Molecular Detection of Medically Important Carbapenemases Genes Expressed by Metallo-β-lactamase Producer Isolates of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*

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Abstract

Background and Objectives: The increase in antibiotic resistance among *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* especially through the production of metallo-β-lactamases (MBLs) puts the use of the carbapenems for therapeutic purposes at risk. The study aimed for preliminary screening and phenotypic confirmatory tests for the production of MBLs. Further, to investigate the role of bla_{VIM}, bla_{NDM}, and bla_{IMP} in addition to bla_{OXA-48} and bla_{KPC}, respectively, in the resistance to carbapenems. Furthermore, to detect gene expression represented coproduction of the assigned enzymes encoding genes using real-time polymerase chain reaction (RT-PCR). **Patients and Methods:** A total of 100 specimens had been taken from 92 patients. All of these specimens were examined by microbiological culture technique. Both double disk synergy (DDS) and combined disk (CD) techniques were used as a confirmatory test for MBL production. Gene expression for Class A, B, and D MBLs was detected by RT-PCR. **Results:** Out of 34 potential MBLs producer clinical isolates of *P. aeruginosa* and *K. pneumonia*, 21 (62.0%) and 6 (18.0%) were positive for DDS test, respectively. All isolates (100%) of the potential MBLs producer isolates of *P. aeruginosa* and *K. pneumonia* were positive for the presence of meropenem alone plus meropenem-EDTA. Molecular assay revealed that a total of 26 (96.0%) of *P. aeruginosa* were confirmed as bla_{OXA-48} producer isolates. Further, out of 27 MBL positive *P. aeruginosa*, 25 (93.0%) were confirmed as bla_{VIM} producer isolates. Further, all isolates of *K. pneumonia* (100%) were confirmed as bla_{VIM} but no gene expression observed for bla_{IMP} P and bla_{KPC} C against *P. aeruginosa* and *K. pneumonia*. Further, our study result yielded that out of 27 *P. aeruginosa* positive isolates for MBL, 21 (78.0%) were confirmed as bla_{NDM} producer isolates. Furthermore, our study result revealed that out of 7 *pneumonia* positive isolates for MBL, 6 (86.0%) were confirmed as bla_{VIM} producer isolates. Coproduction of bla_{VIM} and bla_{OXA-48} encoding genes was produced by all study isolates of *K. pneumoniae*. **Conclusion:** CD test is more preferred than double-disk synergy test for phenotypic confirmatory test for checking carbapenemases production. Further, bla_{VIM}, bla_{NDM}, and bla_{OXA-48} carbapenemases play an important role in resistant to carbapenems represented by imipenem and meropenem among study isolates of *P. aeruginosa* and *K. pneumonia* using RT-PCR. Coproduction of bla_{VIM} and bla_{OXA-48} encoding genes was detected in all study isolates of *K. pneumonia*. The expression of bla_{VIM}, bla_{OXA-48}, and bla_{NDM} genes in the study isolates at high proportion by *K. pneumonia*. bla_{OXA-48} could be the reason why these isolates were endemic in Iraqi patients.

Key words: bla_{IMP}, bla_{KPC}, bla_{NDM}, bla_{OXA-48}, bla_{VIM}

INTRODUCTION

The infections caused by multidrug-resistant *Pseudomonas aeruginosa* and *Klebsiella pneumonia* result in high morbidity and mortality around the world[1] *P. aeruginosa* has numerous intrinsic and acquired mechanisms of drug resistance.[2] The increase in antibiotic resistance among Gram-negative bacteria including *Klebsiella* species

and *P. aeruginosa* especially through the production of metallo-β-lactamases (MBLs) puts the use of the carbapenems for therapeutic purposes at risk. Beta-lactamases are important components of the antimicrobial resistance mechanism found in Gram-negative bacteria including *P. aeruginosa* and *Klebsiella* species.[3] MBLs are a type of carbapenemases that hydrolyze the carbapenems including imipenem, meropenem, ertapenem, and render them inefficacious for treatment. They are β-lactamases that belong to Ambler's Class B type of enzymes, and they degrade a wide variety of β-lactams including penicillins, cephalosporins, and carbapenems by hydrolyzing the amide bond of the β-lactam ring.[4]

Carbapenems are considered the last-line drugs for the treatment of infections caused by multi-resistant Gram-negative bacilli. The emergence of carbapenem-resistant organisms such as *P. aeruginosa* and *Klebsiella* species has become a major therapeutic challenge. Carbapenems resistances may result from decreased outer membrane permeability, exclusion from the cell by efflux pumps, changes of the penicillin-binding protein, and production of β-lactamase.[5] Pathogenic bacteria that produce MBLs are usually susceptible to aztreonam, a monobactam. However, bacterial organisms that express MBLs and other multidrug resistance enzymes are indeed a great threat and of clinical importance, since these organisms are usually resistant to a wide variety of antibiotics especially the β-lactams which are important agents used clinically for the treatment of bacterial related infections.[6]

It is well recognized that imipenem-resistant *P. aeruginosa* (IRPA) is a current and significant concern, especially because of the limited therapeutic options for this pathogen. MBL enzymes may play a critical role in IRPA, given that there is a high possibility of these carbapenemases being spread among nosocomial isolates.[7] The genes responsible for MBL production may be chromosomal- or plasmid-mediated, and they pose a threat of horizontal gene transfer among other Gram-negative bacteria in their environment.[8]

Despite the presence of double disk synergy (DDS) and combined disk (CD) techniques and their ubiquitous use as phenotypic confirmatory tests, but the continuous requirement for more different inhibitor combinations is still dependent. In addition to that, EDTA-based techniques showed high sensitivity but low specificity for detecting carbapenemases.[9] Thus, this poor specificity of EDTA-based tests is worrisome given that a real-time polymerase chain reaction (RT-PCR) is a cutoff diagnostic tool for the production of a variety of MBLs. Therefore, RT-PCR was used in our study to verify whether the isolates harboring MBL genes produced the enzyme.[7] Thus, this study has been undertaken for preliminary screening and phenotypic confirmatory tests for the production of MBLs using both of DDS test and CD. Furthermore, to detect which predictor is more preferred for the detection of MBLs by CD technique using meropenem and/or imipenem. Further, to investigate the role of Class B MBLs which includes verona-integron MBL (VIM), New Delhi MBL (NDM), and imipenemase-MBL (IMP) in addition to Class D and Class A MBLs represented by OXA-carbapenemases and *K. pneumonia* carbapenemases (KPC), KPC, respectively. Furthermore, to investigate the presence or absence of gene expression represents coproduction of enzymes encoding genes using RT-PCR.

