Molecular and Bacteriological Detection of Multi-drug resistant and Metallo- β -Lactamase Producer *Acinetobacter baumannii* in Ramadi City, West of Iraq

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Abstract

Background: One leading factor responsible for resistance in *Acinetobacter baumannii*, is the production of carbapenamases like metallo-β-lactamases (MBLs), which hydrolyze a variety of β-lactams including penicillin, cephalosporins and carbapenems. This study aims to evaluate phenotypic method against genotypic, PCR as gold standard test among carbapenem resistant *A. baumannii* for identifying MBL producers.
Patients and Methods: One hundred and eighty-eight of 213 patients were culture positive (88.26%). Forty-four *Acinetobacter baumannii* clinical isolates were chosen for this study. Phenotypic expression of MBL was detected by IPM-EDTA-disk synergy test and presence of $bla_{\mathbf{IMP\text{-}1}}$ and $bla_{\mathbf{IMP}}$ was detected by PCR for all metallo- β -lactamase producing *Acinetobacter baumannii*.
Results: Forty-one (93.2%) isolates of *Acinetobacter baumannii* (out of 44 isolates) were found to be MBL producers by IPM-EDTA-disk synergy test. Thirty-five (80%) *Acinetobacter baumannii* of 44 presumptive MBL producer isolates (with isolates were negative for MBL producer in phenotypic method used as control) were positive for *bla* $_{\mathbf{IMP\text{-}1}}$ gene by PCR, while twelve (27.3%) *Acinetobacter baumannii* out of 44 presumptive MBL producer isolates (with isolates were negative for MBL producer in phenotypic method used as control) were positive for $bla_{\mathbf{IMP}}$ gene by PCR. The coexistence of $bla_{\mathbf{IMP\text{-}1}}$ with $bla_{\mathbf{IMP}}$ genes in present study 25% (11/44) of cases.
Conclusion: Most isolates of *Acinetobacter baumannii* were found to be metallo beta-lactamase (MBL) producers using IPM-EDTA-disk synergy test. Further, isolates of *A. baumannii* have been produced MBL gene (*bla* $_{\mathbf{IMP\text{-}1}}$). It seems to be the major mechanism of resistance among Iraqi nosocomial isolates of *A. baumannii*.
Key words: **Multidrug-resistant, *Acinetobacter baumannii*, Metallo- β –lactamase (MBL), Imipenem**

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Introduction:

Acinetobacter baumannii is a glucose-non-fermentative, Gram-negative coccobacillus that has emerged in recent years as a leading cause of nosocomial infections associated with elevated morbidity and mortality[1].

An opportunistic pathogen, *A. baumannii* has a high incidence among immunocompromised individuals, particularly those who have long duration in hospital of stay (>90 days). In recent years, it has been designated as a "red alert" human pathogen, generating alarm among the medical fraternity arising

largely from its extensive antibiotic resistance spectrum [2].

More recently, *A. baumannii* has become a major cause for concern in conflict zones, and has gained particular notoriety in the resent desert conflicts in Iraq, earning it the moniker "Iraqibacter." In particular, high incidences of multidrug-resistant (MDR) bacteremia have been noted among US Army service members following military operation in Iraq [3].

The most common and serious MDR pathogens have been encompassed within the acronym "ESKAPE," standing *for Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter spp*. According to CDC (Centre for Disease Control) the six ESKAPE bacteria cause two third of all hospital acquired infections [2, 4].

It is well established that Metallo-β-lactamases (MBLs) are metallo enzymes of Ambler class B and are resistant to clavulanic acid. They require zinc as co-factor for enzymatic activity and their activity is inhibited by ethylene diamine tetra acetic acid (EDTA) and other metal ion chelating agents. *Pseudomonas* spp. and *Acinetobacter* spp. are the most important nosocomial pathogens with multiple drug resistance [5].

Carbapenems are considered the last-line drugs for treatment of infections caused by multiresistant Gram-negative bacilli [6]. Recently, the emergence of carbapenem-resistant organisms such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* has become a major therapeutic challenge. Carbapenem resistance due to acquired metallo-β-lactamases (MBLs) is considered to be more serious than other resistance mechanisms because MBLs can hydrolyze all β-lactam antibiotics except monobactams. Furthermore, the MBL-encoding genes located on integrons can be disseminated easily between strains [7].

Till now, five main types of MBLs have been described throughout the World – IMP "Imipenem hydrolyzing β-lactamase" VIM "Verona integron-encoded metallo-β-lactamases", GIM "German Imipenemase", SPM "Sao Paulo metallo-β-lactamases", and SIM "Seoul imipenemase" enzymes. A new MBL has been recently reported in *Pseudomonas aeruginosa* from Australia – $bla_{\text{AIM-1}}$. There are no standard guidelines by CLSI for detection of these enzymes in various bacteria [8].

