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The Role Of OprD Gene In The Carbapenems Resistance In *Pseudomonas Aeruginosa* Isolated From Burns Infection In Al-Najaf Province

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Abstract

Background: Carbapenems-resistant *Pseudomonas aeruginosa* mutants are disseminating around the globe. 90%-100% of *P. aeruginosa* isolates became Carbapenems-resistant mutants in some countries like Thailand and Brazil respectively. Finding alternative therapeutic options instead of carbapenems is the global duty of researchers and institutions. The OprD gene mutation/s has the main role in carbapenems resistance appearance in *P. aeruginosa* due to the decrease in the outer membrane permeability for carbapenems. **Aim**: This study aims to assess the antimicrobial activity of carbapenems (imipenem, and meropenem) against *Pseudomonas aeruginosa* isolated from hospitalized burn patients in Al-Najaf/Iraq, and the role of OprD gene mutations in the resistance. **Methods and materials**: 76 samples were collected from hospitalized burn patients suffering from burn wounds infections, and the identity of each cultivated microbe was confirmed by VITEK compact system 2 and further confirmation for *P. aeruginosa* isolates via performing molecular identification by OprD gene detection, 10/12 carbapenems resistant isolates, 1/12 sensitive, and 1/12 intermediate resistant were submitted to OprD gene sequencing by Sanger method. **Result**: *P. aeruginosa* is the major cause of burn wound infection in the burn center of Al-Najaf city, 61/76 (80%) of samples shows positive growth, 20/61 (32%) were *P. aeruginosa*, 13/20 of *P. aeruginosa* isolates (65%) were imipenem-resistant the rest 6/20 (30%) imipenem-sensitive and 1/20 (1.5%) was imipenem-intermediate-resistant, also 17/20 of *P. aeruginosa* isolates (85%) were meropenem-resistant the remain 3/20 (15%) were meropenem-sensitive, all carbapenems resistant sequenced isolates contained OprD gene mutations, likely the main factor of resistance appearance and dissemination was antibiotics misuse.

Conclusion: Carbapenems-resistant *Pseudomonas aeruginosa* mutants are prevalent in the Al-Najaf burn center; a new drug is required, also more restrictions on antibiotics usage need to be applied.

Keywords: CRPA (Carbapenems-resistant *Pseudomonas aeruginosa)*, carbapenems (imipenem, and meropenem), OprD mutations, hospitalized burn patients, burn wound infection, antibiotics misuse.

Introduction

Carbapenems-resistant *Pseudomonas aeruginosa* mutants are disseminating around the globe, the disseminating percentage reaches 90% and 100% of isolates in some countries like Thailand and Brazil respectively, the medical significance of this bacterium is that this bacterium causes 10%-20% of nosocomial infections in the hospitals globally and this percent is increasable in the future. The infections publicity which caused by this bacterium is higher among hospitalized burn patients (Vincentia Rizke Ciptaningtyas, 2019), (WHO Team Antimicrobial Resistance Division, 2019), (G P Bodey, 1983), (Robert A. Weinstein, 2003), (Mehmet Faruk Geyik, 2003).

The main molecular mechanism that caused the resistance of the carbapenems is the OprD gene mutation/s, this gene coding for a specific channel (OprD channel) which is the route of entry into the cell for the imipenem and meropenem, mutations in the OprD gene cause reduce/loss of this channel from the OM this lead to prevent the entry of carbapenems into the cell and reach their target in the periplasmic space (José-Manuel Rodríguez-Martínez, 2009).

The last antibiotics of a choice for pertinacious infection caused by gram-negative bacteria including *Pseudomonas aeruginosa* are the carbapenems and carbapenems-resistant *Pseudomonas aeruginosa* mutants became disseminated. Most likely the main factor that plays a major role in the appearance and dissemination of carbapenem-resistant mutants in Al-Najaf city is antibiotics misusage (Meison Abdulbary, 2020), (Ibadi, 2003).

The dissemination of *P. aeruginosa* carbapenem-resistant mutants in hospitals has dangerous futuristic medical consequences and it calls us to find alternative therapeutic options instead of carbapenems.

