Comparison between Phenotypic and Genotypic Detection of Metallo Beta Lactamase Enzyme among Gram Negative Bacteria Isolated from Burn Patient

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Abstract:

Background: Acquired metallo β-lactamases (MBLs) are emerging worldwide as powerful resistance determinants in Gram negative bacteria Rapid dissemination and spread between different bacterial species by large gene transfer favor by globalization and travel represent a high risk of worldwide pandemic among Enterobacteriaceae

Objective:This study design to screen for phenotypic detection of MBL and genes coding for metallo β -lactamase(MBL) such as, blaVIM, blaIMP and bla NDM among infected burn wound patients in Sulaimani city /Iraq.

Materials and Methods: Out of 230 burn wound samples, 201 wound swabs and 29 tissue biopsy were collected from hospitalized burn patients with second and third degrees burnfrom Burn and Plastic Surgery Hospital in Sulaimani city from the period of April to October 2011. According to direct gram stain , bacterial cultural , biochemical tests, analytic profile identification(API) system and vitek 2 Compact system , antimicrobial susceptibility Testing by using Kirby-Bauer disk diffusion method and vitek compact system, phenotypic tests for Metalloβ-lactamase by Double disk synergy test and Combined EDTA disk test and Modified Hodge test and also Molecular detection of Metallo β-lactamase enzyme by using polymerase chain reaction.

Results: Out of 230 burn wound samples177 samples gram negative bacteria were isolated; inflammatory cells showed significantly associated with positive bacterial culture. The most frequent bacteria isolated were Pseudomonas species 48(27.12%), in which Pseudomonas aeruginosa account for a higher percentage 46(25.98%) and one species (0.56%) of each P. stutzeri and P.florescence wasisolated followed by Acinetobacterspecies 44 (24.86%) in which Acinetobacterbaumanniiwas the commonest and accounts for a higher percentage 41 (23.16%) and one species(0.56%) of each of A. ursingi, A. hemolyticasandA.complexwere identified. On the other hand in the family of Enterobacteriaceae,the most common bacteria were Klebsiellapneumoniae44(24.86%), Enterobacter cloacae 18 (10.17%) and Escherichia species 11(6.21%). The susceptibility of bacterial isolates against 18 antibiotics from different classes of antibiotics was tested and it was found that most of the isolated species of non fermenter bacteria such as Acinetobacterand Pseudomonas species show multidrug resistant pattern and high resistance against most of the antibiotics commonly used. The most resistant antibiotics against non fermenter bacteria were Ticarcillin (81.82%) against Pseudomonas species and Ticarcillin, Tazobactam- Pipracillin and Cefoxitin (93.18%) against Acinetobacterspecies .The most resistant antibiotic against K. pneumonia andE.cloacaewere Cefoxitin(84.9%)and Amoxicillin –Clavulanic acid (84.9%).The most resistant antibiotics against Escherichia specieswere Cefotaxime, Trimethoprim and Cefipime (90.91%) but the most effective antibiotic with a highsensitivity rate was Imipenem (90.91).Phenotypic tests were carried out for the detection of MBL enzyme for all studied isolates and it was found that Combined disk test was the most sensitive test giving the highest percentage (31.07%) followed by Double disk synergy test(28.8%), Modified Hodge test (20.9%) .Polymerase chain reaction assay was used for genotypic detection of MBL genes (blaIMP,blaVIM, ,blaNDM) in all isolates and the results revealed that the gene blaIMPwas locatedin 33(18.64%),blaVIM,in19 (10.73%), 2(1.12%)forblaNDM Also these genes were detected in 25 stock cultures of Gram negative bacteria preserved in 25 °C since 2008 to 2010. blaIMP gene was detected in11(44%) , blaVIM in 12(48%) and 2(8%) for blaNDM, all these genes were detected in K. pneumonae,P. aeruginosa and A. baumanniiwith length amplified genes (230) bp for IMP and (390)bp for VIM and(621)bp for NDM.

Conclusions:These results indicate that most of the Meropenem resistant strains from infected burn wound strains in this study were producing MBL enzymes 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 who act as carriers for MBL genes in future

Keywords: Metallo beta lactamase, Gram negative bacteria, Genes.

Introduction:

Mortality rates from burn injury vary across regions of the world. Low and middle income countries suffer higher mortality and morbidty rates from burns as these countries require improved surveillance of burn injury via epidemiology studies, which will determine the incidence and prevalence of burn injury among sub populations [(1)]. One of the significant public health problems in many area of the world is burn injury and despite advances in the use of topical and parenteral antimicrobial therapy, bacterial infection remains a major problem in the management of burn victims today [(2)]. A variety of organisms have been isolated from burn wound colonization such as aerobic bacterial isolates ranging from gram-positive organisms like Staphylococcus aureus, and Enterococcus spp. to gram negative organisms such Acinetobacter spp. which are apparently the only group of gram-negative bacteria that may be natural residents of human skin [(3)]. Non-fermenter Gram negative bacilli are ubiquitous in nature with a wide geographic distribution and they are found in most of the environments; soil, water, plants, food stuff and decay vegetation, therefore they prefer moist environment [(4)]. They are common in hospital setting, and may be found on the surface of humidifiers, ventilator machines, dialysis machines and other equipment, as well as from the skin of hospital personnel especially immune compromised. A report by the Infectious Diseases Society of America specifically addressed three categories of multidrug resistance of Gram negative bacilli, namely extended-spectrum cephalosporin resistance E.coli, Klebsiella spp., Pseudomonas spp. and Carbapenem resistance Acinetobacter spp. Although Acinetobacter spp. are frequently resistant to Fluoroquinolones, Aminoglycosides, and all β-lactams with the exception to Carbapenems [(5)], which were considered to be the last drug of choice for the treatment of infections due to Acinetobacter spp. [(6)]. However, resistance to Carbapenems is emerging and represents a significant threat to the management of MDR isolates in many areas of the world [(7)], which were mediated by mechanisms such as hyper-production of a β-lactamase with weak Carbapenems hydrolyzing activity such as AmpC-type Cephalosporinase or an extended-spectrum β-lactamase respectively [(8)]. Metallo beta lactamase 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. There are two dominant types of transferable MBL genes among clinical isolates, blaIMPand blaVIM, which are frequently present on gene cassettes inserted into integrons located on the chromosome or on plasmids [(9)]. New Dehlimetallo 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 blaNDM producing Enterobacteriaceae have been reported to remain susceptible only to Colistin and Tigecycline [(10)]. Some of the blaOXA type β-lactamase variants also have the ability to hydrolyze ESBL, whereas other variants such as blaOXA type, Carbapenemase are able to hydrolyze Carbapenems. The

occurrence of blaOXA type carbapenemase genes in Acinetobacter spp. represents vertical inheritance in specific species but horizontal acquisition in other species [11]. Class D carbapenemase belong to blaOXA family and are commonly present in Acinetobacter spp. and occasionally in Enterobacteriaceae and Pseudomonas spp. These enzymes hydrolyze Carbapenems weakly and are poorly inhibited by Clavulanate [12] which are able significantly to hydrolyze Aminopenicillins such as Ampicillin, Aamoxicillin and Carboxypenicillins such as Carbencillin and Ticarcillin [13].

Methods:

Study population and specimens

Study was carried out in the Burn and Plastic Surgery Hospital in Sulaimani city at a period from April - October 2011. Two hundred and thirty (230) burned patients with suspected burn wound infection of any total body surface area or burn degree were planned to participate in this study. The swabs samples were obtained from deep areas of the burn wound, the samples were taken from the areas which was appear deep with discharge, and thick eschar while for dry wound, the swab was moistened with sterile saline before swabbing.

Sample Prosessing

Out of 230 burn wound samples, 201 wound swabs and 29 tissue biopsy were collected from hospitalized burn patients with second and third degrees burn from Burn and Plastic Surgery Hospital in Sulaimani city from the period of April to October 2011. Out of 230 samples, According to direct gram stain, bacterial cultural, biochemical tests, analytic profile identification (API) system and vitek 2 Compact system. After taking the samples, four quadrant method was used to strike the surface of blood agar, MacConkey agar and Nutrient agar and the growth was inspected after 24 hours of aerobic incubation at 37°C of all the samples [14].

Antibiotics disks

Two different sets of antimicrobial disks were used according to standards institute for antimicrobial susceptibility Testing. One set (14 antimicrobials) for Enterobacteriaceae The second set (14 antimicrobials) for non-fermenter (Kirby-Bauer disk diffusion method) [15], standard bacterial strains of K. pneumoniae ATCC 700603, E. coli ATCC 25922 and P. aeruginosa ATCC 25853 were used as quality control strains.