PATIENTS AND METHODS

Specimens collection

A total of 100 specimens had been collected from 92 patients admitted to Al-Yarmouk Teaching Hospital and Al Karkh General Hospital during the period from March to December, 2017. The ages of the study patients were ranged from 1 to 78 years with mean 24.46 ± 18.77. The study specimens were distributed as follow: 89 out of 100 specimens (89%) were from a wound (diabetic foot infections, osteomyelitis, and burns infections) and other wound infections; furthermore, 8(8%) urine from catheterized urinary tract infection and 3 (3%) ear swab from a patient with otitis media. All bacteriological examinations and confirmatory biochemical tests were done according to methods and techniques mentioned by Leboffe and Pierce;[10] Procop *et al.*;[11] and Bailey and Scott's.[12]

Antimicrobial susceptibility test

Antimicrobial susceptibility test was done by Kirby–Bauer technique according to the methods mentioned by Clinical and Laboratory Standards Institute, 2017. The bacterial inoculum used in this technique was adjusted and standardized using McFarland turbidity standards. McFarland 0.5 turbidity standard was prepared by added specific volumes of 1% sulfuric acid and 1.175% barium chloride to obtain a barium sulfate solution with a specific optical density. The solution was dispensed into tubes comparable with those used for inoculum preparation, which was sealed tightly and stored in the dark at room temperature. McFarland 0.5 standard provided turbidity comparable with a bacterial suspension containing approximately 1.5×10^8 colony-forming unit/ml.[13,14]

Preparation of EDTA solution

A 0.5 M EDTA solution was prepared by dissolving 0.93 g of disodium EDTA.2H2O in 5 ml of distilled water and adjusting it to PH 8.0 using NaOH. The mixture was sterilized by autoclaving.[15,16] Whatman filter paper No 3 was selected to prepare EDTA and EDTA + Imipenem discs.[17] Using a one-hole puncher, paper discs with an approximate diameter of 6 mm were punched out one by one from a sheet of paper. Since the paper discs had a tendency to curl after punching, these were flattened by spreading them in a single layer on a clean smooth surface then pressed by rolling a bottle

repeatedly. The discs were placed in vials then autoclaved for 15 min at 15 PSI (Ibs) pressure and allowed to cool. The disks were allowed to dry in the incubator at 35°C for 2–3 h without covering them. The disks were stored in a freezer at −14–18°C.[17]

Preliminary screening test for MBL production

The B-lactamase producer isolates were screened for their susceptibility to amoxicillin, amoxicillin-clavulanic acid, cephalexin, cefoxitin, cefotaxime, ceftazidime, piperacillin, and metallo-β-lactams represented by imipenem and meropenem. 5.0 ml of brain heart infusion broth medium was inoculated at 37°C for 3–4 h (mid-log phase). 10 µl of broth suspension was transferred to Mueller–Hinton agar plates and spread with a sterile swab on the agar surface. The selected antibiotic disks were placed with sterile forceps the on the inoculated plates and incubated at 37°C for 18 h in an inverted position. After incubation, the diameter of inhibition zones was noted, measured and interpreted according to Clinical and Laboratory Standards Institute (CLSI).[14]

Confirmatory test for metallo-βeta-lactamase production

MEM and IMP EDTA CD test

EDTA-imipenem or meropenem disks were prepared by adding 10 µl of MBL inhibitor solution (0.5 M EDTA) was added to 10-µg imipenem or meropenem disks to obtain a concentration of 750 µg. The disks were dried immediately in an incubator and stored at 4°C in an airtight vial without desiccant. The study bacterial isolates were adjusted according to McFarland 0.5 turbidity standard and were inoculated to Mueller-Hinton agar. A 10-µg-imipenem or meropenem disk and imipenem or meropenem plus 750 µg EDTA were placed on Mueller-Hinton agar. Another disk containing only 750 µg EDTA was also placed as a control. After overnight incubation, if the difference of inhibition zone between carbapenem disk and carbapenem-EDTA was ≥7mm, the isolate was considered as an MBL-producer.[18,19]

DDS test (DDST)

An overnight broth culture of the test strain (opacity adjusted to 0.5 McFarland opacity standards) was lawn cultured on a Mueller-Hinton Agar plate. 0.5M EDTA was added to the blank filter paper and was left them to dry. 10 µg imipenem or meropenem disk and a blank filter paper disk (6 mm diameter, Whatman filter paper) were placed on the Mueller-Hinton agar plate. The distance between the inhibitor and the substrates was tested at 1.5 cm from center to center. Enhancement of the zone of inhibition in the area between the antimicrobial agents and the inhibitor disk was considered to indicate positivity for MBL.[20-22]

DNA extraction

SaMag bacterial DNA extraction kit was used with SaMag-12 automatic nucleic acid extraction system for extraction of genomic DNA from study isolates (Samaga, Cepheid, Italy). The extraction process was consisted of steps of lysis, binding, washing, and elution. The prepared nucleic acids were suitable for RT-PCR which was used in this study consequently. Sample preparation requirements were highly dependent on the type of starting materials. The buffer BL2 was specialized for bacterial cell wall lyse at which it was used to re-suspend the bacterial pellet before process extraction.

