ORIGINAL ARTICLE

Identification of an OprD and *bla*$_{IMP}$ Gene-mediated Carbapenem Resistance in *Acinetobacter baumannii* and *Pseudomonas aeruginosa* among Patients with Wound Infections in Iraq

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Abstract

Objectives and Background: The presence of *Acinetobacter baumannii* and *Pseudomonas aeruginosa* as nosocomial pathogens in, especially, debilitated patients inside intensive care units creates the risk of multidrug-resistant infections. The study aimed for the molecular analysis for non-enzymatic mechanisms of antibiotic resistance "outer membrane protein (OMP) (OprD Gene)" and the *bla*$_{IMP}$ gene encoding for metallo-beta-lactamase (MBL) with specific primers by polymerase chain reaction, among study isolates of *A. baumannii* and *P. aeruginosa* isolates. **Patients and Methods:** A total of 213 patients were studied during the period from April 2011 to June 2012. 182 of 213 patients were culture positive (88.26%). 44 *A. baumannii* were presumptive MBL producer isolates. Nine *Pseudomonas* ssp. isolates were chosen for this study. **Results:** All isolates (100%) of *Acinetobacter baumannii* of 44 presumptive MBL producer isolates were positive for the OprD gene. Four (44.44%) *Pseudomonas* spp. of 9 presumptive MBL producer isolates were positive for OprD gene using CR. 12 (27.3%) *A. baumannii* of 44 presumptive MBL producer isolates were positive for *bla*$_{IMP}$ gene, while *bla*$_{IMP}$ gene was not detected in *Pseudomonas* spp. **Conclusion:** All isolates of *A. baumannii* appeared to be resistant to carbapenem due to OMP encoded by the OprD gene, while it seems to find in some isolates of *P. aeruginosa* *bla*$_{IMP}$ which was detected in *A. baumannii* only. Some strains of *A. baumannii* possessed two types of resistance mechanisms to carbapenem (*bla*$_{IMP}$ + OprD genes).

Key words: *Acinetobacter baumannii,* metallo-beta-lactamase, OprD, polymerase chain reaction

INTRODUCTION

Acinetobacter baumannii and *Pseudomonas* species are exhibited for their intrinsic resistance to antibiotics and for their capability for acquiring genes encoding resistance. Foremost among the mechanisms of resistance in both of the above bacteria is the production of metallo-beta-lactamases (MBLs) and aminoglycoside-modifying enzymes. Furthermore, diminished expression of outer membrane proteins (OMPs), mutations in topoisomerases, and upregulation of efflux pumps play an essential part in antibiotic resistance.[1,2] The characterization of membrane proteins is consequently critical because of their importance in the antibiotic resistance and virulence.[2] Unfortunately, the accumulation of different mechanisms of resistance leads to the development of multiple-resistant or even "pan-resistant" strains.[1]

Porins are proteins able to create channels allowing the transport of molecules across lipid bilayer membranes that revealed little permeability for hydrophilic solutes. They provide membranes with multiple functions. Porins can do as potential targets for adherence to other cells and binding

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of bactericidal compounds to the surface of Gram-negative bacteria. Variations in their structure as a mechanism to escape from antibacterial pressure or regulation of porin expression in response to the availability of antibiotics are survival strategies that have been emerged from many bacteria.[3]

Three other OMPs have been documented to be missing in the imipenem-resistant strains of *A. baumannii*: One is a 33–36 KDa protein;[4] another is a 29 kDa protein, designated CarO,[5] and finally, a 43 KDa protein, which shows significant peptide homology with OprD from *Pseudomonas aeruginosa*.[6] However, only one of these proteins (CarO) appeared pore-forming properties. No binding site for imipenem could be detected in CarO, suggesting an unspecific monomeric channel function rather than a specific function.[5] It is so important to describe that the protein OprD of *P. aeruginosa* is demonstrated to be involved in the uptake of essential amino acids, small peptides, and of imipenem and meropenem. Therefore, CarO may function as a carbapenem-unspecific channel, and the OprD-like protein may work as a carbapenem-specific channel. *A. baumannii* OprD shows significant peptide homology with OprD from *P. aeruginosa*.[3,6]

