DETECTION OF METALLO B-LACTAMASE ENZYME IN SOME GRAM NEGATIVE BACTERIA ISOLATED FROM BURN PATIENTS IN SULAIMANI CITY, IRAQ

Khanda Abdulateef Anoar
Fattma Abodi Ali
Sherko Ali Omer
Department of Microbiology, Faculty of medical science,
School of Medicine, University of Sulaimani

Abstract

Background: Metallo β-lactamase has emerged worldwide as powerful resistance determinants in Gram-negative bacteria. They hydrolyze virtually all classes of β-lactams except Monobactam, including Carbapenems, which often represent the last option for the treatment of infections with multidrug resistant Gram-negative bacteria.

Objective: the aim of this study was to screen for genes coding for metallo β-lactamase such as, bla_{VIM}, bla_{IMP} *and* bla_{NDM} among infected burn wound patients in Sulaimani city /Iraq.

Methods: This prospective study was carried out in the Burn and Plastic Surgery Hospital in Sulaimani city at a period from April - October 2011. Genes responsible for metallo β-lactamase enzyme such as bla_{VIM}, bla_{IMP} *and* bla_{NDM} were targeted to be screened by multiplex PCR mixture reaction among 177 Gram negative bacteria isolated from 230 burn patients.

Results: One hundred seventy seven Gram negative bacteria were isolated and identified from infected burn wound.

Out of 177 Gram negative bacteria isolated 46(25.9%) isolates were positive for different MBL genes by PCR.

Conclusion : Common causes of multidrug resistant isolates among hospitalized burn patients in burn units was metallo β-lactamase which were not previously established in this hospital .

Keywords: Metallo β-lactamase, Carbapenem, multiplex PCR

Introduction

MBL are metallo enzymes of Ambler class B which are Clavulanic acid resistance enzymes. They require divalent cations of zinc as co-factors for enzymatic activity and are universally inhibited by EDTA as well as other chelating agents of divalent cation (Butt *et al.,* 2005; Walsh and Tolereman 2005).

There are two dominant types of transferable MBL genes among clinical isolates, *bla* $_{IMP}$ and *bla* $_{VIM}$, which are frequently present on gene cassettes inserted into integrons located on the chromosome or on plasmids (Walsh 2011; Fard *et al.,* 2012). Other types of MBLs such as *bla* $_{AIM}$, *bla* $_{GIM}$, and *bla* $_{SPM}$ are found only sporadically in some geographic regions (Athanassios *et al.,* 2009).

Most integrons containing gene cassette for MBL also harbor additional gene cassette classes such as Aminoglycoside or Chloramphinicol, therefore integron transfer might lead to a single step transfer of complex multidrug resistant phenotype (Poirel *et al.,* 2012) and the dissemination of MBL genes among Gram negative pathogens is mediated by mobile elements of DNA which explains why the same gene might be associated with plasmids or interested in chromosome in different strains (Lee *et al.,* 2005).

The origin of acquired MBL genes is unknown, the most kindly sources are environmental bacteria from which Gram negative bacteria, non fermenter and Enterobacteriaceae could acquire the resistant determinants while sharing several common environmental niches (Giuseppe *et al.,* 2011).

it has been reported worldwide with more than 20 different variants in different geographical area , it appears to be the most prevalent MBL type than other types of MBL enzymes (Minond *et al.* 2012).

The latest and novel additions to acquired MBL gene is New Delhi Metallo β-lactamase enzyme with its propensity for international dissemination and was first detected in 2009 from Swedish patient of Indian origin in strain of *Klebsiella pneumoniae* , who traveled to New Delhi city and acquired urinary tract infection and proved to be multiresistant strains against all antimicrobial agents except Colistin (Kumarasmy and Tolerman 2010; Melichercíkováa and Goroncy, 2010).

New Dehli metallo β-lactamase has recently emerged in India, Pakistan and the United Kingdom and it represents a serious threat of rapid dissemination of multiple antibiotic resistances since the majority of *bla*$_{NDM}$ producing

Enterobacteriaceae have been reported to remain susceptible only to Colistin and Tigecycline (Vaux *et al.,* 2011).

