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Occurrence of Metallo-β-lactamase Genes among *Acinetobacter baumannii* Isolated from Different Clinical Samples

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Abstract

Acinetobacter baumannii became a serious endemic and widespread pathogen responsible for causing nosocomial infections due to restricted treatment options. This study was conducted to evaluate the role of both antibiotic resistance and genetic pattern in infection caused by MBLs resistance *A. baumannii* isolated in Baghdad hospitals. Finally, developing selective media in order to detect multidrug resistance *A. baumannii* isolates according to colony shape appearance changing. Collected 124 isolates from various clinical and environmental sources. Biofilm Formation was detected, Antibiotic sensitive for 21 antibiotic discs were determined, MDR index calculated, PCR was performed to investigate MBLs genes *bla* (*IMP2, KPC, DIM, SPM, VIM, GIM, AIM, BIC, SIM, NDM, Pre-NDM-A, GES, Imp-1, and VIM*). Increasing resistant prevalence of *A. baumannii* appear significantly in higher rates to seven antibiotics group and MDR index ranged between 0.29 to 1.0 from 6-21 antibiotics. It was clear in Biofilm formation evaluated 75.8% strong biofilm after 72 hours, indicated that there is a significant negative correlation between biofilm formation capacity and the inherent ability of bacteria to show multidrug resist (*P*< 0.001). Our findings indicated that non-MDR isolates tended to form more robust biofilm formation. Metallo β-Lactamase detection phenotype showed 113/124 isolates able to produced ESBL, a result confirmed by PCR assay to detect the resistance of MBL-genes, appeared that a high percentage of *bla*$_{IMP}$ 79% which left clinicians with limited treatment options. An increasing number of hospital MDR *A. baumannii* has been reported. Furthermore, the study will focus on the evolution and the emergence of multidrug resistance *A. baumannii* in Iraq. The common MBL gene is the *bla*$_{IMP}$.

Keywords: *Acinetobacter baumannii;* Metallo-β-lactamase genes; Biofilm; Multidrug resistance.

INTRODUCTION

Acinetobacter baumannii is one of the most important opportunistic pathogens that cause outbreaks in hospitals, health care and also associated with complications in hospitalized patients[1].

Unstable genetic element, tolerate harsh environments, and Metallo ʎ-lactamases (MBLs) enzyme have led to the development of MDR resistance and epidemic *A. baumannii*[2,4].

Different mechanisms of resistance and resistant to all commercial antibiotic caused alarming problems in a limited choice of antibiotic for the treatment of MDR. *A. baumannii* isolates[3].

Increasing of MDR hospital isolates *A. baumannii* isolate has been stated from many countries around the world. Plus, inter-hospital environment dispersal of multidrug-resistant *A. baumannii* has been observed[5]. The ability of *A. baumannii* to rapid genetic change owing to their ability to acquire genes horizontally and vertically[6].

This study came to highlight the current patterns of resistance to antibiotics as well as the prevailing genetic patterns of resistance in *A. baumannii* in order to achieve effective treatment in Baghdad hospitals for these bacteria with multiple resistance to antibiotics, which represents a threat to public health.

MATERIALS AND METHODS
Sample collection

The study was carried out from December 2017 through March 2018 and involved 6 hospitals: Children's Educational Hospital, Baghdad Teaching Hospital, Al-Hawraq Hospital, Educational Laboratories (Medical City), Imam Ali Hospital and Al-Kindi Hospital. After the patients were examined by the physician, samples were collected from various sources: wounds, burns, ulcers, spinal cord fluid-blood, blood, body fluid and swabs of the hospital environments and tested using conventional microbiological methods.

Laboratory Identification of Isolates
Morphological Examination:

The samples were diagnosed including Gram staining, traditional culture and using the selective media such as the chromo agar Acinetobacter medium and use modified Leeds agar base media by replacing fructose and sucrose and Manitol by xylose and glucose and add crystal violet dry powder dispense from selective media. Furthermore, the results of the identification of *A. baumannii* were confirmed by the API 20E system according to Forbes [7].

