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The outer membrane protein D gene mutations and carbapenem resistance in Pseudomonas Aeruginosa isolated from burns infection in Al-Najaf/Iraq

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***Abstract*---**The Most common mechanism used by *P. aeruginosa* for carbapenem resistance is the decrease of OM permeability, via accumulating mutations cause loss/reduce in the OprD gene expression. This study was designed to detect and determine the OprD gene mutations in carbapenem-resistant isolates of clinical *P. aeruginosa* isolated from burn wound infections. A 12/20 isolates were sequenced by Sanger method and the results were compared with wildtype (POA1) OprD gene sequence. The PCR products of 12/20 isolates were labeled as A and B, A for F1 forward primer and B for F2 forward primer, packed and sent to the Macrogen company for sequencing. All carbapenem-resistant isolates (10/12) contain mutations in their OprD genes, most of mutations are frameshift mutations, 1/12 still carbapenem-sensitive despite containing 40 mutations. All resistant sequenced isolates contain OprD mutations this might be the main cause and most related to carbapenem-resistance trait in the mutant strains, also despite the OprD mutations in isolate 21A it still carbapenem-sensitive indicates that the OprD mutation isn't definitive factor for carbapenems resistance trait.

***Keywords*---**carbapenems-resistant, Pseudomonas aeruginosa, Iraqi patients, OprD gene, mutations, burn wound infections.

Introduction

The OprD gene is one of *P. aeruginosa* (PAO1) chromosomal genes, OprD gene is 1,332 bp in length. Encodes a porin called by the same name OprD channel. The OprD channel inserted in OM, the location of this channel is essential for its function. OprD channel encoded by 1332 bp nucleotides has a molecular weight of 45,000 to 47,000 Dalton (Da), (Trias J, Nikaido H., 1990; Quinn JP *et al.*, 1991; Medhat and Aljanaby, 2022). Crystal structure of OprD by X-ray technique from *Pseudomonas aeruginosa*, reveals 18-stranded β-barrel monomer, contains a very narrow pore, with a positively charged basic ladder on one side and an electronegative pocket on the other side, this OprD channel acts as a specific channel for basic amino acids (like arginine) and some small peptides and contains a binding site for imipenem (Li H *et al.*, 2012) (Biswas S *et al.*, 2007). The studies confirm that the mutation/s occur in this gene that cause loss/reduce in OprD channels give *P. aeruginosa* a resistance trait to imipenem and meropenem because this loss or reduction in OprD channels of OM lead to decrease OM permeability to carbapenems (Trias J, Nikaido H., 1990; Quinn JP *et al.*, 1991; Li H *et al.*, 2012; Hadi and Aljanaby, 2022).

Materials and Methods

The study was conducted in the labs of laboratory investigation department at the faculty of science/ Kufa University/ Al- Najaf province/ Iraq, according to approved ethics from the Al-Najaf health governate. All procedures and protocols followed in this study were according to globally approved and published standards (Abdulla et al., 2022).

Methods of sample collection

76 clinical samples from the burn center of 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 of Al-Najaf province within 1-2 hours, then fresh and sterilized blood and McConkey agar plates were strokes by the media swaps and were incubated in 37 Co for 18-24 hour (Al-Hadraawy et al., 2022).

Method of isolation

Standard striking method was used to isolate a pure bacterial colony. After stroke by media swaps on sterilized blood & MacConkey agar and incubated for 18–24-hour, growth on blood and McConkey agar plates sub-cultured to isolate pure colonies on other fresh blood and McConkey agar plates. Several times of sub-culture conducted until pure colonies were isolated (Alhasnawi and Aljanaby, 2022).

Method of identification

VITEK 2 compact system was used for identity confirmation of bacteria; the instructions of manufacturer were followed accurately. Also, conventional

molecular identification by OprD gene detection (Polymerase Chain Reaction and Agarose gel electrophoresis were preformed) was used to confirm the identity of *Pseudomonas aeruginosa* isolates (Aljanaby et al., 2022). A specific kit was used to extraction the DNA from bacterial cells, procedure was followed accurately according to manufacturer's recommendations, 2 ml of bacterial suspension was taken from pure and fresh culture equal to 0.5 McFarland scale of visual density, placed into sterilized 2ml Eppendorf tube, then procedure followed, the additions of solutions, incubations in the water bath and centrifugations according to steps in the kit's sheet. DNA extractants finally was contained in fresh 2ml Eppendorf tubes sterilized by autoclave, and the extractant stored in -20 Co to be use in PCR procedure latter. Specific primers were used in PCR, also PCR mixture and conditions and primers sequences are listed in the tables below.