Up to my simple knowledge, this is the first study in the identification of the genes (bla_{IMP} and bla_{IMP-1} genes) responsible for carbapenem-resistant by *Acinetobacter baumannii* in Iraq and mostly Arab world**.**

To date, a few reports have described MBLs in Iraq. The aim of this study was to investigate the presence of metallo-beta-lactamase production among beta-lactam resistant *Acinetobacter baumannii* and to compare results gathered from phenotypic and genotypic methods. Also, to extract DNA and quantify from study isolates. Further, for molecular analysis of target genes (bla_{IMP} and $bla_{\text{IMP-1}}$ genes) encoding for metallo-beta-lactamase with specific primers by polymerase chain reaction.

Patients and Methods

Collection of samples

Swabs were taken from different anatomical sites (eg. bone, Joints, connective tissues) of Two Hundred and Thirteen patients with wound infections (diabetic foot infections, osteomyelitis, burn infection, septic arthritis.. etc). The swabs were taken during the period from April, 2011 to June, 2012 from patients admitted to Ramadi Teaching Hospital.

All isolates were bacteriologically identified using conventional and VITEK® 2 system according to criteria mentioned by bioMérieux [9].

Phenotypic Detection of MBLs (IPM-EDTA-disk synergy test)

EDTA-imipenem disks were prepared by adding EDTA solution to 10µg-imipenem disks to obtain a concentration of 750 µg. The disks were dried immediately in an incubator and stored at 4°C in an air tight vial without desiccant. Test study bacterial isolates were adjusted according to McFarland 0.5 turbidity standard and were inoculated to Mueller Hinton agar. A 10-µg-imipenem disk and an imipenem plus 750 µg EDTA were placed on Mueller Hinton agar. Another disk containing only 750 µg EDTA was also placed as a control. After overnight incubation, the established zone diameter difference of ≥ 7 mm between imipenem disk and imipenem plus EDTA was interpreted as EDTA synergy positive [4, 10, and 11].

Detection of MBL Genes

I- *bla*$_{IMP-1}$ gene

Detection of the *bla* $_{IMP-1}$ gene was carried out using primers as described by [12, 13]. *bla* $_{IMP-1}$ primers: *bla* $_{IMP-1}$-F (5′-CATGG TTTGG TGGTT CTTGT-3′) *bla* $_{IMP-1}$-R(5′-ATAATTTGGCGGACTTTGGC-3′).The PCR conditions included:

In a PCR-pre mix tube master mix was prepared as mentioned in the following table:

Table -1 The original PCR reagents and final concentrations used in procedure

Component		concentration	Volume
PCR PreMix master mix tube contain			
a	Top DNA polymerse	1U	
b	Each dNTP	250 µM	
c	Tris–HCL (pH9)	10 µM	
d	KCl	30 µM	
e	$MgCl_2$	1.5 µM	
f	stabilizer and tracking dye		
DNA template sample			2 µl
forward primer		5pmol/µl	4 µl
revers primer		5pmol/µl	4 µl
sterile distilled water			10 µl
Final volume			20 µl

After that, they were mixed well by vortex. All tubes were centrifuged for 30 second at 10000G according to manufacture Company. In a PCR-pre mix tube, master mix was prepared as in table-1; then mixed well by vortex. All tubes were transferred into thermal cycler. The PCR was started as in the following program. Initial denaturation for 4 minute at 94°C. Twenty five cycles of:

A-denaturation at 94°C for 30 second,
B-annealing at 56°C for 30 second, and
C- extension at 72°C for 45 second.

Final extension at 72°C for 7 minutes. Hold temperature at 4 °C for 10 minutes.

II- *bla*$_{IMP}$ gene

Detection of the *bla* $_{IMP-1}$ gene was carried out using primers as described by [14, 15]. *bla* $_{IMP}$ primers: *bla* $_{IMP}$-F (5'-CGGCCG TCAG GAG ACG GTCTTT-3') *bla* $_{IMP}$-R(5'-AAC CA G TTT TGC CT TTAC CTAT-3'). The PCR conditions included:

In a PCR-pre mix tube, master mix was prepared as in Table-1; then mixed well by vortex. All tubes were centrifuged for 30 second at 10000G according to manufacture Company. Two drops from mineral oil were added to the above component. All tubes were transferred into thermal cycler. The PCR was started as in the following program; initial denaturation for 3 minute at 93°C. Forty cycles of,

A- Denaturation at 93°C for 1 minute.
B- Annealing at 55°C for 1 minute.
C- Extension at 72°C for 1 minute.

Final extension at 72°C for 7 minutes. Hold temperature at 4 °C for 10 minutes.