It is well established that MBLs are metalloenzymes of Ambler class B and are resistant to clavulanic acid. They require zinc as cofactor for enzymatic activity, and their activity inhibited by ethylenediaminetetraacetic acid (EDTA) and other metal ion chelating agents. *Pseudomonas* spp. and *Acinetobacter* spp. are the most important nosocomial pathogens with multiple drug resistance.[7] Their high prevalence is of great concern because of their intrinsic and acquired resistance mechanisms, limiting the treatment options. Carbapenems are the drugs of choice for penicillin and cephalosporin-resistant *Pseudomonas* spp. and *Acinetobacter* spp. infections. However, this scenario is changing with the emergence of MBL-producing strains.[8]

Up to our simple knowledge, this is the first study of its kind in the identification of the OprD and bla_{IMP} genes responsible for carbapenem resistance by *A. baumannii* in samples of Iraqi patients. Thus, this study has been designed for molecular analysis for non-enzymatic mechanisms of antibiotic resistance "OMP (OprD Gene) and the bla_{IMP} gene" encoding for MBL with specific primers by polymerase chain reaction, among study isolates of *A. baumannii* and *Pseudomonas* spp. isolates.

PATIENTS AND METHODS

Specimen collection

Swabs were taken from different anatomical sites (e.g., bone, joints, and connective tissues) of 213 patients with wound infections (diabetic foot infections, osteomyelitis, burn infection, and septic arthritis). The swabs were taken during the period from April 2011 to June 2012 from patients admitted to Ramadi Teaching Hospital. All isolates were bacteriologically identified using conventional and VITEK® 2 system according to criteria mentioned by bioMérieux.[9] 188 of 213 patients were culture positive (88.26%).

Forty-four isolates of bacteriological well-defined *A. baumannii* in addition to nine isolates of *Pseudomonas* spp. were subjected to a phenotypic confirmatory test for the detection of MBL production.

Phenotypic confirmatory test for the detection of MBLs (IPM-EDTA-disk synergy test)

EDTA-imipenem disks were prepared by adding EDTA solution to 10 µg imipenem 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. Test study bacterial isolates were adjusted according to McFarland 0.5 turbidity standard and were inoculated to Mueller-Hinton agar. A 10 µg imipenem disk and an imipenem 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, the established zone diameter difference of ≥7 mm between imipenem disk and imipenem plus EDTA was interpreted as EDTA synergy.[8]

Detection of OprD and bla_{IMP} gene

Detection of the OprD gene was carried out using primers as described by Wang and Mi.[10] OprD primers: OprD -F (5'-GCGCA TCTCCAAGACCATG-3') OprD-R (5'-GCC ACG CGA TTT GAC GGA G-3'), while detection of the bla_{IMP} gene was carried out using primers as described by Aktas and Kayacan:[11] bla_{IMP} primers: bla_{IMP}-F (5'-CGGCCGTCAGGAGACGGTCTTT-3') bla_{IMP}-R (5'-AACCAGTTT TG CCTTTAC CTAT-3'). The Polymerase chain reaction (PCR) reagents in a PCR-pre mix tube master mix for the study genes were prepared as mentioned in Table 1.