Table 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 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 3: PCR condition followed in this study

Step	Temperature (Co)	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 4: sequences of the primers used in this study

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

OprD – R	GTCGATTACAGGATCGACAG	20	

Method of primers preparation

Primers used in this study were designed specific to OprD gene, ordered from (Macrogen) South Korea public biotechnology company. Primers were lyophilized by nuclease free water. stoke solution of primers (forward and reverse) was 100 picomole/µl by adding 250 µl from nuclease free water the working solution was 5 picomole/µl. dilution process conducted by using sterilized micropipette tips and Eppendorf tubes by autoclave. The dilutions were made according to following equation: $M_1C_1 = M_2C_2$

Method of PCR

PCR procedure was followed according to (Green MR, 2012). A specific volumes of PCR mixture were poured into sterilized PCR tubes includes premixed master-mix 25µl, forward and reverse primers each 4 µl with final concentration 20 picomole, DNA template 5 µl (extracted previously from bacterial cells by specific DNA extraction kit), and dd water completed to 50 µl (12 µl) by using micropipette (Hasan et al., 2021). The tip of micropipette was replaced after each addition. Micropipette tips were sterilized by autoclave 121 Co for 15 mints. Disposal gloves were worn to prevent contamination. The components of PCR mixed well by pipetting carefully several times. The PCR tubes contain reaction mixture were put in the ice until completing the process of preparation, then the PCR tubes placed in PCR thermoregulator to initiate the reaction. PCR cycle details listed in the following table:

Table 5: PCR condition followed in this study

Step	Temperature (°C)	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

Agarose gel Electrophoresis

PCR products form storage in refrigerator at -20 Co 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 visualized the bands (Green MR, Sambrook J., 2012), the figure (VII) illustrates the visualized bands by UV light.

Method of AST

Standard Kirby-Bauer disk diffusion method was used for AST. Inhibitory zone diameters were measured by electronic ruler. The overnight growth of pure *P.*

aeruginosa culture in 5ml sterile BHI broth incubated in 37 Co and the turbidity was adjusted equal to 0.5 MacFarland, used to inoculate the plates of MH agar. 20 ml in each plate of sterilized 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, horizontally and around the edges, the plate was rotated 90o 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 with leaving enough space between them so inhibition zone could be measured precisely. The plates were inverted upside down and placed in the incubator for 18-24 hour in 37Co. The diameters of inhibition zones were measured after, by using electronic ruler, later the results compared with CLSI (2021, M100-ED31).

Method of OprD gene sequencing

Each PCR products of 12/20 *P. aeruginosa* isolates were divided into two PCR tubes labeled as A and B, A for F1 forward primer and B for F2 forward primer, they packed and sent to Macrogen company in south Korea for sequencing. Two primers were used for sequence the OprD gene, the F1 and F2 forward primers to avoid misreading of DNA polymerase used in the sequencing method. The PCR products which submitted to 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 after the sequencing results of OprD gene for each isolate were sent from the company by email, this software was used to compare the sequencing results of OprD gene of each isolate with OprD gene sequence of wildtype POA1 to detect and determine the mutations.

Results

Type and percent of clinical samples collected from Najaf hospitals

15/76 of collected samples show no growth. The bacterial identities were confirmed by VITEK 2 Compact system, the instructions of manufacturer were followed accurately. Further Molecular identification was performed for *P. aeruginosa* isolates. The remain 61/76 of collected samples showed growth on plates with different bacteria, 20/61 (32.78%) isolate were *Pseudomonas aeruginosa*, 12/61 (19.67 %) isolate *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 figure (II) and table (6):

Table 6: 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%

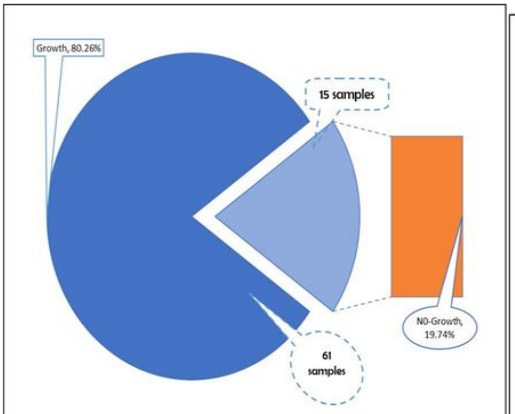

Figure (I): - Growth, and No-Growth percentage of 76 collected samples where 15 samples show no-growth (19.74 %), and 61 samples (80.26 %) show growth on blood & MacConkey agar plates.

