PlantArchives Volume 20 No. 1, 2020 pp. 181-186 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

EFFECT OF SOME SUGARS ON CARBAPENEMASE GENE EXPRESSION IN *PSEUDOMONAS AERUGINOSA*

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Abstract

Forty eight isolates (41.02%) were obtained from 117 wound and burn samples. The isolates that showed high resistance for both antibiotic was two only that represent 4,1% from all isolates. The result of PCR product electrophoresis was referred that the gene is VIM gene. Lactose and raffinose showed double increasing in diameter of inhibition zone of imipenem with 1% that mean showed highest susceptibility that decreased with the concentration increasing, the same result were with meropenem. But no effect were detected on meropenem inhibition zone diameter. Mannose have no effect on the resistance in 1%, 3% and 7%. Results showed that only three case that increase the expression of gene, they were lactose at 1% concentration that increased the expression to 1.815 fold, raffinose 7% that increase it to 1.057 fold. In the other hand, all other cases (sugars and concentrations) were decreased carbapenemase gene expression; the most effect one was mannose on 1% concentration that decrease the expression to 0.018 fold, raffinose at 3% concentration decreases it to 0.0369 fold.

Key words: carbapenemase, *Pseudomonas aeruginosa*, VIM gene.

Introduction

The genus *Pseudomonas*, belonging to Gram negative bacteria, Pseudomonadaceae family, that containing 191 species (Euzéby, 1997). The better studied species include *P. aeruginosa* that represent as the best studied species having role as an opportunistic human pathogen while *P. syringae, P. fluorescens* have an effect on plant, the species of *Pseudomonas* that available in soil is *P. putida* (Colmer-Hamood, 2016). *P. aeruginosa* has few nutritional requirements and can adapt to conditions not tolerated by other organisms. *Pseudomonas aeruginosa* consider as a bacteria with important medical role in human,animal and plant, it's have different type of resistance against antibiotic that make them as difficult pathogens to be cure by ordinary antibiotic so it represent an important hospital acquired pathogen such as ventilator-associated pneumonia and various sepsis syndrome (Madigan and Martinko, 2005).

The low permeability of outer membrane and extensive efflux pumps system of *Pseudomonas aeruginosa* make them as highly ubiquitous in water system and antibiotic resistance responsible for this trend is increased drug resistance to various antibiotics including aminoglycosides, (Olson *et al*., 1985) cephalosporins, (Mulgrave, 1991) and quinolones (Lyobe *et al.*, 1991). *P. aeruginosa* was a U.S. burn center record that the most common organism causing bacteraemia in between 1953 and 1983 was *P. aeruginosa* representing 10.3% (McManus, 1989). Later, improved hygiene application reduced the frequency of *P. aeruginosa* bacterimia (McManus, 1989).

The most common infection according to Swedish study, was burn wound infection (60%) followed by bacterimia (20%), pneumonia (10%), urinary tract infection (10%) (Appelgren *et al*., 2002). This challenging come from its inherent as well as acquired resistance mechanism to many effective groups of antimicrobial agents such as antibiotic (Pirnay *et al*., 2003). The isolate that is resistance to 3 group or more of antibiotic among β-lactams, fluoroquinolones, carbapenems and aminoglycosides named as Multidrug resistance pathogen (MDRPa) (Obritsch *et al*., 2005).

In the past, β-lactams represent as drug choice for *P. aeruginosa* while cefepime, ceftazidime and cephalosporins as well as penicillin/tazobactam were used as preferred therapy for *P. aeruginosa*, In the 1980s and 1990s, (Livermore, 2002; Sordé *et al*., 2011). Latin America, the Middle East and some parts of Asia had

Table 1: Primers sequences.