After that, they were mixed well by vortex. All tubes were centrifuged for 30 s at 10000 G according to manufacture company. In a PCR-premix tube, a master mix prepared as in Table 1; then mixed well by vortex. All tubes transferred into the thermal cycler. The PCR started as in the following program. Initial denaturation for 3 minutes at 93°C followed by 40 cycles for following: (a) Denaturation at 93°C for 1 minute; (b) Annealing at 55°C for 1 minute and (c) Extension at 72°C for 1 minute. The final was an extension for 7 min at 72°C. Hold temperature at 4°C for 10 min. The PCR product of 1100 bp for OprD gene and 587 bp for bla_{IMP} was visualized by 2% agarose gel electrophoresis with Novel Juice.[12]

Table 1: The original PCR reagents and final concentrations used in the procedure

Component		Concentration	Volume
PCR premix master mix tube contain			
a	Top DNA polymerase	1 U	
b	Each dNTP	250 µM	
c	Tris–HCl (pH 9)	10 µM	
d	KCl	30 µM	
e	$MgCl_2$	1.5 µM	
f	Stabilizer and tracking dye		
DNA template sample			2 µl
Forward primer		5 pmol/µl	4 µl
Reverse primer		5 pmol/µl	4 µl
Sterile distilled water			10 µl
Final volume			20 µl

PCR: Polymerase chain reaction

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 of fit test within non-parametric 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 (*A. baumannii* and *Pseudomonas* spp.). All the study graphics (bar chart, scatter diagram, or dot chart) were done using Microsoft Excel 2016-Windows Ten.

RESULTS

41 (93.2%) isolates of *A. baumannii* (of 44 isolates) were found to be MBL producers by IPM-EDTA-disk synergy test (positive) [Figures 1 and 2]. The established zone diameter difference of ≥7 mm between imipenem disk and imipenem plus EDTA was interpreted as EDTA synergy positive (the presence of an enlarged zone of inhibition was interpreted as EDTA-synergy test positive). Three isolates were negative for MBL producers by IPM-EDTA-disk synergy test.

In the present study, the results revealed that all isolates (100%) of *A. baumannii* presumptive MBL producer isolates were positive for the OprD gene by PCR [Figures 1 and 2].

4 (44.4%) *Pseudomonas* spp. (2 *P. aeruginosa*, *Pseudomonas oryzihabitans*, and *Pseudomonas luteola*) were produced OprD gene, while the others 5 (55.6%) were negative as reflected in Figure 3.

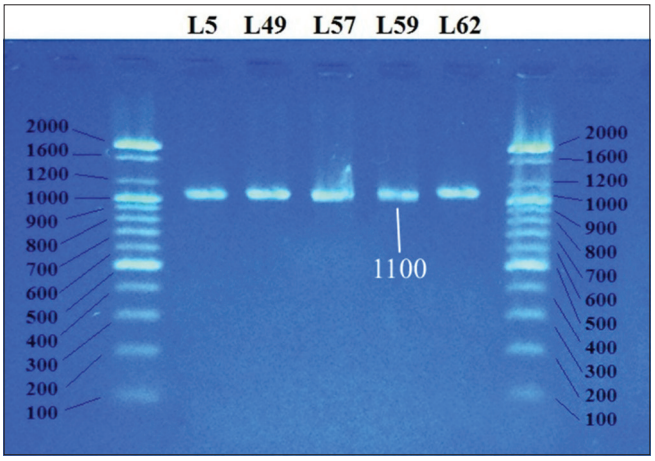

Figure 1: Agarose gel electrophoresis (2%) with Novel Juice dye, bands with OprD gene obtained from *Acinetobacter baumannii* isolates, all isolates were positive results represented in L5-L62. DNA ladder with 100–2000 bp on the left and right used as a DNA molecular weight marker

On the other hand, 12 (27%) *A. baumannii* of 44 presumptive MBL producer isolates were positive for bla_{IMP} gene as represented in Figures 4-7.

The most striking result in this study is that IMP (bla_{IMP}) gene was not detected in *Pseudomonas* spp. [Figure 7].

