Molecular Screening of Some β-Lactam Resistance Genes Producing Clinical Isolates of *Acinetobacter baumannii*

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Abstract

Acinetobacter baumannii is currently as a main opportunistic bacteria to human that posing an rising risk to persons admitted to the hospital, causing them satisfactory infection. Subject of this work aimed to study the dissemination and the frequency of some plasmid genes that encoding Extended Spectrum β-Lactamase (ESBL) and Metallo β-Lactamase (MBL) among *A. baumannii* isolates. A total of 235 of various samples (burns, urine and blood) from hospitalized patients in Al-Hussain General Hospital during a period of five months between June to October 2014.

The isolates were identified using microscopic examination, all positive bacterial samples were submitted to conventional biochemical tests and the suspected isolates were candidate to detect 16-23S rRNA using PCR and finally was diagnosed bacteria to confirm the species level, depending on the results of Vitek 2 System. The results were revealed that only 9 isolates identify as *A. baumannii* and 1 isolate as *A. baumannii* complex.

The results revealed high resistance to different groups of antibiotics, all isolates were resistance 100% to Ampicillin, cefoxitin, ceftazidime, cefotaxime, cefepime and aztreonam, while it was 70% to ciprofloxacin and 60% to gentamicin and amikacin while 50% of isolates were resistant to imipenem.

Molecular screening of tested antimicrobial resistance genes were performed, results revealed that different percent of occurrence as following: *bla TEM-1*(100%), *bla TEM-2*(90%), *bla OXA-1*(90%), *bla OXA-2*(20%), *bla OXA-10*(30%), and *bla IMP*(10%). While *bla* (*OXA-23*, *OXA-48* and *KPC*) genes were no detected in this work.

Conclusion. The spread of β-lactam resistance genes especially ESBLs that produced by *A. baumannii* which detected in this study is a large worry therefore, must take the necessary measures and procedures to reduce this prevalence in the hospitals.

Keyword: Acinetobacter baumannii**,** β-Lactam Resistance Genes**,** PCR

الخلاصة

تعد بكتريا *Acinetobacter baumannii* من المسببات المرضية الانتهازية المهمة التي تزايدت اهميتها من الناحية السريرية خصوصا للأشخاص العاملين او المرضى الراقدين في المستشفى. هدفت الدراسة الحالية الى تقييم انتشار انزيمات البيتالاكتاميز واسعة الطيف وانزيمات الميتالو بيتالاكتاميز المرتبطة بالبلازميدات في عزلات بكتريا *A. baumannii* . جمعت 235 عينة من مصادر سريرية مختلفة شملت الحروق، الادرار والقشع)من عينات مختلفة للمرضى الراقدين لمستشفى الحين الام في محافظة كربلاء المقدسة لمدة خمسة اشهر من حزيران الى تشرين الاول من العام 2014.

زرعت جميع العينات وبعد نموها شخصت باستعمال الفحص المجهري و الاختبارات الكيموحيوية ثم اجري التشخيص التأكيدي للعزلات قيد الدراسة باستعمال جهازي PCR (16–23S rRNA gene) و Vitek 2 عائدية 9 عزلات الى *A. baumannii* و 1 عزلة الى *A. baumannii* complex.

اكدت نتائج اختبار الحساسية وجود مدى واسع من المقاومة المتعددة للمضادات الحيوية، اذ بلغت 100% تجاة المضادات الحيوية (Ampicillin، cefoxitin، ceftazidime ، cefotaxime، cefepime و aztreonam). بينما اظهرت 70 % من العزلات مقاومة للمضاد الحيوي ciprofloxacin، اما المضادان gentamicin و amikacin قد بلغت 60% وكانت نسبة المقاومة 50% عند المضاد الحيوي imipenem . اظهرت الفحوصات الجزيئية للجينات المختلفة نتائج توزعت كالتالي، انزيمات البتالاكتام نوع *TEM–1* (100%)، *TEM–2* (90%), *OXA–1* (90%)، *OXA–2* (20%)، *OXA–10*(30%) و *IMP* (10%) . اما الجينات *OXA–23* ، *OXA–48* و *KPC* فلم تظهر في جميع العزلات.

