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MOLECULAR CHARACTERIZATION OF SOME CARBAPENEM-RESISTANCE GENES AMONG *Pseudomonas aeruginosa* ISOLATED FROM WOUND AND BURN INFECTIONS IN DUHOK CITY, IRAQ

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ABSTRACT

Background *Pseudomonas aeruginosa* is an opportunistic pathogen causes severe nosocomial infections among burn and wound patients. Multi-drug resistant strains namely carbapenems antibiotics have mentioned worldwide.

Objectives The aim of this study was to know the incidence, patterns of antibiotic susceptibility and molecular characterization of *P . aeruginosa* isolates among wound and burn hospitalized patients.

Methods From September 2019 till September 2020, a total of 524 burn and wound swabs were collected from inpatients at Burn and Emergency Hospital, Duhok city, the Kurdistan region, Iraq. The swabs were cultured and bacterial isolates were identified manually through microbiological tests then by Vitek2 compound system. The isolates were checked for their antibiotic susceptibility patterns then subjected to Polymerase Chain Reaction (PCR) assay using a set of specific primers for detection of (*OprD, bla*$_{VIM}$, *bla*$_{IMP}$) carbapenem-resistance genes.

Result About 60 (11.4%) isolates were identified as *P . aeruginosa*; 33 (55%) from female and 27 (45%) from male. Wound isolates exhibited pretty higher resistance over burn against 24 tested antibiotics. Imipenem; meropenem resistance rates were as (33.3 %; 31.7%) and (32.6%; 26.1%) for burn and wound, respectively. High resistance to piperacillin & ticarcillin (75% for both), ticarcillin/clavulanic acid (66.7 %) and tobramycin (63.6 %) were noticed in burn isolates. Colistin and piperacillin/tazobactam exhibited very low resistance. PCR assay indicated that 48 (96%) isolates were contained either single or double genes. *OprD* was predominated 40 (80 %) isolates then *bla* $_{VIM}$ gene 8 (16 %) isolates, while no *bla* $_{IMP}$ gene was detected. About 8 isolates were harbored double resistance genes (*OprD, bla* $_{VIM}$) simultaneously and unexpectedly 1 (12%) of these isolate was phenotypic carbapenem-susceptible. moreover, 5 phenotypic carbapenem-resistance isolates were not contained any target resistance genes by PCR assay.

Conclusion Occurrence of *P . aeruginosa* as a harbor of multiple carbapenemase resistance genes is increasing over time limiting the treatment options to this serious infection. The data support basic mechanism of imipenem resistance could be mostly via the loss of *OprD*. Colistin and piperacillin/tazobactam have high efficacy.

***KEY WORDS**: P . aeruginosa*, Burn and wound, Carbapenem resistance gene, PCR

1. INTRODUCTION

Pseudomonas aeruginosa is a vital opportunistic multi-drug resistance pathogen mainly affects immunocompromised patients; severe burns, wounds infections and others (Moradali *et al*., 2017). This pathogen can tolerate unfavorable environmental factors and somewhat a wide range of temperatures (Moradali *et al*., 2017). Beside intrinsic resistance to several disinfectant and antibiotic, it has the ability to acquire resistance genes and other virulence factors with increase bacterial count for initiation of pathogenicity and infections (Dash *et al*., 2019).

Burns and wounds are defined as a painful traumatic injury of the skin breaching integumentary lowering immunity, increased hospital prolonging and ultimately risks of hospital-acquired infections (Bhatt *et al*., 2015). Frequently, burn and wound infections ends up with sepsis and deaths due to unwise use of systemic antibiotics interventions such as surgical debridement and skin grafting that enables favorable condition for growth of multidrug resistant strains (Dou *et al*., 2017).

Multidrug Resistant (MDR) strains of *P . aeruginosa* in community have severe impact on limited the availability of therapeutic options. Carbapenems are longer prescribing for

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treatment of MDR *P . aeruginosa* infections when other antibiotics have failed. Carbapenems, such as imipenem and meropenem, are used as last-choice antibiotics for the treatment of multidrug-resistant or pan-resistant isolates in the recent period. Expansion of carbapenem resistance may considerably compromise their efficiency with main healthcare issues (Tsao *et al*., 2018). *P . aeruginosa* has the ability to develop chromosomal mutation-related resistance and β-lactamases-encoded resistance gene acquisition (Alma *et al*., 2019). The loss of porin *Opr D* and acquiring resistant genes encoding carbapenem-hydrolyzing (carbapenemase) accounted mainstays of resistance (Al-Khudhairy and Al-Shammari, 2020).