Isolation of genomic DNA from bacterial suspension cultures

1 ml from bacterial culture was pipetted into a 1.5 ml microcentrifuge tube and centrifuged at 5000 × g for 5 min. The supernatant was discarded. After that, 220 µl of buffer BL2 was added to pellets and mixed by vortexing for 5–10 s. A 200 µl suspension had been taken to sample tube.

Isolation of genomic DNA from bacterial plate culture

A bacterial colony was taken from culture plate with an inoculation loop and was suspended in 220 µl of buffer BL2 by vigorous stirring. A 200 µl suspension was taken to sample tube.

Measurement of DNA concentration

Quantus fluorometer was used to detect the concentration of extracted DNA to detect the goodness of sample for downstream applications. For 1 µl of DNA, 9 µl of diluted quantus flour dye was mixed. After 5 min incubated at room temperature, DNA concentration values were detected.[22]

RT-PCR for Class B MBLs (bla_{VIM}, $bla_{NDM,}$ and bla_{IMP})

MBL (bla_{VIM}, $bla_{NDM,}$ and bla_{IMP}) RT-PCR kit uses "hot-start," which greatly reduces the frequency of nonspecifically primed reactions. The MBL VIM, IMP, NDM, and internal control (IC) groups were detected in the FAM/Green, HEX/Yellow, CY5/Red, and Texas/red fluorescent channels, respectively.[23]

All reagents of the kit thawed and vortexed, before starting work. The required number of PCR tubes had been taken for amplification of clinical and control samples (negative control of extraction and negative and positive controls of amplification). The reaction mixture was prepared, mixed in a new sterile tube as follows: 10 µl of PCR-mixed-FRT MBL, 5 µl of RT-PCR-mixed-2, and 0.5 µl of *Taq* DNA polymerase. Then, the mixture thoroughly was vortexed, and 15 µl of the prepared reaction added to each PCR tube. Then, 10 µl of DNA samples isolated from the clinical samples to each PCR tube was added. The control reactions were run as follow: In

negative control, 10 μl of the DNA sample extracted from the negative control was added to the tube labeled NCE (Negative Control of Extraction) while in positive control 10 μl of Pos2 MBL (C+) was added to the tube labeled C+ (positive control of amplification. 10 μl of DNA-buffer was added to the tube labeled as negative control of amplification.

RT-PCR for Class D and Class A MBLs represented by bla_{OXA-48} and bla_{KPC}

MBLs (bla_{OXA-48} and bla_{KPC}) RT-PCR kit uses "hot-start," which greatly reduces the frequency of nonspecifically primed reactions. The carbapenemase group KPC is detected in the FAM/green channel while the carbapenemase group OXA-48-similar (OXA-48 and OXA-162) is detected in the HEX/Yellow channel. The IC is detected in the Texas red channel.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences for Windows (Version 18.0, SPSS Inc., Chicago, IL, USA). Statistical significance was taken with the threshold $P < 0.05$. The significant differences were detected using the goodness fit test within nonparametric statics including Chi-square (cross tabulation) test, Chi-square test was used to study the association (dependence) between β-lactamase production and minimal inhibitory concentration of an imipenem by Vitek2 system, and these types of the genes (bla_{IMP}, bla_{IMP1}, and OprD) were used in the present study for two types of bacterial isolates (*Acinetobacter baumannii* and *Pseudomonas* spp.). All the study graphics (bar chart, scatter diagram, or dot chart) were done using Microsoft Excel 2016 - Windows 10.[24]

RESULTS

A total of 100 specimens obtained from study patients were included in this study. They were admitted to Yarmouk and Al-Karkh Teaching Hospitals and distributed into 58 (58%) male, and 42 (42%) female with a male to female ratio was 1:1.38. The age of these patients was ranged between 1 and 78 years with a mean of 24.46 ± 18.77. Out of these specimens, 65 (65%) were positive for culture while 35 (35%) were negative. Identification of isolates at species level was done by biochemical tests and analytical profile index 20 E system. The negative culture cases were attributed to the reasons that some patients were under antimicrobials chemotherapy during culture time.

Out of 65 (65%) culture-positive specimens, 38 (58.5%) were *P. aeruginosa*, 23 (35.3%) were *K. pneumoniae*, 2 (3.1%) were *Escherichia coli*, and 2 (3.1%) were Gram-positive cocci including *Staphylococcus aureus*. Out of 38 (85.5%) isolates of *P. aeruginosa*, 38 (100%), 37 (97.4%), 38 (100%), 29 (76.3%), and 38 (100%) were resistant to amoxicillin, amoxicillin-clavulanic acid, cephalexin, ceftazidime, and

piperacillin, respectively, while one (2.6%) and one (2.6%) were intermediately resistant to amoxicillin-clavulanic acid and ceftazidime, respectively. Eight (21.1%) out of 38 (85.5%) isolates of *P. aeruginosa* was sensitive to ceftazidime. Regarding *K. pneumoniae*, out of 23 (35.4%) these isolates 23 (100%), 22 (96%), 22 (96%), and 21 (19%) were resistant to amoxicillin, cefoxitin, cefotaxime, and ceftazidime, respectively, while 1 (4%) and 1 (4%) were sensitive to cefoxitin and cefotaxime, respectively. Two (9%) out of 23 (25.4%) of *K. pneumoniae* was intermediate resistant to ceftazidime.

Preliminary screening test for detection of MBLs against clinical isolates of *P. aeruginosa*

Out of 38 clinical isolates of *P. aeruginosa*, 21 (55%) and 29 (76%) were resistant to imipenem and meropenem, respectively, while 14 (37%) and 9 (24%) were sensitive for both of above antibiotic, respectively. On the other hand, three (8%) out of 38 (85.5%) clinical isolates of *P. aeruginosa* were intermediately resistant to imipenem [Figure 1].