Methods:

Different Gram negative bacteria were isolated in this study by culturing wound swab taken from infected burn wound on different culture media such as blood agar , MacConkey agar, nutrient agar,eosin methylen blue and identification was based on colonial morphology, biochemical identification, using Api system and Vitek 2 compact system. All the isolates were screened on bases of susceptibility to carbapenem drug such as Meropenem and Imipenem, and metallo β-lactamase genes such as (*bla* $_{IPM,}$ *bla* $_{VIM}$ and *bla* $_{NDM}$) were screened among all isolates by multiplex polymerase chain reaction(PCR).

Plasmid extractions were done for all the isolated bacteria at the same time by the same procedures and in Multiplex reaction all the isolates were run by using three sets of primers (table 1) , which were first prepared by mixing the reveres and forward primer of each gene then all the primers were mixed together in a specified volume and these were added to other components of the PCR reaction and the PCR condition were corrected as initial denaturation for 2 minute at 95°C, denaturation for 30 second at 95°C, followed by annealing step for 30 second at 48 °C and extension step ; 30 second and final extension at 72 °C for 2 minute.

Table (1) Primers used in the study (Poirerl *et al.*,2011).

Primer name	Sequence of primer	Ann. Temp.	Target gene	Amplicon in bp
VIM-R	TGGTGTTTGGTCGCAAT	48 °C	*bla* $_{VIM}$	390bp
VIM-F	CGAATGCGCACCAG			
IMP-R	GGAATAGAGTGGCTTAACTCTC		*bla* $_{IMP}$	232bp
IMP-F	GTTTAACAAAACAACCACC			
NDM-R	CGGAATGGCTCATCACGATC		*bla* $_{NDM}$	621 bp
NDM-F	GGTTTGGCGATCTGGTTTTC			

Polymerase chain reaction products were resolved using 1% agaros gel electrophoresis and suspected bands were gel purified and the products from agaros gel were recovered by using GeneJETrM Gel Extraction Kit (Fermentas/ Germany). The content of the kit was prepared prior to use as recommended by the Fermentas/ Germany) and sent for sequencing.

Three *bla$_{VIM}$* PCR products from *Pseudomonas aeruginosa* were subjected to sequencing after its purification from agaros in a 1.5 ml Eppendorf tube using their amplification primer sets with 15 µl of both the revers and forward primers . Sequencing of PCR products was done in University of Koya/ Erbil /Iraq, Genome Centre/Sequencing Department. The sequencing reactions were performed using BigDye® TerminatorV3.1 Cycle Sequencing Kit (Applied Biosystem), and

High-performance 4-capillary 3130 Genetic Analyzer pop7 polymer was used for separation.

Data collection software V3.0 was used for sequence analysis in the center and DNAMAN program (Lynnon Corporation, version 4.13) was used for the analysis of sequenced data.

Results:

PCR assay screening for MBL gene was performed for 177 isolated Gram negative bacteria and 46(25.9%) isolates were positive for different MBL genes by PCR , from which 39 (22%) isolates were from Meropenem resistant strains and 7(3.9%) from Meropenem sensitive strains (table 2). Statistically significant correlations were found between PCR results and Meropenem susceptibility ($p < 0.05$).

Table (2) PCR detection of MBL genes in relation to Meropenem susceptibility .

Gram negative isolates	Positive MBL gene No. (%)	negative MBL gene detection No.(%)	Total No.
Meropenem resistant	39 (22.03)	27 (15.25)	66 (37.28)
Meropenem susceptible	7 (3.95)	104 (58.75)	111 (62.71)
Total	46 (25.98)	131(74.01)	177 (100)

P < 0.05 **MBL: metallo β- lactamase**

Gram negative bacteria harboring MBL genes were distributed in all hospital units and the most frequently units in the hospital which harbor these MBL genes were ICU 18 (10.1%), ABU 31(17.5), and PBU 3(1.69) while the least numbers of genes were from PLU 2(1.12%) out of all 46 isolated bacteria with positive MBL genes (table 3).

