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Dissemination of Metallo-β-Lactamase Genes in *Pseudomonas aeruginosa* Isolated from Najaf Hospitals

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Abstract :

Carbapenems are therapeutic choice against infections caused by Gram-negative bacilli including strains of *Pseudomonas aeruginosa*. Efflux pumps, porins, PBPs and β-lactamases mediate resistance to these antibiotics. The aim of this study was to determine the possibility of existence of metallo-β-lactamase (MBL) genes among *P. aeruginosa* isolates collected from Najaf hospitals. During the study period from February to October 2011, thirty-six carbapenem resistant *P. aeruginosa* isolates were collected. The susceptibility to different antibiotics was evaluated by disk diffusion method, MICs of imipenem and meropenem were also determined. PCR performed for detection of bla_{IMP}, bla_{VIM}, bla_{SPM} and bla_{NDM} genes. All isolate were multidrug resistance and 21 produced MBLs. The bla_{IMP}, bla_{SPM}, bla_{VIM} and bla_{NDM} genes were detected among 13, 8, 4 and 2 isolates, respectively. The MICs of isolates to imipenem and meropenem were 4-128 µg/mL and 4-256 µg/mL, respectively. We concluded that production of MBL enzymes presents an emerging threat of carbapenem resistance among *P. aeruginosa* in Najaf city. This is the first report of IMP, SPM, VIM, and NDM β-lactamase producing *P. aeruginosa* in Iraq.

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 (1).The clinical strains of *P. aeruginosa* are usually multidrug resistant to aminoglycosides, fluoroquinolones, ureidopenicillins and third generation cephalosporins. In cases of resistance to β-lactams caused by extended spectrum β-lactamases and AmpC enzyme, carbapenems are often used as the last resort against infections. However, carbapenem- hydrolyzing- β-lactamases of molecular class A, B and D have emerged over the last decade (2).

Class B carbapenemases including IMP and VIM termed as MBLs have been found so far in *P. aeruginosa* and these are encoded by different plasmid types (3;4). Recently, new classes of β-lactams including SPM-1, GIM-1, SIM, and AIM have also been reported (5; 6).

The first nosocomial outbreak with carbapenem-resistant *P. aeruginosa* was reported from the Japan in 1988 (7). Since then, carbapenem-resistant *P. aeruginosa* infections and extensive hospital outbreaks have been reported throughout the world (8). Gram negative bacilli producing acquired IMP, VIM, NDM and SPM has been reported more in Asia and South America, respectively (9; 10). Because of the importance of resistance to β-lactam antibiotics among clinical isolates, understanding the principal genetic mechanisms responsible for the gaining and spread of unique β-lactamase-mediated antibiotic resistance mechanism could eventually assist the improvement of effective preclusion and control measures (11).

The aims of this study were to determine the drug susceptibility patterns of *P. aeruginosa* isolated from patients at different Najaf hospitals and to identify the genes encoding existence of MBL genes among *P. aeruginosa* isolates collected from Najaf hospitals.

Materials and Methods

Bacterial isolates and Antibiotic Susceptibility Assays

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 Holly Najaf province included: Al-Sader Medical City, Al-Hakeem General Hospital, Al-Zahra'a Hospital for Childbirth and Children, over a period between February to October 2011. Isolates were obtained from specimens originated from instrument, floor, burn wound, ear and urinary tract. The isolates were identified by conventional biochemical methods and confirmed by API 20E system.

All isolates were also tested in the Kirby Bauer method of disk diffusion to check their susceptibilities to norfloxacin (NX: 5 µg), ciprofloxacin (CIP: 5 µg), amikacin (AN: 30 µg), gentamicin (CN: 10 µg), 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 (12).

Isolates found resistant to carbapenems (imipenem and meropenem) by disk diffusion test were re-checked in broth micro dilution assay (13). In this study, *P. aeruginosa* isolates were defined as resistant to carbapenems if a MIC for imipenem and meropenem were ≥8µg/ml; intermediate resistant, if the MIC were 8 µg/ml; and sensitive, if the MIC were ≤ 4 µg/ml. The *E. coli* ATCC 25922 strain was adopted as the standard for quality control (14).

Phenotypic detection of carbapenemases production:

a. Double-disc synergy test (DDST): screening for MBLs in tested isolates was performed with DDST. In brief, 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 ≥ 17 mm in zone diameter in the presence 1900 µg of EDTA compared to imipenem alone indicated the presence of an MBL(9).

b. Modified Hodge test (MHT): This test was carried out on tested isolates to detect carbapenemase using imipenem and meropenem as described by the CLSI (15).

c. 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*. (16).