الكلمات المفتاحية: بكتريا *Acinetobacter baumannii*، مقاومةβ–Lactam , جين، تفاعل البوليميراز المتسلسل.

Introduction

The genus, *Acinetobacter*, is a gram-negative, aerobic, non-motile, non-fermenting, non-fastidious, catalase-positive and oxidase-negative (Singh *et al.*, 2013). This genus includes 25 species, among which *A. baumannii* in human, is the most important one (Sharif *et al*., 2014). Nowadays, *A. baumannii* is an one of the most important six opportunistic pathogens that have the capacity to resist many of the antibiotics, making it a real problem in hospitals worldwide. (Luísa *et al*., 2014).

The main problems caused by *A. baumannii* isolates in the hospital environment concern patients hospitalized in intensive care and burn units (Nowak *et al*., 2014). The increasing rate of occurrence is mainly due to the ability of *A. baumannii* to continue to live or exist on environmental surfaces for long time, and it has a great tendency to acquire different antimicrobial resistance genes making nosocomial transmission very hard to stop and control (Gandham, 2014). The beta-lactam antibiotics private carbapenems group are currently first choice used to treat these pathogens; however, in the past decade, unfortunately a vast domain of beta-lactamase enzymes have now been discovered in *A. baumannii*, including AmpC β-lactamases ; extended-β-lactamases ESBL and and metallo-β-lactamases(MBLs) (Villalo´n *et al*., 2013). Hence, the target of this work was to estimate the spread of *A. baumannii* in the burn unit as well as the extent of the genes responsible for antibiotic resistance among these bacteria in the hospital.

Materials and Methods

This study was done at Bacteriology and Molecular Laboratories in Department of Biology, Faculty of Sciences, University of Kufa, Iraq.

Patients and bacterial isolates

During a period of five months between June to October 2014, a total of 235 isolates were recovered from different clinical sources (burns 100, urine 100 and sputum 35) collected from patients being treated in Al-Hussain General Hospital in Karbala Province. Under a septic conditions all samples were cultured directly on blood agar and MacConkey agar for isolation of aerobic bacteria. All positive growth samples were done primarily using gram stained smear, Conventional biochemical tests (catalase and oxidase tests, motility, Indole test , citrate test and acidity or alkalinity in triple sugar iron (TSI), urease test, Voges-Proskauer test, methyl red test and hemolysis and followed by confirmed diagnosed to all suspected clinical isolates of *A. baumannii* were evaluated using PCR for the 16S-23S rRNA gene. As well as, these isolates were confirmed as *A. baumannii* using Vitek 2 automatic system (Biomerieux, France) for full identification using the identification card for Gram negative strains (ID-GNB) according to the manufacturer's recommendations. All Positive isolates were preserved in freezing at -20ºC in brain heart infusion broth with 20% glycerol until need in the screening test.

Antibiogram susceptibility testing

The antibiogram of the isolates were performed according to method of Kirby-Bauer´s disc-diffusion on Mueller-Hinton Ager (Bauer *et al*., 1966). The inoculation of all isolates had attended through suspending the overnight growth of tested isolates in sterile normal saline adjusted to a 0.5 McFarland standard tube. Routine antibiotic discs (Bioanalyse, Turkey) ampicillin(10µg), cefoxitin (30µg), cefotaxime (30µg), ceftazidime (30µg), cefepime (30µg), aztreonam (30µg), gentamicin (10µg), amikacin (10µg), ciprofloxacin (5µg), and imipenem (10µg).The zone diameters were interpreted as per Clinical Laboratory Standards Institute (CLSI) recommendations (CLSI,2012). Escherichia coli ATCC 25922 strain was used as negative control.