Genes responsible for metallo β-lactamase (MBL) was detected in this study by using PCR, and three possible genes such as bla_{VIM}, bla_{IMP} *and* bla_{NDM} according to their amplicon size were identified on agaros gel (figure 1). Detection of bla_{IMP} gene was observed in 33 (18.6%) isolates while bla_{VIM} *gene* was detected from 19 (10.7 %) isolates. Concerning $bla_{NDM\ (621\ bp)}$ gene, two possible products were amplified and one of them was isolated from multidrug resistant *K. pneumonia* and the other one from Meropenem sensitive strain of *K. pneumonia* (table 4) .

Table (3) Distribution of MBL genes among Gram negative isolates in different hospital units.

Hospital Units	MBL genes No. (%)			Total No. of MBL gene isolated No.(%)	Total No. of bacteria in unit No. (%)
	bla$_{VIM}$	*bla*$_{IMP}$	*bla* $_{NDM}$		
ICU	10 (5.64)	7 (3.95)	1 (0.56)	18 (10.1)	32 (18)
ABU	6 (3.38)	24 (13.55)	1 (0.56)	31 (17.5)	55 (31)
PBU	1 (0.56)	2 (1.12)	-	3 (1.69)	50(28.24)
PLU	2 (1.12)	-	-	2 (1.12)	40 (22.59)
Total	19 (10.73)	33 (18.64)	2 (1.12)	54 (30.5)	177 (100)

Three *bla*$_{VIM}$ from *p. aeruginosa* were subjected to sequencing using their amplification primer sets. The results of forward and reverse primers sequencing data were subjected to BLAST service available at National Center for Biotechnology Information (http://blast.ncbi.nlm.nih. gov/Blast.cgi).

When sequence data from three *bla* $_{VIM}$ gene products were aligned together using DNAMAN program using both forward sequence and reverse sequence data the following results were obtained showing homology ranging from 96.77 % for forward sequences to 98.14% for reverse sequences (figure 2 and 3).