Phenotypic and molecular-based methods have been employment worldwide to find Carbapenem resistant isolates (Miriagou *et al*., 2010; Nordmann & Poirel, 2014). Therefore, the purposes of this study were; bacteriological characterization of *P . aeruginosa* isolates from burns and wound hospitalized patients in Duhok city, Kurdistan of Iraq. Also, to investigate the molecular characterization of carbapenem resistance genes by PCR assay using different primers.

2. MATERIAL AND METHODS

2.1 Study setting and subjects

This prospective study was conducted in the Burn and Emergency Hospital, Duhok city from September 2019 till September 2020. A total of 524 inpatients with various genders and ages having burn injuries and wounds were included. Inclusions were involved wound patient that suffering from diabetic, accident and surgery and not taking antibiotics three days ago also samples taken from burns before applying of disinfectants.

2.2 Ethical consideration

Study proposal and informed consent was approved by ethics committee of the College of Medicine, Duhok University and Duhok Public Health Directorate, the Kurdistan Region, Iraq. From all patients, formal consent was taken from their parents/guardians before collecting the samples.

2.3 Sample collection and processing

A total of 222 (146 males : 76 females) burn swabs and 302 (126 males : 176 females) wounds swabs (pus or discharge) were aseptically collected. The samples were transported to laboratory within 30 minutes to one hour for bacteriological processing. Swabs were cultured on Blood agar and MacConkey agar and incubated at 37 °C for 24 hours and extended to 48 hrs if no growth appeared. Purified colonies were first identified through bacteriological reactions and cultural traits (Garcia, 2013; Mushtak *et al*., 2018). All isolates were then subjected to Vitek2 automated bacterial species identification system (Versalovic *et al*., 2011) for identification of bacterial species. Confirmed isolates of *P . aeruginosa* were stored at -20 °C in nutrient broth supplemented with 20% glycerol until further processing (Nanvazadeh *et al*., 2013).

2.4 Antimicrobial susceptibility Assay

Most of *P . aeruginosa* isolates subjected to antibiotic susceptibility test through Kirby-Bauer disc diffusion technique (Bauer *et al*., 1966) according to reference of Clinical and Laboratory Standards Institute (CLSI) (Weinstein *et al*., 2017). Set of 24 antibiotics discs have been tested (Oxoid) that included: cefoxitin (30µg), amikacin (30µg), ceftriaxone (10µg), cefixime (5µg), gentamicin (10µg), ciprofloxacin (10µg), imipenem (10µg), trimethoprim/ sulphamethoxazole (10/50µg), ampicillin (20µg), meropenem (10µg), piperacillin (100µg), peracillin/tazobactam (100/10µg), colistin (10µg), ceftazidime (30µg), cefepime (30µg), cefuroxime (30µg), nitrofurantoin (100µg) cefuraxime-axitil (30µg), tobramycin (10µg), ticarcillin (75µg), ticarcillin/clavulanic acid (75/10 µg), tigecycline (15µg), cefazolin (30 µg) and lefofoxacillin (5 µg). Briefly, an overnight suspension broth culture that corresponding to 0.5 MacFarland Standard were evenly inoculated entire surfaces of Muller-Hinton agar by cotton swab and incubated at 37 °C for 24 hour. Dishes were later read by detection of growth inhibition zone diameters (millimeter) around each of the antibiotic discs. The zone diameter of drugs was inferred according to Clinical and Laboratory Standards Institute criteria (CLSI) (Weinstein *et al*., 2017).