Out of 23 clinical isolates of *K. pneumoniae*, 6 (28.57%) and 18 (78.3%) were resistant to imipenem and meropenem, respectively, while 8 (38.09%) and 4 (17.4%) were sensitive for both of above antibiotics, respectively. Seven (33.3%) and one (4.4%) out of 23 (35.3) isolates of *K. pneumoniae* were intermediately resistant to imipenem and meropenem, respectively [Figure 2].
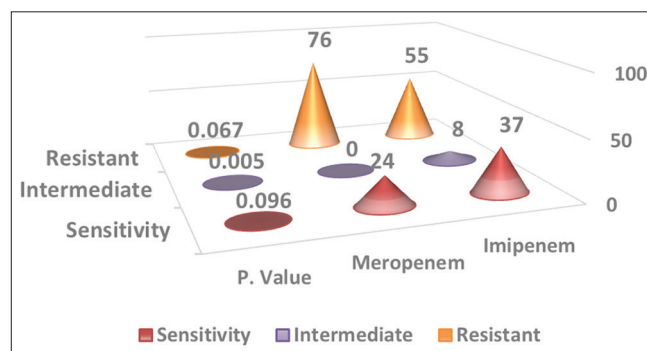

Figure 1: The screening test for metallo-β-lactamases among study isolates of *Pseudomonas aeruginosa*
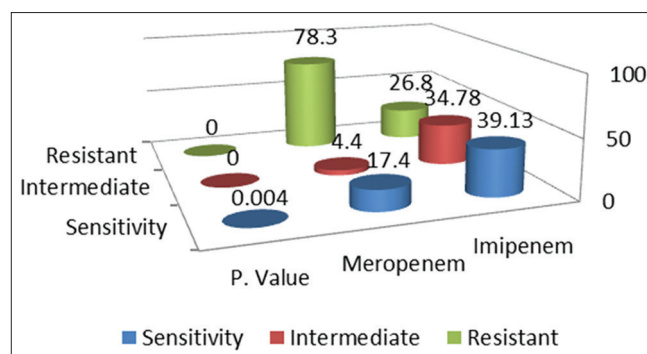

Figure 2: The screening test for metallo-β-lactamases among study isolates of *Klebsiella pneumonia*

Phenotypic confirmatory test for detection of MBLs

DDS

Out of 34 potential MBLs producer clinical isolates of *P. aeruginosa* and *K. pneumonia*, 21 (62%) and 6 (18%), were positive for DDS test, respectively, while the remaining 6 (18%) and 1 (3%) of *P. aeruginosa* and *K. pneumonia* were negative for this test, respectively [Figure 3].

CD test

A total of 34 of potential MBLs producer isolates of *P. aeruginosa* and *K. pneumoniae* were examined by three techniques in a CD as follows:

Imipenem only or imipenem-EDTA

Out of the above isolates, 16 (47%) were positive for this test while 18 (53%) were negative. The mean of zone of inhibition of imipenem alone was 8.2 ± 2.0, and the distance for imipenem plus EDTA was 18.93 ± 2.25, and the mean of differences between imipenem alone and imipenem plus EDTA was 10.73 ± 2.12, and this result is supported by statistical analysis at which there are huge significant differences ($P = 0.000$) [Figures 4 and 5].

Meropenem only or meropenem-EDTA

The results revealed that all isolates (100%) of potential MBLs producer of both *P. aeruginosa* and *K. pneumoniae* were positive for the presence of meropenem only or meropenem-EDTA (The difference between imipenem alone and imipenem-EDTA was ≥ 7 mm) [Figures 4 and 5].
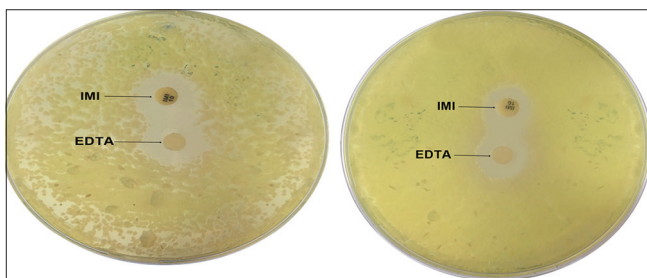

Figure 3: Phenotypic confirmatory test for production of metallo-β-lactamases among study isolates using double-disk synergy test

Imipenem only or imipenem-EDTA and meropenem only or meropenem-EDTA

In this section, the study results revealed that 16 (47%) of the studied clinical isolates of *P. aeruginosa* and *K. pneumoniae* were positive for this test when they were exposed to imipenem only or imipenem-EDTA and meropenem only or meropenem-EDTA at the same time. The remaining 18 (53%) were positive in the presence of meropenem plus meropenem-EDTA, but at the same time, they were negative when they were submitted to imipenem only or imipenem with EDTA.

Molecular detection of the MBLs producer clinical isolates of *P. aeruginosa* and *K. pneumoniae*

The concentration of extracted DNA measured by an automated machine, Quantus fluorometer was ranged from 7.3 to 94 with mean 47.70 ± 24.9. The extracted DNA was submitted to amplification to most medically important Classes (A, B, and D) MBLs and the results as follows:

Class D (bla$_{OXA-48}$) and Class A MBLs (bla$_{KPC}$)

The results showed that out of 27 MBL positive isolates of *P. aeruginosa*, 26 (96%) were confirmed as bla_{OXA-48} producers while one (4%) had no detectable expression for this gene. On the other hand, all isolates (100%) of *K. pneumoniae* were produced bla_{OXA-48}. The most striking result is that no gene expression was observed for Class-A KPC against clinical isolates of *P. aeruginosa* and *K. pneumoniae*. This result was reflected by Figures 6-8.