The PCR product of 620 bp for *bla*$_{IMP-1}$ and 587 bp for *bla* $_{IMP}$ was visualised by 2% agarose gel electrophoresis with Novel Juice [16].

Results:

Forty-one (93.2%) isolates of *Acinetobacter baumannii* (out of 44 isolates) were found to be MBL producers by IPM-EDTA-disk synergy test (positive) (Fig-1 & Fig-2). The established zone diameter difference of ≥ 7 mm between imipenem disk and imipenem plus EDTA was interpreted as EDTA synergy positive (the presence of an enlarged zone of inhibition was interpreted as EDTA-synergy test positive). Three isolates were negative for MBL producers by IPM-EDTA-disk synergy test.

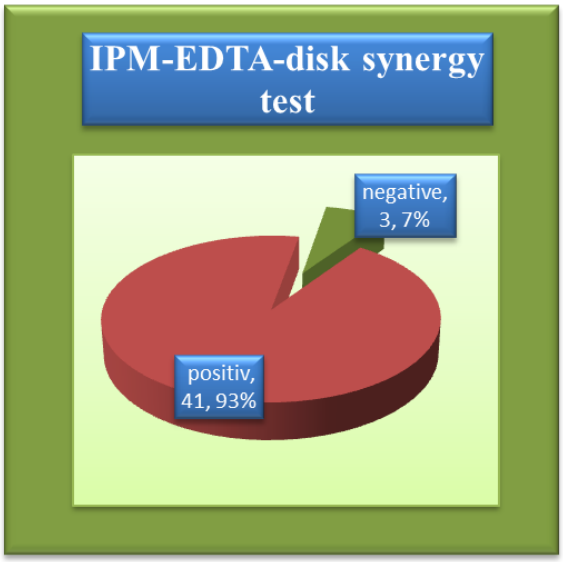
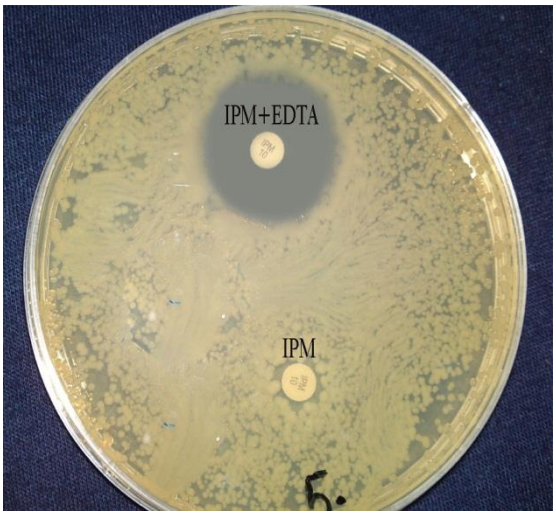
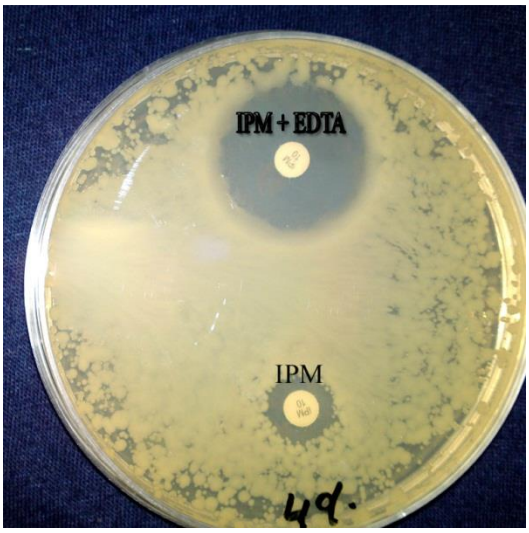


Figure-1 Result of IPM-EDTA-disk synergy test for metallo beta-lactamase production for *Acinetobacter baumannii* .





Isolate No.(5) **Isolate No.(49)**

Figure-2 Positive result for IPM-EDTA-disk synergy test for metallo beta-lactamase production (mm) by *Acinetobacter baumannii*

Thirty-five (80%) *Acinetobacter baumannii* of 44 presumptive MBL producer isolates (with isolates were negative for MBL producer in phenotypic method used as control) were positive for *bla* $_{\text{IMP-1}}$ gene by PCR (Fig-3, 4, 5, 6, 7 & 8). No *bla* $_{\text{IMP-1}}$ genes were found in isolates negative by the phenotypic test (IPM-EDTA disk synergy test).