DISCUSSION

A significant problem with this critical emerging pathogen is the shortage in the information concerning the OMPs. Today, only a few OMPs were reported, and there was no evident function associated with the described proteins. In this study, OprD gene which encodes for 43 KD OMPs selected due to significant peptide homology with *P. aeruginosa*. By electrophoresis and MALDI-MS analyses, the 43 kDa OMP

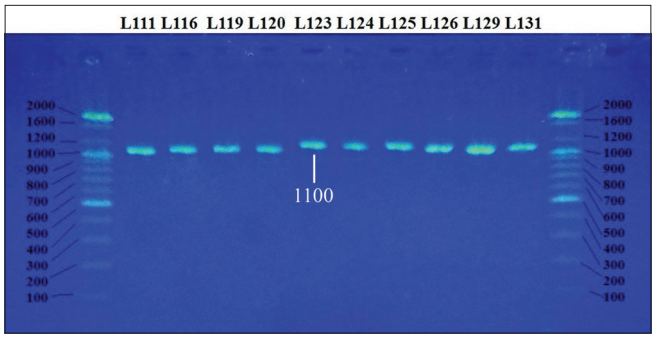

Figure 2: Agarose gel electrophoresis (2%) with Novel Juice dye, bands with OprD gene obtained from *Acinetobacter baumannii* isolates, all isolates were positive results represented in L111–L131. DNA ladder with 100–2000 bp on the left and right used as a DNA molecular weight marker

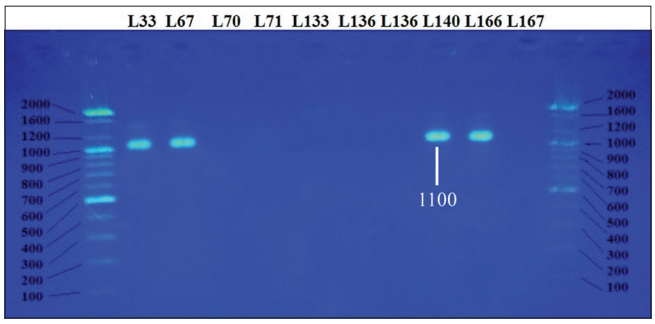

Figure 3: Distribution of OprD gene in *Pseudomonas* spp. isolates. Agarose gel electrophoresis (2%) with Novel Juice dye, bands with OprD gene obtained from *Pseudomonas* spp. isolates, which showed that positive results are represented by (L33, L67, L140, and L166), while L70–L136 and L167 were negative results. DNA ladder with 100–2000 bp on the left and right used as a DNA molecular weight marker

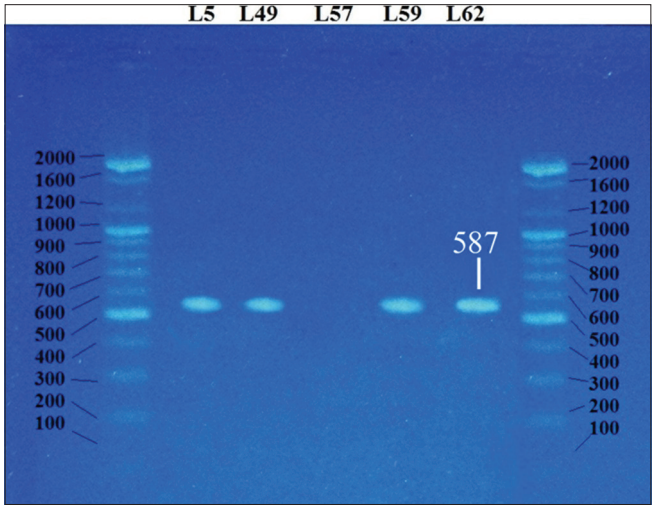

Figure 4: Distribution of bla_{IMP} gene in *Acinetobacter baumannii* isolates. Agarose gel electrophoresis (2%) with Novel Juice dye, bands with bla_{IMP} gene obtained from *A. baumannii* isolates, which showed that positive results are represented in L5, L49, L59, and L62, while L57 was a negative result. DNA ladder with 100–2000 bp on the left and right used as a DNA molecular weight marker