Methods

The study was conducted in the labs of the laboratory investigation department at the faculty of science/Kufa University, according to the approved ethics from the Al-Najaf health governate. All procedures and protocols followed in this study were according to globally approved and published standards.

Methods of sample collection

76 clinical samples from the burn center in Al-Najaf province of hosted burn patients suffering burn infection were collected between 1 October 2021 and 31 January 2022. All specimens were collected by sterile cotton media swaps, labeled, and transported to the central lab within 1-2 hours, then blood and McConkey agar plates were strokes by the media swaps and the plates were incubated in 37 C^o for 18-24 hour.

Method of isolation

The standard striking method was used to isolate a pure bacterial culture. After stroking by media swaps on sterilized blood & MacConkey agar and incubated for 18–24 hours, growth on blood and McConkey agar plates sub-cultured to isolate pure colonies on other sterile blood and McConkey agar plates. Several times sub-culture was conducted until pure colonies were isolated.

Method of identification

VITEK 2 compact system 2 was used for identity confirmation, and the instructions of the manufacturer were followed accurately. Also, conventional molecular identification by OprD gene detection (Polymerase Chain Reaction and Agarose gel electrophoresis were performed) was used to confirm the identity of *Pseudomonas aeruginosa* isolates. A specific kit was used to extract the DNA from bacterial cells, the procedure was followed accurately according to the manufacturer's recommendations, 2 ml of bacterial suspension was taken from pure fresh culture equal to 0.5 McFarland scale of visual density, placed into sterilized 2ml Eppendorf tube, then procedure followed, the addition of solutions, incubate in the water bath and centrifugations according to steps in the kit's sheet, the components of DNA extraction Kit are listed in the table (1-1) below. DNA extractants finally were contained in fresh 2ml Eppendorf tubes sterilized by autoclave and the extractant was stored in -20 C^o to be used in the PCR procedure. Specific primers were used in PCR, also PCR mixture, conditions, and primers sequences are listed in the tables below (1-2), (1-3), and (1-4).

Agarose gel Electrophoresis

PCR products from storage in the refrigerator (-20 C^o) were loaded into wells of 1% agarose solubilized in 100 ml of 1X TBE buffer, agarose contains 5 ml of fluorescent gel red dye. Agarose immersed in 1X TBE buffer, the voltage was 70 volts for 60 mints. Using UV light illuminator to visualize the bands (Michael Richard Green, 2012).

Method of AST

Standard Kirby-Bauer disk diffusion method was used for AST. Inhibitory zone diameters were measured by an electronic ruler. Overnight growth of pure *P. aeruginosa* cultured in 5ml sterile BHI broth incubated in 37 C^o and the turbidity was adjusted equal to 0.5 MacFarland. 20 ml of sterile MH agar media was prepared. sterile cotton swap dipped in BHI broth rubbed against the tube's wall several times to remove excess inoculum, then the Muller Hinton agar's surface was stroked vertically and horizontally and around the edges, and the plate was rotated 90^o until the whole MH agar's surface cover with bacterial suspension, after that the plate lift to dry for a while about 10 mints, then antibiotic diffusion discs were applied by sterile metal forceps burned by burner flame each time. 7 antibiotics were applied to leave enough space between them so the inhibition zone could be measured precisely. The plates were inverted upside down and placed in the incubator for 18-24 hours in 37C^o. The diameters of inhibition zones were measured by using an electronic ruler, later the results were compared with CLSI (2021, M100-ED31), figures (4-4), and (4-6) below show the picture for AST.

Method of resistance gene sequencing

The PCR products of 12/20 isolates labeled as A and B, A for F1 forward primer, and B for F2 forward primer were packed and sent to Macrogen company in south Korea for sequencing. 10/12 isolates were carbapenem-resistant, 1/12 was susceptible to carbapenems, and 1/12 was intermediate resistant to carbapenems. Two primers were used for the sequence of the OprD gene, the F1 and F2 forward primers to avoid misreading of DNA polymerase used in the sequencing method. The PCR products

submitted to the Sanger sequencing method were sent along with diluted primers 20 picomole/µl contained in sterilized Eppendorf tubes.

Method of sequencing result analysis

Finch TV software was used for sequencing results analysis, which was used to compare the sequencing result of OprD from isolates with the OprD gene sequence of wildtype POA1.