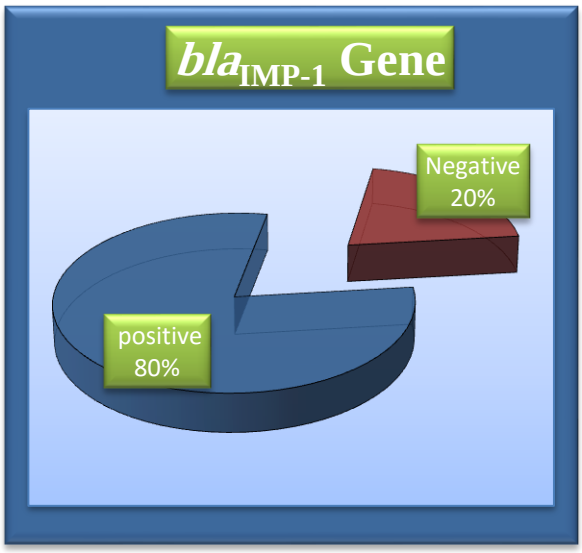


Figure-4.3 Distribution of *bla* $_{\text{IMP-1}}$ gene in *Acinetobacter baumannii* isolates

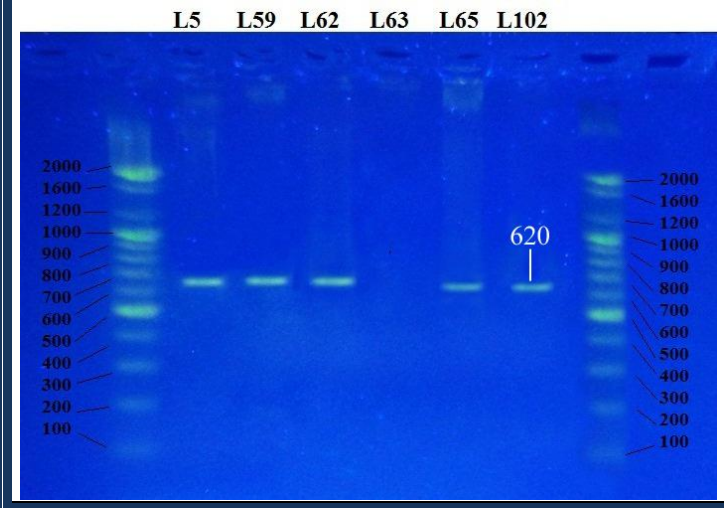


Figure-4 Agarose gel electrophoresis (2%) with Novel Juice dye, bands with *bla*$_{IMP-1}$ gene obtained from *Acinetobacter baumannii* isolates, which showed that positive results are represented in (L5,L59,L62,L65,L102), while (L63) was negative result. DNA ladder with (100-2000 bp) on the left and right were used as DNA molecular weight marker.

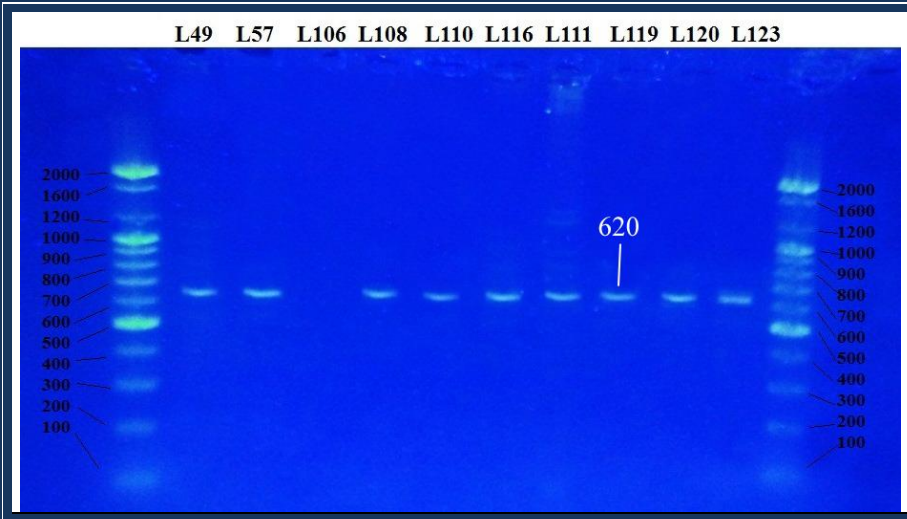


Figure-5 Agarose gel electrophoresis (2%) with Novel Juice dye, bands with *bla*$_{IMP-1}$ gene obtained from *Acinetobacter baumannii* isolates, which showed that positive results are represented in (L49,L57,L108-L123), while (L106) was negative result. DNA ladder with (100-2000 bp) on the left and right were used as DNA molecular weight marker.