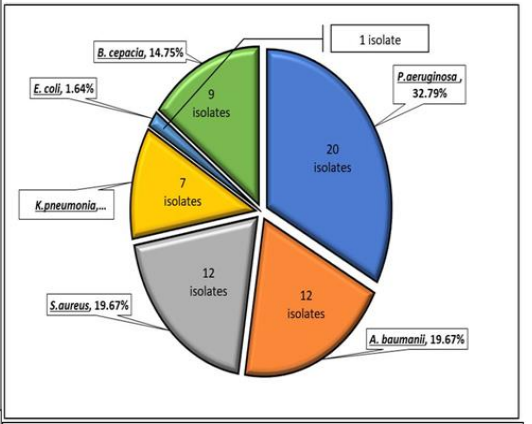

Figure (II): - percentage of bacterial type from total 61 samples were collected from burn center from admitted burn patients were suffering burn infection.

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

According to comparison of measured inhibition zone of antibiotic diffusion disks on Muller Hinton agar with antimicrobial susceptibility guide of CLSI (2021, M100-ED31) for Imipenem (10 µg) and Meropenem (10 µg), there are 13 of 20 isolates (65%) were resistant to Imipenem, 6 of 20 isolates (30%) were sensitive to Imipenem, and 1 of 20 (1.5%) isolate was intermediate resistant to imipenem, also 17 of 20 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:

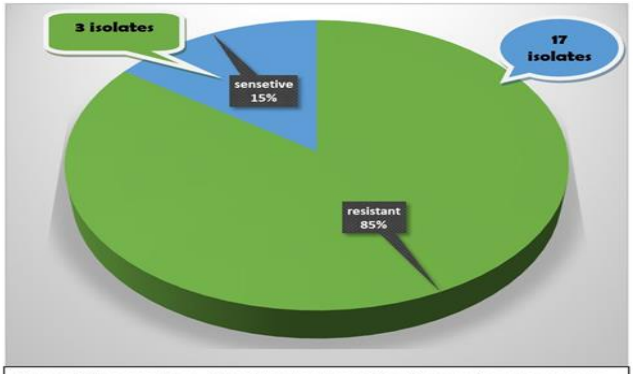

Figure (III): percentage of resistant and sensitive *P. aeruginosa* isolates to Meropenem, where 17 isolates were resistant and 3 isolates were sensitive.

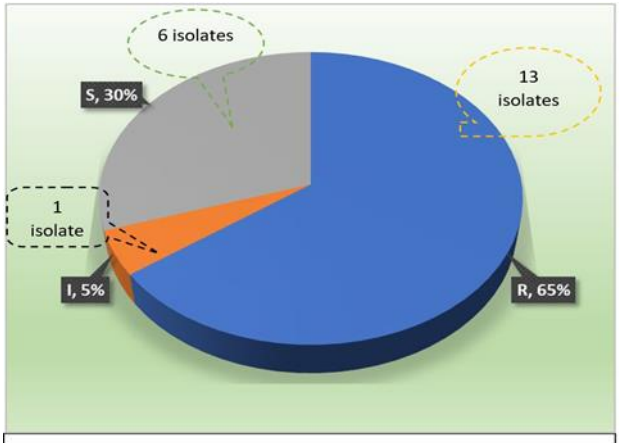

Figure (IV): percentage of resistant and sensitive *P. aeruginosa* isolates to Imipenem, where 13 isolates were resistant and 6 isolates were sensitive, and 1 intermediate.