PCR Preparation

a. Plasmid Extraction and Purification

A single colony of cultivated *A. baumannii*, which had been incubated overnight, transfer to 2 ml of sterile brain heart infusion broth then incubate at 37 ◦C for 18-20 hours .Plasmid DNA was extracted and purified using High-Speed mini DNA plasmid extraction kit (Geneaid Biotech.) according to the manufactures protocol. It was used to detect *bla TEM-1, bla TEM-2, bla OXA-1, bla OXA-2, bla OXA-10, bla OXA-23, bla OXA-48, bla KPC* and *bla IMP* genes.

b. Genomic Extraction and Purification

A single colony of cultivated *A. baumannii*, which had been incubated overnight, transfer to 2 ml of sterile brain heart infusion broth then incubate at 37 ◦C for 18-20 hours. All genomic DNA was extracted and purified using Genomic DNA Mini plasmid kit (Geneaid Biotech.) according to the manufactures protocol. It was used to detect 16-23S *rRNA* gene.

PCR amplification and gel electrophoresis

PCR amplification to all confirmed isolates were performed using monoplex PCR to screened for presence of genes of *16-23S rRNA, bla TEM-1, bla TEM-2, bla OXA-1, bla OXA-2, bla OXA-10, bla OXA-23, bla OXA-48, bla KPC* and *bla IMP* genes. The specific primers and reaction conditions used in the study are shown in tables 1 and 2)

PCR products (amplicon), were run 1.5% agarose gel. The gel was stained with 4 µLof 10mg/mL ethidium bromide (Sigma, USA) and it run at 80v for 1.5h. A single band was observed at the desired position on ultraviolet light transillumintor (Cleaver, UK); bands were photographed using gel documentation system (Cleaver, UK). A 100bp ladder (Bioneer, Korea) was used to measure the molecular weights of amplified products (Chaudhary and Payasi, 2014).

Table 1. PCR primers used in this work

Name	Sequence (5' to 3')	product size (bp)	Reference
16-23S rRNA-F	GTC GTA ACA AGG TAG CCG TA	800	(Sarhan *et al*., 2014)
16-23S rRNA-R	GGG TTC CCC CRT TCA GAA AT		
TEM-1F	ATG AGT ATT CAA CAT TTC CG	867	(Kaye *et al*., 2004)
TEM-1R	CTG ACA GTT ACC AAT GCT TA		
TEM-2F	ACT GCG GCC AAC TTA CTT CTG	374	
TEM-2R	CGG GAG GGC TTA CCA TCT G		
OXA-1F	ACA CAA TAC ATA TCA ACT TCG C	813	
OXA-1R	AGT GTG TTT AGA ATG GTG ATC		
OXA-2F	TTC AAG CCA AAG GCA CGA TAG	702	
OXA-2R	TCC GAG TTG ACT GCC GGG TTG		
OXA-10F	CGT GCT TTG TAA AAG TAG CAG	651	
OXA-10R	CAT GAT TTT GGT GGG AAT GG		
OXA-23 F	AAG CAT GAT GAG CGC AAA G	1066	(Senkyrikova *et al*., 2013)
OXA-23R	AAA AGG CCC ATT TAT CTCAAA		
OXA-48F	TTG GTG GCA TCG ATT ATC GG	744	
OXA-48R	GAG CAC TTC TTT TGT GAT GGC		
KPC-F	ATG TCA CTG TAT CGC CGT CT	893	
KPC-R	TTT TCA GAG CCT TAC TGC CC		
IMP-DIA-F	GGA ATA GAG TGG CTT AAT TCT C	361	(Docquier *et al*., 2003)
IMP-DIA-R	GTG ATG CGT CYC CAA YTT CACT		