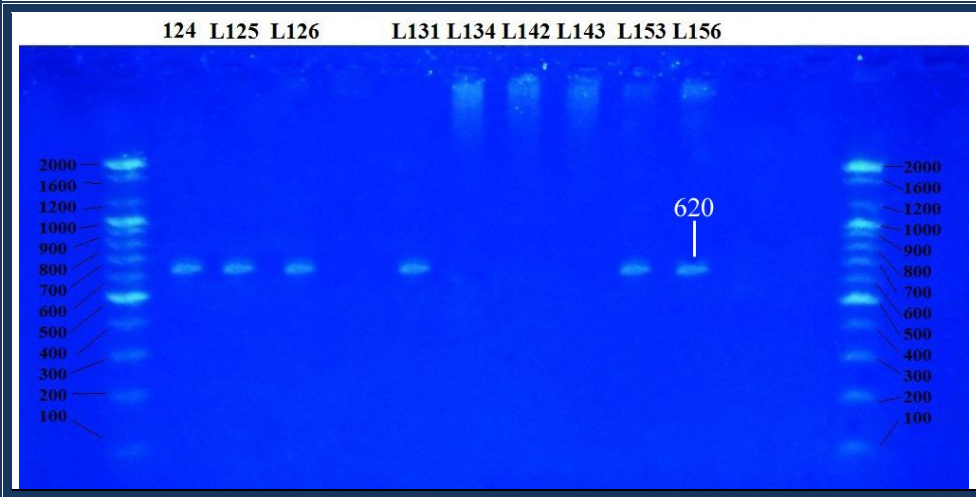


Figure-6 Agarose gel electrophoresis (2%) with Novel Juice dye, bands with *bla*$_{IMP-1}$ gene obtained from *Acinetobacter baumannii* isolates, which showed that positive results are represented in (L24,L25,L126,L131L153,L156), while (L134,L142,L143) were negative results. DNA ladder with (100-2000 bp) on the left and right were used as DNA molecular weight marker.

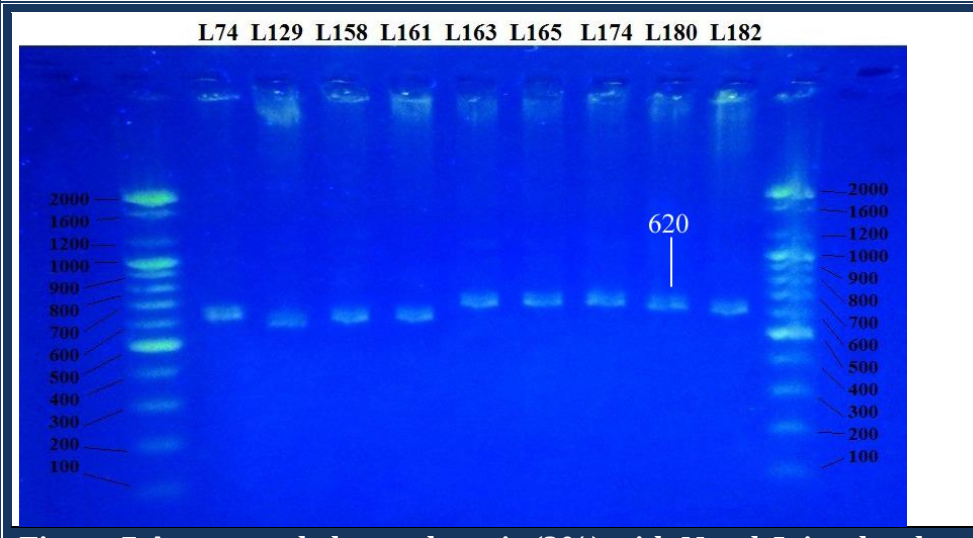


Figure-7 Agarose gel electrophoresis (2%) with Novel Juice dye, bands with *bla*$_{IMP-1}$ gene obtained from *Acinetobacter baumannii* isolates, all isolates were positive results are represented in (L74-L182). DNA ladder with (100-2000 bp) on the left and right were used as DNA molecular weight marker.