Phenotypic tests for Metalloβ-lactamase

Double disk synergy test (DDST)

This test is performed by inoculating the tested organism onto muller hinton agar plate. A 10 µgMeropenem disk and a blank filter paper disk 6 mm in diameter were placed 10 mm apart from edge to edge, then, 10 µl of 0.5 M EDTA solution was applied to the blank disk, after 18hours of incubation at 37ºC, the presence of extension of zone towards the impregnated EDTA disk was interpreted as EDTA synergy test positive [16].

Combined EDTA disk test

An overnight broth culture of the test strain with an opacity adjusted to 0.5 McFarland standards was used to inoculate a plate of Mueller-Hinton agar. 4 µml of the sterilized EDTA solution was added to 10 µg Meropenem disk, then the EDTA impregnated antibiotic disks were dried immediately in an incubator and stored at -20 ºC in airtight vials without any desiccants until used after drying of MHA plate, a 10 µgMeropenem disk and meropenem disk combined with EDTA was placed

20 mm apart. After 24 hours incubation at 37 ºC an increase in the zone size of at least 7 mm around the Meropenem combined EDTA impregnated disk compared to Meropenem disks alone was recorded as MBL producing strains[17].

Modified Hodge test

All tested gram negative bacteria were subjected to MHT test according to what was described by [18]. An overnight culture suspension of pan sensitive strain of E.coli was prepared by adding two to three isolated colonies of E.coli strain to 5 ml of normal saline, and the suspension was further diluted by adding 1 ml of suspension to 4 ml of (0.85%) NaCl and the mixture were adjusted to 0.5 McFarland's standard and this suspension was streaked across the entire plate of MHA plate. After drying 10 µg of Meropenem disk was placed at the center of the plate and up to 4 different isolates of gram negative bacteria were streaked linearly from the periphery of the plate into the direction of Meropenem disk at the center and the test plate was incubated for 18 hours at 37ºC. The presence of a 'cloverleaf shaped' zone of inhibition around each tested strain is interpreted as Carbapenemases producing strain or a positive result.

Molecular detection of Metallo β-lactamase enzyme.

All gram negative bacteria isolated from hospitalized burn patient and 25 clone stocks randomly selected from Bacteriology Department reservoir culture of Burn and Plastic Surgery Hospital in Sulaimani in which they were isolated from MDR strains of severely burned patients since 2008 and cultured on clone stock culture and preserved at 25ºC were screened for presence of MBL gene (blaIPM, blaVIM and blaNDM) by multiplex PCR reaction.

Reactivation of bacterial stock culture

For reactivation of bacterial strain, the frozen surface of the a stock culture was scraped with a sterile inoculating needle or tip, and immediately immersed in 2 ml of Tryptic Soya broth and incubated for 24 hours in a shaking incubator with 200 rpm at 37 ºC [19].

Plasmid extraction

Plasmid extraction was performed as recommended by the manufacturer of Gene JET plasmid Miniprep Kit. The column was discarded and purified plasmid DNA concentration was read by Nanodrop®spectrophotmetery via the program of Nanodrp 2000/2000c program. The extracted DNA samples were stored at -20 ºC. All bacterial plasmids were run on (1%) agaros gel for the detection of their pattern. 15 µl of extracted plasmid was mixed with 3 µl of loading dye (6×) and the mixture was loaded into prepared agaros gel and the gel was passed in to the gel tank for 30 minute at a voltage of 1 to 5 volts/ cm2 of the gel size. The results were read using ultra violet light in Gel Documentation System [20].

Polymerase Chain Reaction (PCR)

Polymerase chain reaction mixtures were prepared in duplicates and negative controls were included in each run to validate the reaction. Each reaction mixture was prepared to a volume of 25µl in a sterile PCR tube table (1). Lyophilized primers provided by Cinagen were processed in order to produce a stock concentration of 100 µm by mixing the concentrated lyophilized primer with specific volumes of nuclease free deionized

water according to the primer manufacture (Cinagen). From this stock concentration a working primer concentration of 10µm was prepared by mixing 90µl of deionized H2O with 10µl stock primer.

Table (1): Recommended volumes used to prepare the PCR mixture.

Mixture component	Volume	Final concentration
Green master mix 2 X	12.5 µl	1X
Forward primer 10 µm	1µl	0.1-1 µg/ml
Reveres primer 10 µm	1µl	0.1-1 µg/ml
DNA template	3µl	< 250 ng
Nuclease free Deionized water	To reach 25 µl	

In Multiplex PCR, the reaction was prepared to contain three sets of primers which were first prepared by mixing the revers and the forward of each primer then all the primers were mixed together in a specified volume and these were added to other components of the PCR reaction. Polymerase chain reaction amplification conditions were modified according to the annealing temperature of the primer sets table (2).

Table (2): Primers used in the study.

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			

Agaros gel preparation and gel purification of DNA products

The agaros gel was prepared according to what was performed by Ozeret al., [21], PCR products from agaros gel were recovered by using GeneJETrM Gel Extraction Kit (Fermentas). The content of the kit was prepared prior to use as recommended by the manufacturers.

Sequencing of PCR products

Sequencing of PCR products was done in University of Koya/ Erbil/ Iraq, Genome Centre, and Sequencing Department. The purified PCR product was sent after its purification from agaros in an 1.5 ml Eppendorf tube and 15 µl of both the revers and forward primers was also sent in a separate tube. The sequencing reactions were performed using BigDye® Terminator V3.1 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.

Statistical analysis

Statistical analysis was done by using SPSS program (Statistical Package for Social Science) version 18 Inc., Evanston. Type of work was a descriptive study in which the data were analyzed between factors as categorical

using 2-sided Pearsonchi-square test to see significant differences between them at p value of < 0.05, correlation was performed at (99%) confidence interval.

Result:

Primary cultivation was done for all 230 burn wound specimens in which 201specimens were wound swabs and 29 were tissue biopsies and the growth was detected in 211(91.74%) samples and 19(8.26%) samples were found to be negative table (3) and both types of specimens showed positive cultures that is statistically significant ($p<0.05$).

Out of 211 samples which give positive cultures, 177 isolates of Gram negative bacteria were isolated from both wound swab and tissue biopsies. The most frequently isolated bacteria among burn patients was the group of non glucose fermenting Gram negative bacilli followed by family of Enterobacteriaceae in which Pseudomonas species 48(27.12%) was the most frequently isolated out of all Gram negative isolates. The most frequently isolated species was P. aeruginosa 46 followed by one isolate of each of P. florescence and P. stutzeri. Among the 46 species of P.aeruginosa, 6 isolates showed blue green pigment (pyocyanin) and 2 isolates showed red pigmentation (pyoverdin) with appearance of β-hemolysis over blood agar plate in 32 species. The second common isolated bacteria from burn wound was Acinetobacterspecies account for 44(24.86%) From the family of Enterobacteriaceae, K.pneumoniae was the most frequently isolated bacteria (24.86%) followed by Enterobacter cloacae (10.17) asin figure(1).

Antimicrobial susceptibility tests

All Gram negative isolates were tested for antimicrobial susceptibility testing using disk diffusion methods and the results were interpreted according to standard values provided by Clinical and Laboratory Standard Institute of antimicrobial susceptibility testing (15). Tables (4, 5) illustrate the susceptibility profile for all Gram negative isolates by using 14 antimicrobial agents. Most of Gram negative isolates in this study showed resistance to at least eight of antimicrobial agentswere considered to be multidrug resistant (MDR) isolates. Pseudomonas spp. shows resistance to at least 3 classes and most of resistance were observed against Ticarcillin (81.2 %), followed by Pipracillin – Tazobactam (72.9%), Gentamicin and Tobramycin (70.83%), Cefoxtin (68.75%), Acinetobacter species which revealed high resistant pattern against Cefoxitin (93.18%), Ticarcillin (93.1%) Pipracillin–Tazobactam (93.1%), Trimethoprim (90.9%), Amikacin (90.9 %), and Cefipime (88.4 %). From the family of Enterobacteriaceae, the susceptibility pattern was different. Klebsiella pneumonia showed resistance to Amoxicillin–Clavulanic acid and Cefoxitin (84%), followed by Ticarcillin and Cefazolin (81.8%) and Ceftazidime (79.5).

Out of 177 gram negative isolates that were screened for susceptibility to carbapenem by disk diffusion method, 70(39.5%) bacterial species were carbapenem resistant and from these 66(37.2 %) were Meropenem resistant strains table (6).

IMP: Imipenem, MEM: Meropenem

Double disk synergy test (DDST) were performed by observing synergism between Meropenem disk and EDTA disk figure (2), and this test showed that 51(28.81%) bacteria from all isolated

bacteria isolated from burn patients were positive by this test, and Acinetobacterspecies (15.82%) were the most frequently isolated bacteria observed to be positive by this test among all gram negative bacteria followed by pseudomonas species (8.47%) as in table (7).