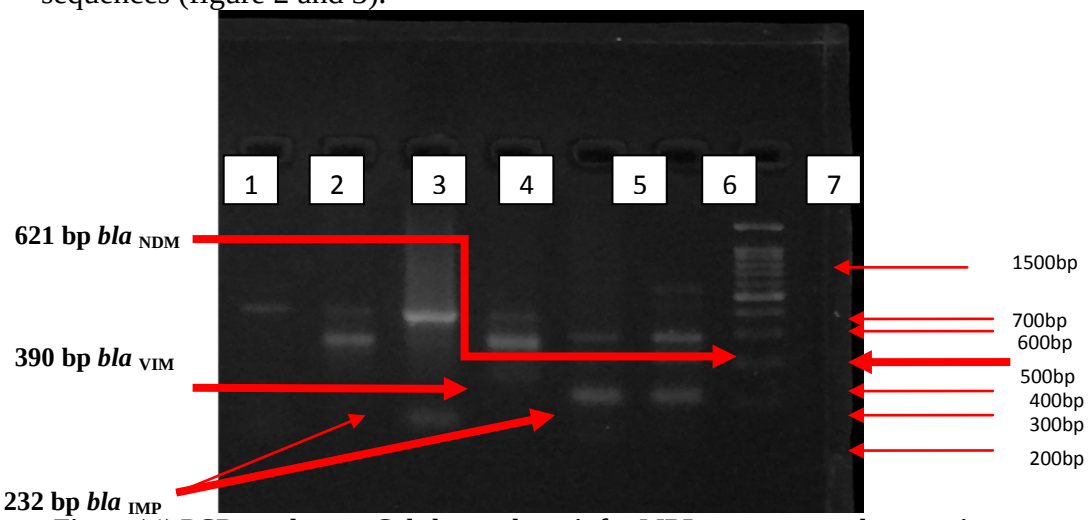
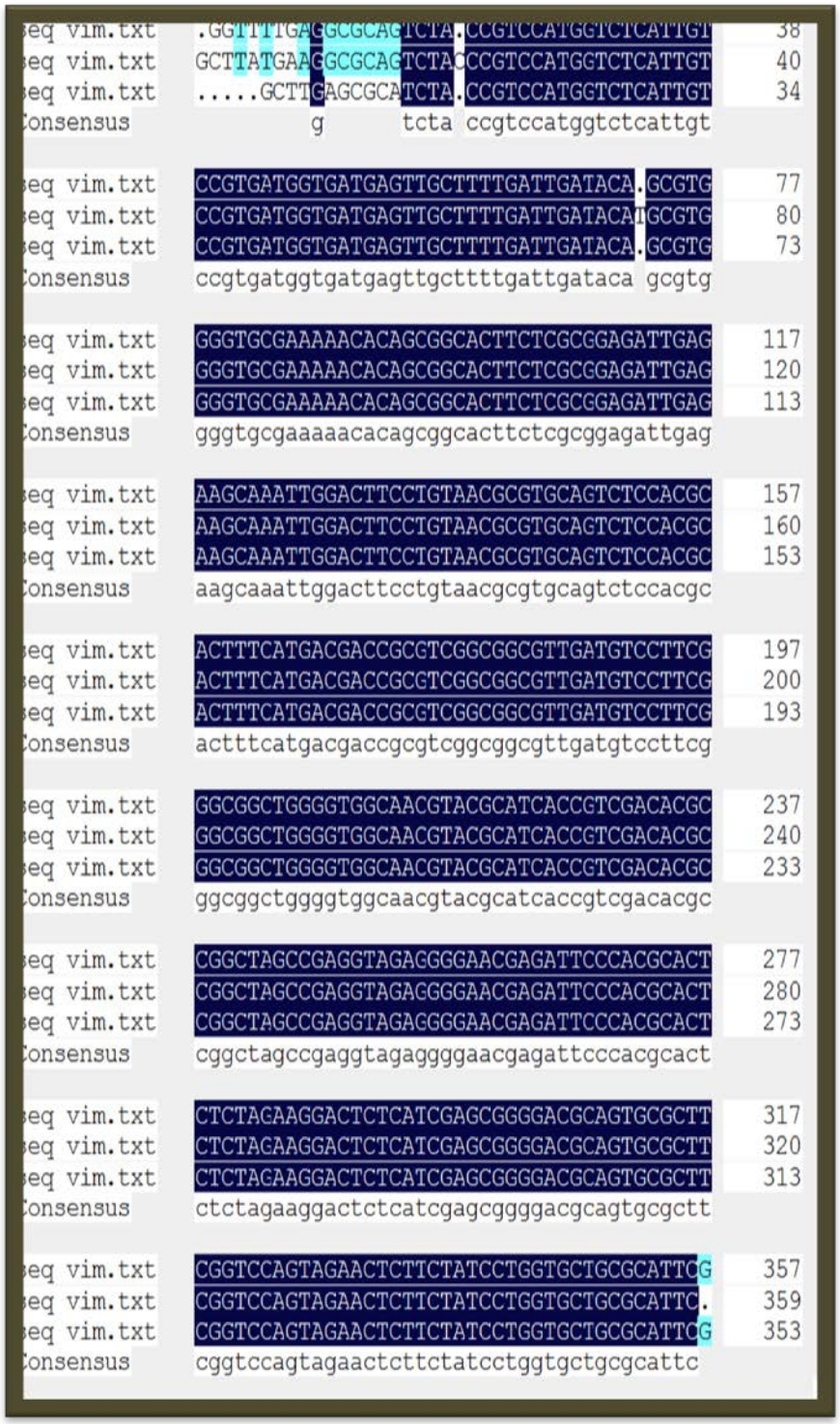


Figure (1) PCR product on Gel electrophoresis for MBL genes among burn patients. lane 7: DNA ladder , lane 6 : *bla* $_{NDM}$ positve *K.pneumoniae* , lane 2, 3,5,6 *bla* $_{IMP}$ positive *P. aeruginosa* and *A. baumannii* ,lane (4,1)390 bp *bla* VIM positive (red arrow) *P. aeruginosa.*