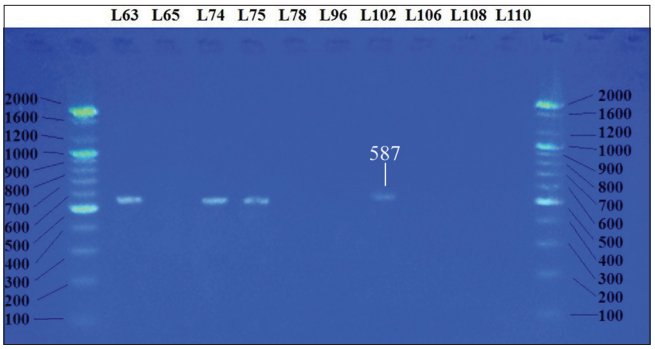

Figure 5: Agarose gel electrophoresis (2%) with Novel Juice dye, bands with bla_{IMP} gene obtained from *Acinetobacter baumannii* isolates, which showed that positive results are represented in L63, L74, L57, and L102, while L65, L78, L96, and L106–L110 were negative results. DNA ladder with 100–2000 bp on the left and right used as a DNA molecular weight marker

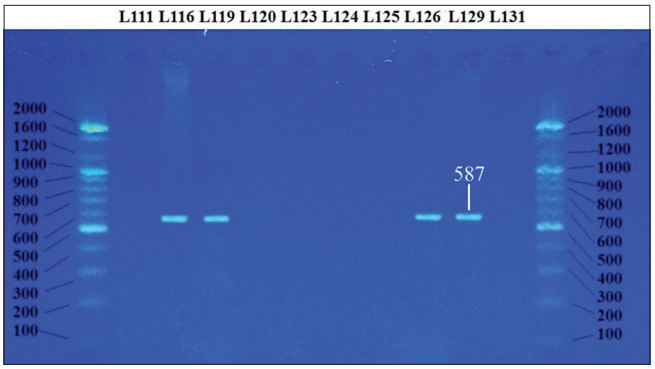

Figure 6: Agarose gel electrophoresis (2%) with Novel Juice dye, bands with bla_{IMP} gene obtained from *Acinetobacter baumannii* isolates, which showed that positive results represented in L116, L119, L126, and L129, while L111, L120–L125, and L131 were negative results. DNA ladder with 100–2000 bp on the left and right used as a DNA molecular weight marker

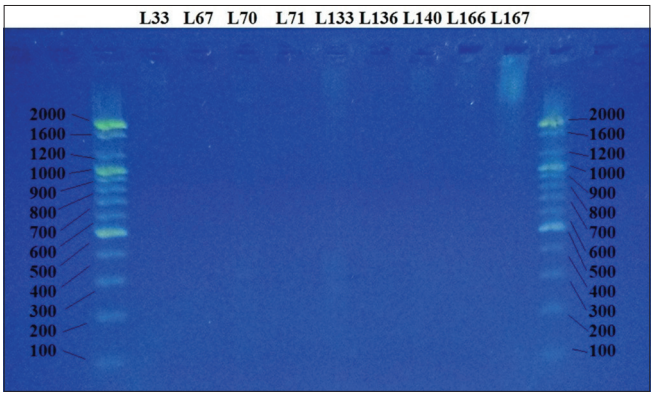

Figure 7: Agarose gel electrophoresis (2%) with Novel Juice dye, bands with bla_{IMP} gene obtained from *Pseudomonas* spp. isolates, all isolates were negative results represented in L133–L167. DNA ladder with 100–2000 bp on the left and right used as a DNA molecular weight marker

identified as a protein belonging to the OprD family, essential amino acid, and imipenem porin.[6]

In the present study, all isolates (100%) *A. baumannii* of 44 presumptive MBL producer isolates were positive for the OprD gene by PCR. Up to our pure knowledge, no previous studies were reported on OprD protein identified in *A. baumannii* in Iraq. In the past two decades, an observed number of *Acinetobacter* nosocomial infection outbreaks, caused mainly by MDR strains, has been reported, causing increasing concern.[12] Until now, no specific porin protein has been identified in *A. baumannii,* and hence, we report, for the first time, the presence of an OprD protein in the outer membrane.[6]