Phenotypic detection of MBL enzyme by using combined EDTA disk test CMDT.

This test was performed to detect MBL production. If the zone of inhibition around Meropenem with EDTA was more than 7 mm compared to Meropenem disk alone, the test is considered to be positive figure (3). Among 177 isolates, 55(31.07%) were found to be positive by this test, and (95%) of positive bacteria by this test showed a high rate of zone of inhibition > 20 mm and Acinetobacter species were the most common bacteria observed to be positive by this test (14.69%), followed by Pseudomonas species (14.12), as in table (7). Out of the 177 isolated bacteria tested by this method, only 2 isolates were found to be positive in which both of the strains were Meropenem resistant, while 175 samples were negative, but 51 isolates showed entire growth along the strip and this will be regarded as undermined results.

Phenotypic detection of MBL enzyme by using Modified Hodge test

Modified Hodge test was performed as a screening test for all Gram negative bacilli isolated from burn patients including both meropenem resistant and sensitive strains. Positive test was recorded as indentation of the zone of inhibition along the tested bacteria towards the Meropenem disk as seen in figure (4). MHT test also was detected in 37(20.9%) out of all 177 Gram negative bacteria, from which only 6 isolates were from Meropenem resistant strains and the reaming 31 samples were Meropenem sensitive strains table (7).

Molecular detection of MBL genes by PCR

Plasmid extractions were performed for all 177 isolated Gram negative bacteria from infected wounds of burn patients and 25 bacterial stocks. Genes responsible for metallo β-lactamase (MBL) can be detected by using polymerase chain reaction (PCR). Several possible genes in this study such as blaVIM, blaIMP and blaNDMwere targeted to be screened by multiplex PCR mixture figure (5).

Polymerase chain reaction products were resolved using (1%) agarose gel electrophoresis and suspected bands were gel purified and sent for sequencing. Three blaVIMfromP. aeruginosawere 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. The amplified PCR of blaVIM product belongs to one of several genes of MBL integrated into P. aeruginosa, Elizabeth kingia meningo septica, A. bereziniaer or other organisms. When the reverse primers sequence data were uploaded into the BLAST query, the same results of MBL were obtained. When sequence data from three blaVIM 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, figures (6, 7). Concering sequencing blaIMP a nd

NDM gene products, sequencing failed to show any results and this will need further work to confirm these products. Detection of blaIMP gene was observed in 33(18.6%) Gram negative isolates with implified gene (230) bp from which 29 isolates were Meropenem resistant and 4 were Meropenem susceptible while blaVIM gene was detected from 19(10.7%) Gram negative bacteria with implified genes (390) bp from which 14 isolates were Meropenem resistant and 5 were Meropenem susceptible strains as presented in table 8. Concerning blaNDM with implified genes (621) bp. Two possible products were amplified and one of them was isolated from multidrug resistant Klebsiella pneumonia and the other one from Meropenem sensitive strain of Klebsiella pneumonia figure (8). PCR assay screening for MBL gene was performed for all 177 isolated Gram negative bacteria and 25 Gram negative stock samples which were stored at 25 °C since 2008-2010 in laboratory of Emergency and Plastic Surgery Hospital in Sulaimani. Out of 177 Gram negative bacteria isolated from burn patients, 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 as in table (9). Statistically significant correlations were found between PCR results and Meropenem susceptibility ($p< 0.05$).

Genotypic detection of MBL gene in Gram negative bacteria isolated from stocks culture.

Polymerase chain reaction was performed for the detection of different MBL genes (blaIMP, VIM, NDM) in 25 stock samples since 2008, and all these stocks were from multidrug resistant strains of severely burned patients from ICU and ABU in Emergency and Plastic Surgery Hospital in Sulaimani. MBL genes were isolated form 17 Gram negative out of 25 Gram negative stock cultures. Out of 25 stock samples from different Gram negative bacteria, 17(68%) strains were MBL producers by PCR from which blaIMP, account for 11(44%), bla VIM12 (48%) and 2(8%) for blaNDMas clarified in table (9) with length amplified genes (230) bp for IMP and (390) bp for VIM and (621) bp for NDM. The times of isolation of five strains date back into 2008, three strains from 2009, and nine strains from 2010. MBL genes were detected among 6(24%) isolates of P. aeruginosa, 5(16) of A.baumannii and 14(32%) were K. pneumoniae as figure (9) and table (9).

Comparing the phenotypic and gene detection methods, it was found that each test showed different results for MBL. The highest percentage of phenotypic detection of MBL (20.9%) was observed by using CMDT, and the highest percentage of MBL gene such as blaIMP 30(16.9) was mostly observed among A.baumannii 19(10.7) as seen in table 10. while bla VIM genes were detected in 19(10.73%) among all Gram negative isolates specifically P. aeruginosa (5%). Statistically, no significant relations were observed between each gene with phenotypic test ($p>0.05$).

Table (3): Relation of bacterial culture with types of specimens.

Specimens	Positive culture	Negative culture*	Total
	No.(%)	No.(%)	No. (%)
Wound swab	192 (83.48)	9 (3.91)	201 (87.39)
Tissue biopsy	19 (8.26)	10 (4.35)	29 (12.61)
Total	211 (91.74)	19 (8.26)	230 (100)

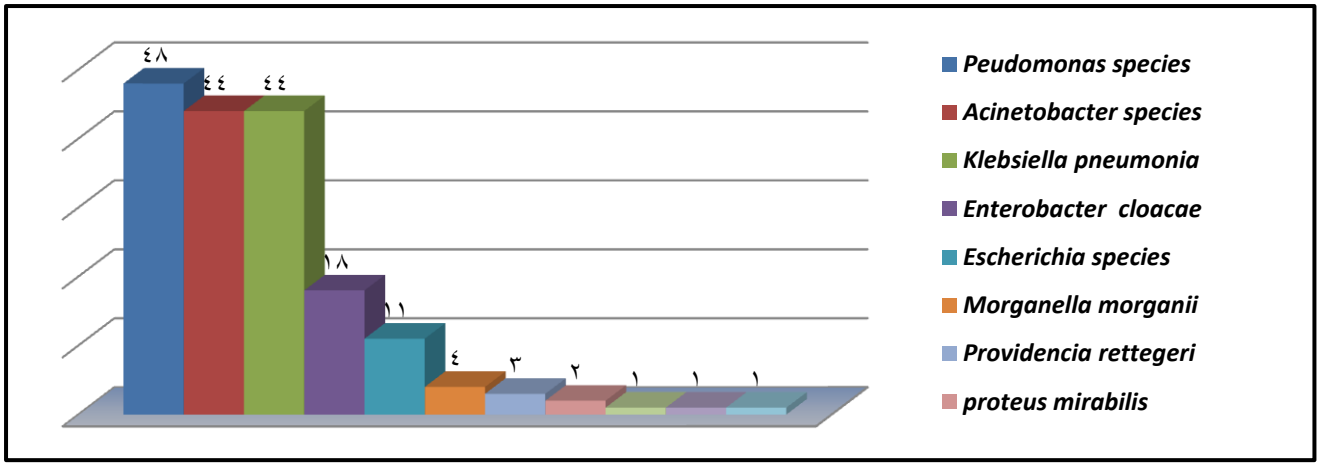


Figure (1): Number of Gram negative bacteria isolated from hospitalized burn patients.

Table (4): Antibiotics Susceptibility of Enterobacteriaceae.