Temple preparation for PCR

Template DNA was prepared by the boiling method as described by[8].

Molecular detection of *A. baumannii*

In order to confirm the detection, isolates were subjected to PCR against the *16s rRNA*, oxa51 and RecA, PCR mixture and amplification were done as explained by[9,10,11] respectively.

Antimicrobial susceptibility and ʎ-Lactamase detection

Isolates were plated on Mueller–Hinton agar and their susceptibilities to different antibiotics:Ampicillin/Sulbactam 20-10g (SAM30),_Piperacillin/Tazobactam 100-10g (PTZ110),piperacillin (PRL100), Ticarcillin / Clavulanic acid 75-10ug (TIM85), Ceftazidime 30ug (CAZ30), Cefotaxime 30ug (CTX30), Ceftriaxone 30ug (CRO30), Imipenem 10ug (IMI10), Meropenem 10ug (MEM10), Colistin Sulphate 25 ug(CO25), Gentamicin 30 mg(GN 30), Tobramycin 10ug (TN10), Amikacin 30µg (AK30), Netilmicin 30ug (NET30), Doxycycline 30ug (DXT30), Tetracycline 30ug (T30), Lofloxacine 5ug (LEV5), Ciprofloxacin 5µg (CIP 5), Trimethoprim - sulfamethoxazole1.25/23.75µg (SXT1.25/23.75), were tested by disk diffusion method according to the Clinical and Laboratory Standard Institutes guidelines (2017).

Modified Hodge test (MHT)

Performed according to Bonnin[12], for detection of carbapenemase production, Metallo β-Lactamase and ESBLs production among isolates by using modified indirect three-dimensional methods according[3].

Biofilm Formation

Congo red agar (CRA) method (qualitative Biofilm production assay): A simple qualitative assay for detection of biofilm was described by Mathur[13].With modification as follows: (40) g\l blood agar base, (0.8) mg Congo red,(5)g\l d-glucose, (5) g/l xylose. These components were dissolved in 1L of distilled water and autoclaved. The agar then was dispensed into sterile Petri dishes and kept at 4°C until being used. Following inoculation, the agar plates were incubated at 37°C for 24–48 h. The appearance of black colonies with

a dry crystalline consistency could be considered as strong evidence for the ability to form a biofilm. Each experiment was conducted in three repeats

Tissue culture plate method (quantitative biofilm production assay)

Generally considered to be the gold-standard technique for biofilm detection, Adopted the prescribed route by Lotfi[14].

Motility Assay

Motility Assay Performed as per[15].

PCR amplification of **Metallo β –lactam genes**: PCR method was used for screening of the Metallo β-lactam genes: *bla* (*IMP2, KPC, DIM, SPM, VIM, GIM, AIM, BIC, SIM, NDM, Pre-NDM-A, GES, Imp-1, and VIM*). The primers and PCR programs used in this study were as previously described by (16; 17; 18 and 5). PCR product bands were analyzed after electrophoresis in a 1% agarose gel at 95 V for 45 min in 1X TBE containing Ethidium Bromide under UV radiation[8].

Statistical analysis

One-way ANOVAs was applied exploitation SPSS 21 so as to match variations among Microtiter plate below standard and changed conditions. The experiments were performed in triplicate. P values of ≤ 0.05 were thought of as significant[31].

Table 1. Significance and Antibiotic groups distribution according to the number and percentage of resistance

