

## First Report of New Delhi Metallo-beta-Lactamase (NDM-1) Producing *Pseudomonas aeruginosa* in Iraq

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### Abstract

Carbapenems are therapeutic choice against infections caused by Gram-negative bacilli including strains of *Pseudomonas aeruginosa*. New Delhi metallo- $\beta$ -lactamase (NDM-1) is a novel metallo- $\beta$ -lactamase (MBL). That confers resistance to all  $\beta$ -lactamases. Sporadic cases of NDM-1 positive strains have been reported from different countries. The aim of this study was to determine the possibility of existence of NDM-1 gene among *P. aeruginosa* isolates collected from Najaf hospitals. Thirty-six carbapenem resistant *P. aeruginosa* isolates were collected from various clinical samples. The susceptibility to different antibiotics was evaluated by disk diffusion method, MICs of imipenem and meropenem were determined. The *bla*<sub>NDM</sub> was detected by PCR. All isolate were multidrug resistance. Two isolates were harbored *bla*<sub>NDM</sub> genes. The MICs of isolates to imipenem and meropenem were 4-128  $\mu$ g/mL and 4-256  $\mu$ g/mL, respectively. The study concluded that production of MBL enzymes presents an emerging threat of carbapenem resistance among *P. aeruginosa* in Najaf province. This is the first report of NDM  $\beta$ -lactamase producing *P. aeruginosa* in Iraq.

**Keywords:** *P. aeruginosa*; Carbapenems; metallo- $\beta$ -lactamase; *bla*<sub>NDM</sub>.

### Introduction

*Pseudomonas aeruginosa* is an important cause of nosocomial infections and has been associated with a wide variety of illnesses in hospitalized patients, especially patients in the intensive care units (Brooks *et al.*, 2007). The clinical strains of *P. aeruginosa* are usually multidrug resistant to aminoglycosides, fluoroquinolones, ureidopenicillins and third generation cephalosporins. In cases of resistance to  $\beta$ -lactams caused by extended spectrum  $\beta$ -lactamases and AmpC enzyme, carbapenems are often used as the last resort against infections. However, carbapenem- hydrolyzing-  $\beta$ -lactamases of molecular class A, B and D have emerged over the last decade (Strateva & Yordanov 2009).

The emerging NDM-1, an acquired class B carbapenemase that was first clinically detected in a patient at a hospital in New Delhi, India, has brought up worldwide public attention again (Bonomo, 2011; Rolain *et al.*, 2010). Although current reports indicate that NDM-1 does not hydrolyze monobactams, most of NDM-1-carrying strains also express enzymes that could hydrolyze monobactams, making NDM-1-producers very difficult to control (Shakil *et al.*, 2011). The rapid dissemination of NDM-1-producing gram-negative species also contributes to this major concern of public health. It has been reported in 50 countries across five continents, in the last 2 years (Bonomo, 2011; Rolain *et al.*, 2010). NDM-1 is detected mainly in Enterobacteriaceae but occasionally in *Acinetobacter baumannii*, *Stenotrophomonas maltophilia* and *P.aeruginosa* ( Jovcic *et al.*, 2011). There is no evidence of the emergence of NDM-1-producing Enterobacteriaceae and *P.aeruginosa* in Iraq at this point. In the present study, we report the first isolation in Najaf of a NDM-1-producing *P.aeruginosa*.

The aims of this study were to determine the existence of NDM  $\beta$ -lactamase producing *P. aeruginosa* isolates and their antibiotic susceptibility patterns in Najaf hospitals.

### Materials and Methods

#### Bacterial isolates

A total of 36 non-repetitively meropenem and/or imipenem resistant *P. aeruginosa* isolates were obtained from hospital environmental samples and patients visited/or admitted to different hospitals in the Najaf province included: Al-Sader Medical City, Al-Hakeem General Hospital, Al-Zahra'a Hospital for Childbirth and Children, over a period from February to October 2011. Isolates were acquired from specimens originated from instrument, floor, burn wound, ear and urinary tract. The isolates were identified by conventional biochemical methods and confirmed by using API 20E system.