Antibiotic names		*Klebsiella*			*E.coli*			*Enterobacter*			*Morganella*			*Providencia*			*Proteus*		
		R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
AN	No.	21	4	19	5	0	6	6	1	11	0	2	2	1	0	2	0	0	2
	%	47.73	9.09	43.18	45.45	0	54.55	33.33	5.56	61.11	0	50	50	33.33	0	66.67	0	0	100
ATM	No.	34	1	9	9	1	1	12	0	6	4	0	0	1	0	2	2	0	0
	%	77.27	2.27	20.45	81.82	9.09	9.09	66.67	0	33.33	100	0	0	33.33	0	66.67	100	0	0
CZ	No.	36	1	7	9	0	2	15	0	3	4	0	0	1	0	2	2	0	0
	%	81.82	2.27	15.91	81.82	0	18.18	83.33	0	16.67	100	0	0	33.33	0	66.67	100	0	0
CTX	No.	34	2	8	10	0	1	13	0	5	4	0	0	1	0	2	2	0	0
	%	77.27	4.55	18.18	90.91	0	9.09	72.22	0	27.78	100	0	0	33.33	0	66.67	100	0	0
IMP	No.	5	1	38	1	0	10	0	1	17	1	0	3	0	0	3	1	1	0
	%	11.36	2.27	86.36	9.09	0	90.91	0	5.56	94.44	25	0	75	0	0	100	50	50	0
MEM	No.	6	1	37	2	0	9	1	0	17	0	0	4	0	0	3	0	0	2
	%	13.64	2.27	84.09	18.18	0	81.82	5.56	0	94.44	0	0	100	0	0	100	0	0	100
CAZ	No.	35	1	8	8	1	2	11	0	7	4	0	0	1	0	2	1	1	0
	%	79.55	2.27	18.18	72.73	9.09	18.18	61.11	0	38.89	100	0	0	33.33	0	66.67	50	50	0
TMP	No.	31	4	9	10	0	1	8	3	7	1	0	3	1	0	2	1	1	0
	%	70.45	9.09	20.45	90.91	0	9.09	44.44	16.67	38.89	25	0	75	33.33	0	66.67	50	50	0
TZP	No.	31	0	13	6	1	4	14	0	4	0	3	1	1	0	2	2	0	0
	%	70.45	0	29.55	54.55	9.09	36.36	77.78	0	22.22	0	75	25	33.33	0	66.67	100	0	0
AMC	No.	37	0	7	9	1	1	16	0	2	4	0	0	1	0	2	2	0	0
	%	84.09	0	15.91	81.82	9.09	9.09	88.89	0	11.11	100	0	0	33.33	0	66.67	100	0	0
CIP	No.	9	5	30	5	1	5	6	5	7	0	1	3	1	0	2	1	0	1
	%	20.45	11.36	68.18	45.45	9.09	45.45	33.33	27.78	38.89	0	25	75	33.33	0	66.67	50	0	50
FEP	No.	34	0	10	10	0	1	12	1	5	1	2	1	1	0	2	2	0	0
	%	77.27	0	22.73	90.91	0	9.09	66.67	5.56	27.78	25	50	25	33.33	0	66.67	100	0	0
FOX	No.	37	0	7	7	1	3	13	2	3	0	4	0	1	0	2	2	0	0
	%	84.09	0	15.91	63.64	9.09	27.27	72.22	11.11	16.67	0	100	0	33.33	0	66.67	100	0	0
CM	No.	34	0	10	7	0	4	13	1	4	1	0	3	1	0	2	2	0	0
	%	77.27	0	22.73	63.64	0	36.36	72.22	5.56	22.22	25	0	75	33.33	0	66.67	100	0	0
TC	No.	36	0	8	7	0	4	12	1	5	4	0	0	0	0	3	0	0	2
	%	81.82	0	18.18	63.64	0	36.36	66.67	5.56	27.78	100	0	0	0	0	100	0	0	100

Table (5): Antibiotics Susceptibility of non fermenter gram negative bacteria.

Antibiotic Name		*Pseudomonas*			*Acinetobacter*			*Barkholderia*			*Achromobacter*			*Ralastonia*	
		R	I	S	R	I	S	R	I	S	R	I	S	R	S
AN	No	29	2	17	40	0	4	1	0	0	1	0	0	1	0
	%	60.42	4.17	35.4	90.91	0	9.09	100	0	0	100	0	0		
FEP	No	32	1	15	39	2	3	0	0	1	1	0	0	0	0
	%	66.67	2.08	31.3	88.64	4.55	6.82	0	0	100	100	0	0		
COL	No	3	0	45	5	1	38	0	0	1	0	0	1	0	1
	%	6.25	0	93.8	11.36	2.27	86.4	0	0	100	0	0	100	0	100
IMP	No	11	5	32	21	5	18	0	0	1	0	0	1	0	0
	%	22.92	10.4	66.7	47.73	11.36	40.9	0	0	100	0	0	100	0	0
MEM	No	25	3	20	30	4	10	0	0	1	0	0	1	0	1
	%	52.08	6.25	41.7	68.18	9.09	22.7	0	0	100	0	0	100	0	100
TZP	No	35	1	12	41	0	3	1	0	0	1	0	0	1	0
	%	72.92	2.08	25	93.18	0	6.82	100	0	0	100	0	0	100	0
TOB	No	34	0	14	36	0	8	1	0	0	1	0	0	1	0
	%	70.83	0	29.2	81.82	0	18.2	100	0	0	100	0	0	100	0
CIP	No	24	5	19	36	5	3	0	1	0	0	1	0	1	0
	%	50	10.4	39.6	81.82	11.36	6.82	0	100	0	0	100	0	100	0
CAZ	No	31	0	17	36	5	3	1	0	0	0	1	0	1	0
	%	64.58	0	35.4	81.82	11.36	6.82	100	0	0	0	100	0	100	0
CM	No	34	3	11	35	1	8	1	0	0	1	0	0	1	0
	%	70.83	6.25	22.9	79.55	2.27	18.2	100	0	0	100	0	0	100	0
TC	No	39	0	9	41	0	3	1	0	0	1	0	0	1	0
	%	81.25	0	18.8	93.18	0	6.82	100	0	0	100	0	0	100	0
CTX	No	33	0	15	43	0	1	1	0	0	1	0	0	1	0
	%	68.75	0	31.3	97.73	0	2.27	100	0	0	100	0	0	100	0
AMS	No	25	4	19	11	4	29	1	0	0	1	0	0	0	1
	%	52.08	8.33	39.6	25	9.09	65.9	100	0	0	100	0	0	0	100
TMP	No	26	5	17	40	2	2	0	1	0	1	0	0	1	0

Table (6): Numbers and percentages of Carbapenem resistance among Gram negative bacteria.

Bacterial species	IMP No. (%)	MEM No.(%)	Both No.(%)	Carbapenem resistance No. (%)	Total
Pseudomonas species	1(0.56)	17(9.6)	10(5.65)	28(15.82)	48 (27.12)
Acinetobacter species	1(0.56)	10 (5.65)	20(11.3)	31(17.51)	44 (24.86)
Klebsiellapneumonia	1(0.56)	2(1.12)	4(2.25)	7 (3.95)	44 (24.86)
Enterobacter cloacae	-	1(0.56)	-	1 (0.56)	18 (10.17)
Escherichia coli	-	1(0.56)	1(0.56)	2 (1.11)	11 (6.21)
Morganellamorganii	1(0.56)	-	-	1 (0.56)	4 (2.25)
Providenciarettegeri	-	-	-	-	3 (1.69)
Proteus mirabilis	-	-	-	-	2 (1.12)
Barkholderiapseudomallei	-	-	-	-	1 (0.56)
Achromobacterxylosoxidans	-	-	-	-	1 (0.56)
Ralastoniapauculae	-	-	-	-	1 (0.56)
Total	4 (2.26%)	31 (17.51%)	35 (19.77%)	70 (39.55)	177 (100)

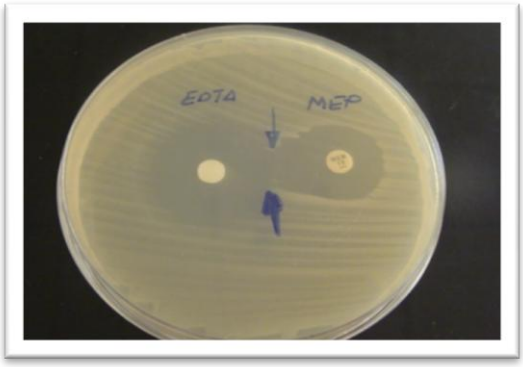
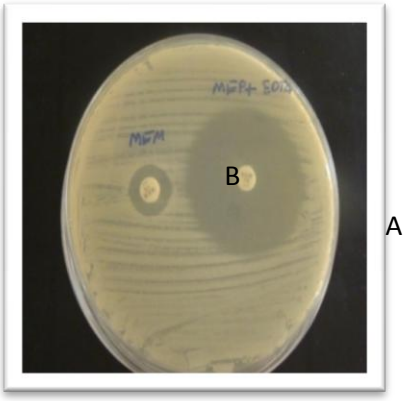
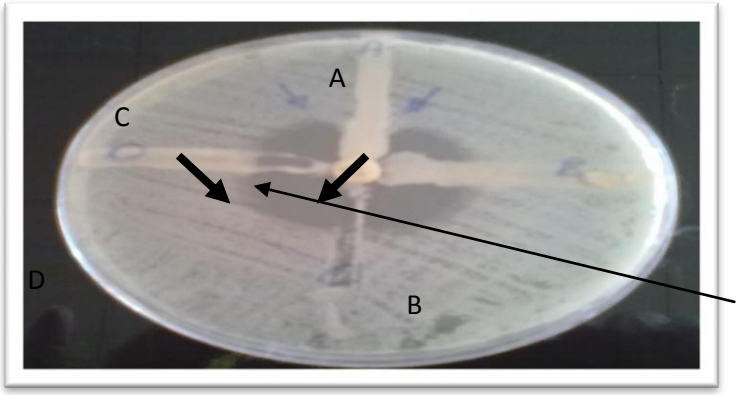


Figure (2): Double disk synergy test for MBL.positive MBL test indicated by synergy between Meropenem disk and EDTA disk (black arrow).



