current finding indicated that higher resistance against imipenem and meropenem have compared with study in Najaf by Al-Shara in 2013 [23], who reported that the resistance rate was 7.4% and 14.8%, respectively. The percentage of fluoroquinolone-resistant isolates was 83.50% and 60.19% of isolates resistant to ciprofloxacin and Levofloxacin, respectively identified in this study is considerably higher than that reported in study conducted in Najaf Hospitals, in which resistance were 73.4 % for ciprofloxacin and 55.5% for Levofloxacin [23] also it is in harmony with previous study in Najaf [24]. Fluoroquinolone resistance among *P. aeruginosa* isolates looks to be increasing in the Wasit hospitals, perhaps because of high increasing fluoroquinolone use, and the lack of adherence to approved infection control practices by hospitals. The *P. aeruginosa* isolates were most resistant to amikacin (85.44%) and gentamicin (91.26%), the resistance rate was higher when compared with other study reported by Al-Shara [23] in Najaf, who revealed that only 64.8% of the P. *aeruginosa* isolates resistant to this antibiotic. However, the findings of *P. aeruginosa* antibiogram in the present study disagree with a study done in United States of America [25]. Results in the current research, showed that 51.46% of the *P. aeruginosa* isolates were resistant to aztreonam, Present findings are higher than previous study done by Abdullah and Mehdi, who showed low rate of aztreonam resistance among *P. aeruginosa* clinical isolates [26]. Colistin resistance is not dependent upon bacterial metabolic activity and acquired resistance is rare [27]. In present study, the resistance of the isolates to Colistin was 2.78%, this result disagreed with the study in Turkey, who mentioned that all multidrug-resistant strains were 100% susceptible to Colistin [28]. The present investigation showed that Colistin was only antibiotic that may remain highly active against carbapenem resistance *P. aeruginosa* (CRPA) isolates, these results accepted with Goli et al. study in Iran [28]. This might be explained by the high cost of Colistin and limited use out of the hospitals. The high rate of resistance observed in *P. aeruginosa* isolates in this study, may be explanted by incorrectly prescribed antibiotics, extensive of antibiotics in animal food which in turn transfers to humans by meat and egg consumption, and availability of few new antibiotics.

The production of MBLs is the most common mechanism for carbapenem resistance in *Enterobacteriaceae* and *P. aeruginosa* isolates [29,30]. The resistant isolates were tested by MHT revealed 16 (44.44%) were positive isolates, in addition double disc synergy test showed that 32 (88.89%) isolates out of 36 *P. aeruginosa* (CRPA) were positive. Present study revealed that these two tests may be useful in screening for MBL, but these tests cannot be routinely performed in all national laboratories. The current results showed that 50% percentage of *P. aeruginosa* (CRPA) isolates have $bla_{\text{NDM-1}}$ gene in plasmid DNA, the percentage of $bla_{\text{NDM-1}}$ gene in the current study was higher than previous study in Najaf who showed only 2 (5.6%) isolates harbored $bla_{\text{NDM-1}}$ gene [23]. In Slovakia, study was reported $bla_{\text{NDM-1}}$ gene in 6 isolates; 20%, this result disagreed with current study [30]. The spread of $bla_{\text{NDM-1}}$ gene on a large scale of serious things because it leads to the absence of any effective antibiotic against MDR bacteria [31]. The current result of $bla_{\text{NDM-1}}$ gene represent highest ratio recorded in Iraq, 50% of *P. aeruginosa* (CRPA) isolates, this percentage higher than the result recorded by Al-Shara, 2013 in Najaf who was showed low findings that 2 (5.6%) isolates harbored $bla_{\text{NDM-1}}$ gene [23], that's because it was easily transferred and

rapidly disseminated to other *Enterobacteriaceae* as it is plasmid borne. Moreover, it contained a variety of other resistance determinants, including a gene encoding another broad-spectrum β-lactamases and genes inactivating ciprofloxacin, erythromycin, chloramphenicol and rifampicin. In addition, the genetic element encoded an efflux pump that capable of producing additional antimicrobial resistance and growth promoters that insured the transcription of the genes contained in the genetic element [31].

This study concluded that the rate of occurrence of bla_{NDM-1} producers is highest among carbapenem-resistant *P. aeruginosa* isolated from clinical samples in Wasit hospitals. Therefore, its recognizable proof in clinical bacterial diseases will be suspected in any carbapenem resistance *P. aeruginosa*.

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Authors contribution

Hussein: conducted the sampling, isolation, and diagnosis, the molecular work and writing the manuscript. Dr. Kadhim and Dr. Hassan supervised the work, edit and finalize the writing of the study.

Conflict of interest

Authors declare no conflict of interest.

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