Molecular detection of MBL genes

P. aeruginosa DNA extraction was done according to the Pospiech and Neumann method (17). Specific primers and protocols were used to detect bla_{SPM}, bla_{IMP}, bla_{VIM}, bla_{SIM-1} and bla_{NDM-1}(18, , 19, 20).

Results

The isolates resistant to meropenem and /or imipenem 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 Tab.(1). 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 1, resistant to all antibiotics (12 isolates, 33.3%), phenotype 2, susceptible only to imipenem (11 isolates, 30.6%); phenotype 3, 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.

The antipseudomonal potency and spectrum for 18 selected antimicrobial agents of different classes against the isolates are summarized in Fig.(1).

Antibiotic susceptibility pattern revealed that all **the** isolates were resistant to at least three classes of antibiotics Tab.(2).

Imipenem and meropenem MICs of isolates (documented by disk diffusion method) were established according to Clinical and Laboratory Standards Institute criteria (13) by a standard agar dilution method on Mueller-Hinton medium containing antibiotics Tab.(3).

The MIC of imipenem showed a varied range from ≥4 to ≥128 µg/ml. Two isolates (5.6%) showed maximum MICs of 128 µg/ml; one (2.8%) isolate with MIC of 64 µg/ml, while 4(11.1%), 7(19.4%) and 14(38.9%) isolates exhibited MICs of 32µg/ml, 16µg/ml and 8µg/ml, respectively. 8 (22.2%) isolates showed MIC level equal to 4µg/ml. In particular, none of the isolates showed an MIC value ≥256 µg/ml for imipenem. Similarly, MIC of meropenem showed a varied range from ≥8 µg/ml for 34(94.4%) isolates, with a maximum of ≥256 µg/ml for two (5.6%) isolates. Results found that the MIC of meropenem varied slightly; 3(8.3%) isolates it was 8µg/ml, for 1 (2.8%) isolate it was 32 µg/ml, for 2 (5.6%) isolates it was 32 µg/ml, whereas for 6 (16.7%), 13 (36.1%), and 2 (5.6%) isolates they were 64, 128 and ≥256 µg/ml, respectively.

28 isolates (77.8%) were positive according to imipenem-meropenem-EDTA disk synergy test Fig.(2), while no remarkable distinct change was noticed in the remaining 8 (22.2%) isolates Tab. (4).

Only 14 isolates (38.9%) tested positive in modified Hodge test, indicating the production of carbapenemases in these isolates Tab.(5).This test did not give any information on the type of carbapenemase enzyme produced by the isolates tested.

EDTA-imipenem microbiological assay was document 28 (77.8%) bacterial extract with and without $ZnSO_4$ 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, Fig.(3) and Tab.(6).

The MBL genes, was present in a total of 58.3 % (21/36) isolates. The bla_{IMP} (578 bp) was the most frequent MBL gene and documented in 13 (36.1%) of the isolates Fig. (4). A bla_{IMP}-type gene alone or in addition with bla_{SPM} or with bla_{NDM} genes was detected in nine (25.0%), 3 (8.3%), and 1 (2.8%) of the isolates, respectively. 4 (11.1%) isolates carried a bla_{VIM} -type gene Fig.(5), of these, one also harbored bla_{SPM} and bla_{NDM} genes. It was found that 8 (22.2%) of the tested isolates harbored bla_{SPM} gene Fig.(6), 4 of them had a bla_{SPM}-type gene alone and 4 had a bla_{SPM}-type gene in combination with other MBL genes. The PCR amplification of MBL genes confirmed that 2 (5.6%) isolates harbored bla_{NDM} gene Fig.(7), since one isolate was also carried bla_{IMP} gene and other isolate carried bla_{VIM} and bla_{SPM} genes. No PCR products were produced from other isolates. All MBL- producing isolates exhibited high imipenem and meropenem MICs and had a MDR resistant phenotype Tab.(7).

Discussion

P. aeruginosa classically considered as one of the leading Gram-negative pathogen that causes nosocomial infections worldwide (21). It is one of the most important bacterial pathogen seriously contributing to the problem of hospital infections (22), so has received most attention in Najaf hospitals.