Primer	Nucleotide sequence (5—3)	Length	GC %	PCR product
IMP F	GAATAGRRTGGCTTAAYTCTC	21	40.5%	188 bp
IMP R	CCAAACYACTSGTTATC	18	41.7%	
VIM F	GTTTGGTCGCATATCGCAAC	20	50%	382 bp
VIM R	AATGCGCAGCACCAGGATAG	20	55%	
GIM F	TCAATTAGCTCTTGGGCTGAC	21	47.6%	72 bp
GIM R	CGGAACGACCATTTGAATGG	20	50%	
SIM F	GTACAAGGGATTCGGCATCG	20	55%	569 bp
SIM R	TGGCCTGTTCCCATGTGAG	19	57.9%	
SPM F	CTAAATCGAGAGCCCTGCTTG	21	52.4%	798 bp
SPM R	CCTTTTCCGCGACCTTGATC	20	55%	
PASSF	GGGGGATCTTCGGACCTCA	19	63%	956 bp
PASSR	TCCTTAGAGTGCCCACCCG	19	57%	
16SrDNA F	ACCTGGACTGATACTGACACTGA	23	47%	
16SrDNA R	GTGGACTACCAGGGTATCTAATCCT	25	48%	

the higher rates of resistance (Rosenthal *et al*., 2012; Gill *et al*., 2016; Labarca *et al*., 2016). Carbapenems and fluoroquinolones resistance appeared as emergence resistance against β-lactams therapy (Lynch *et al*., 2017).

Materials and Methods

Samples Collection

Samples of wound and burns swabs were collected from Al-Kindi hospital, Al-Yarmook hospital and Teaching Hospital of Baghdad Medical city between September, 2018 to January, 2019. Swabs were cultured in the hospital laboratory by using Cetramide agar and MacConkey agar plates and transported to the laboratory, then incubated at 37°C for 24 hours.

Identification of Bacteria

• Cultural Characteristics:

Isolates were inoculated on different culture media including Blood agar, Cetrimide agar, MacConkey agar, milk agar and Nutrient agar; the media were incubated at 37°C for 24 hrs. Suspected colonies were identified morphologically and biochemically.

• Microscopic Characteristics:

The suspected colonies were tested by Gram staining, to identify its reaction with crystal violate-iodine complex and its shape and arrangement.

Table 2: Conventional PCR program.

Component	Volume (µl)
PCR premix	Lyophilized
Forward primer (10pmol)	1µl
Reverse primer (10pmol)	1µl
Distilled water	15 µl
Template DNA	3 µl4
Final volume	20 µl

Biochemical Tests

Catalase Production Test (Benson, 2002), Indole Test (MacFaddin, 2000), Growth at 4°C and 42°C (Holt *et al*., 1994) and Oxidase Test (Benson, 2002).

Identification with VITEK-2-compact

This device is used to diagnose bacterial isolates and confirm that is *Pseudomonas areginosa* after being confirmed by first biochemical test as well as testing their sensitivity to antibiotic.

Antibiotic Susceptibility Testing

The modified Kirby-Bauer method (Vandeppitte *et al*., 2003) to estimate the resistance of isolates against carbapenems discs (imipenem 10µg, meropenem 10µg), then the highest resistance one was examined on modified Muller Hinton agar (Muller Hinton media that the starch was replaced by different sugars in different concentrations 1, 3 and 7%) to estimate the effect of sugars and its different concentration on resistance against carbapenems.

Molecular Assay

• Extraction of Genomic DNA:

DNA was extracted from *P. aeruginosa* isolates using a commercial extraction kit (BIOBASIC, USA), following the manufacturer's instructions, for DNA purification from gram negative bacteria.

PCR Amplification

The extracted DNA, primers (Table 1) and distributed maxime PCR premix (i-taq) (intron, Korea), were thawed at 4°C, vortexed to have homogenous contents, a PCR mixture was made in a total volume as described in table 2. A negative control contained all materials with the substitute of distilled water instead of template DNA.

PCR reaction tubes were placed in a thermo-cycler PCR instrument, DNA was amplified as in the conditions indicated in table 2-6. The temperature and time of PCR program were optimized by using gradient PCR.

Electrophoresis

Gels containing 2µl (5%) red safe dye, were run

Table 3: qRT-PCR program.

qPCR	Temp	Times	Cycle (s)
cDNA Synthesis	42ºC	15 minutes	1
Enzyme Activation	95ºC	10 minutes	1
Denaturation	95ºC	15 seconds	30-35
Annealing/Extension*	60ºC	60 seconds	
Melting curve	Refer to specific guidelines for instrument used		

Table 4: Characteristic of culture on different media.

