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Original Article

Molecular analysis for the *OprD* gene among *Pseudomonas aueroginosa* clinical isolates obtained from hospitals in Jordan

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Abstract

Introduction: *Pseudomonas aeruginosa* has increasingly been associated with the emergence of antibiotic resistance. Antibiotic resistance among *P. aeruginosa* isolates is an ambiguous and complicated mechanism utilizing several enzymes and structural proteins. This study was conducted to investigate the prevalence of mutations in the chromosomal *OprD* gene that show resistance to carbapenems among clinical isolates of *P. aeruginosa*.
Methodology: Sixty-three clinical isolates of *P. aeruginosa* resistant to meropenem were collected from public hospitals in Irbid city, north of Jordan. Analysis of antimicrobial susceptibility was carried out and their susceptibility was categorized. Molecular analysis of mutations in the *OprD* gene was performed using restriction fragment length polymorphism (RFLP) and DNA sequencing.
Results: Molecular analysis of *P. aeruginosa* isolates showed 52% of the common molecular modifications among the collected isolates. These alterations could be associated and affect meropenem-susceptibility rather than imipenem. The most frequent molecular changes among the resistant isolates were the F170L substitution mutation. This was detected in 22 (35%) of the isolates with an unusual insertion sequence (IS) of 100 bp within the 590 bp DNA segment downstream of the restriction site. The divergent sequence of 10 amino acids 372(VDSSSSYAGL)383 was detected in 7 (11%) of the isolates.
Conclusions: A significant alteration in the *OprD* gene in *P. aeruginosa* clinical isolates was found. Alterations in the *OprD* gene could be linked to protein permeability of the outer membrane of *P. aeruginosa* associated with meropenem resistance. Further investigations with a larger number of bacterial isolates are needed to validate the proposed association.

Key words: *P. aeruginosa*, *OprD*-gene, Insertion-sequence, OprD-porin , F170L.

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Introduction

Pseudomonas aeruginosa is an aerobic Gram-negative opportunistic pathogen implicated in a variety of acute and chronic infections such as respiratory, urinary tract, and gastrointestinal infections as well as bacteremia. *P. aeruginosa* is commonly isolated from patients suffering from severe infections and usually leads to unpredictable complications [1,2]. This species is widely distributed and transmitted to patients in clinics and hospitals from contaminants and environmental sources [3]. Conventional antibiotics including aminoglycosides, β-lactams, and fluoroquinolones are widely used in the treatment of *P. aeruginosa* infections [4]. *P. aeruginosa* is characterized by its rapid evolution, compared to other Gram-negative bacteria, leading to high resistance rates to the most antibacterial agents. A broad spectrum of resistance to a variety of antibiotics has been reported for several strains of *P. aeruginosa* [4]. The emergence of antibiotic resistance among *P. aeruginosa* is an abruptly complicated mechanism. *P. aeruginosa* utilizes many enzymes and proteins in developing antibiotic resistance [5].

Carbapenem is one of the antimicrobial agents used in the treatment of *P. aeruginosa* infections including those resulting from multidrug resistant strains. It is characterized by high stability in the presence of β-lactamase and it exerts its bactericidal action by inhibiting the peptidoglycan-assembling transpeptidase [6]. The carbapenem's effect depends on a permeable outer membrane protein (OMP) known as OprD porin. In *P. aeruginosa*, OprD porin plays a major role in the uptake of certain basic amino acids and other nutrients [6,7]. OprD porin of *P. aeruginosa* is a semipermeable

OMP that has a 16-strand transmembrane-barrel structure encoded by the chromosomal *OprD* gene [7,8]. Structural alterations in the OprD protein usually occur as a natural response to antimicrobial exposure after the emergence of specific mutational changes in the corresponding gene. Previous studies have demonstrated that mutational changes in the *OprD* gene may modify protein permeability associated with carbapenem resistance in *P. aeruginosa* [7,9]. Moreover, malfunction of the OprD porin was observed among *P. aeruginosa* clinical isolates which are susceptible or intermediate in susceptibility to carbapenems and imipenems [10,11]. Knowledge of resistance mechanisms in *P. aeruginosa* isolates may help in the regulation of infection control strategies and in enhancing the efficacy of imipenem for the treatment of infections caused by these bacteria. In the present study, we explored the prevalence of the most common mutations in the *OprD* gene among *P. aeruginosa* clinical isolates from three Jordanian hospitals. The possible association between these mutations and carbapenem resistance patterns was evaluated.