Anti-biotics	Resp.	No.	%	P-value	Anti-biotics	Resp.	No.	%	P-value
TE	S	53	42.7	0.704	AK	S	9	7.3	0.000
	I	13	10.5	NS		I	10	8.1	
	R	58	46.8			R	105	84.7	
Doxt	S	69	55.6	0.099	NET	S	25	20.2	0.000
	I	5	4	NS		I	8	6.5	
	R	50	40.3			R	91	73.4	
SXT	S	13	10.5	0.000	PTZ	S	7	5.6	0.000
	I	8	6.5	HS		I	8	6.5	
	R	103	83.1			R	109	87.9	
LEV	S	37	29.8	0.002	TIM	S	14	11.3	0.000
	I	17	13.7	HS		I	5	4	
	R	70	56.5			R	105	84.7	
CIP	S	16	12.9	0.000	SAM	S	13	10.5	0.000
	I	6	4.8	HS		I	16	12.9	
	R	102	82.3			R	95	76.6	
CTX	S	4	3.2	0.000	IMP	S	28	22.6	0.000HS
	I	14	11.3	HS		I	3	2.4	
	R	106	85.5			R	93	75	
CFM	S	7	5.6	0.000	MEM	S	20	16.1	0.000HS
	I	3	2.4			I	2	1.6	
	R	114	91.9			R	102	82.3	
CPM	S	11	8.9	0.000	PIP	S	3	2.4	0.000HS
	I	10	8.1	HS		I	3	2.4	
	R	103	83.1			R	118	95.2	
CRO	S	2	1.6	0.000	AUG	S	4	3.2	0.000HS
	I	7	5.6			I	7	5.6	
	R	115	92.7			R	113	91.1	
CN	S	5	4	0.000	CAZ	S	2	1.6	0.000HS
	I	4	3.2	HS		I	17	13.7	
	R	115	92.7			R	105	84.7	
TOB	S	8	6.5	0.000	S: Sensitive				
	I	9	7.3		R: Resistance				
	R	107	8603		I: Intermediate				

RESULTS

A total of 124 *A. baumanii* isolates were collected from patients infected with significant and life-threatening diseases show in Fig. (1&2). The specimens were as follows: C.S.F 44/124 (35.5%), blood 32\124 (25.8%), sputum7/124 (5.65%), urine 5/124 (4.03%), burn and wounds and post-surgical swab 23/124 (18.54%), body fluid 3\124(2.42%), and swab from environment swab 10\124(8.06%). The diseases from which *A. baumanii* bacteria isolated were wound and urinary tract infection, septicemia and bacterima, pneumonia, and meningitis. Hence, the most prevalent disease related to *A. baumanii* was meningitis followed by septicemia. The age of patients ranged from 1 day to 70 years and male to female ratio was 1.375. And the child\adult ratio of 1.19.

Antimicrobial susceptibility detection

The overall susceptibility patterns of *A. baumannii* isolates from various clinical sources is displayed in Table 1. Isolates showed high XDR 75%, MDR 23.4%, and PDR 1.6%, The MAR index for experimental isolates (6-21 from 21 antibiotics) ranged between 0.29 to 1.0.

β-Lactamase detection

Metallo β-Lactamase detection phenotype showed (n=113)91.1% isolates able to produced while only (n=1)0.8% overall the isolates produce ESBL.

Correlation between biofilm formation and multidrug-resistant isolates

The simple logistic regression test analysis indicated that there is a significant negative correlation between biofilm formation capacity and the inherent ability of bacteria to show multidrug -resistance index ($P < 0.001$). Our findings indicated that non-MDR isolates tended to form more robust biofilm formation Fig. (3). Among 124 strong biofilm producers, 94% (n = 91) were non-MDR and 6.2%% (n = 6) were MDR isolates. All of the negative biofilm isolates were MDR 100% (n=3) and weak biofilm producers were 50%MDR index isolates and 50% non-MDR.
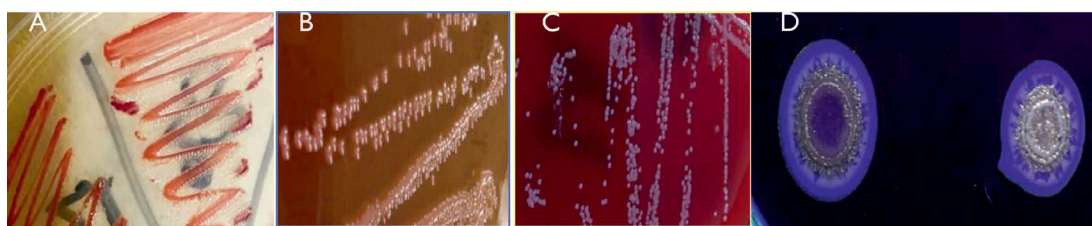