2.5 Extraction of DNA

Fifty isolates of *P . aeruginosa* were chosen according to their susceptibility patterns to meropenem and imipenem and subjected to molecular characterization (PCR assay). The DNA extraction was done by sweeping a few purified bacterial colonies on blood agar media by utilizing the boiling method (Khosravi *et al*., 2017). Briefly about 200 microliter of bacterial

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suspension was heated for 20 minutes at 120 ˚C followed by high-speed centrifugation (3500 rpm for 20 minutes). Supernatants considered as DNA template for PCR amplification. DNA concentration was measured by microvolumes through using the NanoDrop spectrophotometer (Cambridge, England) instrument as described by (Sukumaran, 2011), while purity of DNA were measured by reading the 260/325 absorbance ratios (Desjardins *et al*., 2011**)** .

2.6 PCR Amplification protocol

Monoplex PCR for carbapenem resistance genes was performed using published primers (Fatemeh and Fereshtesh, 2014; Shariati *et al*., 2018) for detection of *Opr D, bla* $_{VIM}$ and *bla* $_{IMP}$, respectively as shown in Table (1).

The master mix of PCR reaction was performed in the final reaction of a total volume of 50μL .Each reaction contained PCR Master Red Load Taq Master (2X) (Germany) which contains 200Mm of each deoxy nucleoside triphosphate (dNTP), (15Mm $MgCl_2$) , PCR Buffer (100Mm Tris-HCl, pH 8.8, 500 Mm, KCl, 0.1% Tween 20), 1.5 U Taq DNA polymerase, 4 μL of primer (10 pmol μl^{-1}) mixture (2 μL for each forward and reverse primer) and 5 μL of DNA template (50ng/ ml). Amplification of products was done for for *bla VIM* as following thermal steps and cycling conditions: initial denaturation at 95°C for 3 minutes, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min; and final extension at 72°C for 7 min. for *bla IMP*: initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 53°C for 30 sec, and extension at 72°C for 1 min; and final extension at 72°C for 8 min. For *OprD* initial denaturation was at 95°C for 2 min, then followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 51°C for 30 sec, and then extension at 72°C for 1 min; and final extension at 72°C for 1 min. The amplified products were then run in1 % agarose gel to show specific molecular DNA bands by comparing them to the known molecular weight of the ladder marker.

Table (1): Specific genes, primers sequences and expected products for PCR assays used for carbapenem resistance genes.

Primer name	Sequence 5-3	Amplicon size (pb)	Optimum annealing temperature	References
OprD	F 5´-ATGAAAGTGATGAAGTGGAG-3´ R 5´-CAGGATCGACAGCGGATAGT-3´	1329	51°C	(Shariati *et al*., 2018)
bla IMP	F 5´-GAAGGCGTTTATGTTCATAC-3´ R 5´-GTATGTTTCAAGAGTGATGC-3´	587	53°C	(Fatemeh and Fereshtesh *et al*., 2014)
blaVIM	F 5´-GTTTGGTCGCATATCGCAAC-3´ R 5´-CTACTCGGCGACTGAGCGAT-3´	645	55°C	Fatemeh and Fereshtesh *et al*., 2014)

3.7 Statistical Analysis

Statistical analysis was carried out using Graph Pad Prism program (Version 6.01) (Graph Pad Software, USA). The significant difference between variables was determined using Fisher and Chi-square test (Sokal and Rohlf, 2013). P-value < 0.05 was considered a significant.

3. RESULTS

A total of 60 (11.4%) isolate of *P* . *.aeruginosa* were isolated and identified over 524 burns and wound swabs that distributed in various rates among genders, ages and involved hospitals as shown in table (2).

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Table (2): Shows rates of *P . aerugenosa* among genders, ages and involved hospitals

Variable	Specimens					
	Burn (26)	Wound (34)	Total (60)	p-value		
Gender				0.368		
Male	9 (33.3%)	18 (66.7%)	27			
Female	17 (51.5%)	16 (48.5%)	33			
Age					**Total**	**P.Value**
1-9	6 (66.7%)	3 (33.3%)	9	0.02*		0.368
10 19	5 (62.5%)	3 (37.5%)	8		27	
20-29	8 (61.5%)	5 (38.5%)	13		33	
30-39	3 (37.5%)	5 (62.5%)	8			
40-49	3 (42.9%)	4 (51.1%)	7			
>=50	1 (6.7%)	14 (93.3%)	15			
Hospital						
Burn	26 (43.3 %)					
Emergency	34 (56.7 %)					

*- Statistically significant

In current study, burns samples were more infected with *P . aerugenosa* especially among females gender while wounds swabs among males were more harbored isolates but no significant differences between variable genders were noticed (p- value 0.368). The highest burn infection rate (66.7 %) was recorded among age group (0-9) years and the lowest rate (6.7 %) was in age (>=50 years). Inversely, in wounds the age (>=50 years) was the highest rate (93.3%) and the lowest rate (33.3%) was seen among (0-9 years) and statistically was significant (P. value 0.02).