Culture media	Characteristic
Blood agar	β or γ haemolysis, elevated colonies
Cetrimide agar	Growth with fluorescent green colour, elevated colonies, and grape-like odour
MacConkey agar	Pale non-lactose fermenter elevated colonies
Nutrient agar	Growth with fluorescent green colour, elevated colonies, and grape-like odour
HiChrome agar	Green colony

horizontally in TBE buffer (1X), 3µl of genomic DNA was mixed with 2µl of loading dye and loaded into the wells of the gel. TBE was added until submerging the gel and run for 60 min. at 80v. A 1000bp or 100bp DNA ladder was added with each run to detect the product size, DNA bands were visualized by UV trans-illuminator documentation system.

Extraction of Genomic RNA

RNA was extracted from *P. aeruginosa* isolates using a commercial extraction kit (GENEzol™ TriRNA Pure Kit, Japan), following the manufacturer's instructions, for RNA purification from gram negative bacteria.

Quantitative RT-PCR for RNA of carbapenemase gene expression isolated from carbapenems resistant *Pseudomonas aeruginosa* grow with different sugars in different concentrations

The RNA extracted from carbapenems resistant *Pseudomonas aeruginosa* that grow in different sugars and concentrations were used to determine the gene expression of carbapenemase gene by using appropriate primer and quantitative real time-polymerase chain reaction as in the program (Table 3).

Results and Discussion

Identification of Bacteria

- **Cultural Characteristics:**

Forty eight isolates (41.02%) were obtained from 117 wound and burn samples, all isolates were characterized on different media as illustrated in table 4.

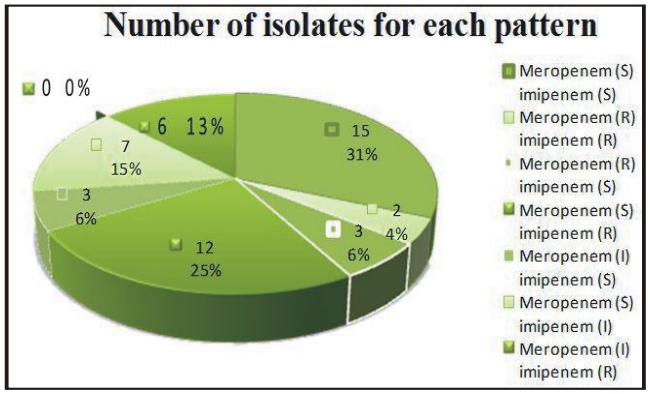

Fig. 1: Pattern percentage of Psuedomonas aeruginosa isolates.

Table 5: Effect of sugar type on bacterial growth.

Sugars	Plate 1 & 2 (CFU)		Plate 3 & 4 (CFU)		Mean (CFU)	Percent (%)
Glucose	37	41	52	47	44	27
Mannose	163	159	167	171	165	105
Raffinose	164	167	173	171	169	107
Lactose	152	157	171	167	162	103
Saccharose	68	74	86	80	77	49

The isolates that sensitive for both antibiotics (meropenem and imipenem) showed that highest percentage (31.2%) among the isolates, while no isolate were exhibited the pattern that characteristic with resistance to imipenem and intermediated to meropenem.

The isolates with pattern that characterize as resistance to meropenem and sensitive for imipenem that gave the second percentage between the isolates pattern (25%). The isolates that showed high resistance for both antibiotic was two only that represent 4.1% from all isolates (Fig. 1).