#### Antibiotic Susceptibility Assays:

All isolates were tested in the Kirby Bauer method of disk diffusion to check their susceptibilities to norfloxacin (NX: 5  $\mu$ g), ciprofloxacin (CIP: 5  $\mu$ g), amikacin (AN: 30  $\mu$ g), gentamicin (CN: 10  $\mu$ g), tobramycin (TOB: 10

µg) aztreonam (ATM: 30 µg), meropenem (MEM: 10 µg), imipenem (IMP: 10 µg), cefepime (FEP: 30 µg), ceftriaxone (CRO: 30 µg), cefotaxime (CTX: 30 µg), ceftazidime (CAZ: 30 µg), ticarcillin- clavulanat (CTC: 75/10 µg), piperacillin-tazobactam (PTZ: 110 µg), ticarcillin (TIC: 75 µg), piperacillin (PRL: 100 µg), carbenicillin (PY: 100 µg) (Himedia-India). Multidrug resistance (MDR) was defined as resistance to 3 or more classes of drugs that would otherwise serve as treatments for *P. aeruginosa* infection (Falagas *et al.*, 2008). [7] Isolates found resistant to carbapenems (imipenem and meropenem) by disk diffusion test were re-checked in broth micro dilution assay (Clinical and Laboratory Standards Institute 2012). [8]. In this study, *P. aeruginosa* isolates were defined as resistant to carbapenems if MIC for imipenem and meropenem were  $\geq 8 \mu\text{g/ml}$ . The *E. coli* ATCC 25922 strain was adopted as the standard for quality control (Yong *et al.*, 2002).

#### *Phenotypic screening for carbapenemases production:*

**Double-disc synergy test (DDST):** Screening for MBLs was performed using disks containing 1900 µg of EDTA plus 10 µg of imipenem disk were placed on the inoculated plates containing Muller Hinton agar. An increase of  $\geq 17$  mm in zone diameter in the presence 1900 µg of EDTA compared to imipenem alone indicated the presence of an MBL (Lee *et al.*, 2003).

**Modified Hodge test (MHT):** This test was carried out to detect carbapenemase using imipenem and meropenem as described by Clinical and Laboratory Standards Institute (2010).

**Carbapenemase bioassays:** In order to determine whether resistance to imipenem and meropenem was likely caused by production of carbapenemase, a disk diffusion bioassay was performed on Mueller-Hinton agar with imipenem and meropenem disks as described by Walsh *et al.*, (2002).

#### *PCR amplification*

DNA was extracted from the isolates by boiling five colonies in 250 µl of sterile ultrapure water for 10 minutes, followed by cooling in ice for 10 minutes and centrifugation for 1 min at 14,000 rpm. Supernatants were conserved at -20°C until amplification (Hall & Barlow 2005). [13]. Amplification of *bla<sub>NDM</sub>* gene was performed using primer pairs (F) 5'- ACCGCCTGGACCGATGACCA -3' and (R) 5'- GCCAAAGTTGGGCGCGGTTG -3'. This set of primers gave rise to an amplicon of 264 bp. After initial denaturation for 2 min at 94°C, 35 cycles were performed (the conditions for each cycle were: 30 s at 95°C, 30 s at 58°C and 30 s at 72°C). The final cycle was followed by 72°C incubation for 10 min. The PCR product was electrophoresed on 1.5% agarose gel and following ethidium bromide staining was viewed under UV light (Zarfel *et al.*, 2011).

## **Results**

Meropenem and /or imipenem resistant isolates were collected as part of the first national study on the distribution and evolution of carbapenem resistance *P. aeruginosa* in Najaf hospitals. The frequency of the isolates and their sites of isolation are listed in table (1). High percentage of resistant carbapenemase isolates 19/36(52.7%) were received from burin wound samples in compared with others. Majority of the isolates were obtained from Al-Sader Medical City (80.6%) followed by Al-Hakeem General Hospital (11.1%), since only a minority of these isolates were from Al-Zahra'a Hospital for Childbirth and Children (8.3%).