Fig. 1. Colony of *Acinetobacter baumanii*. A: On Chromo agar; B: OnMacConckey aga; C: On Blood agar r; D: On Modified Media
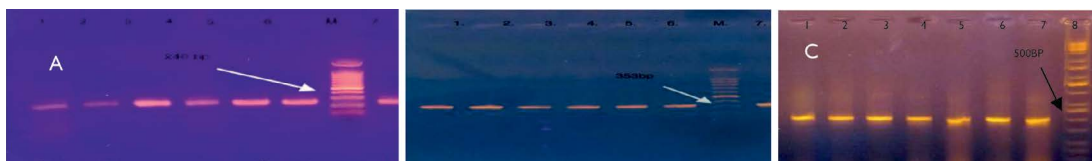

Fig. 2. Agarose gel electrophoresis (1% agarose, 7 v/cm^2) and Ethidium bromide staining to detect A: *16S rRNA* gene size product (band 240bp); B: *OXA51* gene size product (band 353bp) c- rec A gene .using template DNA prepared by boiling method. Right, Lane, molecular size DNA ladder (100 bp DNA Ladder); Left lanes DNAs isolated from *A.baumannii* sample showed Positive PCR.

Fig. 3. Biofilm formation by A -Congo red agar B- modified Congo red agar

Twitching motility pattern

The results showed that 6/124 isolates were non-motile (4.8%), while 118/125 (95.2%) isolates of *A. baumannii* had positive results, 95/124 (75%) of isolates had high mobility isolates with a large area of migration <25 mm after 24 h. of incubation period; while 23/124 (18.5%) isolates

Table 2. Grouping of *A. baumannii* according to antibiotic sensitivity, antibiotic genes patteren and bioflim formation

Biofilm Formation	N0.	Beta lactam antibiotic lactam antibiotic	Non Beta and intermediate sensitive and intermediate	metallo-β-lactamase sensitive genes	Predominate pattern
Negative	3	(IMP, MEM, TIM AUG-+)	DXT, TE(LEV, CN (SXT, NET_+)	IMP(DIM, SPM SIM,VIM, GIM, BIC, NDM, PRE NDM +_)	Non
Weak	4	CIP, CTX, CPM(SAM, IMP, CFMPTZ, TIM, MEM, AUG +_)	TE,DXT,LEV, CN (SXT+-)	IMP(VIM, BIC KPC, GES, SIM, GIM, NDM_+)	*1-bla* GIM, IMP ,BIC(50%) 2- *bla* VIM, KPC (50%) 3- *bla* DIM (50%), 4-*bla*GES, SIM,NDM,SPM (33.3%)
Moderate	23	(CIP, CFM, MEM, SAM, TIM, CTX , CRO, PIP, IMP, CPM, PTZ, CAZ+_)	(TE, DXT, AK, LEV, NET SXT, TOB SXT, CN+_)	(IMP, VIM, SIM, GIM, GES, DIM, NDM, KPC, SIM, SPM, BIC, PRE NDM, VIM-1, IMP-1_+)	1. *bla*IMP, VIM (30.4%) *2-bla* IMP GES (30.4%) *3-bla* IMPalone (21.7%) 4- *bla* VIM, SIM 5-*bla*DIM, GIM, DIM (17.3%) 6- *bla* IMP SIM ,GIM ,GES (8.7%) 7- *bla* GIM VIM-1(8.6%) 8 - TE, DXT, AK (1.6%) 9- TE, DXT, NET (4.8%) 10- (4.3%) for nine patterns
Strong	94	(CIP, CFM, SAM, TIM, MEM, CPM, IMP, CRO, AUG, PIP, CN, CTX, PTZ, CAZ_+)	AK, CN(TE,DXT,LEV, SXT, NET, TOB_+)	IMP (VIM, SIM, GIM, GES, DIM, NDM, KPC, SIM ,SPM, BIC, PRE NDM , VIM-1, IMP- 1_+)	1- *bla* IMP, AIM, IMP-1 (21.1%) 2-*bla*I MP, DIM (19.1%) 3-*bla* IMP, BIC, AND GES (17.02%) 4- *bla* IMP, GES (15.9%) 5-*bla* IMP, SIM (14.9%) 6-*bla* IMP, VIM-1 (14.06%) 7- *bla* IMP, IMP-1 (13.8%) 8-*bla* DIM, GIM (12.8% 9-*bla* IMP, SIM ,GIM(12.8%) 10-*bla* IMP, KPC (11.7%) 10- *bla* VIM, SIM (10.6%) 11-*bla* TE (61.6%) 12*bla*- DXT (53.2%) 13- *bla*LEV (47.9%) 14-, *bla*NET (44.7%) 15-*bla* TE, DXT (31.9%) 16 *bla-DXT, NET (23.4%)* 17- *bla*TE, LEV (20.2%) 18- *bla* TE,DXT,LEV(16.2) 19- *bla* DXT, LEV (17.02%) 20-*bla*TOB (15.96%) 21-, *bla NET*, DXT (11.7%) 22- *bla*TE, DXT, NET (10.6%) 23- *bla*TE,SXT,LEV(8.5%) 24-*bla*DXT, SXT (7.4%) 25- *bla*TE, DXT, AK (6.4%) 26-*bla* -AK, NET (6.4%) 27- *bla* DXT, AK (6.4%) 28- *bla* LEV, TOB (5.3%) 29- *bla* TE, DXT, LEV, NET (3.2%) 30- *bla* LEV, CN (2.1%) 31 −*bla*TE,DXT,LEV(2.1%) 32- *bla* non predominate pattern show 1.06%