Methodology

Ethics declarations

The study was approved by the administrations of the Princess Basma Teaching Hospital, Princess Rahma Hospital, and Princess Badea Hospital. In this study, the patient and the human samples were not examined directly, and the bacterial samples were collected from the teaching hospitals.

Bacterial isolates

Sixty-three clinical isolates of *P. aeruginosa* were collected from clinical laboratories of three public hospitals: Princess Basma Teaching Hospital, Princess Rahma Hospital, and Princess Badea Hospital in Irbid city, north of Jordan. The *P. aeruginosa* clinical isolates were collected from different infection sites in the body and the distribution of the samples was as follows: ear 41, wound discharge 12, nipple discharge 4, urine 4, and blood 2. The isolates were grown on blood agar and MacConkey agar as a selective medium following routine protocol. The plates were incubated at 37 °C for 48-72 h. Thereafter, they were examined for *P. aeruginosa* growth. Characterization and identification of the isolates were performed in the microbiology division of the medical laboratory of each hospital, based on colony morphology on MacConkey agar, pigment production, and oxidase reaction. All isolates were identified by the conventional biochemical tests that are commercially available (API 20 NE system (BioMerieux, Paris, France) and 16S rRNA gene sequence analysis). After identification, the isolates were subcultured on nutrient agar plates and incubated at 37 °C for 18-24 h under aerobic conditions for further study and analysis.

Antibiotic susceptibility testing and Metallo-beta-lactamase screening

Resistance to antibiotics was determined by the Kirby-Bauer disk diffusion agar method on Mueller-Hinton agar plates using antibiotic-impregnated disks. Overnight culture (OD600: 0.8 to 1.0) grown in a Mueller-Hinton broth (~106 CFU/mL) was spread on the agar. Disks of eight different antibiotics (Figure 1) were applied on the surface of inoculated plates. After 1 h of diffusion at room temperature, plates were incubated overnight at 37 °C. The inhibition zone for each antibiotic and antimicrobial agent tested was measured and compared with control strains. The isolates were categorized as susceptible, intermediate, or resistant according to Clinical and Laboratory Standards Institute guidelines [12]. *P. aeruginosa* strain ATCC 27853 was used as a control. The isolates were screened for Metallo-beta-lactamase (MBL) production using the combined-disk method with ethylenediaminetetraacetic acid (EDTA) [13,14]. An overnight culture of tested isolates in Luria-Bertani (LB) broth was inoculated on a Mueller-Hinton agar plate. One imipenem (10 µg) disk and one EDTA impregnated imipenem disk was placed on inoculated Mueller-Hinton agar plate. The plate was incubated at 37 °C for 18 h. An increase greater than 7 mm in the inhibition zone around the imipenem-EDTA disk compared to the imipenem disk (without EDTA), was considered a positive result for the Metallo-beta-lactamase activity. The values of minimum inhibitory concentrations (MICs) of imipenem and meropenem were determined by the broth microdilution method and Etest® (BioMérieux, Paris, France) according to the manufacturer's instructions.

Molecular Analysis

Bacterial isolates were grown in 5 mL LB broth at 37 °C with vigorous shaking at 180 rpm for 18 h. The total chromosomal DNA of *P. aeruginosa* isolates was extracted using the Wizard Genomic DNA purification kit (Promega, Wisconsin, USA). The purity of the extracted DNA was tested by running samples on agarose gel electrophoresis and the isolated DNA was stored at -70 °C. The *OprD* gene was amplified by thermal cycling for polymerase chain reaction (PCR) amplification from genomic DNA using specific