Present results revealed that a total of 10 different antibiotic resistance phenotypes were seen among the carbapenem-resistant isolates, although some of these were minor variants. Three phenotypes were predominant, as follows: phenotype class I, resistant to all antibiotics (12 isolates, 33.3%), phenotype class II, susceptible only to imipenem (11 isolates, 30.6%); phenotype class III, susceptible to imipenem and amikacin but resistant to all other antimicrobials tested (5 isolates 13.9%). The remaining 8 phenotypes accounted for 19.4% of the meropenem-resistant isolates.

Antibiotic susceptibility pattern revealed that 4(11.1%) isolates were resistant to at least three classes of antibiotics, hence they were considered to be MDR. The current study also reported that 20 (55.5%) and 12(33.3%) of carbapenem resistance *P. aeruginosa* isolates were extensive drug resistant (XDR) and pandrug resistant (PDR) isolates respectively (Table 2). The MIC of imipenem showed a varied range from  $\geq 4$  to  $\geq 128 \mu\text{g/ml}$ . Twenty eight (77.8%) isolates showed imipenem resistance (8-128) µg/ml. In particular, none of the isolates showed an MIC value  $>4 \mu\text{g/ml}$  for imipenem. Similarly, MIC of meropenem  $\geq 8 \mu\text{g/ml}$  for 34(94.4%) and 2 isolates were  $\geq 256 \mu\text{g/ml}$ .

The phenotypic technique for detecting carbapenemase activity. Among over all carbapenem resistant isolates (36), table (4) shows only 28 (77.8%) and 14 (38.9%) were positive according to imipenem-meropenem-EDTA disk synergy test (Figure 1) and modified Hodge test respectively. EDTA-imipenem microbiological assay was document 28 (77.8%) bacterial extract with and without ZnSO<sub>4</sub> indicated the presence of carbapenemase activity. Moreover, indicator strain growth was inhibited around the same 28 (77.8%) bacterial extract supplemented with EDTA confirmed the presence of MBLs (Figure 2).

The PCR amplification of NDM gene confirmed that 2 (5.6%) isolates harbored *bla*<sub>NDM</sub> gene (Pa9 and Pa22) Figure (3). All NDM- producing isolates exhibited high antibiotic resistance and had a XDR phenotype (Table 2).

## Discussion

*P. aeruginosa* classically considered as one of the leading Gram-negative pathogen that causes nosocomial infections worldwide (Meenakumari *et al.*, 2011). It is one of the most important bacterial pathogen seriously contributing to the problem of hospital infections (Fonseca *et al.*, 2007), so has received most attention in Najaf hospitals.

In the present study, a high rate of resistance among isolates recovered from clinical and hospital environments from three hospitals in Najaf city was documented. The largest carbapenem-resistant isolates taken hold of Al-Sader Medical City afterwards Al-Hakeem General Hospital and in the wake of little from Al-Zahra'a Hospital for Childbirth and Children. Carbapenems are potent agents against multiresistant *P. aeruginosa*, in spite of the slightly use of carbapenem therapy in these hospitals, but survey data show emerging resistance in *P. aeruginosa* isolates.

The emergence of carbapenem resistant by acquired carbapenemase including MBLs among *P. aeruginosa* represents an epidemiological risk for at least two reasons. Firstly, carbapenemase confer resistance not only to carbapenems but to virtually all  $\beta$ -lactams and are frequently associated with resistance to numerous aminoglycosides; and secondly, genes encoding for carbapenemase enzymes are most commonly carried on mobile genetic elements (integrons, plasmids, transposons) that can spread horizontally among unrelated strains (Walsh, 2005; Hammami *et al.*, 2010).