Class B-MBLs (bla$_{VIM}$, bla$_{NDM}$, and bla$_{IMP}$)

Class B MBLs producer isolates of *P. aeruginosa* and *K. pneumonia* were submitted to RTPCR to confirm whether these isolates encoded by Verona integron-encoded-MBL (bla_{VIM}) genes. The study result revealed that out of 27 *P. aeruginosa* positive isolates for MBL, 25 (93%) were confirmed as bla_{VIM} producer isolates, while two (7%) of these isolates were not expressed these genes [Figure 9]. No bla_{IMP} encoding genes for all study isolates were observed. Further, all isolates of *K. pneumonia* (100%) were genetically expressed bla_{VIM} encoding genes.
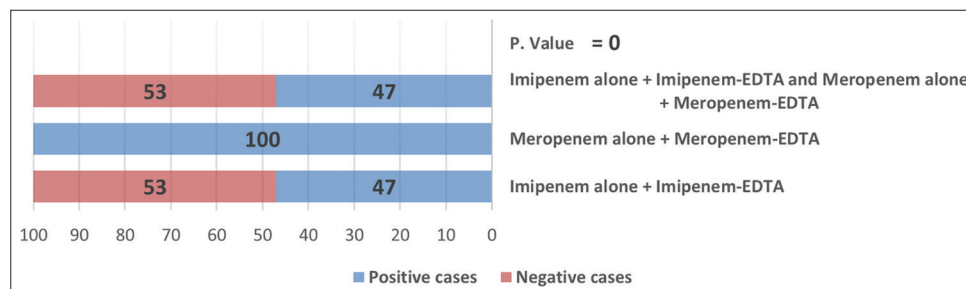

Figure 4: Optimization of a combined disk using three techniques among study isolates of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*
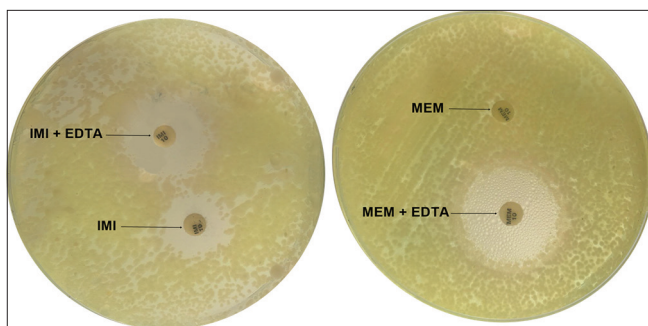
Figure 5: Phenotypic confirmatory test for production of metallo-β-lactamases among study isolates using combined disk
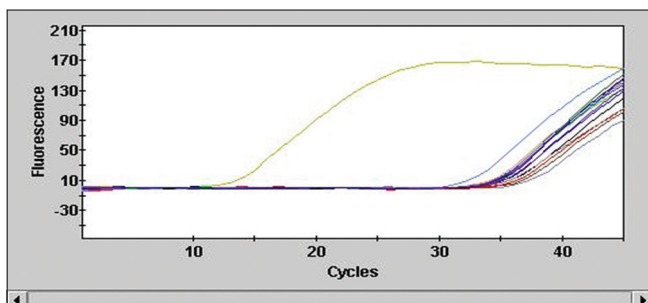

Figure 6: Detection of profile carbapenemase bla$_{OXA}$-48 cases in HEX fluorescent channel, yielded by real-time polymerase chain reaction
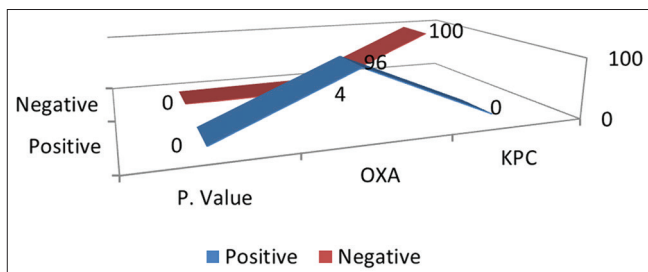

Figure 7: Distribution of positive and negative cases of bla$_{OXA}$-48 and bla$_{KPC}$ among study isolates of *Pseudomonas aeruginosa*

It was found that out of 27 *P. aeruginosa* positive isolates for MBL, 21 (78%) were confirmed as *bla*$_{NDM}$ producer isolates, while six (22%) had no expression for this gene. Furthermore, the result showed that out of seven *K. pneumoniae* positive isolates for MBL, 6 (86%) were confirmed as *bla*$_{NDM}$ producer isolates. It was found that out of 27 P. aeruginosa positive isolates for MBL, 21 (78%) were confirmed as blaNDM producer isolates, while six (22%) had no expression for this gene. Furthermore, the result showed that out of seven K. pneumoniae positive isolates for MBL, 6 (86%) were confirmed as blaNDM producer isolates [Figure 10].

According to the study result of RT-PCR against clinical isolates of *P. aeruginosa* for Class A, B, and D carbapenemases, the results can be grouped as follows: Group I: 18 (66.66%) of clinical isolates were positive for *bla*$_{VIM}$, *bla*$_{NDM}$, and *bla*$_{OXA-48}$ and negative for *bla*$_{IMP}$ and *bla*$_{KPC}$ genes. Group β: 1 (3.70%)
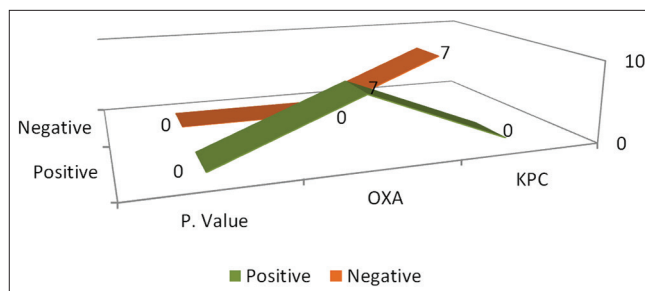