A

Figure (3): Combined EDTA disk testA: EDTA mixed with Meropenem disk, B: Meropenem disk, positive MBL test indicated by zone of inhibition around combined disk is > 7mm to that of meropenem disk alone.



Meropenem disk

Figure (4): Photograph of Modified Hodge test.
A: *Klebsiellapneumoniae* (positive), B: *Acinetobacterbaumannii* (negative), C: *Pseudomonas aeruginosa* (negative), D: *E. coli* (negative). Positive MBL: indentation of the zone of inhibition along the tested bacteria (A) designated by black arrow towards Meropenem disk.

Table (7): Phenotypic test for MBL detection.

Meropenem resistance Bacterial species	Positive phenotypic test		
	Hodge test	DDST	CMD
pseudomonas aeruginosa	8	12	25
Acinetobacterbaumannii	7	22	26
Barkholderiapseudomallei	0	0	0
Klebsiella pneumonia	4	3	3
Enterobacterclocca	0	0	0
Escherichia coli	1	1	1
Total	20	38	55

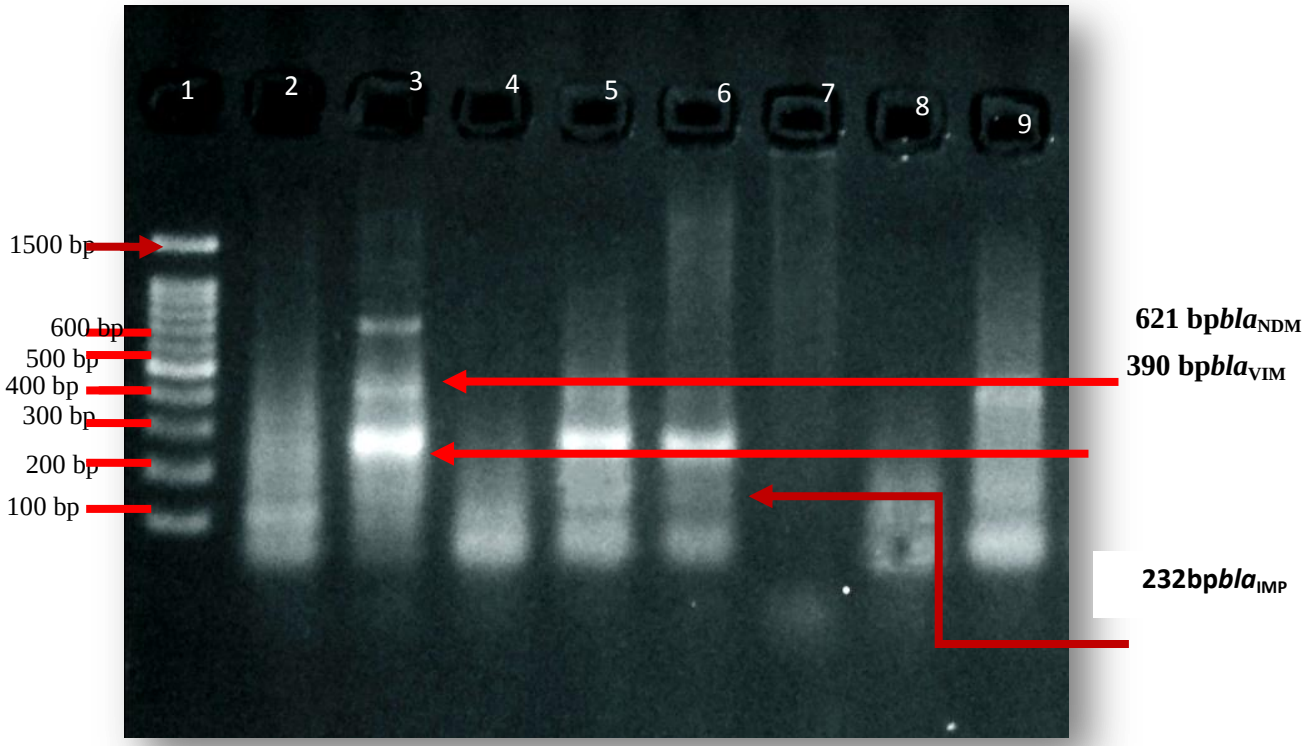


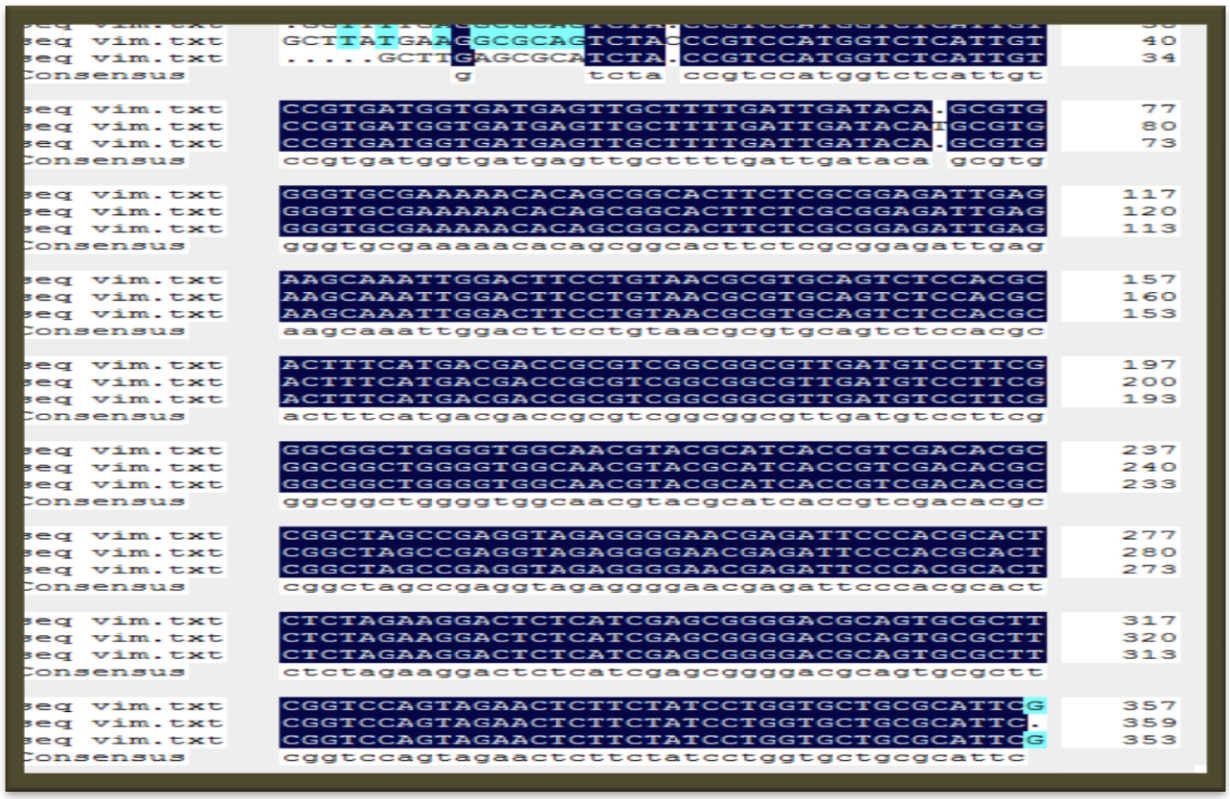
Figure (5): PCR products for MBL genes.

A: 1. DNA ladder (1 Kbp), 2: Klebsiellapneumoniea negative, 3. K. pneumoniae positive blaIMP, VIM, NDM (red arrow), 4. Pseudomonas aeruginosanegative, 5. AcinetobacterbaumanniiblaIMP, bla VIM positive, 6. AcinetobacterbaumanniiblaIMP positive, 7 and 8: Acinetobacterbaumanniinegative, 9. Pseudomonas aeruginosapositive blaVIM.