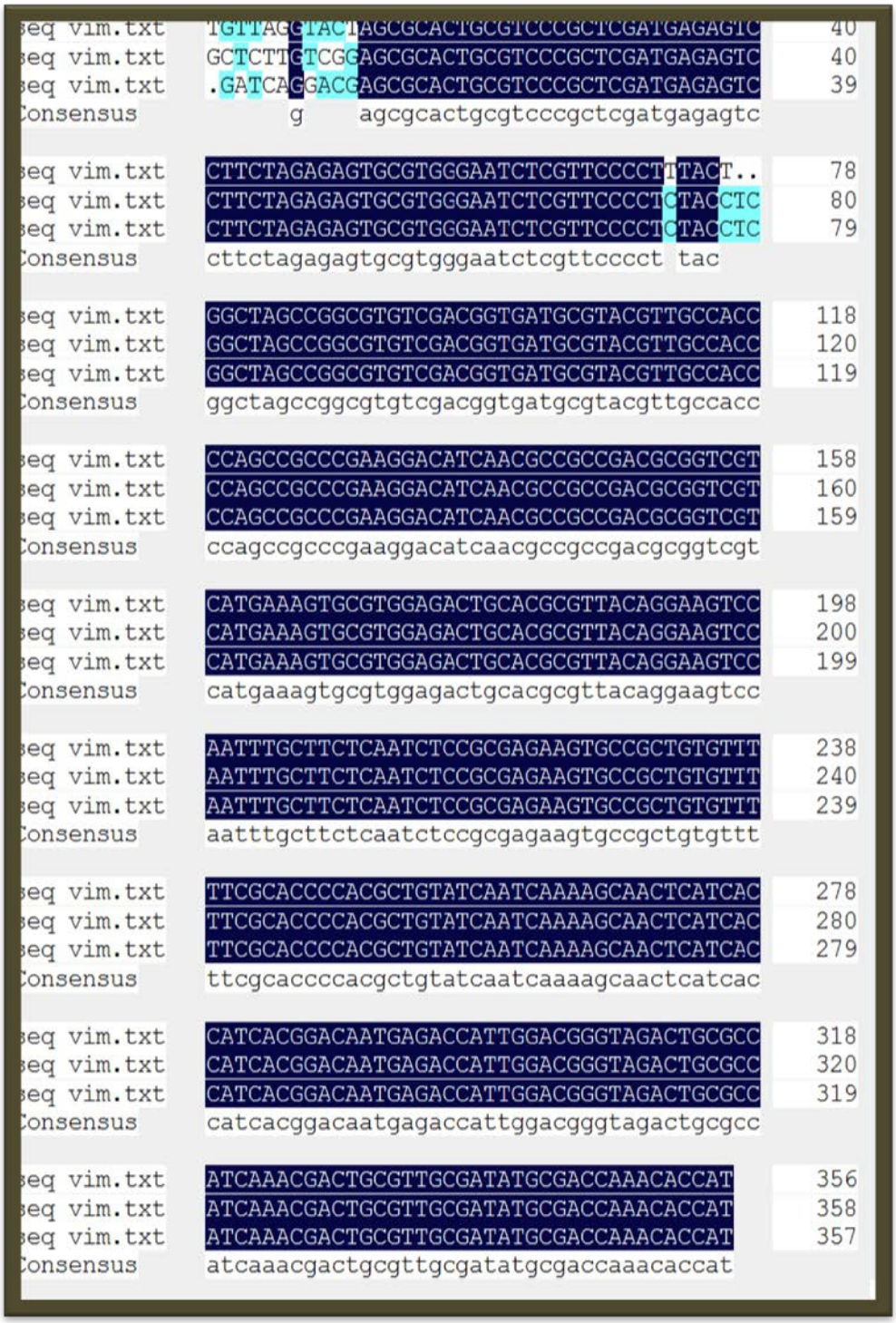
Table (4) Different MBL genes amplified using PCR.

Bacterial species	MBL genes				
	bla $_{IMP,}$ **No. (%)**	***bla*** $_{VIM}$ **No.(%)**	***bla*** $_{NDM}$ **No.(%)**	**Total gene isolated**	**Total No. of bacteria**
Pseudomonas species	7 (3.95)	9 (5.08)	-	16 (9.04)	48 (27.12)
Acinetobacter species	19 (10.73)	5 (2.82)	-	24 (13.56)	44 (24.86)
Klebsiella pneumoniae	7 (3.95)	5 (2.82)	2 (1.12)	14 (7.91)	44 (24.86)
Enterobacter cloacae	-	-	-	-	18 (10.17)
Escherichia species	-	-	-	-	11 (6.21)
Morganella morganii	-	-	-	-	4 (2.25)
Providencia rettegeri	-	-	-	-	3 (1.69)
proteus mirabilis	-	-	-	-	2 (1.12)
Barkholderia pseudomallei	-	-	-	-	1 (0.56)
Achromobacter xylosoxidans	-	-	-	-	1 (0.56)
Ralastonia paucula	-	-	-	-	1 (0.56)
Total	33 (18.64)	19 (10.73)	2 (1.12)	54 (30.51)	177 (100)

bla **:β- lactamase gene , VIM: Verona integron metallo β- lactamase, IMP: Imipenemase, NDM: new delhi metallo β- lactamase.**