Concerning antibiotic susceptibility test, generally all isolates from wound sources were exhibited relatively higher resistance rates over burn isolates against (24) tested antibiotics. Nearly, all burn swabs isolates showed lower resistance rates except for piperacillin and ticarcillin (75%), ticarcillin/clavulanic acid (66.7 %) and tobramycin (63.6 %). While, more resistance rates were seen among wound swabs isolates notably to tigecycline (86.7%), cefazolin (76.9%), ampicillin (62.5%) and levofloxacin (60%). Colistin and piperacillin/tazobactam were more efficient antibiotics against all isolates from burn followed by wound swabs as in table (2).

Table (3) shows molecular detection of carbapenem resistance genes among 50 isolates that were phenotypically resistant and sensitive to meropenem and imipenem and subjected to PCR assay for presence and absence of *Opr D, bla* $_{VIM}$ and *bla* $_{IMP}$ genes. *Opr D* was common accounted 32 (85%) mainly among meropenem and imipenem resistant isolates while sensitive isolates also contained it. *bla* $_{VIM}$ gene was found among 7 (17.5%) isolates , while *bla* $_{IMP}$ was not detected among studied isolates.

Table (3): Antibiotic susceptibility patterns of *P. . aeruginosa* from burns and wounds

Antibiotics	Burns Isolates		Wounds Isolates	
	Resistance (%)	Susceptible (%)	Resistance (%)	Susceptible (%)
Amikacin	33.3	66.7	33.3	66.7
Ampicillin	37.5	72.5	62.5	37.5
Cefazolin	23	77	76.9	23.1
Cefepime	38.5	61.5	26.9	73.1
Cefixime	39.4	60.6	54.5	45.5
Cefoxitin	36.9	63.1	63	37
Ceftazidime	30	70	23.3	76.7
Ceftriaxone	37	63	56.5	43.5
Cefuraxime	45.2	54.8	54.8	45.2
Cefuraxime axetil	45.2	54.8	54.8	45.2

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Ciprofloxacin	35	65	30	70
Colistin	0	100	8.3	91.7
Gentamicin	39	61	23.7	72.3
Imipenem	33.3	66.7	31.7	68.3
Levofloxacin	13.3	86.7	60	40
Meropenem	32.6	67.4	26.1	73.9
Nitrofurantion	37	63	60.9	39.175
Piperacillin	75	25	25	
Piperacillin/ Tazobactam	11.1	88.9	11.1	88.9
Ticarcillin	75	25	25	7575
Ticarcillin/ Clavulanic acid	66.7	33.3	25	
Tigecycline	13.3	86.7	86.7	13.3
Tobramycin	63.6	36.8	18.7	81.3
Trimethoprim/ Sulfamethoxazol	36.2	63.8	57.4	42.6

Table (4): Molecular Detection of Carbapenem resistance genes in 50 isolates of *P . aeruginosa*

Results	**50 isolate of *P . aerugenosa***					
	40 isolate imipenem & meropenem resistant			10 isolates imipenem susceptible		
	bla VIM	*Opr D*	*bla IMP*	*bla VIM*	*Opr D*	*bla IMP*
+ ve	7 (17.5 %)	32 (80%)	0	1 (10 %)	8 (80 %)	0
- ve	33 (82.5 %)	8 (20 %)	0	9 (90 %)	2 (20 %)	0

From another hand, about 8/100 isolates were found to contain double resistance genes (*Opr D+ bla$_{VIM}$*) simultaneously that occurred more within imipenem resistant isolates as in table (4). Some of the isolates that expressed resistance phenotypically to both imipenem and meropenem but they were not contained any target resistance genes as in table (5).