In general, Gram-positive cocci is more susceptible to imipenem while Gram-negative bacilli is more susceptible to meropenem. They display similar action of pharmacokinetics. Meropenem is used for bacterial meningitis treatment, whereas imipenem is not. Cephalosporins and penicillins, due to their zwitterionic nature and low molecular weight, readily penetrate the outer membrane through porin proteins of Gram-negative bacilli (Satake *et al.*, 1990). Outer membrane proteins (OMPs) F and C represent as the major porins involved while OMP D2 represent as an unconventional route for Carbapenems entry (Margaret *et al.*, 1989). Kayser *et al.*, (1989). Carbapenems also are not very susceptible to hydrolysis by penicillinases and cephalosporinases, so, cross-resistance between cephalosporins and penicillins and carbapenems does not occur (Satake *et al.*, 1990). Imipenem resistance may causes as a result to mutational loss of the D2 porin in *Pseudomonas aeruginosa*. This mechanism could function only when the chromosomal beta-lactamase was expressed. Mutants lacking both the D2 porin and the beta-lactamase were almost as

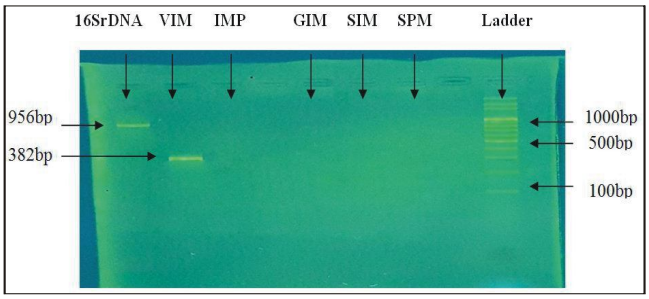

Fig. 2: Electrophoresis of PCR product for different primers.

Table 6: Effect of sugars concentrations on carbapenems inhibition zone.

Sugars	Antibiotics	1 %					3 %					7 %				
		diameter				ratio	Diameter				ratio	diameter				ratio
Mannose	Imi	13	14	12	13	1	15	16	14	13	1.11	15	14	14	14	1.09
	Mero	9	11	11	10	1.13	11	12	11	10	1.22	12	13	11	13	1.36
Raffinose	Imi	26	25	26	24	1.94	23	22	23	24	1.76	20	22	21	19	1.57
	Mero	16	17	16	15	1.77	11	12	11	10	1.22	8	8	9	8	0.91
Lactose	Imi	27	28	25	26	2.03	22	21	24	20	1.67	17	16	17	17	1.28
	Mero	18	15	19	21	2.02	14	15	14	15	1.61	9	10	8	10	1.03

susceptible as those that retained the porin but lacked the beta-lactamase. Thus, imipenem resistance reflected an interplay of the impermeability and enzyme, not either factor alone. These suggesting that the activity of a carbapenem more beta-lactamase stable than imipenem should be less affected by the porin loss.

VITEK

The resistance isolate was identified by VITEK to confirm its species and sensitivity, the result probability showed 99% matching with *Pseudomonas aeruginosa* and also confirmed the result of antibiotics sensitivity by disc diffusion method, that it appeared resistant to both imipenem and meropenem.

Molecular assay

PCR product electrophoresis showed that the product size was estimated 375 bp (Fig. 2). The weight of product was calculated by the standard curve that came from the relation between the relative mobility of each band in ladder with the weight of the corresponding band. The relative mobility of our band was calculated by dividing the distance of band on the distance of the stain. From this equation we got a value that can revealed to the weight of our band from standard curve. The result was referred that the gene is VIM gene that it near with what Mendes and coworker revealed when they used the same primers for different carbapenems genes (Mendes *et al.,* 2007).

Mannose, raffinose and lactose gave no difference in bacterial growth comparing with Muller hinton agar, some sugars showed decreasing in growth that reach to minimize the bacterial colony number to 27% with glucose. While other sugars showed slightly effect on bacterial growth that reach to 49% as with saccharose (Table 5).

Determination of sugars effect on bacterial susceptibility against carbapenems

Sugars that gave the same supporting for growth with the original MHA were examined for their effect on carbapenems sensitivity with different concentrations. Table 6, showed the effect on susceptibility by comparing the inhibition zone diameter with the original one on Muller hinton agar.

Lactose and raffinose showed double increasing in diameter of inhibition zone of imipenem with 1% that mean showed highest susceptibility that decreased with the concentration increasing, the same result were with meropenem. But no effect were detected on meropenem inhibition zone diameter. Mannose have no effect on the resistance in 1%, 3% and 7%.

Gene expression with different sugars

RNA extracted from isolate that grow in the same media but with different carbon source (sugars) and concentrations (1%, 3% and 7%) were used to determine the effect on gene expression of carbapenemase by using quantitative real time PCR.