In the present study, a high rate of increased resistance among isolates recovered from clinical and hospital environments from three hospitals in Najaf city was documented. 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 β-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 (23;24). The high incidence of MDR, XDR and PDR observed among *P. aeruginosa* isolates underlines the strict consideration in antibiotics use at Najaf hospitals.

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*, but survey data show

emerging resistance in *P. aeruginosa* isolates from samples in the slightly of carbapenem therapy in these hospitals.

The production of MBL is the most common mechanism for carbapenem resistance in *Enterobacteriaceae* and *P. aeruginosa* isolates (25; 26). 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. Lee *et al.* (27) reported that only 66% of the 39 MBL producing isolates of *P. aeruginosa* and *Acinetobacter* spp. gave positive results by the Hodge test. 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 (28; 29).

The resistant isolates were tested by the imipenem-meropenem-EDTA disk synergy test and 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* (7). 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 cannot be routinely performed in all nation laboratories. This report was in concordance with the results of two Indian studies on the detection of MBL by DDST; Uma *et al*. (30) 70% and Gupta *et al*. (31) 75%.

In this study, EDTA-imipenem microbiological assay was used in order to detect metalloenzymes in all isolates with cellular extracts of the studied bacteria. The study revealed that 28 isolates were positive, 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.

PCR analysis confirmed MBLgenes in 21 isolates (58.3%) among which 13 (61.9%) isolates were found to carry the bla_{IMP} gene. This result suggests that the most common carbapenemase-type genes in nation hospitals were bla_{IMP}. This is the first time present study detect IMP enzyme in Najaf province. The emergence of IMP-type enzymes have been described, mostly in Japan, China, Taiwan, Australia, USA, Canada, Brazil and Europe (23; 32). Gene encoding IMP is generally located within integrons, together with those encoding aminoglycoside-modifying enzymes that confer multidrug resistance (33). Furthermore, the integrons harboring IMP determinants are frequently located on plasmids, certainly facilitating their intra- and interspecies spread (34). However, the spread of bla_{IMP} gene to *P. aeruginosa* or may be even to other Gram-negative bacteria in Iraqi hospitals is a cause for concern and warrants continuous surveillance to control the further spread of this resistance mechanism.

The second dominant type of acquired MBL in *P. aeruginosa* was SPM, 8 (22.2%) of the carbapenem-resistant isolates tested were found to be positive in PCR for the presence of bla_{SPM} genes. SPM-1, a previously discovered MBL, was isolated from a juvenile leukemia patient residing in a hospital in São Paulo, Brazil just prior to the patient succumbing to septicemia brought on by *P. aeruginosa* expressing SPM-1 (35). SPM-1 is the predominant MBL in Brazil (36). It has recently been reported in Europe and Iran (37; 38). Present study observed that bla_{SPM} isolates accounted for a considerable number of carbapenem-resistant cases .

Present investigation revealed that bla_{VIM} 4 (11.1%) is the third gene encoding MBL among the isolates of *P. aeruginosa* in this study. This is the first report in Iraq of *P. aeruginosa* isolates producing a VIM-type carbapenemase, confined in Najaf hospitals. However, VIM was first isolated in Verona, Italy, in 1997 with the identification of VIM-2 in France in 1996 subsequently reported (33). The VIM family currently consists of 23 members (39), with occurrences mostly in *P. aeruginosa* within multiple-integrons cassette structures. VIM-2 has the dubious distinction of being the most-reported MBL worldwide (23). Present study found the incidence rate of VIM enzyme in carbapenem-resistant *P. aeruginosa* isolated from Najaf hospitals is undoubtedly high.

Present study report here the first two cases of infections due to NDM-1-producing *P. aeruginosa* in Iraq. To date, only three other cases of colonization or infection by NDM-1-producing *P. aeruginosa* have been reported worldwide, occurring in three patients hospitalized in Belgrade, Serbia and France. All had undergone invasive surgical interventions and none of them had travelled outside of their countries (40; 41). In recent years, many Iraqi patients were travelled to other countries for treatment purpose, which may have helped in obtaining this gene. The gene encoding NDM-1 is located in a very mobile genetic element, resulting in rapid spread across the species and countries (5; 42). However, the *bla*$_{\text{NDM-1}}$ gene positive *Enterobacteriaceae* has spread from India, Pakistan and Bangladesh to UK, US, France (41), Kenya, Japan, Canada, Belgium, the Netherlands, Serbia (43), Taiwan (44), Singapore, Sultanate of Oman (45) and Iraq (46). A substantial number of infected/colonized patients have been part of the so-called "medical tourism."