Figuers (2) Sequence alingment of three sequence data from forward primers of *bla* $_{VIM}$.



Figuers(3) Sequence alingment of three sequnece data from revers Primers of *bla* $_{VIM}$ gene.

Discussion

In this study PCR detection of three MBL genes yielded many positive results. Obtaining the expected size of amplicon was considered as an indicator for the gene presence. These amplicon sizes were consistent and the same results were obtained when PCR was repeated on the same samples. The prevalence of MBL gene was different according to each Gram negative bacteria isolated among burn patients but in general it was demonstrated in 46 (25.9 %) isolates in which 39 (22 %) isolates were from Meropenem resistant strains and 7 (3.9%) from Meropenem sensitive strains.

These results indicate that most of the Meropenem resistant strains isolated in this study were producing MBL enzymes which were distributed in all the burn units of the hospital especially in the ICU (41.3%) , ABU (39.1 %) , and PBU (13 %) but plastic units harbor the least number of MBL producers that indicates most of the MBL produce strains distributed among severely burned patients with weak immune system and they are at the risk of acquiring these resistance genes. Similar studies were done in other countries with different prevalence rates such as 16% in Italy, 17.8% in UK and 30% in India (Lagatolla *et al.,* 2006; Ellington *et al.,* 2007; Rajput *et al.,* 2010).

The presence of MBL genes among Meropenem sensitive strains indicates that there might be a hidden MBL gene among isolated strains which cannot be diagnosed by phenotypic tests, leading to the dissemination of these genes in the hospital silently among patients even within normal health workers whom act as carriers for MBL genes in future. Other causes are due to the fact that in this study Meropenem and Imipenem were selected to be tested against isolated bacteria as an example of Carbapenem agent, but there were other generations such as Etrapenem and Doripenem which were not used and resistance might be detected in these 7 cases of MBL positive strains.

Current results revealed that there was a difference in the prevalence of MBL production among each isolated Gram negative bacteria such as 56.8% of *A.baumannii* were MBL producer by PCR reaction which was higher in comparison to other bacteria in the hospital as this bacteria is more prevalent in the hospital environment and even in soil which comes from the visitors foots to the hospital, although the main mechanism of Carbapenem resistance in *Acinetobacter* species is class D *bla* $_{\text{OXA}}$ carbapenemase specifically *bla* $_{\text{OXA 51 like}}$ which is intrinsic to the most species of *A. baumannii* (Manchanda *et al.,* 2010) .

MBL producers were 33.3% for *P.aeruginosa* while 31.8% of *K.pneumoniae* were recorded among all isolated *Klebsiella* species. These findings disagree with what were found by other workers in Iran (Saderi *et*

al.,2008; Tanzinah *et al.*, 2010) in which *Pseudomonas* species was the most frequent MBL producer among Gram negative bacteria and even within its own species at the rate of 68%.

Regarding MBL gene, three types of MBL genes *bla* $_{IMP,}$ *bla* $_{VIM,}$ and *bla* $_{NDM}$ were isolated from the current study. The most frequently detected gene was *bla* $_{IMP}$ gene 33 (18.6 %) among all isolates. Similar results were recorded in Turkey and Spain (Ozgumus *et al.*, 2007; Duljasz *et al.*, 2009) in which the detection of MBL genes (*bla* $_{IMP,}$ *bla* $_{VIM}$) were observed but in lower percentages to that of our study being 2.5% and 11% respectively.