F: Sense primer; R: Antisense primer

Table 2. PCR conditions used in this work

monoplex gene	Temperature (c) / Time					Cycle number
	Initial denaturation	Cycling condition			Final extension	
		Denaturation	Annealing	Extension		
16-23S rRNA	95°C/ 3 min	95°C/60 sec	55°C /60 sec	72°C/ 1 min	72°C/5 min	35
TEM-1	95°C/ 5 min	94°C/ 60 sec	58°C/ 60 sec	72°C/1 min	72°C/ 10min	35
TEM-2	95°C/5 min	94°C /30sec	62°C/30 sec	72°C/30 sec	72°C/10min	35
OXA-1	96°C/ 5 min	96°C/60 sec	61°C /60 sec	72°C/ 2 min	72°C/10min	35
OXA-2	96°C/5 min	96°C /60 sec	65°C /60 sec	72°C / 2 min	72°C/10min	35
OXA-10	96°C/5 min	96°C/60 sec	61°C /60 sec	72°C / 2 min	72°C/10min	35
OXA-23	94°C/5 min	94°C/60 sec	50°C /50sec	72°C / 1 min	72°C/10min	35
OXA-48	94°C/5 min	94°C/60 sec	55°C / 45sec	72°C / 1 min	72°C/7min	35
KPC	96°C/5 min	96°C/60 sec	60°C / 45sec	72°C / 2 min	72°C/8 min	35
IMP	95°C/ 5 min	94°C/60 sec	53°C/ 60 sec	72°C/1.5min	72°C/ 10min	35

Statistical Analysis

The electrophoresis results were analyzed size of DNA bands (PCR amplicons) were measured by using gel analyzing program (UV band software version12.14) in comparison with DNA ladder (100 bp).

Results and Discussion

Patients and bacterial isolates

Only ten clinical isolates of *Acinetobacter* genus have been identified using PCR technique that indicate that the diagnostic 16S-23S rRNA gene was a precise and accurate method for diagnosis *A. baumannii* through all isolates gave positive band according to specific product size (Figure 1). The results of Vitek 2 system were revealed that only 9 isolates identify as *A. baumannii* and 1 isolate as *A. baumannii* complex. Biochemical tests showed that all 10 isolates gave features of the genus of *Acinetobacter*, they are summarized in Table 3.

Table (3): diagnostic results of biochemical tests for *A. baumannii* isolates

Test	Result	Test	Result
Oxidase	-	Lactose fermentation	-
catalase	+	Citrate	+
Gram stain	-	VP	-
motility	-	MR	-
urease	-	Hemolysis	-
Kliglar iron agar	Alkaline slant/ no change bottom,	H_2S production	-

VP Voges-Proskauer, MR methyl red , + positive result, - negative result

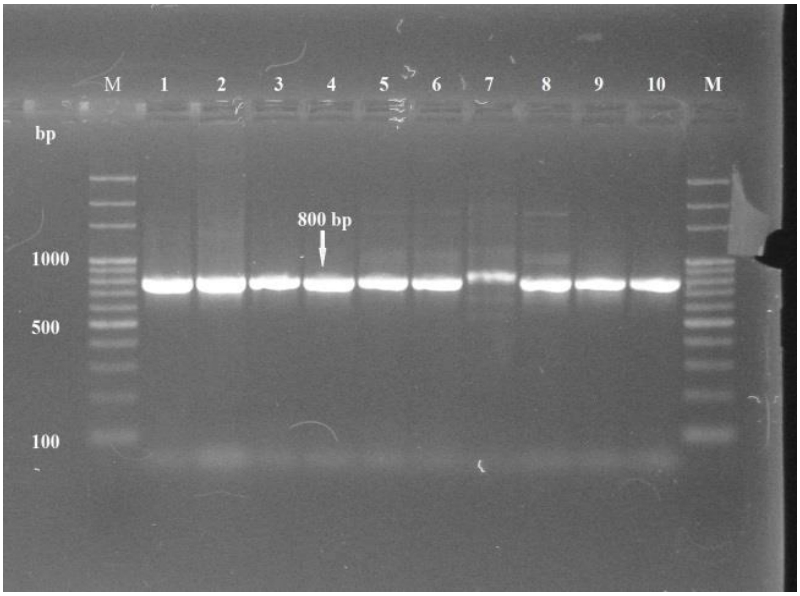


Figure (1): Agarose gel electrophoresis of PCR product for 16-23S rRNA gene in *A. baumannii* isolates. L= DNA molecular size markers (100 bp), lanes, 1 to 10 represent number of isolates

Data in table 4 showed that majority of *A. baumannii* isolates were recovered from the burn specimens (n=6; %), whereas 3 isolates were gained from urine (n=3), The remaining isolate from sputum (n=1).