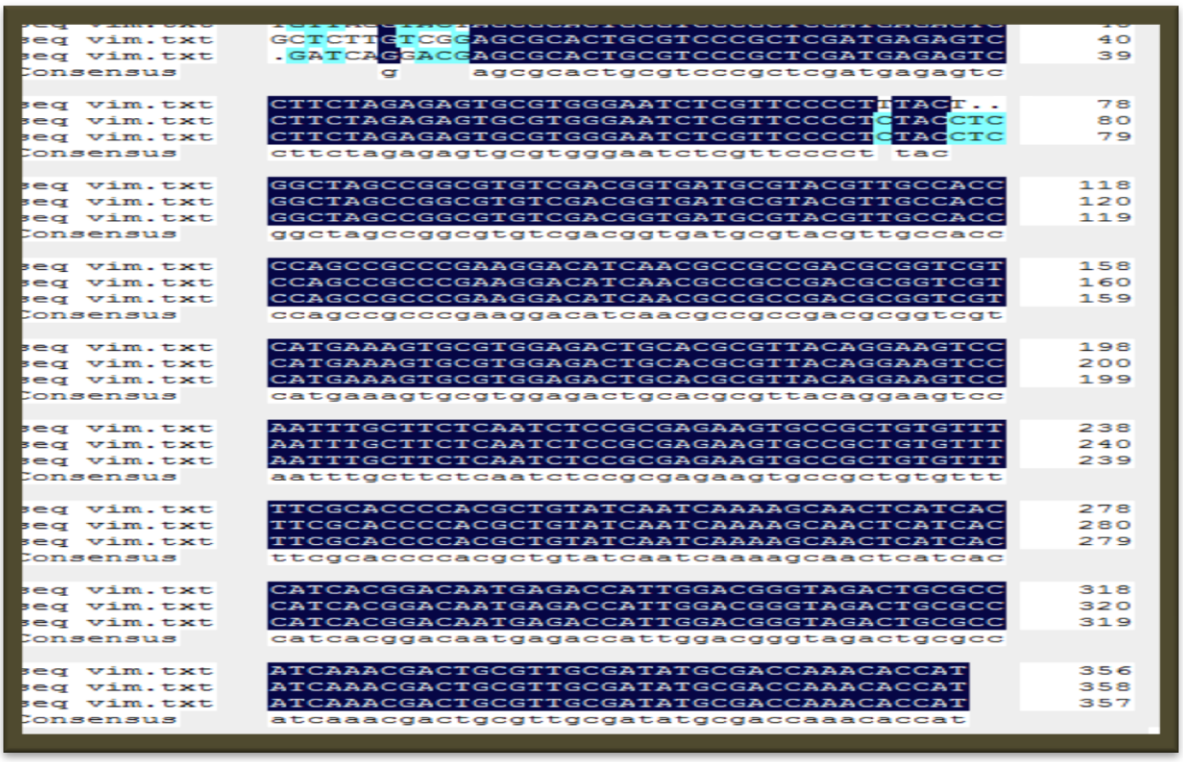
Table (8): Different MBL genes amplified using PCR .

		MBL	genes		
Bacterial species	*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 pneumonia	7 (3.95)	5 (2.82)	2 (1.1)	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 (6): Sequence alingment of three sequence data from forward primers of *bla*$_{VIM}$. Using DNAMAN sequence alingment, A homology of (96.77%) was obtined.



Figuers (7): Sequence alingment of three sequnece data from revers Primers of *bla*$_{VIM}$ gene. DNAMAN sequence alingment. A homology of (98.14%) was obtined.

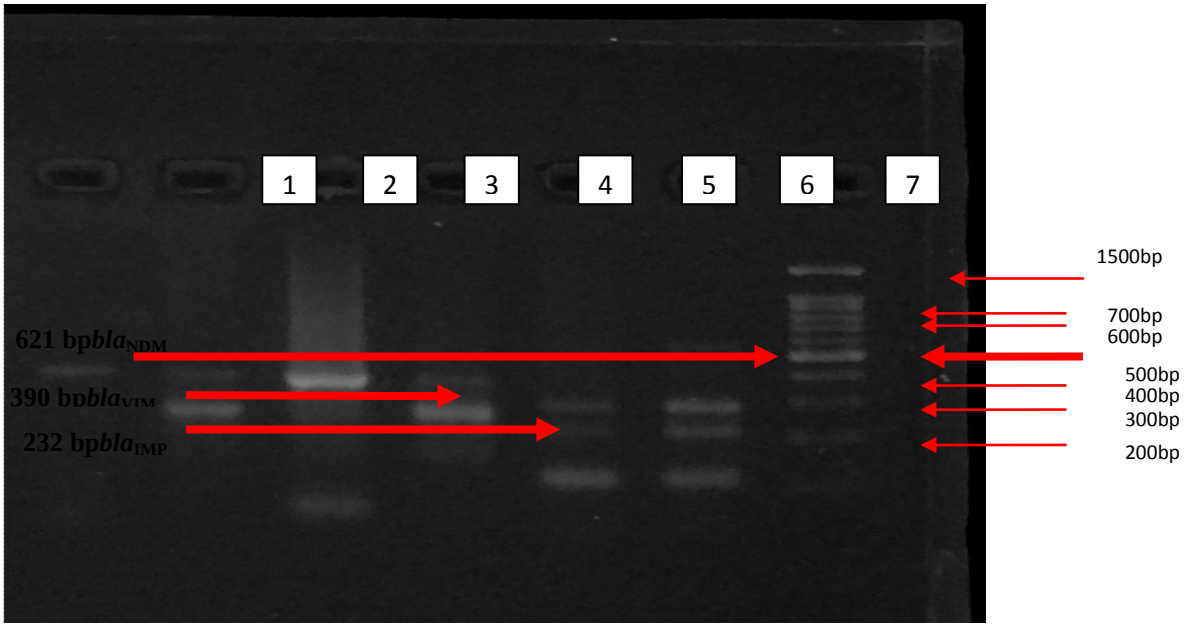


Figure (8): PCR product on Gel electrophoresis for MBL genes among burn patients. lane 7: DNA ladder , lane 6 : *bla*$_{NDM}$ positive *K.pneumoniae*, lane2, 3, 5, 6 *bla*$_{IMP}$ positive *P. aeruginosa*and *A. baumannii*, lane 4,1 :390 bp*bla* VIM positive (red arrow) *P. aeruginosa.*

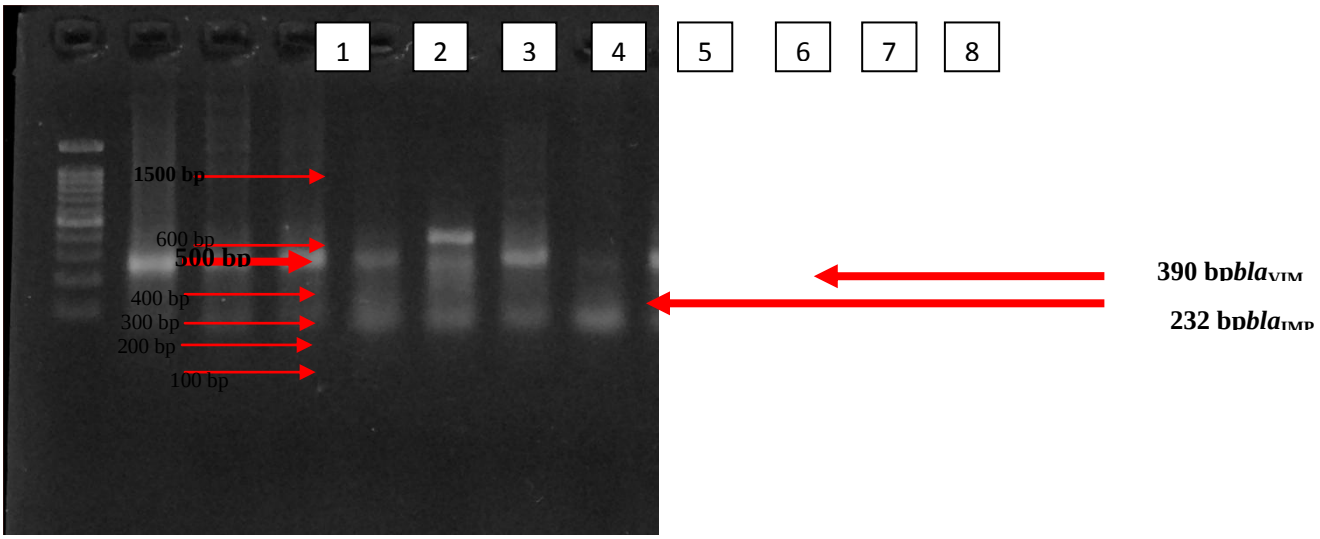


Figure (9): PCR product among stock cultures.
Lane 1: DNA ladder, 2, 3, 4, 5, bla_{IMP} positive *A.baumanii* and *P.aeruginosa* , lane 6: bla_{VIM} positive *K.pneumonia*,

Table (9): MBL genes detection by PCR among stock culture.