The rate of resistance to  $\beta$ -lactams was high in this study. It seems that other mechanisms such as decreased permeability, over expression of efflux pump, production of carbapenemases probably are involved in resistance to  $\beta$ -lactams. Understanding the underlying genetic mechanisms responsible for the acquisition and spread of this unique  $\beta$ -lactamase mediated antibiotic resistance mechanism could eventually facilitate the development of effective prevention and control strategies and thereby allowing more effective drug usage and treatment of disease, and reducing resistance development (Yong *et al.*, 2002). This may confer with high incidence of MDR, XDR and PDR observed among *P. aeruginosa* isolates underlines the strict consideration in antibiotics use at Najaf hospitals.

The production of MBL is the most common mechanism for carbapenem resistance in *Enterobacteriaceae* and *P. aeruginosa* isolates (Lledo *et al.*, 2009; Munoz-Price & Quinn 2009). Present study revealed that 14 isolates were modified Hodge's test positive which optimistically indicate that they may possess the carbapenemase gene, which mediated the carbapenem resistance. MBLs have been identified from clinical isolates worldwide with increasing frequency over the past few years and strains producing these enzymes have been responsible for prolonged nosocomial outbreaks that were accompanied by serious infections (Hemalatha *et al.*, 2005; Marra *et al.*, 2006).

The resistant isolates were tested by the imipenem-meropenem-EDTA disk synergy test, 77.8% were found to be MBL producers phenotypically. Several studies reported that imipenem-EDTA combination was most sensitive in detection of MBL in *P. aeruginosa* (Queenan & Bush 2007). A high proportion of isolates produced MBL was found in Najaf hospitals. Present study revealed that this test may be useful in screening for MBL. This report was in concordance with the results of two Indian studies on the detection of MBL by DDST reported that 70% and 75% were MBL positive (Uma *et al.*, 2009; Gupta & Chander 2006), respectively. In this study, EDTA-imipenem microbiological assay was used in order to detect metalloenzymes in all isolates with cellular extracts. The positive isolates suggesting the presence of MBLs. This result displayed a performance comparable to that of imipenem-meropenem-EDTA disk synergy test, concerning the high sensitivity of these methods for the detection of MBL producers.

The present study report here the first two cases of infections due to NDM-1-producing *P. aeruginosa* in Iraq. The gene encoding NDM-1 is located in a very mobile genetic element, resulting in rapid spread across the species and countries (Yong *et al.*, 2009; Cholley *et al.*, 2011). However, the *bla*<sub>NDM-1</sub> gene positive *Enterobacteriaceae* has spread from India, Pakistan and Bangladesh to UK, US, France (Fleateau *et al.*, 2012), Kenya, Japan, Canada, Belgium, the Netherlands, Serbia (Leverstein-van *et al.*, 2010), Taiwan (Wu *et al.*, 2010), Singapore, Sultanate of Oman (Poirel *et al.*, 2011a), Australia (Poirel *et al.*, 2010) and Iraq (Poirel *et al.*, 2011b). A substantial number of infected/colonized patients have been part of the so-called "medical tourism." The antibiotic resistant pattern revealed that isolates harbored *bla*<sub>NDM</sub> were XDR phenotype. NDM-1 producing

bacteria are usually extended drug to antibiotics because *bla*<sub>NDM-1</sub> encoding plasmids co-harbor multiple resistance determinants. Moreover, *P. aeruginosa* shows a high level of intrinsic resistance to antimicrobial agents. Its ability to acquire and combine different resistance determinants represents a major threat, compromising therapeutic options (Moriyama *et al.*, 2010; Prevotat *et al.*, 2010).

### Conclusion

In conclusion, the detection of NDM-1-positive *P. aeruginosa* isolates in this study indicates immediate importance of strengthening surveillance to prevent the nosocomial infection and dissemination of NDM-1 in Najaf.