primers (Table 1, row 1). The primers were designed using the *OprD* gene sequence of *P. aeruginosa* PAO1 wild type (GenBank Accession Number Z14065.1). Primer's syntheses were performed at (SIGMA ALDRICH, Taufkirchen, Munich, Germany). The final PCR reaction mixture consisted of 25 µL with 12.5 µL of PCR 2x master mix (Taq DNA polymerase 2x Master Mix Red, Ampliqon) and 2 µL of 0.5 M of each primer, and 2-3 µL of DNA template. The cycling conditions were 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 62.5 °C for 30 s, and extension for 1 min at 72 °C followed by a final extension step at 72 °C for 5 min. The PCR products were analyzed using 1% w/v agarose gel electrophoresis for each reaction to determine and ensure the generation of amplified 2.1 kb DNA fragment of the *OprD* gene. The amplified PCR product of the *OprD* gene was purified using either a manual protocol or the Wizard Plus SV minipreps DNA purification system (Promega, Wisconsin, USA). The nested PCR was the second round of PCR in which the first-round amplification product was used as a template. The primers (Table 1) for nested PCR reaction were internal to the primers used in the initial amplification and were designed for the wild type.

Detection of the Deletion Mutations in the OprD Gene

To detect the common deletion of ten amino acid residues of the *OprD* gene from the position 433(LIVDYPLSIL)44, nested PCR was carried out using *OprD* deletion primers (Table 1 row 4). Nested PCR was employed to enhance sensitivity and specificity in the screening of the 433(LIVDYPLSIL)443 deletion using primers internal to those used for the initial amplification of the *OprD* gene.

Detection of a Divergent Sequence in the OprD Gene

The amplified product of *the OprD* gene was also screened for the occurrence of a divergent sequence of ten amino acid residues, 372(VDSSSSYAGL)383, using the specific primers (Table 1, row 3). The nested PCR product for the amplified internal DNA segment that contains the usual 12 amino acids 372(MSDNNVGYKNYG)385 was estimated to be 323bp. The nested PCR products were resolved on 1% agarose gel and the negative product was taken to determine the replacement of the normal 12 amino acids stretch 372(MSDNNVGYKNYG)385 with the divergent 10 amino acids sequence.

Detection of a Single Nucleotide Polymorphism (SNP) in the OprD Gene

A 590 bp DNA stretch of the *OprD* gene was amplified by nested PCR using specific primers (Table 1, row 2). This fragment was found to contain the C1245G/A SNP that results in a single amino acid residue substitution (F170L) in imipenem susceptible *P. aeruginosa* isolates. DNA sequencing of both strands of each gene was carried out using an external resource (Macrogen Inc., Seoul, South Korea).

Restriction Fragment Length Polymorphism (RFLP) analysis for F170L

The nested PCR 590 bp amplified product was subjected to the digestive action of the EcoR1 restriction endonuclease and the restriction digests were resolved on 2% agarose gel in 1x Tris borate EDTA (TBE) buffer for 1 h. Restriction analysis was conducted using the EcoR1 enzyme. EcoR1 digests the wild type 590 bp fragment (genotype C1245) into two (500 bp + 90 bp) fragments. On the other hand, the mutant genotype fragment (A or G) is not digested by EcoR1 and remains in a single 590 bp fragment. RFLP cluster analysis of the isolates and banding patterns of the restriction PCR products for the nested PCR 590 bp were examined by estimating the size of each fragment on the electrophoresis gel [15].