Results

Type and percent of clinical samples collected from Al-Najaf hospitals

15/76 samples show no growth. The identity of remain 61/76 bacterial isolates was confirmed by VITEK 2 Compact system and the manufacturer's instructions were followed accurately. Molecular identification was also performed for *P. aeruginosa* isolates. 61/76 of samples showed growth on plates with different bacteria, they were 20/61 (32.78%) of burn patients' samples were *Pseudomonas aeruginosa*, 12/61 (19.67 %) samples *Acinetobacter baumanii*, 12/61 (19.67 %) *Staphylococcus aureus*, 7/61 (11.47 %) *Klebsiella pneumonia*, 1/61 (1.63 %) *Escherichia coli*, and 9/61 (14.75 %) were *Burkholderia cepacia*, they are represented in figures (4-1), (4-2) and the table (1-5) below.

Antibiotic susceptibility test (AST) of *P. aeruginosa*

According to the comparison of measured inhibition zone of antibiotic diffusion disks on Muller Hinton agar and antimicrobial susceptibility guide of CLSI 2021 (M100-ED31) for Imipenem (10 µg) and Meropenem (10 µg), there are 13 of 20 isolates (65%) resistant to Imipenem, 6 of 20 isolates (30%) sensitive to Imipenem, and 1 isolate (1.5%) is intermediate resistant to imipenem, also 17 of 20 of *P. aeruginosa* isolates (85%) were resistant to Meropenem and 3 of 20 isolates (15%) were sensitive to Meropenem. According to CLSI 2021 (M100-ED31) for Imipenem (10 µg): ≤ 18 mm inhibition zone/R, among 19-21 mm inhibition zone/ I, and ≥22 mm inhibition zone/ S. for Meropenem (10 µg): ≤ 14 mm inhibition zone/R, among 15-17 mm inhibition zone/I, and ≥18 mm inhibition zone/S, figures (4-3), (4-5) below.

Molecular screening of OprD gene

All 20 *P. aeruginosa* isolates were submitted to conventional PCR (Polymerase Chain Reaction) for OprD gene (resistance gene) amplification by using a specific primer, all isolates tested positive for OprD gene. The OprD gene 1,332 bp in length and the ladder of 10Kbp were used as shown in the figure (4-8) below.

Sequencing results

According to the sequencing results of the OprD, all 10/12 (83.33%) of carbapenems resistant isolates of *P. aeruginosa* in this study contained OprD gene mutations most of them frameshift mutations in comparison with the sequence of the OprD gene in PAO1 wildtype.

Discussion

The significance of burn infection that is 11 million burn patients sought medical care for their wounds in 2004 globally. In a study conducted by Iraqi National Injury

Mortality Surveillance System during the period from 1-1-2010 to 31-12-2015, 5150 deaths from unintentional injuries, were primarily due to burns 2369 (46%) and electrical injuries (31%). *P. aeruginosa* is the first in causing burn wound infections and deaths in burn patients, (Mehmet Faruk Geyik, 2003), (Robert A. Weinstein, 2003), (Monika Kristaq Belba, 2013), (David G. Greenhalgh, 2019), (Matthew Goers, 2020).

Misusage of antibiotics leads to the dissemination of *P. aeruginosa* mutants that are resistant to all antibiotics used in this study including carbapenems, in Al-Najaf city, (Abdulbary, 2020), (Ibadi, 2003), (Al-Naseri, 2019).

Carbapenems-resistant *Pseudomonas aeruginosa* mutants are disseminating globally for instance In Brazil, the isolates of *Pseudomonas aeruginosa* were 100% resistant to Carbapenems (imipenem and meropenem) in Brazilian hospitals, in Japan carbapenems-resistant *P. aeruginosa* isolates was 31.5% for (imipenem and meropenem) in 2015, In Germany (25.1%) isolates were resistant to carbapenems, samples were collected from January 2016 to April 2017, in Chinese hospitals about 40% of isolated *P. aeruginosa* were carbapenem-resistant, in Iraq from hospitalized burn patients from December 2012 to June 2013 in Karbala province\Iraq, 58.33% (35) of *P. aeruginosa* isolates were resistant to Imipenem, and 66.67% (40) of *P. aeruginosa* isolates were resistant to Meropenem (Shilba, 2015), (Gales, 2003), (Terahara, 2019), (Hong, 2015), (Kresken, 2020).