The emergence of acquired MBLs among *P. aeruginosa* represents an epidemiological risk for at least two reasons. Firstly, MBLs confer resistance not only to carbapenems but to virtually all β-lactams and are frequently associated with resistance to aminoglycosides; and secondly, genes encoding for MBL enzymes are most commonly carried on mobile genetic elements (integrons, plasmids, transposons) that can spread horizontally among unrelated strains. There should be more comprehensive investigations to reach appropriate conclusions about a possible link between MBLs and antibiotics resistance.

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

Isolate symbol	Hospital	Sample source	Piperacillin	Ticarcillin	Carbenicillin	Ticarcillin - clavulanic	Piperacillin - tazobactam	Ceftriaxone	Ceftazidime	Cefotaxime	Cefepime	Aztreonam	Cefoxitin	Imipenem	Meropenem	Gentamicin	Amikacin	Tobramycin	Ciprofloxacin	Norfloxacin	No. of antibiotic resistant
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
Pa6	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	17
Pa7	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18
Pa8	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	17
Pa9	Zh *	IN **	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	17
Pa10	Sh	FL **	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	17
Pa11	Zh	FL	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18
Pa12	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	17
Pa13	Sh	Ea **	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S	R	R	R	16
Pa14	Sh	IN	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	17
Pa15	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18
Pa16	Sh	FL	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S	R	R	R	16
Pa17	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S	R	R	R	16
Pa1	Sh	Ea	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18

8																					
Pa19	H*	UR**	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	17
Pa20	Sh	UR	S	R	R	R	S	R	S	R	R	R	R	S	R	R	S	R	S	R	12
Pa21	Hh	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
Pa23	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18
Pa24	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18
Pa25	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18
Pa26	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S	R	R	R	16
Pa27	Hh	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
Pa30	Hh	UR	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18
Pa31	Sh	UR	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	17
Pa32	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18
Pa33	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18
Pa34	Sh	FL	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	18
Pa35	Zh	FL	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	17
Pa36	Sh	BW	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S	R	R	R	16
Total resistant (%)			34 (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 **BW, Burin wound; Ur, Urine; Ea, Ear; IN, Instrument; FL, Floor.