Red color means constant

showed an average migration area of between 5 and 25 mm**.**

Beta-lactam genes distribution

From all isolates among them, 0.8% (n=1) isolates were phenotypically confirmed ESBL producers while 99.2% (n=123) were phenotypically confirmed by non-ESBL producers. While Metallo beta-lactamase present in 91.2% (n=113), and8.9 % (n=11) non Metallo beta-lactamase, Out of the 14 beta-lactam (*bla*) genes studied, *bla*$_{IMP}$ was detected among (79%), followed by *bla*$_{GIM}$ in (33.9%) and variable detection of other genes range between 28.2% - 8.1%. (Fig. 4&5).

Association between bla genes, antibiotic sensitivity, and biofilm formation

The result can be divided to[4] group description as shown in Table 2, Hence, the most prevalent group was D include strong bioflim production with highly MDR index and different patterns of gene resistant.

Allocation of specimens of *A. baumannii* may diverge with each hospital as each hospital aperient has a different environment associated with it. More than 91.94% of the *A. baumannii*

isolates were obtained from CSF culture, Blood culture, burn swab, postoperative wound swab, sputum, urine, and body fluid Similar results had been obtained in different studies reported in Iraq by Hussein (19; 5) and (15). As well in different countries such as Doughari[20,21,22].

This consideration reveals that a total 76/124 *A. baumannii* were isolated, d from systemic infection CSF and blood culture, This may be due to the fact that bacteria are opportunistic pathogens capable of penetrating the defenses of the body, although ability to adhere to the instruments or hands of workers, which makes it easier to transmitted between patients who are in hospitals, from above indicated horizontal transition of bacteria from the skin or infected organs to the blood causing Bacteremia.