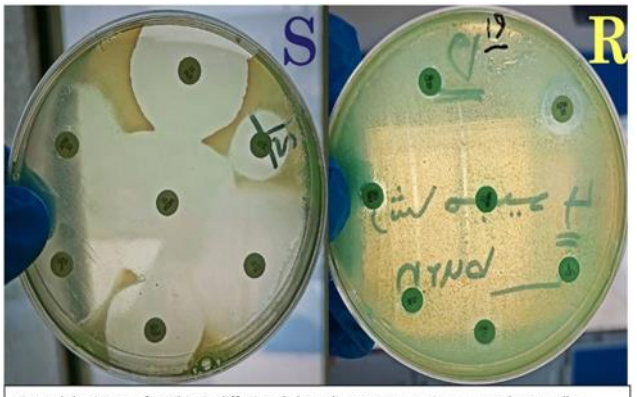

Figure (V): picture of antibiotic diffusion disk applies on *P. aeruginosa* growth on Muller Hinton agar, R refers to Resistant to Meropenem, S refers to sensitive to Meropenem, the pictures were taken by mobile Camera 48 MP.

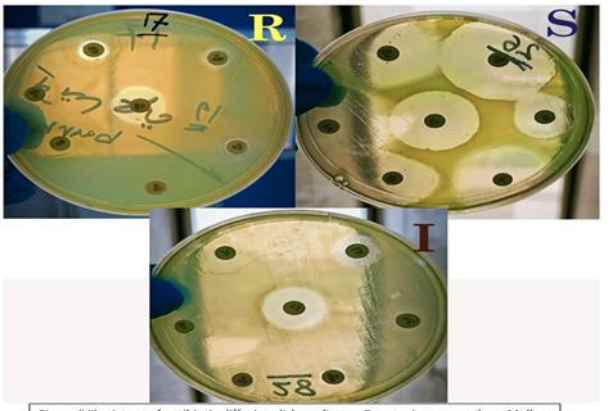

Figure (VI): picture of antibiotic diffusion disk applies on *P. aeruginosa* growth on Muller Hinton agar, R refers to Resistant to Imipenem, S refers to sensitive to Imipenem, and I refer to Intermediate resistance to Imipenem, the pictures were taken by mobile Camera 48 MP.

Molecular screening of OprD gene

All 20 resistant *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 by agarose gel electrophoresis. The OprD gene of 1,332 bp length and the ladder of 10K bp were used as shown in the figure below:

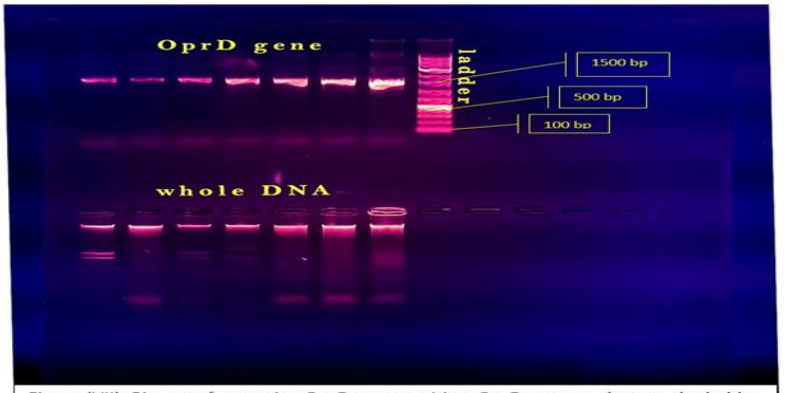

Figure (VII): Picture of screening OprD gene positive, OprD gene on the top, the ladder is 10K bp long, the OprD in 1400- 1500 bp band's region consider as positive for OprD gene. The pic. Was taken by mobile Camera 48 MP.

Sequencing results

All 12/20 sequenced isolates contain OprD gene mutations. 10/12 (83.33%) of sequenced isolates were carbapenems-resistant, 1/12 (8.33%) was carbapenem-sensitive, and 1/12 (8.33%) was intermediate-carbapenem-resistant. Despite 40 mutations in OprD gene of isolate 21A and most of them were frame shift mutations but it remains carbapenem-sensitive. Also, isolates 9 and 14 still meropenem-sensitive despite mutations in their OprD genes, and isolate 10 despite OprD mutations still imipenem-sensitive, the table (7) shows the mutations profile in detail:

Table 7: Mutations profile of OprD gene of this study, by Sanger sequencing method