Table (5): Distribution of double resistance genes (*Opr D+ bla$_{VIM}$*)

Target gene	**Phenotypic Resistance spectrum**	**No. (%)**
Opr D+ bla$_{VIM}$	imipenem(R)+ meropenem(R)	5 (62.5)
	imipenem(R)+ meropenem(S)	2 (25)
	imipenem(S)+ meropenem(S)	1 (12.5)
	Total	8 (100)

R: resistance, S:sensitive

Table (6): Carbapenem-resistance Isolates without target-resistance genes

Target genes	**Phenotypic Resistance spectrum**	**No. (%)**
Opr D –ve	imipenem(R)+ meropenem(R)	2 (33)
bla$_{VIM}$ -ve	imipenem(R) only	4 (67)
bla$_{IMP}$ –ve	Total	6 (100)

4. DECUSSION

The present research is useful in providing data on the prevalence of carbapenem resistant *P . aeruginosa*, particularly in burn and wounds of nosocomial infections. Sixty (11.4 %) *P . aeriginosa* isolate were diagnosed from burns and wounds infections. Similar study done in Duhok city; Iraq found 12.7 % of *P . aeruginosa* isolates were from wounds (Yassin *et al*., 2014). Other studies in Iraq, announced 12.4 % in Najaf, 12 % in Baghdad and 18% in Wasit city (Al-Huraishi, 2016; Hussein & Shamkhi, 2018; Al-khudhairy & Al-Shammari, 2020). Low rate 3.95 % was reported by Anoar *et al*., 2014, in Sulaimaniyah City, Iraq. These differences and diversity in proportions might be due the type of inclusion criteria for enrolled patients with various as gender, specimen types, kind of test used in isolation and usage of preventive handling that determines the percentage (Hussein & Shamkhi, 2018). In the current study males were more affected than females and this in line with a study done in

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Sulaimaniyah city, Iraq showed female accounted 57 % and male 43% (Othman *et al*., 2014). Our results were in line with two studies done in Iran; in Shiraz and Ahwaz city they found female; male were 53 %; 26% and 56% and 41% (Anvarinejad *et al*., 2014; Khosravi *et al*., 2017) respectively. Disagree to Qader *et al*., 2020, who recorded 63.5% and 36.4% among males and females respectively, in Duhok and Erbil Hospitals/Iraq. Female gender is spending more their times in the kitchen in our society so they are more likely to be at risk to getting burns, as well as they are more exposed to suicide because of either domestic violence or marital issues. Two hospitals located in Duhok city were involved in the current study; Emergency Hospital comprised 56.7 % isolates and Burn Hospital 43.3 % isolates, indicating that large hospital was harbored more isolates of this pathogen. Similar results reported by studies done performed by (Aljanaby & aljanaby, 2017) in Kufa city, Iraq and (Sales *et al*., 2017; Shaaban *et al*., 2017) in Iran. Many staff members, carries states and infected patients belong large hospitals may transiently become contaminated and shed pathogens to immunocompromised patients (Douglas, 2001)

In this study less resistance rates were seen toward imipenem; meropenem (33.3 %; 31.7%) and (32.6%; 26.1%) from burn and wound isolates, respectively. This results needs to be taken in considerations in this setting. Similarly, Jaafar *et al*., (2014) in Baghdad city, Iraqi announced that imipenem resistance was 24.4% while meropenem was17.24%. Hussein *et al*., (2018) in Wasit city, Iraqi, revealed the same percentage (34.95%) of resistance found toward imipenem and meropenem. Different from results of the present study, Al-Shara, (2013) in Najaf, Iraq, reported less resistance against imipenem and meropenem 7.4 % and 14.8 %, respectively. A recent study by Al-khudhairy & Al-Shammari, (2020) in Iraq stated that imipenem resistance rate was 12.4 %. Farajzadeh Sheikh *et al*., (2014) in Ahwaz city, Iran mentioned meropenem resistance was 58.7%. later, another two studies in Iran showed alarming increasing rates as 97% and 96% in Isfahan and Tehran (Radan *et al*., 2016; Mahmood Saffari *et al*., 2016) respectively. Some reports from European countries stated increased frequency of carbapenem-resistant *P . aeruginosa* from 1% to 28 % between 2002 till 2006 (Hong *et al*., 2015; Bassetti *et al*., 2018). Unfortunately, carbapenemase -producing organisms will develop a resistance to both imipenem and meropenem in addition to the quality of hygiene and duration of hospital stays for patients (Chairat *et al*., 2019). The resistance rate to colistin was 8.3 % in this work. Colistin resistance is not dependent upon bacterial metabolic activity and also the acquired resistance is rare (Ece *et al*., 2014). The current data agree with Hussein *et al*., 2018, who recorded colistin resistance rate 2.78 % in Wasit city, Iraq. Less rate of colistin resistance found in study in Tabriz, Iran revealed a resistance rate 2% (Goli *et al*., 2016). Additionally good data obtained by Al-khudhairy & Al-Shammari, 2020 in Iraq claimed no resistance rate to colistin 0 %. The high price of colistin, its nephrotoxicity and limited use outside of the hospitals might explain its higher effectivity (Hussein *et al*., 2018). Effective MDR *P . aeruginosa* therapies may be limited, requiring doctors to be familiar with older antibiotics (*i.e*. colistin) which are expected to be released in the near future for general use.