Percentage of resistant isolates

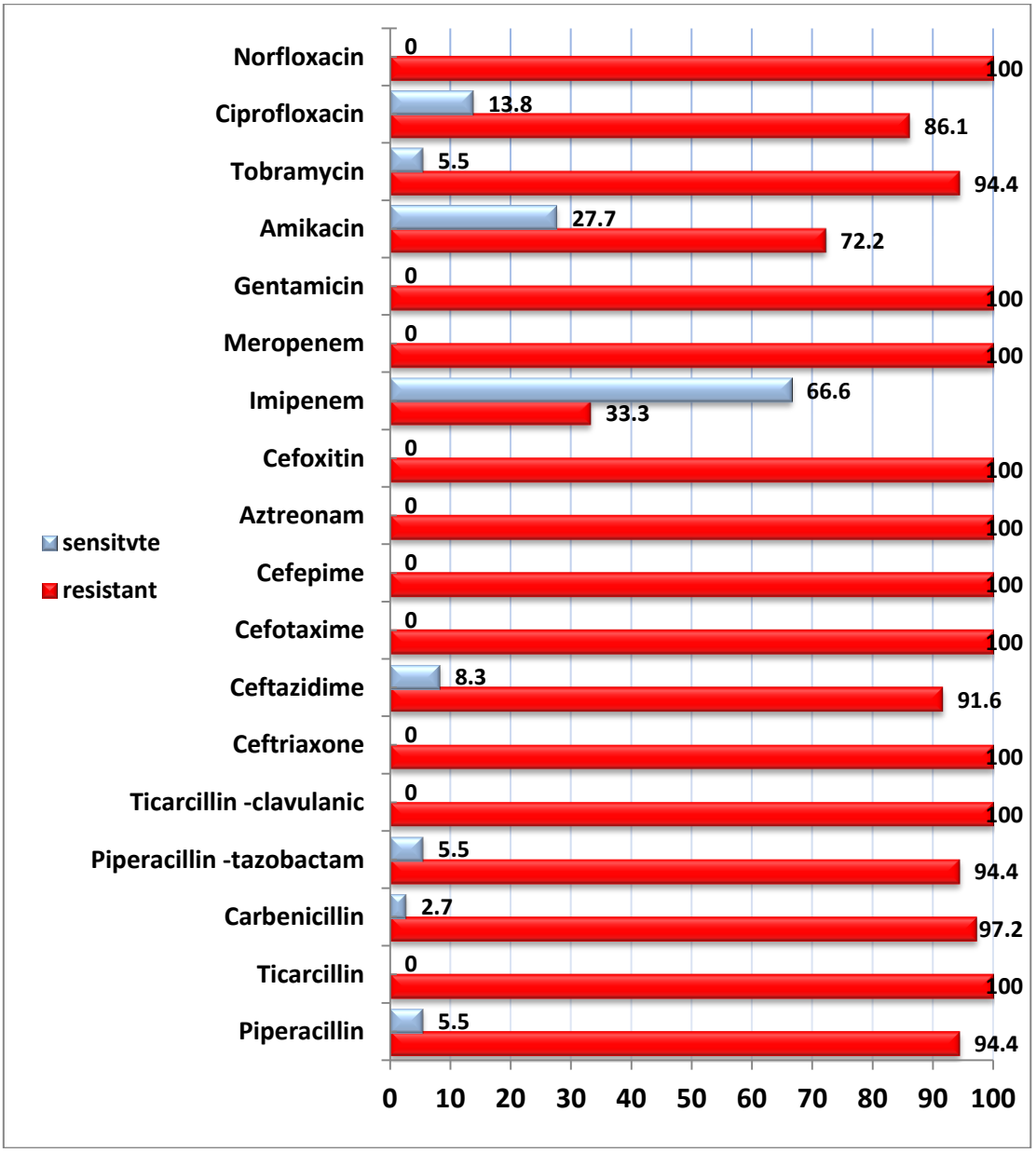

Figure (1): Antibiotic susceptibility patterns of carbapenem-resistant *P. aeruginosa* isolates (n= 36). (L.S.D. 0.05 = 2.11)

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 (µg/ml) and susceptibility for carbapenems according to United States (CLSI, 2012)

Isolate	MIC_{IMP}	MIC_{MRP}	Susceptibility according to CLSI ≥ 8		Disk diffusion method IMP	Disk diffusion method MRP
			IMP	MRP		
Pa1	8	32	R	R	S	R
Pa2	16	32	R	R	S	R
Pa3	8	32	R	R	S	R
Pa4	8	32	R	R	S	R
Pa5	4	8	I	R	S	R
Pa6	8	64	R	R	S	R
Pa7	16	32	R	R	R	R
Pa8	4	8	I	R	S	R
Pa9	8	32	R	R	S	R
Pa10	32	64	R	R	S	R
Pa11	16	128	R	R	R	R
Pa12	32	128	R	R	S	R
Pa13	8	64	R	R	S	R
Pa14	8	64	R	R	S	R
Pa15	8	64	R	R	R	R
Pa16	8	32	R	R	S	R
Pa17	8	32	R	R	S	R
Pa18	32	128	R	R	R	R
Pa19	4	8	I	R	S	R
Pa20	16	32	R	R	S	R
Pa21	8	128	R	R	S	R
Pa22	32	128	R	R	S	R
Pa23	16	32	R	R	R	R
Pa24	32	128	R	R	R	R
Pa25	64	128	R	R	R	R
Pa26	4	16	I	R	S	R
Pa27	8	64	R	R	S	R
Pa28	128	256	R	R	S	R
Pa29	4	128	I	R	R	R
Pa30	16	128	R	R	R	R
Pa31	8	128	R	R	S	R
Pa32	8	128	R	R	R	R
Pa33	4	128	I	R	R	R
Pa34	128	256	R	R	R	R
Pa35	4	128	I	R	S	R
Pa36	4	32	I	R	S	R
Resistant			28(77.8%)	36(100%)	12(33.3%)	36(100%)
Intermediate			8(22.2%)	0(0.0%)	0	0
Susceptible			0(0.0%)	0(0.0%)	24(66.7%)	0 (0.0%)
L.S.D.(0.05)			6.26		9.23	