Isolate	Mutation	Type & Number
2	C199/Deletion, A324C, C375T, C 400T, G438T, G462A, C517CT, G520A, C529G, C535T, T551C, A560G, C568G, G571C, G572C, C573G, A580G, C582T, C586T, C606T, G609C, T617C, G620A, C624G, G642C, G643T, G649A, C650A, C651T, C656G, G668A, C678T, T693C, T701C, G709A, G714C, A715G, C727T, C735T, C746G, T759C, G762A, G768A, G769T, A770C, A771C, T774C, G777A, C792T, C810A, C812A, C816T, T826G, G831A, C855T, T856A, C867G, G869C, C870A, G873C, C879T, C885T, A888G, C891A, C894T, C902T, C904G, C906A, C914A, C928T, G929C, C937T, C943T, A944T.	75 mutations: 1 deletion mutation 74 substitution mutation
11	C199/Deletion, T364A, A367G, C375T, C385T, A397/Insertion, C400T, G438T, T449G, C450T, T451C, A504G, C506T.	13 Mutations: 1 Deletion 1 Insertion 11 substitutions
3	A1/Insertion, G92A, A168T, G249C, C282T, G305C, C313G, C314T, C317T, G332A, A349C, G354C, C357T, T364A, A367G, C378T, G382C, C385G, G393A, C413T, A428G, A435G, G444T, A468C, C474T, G492A, C502A.	27 Mutations: 1 insertion 26 substitutions
20A	C282T, C313G, C317T, A349C, G354C, C357T, C363T, T364A, C378T, C385G, G391A, G393A, C413T, G429/Insertion, A435G, G444T, G465T, A467G, G468C, T474/Insertion, T479C, C491G, G492A, C498T, A503C, A504T, C506T.	27 Mutations: 2 insertions 25 substitutions
21A	G92A, G249C, C282T, G305C, C313G, C317T, G332A, A349C, G354C, C357T, C378T, C413T, G393A, A428G, A435G, G444T, G462/Deletion, G466C, A468C, G472C, C474T, T479C, C494G, T505/Insertion, C514A, A515G, G516C, C517G, G518A, G520C,	40 mutations: 1 deletion 2 insertions 37 substitutions

	T521C, G524A, A526G, C529T, C535T, C543G, T550/Insertion, A555G, A565G, A569G.	
22A	A45G, C143/Deletion, G180T, G189C, C191/Insertion, A193G, C200A, C201G, A202T, T221G, G246/Insertion, T248G, G262T, G265C, C279G, C282T, T284C, T285/Insertion, G286/Insertion, G287A, G306A, C313G, C317T, T334A, G335A, G342A, A343C, A348C, G350C, C360T, C375A, G393A, C400T, G418A, A428G, A435G, G444A, A504G, C506T.	39 mutations: 1 deletion 4 insertions 34 substitutions
8	G66/Deletion, C128G, G129A, A171G, G172A, C173A, C176T, A177C, C179G, G182T, C185T, C194T, A203G, T221C, C236T, C279G, G305C, C360T, C385A, T386G, A428G, G444C, C460T, C474T, C489G, A504G, A510G, C514G, A515C, G518A, G520A, C529G, A541G, G544A, T551C, C568G, G571C, G572C,C573G, A580G, C582T, G586A, C595T, G596T,A597G, A598G, T600C, C601T, T602C, A603/Insertion, A604/Insertion, G609C, T613A, T615/Insertion, C616/Insertion, T617C, G618C, G621A, G622C,C624A,G626A,C629A, G635A, G637A, G638/Insertion, G640C, G642C, G649C, C650G, T652/Insertion, G653/Insertion, C659A, G668A, A669T, C675G, C678A, C681/Insertion, A686C, C688G, G689C, C690/Insertion, T693C, C696T, G698T, G702/Insertion, C708A, G709A.	87 mutations: 1 deletion 10 insertions 76 substitutions
24A	G92A, A168T, G249C, C282T, G305C, G306A, C313G, C314T, C317T, G332A, A349C, C351/Insertion, G354C, C357T, T364A, A367G, C370G, G373C, G377A, C378T, C381A, G382C, C384T, C385T, T386C, G387A, G393C, C409T, A410T, C413T, A421/Insertion, G426A, A427G, A428G, A435G, G438A, G444T, T451C, G453/Insertion, G455T,	53 mutations: 4 insertions 49 substitutions