The results of qRT-PCR with the home gene primer for 16SrDNA gene showed pure product for this gene that exhibited by melting point value that seems very closed to each others in all cases of sugars and their concentrations, they ranged for Mannose ranged from 86.23°C to 86.65°C, lactose sugars melting point ranged from 86.33°C to 86.72°C, for raffinose sugar the values ranged from 86.08°C to 86.43°C.

The results of melting point for VIM gene product exhibited one peak in melting point curve in all processes, that meant it was pure in all of them, the melting point values were also very closed to each others. It ranged for mannose 77.26°C to 77.81°C, lactose ranged from 77.22°C to 77.58°C and raffinose ranged from 77.25°C to 77.95°C.

Table 7: Gene expression values in different sugars concentrations.

Sugar	16S	VIM	DCT	DDCT-2	Fold
Mannose 1%	11.59	27.24	15.65	3.83	0.070316
Mannose 3%	12	26.4	14.4	2.58	0.167241
Mannose 7%	9.49	27.09	17.6	5.78	0.018199
Lactose 1%	14.67	25.63	10.96	-0.86	1.815038
Lactose 3%	13.27	26.36	13.09	1.27	0.41466
Lactose 7%	13.08	27.07	13.99	2.17	0.222211
Raffinose 1%	11.87	27.2	15.33	3.51	0.087778
Raffinose 3%	10.63	27.21	16.58	4.76	0.036906
Raffinose 7%	15.54	27.28	11.74	-0.08	1.057018
MCTest	15.31	27.13	11.82	0	1

Quantification cycle (Cq) of the 16SrDNA exhibited slightly difference among all processing, for mannose Cq ranged from 9.49 to 12 cycle, while lactose Cq values were ranged from 13.08 to 14.67 cycle and raffinose Cq was 10.63 to 15.54 cycle. Quantification cycle (Cq) of the VIM gene exhibited slightly difference among all processing also, it ranged from 25.63 to 27.78 cycle, for mannose Cq ranged from 26.81 to 27.78 cycle, while lactose Cq values were ranged from 25.63 to 27.07 cycle and raffinose Cq was 27.2 to 27.28 cycle. From the table 7 of gene expression of carbapenemase in *Pseudomonas aeruginosa* that grew in different sugars with different concentrations (1, 3 and 7%) the results showed.

The following: cmparison was between carbapenemase gene expression of isolate grew on different sugar and concentrations with gene expression of the same isolate grew on Muller Hinton agar with carbapenem discs. Results showed that only three case that increase the expression of gene, they were lactose at 1% concentration that increased the expression to 1.815 fold, raffinose 7% that increase it to 1.057 fold. In the other hand, all other cases (sugar and concentrations) were decreased carbapenemase gene expression, the most effect one was mannose on 1% concentration that decrease the expression to 0.018 fold, raffinose at 3% concentration decrease it to 0.0369 fold. In general, sugars on 1% concentration exhibited decreasing in carbapenemase gene expression with higher effect for mannose then raffinose. Lactose represent as exception in 1% concentration that exhibited activation of the gene. Further analyze *P. aeruginosa* physiology in a burn wound environment, the burne wound exudates (BWE) chemical composition was determined before and after 24 h., of bacterial growth. Results have shown a full consumption of lactate (>96%) by *P. aeruginosa*, whereas the glucose level decreased only by 10.3%. These data are consistent with *P. aeruginosa* carbon source preferences (Palmer *et al*., 2007). The low glucose consumption was further supported by the RNAseq data that showed no induction of the main genes involved in the glucose catabolism (Singh *et al*., 2016). Interestingly, despite stable expression of glucose porin oprB (PA3186), both glucose dehydrogenase gcd (PA2290) and gluconate permase gnuT (PA2322) showed induction in BWE compared to LB control conditions. These data suggest that P. aeruginosa stimulates the pathway involved in gluconate production, whereas glucose catabolism, *via* glucose-6-P and 2-keto-6P-gluconate branches, as well as glycolysis, were repressed (Gonzalez *et al.*, 2018).

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