Our study indicates similar results, 32% of burn patients were infected with *P. aeruginosa* caused burn wound infection, *P. aeruginosa* isolates 20/61 (32%), a 13/20 (65%) were imipenem-resistant the rest 6/20 (30%) imipenem-sensitive and 1/20 (1.5%) was imipenem-intermediate-resistant, and 17/20 (85%) were meropenem-resistant the remain 3/20 (15%) were meropenem-sensitive. All 10/12 carbapenem-resistant isolates contain OprD gene mutations in comparison with the OprD gene sequence of PAO1 wildtype.

Conclusion

P. aeruginosa is the major cause of burn wound infection in the burn center of Al-Najaf city. Carbapenems-resistant *Pseudomonas aeruginosa* mutants are disseminating in Al-Najaf burn center, most likely the main factor that plays the major role in the appearance and the dissemination of carbapenem-resistant mutants in Al-Najaf city is antibiotics misusage, find alternative therapeutic options instead of carbapenems required, more restrictions on antibiotics usage need to be applied.

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Table (1-1): DNA extraction kit component

Component	N1151/50 preps.
Solution DS	15 ml
Solution MS	20 ml
Proteinase K (20mg/ml)	1 ml
Wash buffer PS	18 ml
Wash buffer PE	15 ml
Elution buffer TE (10mM Tris-HCl, 1M EDTA, PH 8.5)	5 ml
Spin columns	50 each

Table (1-2): PCR reaction mixture component

Master-mix	25 µl
Forward primer	4 µl
Reverse primer	4 µl
DNA sample	5 µl
Deionized distilled water	12 µl

Table (1-3): PCR condition followed in this study

Step	Temperature (C^o)	Time(second)	No. of cycles
1-intiatal Denaturation	95	300	1
2-Denaturation	95	300	30
3-Anneling	63	30	
4-Extention	72	90	
5-termination	4	∞	1

Table (1-4): sequences of the primers used in this study

Primer name	Sequence 5'—3'	N0. of nucleotides	Reference
OprD – F	CGCCGACAAGAAGAACTAGC	20	(Rodríguez-Martínez, 2009)
OprD - F2	GCCGACCACCGTCAAATCG	19	
OprD – R	GTCGATTACAGGATCGACAG	20	

Table (1-5): the bacterial genus and spices were found in 61 samples of this study

Genus	Spices	Number	Percentage
Pseudomonas	*aeruginosa*	20	32.78%
Acinetobacter	*baumannii*	12	19.67%
Staphylococcus	*Aureus*	12	19.67%
Klebsiella	*pneumonia*	7	11.47%
Escherichia	*Coli*	1	1.63%
Burkholderia	*Cepacia*	9	14.75%

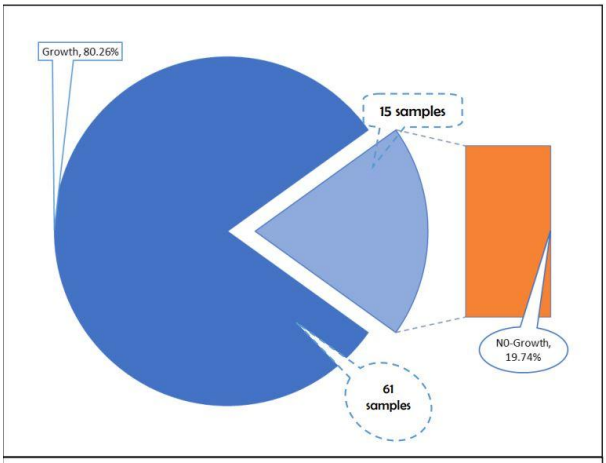

Figure (4-1): - Growth, and No-Growth percentage of 76 collected samples where 15 samples show no-growth (19.74 %), and 61 samples (80.26 %) show growth on blood & MacConkey agar plates.