	A461/Insertion, C464T, A468C, G472C, C473T, C474T, T479C, C482T, G486A, C489G, C494T, A504G, C506T.	
14	T32A, C128G, G129A, A171G, G172A, C173A, C176T, A177C, C179G, C282T, C313G, C318T, A349C, G354C, C357T, C378T, C385G, G393A, C413T, A428G, A435G, G439T, G520A, T521C, C529G, C535T, T551C, G609A, G620A, C624G, G642C, C645T, G649A, C650T, C651T, C656G, G668A, C678T, T701C,C705T, C708T, G714C, A715G, C729T, C735T, C746G, T759C, G762A, G768A, G769T, A770C, A771C, T774C, G777A, C792T, C810A, C812A, C816T, T826G, C827/Deletion,C828A, C855T, C867G, G873C, C879T, C885T, A888G, C894T, C902T, C904G, C914A, C928T, G929C, C937T, C943T, G948A, A962G, A963G, T973C, C979T, G980T, C988T, A1005G, C1007G, C1030G.	85 mutations: 1 deletion 84 substitutions
9	T32A, T38G, C29/Deletion, C375T, A398T, C400T, C402T, C403T, G438T, C517T, G520A, C529G, C535T, C538T, C551C, G609A, G620A, C623T, C682T, C712T, C735T, T759C, G768A, G769T, A770C, A771C, T774C, C816T, G825A, T826G, A853G, A888G, T895/Insertion, C904G, C919G, T940C, A962C, A963T, T973C, C979G, C988T, C994/Insertion, A1005G.	43 mutations: 1 deletion 2 insertions 40 substitutions
10	C128G, G129A, G166T, A171G, G172A, C173A, C176T, A177C, C179G, G182T, C185T, C194T, A203G, T221C, C236T, T253G, C279G, G305C, C360T, C385G, T386G, G438A, G444C, C460T, A504G, C506T.	26 mutations: 26 substitutions
12	T84/Insertion, A171G, G172A, C173A, C176T, A177C, C179G, C182C, C185T, C194T, C203T, T221C, C236T, C279G, G290A, C299T, C375T, C400T, G438T,	42 mutations: 4 insertions 38 substitutions

	A468G, G486A, C502G, A515G, G516A, C517T, C529G, C535T, A564/Insertion, A569G, C581/Insertion, C595G, G609A, C612G, G618T, C619G, G621A, C623A, C624T, C631T, G635A, A636G, C638/Insertion, C651/Deletion, C656G, G658A, C659G, T670/Insertion, A673G, C675G, T681/Insertion, C682T, C696T, G704A, G706C, C712T.	

Discussion

Many studies indicate that the OprD gene mutations in *P. aeruginosa* involved in reduce/loss of OprD channel located in OM reduces the OM permeability for carbapenems antibiotics, considering that the OprD channel is the carbapenems route of entry into cell this leads to carbapenem resistance, *P. aeruginosa* mutants that have OprD gene mutation/s shows a carbapenems resistance in compare with wild type POA1 (Li H *et al.*, 2012), (Quinn JP *et al.*, 1991), (Trias J and Nik aido H., 1990). Same finding of this study, all resistant sequenced isolates 10/12 (83%) contain OprD gene mutations, but despite the number of mutations in isolate number 21A it remains sensitive to imipenem and meropenem. Misusage of antibiotics including not committing to the dosages prescribed or antibiotics administration without AST provides *P. aeruginosa* with low concentrations of antibiotics not enough to kill them instead these concentrations enough to induce bacterial molecular machinery to develop resistance against these antibiotics and cross-resistance could be produced also, that means the mutation rate accelerated by misuse of antibiotics this can push bacteria to evolve faster multiple times than the bacteria have no exposure to these low ineffective antibiotics concentrations (antibiotics misuse) (CDC, 2019), (C. Lee Ventola and MS, 2015), (Voor in 't holt AF *et al.,* 2014).

Conclusion

All carbapenem resistant isolates contain mutations in their OprD gene sequence, considering the case of isolate number 21A the mutations in OprD gene don't consider a definitive in *P. aeruginosa* carbapenem-resistance trait. The high number of mutations in isolates indicates that isolated *P. aeruginosa* from clinical samples continuously create and accumulate mutations in their OprD gene attempt to evolve into carbapenem-resistant mutants this accelerated by antibiotics misusage which exert selective pressure (natural selection) pushes the bacterium to evolve into resistant strains which are spreading until reach full dissemination.

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