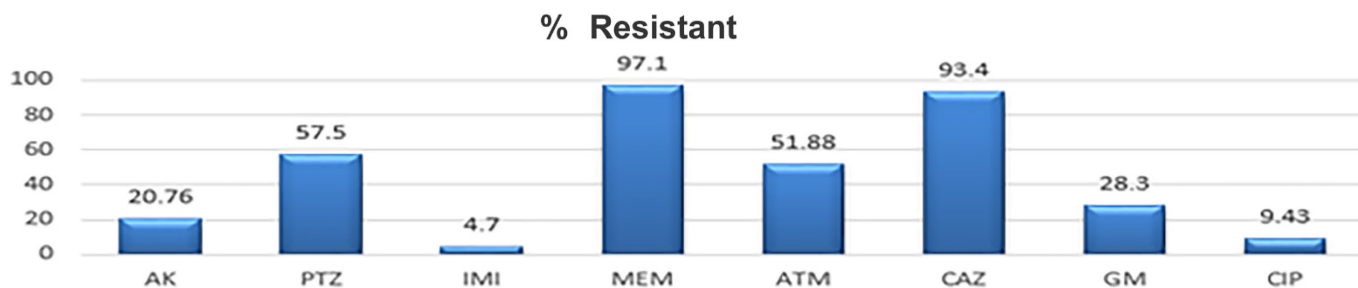
Data analyses

Statistical analyses were generated using SPSS 23.0 (IBM, New York, USA). Processing and analysis of the data were conducted using Microsoft Excel (Microsoft Inc., Redmond, WA, USA).

Table 1. The primers for *Pseudomonas aeruginosa* PAO1 wild type used in this study.

Primer Name	Sequence 5'-3'	Size (bp)
OprD gene	F: 5' GGATCCAAAGCGAACATA 3' R: 5' ATTCGAGCTCGGTACCTA 3'	2100
OprD SNP	F: 5' AGT GAT GAA GTG GAG CG R: 5' AGG TGG CAT AGA GTT CG	590
OprD S	F:5' AAC ACC ACT TGG TCC CT 3' R: 5' GCC GTA GTT CTT ATA GCC G 3'	323
OprD Del +	F: 5' GAT GGC AAG CAC CAC GA 3' R: 5' CAG GAT CGA CAG CGG ATA 3'	171

Figure 1. Antimicrobial resistance rates.



Distribution of antimicrobial resistance rates among *P. aeruginosa* clinical isolates used in this study AK; amikacin, PTZ; pipracillin-tazobactam, IMI; imipenem, MEM; meropenem, ATM; aztronam, CAZ; ceftazidime, GM; gentamicin; CIP; ciprofloxacin.

Results

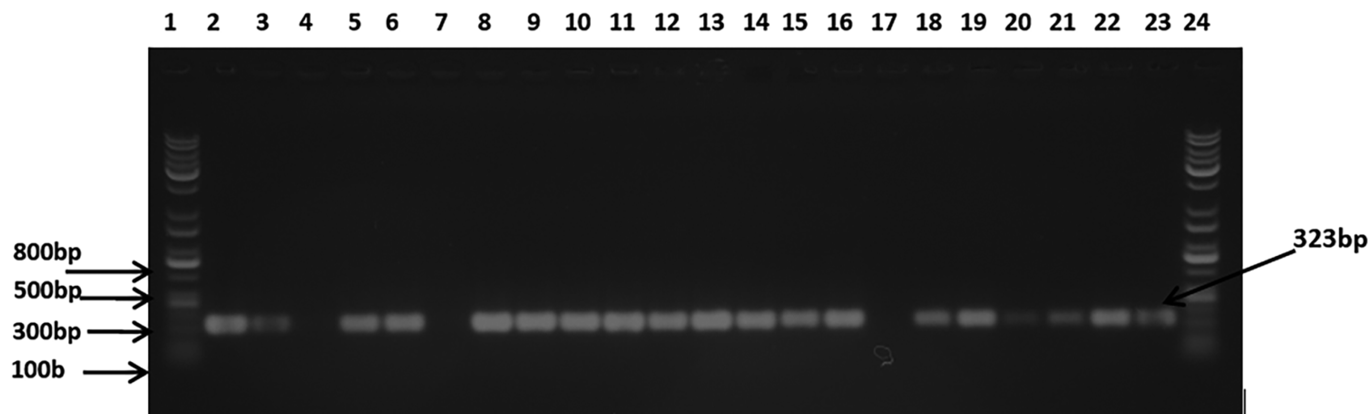
Antibiotic susceptibility testing

The findings revealed a susceptible rate to imipenem of 97% of the 63 clinical isolates of *P. aeruginosa.* All were meropenem resistant (Figure 1). Resistance to imipenem was in less than 5% of the isolates. However, various combination patterns of susceptibility to the other tested antibiotics were observed (Figure 1). Forty-one isolates (65%) showed multidrug resistance (MDR), resistance to three or more antibiotics. Fourteen (22%) of the isolates tested positive for Metallo-beta-lactamase activity. The rates of resistance toward other antimicrobial agents appear in Figure 1. The highest resistance rate was confirmed for ceftazidime at 94%, followed by piperacillin-tazobactam combinations at 57%. In addition, approximately 10% of the strains were resistant to ciprofloxacin.