In *P. aeruginosa*, this protein is involved in the uptake of essential amino acids, small peptides, and of imipenem and meropenem. Furthermore, a decreased expression of OprD, resulting in imipenem resistance, has been reported in MDR mutants of *P. aeruginosa*.[13]

In a study laid down by Dupont *et al*.[6] approached the current study, a homolog of OprD, a 43-kDa protein, was identified in all *A. baumannii* isolates. OprD-like protein identified in *A. baumannii*; the corresponding sequence of this putative *A. baumannii* OprD exhibited a significant homology with the *Pseudomonas* OprD group.[3,6]

In another study performed by Lin and co-workers[15] who reported that the complete OprD sequences from four strains of Acinetobacter spp. were amplified by PCR. They were fully sequenced and BLASTed in Gen Bank. The result showed that the OprD of these four *Acinetobacter* strains contained the conserved domains of the OprD family from *P. aeruginosa*, and a BLAST at NCBI showed 33–34% sequence identity with OprD from *P. aeruginosa* PAO1.[14]

The OprD of *A. baumannii* has shown to be involved in essential amino acid uptake and carbapenem diffusion, and loss of OprD in *A. baumannii* correlated with carbapenem resistance.[6] Furthermore, in *P. aeruginosa*, carbapenem resistance is driven mainly by mutational inactivation of OprD.[15] The OprD of *Acinetobacter* spp. shared conserved domains with the OprD of *P. aeruginosa*, suggesting similar functions of this protein across species. Therefore, the evidence gathered in this study indicates that OprD was associated with imipenem resistance in *Acinetobacter* spp.[14]

The study suggested that A. baumannii as well as P. aeruginosa were possessed restricted outer membrane permeability which modulates it's membrane channel capacity by reducing the expression of a porin that belong to the OprD family. *Acinetobacter* exhibits an exceptional catabolic capability and a high competence for natural transformation associated with genetic flexibility and versatility.

The size of porins and a small number could reflect the decrease in *A. baumannii* outer membrane permeability (<5%) when it is compared with other Gram-negative organisms. The outer membrane of *A. baumannii* is less permeable to antimicrobials than that in *Escherichia coli*. In accordance with Sato and Nakae,[16] the coefficient of permeability to cephalosporins is between 2- and 7-fold larger in *P. aeruginosa* than in *Acinetobacter* spp.[3] They, therefore, suggested that the natural cause of the resistance to antimicrobial agents may be due to a small number of porins as well as their small size. However, another possibility to maintain this intrinsic resistance to antimicrobials could be the low level of constitutive expression of one or several active efflux systems in *A. baumannii* or to the interplay between both low permeability and constitutive expression of the efflux pump(s).[6]

Nine isolates were positive for the OprD gene. Imipenem, and to a certain degree also meropenem, enters *P. aeruginosa* through an OMP, OprD. Loss of this membrane protein has shown to play a significant role in the acquired resistance to imipenem followed by meropenem. Furthermore, imipenem selects for OprD-deficient mutants in 15–20% of patients treated with this antibiotic.[17]

Many types of research have reported that there is a presence of OprD gene in the *Pseudomonas* species in different percentages. In a study performed in Spanish hospitals, OprD genes from 10 randomly selected imipenem-resistant clones were PCR amplified and fully sequenced. Nine of the 10 clones indeed contained inactivating mutations in the OprD gene.[14] In other study performed by Ambast *et al*.,[18] they documented that a total of eight isolates of *P. aeruginosa* were carbapenem resistant. OprD gene of all eight carbapenem-resistant strains of *P. aeruginosa* was sequenced and observed for defective chromosomal mutations. The most common causes of OprD mutational inactivation were frameshift mutations produced by 1-bp insertions or deletions and point mutations leading to the creation of premature stop codons.[14,18,19]