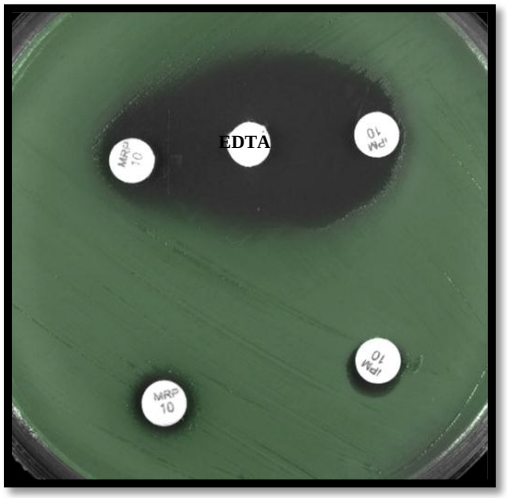

Figure (4): Performances of imipenem-meropenem-EDTA disk synergy test using disks of carbapenems (imipenem, IMP and meropenem, MRP) and disk of EDTA (1.900 µg). Panel show results for *P. aeruginosa* isolate Pa34. Carbapenems disks and an EDTA disk produced large synergistic inhibition zones for isolate (top). An IMP disk and a MRP disk failed to produce inhibition zones (bottom).

Table (5): Results of imipenem-meropenem-EDTA disk synergy test for detection of metallo-β-lactamases in 36 carbapenem-resistant *P. aeruginosa* isolates

Phenotypic result	No. (%) of isolates	Isolate symbol
Positive	28 (77.8)	Pa1, Pa2, Pa3, Pa4, Pa6, Pa7, Pa9, Pa10, Pa11, Pa12, Pa13, Pa14, Pa15, Pa17, Pa18, Pa20, Pa22, Pa23, Pa24, Pa25, Pa27, Pa28, Pa29, Pa30, Pa31, Pa32, Pa34, Pa35
Negative	8 (22.2)	Pa5, Pa8, Pa16, Pa19,Pa21, Pa26, Pa33, Pa36

Table (6): Results of modified Hodge test for detection of carbapenemase in 36 carbapenem-resistant *P. aeruginosa* isolates

Phenotypic result	No. (%) of isolates	Isolate symbol
Positive	14 (38.9)	Pa2, Pa11, Pa12, Pa13, Pa15, Pa16, Pa18, Pa24, Pa25, Pa29, Pa30, Pa31, Pa34, Pa35
Negative	22 (61.1)	Pa1, Pa3, Pa4, Pa5, Pa6, Pa7, Pa8, Pa9, Pa10, Pa14, Pa17, Pa19, Pa20, Pa21, Pa22, Pa23, Pa26, Pa27, Pa28, Pa32, Pa33, Pa36

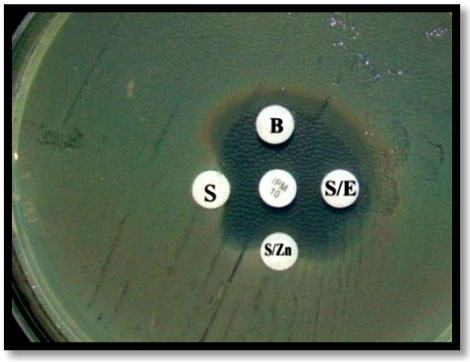

Figure (5): Detection of metallo-β-lactamases in *P. aeruginosa* isolate Pa34 by EDTA-imipenem microbiological assay. The presence of enzymes hydrolyzing imipenem (IPM) in bacterial extracts was tested by the growth of the *E. coli* ATCC 25922-indicator strain. The different disks were as follows: S, containing bacterial extract; S/Zn, containing bacterial extract supplemented with $ZnSO_4$; S/E, containing bacterial extract supplemented with EDTA; B, containing buffer. The growth of *E. coli* cells around the bacterial extract with and without $ZnSO_4$ indicated the presence of imipenemase activity. MBLs differentiated from nonmetalloenzymes by indicator strain growth inhibition around a bacterial extract supplemented with EDTA.

Table (7): Results of EDTA-imipenem microbiological assay for detection of metallo-β-lactamases in 36 carbapenem-resistant *P. aeruginosa* isolates

Phenotypic result	No. (%) of isolates	Isolate symbol
Positive	28 (77.8)	Pa1, Pa2, Pa3, Pa4, Pa6, Pa7, Pa9, Pa10, Pa11, Pa12, Pa13, Pa14, Pa15, Pa17, Pa18, Pa20, Pa22, Pa23, Pa24, Pa25, Pa27, Pa28, Pa29, Pa30, Pa31, Pa32, Pa34, Pa35
Negative	8 (22.2)	Pa5, Pa8, Pa16, Pa19, Pa21, Pa26, Pa33, Pa36