Compared with other local studies, this finding was lower than a study of Nassar *et al*., (2013) who mention that 26(23%) of *A. baumannii* were isolated from 130 wound and burn patients in Al-Ramadi Teaching Hospital. These results nearly in agree with the results obtained by Jabur (2014) who reported that a total of 75 specimens taken from a variety of clinical sources (urinary tract infections (UTIs), wounds, burn and sputum) from inpatients in Hilla Teaching Hospital.

The majority of isolates in this work was from burn units, this is perhaps resulted from high exposure of burn patients to nosocomial infection due to their broken skin barrier and suppressed the defenses of immune system (Jubar,2014). As well as ability

of *A. baumannii* to survive for long periods of time in the hospital equipment's and capacity to acquired drug resistance which helps it in the spread of the infection (Nasser *et al*., 2013; Manchanda *et al*., 2010).

Table (4): Distribution of *A.baumannii* isolates according clinical sources

Specimen source	Specimen no.	*A.baumannii* usingVitek 2 (no., %)	*A.baumannii* using 16-23S rRNA gene
burns	100	*A. baumannii* (6, 60%)	+ (6, 60%)
urine	100	*A. baumannii* (3, 30%)	+ (3, 30%)
sputum	35	*A. baumannii* complex (1, 10%)	+ (1, 10%)
Total (%)	235	10(100%)	10(100%)

Antibiogram susceptibility profile

The resistance patterns of *A. baumannii* towards various classes of antibiotics were determined using disc diffusion method. Data in table 5 exhibited the highest resistance (100%) against ampicillin, cefoxitin, cefotaxime, ceftriaxone, cefepime and aztreonam. However this data agree with a previous local study done in Baghdad by Ali (2010) revealed that resistance percentage of *A. baumannii* isolates to ampicillin, ceftriaxone and cefotaxim were 100%. Another study in Egyptian hospitals showed that *A. baumannii* isolates were 100% resistant to amoxicillin–clavulanate, aztreonam, cefepime, cefotaxime, and ceftazidime, while the isolates, were resistant 5% to colistin, 45% to amikacin, 70% to imipenem, and 85% to ciprofloxacin (Al-Agamy *et al*., 2014)

This study also revealed moderate resistance to gentamicin 60% , amikacin 60% , imipenem 50% while 7of isolates 70% recorded resistance to ciprofloxacin antibiotic. These result are closer to a study done by Hussein *et al.*, 2013 revealed that all *A. baumannii* clinical isolates had 100% resistance to amoxicillin-clavulanic acid, cefepime, cefotaxime and rifampin. and aztreonam 97.39% , ceftriaxone 97.39%, ceftazidime 89.57%, gentamicin 87.83% , 86.09% , ciprofloxacin (83.48%), amikacin 72.17% and 58.26% were resistant to both imipenem and meropenem.

Several international and local studies indicate that *Acinetobacter* spp. has a great capacity to develop antibiotic resistance in response to challenge with new antibiotics (Fazeli *et al*., 2014 and Nasser *et al*., 2013) . However, In this study showed all isolates (100%) were resistance to at least one agent in ≥ 3 antimicrobial categories, it means they were MDR (Al-Hamadani *et al*., 2014). Therefore, they are difficulties in antibiotic therapy. This result was agree with several local studies that refer to prevalence of multi-drug resistance of *A.baumannii* isolates among Iraqi hospitals (Al-Hamadani *et al*., 2014 and Shali, 2012). The increasing height of the bacterial resistance to drugs is a concern among health communities therefore, their rapid identification is necessary and important for healthcare centers. Mismanaged repeatedly for the antibiotics play a role in increment selective pressure among microbial population that lead to the evolution of multidrug-resistant isolates then difficult and limitation in antimicrobial therapy (Thiraviam *et al*., 2014).