Till now there are no published works in Iraq reporting *bla* $_{IMP,}$ gene. In this study, it was commonly detected in 19 (75 %) of *A. baumannii* isolates while 7 isolates from each *P. aeruginosa* and *K. pneumoniae* were found to be positive for *bla* $_{IMP}$ gene which is consistent with a study done in Turkey at different periods observing 39% which are much lower than our results (Eser *et al.*, 2009).

On the other hand, *bla* $_{VIM}$ gene was recorded in 19 (10.7 %) among all isolates. In this context, it was clear that the gene was present in all hospital wards especially in the ICU and ABU, as most of the Meropenem resistant cases were from these two units. This is consistent with the study in Tehran by Bahar and Samadikuchaksaraei, (2010) and the first report published from newly hospital in Iraq from Baghdad by Huang *et al.*, (2012) in which they recorded *bla* $_{VIM}$ to be 11% and 12.3% respectively. Though *bla* $_{VIM}$ is distributed all over the world, there was a study done in Esfahan (Fard *et al.*, 2012) in which *bla* $_{VIM}$ was not recorded entirely by *Pseudomonas aeruginosa*.

Different areas in the world were recorded to carry this gene *bla*$_{VIM}$ especially in neighboring countries at various prevalence rates lower than current study which were 2.3% for *bla* $_{VIM-2}$ in Teheran (Sepehriseresht *et al.* 2012), and 6.7% in India (Bandekar *et al.*, 2011). The cause of lower prevalence rate of *bla* $_{VIM}$ in those studies in spite of larger sample size is due to restriction of work in *P. aeruginosa* only from burn unit.

References:

Athanassios T. , Kristo,I., Pittaras, T., Spanakis, N., and Pournaras, S.(2009). Large Dissemination of VIM-2Metallo β-lactamase producing *Pseudomonas aeruginosa* strains causing health care associated community onset infections. Journal of clinical Microbiology. 47(11): 3524–3529.

Bahar, M.A, and Samadikuchaksaraei A.J. (2010).Imipenem resistant *Pseudomonas aeruginosa* strains carry metallo β-lactamase gene *bla*(VIM) in a level I. Iranian burn hospital. Epub.36 (6): 826-830.

Bandekar, N., Vinodkumar, C.S, Basavarajappa, K.J., Prabhakar,P.J., and Nagaraj,P.(2011).β-lactamases mediated resistance amongst Gram negative bacilli in Burn infection. Int J Biol Med Res.2(3): 766-770.

Butt T, Usman, M., and Ahmed, R.N. (2005).Emergence of Metallo β-lactamase producing *Pseudomonas aeruginosa* in Pakistan. J Pak Med Assoc. 55: 302–304.

Duljasz, G. M., Sitter, S., Wojna, A., and Jebelean, C. (2009).First organisms with acquired metallo β-lactamases (IMP-13, IMP-22, and VIM-2) reported in Austria. Antimicrob Agents Chemother. 53 (2221): 23-28.

Eser, O., Ergin, A., and Hasçelik, G. (2009).Antimicrobial resistance and existence of metallo β-lactamase in *Acinetobacter species* isolated from adult patients. Mikrobiyol Bul. 43(3): 383-390.

Ellington, M. J., Kistler, J., Livermore , D.M. and Woodford,N. (2007).Multiplex PCR for rapid detection of genes encoding acquired metalloβ-lactamases. J. Antimicrob. Chemother. 59: 321–322.

Fard , F., Irajian, M., Moslehi,G., Takantape Z, Fazeli H, and Salehi M. (2012). Drug resistance pattern of *Pseudomonas aeruginosa* strains isolated from cystic fibrosis patients at Isfahan AL Zahra hospital, Iran (2009-2010). Indian J Med Microbiol. **4**(2): 94-97.

Giuseppe , C. , Helen. G ., and Gian M. R.(2011).Metallo β-lactamse a last forntier for β-lactamse .Lancet Infect Dis. 11: 381-393.

Huang, X., Frye,J., Chahine,M., Glenn,L., Ake,J., Nikolich, M., and Lesho, E.(2012). Characteristics of plasmids in multi-drug-resistant Enterobacteriaceae isolated during prospective surveillance of a newly opened hospital in Iraq. PLoS One. 7(7).

Kumarasmy, K.K. , and Tolereman, M.A. (2010).Emergence of new antibiotic resistant mechanisms in India, Pakistan, and UK : Molecular biological and epidemiological study. Lancet Infect Dis. 10: 597-602 .