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Table 1: Sample demographics and antibiotics susceptibilities of carbapenem-resistant *P. aeruginosa* isolates

Isolate symbol	Hospital	Sample source	Piperacillin	Ticarcillin	Carbencillin	Ticarcillin - clavulanic	Piperacillin - tazobactam	Ceftroxone	Ceftazidime	Cefotaxime	Cefepime	Aztreonam	Cefoxitin	Imipenem	Meropenem	Gentamicin	Amikacin	Tobramycin	Ciprofloxacin	Norfloxacin	No. of antibiotic resistant and phenotype class
Pa1	Sh*	BW**	R	R	R	R	R	R	S	R	R	R	R	S	R	R	S	R	S	R	14
Pa2	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S	R	S	R	15
Pa3	Sh	BW	R	R	R	R	R	R	S	R	R	R	R	S	R	R	S	S	R	R	14
Pa4	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	S	R	R	16
Pa5	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	17-II
Pa6	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	7
Pa7	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18-I
Pa8	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	17-II
Pa9	Zh*	IN**	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	17-II
Pa10	Sh	FL**	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	17-II
Pa11	Zh	FL	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18-I
Pa12	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	17-II
Pa13	Sh	Ea**	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S	R	R	R	16-III
Pa14	Sh	IN	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	17-II
Pa15	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18-I
Pa16	Sh	FL	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S	R	R	R	16-III
Pa17	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S	R	R	R	16
Pa18	Sh	Ea	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18-I
Pa19	H*	UR**	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	17-II
Pa20	Sh	UR	S	R	R	R	S	R	S	R	R	R	R	S	R	R	S	R	S	R	12
Pa21	H*	UR	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	S	R	16
Pa22	Sh	UR	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	17-II
Pa23	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18-I
Pa24	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18-I
Pa25	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18-I
Pa26	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S	R	R	R	16-III
Pa27	H	Ea	S	R	S	R	S	R	R	R	R	R	R	S	R	R	S	R	R	R	13
Pa28	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	S	R	16
Pa29	Sh	Ea	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18-I
Pa30	H*	UR	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18-I
Pa31	Sh	UR	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	17-II
Pa32	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18-I
Pa33	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18-I
Pa34	Sh	FL	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18-I
Pa35	Zh	FL	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	17-II
Pa36	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S	R	R	R	16-III
		Total resist-ant (%)	36 (94.4)	36 (100)	35 (97.2)	36 (100)	34 (94.4)	36 (100)	33 (91.6)	36 (100)	36 (100)	36 (100)	36 (100)	12 (33.3)	36 (100)	36 (100)	26 (72.2)	34 (94.4)	31 (86.1)	36 (100)	

\*Sh, Al-Sader Medical City; Hh, Al-Hakeem General Hospital; Al-Zahra'a Hospital for Childbirth and Children<sup>(1)</sup>

\*\*BW, Burin wound; Ur, Urine; Ea, Ear; IN, Instrument; FL, Floor.<sup>(2)</sup>

I, II,III, phenotype class 1,2 and 3.<sup>(3)</sup>

Table 2: Multidrug resistance (MDR), extensive drug resistance (XDR) and pandrug resistance (PDR) of carbapenem-resistant *P.aeruginosa* isolates (n=36).

Type of resistance	Isolate symbol	No. of antibiotics classes(n= 7)
MDR (n=4, 11.1%)	Pa20, Pa27	3
	Pa1, Pa2	4
XDR (n=20, 55.5%)	Pa3, Pa13 , Pa16, Pa17, , Pa21, Pa26, Pa28, Pa35, Pa36	5
	Pa4, Pa5, Pa6, Pa8, Pa 9, Pa10, Pa12, Pa14, Pa 19, Pa22, Pa31,	6
PDR (n=12, 33.3%)	Pa7, Pa 11, Pa15, Pa18, Pa23, Pa24, Pa25, Pa29, Pa30, Pa32, Pa33, Pa34	7