Species of *Acinetobacter* was showed its real resistance to antimicrobial agents and their possibility to get some genes that encoding resistance factors. Primarily among the methods of resistance is enzyme production especially β-lactamases and aminoglycoside-modifying . As well as, reduced antimicrobial uptake due to mutations in the regulatory genes that are responsible for the membrane permeability , and efflux pumps that effectively remove the drug. All these mechanisms play a serious role in drug resistance. Tribulation is occur when the pathogen become multidrug resistance or sometimes pan drug resistant isolate (Perez *et al*., 2007).

Table 5: Antimicrobial susceptibility of *A.baumannii* isolates using disc-diffusion method

Isolate	Source	Antibiotic susceptible									
		AMP	**FOX**	**CAZ**	**CTX**	**FEP**	**AZM**	**CIP**	**CN**	**AK**	**IMP**
Ab1	burns	R	R	R	R	R	R	R	R	R	R
Ab2	burns	R	R	R	R	R	R	R	R	R	R
Ab3	burns	R	R	R	R	R	R	R	R	R	R
Ab4	burns	R	R	R	R	R	R	R	R	R	R
Ab5	sputum	R	R	R	R	R	R	S	S	S	S
complex											
Ab6	burns	R	R	R	R	R	R	R	R	S	S
Ab7	burns	R	R	R	R	R	R	R	R	R	R
Ab8	urine	R	R	R	R	R	R	S	S	S	S
Ab9	urine	R	R	R	R	R	R	S	S	S	S
Ab10	urine	R	R	R	R	R	R	R	S	R	S
Total		100	100	100	100	100	100	70	60	60	50

Ab *A.baumannii*

Distribution of ESBLs and MBLs-producing isolates

Plasmids were isolated from 10 isolates and were used as templates for screening of the possibility plasmid-mobile bla genes using conventional PCR depending on specific primers.

Resistance to β-lactams drugs in gram-negative bacteria including *Acinetobacter* spp. is increasingly correlated with ESBLs (Slama, 2008). In Iraq, several studies have revealed that the distribution of ESBLs has been observed in different rates (Tuwaij, 2014). Data of PCR revealed 10 of *A.baumannii* isolates(100%) gave positive bands for TEM-1 gene (figure 2). these results agree with Ting *et al*. (2013) They detected TEM (100%) gene among *A. baumannii* isolates. However, this finding agrees with many studies which were reported to refer to TEM β-lactamase spread worldwide .

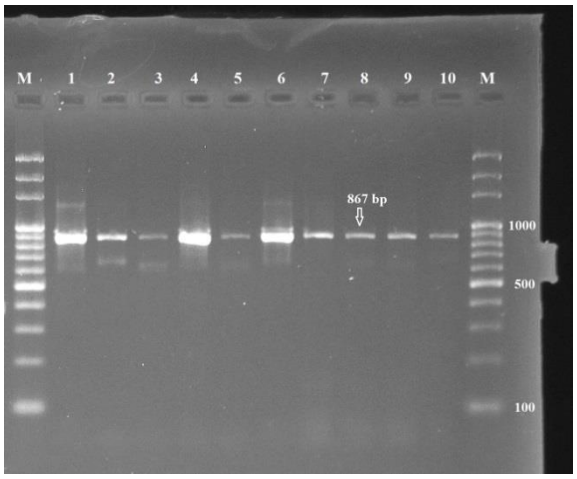
The results of the current study showed that 9 (90%) of *A.baumannii* isolates were positive for TEM-2 gene (figure 3). On other hand, PCR results showed 9 (90%) isolates were positive for OXA-1 ESBLs gene (figure 4), while the results in figure 5 and 6 revealed that only 2 (20%) and 3(30%) isolates were positive for OXA-2 and OXA-10 ESBLs genes respectively, where two of them were positive for OXA-1,OXA-2 and OXA-10 genes at the same time. One isolate (10%) was negative for OXA genes using PCR technique (table 6). Compared with another study done in Iran by Forajnia *et al*., (2012) who indicated that OXA-2 and OXA-10 genes were prevalence in *A. baumannii* isolated from patients admitted to hospital of Imam Reza in Tabrizi

province. However, The first mechanism of resistance in *Acinetobacter* genus to β-lactam drug is usually because of the making of β-lactamases which mediated by plasmids or chromosome that protect bacteria from the fatal effects of antimicrobial agents. (Dortet *et al*., 2006).