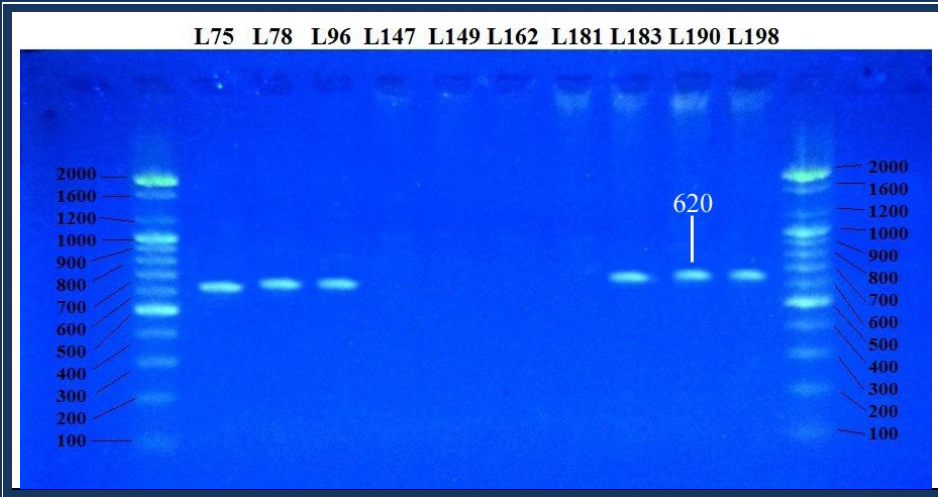


Figure-8 Agarose gel electrophoresis (2%) with Novel Juice dye, bands with *bla*$_{IMP-1}$ gene obtained from *Acinetobacter baumannii* isolates, which showed that positive results are represented in (L75-L96,L57,L183-L198), while (L147-L162) were negative results. DNA ladder with (100-2000 bp) on the left and right were used as DNA molecular weight marker.

Twelve (27.3%) *Acinetobacter baumannii* out of 44 presumptive MBL producer isolates (with isolates were negative for MBL producer in phenotypic method used as control) were positive for *bla* $_{IMP}$ gene by PCR (Fig-10, 11, 12, & 13). No *bla* $_{IMP}$ genes were found in isolates negative by the phenotypic test (IPM-EDTA disk synergy test).

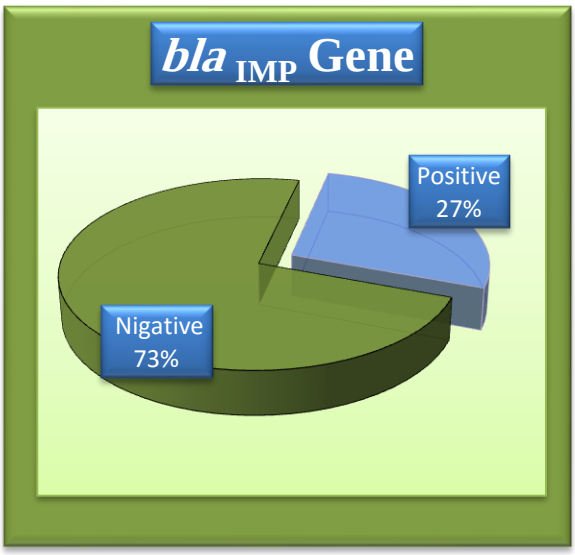


Figure-10 Distribution of *bla* $_{IMP}$ gene in *Acinetobacter baumannii* isolates

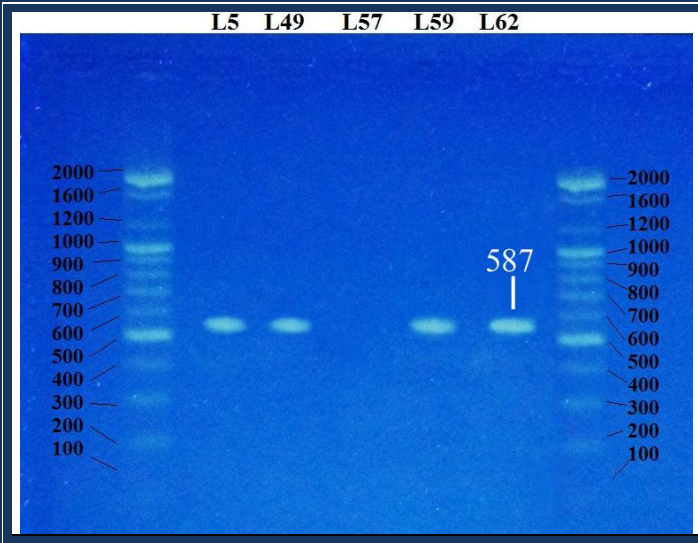


Figure-11 Agarose gel electrophoresis (2%) with Novel Juice dye, bands with *bla*$_{IMP}$ gene obtained from *Acinetobacter baumannii* isolates, which showed that positive results are represented in (L5,L49,L59,L62), while (L57) was negative result. DNA ladder with (100-2000 bp) on the left and right were used as DNA molecular weight marker.