		MBL	genes		
Bacterial species	$bla_{IMP,}$ No. (%)	bla_{VIM} No.(%)	bla_{NDM} No.(%)	Total gene isolated	Total bacterial No.
Pseudomonas aeruginosa	3 (12)	3 (12)	-	6 (20)	6 (24)
Acinetobacterbaumannii	2 (8)	3 (12)	-	5 (16)	5 (20)
Klebsiellapneumonae	6 (24)	6 (24)	2 (8)	14 (32)	8 (32)
Enterobacter cloacae	-	-	-	-	3 (12)
Escherichia coli	-	-	-	-	3 (12)
Total	11 (44)	12 (48)	2 (8)	25 (100)	25 (100)

Relation of PCR with different phenotypic tests used for the detection of MBL genes.

Table (10): Prevalence rate of MBL enzyme by phenotypic and PCR assay among burn patients.

Gram negative bacteria	Positive MBL genes			positive phenotypic test of MBL			Total No. of Gram negative
	No. (%)			No. (%)			
	bla_{VIM}	bla_{IMP}	bla_{NDM}	DDST	CMDT	MHT	
Pseudomonas species	9 (5.08)	7 (3.95)	-	15(8.47)	25(14.12)	8(4.51)	48 (27.12)
Acinetobacter species	5 (2.82)	19 10.73)	-	28(15.82)	26(14.69)	9 (5.08)	44 (24.86)
Klebsiella pneumonia	5 (2.82)	7 (3.95)	2 1.13)	7 (3.95)	3 (1.69)	18 (10.17)	44 (24.86)
Enterobacter cloacae	-	-	-		-	1 (0.56)	18 (10.17)
Escherichia species	-	-	-	1 0.56)	1 (0.56)	1 (0.56)	11 (6.21)
Morganellamorganii	-	-	-	-	-	-	4 (2.26)
Providenciarettegeri	-	-	-	-	-	-	3 (1.69)
Proteus mirabilis	-	-	-	-	-	-	2 (1.12)
Burkholderiapseudomallei	-	-	-	-	-	-	1 (0.56)
Achromobacterxylosoxidans	-	-	-	-	-	-	1 (0.56)
Ralastoniapaucula	-	-	-	-	-	-	1 (0.56)
Total	19(10.73)	33(18.64)	2 1.12)	51 (28.8)	55 (31.07)	37 (20.9)	177 (100)

DDST: Double disk synergy test, CMDT: combined disk test, MHT: Modified hodge test, E-test: Episilometer test, *bla* :β- lactamase gene , VIM: Verona integrin metallo β- lactamase, IMP: Imipenemase, NDM: new delhimetallo β- lactamase. p >0.05

Discussion:

One of the risk factors for bacterial infection of burn is the duration of hospitalization, as the mean of hospital stay in this study was 9 days which was a too long period for the acquisition of bacteria from different sources such as the environment or patients in burn units. This was in agreement with study done in Tunisia by Fekih et al. [22]. Other risk factors are acquisition of infection from relatives of patients or health care workers, inadequate sterilization of the burn surface which provides a suitable site for bacterial colonization and multiplication that is rich in protein as it was opened into environment and is easily contaminated [23]. The risk factors for nosocomial infection among burn patients are unlike to what were concluded in Turkey by Alp et al., [24] in which advanced age, and underlying disease were found to be risk factors for nosocomially infection among burn patients. The relative frequency of bacterial isolation in burn patients varies among different studies and different countries. In this study, Gram negative bacteria (76.9%) were isolated from burn wound infection but it was higher than what was recorded in Erbil (43%) by Hussen [25], in Karbalae (67.44%) by Kawkab, [26], in which Gram negative bacteria were regarded as a common microorganism isolated from burn patients but with different prevalence rate that may be due to differences in samples size.

In the present study, P. aeruginosawas the most frequently isolated gram negativebacteria (27.1%) over all gram negative bacteria from burn patients, which was in agreement with studies done in Hilla from Iraq by Nktel et al., [27] and in India [28] in which Pseudomonas was the most commonly isolated microorganism but with high prevalence rate in India (55%) and in Iraq (32%),The second bacteria isolated in this study wasAcinetobacter spp. (24.86%) as A. baumanniiwas the commonest isolates and mostly occur among seriously ill patients indicating that the group of non- glucose fermenting gram negative bacteria was the most frequently isolated among hospitalized burn patients. This is similar to what was concluded by Vitkauskienėet al. [29] in which prevalence rate of (34%) among burn patients, This results disagree entirely to what was identified in United Kingdom regarding types of microorganisms isolated among burn patients as a cause of burn wound infection such as Stenotrophomonasmaltophilia, Vibrio species Chryseobacteriumspecies, Alcaligenesxylosoxidans [30] which might be due to changing microorganisms in this country regarding microbial flora or environmental distribution of Gram negative bacteria.

Colonization of skin also remains one of the risk factors for infection by Acinetobacterspecies as recorded in the studies done by Balkhyetal., [31] in Sudia Arabia in which colonization of the skin prior to the burn injury was regarded as one of the risk factors for burn wound infection. The family of Enterobacteriaceae comes after the non fermenter group in which K. pneumonia was the most frequent bacteria (24.8%) followed by E. cloaca (10.2%), E. coli, M. morganiiand Proteus species. Most of the bacterial isolates in this study were similar in pattern to bacteria isolated from other studies in Iraq such as Baghdad and Mosel and Saudi Arabia [32] in that Enterobacteriaceae comes

after the group of non fermenter apart from absence of Acinetobacterspecies while there are other studies done in neighboring countries such as Turkey by Oncul et al., [33] which recorded the pattern of gram negative bacteria among burn patients with P. aeruginosaasthe commonest isolates followed by K. pneumoniae, A. baumanniiandproteusspp. But in Iran the same patterns of Gram negative bacteria were recorded with addition of E.coli [34]. The reason for this high prevalence of gram negative microorganisms may be related to their virulence factors, and prevalence in hospital environment, to the source of infection that may arise from overcrowding in the hospital wards, frequent visitors to the burn patients, colonization by normal flora such Acinetobacter species.

The result of antimicrobial sensitivity showed that P. aeruginosa and all other non-fermenter gram negative bacilli were highly resistant to Pipracillin, Tazobactum and Ticarcillin in the range of (72-81%) and for third generation cephalosporin from (63-69%). This is in agreement with a study done in India in which resistance against third generation Cephalosporin such as Ceftazidim (68%) was recorded [28] while Pseudomonas species were sensitiveagainst Colistin and Imipenem. Our result in this study that in agreement with what was done by Branskiet al., [35] in Texas in which Colistin was the sensitive drug (100%) against all MBL producing Pseudomonasspecies. Resistance was highly detected against most of the currently used antimicrobial drugs such as Cefotaxim, Cefoxintin, Ciprofloxacillin making the outcome serious regarding treating the patients in burn unit. These results were in agreement with those done in USA, Iran and Nigeria in which most of the bacteria isolated from burn unite were MDR and posses resistance to floroquinolon and third generation cephalosporin drugs while Imipenem drugs give sensitivity (66.67%) for Pseudomonas specieswhich was higher to a study done in Tunisia by Zoghlamiet al,. [36] as they were recording (31%) sensitivity to Imipenem drug.

Carbapenems still remain a major issue in the management of hospitalized patients and are increasingly used because of the emergence of ESBL in Enterobacteriaceae, particularly among E. coli and K. pneumonia [37] also as the last resort for treating infections caused by organisms producing ESBL such as P. aeruginosa and antibiotic resistant Acinetobacterspp. [38]. This jeopardizes the effective use of Carbapenem and may lead to the generation of a new class of Gram negative “superbugs” of particular concern which are often commensals. So, the risk of spread from asymptomatic carriers outside of the hospital setting is high [39]. In this study, Carbapenem resistance was detected in 70(30.4%) among Gram negative bacteria isolated from burn patients and 66(28.6%) of these isolates were Meropenem resistant strains. These results unlike the study done in Turkey in which Carbapenem still remains the therapeutic choice in the burn hospital [40]. Out of 70 Carbapenem resistant strains, 30 isolates of Acinetobacter spp. and 28 of pseudomonas spp. were meropenem resistant strains. This indicates that among Gram negative bacteria isolated from burn patients, 58(82.8%) were non fermenter Gram negative bacilli with Meropenem resistant strains which disagrees with

what was recorded in India by Goel et al., [41] in which Meropenem resistant was only observed among Acinetobacterspecies (18%) while all Pseudomonas specieswere sensitive to it. Another study done in China [24] revealed that all A. baumannii isolated from burn patients were (100%) sensitive to Meropenem. It was clear that most of the Meropenem resistance isolates in this study showed resistance to more than 6 drugs from different classes of antibiotics that were designated as MDR strain. This finding was the same as other studies done in Tehran, Israel, Sweden and Sanandaj from Iran [4] and the reasons for the increase in prevalence of MDR are multi factorial and it will differ according to the geographical area such as endogenous colonization or exogenous spread from hospital environment having a high burden of MDR patients, ground water or other water sources contaminated because of inadequate waste management or sewage disposal system and miss uses of antibiotic [43]. The importance of surveillance is underscored by the fact that the appearance of acquired carbapenemase in different countries has been associated with imported cases, mainly due to the transfer of patients from geographical areas where this problem is widely established. Demographic change, environmental changes, improved medical technology, bacterial evolution, and the breakdown of public health systems, urban migration.