Table 3: MIC values ( $\mu\text{g/ml}$ ) and susceptibility of *P. aeruginosa* isolates (n=36) for carbapenems according to (CLSI, 2012)

Status	Imipenem		Meropenem	
	Range ( $\mu\text{g/ml}$ )	No.(%) of isolate	Range ( $\mu\text{g/ml}$ )	No.(%) of isolate
Resistant	8-128	28(77.8%)	8-256	36(100%)
Intermediate	4	8(22.2%)	-	0(0.0%)
Susceptible	-	0(0.0%)	-	0(0.0%)

Table 4: Phenotypic screening for carbapenemase production in 36 *P. aeruginosa* isolates

Test	Positive	Negative
imipenem-meropenem -EDTA disk synergy test	28 (77.8)	8 (22.2)
modified Hodge test	14 (38.9)	22 (61.1)
EDTA-imipenem microbiological assay	28 (77.8)	8 (22.2)

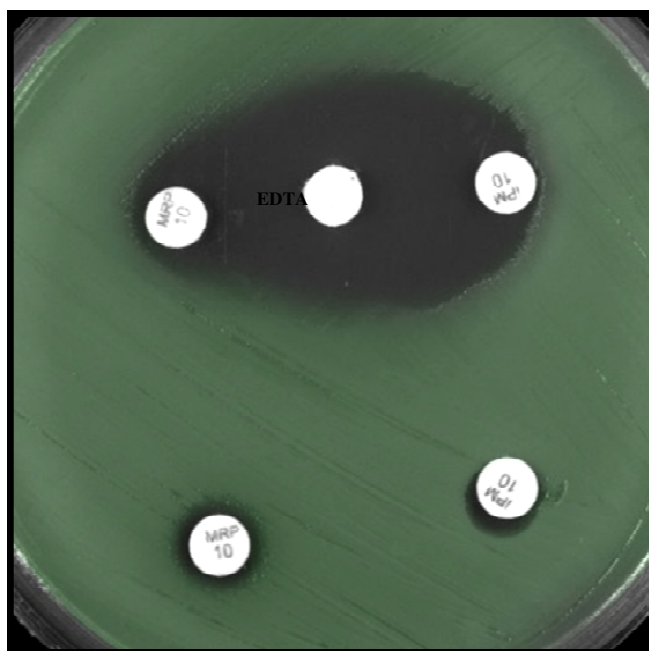


Figure 1: Performances of imipenem-meropenem-EDTA disk synergy test using disks of carbapenems (imipenem, IMP and meropenem, MRP) and disk of EDTA (1.900  $\mu\text{g}$ ). Panel show results for *P. aeruginosa* isolate Pa34. Carbapenems disks and an EDTA disk produced large synergistic inhibition zones for isolate (top). An IMP disk and a MRP disk failed to produce inhibition zones (bottom).