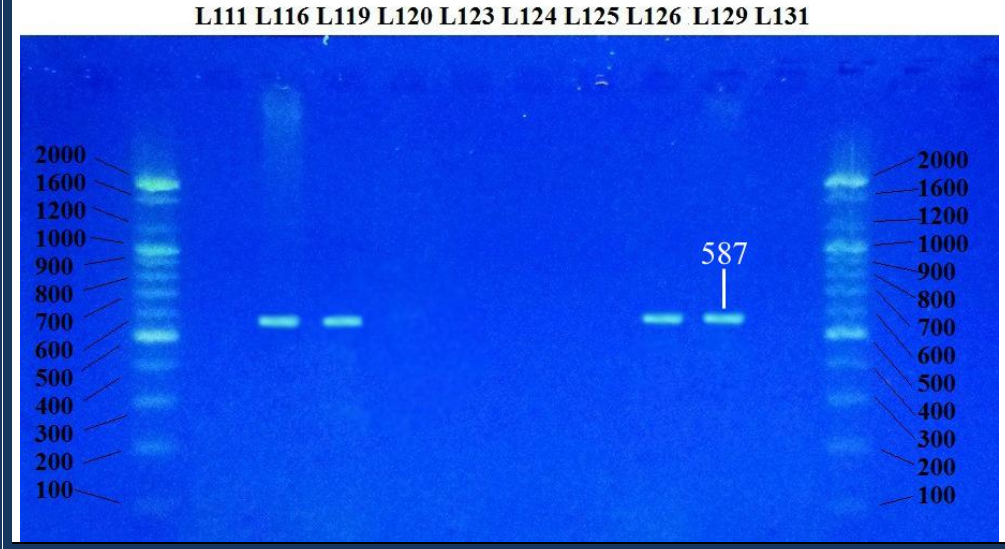


Figure-12 Agarose gel electrophoresis (2%) with Novel Juice dye, bands with *bla*$_{IMP}$ gene obtained from *Acinetobacter baumannii* isolates, which showed that positive results are represented in (L١١٦,L119,L١26,L129), while (L111,L120-L125,L131) were negative results. DNA ladder with (100-2000 bp) on the left and right were used as DNA molecular weight marker.

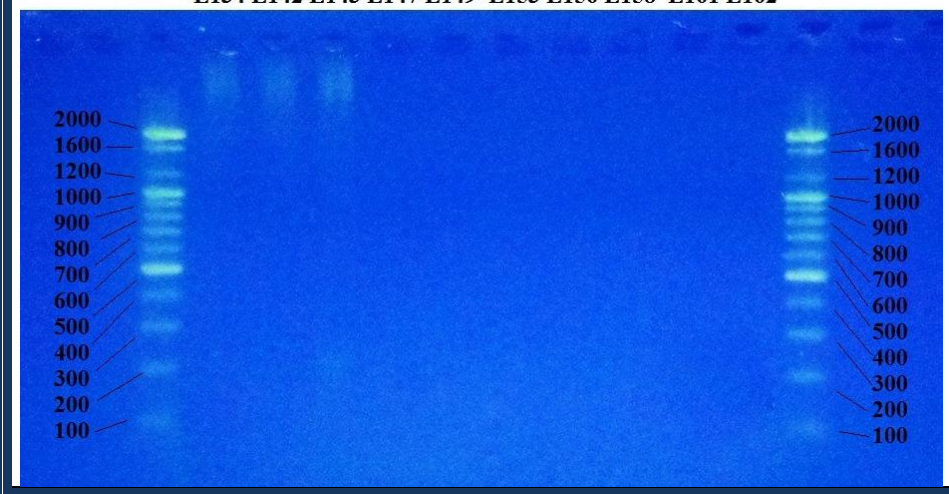


Figure-13 Agarose gel electrophoresis (2%) with Novel Juice dye, bands with *bla*$_{IMP}$ gene obtained from *Acinetobacter baumannii* isolates, all isolates were negative results are represented in (L134-L162). DNA ladder with (100-2000 bp) on the left and right were used as DNA molecular weight marker.

Discussion

Several phenotypic methods are available for detection of MBL-producing isolates, but Clinical and Laboratory Standards Institute (CLSI) has not recommended any standardized protocol for screening of MBLs. Currently, the method using a disc with Imipenem plus 750 µg of EDTA (combined disc method) is simple to perform and highly sensitive in differentiating MBL-producing isolates [7, 11].

Forty-one (93.2%) isolates of *Acinetobacter baumannii* (out of 44 isolates) were found to be MBL producers by IPM-EDTA-disk synergy test (positive) (Fig-1 & Fig-2). Lee *et al*., 2001[10] and Yong *et al*., 2002[11] were concluded that.

Forty-one (93.2%) isolates of *Acinetobacter baumannii* displayed unusually high-level of imipenem resistance.

In the Middle East, the occurrence of imipenem-resistant *A. baumannii* is alarmingly recognized.

In Saudi Arabia, the susceptibility rate of *A. baumannii* isolated from a tertiary care hospital to imipenem was reported to be as low as 10% [17]. In Bahrain, 58% of 454 *A. baumannii* isolated from a 1000-bed tertiary care centre were resistant to imipenem [18]. In United Arab Emirates and Qatar, 100% resistance to imipenem was observed in the tested *A. baumannii* isolates [19, 20, and 21]. The extensive use of carbapenems in this part of the world has likely created a selective antibiotic pressure which in turn has resulted in an increased prevalence of carbapenem-resistant *A. baumannii*.