Divergent sequence 372(VDSSSSYAGL)383 and deletion mutation 433(LIVDYPLSIL)443

Nested-PCR reaction was carried out for all the *P. aeruginosa* isolates to investigate the entire *OprD* gene for a common deletion mutation of ten amino acids sequence 433(LIVDYPLSIL)443. A deletion mutation resulting in the loss of ten amino acid residues at the 433-443 position of OprD porin is usually encountered in carbapenem-resistant and carbapenem-susceptible isolates. The results showed the absence of the 433(LIVDYPLSIL)443 deletion. Moreover, a nested PCR reaction was carried out to analyze the *OprD* gene of all *P. aeruginosa* isolates for the divergent sequence 372(VDSSSSYAGL)383. Results of nested-PCR for the amplified internal gene segment that contains the stretch sequence of 12 amino acids 372(MSDNNVGYKNYG)385 revealed the occurrence of the corresponding mutations. The finding revealed a negative PCR product corresponding to the gene segment that indicates substitution occurrence of the usual 12 amino acids stretch by the stretch sequence of 10 amino acids (VDSSSSYAGL). However, the divergent amino sequence acids

Figure 2. Agarose gel analysis.



Agarose gel analysis for the nested PCR product used for screening for the occurrence of divergent sequence 372(VDSSSSYAGL)383. Lanes 1 and 26: universal DNA ladder. Lanes in between are PCR products of 24 *Pseudomonas aeruginosa* clinical isolates. Lanes 4, 7 and 17, lack the PCR product indicating the occurrence of the divergent sequence mutation.

372(VDSSSSYAGL)383 were detected in 7 of the 63 (11%) of the collected *P. aeruginosa* isolates (Figure 2).

Substitution mutation

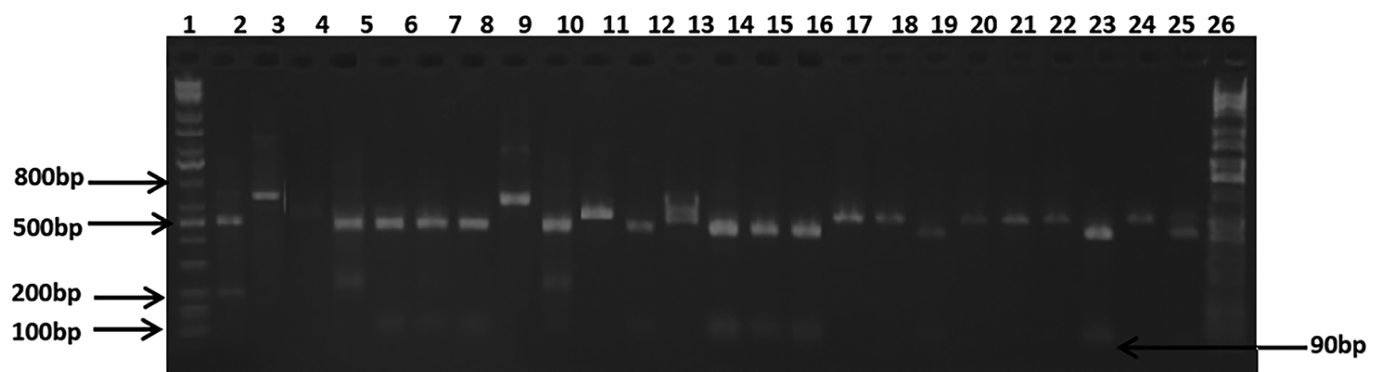
Nested PCR reaction was carried out using the primers listed in Table 1 to analyze the *OprD* gene of all *P. aeruginosa* isolates for the amino acid substitution F170L. The amplified nested PCR product was digested with appropriate *EcoR1*, digestion of 590 bp PCR product of the genotype C1245 (wild type) at position 505 resulted in two fragments, 500 bp and 90 bp. The mutant genotype (A or G) did not respond to digestion by *EcoR1* and produced one continuous fragment of 590 bp. PCR-RFLP analysis of the F170L C1245G/A single nucleotide polymorphism was carried out on *P. aeruginosa* isolates. Single nucleotide polymorphism resulted in the substitution of one amino acid residue F170L. The F170L substitution mutation was detected among 22 (35%) of the total *P. aeruginosa* isolates (Figure 3). The results show an unexpected PCR product for another 12 isolates in the DNA fragment that contains this SNP. Instead of the expected 500 bp + 90 bp wild type DNA digest, a DNA fragment of approximately 200 bp is found. Therefore, the digestion of these 12 isolates resulted in a single 700 bp fragment instead of the 590 bp fragment expected in the F170L SNP mutants (Figure 3).