Figure 8: Distribution of positive and negative cases of bla$_{OXA}$-48 and bla$_{KPC}$ among study isolates of *Klebsiella pneumonia*
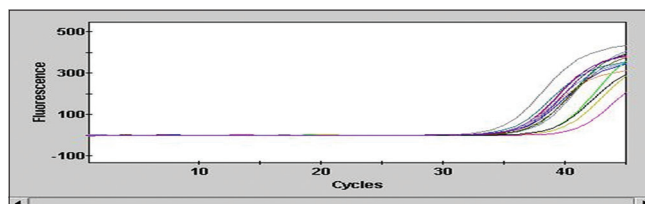

Figure 9: The amplification curve of carbapenemases bla$_{VIM}$ cases in FAM fluorescent channel detected by real-time polymerase chain reaction
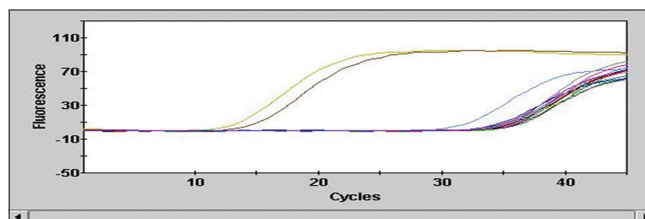

Figure 10: The amplification curve of bla$_{NDM}$ in RED fluorescent channel detected by real-time polymerase chain reaction

of clinical isolates revealed which were positive for *bla*$_{VIM}$ and *bla*$_{OXA-48}$ and negative for *bla*$_{IMP}$, *bla*$_{NDM}$, and *bla*$_{KPC}$ genes. Group χ: 1 (3.70%) of clinical isolates were positive only for *bla*$_{OXA-48}$ and negative for *bla*$_{VIM}$, *bla*$_{IMP}$, *bla*$_{NDM}$, and *bla*$_{KPC}$ genes. Group β: 1 (3.70%) of clinical isolates were positive for *bla*$_{NDM}$ and *bla*$_{OXA-48}$ and negative for *bla*$_{VIM}$, *bla*$_{IMP}$, and *bla*$_{KPC}$ genes. Group δ: 6 (22.22%) of clinical isolates were positive for *bla*$_{VIM}$ and *bla*$_{OXA-48}$ and negative for *bla*$_{IMP}$, *bla*$_{NDM}$, and *bla*$_{KPC}$ genes. There is high significant difference ($P = 0.000$) [Table 1].

Furthermore, two groups were done according to the results of RT-PCR against clinical isolates *K. pneumonia* for Class A, B, and D carbapenemases divided into two groups as follows: Group I: 6 (85.71%) of clinical isolates were positive for *bla*$_{VIM}$, *bla*$_{NDM}$, and *bla*$_{OXA-48}$ and negative for *bla*$_{IMP}$ and *bla*$_{KPC}$ genes. Group α: 1 (14.28%) of clinical isolates were positive to *bla*$_{VIM}$ and *bla*$_{OXA-48}$ and negative for *bla*$_{NDM}$, *bla*$_{IMP}$, and *bla*$_{KPC}$ genes. The statistical difference between groups is high significant ($P < 0.01$) [Table 2].

Table 1: The grouping of study clinical isolates of *P. aeruginosa* according to their response to Class A, B, and D MBLs

P. aeruginosa	Group I	Group II	Group III	Group IV	Group V	*P*
n (%)	18 (66.6)	1 (3.7)	1 (3.7)	1 (3.7)	6 (22.2)	0.000

P. aeruginosa: Pseudomonas aeruginosa, MBLs: Metallo-β-lactamases

Table 2: The grouping of study clinical isolates of *K. pneumoniae* according to their response to Class A, B, and D MBLs

K. pneumoniae	Group I	Group II	*P*
n (%)	6 (85.71)	1 (14.28)	0.000

K. pneumoniae: Klebsiella pneumoniae, MBLs: Metallo-β-lactamases

DISCUSSION

It is well recognized that *P. aeruginosa* is one of the most important opportunistic pathogens that have been associated with community and hospital-acquired infections such as respiratory tract infections, burns, wounds, otitis media, and nosocomial infections.[25] Carbapenems (e.g., imipenem and meropenem) are members of the β-lactam antibiotics which are used for the treatment of infections caused by cephalosporin resistant Gram-negative bacilli due to their broadspectrum of activity and stability to hydrolysis by most beta-lactamases.[26,27] This scenario is now changing with the emergence of MBL producing strains, especially among non-fermenting Gram-negative bacilli like *P. aeruginosa*.[21] Carbapenems have been kept as a last resort therapy for the control of multidrug-resistant *P. aeruginosa* infection. Such infections are associated with limited therapeutic options and high rates of mortality and morbidity especially in hospitalized and immunocompromised patients.[28]

On the other hand, during the past years, the distribution of carbapenemases producing Enterobacteriaceae has emerged almost globally.[29] KPCs producing bacteria are also causes of nosocomial and systemic infections. The three most studied chromosomally encoded resistance mechanisms against carbapenems in *P. aeruginosa* are (1) inactivation of outer membrane protein (OprD), (2) overexpression of a variety of beta-lactamases including metallo ones, and (3) overproduction of multidrug efflux pumps such as MexAB-OprM and MexXY-OprM.[27] In our study designed in Iraq, the research plan was to investigate the role of five modern and updated MBLs (Enzymatic causes) divided into two main groups: (1) Class B MBLs which includes bla_{VIM}, bla_{NDM}, and bla_{IMP}; (2) Class D and Class A MBLs (bla_{OXA-48} and bla_{KPC}) against potent β-lactamases producer isolates of *P. aeruginosa* and *K. pneumoniae*.