PCR assay was carried out by using previously published primers for amplification of genes encoding carbapenemases (*bla*$_{IMP}$ and *bla*$_{IMP-1}$ genes).

Up to my simple knowledge, this is the first study of its kind in country and Arab world in the identification of the genes (*bla*$_{IMP}$ and *bla*$_{IMP-1}$ genes) responsible for carbapenem-resistant by *A. baumannii*.

MBLs are less commonly identified in *A. baumannii* than the OXA-type carbapenemases but their carbapenem-hydrolysing activities are 100–1000-fold more potent. Their presence in MDR *A. baumannii* isolates is in some instances difficult to detect , indicating that their contribution to the carbapenem resistance may be underestimated [1].

The high percentage for *bla* $_{IMP1}$ gene in present study (٨٠%), confirmed by many studies in different percentages, in a surveillance study in 2003-2004, MBLs were detected in 135 of 545 (24.8%) imipenem-resistant *Acinetobacter spp*. isolates, the proportion of IMP-1 was 61% [22]. A study in 2006 showed that, among 31 carbapenem-resistant *Acinetobacter spp.*, IMP-1 was detected in 15 (48.4%) isolates [23]. In another study performed by [24] in Brazilian Teaching Hospital; more than half of the isolates (55%) had a positive *bla*$_{IMP-1}$, In the same study, the proportion of IMP-1–producing *Acinetobacter* isolates among carbapenem-resistant strains increased from 0% in the 1993-1997 period to 29% in 1998 and 100% in the 1999-2001 period.

The high percentage for IMP-1–producing strains of *Acinetobacter baumannii* in present study (80%), indicating that this important mechanism of antimicrobial resistance was disseminated among distinct clones. A major contributing factor in the emergence of multidrug-resistant strains of *Acinetobacter* is the acquisition and transfer of antibiotic resistance via plasmids and mobile genetic elements, including transposons and integrons [23].

Most references until the year 2008 showed that the IMP-1gene carried on plasmid [25, 26] but the modern references confirmed the presence of the IMP-1gene on cassettes in Class 1 integron [1, 27].

Twelve (27.3%) *Acinetobacter baumannii* out of 44 presumptive MBL producer isolates (with isolates were negative for MBL producer in phenotypic method used as control) were positive for *bla* $_{IMP}$ gene by PCR (Fig-10, 11, 12, & 13). No *bla* $_{IMP}$ genes were found in isolates negative by the phenotypic test (IPM-EDTA disk synergy test).

Previous researches have reported that the presence of *bla* $_{IMP}$ gene in the *Acinetobacter* species in low percentages. In the study Hwa, 2008[28] was 5.12%.The prevalence of the metallo-β-lactamase genes(*bla* $_{IMP}$ gene) is generally low within *A. baumannii* isolates as illustrated in a study by Mendes *et al*.,2009 [29] where the prevalence was 0.8% in Taiwan. Other researches were having not been detected *bla* $_{IMP}$ genes [30, 31, 32, and 33].

The isolates which were positive MBL production by confirmatory test but negative for *bla*$_{IMP}$ amplification may have variant *bla*$_{IMP}$ or *bla*$_{SIM}$ genes [34]. This was confirmed by the current study, by the presence of the *bla*$_{IMP1}$ gene in proportion (80%).The imipenem-resistant *Acinetobacter baumannii* strains in present study with no phenotypic or genotypic sign of MBL production may possess other enzymes mediating carbapenem resistance, such as OXA-type lactamases (class D) or AmpC b-Iactamases and/or other mechanisms such as outer-membrane permeability and efflux mechanisms [31]. The mechanism of cleavage of the ß-lactam ring is different for MBL's as compared to ß-lactamases; however, both gene products still share a unique αßßα fold in the active sites of the enzymes.

The bla_{IMP} is a foreign gene that is introduced from another species of bacteria and *A. baumannii* only retain the gene in environments where there is selective pressure in the form of the presence of imipenem [32].

The coexistence of bla_{IMP-1} with bla_{IMP} genes in present study 25% (11/44) of cases exemplify the extraordinary ability presented by *A. baumannii* to acquire multiple resistance mechanisms.

The study concluded that, resistance to imipenem was found to be a better indicator of MBL production. IPM-EDTA-disk synergy test appears to be useful in differentiating MBL and non-metalloenzyme producers. Most isolates of *Acinetobacter baumannii* were found to be metallo beta-lactamase (MBL) producers using IPM-EDTA-disk synergy test. Also, isolates of *A. baumannii* have been produced MBL gene (bla_{IMP-1}). It seems to be the major mechanism of resistance among Iraqi nosocomial isolates of *A. baumannii*.

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