Lagatolla, C., Edalucci,E., Dolzani, L., Riccio, M.L., DeLuca,F., Medessi, E., Rossolini , G.M., and Tonin, E.A.(2006).Molecular evolution of metalloβ-lactamase producing Gram negative in a nosocomial setting of high-level endemicity. J. Clin. Microbiol. 44: 2348–2353.

Lee, K., Yum, JH. , Yong, D., Lee, H.M., Kim, H.D. Docquier, J.D., Rossolini, G.M. (2005).Novel acquired metallo-β-lactamase gene, blaSIM-1, in a class 1 integron from *Acinetobacter baumannii* clinical isolates from Korea. Antimicrob Agents Chemother . 49: 4485–4491.

Manchanda, V., Sanchaita, S., and Singh, N. (2010).Multidrug resistant *Acinetobacter* spp. J. Glob Infect Dis. 2 (3): 291-304.

Melichercíkováa, U.V., and Goroncy B.P.(2010). New Delhi metalloβ-lactamase: a cautionary tale. Journal of Hospital Infection.75: 236–246.

Minond, D., Saldanha,S.A., Spicer, T., Mercer, B.A., Roush, W.R., and Hodder, P. (2012).Prevalence and Risk Factors of Metallo β-lactamase

Producing *Pseudomonas aeruginosa* and *Acinetobacter species* in Burns and Surgical Wards in a Tertiary Care Hospital. J Lab Physicians. 4(1): 39-42.

Ozgumus, O.B, Tosun , C.R., Sandalli, C., Aydin, K., and Koksal,I. (2007).Molecular epidemiology of clinical *Pseudomonas aeruginosa* isolates carrying IMP-1 metallo β-lactamase gene in a University Hospital in Turkey.Microb Drug Resist. 13 (3): 191-198.

Poirel, L, Ozdamar, M, Ocampo-Sosa ,A.A, Türkoglu, S., and Ozer, U.G. (2012).NDM-1-producing *Klebsiella pneumoniae* now in Turkey. Antimicrob Agents Chemother. 56(5).

Poirel, L., Walsh,T.R., Cuvillier, V., Nordman,P.(2011). Multiplex PCR for detection of acquired carbapenemase genes.Diagnostic Microbiology and Infecious Diseas; 70**:**119-123.

Rajput, A., Saxena,r., Singh, K.P., Kumar,V., Singh, S., Gupta, A., and Singh, R.K.(2013). Prevalence and antibiotic resistance pattern of metalloβ-lactamase-producing *Pseudomonas aeruginosa* from burn patients experience of an Indian tertiary care hospital. J Burn Care Res; 31(2): 264-268.

Saderi, H., Zohreh, K. , Parviz, O. , Bahar,M.A., Baghr, S.M. (2008).Phenotypic Detection of Metallo-beta-lactamase Producing *Pseudomonas* aeruginosa strains isolated from burned patients. Iranian Journal of Pathology.;3 (1): 20-24.

Sepehriseresht, S., Pourgholi,L., Habibi,E., Sotoudeh M.A. and Sattarzadeh, T.(2012).Detection of VIM and IMP type metallo β-lactamases in *Pseudomonas aeruginosa* clinical isolates. Arch Iran Med. 15(11): 670 -673.

Tanzinah , N., Shariful, A. Jilani, Lovely B. , and Ashraful, j. H. (2010). Metallo-ß-lactamase Producing *Pseudomonas species* in a Tertiary Care Hospital of Dhaka City.Bangladesh J Med Microbiol . 4 (1): 43-45.

Vaux, S., Carbonne,A., Thiolet, J.M., Jarlier, V., and Coignard, M. (2011). Emergence of Carbapenemase producing Enterobacteriace in France from (2004 to 2011), Surveillance and outbreak reports. 16(22).

Walsh, T. R., Tolereman, M.A. (2005). Metalloβ- lactamase:The quite befor the storm. Clinical Microbiological Review .18: 306-325.

Walsh, T. R. (2011). New Delhi metallo-beta-lactamase-1: detection and prevention. CMAJ. 183(11): 1240-1241.