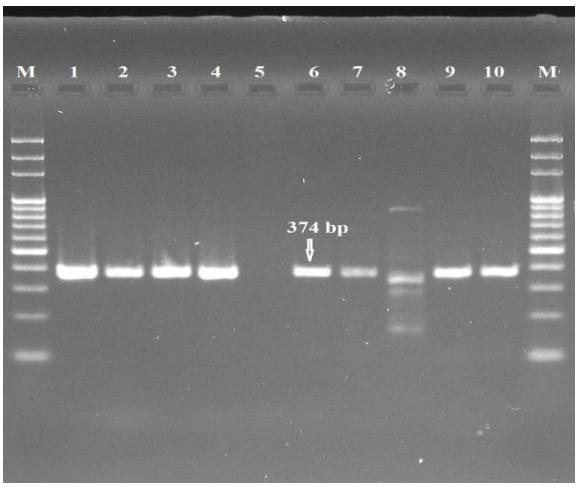
Only one isolate harboured one β-lactamase gene (TEM-1) while other isolates harboured three or more than one types of β-lactamase genes. However, the OXA enzyme is the major product of *A. baumannii*. by different ways,, the newer type of OXA enzymes has similar characteristics with those enzymes of ESBL enzymes of ESBL, that typically possess small sequence substitutions as compared with the original genes of β-lactamases. The alterstions in this sequence may be express vastly increment the spectrum of activity of the β-lactamases against antimicrobial agents like ceftazidime or aztreonam. The mutants in OXA-10 gene express ESBL efficiency and able to break down the third-generation of cephalosporin's and monobactam (aztreonam), (Bonomo and Szabo, 2006).

One of this work aim was carried out to detect dissemination of MBLs in *A.baumannii* isolates. Interestingly, the presence of the IMP gene has been observed in one isolates (figure 7). None of the isolates had bla (OXA-23, OXA-48, and KPC) genes. Several researchers noted in their studies on *A. baumannii* that more prevalent enzymes of metallo–β-lactamases were back to two main groups included IMP and VIM type (Walsh *et al*., 2005).

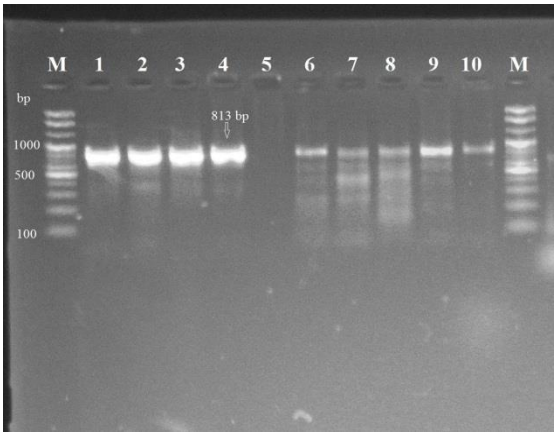
In this research, in spite of raise resistance average against imipenem drug among *A. baumannii* isolates, the little average of genes producing MBLs have been revealed, actually perhaps some other genes no screening are participated in phenotypic production of MBLs lead to raise carbapenem resistance in Al-Hussain General hospital, Iraq.



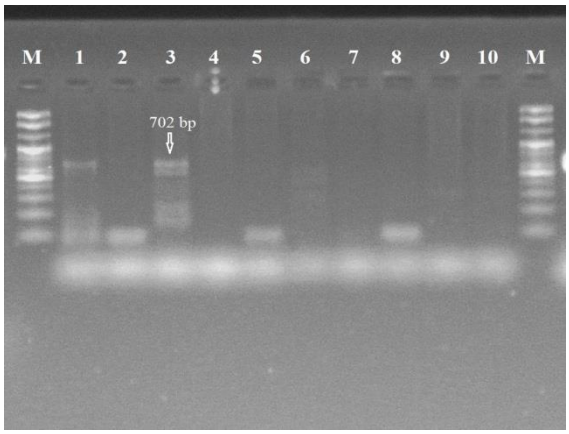
Figure(2): Agarose gel electrophoresis of PCR product for bla TEM-1 gene in *A. baumannii* isolates. L= DNA molecular size markers(100bp), lanes, 1 to 10 represent number of isolates