Discussion

P. aeruginosa is widespread in the environment, particularly the healthcare facilities. In the current study, 63 clinical isolates of *P. aeruginosa* were investigated to explain and understand the association between antibiotic resistance rates and alteration in the *OprD* gene. The results of this survey highlight the importance of continuous monitoring and good maintenance of hospitals to prevent pathogen contamination. It further points to the importance of adequate chemical and microbiological quality to control and prevent the occurrence of undesirable multi-drug resistance strains.

Whether the *OprD* gene alterations can contribute to the highly resistant and diverse *P. aeruginosa* strains is controversial. Several studies revealed an increase in the *P. aeruginosa* resistance to most classes of antibiotics that are usually used in the treatment of bacterial infections [2,4]. The majority of the *P. aeruginosa* isolates from various samples were resistant to ceftriaxone, cefotaxime, meropenem, and piperacillin/tazobactam. Studies have reported more than 40% of the *P. aeruginosa* clinical isolates as multidrug-resistant, including resistant to third generation cephalosporins, aminoglycosides, and carbapenems [2,4]. The resistance pattern obtained for *P. aeruginosa* clinical isolates in this study was similar to reports from different countries [2,3]. In 2016, Sharma and Srivastava showed that all the *P. aeruginosa* isolates studied were sensitive to imipenem and meropenem [4]. Among the antimicrobial agents are carbapenems which are commonly used in counteracting complicated infections caused by *P. aeruginosa*. Carbapenems including imipenem and meropenem can penetrate the bacterium's outer membrane through aqueous channels called porin proteins [18,19]. *OprD* loss or alteration due to an inactivating mutation is one of the most important mechanisms that is supposed to be involved in the emergence of carbapenem-resistant *P. aeruginosa*

Figure 3. Agarose gel of the F170L.



Agarose gel of the F170L C1245G/A PCR-RFLP analysis. Nested PCR amplification and their digestion with EcoR1 resolved in 2 % agarose gel. Lanes 1 and 26 are universal ladders. Lanes 6, 7 and 8 show a 500 bp DNA fragment and a 90 bp DNA fragment (the wildtype). Lanes 5 and 10 have a 500bp fragment and a ~200 bp fragment (inferred to represent a ~100 bp insertion segment, IS, in the wildtype). Lanes 11, 17 and 18 show a continuous a single 590 bp fragment (inferred to be F170L mutant without IS). Lane 9 shows a single 700 bp DNA segment (inferred to represent a ~100bp IS in an F170L mutant).