In this study, 48 (96%) isolate were contained either single or double genes. *OprD* gene was common comprised 40 (80 %) isolate included both resistant and susceptible isolates. This result coincides with other studies that searched for *OprD* gene among resistant and sensitive isolates for instance; Amin *et al*., (2005) in Sweden; Ocampo-Sosa *et al*., (2012) in Spanish and Shariati *et al*., (2018) in Iran. Every loss of *OprD* expression from the outer membrane greatly reduces the susceptibility of *P . aeruginosa* to carbapenem (El Amin *et al*., 2005). In addition, multi-drug efflux pumps properties also play a significant role in resistance (Livermore, 2001). In our results, the incidence of *bla* $_{VIM}$ gene was detected among 8 (16 %) isolates and it was illustrated that this gene was more distributed among those isolates that were resistant to both imipenem and meropenem. This already was higher as 75% that stated by Shaaban *et al*., (2017) in Egypt. More high rate in Wasit city; Iraq was 94 % (Hussein & Shamkhi, 2018). In contrast, only 1 out of 81 isolates mentioned by van Burgh *et al*., 2019 in Kurdistan, Iraq. In Turkey, 1% of isolates were harbored *bla* $_{VIM}$ gene (Ozgumus *et al*., 2007). Because *P . aeruginosa* is a leading cause of nosocomial infections, thus plasmids carrying this gene can be circulated and easily transferred between the strains in close proximity, leading to multi-drug resistant microorganisms (Turk, 2011). Regardless of

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their phenotypic resistance patterns, *bla* $_{IMP}$ gene was not detected among all selected isolates by PCR assay. Another study by Al-Ouqaili, (2016) in Iraq, also not detected this gene. The results agree with Rodríguez-Martínez *et al*., (2009) that have not detected it. While disagreeing with two studies in Iran; Radan *et al*., (2016) reported 74.3% and Salimi & Eftekhar, (2014) accounted 56.25%. Not detecting of this gene might be due to geographical variations and use of certain types of antibiotics that playing a role in selecting the type of Metallo-β-Lactamase (MBL) producing strains (Turk, 2011). In this study, 8 isolates were found to contain double resistance genes (*OprD*+*bla* $_{VIM}$) simultaneously that occurred more frequently among isolates that showed resistance to both imipenem and meropenem and remarkably within one susceptible isolate. The striking finding is that some isolates that expressed resistance phenotypically to imipenem and meropenem together and individually were not contained any target resistance genes. This might be due to the presence of other resistance genes than targeted or other carbapenem resistance mechanisms as *P . aeruginosa* often accumulate different resistance mechanisms that contribute to carbapenem resistance (Rodríguez-Martínez *et al*., 2009). The differences in the prevalence of *P . aeruginosa* genes between geographic locations could be because of climate (moisten) of each place, civilizations, nutrition, and hospital's hygiene (Karimian *et al*., 2012; Rasol, 2013; Qader *et al*., 2020).

The study concludes that the emergence of *P . aeruginosa* as a reservoir of multiple carbapenemases resistance genes is increasing over time limiting the treatment options to this serious infection. The data support basic mechanism of imipenem resistance could be mostly via the loss of *OprD*. Colistin and piperacillin/tazobactam have high efficacy.

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