Regarding the result of preliminary screening tests for detection of MBLs using imipenem and meropenem against clinical isolates of *P. aeruginosa* and *K. pneumoniae*, higher resistance rates (76.0% and 78.3%) were observed to meropenem for *P. aeruginosa* and *K. pneumoniae,* respectively, in contrast to lower resistance rates, when using imipenem (55.0% and 28.57%) for the above bacteria. In Iran, 75 (70.1%) of *P. aeruginosa* were resistant to imipenem and 51 (47.7%) to meropenem.[26] In India, two important studies were achieved in this field, in the first one laid down by Shaerma and Chauhan[16] who documented that 38.29% isolates of *P. aeruginosa* were resistant to imipenem, and 20.57% isolates were resistant to meropenem while 15.60% and 4.96% showed intermediate resistance for Imipenem and Meropenem, respectively.

In the second study published by Minhas and Sharma,[30] they founded that the majority of clinical isolates of *P. aeruginosa* 33.89% were resistant to imipenem while lower proportions of isolates were recorded resistant to doripenem and meropenem and ranged from 6.67% to14.44%, respectively. From the results of our study and other studies in the related field worldwide, we have noticed that the study results were similar to some extent to those observed in Iran and different from two Indian studies. This may be attributed to the fact that in Iraq, meropenem was used ubiquitously in Governmental Hospitals and Private Pharmacies in irrational use, thus, the antimicrobial agent is work as a selective pressure which encourages selection and emergence of meropenem-resistant mutants that emerged as new resistant population for the original drug while imipenem was not introduced into Iraq. Therefore; based on the fact that, the role of antimicrobial chemotherapy is to suppress bacterial growth to the level at which the immune system can overcome the residual bacteria (resistant mutant) which initially present. The later was not intensively exposed to antimicrobial selective pressure,[31] and while the study patients were immunocompromised, thus, meropenem was selected, and a high resistance ratio was resulted. Accordingly, imipenem could be an active drug to treat *P. aeruginosa* infection in our country but should be appropriately used. On the other hand, CLSI[32] documented that imipenem is a poor screening agent for *in vitro* detection of MBL among Enterobacteriaceae. The use of novel resistant mechanisms by these organisms, coupled with the fact that these soil microbes not as intensively exposed to antimicrobial selective pressures as are the clinical pathogens, emphasize the fact that resistance is the natural part of the microbial ecosystem and highlights the evolutionary possibilities for novel antimicrobial resistance determinants.[31]

As the standard guidelines for detection of MBL producers are not clearly defined, different workers have employed various methods of detection such as Modified Hodge Test and Spectrophotometrically.[16,33] Some researchers have been used

screening methods for detection of metallo-beta-lactamases which utilize metal chelators such as EDTA either by Imipenem-EDTA combined test[34] or by DDS test as mentioned by Franco *et al.*[7,35] They were reported the sensitivity and specificity for detection of such enzymes. Therefore, in this study, both of imipenem and/or meropenem-EDTA CD and DDS test were used as phenotypic confirmatory techniques for detection of MBL production test. On another hand, to detect which of them is more preferable in term of sensitivity and specificity. As observed in the study result, in DDS test, *P. aeruginosa* has yielded this phenomenon in 62.0% of suspected isolates while it was noted in 18.0% of alleged clinical isolates of *K. pneumoniae*. It is well known that DDS is a feasible option for routine laboratory testing, but there is a need for different inhibitor combinations tests and performance may vary among different enzymes. In this study, MAA demonstrated the best sensitivity and specificity for detecting the SPM-1 enzyme. MPA demonstrated the best performance for detecting VIM-2. Even in the case of the E test MBL strip, EDTA showed high sensitivity but low specificity for detecting both enzymes.[7]

In a Brazilian study, Pica˜o *et al.*[36] published that very similar results for the sensitivity and specificity of DDS phenotypic tests, using disk-inhibitor combinations among IRPA-MBL producing strains. In another recent Brazilian study, Marra *et al.*[37] found a 69.6% false MBL detection rate with EDTA. Chu *et al.*[38] also suggested that methods using EDTA are highly sensitive but not specific.

In the combined test, two antimicrobial agents from the same family were used, imipenem and meropenem to provide an impression which of them is the best indicator to be a target in this technique. The result revealed that all isolates (100%) of suspected MBLs producer isolates of *P. aeruginosa* and *K. pneumoniae* were positive for the presence of meropenem only or meropenem-EDTA. When the study used both of imipenem only or imipenem-EDTA and meropenem only or meropenem-EDTA, the results revealed that 16 (47.0%) of the clinical study isolates of *P. aeruginosa* and *K. pneumoniae* were positive for this test when they were exposed to imipenem only plus imipenem-EDTA and meropenem only or meropenem-EDTA at the same time. The remaining 18 (53.0%) were positive in the presence of meropenem plus meropenem-EDTA, but at the same time, they were negative when they were exposed to imipenem plus imipenem plus EDTA. It is well documented that in Iran, among 169 carbapenem-resistant isolates, the researchers reported that 102 (60.4%) isolates exhibited ≥ 7 mm zone diameters by IMI disk/750µg EDTA, 81 (47.9%) isolates by MEM disk/750µg EDTA (the same concentration used in this study). All of the imipenem-resistant isolates were MBL E-test positive, except one strain that showed the IMI/IMD ratio $\leq$ eight µg/mL (2.6µg/mL) and five intermediate imipenem-resistant strains were MBL E-test positive. The sensitivities obtained for the CD test with IMI, MEM, and DOR disk/750 µg EDTA and MBL E-test were 100% yet the result for the CD test with the ERT disk/750µg EDTA was less than other carbapenems.

On the other hand, the study result was in agreement with those observed in a study published by Minhas and Sharma[30] in India who noted that a total of 40/180 (22.22%) isolates of *P. aeruginosa* were resistant to one or both carbapenems (imipenem and meropenem).