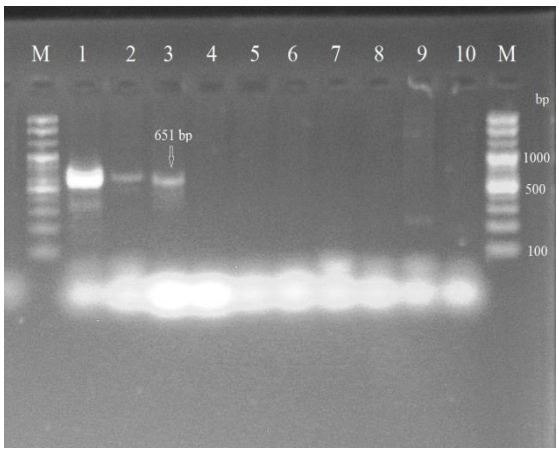
Figure(3): Agarose gel electrophoresis of PCR product for bla TEM-2 gene in *A. baumannii* isolates. L= DNA molecular size markers(100bp), lanes, 1 to 10 represent number of isolates



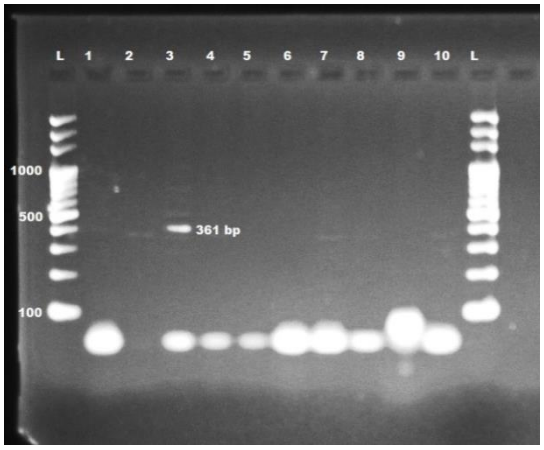
Figure(4): Agarose gel electrophoresis of PCR product for bla OXA-1 gene in *A. baumannii* isolates. L= DNA molecular size markers(100bp), lanes, 1 to 10 represent number of isolates



Figure(5): Agarose gel electrophoresis of PCR product for bla OXA-2 gene in *A. baumannii* isolates. L= DNA molecular size markers(100bp), lanes, 1 to 10 represent number of isolates



Figure(6): Agarose gel electrophoresis of PCR product for bla OXA-10 gene in *A. baumannii* isolates. L= DNA molecular size markers(100bp), lanes, 1 to 10 represent number of isolates



Figure(7): Agarose gel electrophoresis of PCR product for bla IMP gene in *A. baumannii* isolates. L= DNA molecular size markers(100bp), lanes, 1 to 10 represent number of isolates

Table (6): Occurrence of β-lactamases in clinical isolates of *A. baumannii* (n=10)

Isolate no.	Source	TEM-1 gene	TEM-2 gene	OXA-1 gene	OXA-2 gene	OXA-10 gene	OXA-23gene	OXA-48 gene	IMP gene
Ab 1	burns	+	+	+	+	+	-	-	-
Ab 2	burns	+	+	+	-	+	-	-	-
Ab 3	burns	+	+	+	+	+	-	-	+
Ab 4	burns	+	+	+	-	-	-	-	-
Ab5 complex	sputum	+	-	-	-	-	-	-	-
Ab 6	burns	+	+	+	-	-	-	-	-
Ab 7	burns	+	+	+	-	-	-	-	-
Ab 8	urine	+	+	+	-	-	-	-	-
Ab 9	urine	+	+	+	-	-	-	-	-
Ab 10	urine	+	+	+	-	-	-	-	-

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