The use of selective cultures in the diagnosis of *A. baumannii* in principle is one of the quickest methods and gives a good percentage of diagnosis. This study proved the modified media in the accurate diagnosis of *A. baumannii* compared to the routine culture media. On the other hand, the study clarified that the diagnosis of genus and species together by using the PCR reaction is quick,
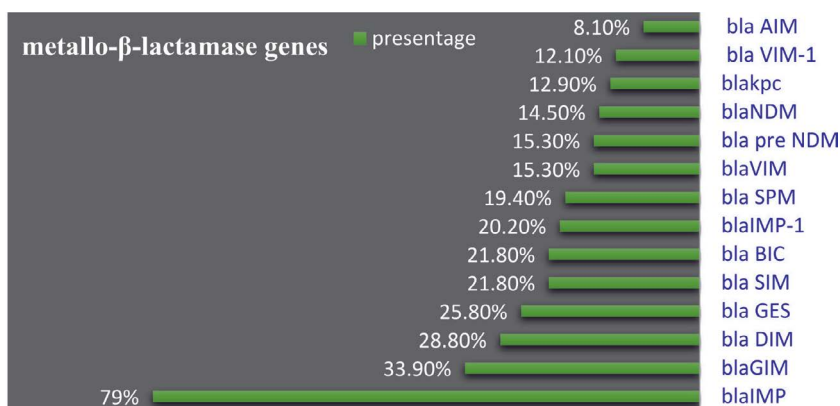

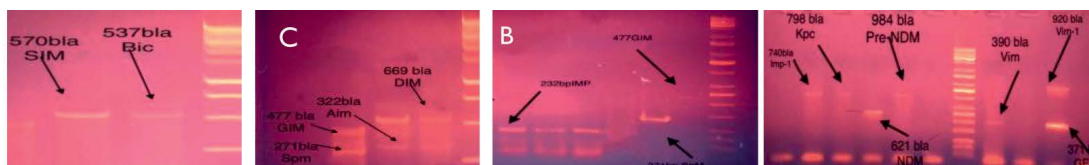**Fig. 4.** distributing Metallo-β-lactamase genes among *A.baumannii*

Fig. 5. Agarose gel electrophoresis (1% agarose, 7 v/cm[2)] and Ethidium bromide staining to detect A, B, C, D Metallo gene using template DNA prepared by boiling method. Right Lane, molecular size DNA ladder A and B (100 bp DNA Ladder); C and D (250 bp ladder) Left lanes DNAs isolated from *A. baumannii* sample showed Positive PCR

accurate and highly sensitive.

It is clear that the emergence of resistant *A. baumannii* isolates is increasing worldwide. In this research, the resistance of *A. baumannii* isolates to 15 out of 21 tested antibiotics was above 80%.

The frequency of MβLs among isolates of *A. baumannii in* this study was 91.1%. In M λL-producing *A. baumannii* were multidrug resistant, the lowest resistance was observed against Tetracyclin and doxycycline which can be used as a treatment option. A significant increase in resistance to carbapenem antibiotics was observed, In this regard[23] pointed out that the most important reasons for the emergence of resistance to carbapenem antibiotics are including the production of enzymes Carbapene-mases belonging to β-lactamase enzymes, and loss of protein in the outer membrane has been associated with anti-microbial resistance to Imipenem and Meropenem. It is worth mentioning that the resistance of Meropenem was higher than Imipenem. The high rate of resistance to most cephalosporins antibiotics tested indicates these bacteria possess multiple resistance mechanisms. In addition to their production enzymes β-lactamase and extend β-lactamase and have the ability to alter outer membrane proteins and efflux pump system that acts on efflux the antibiotic extracellular extrusion[24]. The resistance of *A. baumannii* to the quinolones and aminoglycoside was increased even to an active antibiotic such as Amikacin.

The study demonstrated the increase of antibiotic resistance to most classes of antibiotics, especially the antibiotics of choice for the treatment of *A. baumannii* infections include the fluoroquinolones, aminoglycosides, and carbapenems.

The MAR index values for all isolates showed high MDR, ranged between 6-21 from 21 antibiotics. The MAR index for experimental isolates ranged higher than 0.29 reach to 1.0. Suggesting the source of these isolates is from highly contaminated environments where it is excessive and randomly use of antimicrobial agents. Development of MAR pathogenic isolates of *A. baumannii* determined potential nosocomial infection in the hospital environment. Most isolates from CSF and respiratory system had MAR indices over (0.43), and over (0.76) show in UTI infection while the lowest MDR index (0.29) in body fluid confirming that there was a high antibiotic use and high selective pressure in these environments MDR strains become established in the hospital environment, these can persist for months.