strains. Many studies have investigated the association between carbapenems susceptibility and OprD porin alterations [18,20]. Several studies reported genetic diversity and heterogeneity among *P. aeruginosa* clinical isolates. Therefore, researchers focus on the elucidation of the molecular basis of this association by identifying the alterations that occur within OprD porin [18,19]. Association between carbapenem susceptibility and OprD porin alteration in *P. aeruginosa* clinical isolates was demonstrated by reports from different countries [22,23]. Other studies reported the association between the levels of expression of the *OprD* gene and the degrees and patterns of susceptibility to both carbapenems [16,21]. The most frequent OprD porin alteration in *P. aeruginosa* clinical isolates is F170L substitution mutation which results from a single nucleotide polymorphism. Such amino acid change was detected earlier in clinical isolates showing the variable degree of susceptibility to both meropenem and imipenem [16]. F170L substitution mutation occurs in putative loop L3 which has been proposed to be involved in specific binding to basic amino acids and imipenem [21]. Our study demonstrated the F170L substitution mutation among 22 (35%) *P. aeruginosa* clinical isolates. Imipenem-resistance in *P. aeruginosa* clinical isolates was proposed to be mainly due to point mutations and deletions in the *OprD* gene [24]. Accordingly, we can conclude that F170L substitution mutation does not affect the susceptibility to imipenem. This conclusion is supported by our finding which revealed that all the isolates harboring the F170L substitution mutation were susceptible to imipenem and resistant to meropenem. Previous studies reported F170L substitution mutation among imipenem-resistant *P. aeruginosa* clinical isolates [15,19]. It was indicated that alteration of porin OprD and efflux proteins don't satisfactorily explain the resistance pattern observed in clinical isolates [10]. A divergent sequence 372(VDSSSSYAGL)383 was detected in eight isolates of *P. aeruginosa* in our study. The isolates which harbor this divergent sequence were meropenem resistant. Previous studies found that divergent sequence mutation that led to shortening of two amino acids in Loop 7 of the OprD porin was occurring frequently among *P. aeruginosa* isolates with variable degrees of resistance to meropenem [18,19,24]. These findings are consistent with the results indicating the association between divergent sequence 372(VDSSSSYAGL)383 and meropenem susceptibility obtained for *P. aeruginosa* clinical isolates [19]. The results regarding the divergent sequence 372(VDSSSSYAGL)383 are inconsistent with the results obtained by Epp *et al*., who proposed that the shortening of L7 by two amino acids because of divergent sequence 372(VDSSSSYAGL)383 allows sufficient penetration of meropenem into the bacterial cells leading to more meropenem bacteriostatic activity [20]. These findings deny the conclusion that structural alteration in the OprD porin changes the specificity and the porin conformation leading to OprD channel constriction.

A deletion mutation resulting in the loss of ten amino acid residues at the 433-443 position of OprD porin was usually reported among *P. aeruginosa* isolates for carbapenem-resistant or carbapenem-susceptible. Sequencing data showed that a ten amino acid sequence at 434 position is conserved among clinical *P. aeruginosa* isolates with a variable pattern of susceptibility to imipenem and meropenem. The results we obtained herein do not suggest the occurrence of 433(LIVDYPLSIL)443 deletion mutation in any of the 63 collected isolates. This is consistent with the susceptibility results to the imipenem and supports that meropenem enters through another route [20]. Our results from the DNA restriction enzyme analysis suggested the presence of an alteration in the *OprD* represented an IS element of approximately 100 bp within the 590 bp DNA segment downstream from the restriction site in 12 (19%) of the isolates. *P. aeruginosa* is a well-known pathogenic microorganism possessing a flexible genome with a high probability of uptake of IS elements [5,20]. The uptake of IS elements provokes insertional inactivation in the *OprD* gene leading to antibiotic resistance. Clinical and nonclinical *P. aeruginosa* isolates have previously been reported to show a significant association between antibiotic resistance and uptake of IS elements in the *OprD* gene [5,24-29]. However, Segal and his colleagues showed that imipenem resistance in *P. aeruginosa* clinical isolates was due to point mutations and deletion in *OprD* rather than missing IS elements in the studied isolates [16].

Conclusions

The current study tries to shed light on the genetic alteration in the OprD porin encoding gene among clinical isolates of *P. aeruginosa* isolated from hospitals in Jordan. The association between antimicrobial resistance and OprD porin structural alteration was studied. The results showed correlation between *OprD* porin structural changes and susceptibility to meropenem, but not imipenem. OprD porin structural alteration may increase the emergence of the antibiotic-resistant strain of *P. aeruginosa* and increase the

incidence rate of infections by multidrug resistance strain, considering that hospitals are the residence of a people who are potentially susceptible to opportunistic pathogens. This work underlines the role of porins in antimicrobial susceptibility establishing a basis for future studies elucidating the mechanisms of drug resistance in *P. aeruginosa* and other Gram-negative bacteria from clinical and environmental isolates.

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