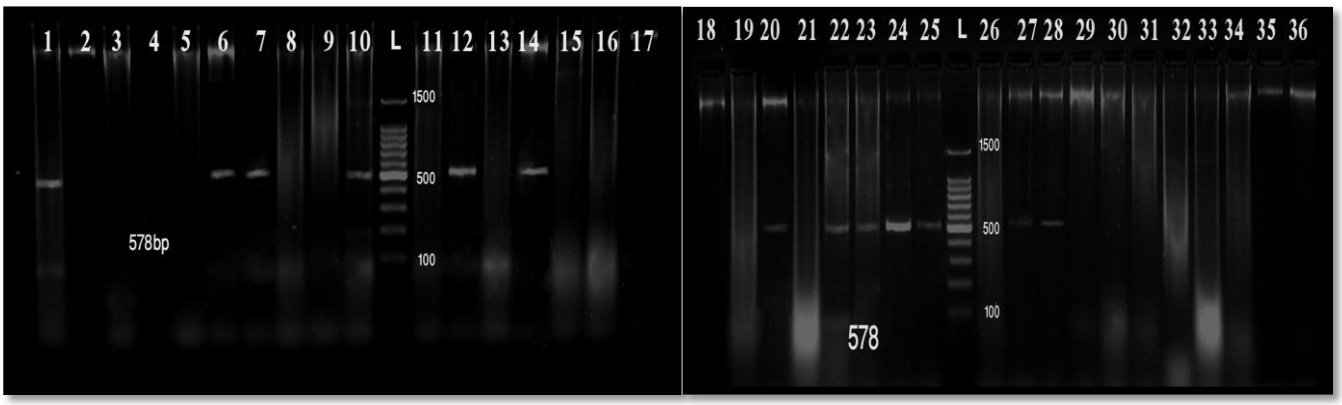

Figure (6 a and b): Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of *P. aeruginosa* amplified with bla_{IMP} gene primers (forward and reverse). The electrophoresis performed at 60 volt for 2 hr. Lane (L), DNA molecular size marker (l500-bp ladder). Lanes (1, 6, 7, 10, 12, 14, 20, 22, 23, 24, 25, 27 and 28) of *P. aeruginosa* isolates show positive results with bla_{IMP} (578 bp).

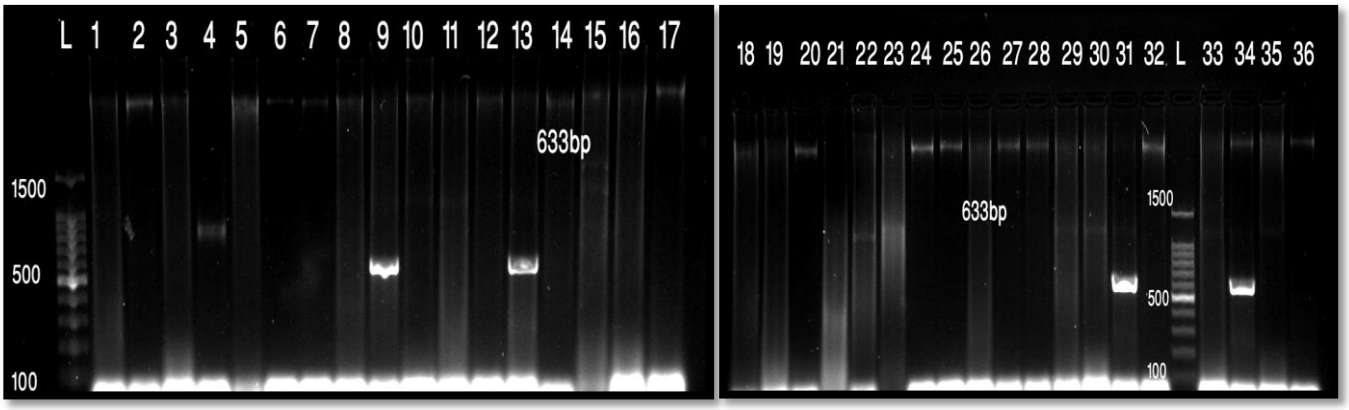

Figure (7a and b): Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of *P. aeruginosa* amplified with bla_{VIM} gene primers (forward and reverse). The electrophoresis performed at 60 volt for 2 hr. Lane (L), DNA molecular size marker (l500-bp ladder), Lanes (9, 13, 31 and 34) of *P. aeruginosa* isolates show positive results with bla_{VIM}(633 bp).