Detection of biofilm formation
Microtiter plate assay

A. baumannii isolates were classified into strong, moderate, weak, and none biofilm producers, respectively at 550nm.Gentile[25]. demonstrated that there was a high rate of biofilm production between 60% of highly resistant *A. baumannii* isolates, our results agreed with Iraqi study[15].

Twitching motility pattern

It was believed *in A. baumannii* strains that display a motility phenotype depending on autoinducer molecules related to phenomenon quorum sensing.McQueary[26]. Further revealed that the production of lipopolysaccharide (LPS) Pili has an important role in twitching motility and adhesion (inert or living surface) and contributes indicating biofilm formation. The results indicated that when the resistance to antimicrobial agent rate is relatively high, there is an increase in productivity of adhesion factors gradually, on the contrary of Ali and Khudhair[27] study.

Genotype detection of the studied genes for MBLs

In our study, *blaBIC*were identified only in (21.8%) of *A. baumannii* isolates by PCR. In the other hand, the bla_{KPC} gene was identified among 16 /124 isolate (12.9%) of *A. baumannii*. Also, the bla_{GES} gene was detected among 32/124 isolate (25.8%) *A. baumannii*. Class A β-lactamases in *A. baumannii* may be considered a major problem in comparison to other carbapenemases and was usually not-carbapenem specific and often hydrolyze most compounds of the lower beta-lactam classes and take part in the high-level resistance to carbapenems[28].

The high frequency of Class A carbapene-mases genes incidence may be because they are plasmid mediated so bacteria can gain this plasmid from another gram-negative bacteria presented in hospital environmental.

The most frequent Class B Metallo-β-lactamases genes detected in *A.baumannii* were $bla_{IMP,\ IMP-1,\ VIM,\ VIM-1,\ GIM,\ SIM,pre-NDM\ and\ NDM}$. All these genes in this group chromosome encoded or carried by the plasmid. Among MBL genes, IMP is more important, especially in Iraq, because of its high presence 79% in *A. baumannii* isolates. The prevalence of β-lactamase-producing isolates is increasing at an alarming rate worldwide[4].

The Metallo-beta lactamase (MBL)gene bla_{VIM} and $_{VIM-1}$ was the only carbapenemase gene detected among 15.3% and 12.1% *A. baumannii* isolate respectively, previous studies showed that 42.8 % of *A. baumannii* isolates from Baghdad/Iraq were $_{VIM}$ type positive[3]. Similar to this observation, AL-Jubori in 2016 reported 25% of *A. baumannii* isolates carried bla_{VIM}[11]. Further bla_{VIM} gene screening in Sulaimani/Iraq noticed only in 2.8% of *A.baumannii* isolates[29].

The accurate identification of MDR-positive *A. baumannii* isolate represents an important step in infection control and prevention of the spread of multidrug-resistant isolates (30). First large series of clinical isolates from Asia and the UK was bla_{NDM} have been reported by Yong[31], since this publication, $_{NDM-1}$ has an extensive in worldwide and now it is one of the most important carbapenemases in all Enterobacteriaceae and in *A. baumannii*[6]. While the $_{NDM-1}$ reported in Iraq is found in 20%to 40% of *A. baumannii*[1].

Carefully monitoring the presence of MDR *A. baumannii* among hospitalized patients is recommended to prevent wide dissemination of antibiotic resistance and limit the indiscriminate use of cephalosporins and carbapenems antibiotic in the hospital. Antibiotics Program, infection control, and monitor hospital environment should be applied to minimize the emergence of multiple drug-resistant bacteria.

The gaining and spread of unique ^{2}-lactamase-mediated antibiotic resistance mechanism could finally assist the development of effective protection and control measures.

In conclusion, we have shown that metallo-beta-lactamase producing *A. baumannii* isolates is an emerging threat in Baghdad hospitals and Finally, in a proof of concept experiment the novel selective media were applied to determine the *A. baumannii* isolates as well as distinguish sensitive isolates from multiple antibiotic resistance.

CONFLICT OF INTEREST

The authors declares that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors have made substantial, direct and intellectual contribution to the work and approved it for publication.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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