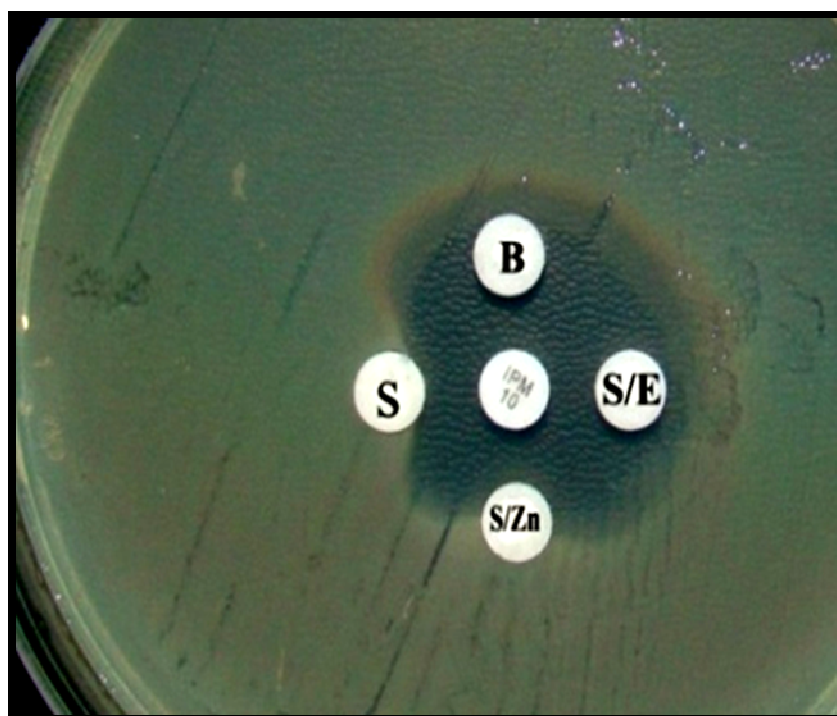


Figure 2: Detection of metallo- $\beta$ -lactamases in *P. aeruginosa* isolate Pa34 by EDTA-imipenem microbiological assay. The presence of enzymes hydrolyzing imipenem (IPM) in bacterial extracts was tested by the growth of the *E. coli* ATCC 25922-indicator strain. The different disks were as follows: S, containing bacterial extract; S/Zn, containing bacterial extract supplemented with ZnSO<sub>4</sub>; S/E, containing bacterial extract supplemented with EDTA; B, containing buffer. The growth of *E. coli* cells around the bacterial extract with and without ZnSO<sub>4</sub> indicated the presence of imipenemase activity. MBLs differentiated from nonmetalloenzymes by indicator strain growth inhibition around a bacterial extract supplemented with EDTA.

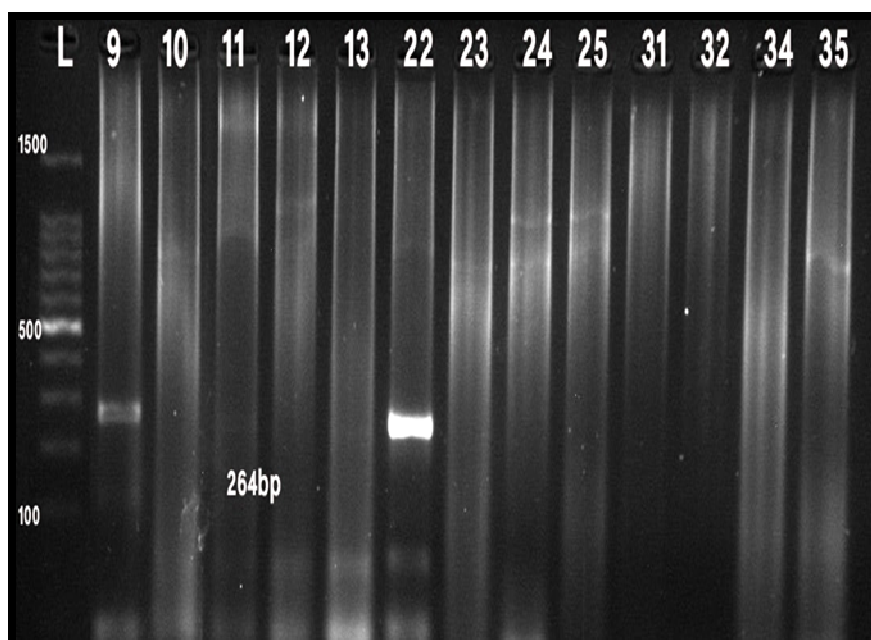


Figure 3: Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of *P. aeruginosa* amplified with *bla*<sub>NDM</sub> gene primers (forward and reverse). The electrophoresis performed at 60 volt for 2 hr. Lane (L), DNA molecular size marker (1500-bp ladder), Lanes (9 and 22) of *P. aeruginosa* isolates show positive results with *bla*<sub>NDM</sub> (264 bp).



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