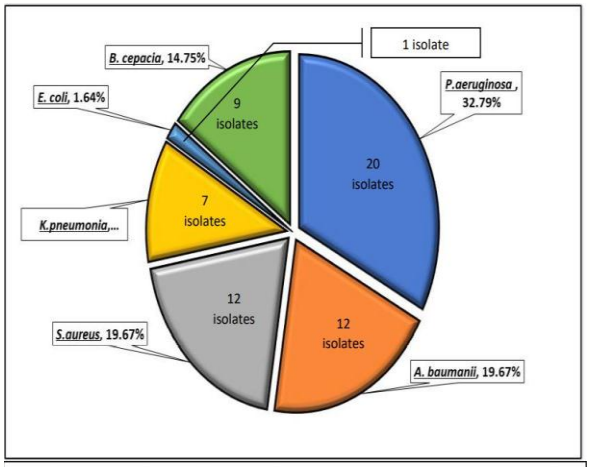

Figure (4-2): - percentage of bacterial type from total 61 samples were collected from burn center from admitted burn patients were suffering burn infection.

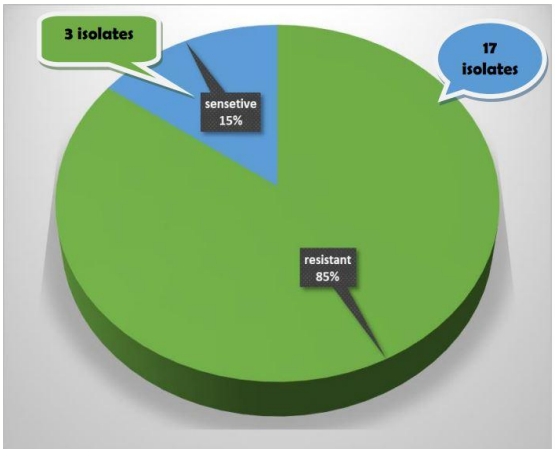

Figure (4-5): percentage of resistant and sensitive P. aeruginosa isolates to Meropenem, where 17 isolates were resistant and 3 isolates were sensitive.

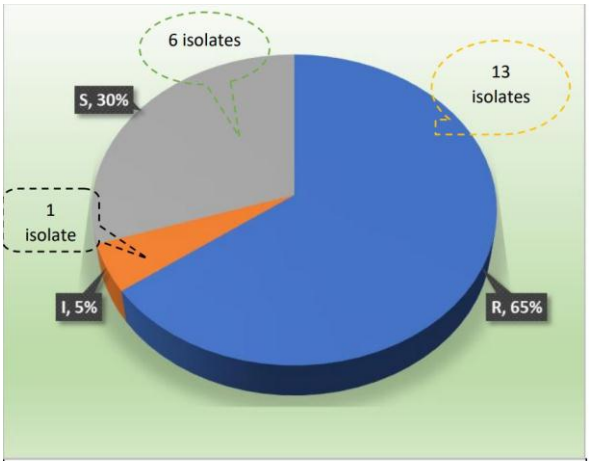

Figure (4-3): percentage of resistant and sensitive P. aeruginosa isolates to Imipenem, where 13 isolates were resistant and 6 isolates were sensitive, and 1 intermediate.

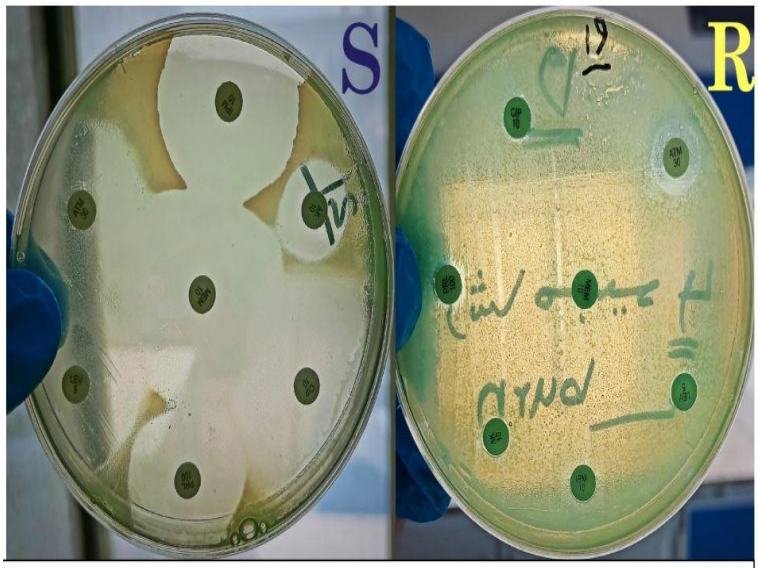

Figure (4-6): picture of antibiotic diffusion disk applies on *P. aeruginosa* growth on Muller Hinton agar, R refers to Resistant to Meropenem, S refers to sensitive to Meropenem, the pictures were taken by mobile Camera 48 MP.