In the CD assay, the best separation between MBL and/or meropenem positive and negative isolates was obtained using 750 µg of EDTA/disk with a breakpoint of 6 mm. It is well known that EDTA may increase bacterial cell wall permeability and that zinc (chelated by EDTA) accelerates IPM decomposition and decreases OprD expression of *P. aeruginosa*.[39] These nonspecific effects might cause false-positive MBL results in the CD assay with 930 g EDTA added but not in that with 290 g and 750 g EDTA added. Interestingly, the zone diameter increases for VIM-2-producing *P. aeruginosa* isolates were found to be higher than those for IMP-9-producing *P. aeruginosa* isolates in the CD assay (IPM-EDTA(with 290 g and 750 g EDTA.

The phenotypic difference may be associated with the difference in inhibition ability of EDTA between the VIM- and IMP-type MBLs. This presumption needs to be confirmed by more MBL producers.[20] These findings suggest that caution must be taken in using only EDTA as the inhibitor agent when analyzing MBL production, as this method may lead to false-positive results. Some authors have stated that EDTA concentration is critical and that this compound may have its bactericidal activity that leads to expanded inhibition zones (synergy) of carbapenems that are not associated with actual MBL production. EDTA may also act on membrane permeability, increasing susceptibility to several antimicrobials including imipenem, which would also lead to false interpretations of MBL synergy tests.[40] As another possibility of false MBL phenotypic detection, OXA enzymes that act like carbapenemases may also be affected by the EDTA inhibitory effect.[41] Franco *et al.*[7] reported the requirement to focus light on the argument that the tests with low specificity may have a negative impact on the range of therapeutic options available and could increase the cost of nosocomial isolation precautions.

Even if E-test is used, the false-negative results of E-test for MBLs have been reported in a study laid down by Walsh *et al.*[9] Thus, this poor EDTA specificity is worrisome given that an EPCR is a cutoff diagnostic tool for the production of a variety of MBLs. Therefore, RT-PCR was used in our study to verify whether the isolates harboring MBL genes produced the enzyme and it was considered the standard for evaluation of the methodology in this study. The study genes encoded enzymes were included Class D (OXA-carbapenemases, Class A MBLs (KPC), and Class B MBLs which contain (1) Verona integron-encoded-MBL (VIM), (2) NDM, and (3) IMP.

Our result showed that all isolates of *K. pneumoniae* were produced MBL OXA-carbapenemases. Further, the result obtained revealed that all isolates of *K. pneumoniae*

were confirmed as MBL (VIM). Coproduction of bla_{VIM} and bla_{OXA-48} enzymes was producing all study isolates of *K. pneumoniae*. This result was in agreement with those observed by Cakirlar *et al.*[42] who suggested significant effort must be made to prevent the spread of carbapenem producing *K. pneumoniae* is necessary for our clinical settings. Our study result was sharing with four other international studies at which each one discovered a clinical case of coproduction of the above enzymes starting from Barguigua *et al.*[43] who have been reported a case of *K. pneumoniae* strain coproducing NDM-1 and OXA-48 in an elderly male's urine sample in Morocco. The other study laid down by Nasr *et al.*[44] in Tunisia, a country where OXA-48 producers clinical isolates were already endemic as well as in Turkey followed by a third study in Serbia laid down Seiffert *et al.*[45] The researchers reported the presence of *K. pneumoniae* isolated from the rectal swab of a patient transferred from the intensive care unit of a hospital located in Belgrade of Serbia to Bern University Hospital in Switzerland and this isolates harboring both of OXA-48 and NDM genes. The fourth *K. pneumoniae* isolates that coproduced the carbapenemases were documented by Rafei *et al.*[46,47] The above isolate was obtained from a patient who was transferred from Sanliurfa (on the border between Syria and Ankara). From the above, we hypothesized that Iraq was endemic by OXA-48 which was found in all carbapenemases producing *K. pneumoniae* study isolates. In all isolates as coproducers in addition to six of them harboring NDM enzyme-encoding genes refers to a low hygienic condition in the hospitals which create a considerable proportion of an excellent media for transferring these problematic bacteria between patients.

Out of 27 MBL-Positive *P. aeruginosa*, 25 (93.0%) were confirmed as MBL VIM producer isolates. Further, 21 (78.0%) isolates were confirmed to be MBL New Delhi MBL (NDM) producer isolates. The study result coincides with the results mentioned by Shaaban *et al.*[25] who concluded that all carbapenem-resistant *P. aeruginosa* isolates harbor carbapenemase genes especially MBLs (bla_{NDM-1} and bla_{VIM}). One of the findings in this study was no expression for Class-A KPC and imipenemase-MBL (IMP) against *P. aeruginosa* and *K. pneumoniae* was observed in our study. The study result is an agreement with those found by Shaaban *et al.*[25] who reported bla_{KPC} and b bla_{IMP} were not detected in the carbapenem-resistant isolates and the mechanisms of resistance may be due production of other types of enzymes such as extended spectrum β-lactamases and/or ambler Class C β-lactamases as concluded in a study laid down by Al-Ouqaili *et al.*[48] On the other hand, one of the most important mechanisms of resistance of pseudomonal infection to the carbapenems may be due to diminished expression of OprD-like protein which works as a carbapenem-specific channel and expressed by the OprD gene.[49]

The study suggested that meropenem was a potent and strong predictor for the reliability of phenotypic confirmatory results in Iraq, while imipenem is considered as a poor screening agent for MBL detection. Furthermore, CD test is more preferred than double-disk synergy test for phenotypic confirmatory test for checking carbapenemases production. Further, it has been suggested that VIM, NDM, and OXA-48 carbapenemases play an essential role in resistant to carbapenems among study isolates of *P. aeruginosa* and *K. pneumoniae* using RT-PCR. All study isolates of *K. pneumoniae* produced coproduction of bla_{VIM} and bla_{OXA}-48 encoding genes. Furthermore, coproduction of bla_{VIM}, bla_{OXA-48}, and bla_{NDM} encoding genes was harboring at such high proportion by study isolates of *K. pneumoniae*. The study also concluded that OXA-48 carbapenemase is endemic in Iraq.

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