Loss of OprD does not necessarily confer resistance to β-lactams other than the carbapenems. Mutational loss of OprD is common through imipenem therapy. In many types of clinical studies, imipenem resistance has emerged during treatment of *P. aeruginosa* infections in ~25% of patients treated with that drug.[20]

The main function of OprD is the uptake of essential amino acids that means that OprD is vital for bacterial adaptability to changing environments, and it is known that the so-called contingency genes are highly mutable as compared with housekeeping genes, which are relevant for essential bacterial metabolism and structure, and which mutate at an expected low frequency.[18] In the present study, the detection of OprD gene in *Pseudomonas* spp. and *A. baumannii* was 44.4% and 100%, respectively. The large variety of OprD homologs in *P. aeruginosa* and other bacteria presents opportunities for intrachromosomal exchange and horizontal gene transfer.[19]

Twelve (27.3%) *A. baumannii* of 44 presumptive MBL producer isolates were positive for bla_{IMP} gene by PCR.

Previous researches reported that the presence of the bla_{IMP} gene in the *Acinetobacter* species in low percentages. In a study conducted by Hwa,[21] there was 5.12% of these genes. The prevalence of the MBL gene (bla_{IMP} gene) is generally low within *A. baumannii* isolates as illustrated in a study by Mendes *et al*.[22] where the prevalence was 0.8% in Taiwan. Other researches had not detected bla_{IMP} genes.[23-26]

The isolates which were actual MBL production by confirmatory test but negative for bla_{IMP} amplification may have variant bla_{IMP} or bla_{SIM} genes.[27] It was confirmed by the current study, by the presence of the bla_{IMP1} gene in proportion (80%). The imipenem-resistant *A. baumannii* strains in the present study with no phenotypic or genotypic sign of MBL production may possess other enzyme-mediating carbapenem resistance, such as OXA-type lactamases (class D) or AmpC b-Lactamases and/or other mechanisms such as outer-membrane permeability and efflux mechanisms.[24] The mechanism of cleavage of the ß-lactam ring is different for MBLs as compared to ß-lactamases; however, both gene products still share a unique fold in the active sites of the enzymes. The bla_{IMP} is a foreign gene that is introduced from another species of bacteria and *A. baumannii* only retains the gene in environments where there is selective pressure in the form of the presence of imipenem.[25] Furthermore, in the present study, 25% (11/44) of *A. baumannii* strains possess two types of resistance mechanisms to carbapenem (bla_{IMP} plus OprD Genes). It is likely that beta-lactamases and outer-membrane alterations work together to confer resistance to beta-lactam agents (carbapenem).

The mechanisms of antimicrobial resistance in *A. baumannii* are classified into three broad categories: (1) Antimicrobial-inactivating enzymes, (2) reduced penetration to bacterial targets (due to decreased outer membrane permeability caused by the loss or reduced expression of porins, overexpression of multidrug efflux pumps), and (3) mutations that change targets or cellular functions penicillin-binding proteins). A combination of different mechanisms may be present in the same microorganism as has also been observed in other Gram-negative bacteria.[28]

The intrinsic impermeability of their outer membranes coupled with the close relationship of *A. baumannii* and *P. aeruginosa* to the soil and aquatic environment has made it possible for these organisms to acquire highly effective resistance determinants in response to multiple challenges.[29] In a study by D'Costa *et al*., the soil was demonstrated to be a reservoir of resistance genes.[1,30]

The IMP (bla_{IMP}) gene was not detected in *Pseudomonas spp.* in the present study by PCR. The result above was confirmed in numerous researches[23,31,32] while it was observed clearly in *A. baumannii* isolated from wound infections in the same area.[33]

The study concluded that all Iraqi isolates of *A. baumannii* were appeared to be resistant to carbapenem due to OMP (porin) changes (OprD Gene), while it seems in some isolates of *Pseudomonas* spp. Further, bla_{IMP} was detected in *A. baumannii* only. Furthermore, some strains of *A. baumannii* possessed two types of resistance mechanisms to carbapenem (bla_{IMP} plus OprD genes).

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