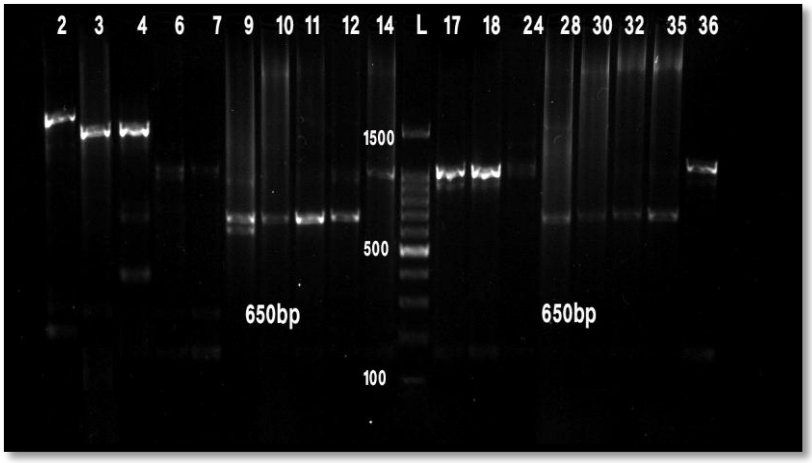

Figure (8): Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of *P. aeruginosa* amplified with bla_{SPM} gene primers (forward and reverse). The electrophoresis performed at 60 volt for 2 hr. Lane (L), DNA molecular size marker (l500-bp ladder), Lanes (9, 10, 11, 12, 28, 30, 32 and 35) of *P. aeruginosa* isolates show positive results with bla_{SPM}(650 bp).

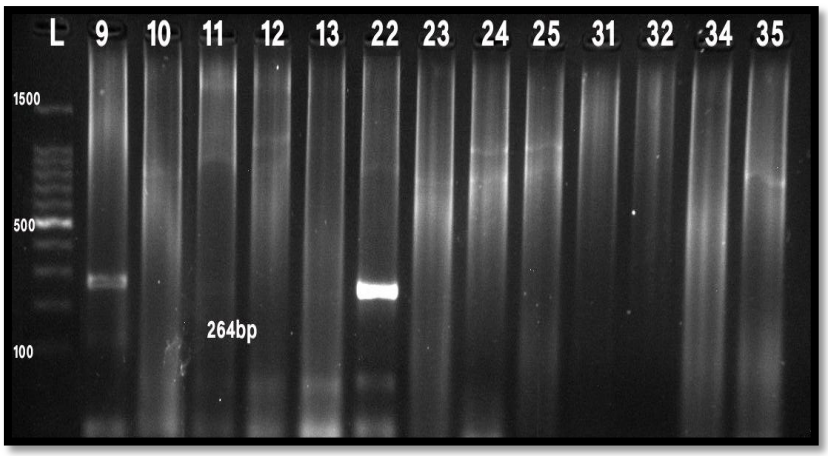

Figure (9): Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of *P. aeruginosa* amplified with bla_{NDM} gene primers (forward and reverse). The electrophoresis

performed at 60 volt for 2 hr. Lane (L), DNA molecular size marker (l500-bp ladder), Lanes (9 and 22) of *P. aeruginosa* isolates show positive results with bla_{NDM} (264 bp).

Table (8): Distribution of various MBL genes and their combinations in 36 carbapenem-resistant isolates

Occurrence of MBL genes	No.(%) of isolates	Isolate code No.	Imipenem MIC range µg/ml	Meropenem MIC range µg/ml
bla_{IMP}	9 (25.0%)	Pa1, Pa6, Pa7, Pa14, Pa20, Pa23, Pa24, Pa25, Pa27	8-64	32-128
bla_{SPM}	4 (11.1%)	Pa11, Pa30, Pa32, Pa35	4-32	128
bla_{IMP}+ bla_{SPM}	3 (8.3%)	Pa10, Pa12, Pa28	32-128	64-256
bla_{VIM}	3 (8.3%)	Pa13, Pa31, Pa34	8-128	64-256
bla_{IMP} + bla_{NDM}	1 (2.8%)	Pa22	32	128
bla_{VIM} + bla_{SPM} + bla_{NDM}	1 (2.8%)	Pa9	8	32
No MBLs genes positive isolate	15 (41.7%)	Pa2, Pa3, Pa4, Pa5, Pa8, Pa15, Pa16, Pa17, Pa18, Pa19, Pa21, Pa26, Pa29, Pa33, Pa36	4-32	8-128

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