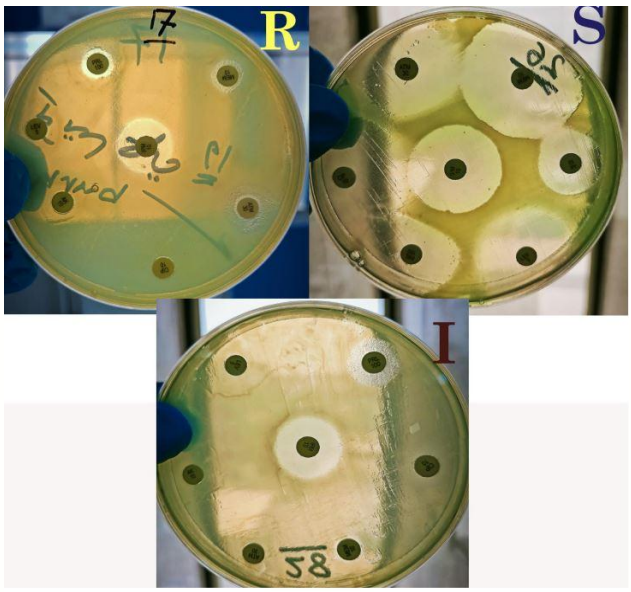

Figure (4-4): picture of antibiotic diffusion disk applies on *P. aeruginosa* growth on Muller Hinton agar, R refers to Resistant to Imipenem, S refers to sensitive to Imipenem, and I refer to Intermediate resistance to Imipenem, the pictures were taken by mobile Camera 48 MP.

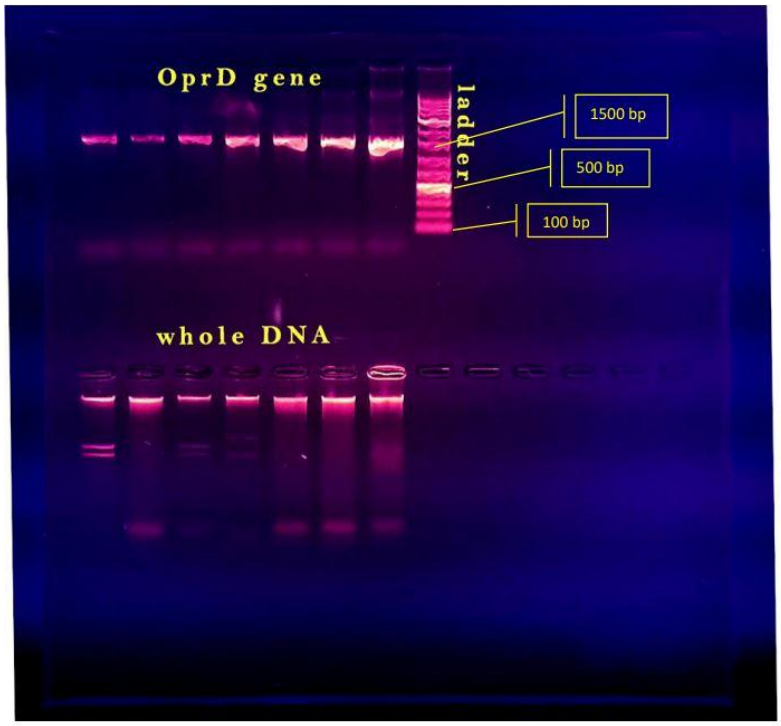

Figure (4-8): Picture of screening OprD gene positive, OprD gene on the top, the ladder is 10K bp long the OprD in 1400- 1500 bp band's region consider as positive for OprD gene. The pic. Was taken by mobile Camera 48 MP.