Double disk synergy test detect MBL in 51(28.8%) out of all gram negative bacterial strains in which (95%) of Meropenem resistant isolates showed a significant zone of enhancement toward EDTA disk which was in agreement with a study done in Ahwaz and Teheran [44] which detected (26.5%), Beside using DDST, other tests, such as CMDT, were used which show high positive results in (98%) of Meropenem resistance strains with zone of inhibition > 7 mm. Interpretation of the CMDT assay results is more objective than that of the DDST results according to what was concluded previously [45], because the DDST depends upon the technician's expertise in discriminating true synergism from intersection of inhibition zone whereas CMDT is easy to be interpreted as it needs no synergisms just an increase zone of inhibition around the combined Meropenem disk by EDTA. The prevalence rate of CMDT in the current study was 55(31.07%) among all Gram negative bacteria which was in agreement with that done by Eser et al., [46] which found (76%) positive CMDT among Gram negative bacteria but it disagrees with what was recorded by Guptaet al., [47] in which the sensitivity of CMDT was equal to that of E-test. MHT was detected in 37(20.9%) isolated Gram negative bacteria from which 20(30.3%) isolates were from Meropenem resistance strains and 19(17.1%) from Meropenem sensitive strains that were isolated from different units in burn hospital. These results indicate that the positive MHT test among Meropenem sensitive strains was indicative of MBL positive strains or presence of other types of β-lactamase enzymes such as class A Carbapenemase KPC which is in agreement to studies done in India and Iran [48] which concluded that MHT test is a good tool for the diagnosis of MBL even in small percentages (12% and 16%). Out of all Gram negative isolates, K. pneumonia was found to be positive for MHT test at 17(9.6%) out of all

Meropenem sensitive and resistant strains. This result was similar to study done in Pakistan but with a higher MHT positive (27%) among Gram negative bacteria isolated from burn patients [18] while lower percentage of MHT positive test was observed among non fermenter group such as Acinetobacter and Pseudomonas species which were lower than K. pneumonia in the current 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. For blaVIM gene, three different amplicons from different isolates were sequenced and the data showed homology to genes documented to be for MBL resistance gram negative bacteria and all three amplicons, when aligned together, show homology of more than (96%) indicating to be for the same gene in different isolates. In this study, the prevalence of MBL production by using PCR was different according to each gram negative bacteria isolated among burn patients but in general it was demonstrated in 46(25.9 %) isolated among all gram negative isolates in which 39(22 %) isolated were from Meropenem resistant strains and 7(3.9%) from Meropenem sensitive strains. These results indicate that most of the Meropenem resistant strains from infected burn wound strains 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 were 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 [28]. 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 who 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. Our results revealed that there was a difference in the prevalence of MBL production among each Gram negative bacteria isolated. (56.8%) of A.baumannii were MBL producer by PCR reaction which was higher in comparison to other bacteria in the hospital although the main mechanism of Carbapenem resistance in Acinetobacter species is class D blaOXAcarbapenemase specifically blaOXA51 like which is intrinsic to the most species of Acinetobacterbaumannii [49]. 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 [50] 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 blaIMP, bla VIM, and bla NDM were isolated from the current study. The most frequently detected gene was blaIMP gene 33(18.6%) isolate among all Gram negative bacteria isolated from burn patients. Similar results were recorded in Turkey and Spain [51] in which the detection of MBL genes (blaIMP, bla VIM) were observed but in lower percentages to that of our study being (2.5%) and (11%) respectively. 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 blaIMP gene which is consistent with a study done in Turkey at different periods observing (39%) which are much lower than our results [46]. On the other hand, bla VIM gene was recorded in 19(10.7%) among all Gram negative bacteria isolated from burn patients. 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, [52] and the first report published from newly hospital in Iraq from Baghdad by Huang et al., [53] in which they recorded bla VIM to be (11%) and (12.3%) respectively. Though bla VIM is distributed all over the world, different areas in the world were recorded to carry this gene blaVIM especially in neighboring countries at various prevalence rates lower than our study which were (2.3%) for bla VIM-2 in Teheran [54], and (6.7%) in India [55]. 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. There were 2 cases of newest superbug gene blaNDM (NDM) depending on the amplicon size of the PCR products (621bp). All of the two isolates were from Klebsiella pneumonia thatneed further work, due to the fact that the PCR reaction for MBL detection in this study was multiplex reaction which faced difficulty during sequencing process. This superbug gene disseminates rapidly in all the parts of the world as it was identified in thirteen countries in Europe [56] over the last three years, and recorded by researchers in the world, majority of cases were in the UK [57]. Apart from European countries, blaNDM gene was disseminated in all other neighboring countries such as one case report in Iran by Gaibani et al., [58], Turkey by Poirel et al., [59] and there was a single report from each of Israel, Oman, United Arab Emirates and Iran, but they were few in numbers in comparison to blaVIM and blaIMPgenes. Although blaNDM gene is a new type of MBL gene but it is present and it is likely that the number of cases reported is underestimated in most countries in which they do not have an idea about the gene and confirmation tests that are routinely performed for MBL or there may be diagnosed cases but they were not notified by Reference Laboratories. Till now there are no published cases in Iraq but there are two cases reports published in France and Lebanon from Iraqi patients travelling via medical tourists to these countries. They were diagnosed as MDR strains with blaNDM gene positive. This result indicates that this new superbug gene was present in Iraq and is regarded as a source of international disseminations and may be

this MDR gene is transmitted to our locality from tourists of other neighboring countries such as Iran, Turkey and South and Middle of Iraq and transmission mostly occurs by plasmid caring genes encoding for MBL enzyme. Regarding those Meropenem resistant bacterial strains that give negative results for PCR reaction for MBL genes, the causes might be due to the presence of other types of MBL gene that are responsible for β-lactamase production such as class A Klebsiella pneumonia carbapenemase (KPC) enzyme or the presence of blaoxagenes which are specific to A.baumanniiespecially blaoxa 23, and blaoxa 48, and blaoxa 51(60). Apart from being Carbapenem-resistant, all MBL positive strains were resistant to important groups of tested antimicrobials, including third generation Cephalosporins, Aminoglycosides and which are a characteristic feature of MBL-producers which supports the results recorded in Iran by Peshattiwar and Peerapur [(61)] as they recorded resistant isolates to multiple classes of antimicrobial agents in spite of MBL positive. All positive MBL in this study had hospital stay for more than 9 days and with an invasive device such as IV line, urinary catheter which was regarded as a major risk factor in combination of TSAB of the MBL patients.

MBL genes were also isolated from different Gram negative bacterial stock culture from previous years since 2008 in Emergency and Plastic Surgery Hospital in Sulaimani indicating that MBL genes were present in this hospital and were circulating in environment of the hospital. MBL genes were isolated form 17 samples out of 25 stock cultures selected randomly among Gram negative bacteria, dating back to 2008, 2009 and 2010 indicating that these genes were present since these years and continuously circulate in the hospital environment that acts as source of MBL genes in this hospital. The microorganisms that were positive for MBL gene from stock culture were the same of the current study.

In this study, the evaluation of simple phenotypic tests for MBL detection such as DDST, CMDT, MHT was done for the comparison of the efficacy and sensitivity of PCR for MBL gene detection. It was found that (94.8%) of all Gram negative bacteria with MBL positive by PCR were positive by CMDT and (64.1%) were recorded for DDST, but only (28.2%) of MBL positive by PCR were positive by MHT test which is in agreement with two studies performed in India. One of them observed the strong relation between the phenotypic tests and PCR results but with restriction of the study to the Pseudomonas species only, and (100%) sensitivity of DDST observed among PCR positive and (92%) for E-test [(62)], The rate of sensitivity was higher than this study which may be due to the fact that all the tested strains were MDR non fermenter group in contrast to this study in which all isolated Gram negative bacteria (Meropenem resistant and Meropenem sensitive strains) were screened by PCR.

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