Pharmacophore

ISSN-2229-5402

Journal home page: http://www.pharmacophorejournal.com

MOLECULAR CHARACTERIZATION OF CARBAPENEM-RESISTANCE GENES AMONG *Pseudomonas aeruginosa* AND *Acinetobacter baumannii* CLINICAL ISOLATES IN RIYADH

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ARTICLE INFO

Received:
16 Nov 2020
Received in revised form:
14 Dec 2020
Accepted:
14 Dec 2020
Available online:
28 Dec 2020

Keywords: Antimicrobial resistance, molecular characterization, carbapenem-resistance, *pseudomonas aeruginosa, Acinetobacter baumannii*, genes

ABSTRACT.

Background: Antimicrobial resistance in Gram-negative bacteria has been a serious threat and a global problem. *Acinetobacter baumannii* and *Pseudomonas aeruginosa* are considered a leading cause of nosocomial infections worldwide and in the KSA. **Objectives:** The present study aimed to recognize the genes encoding Carbapenem resistance in two different non-fermentative, Gram-negative, non-fastidious pathogens, such as *P. aeruginosa* and *A. baumannii* strains isolated from a tertiary care hospital in Riyadh, Saudi Arabia. **Materials/Methods:** A total of 115 clinical isolates (80 *P. aeruginosa* and 35 *A. baumannii*) were obtained from different body sources at the clinical microbiology laboratory at King Khalid University Hospital, Riyadh, Saudi Arabia, from June to December 2017. The identification and antibiotic susceptibility testing were made for all the isolates using conventional (E-test) and the automated Vitek®2 system. The antibiotic susceptibility profiles of the isolates were determined as recommended by the Clinical and Laboratory Standards Institute (CLSI 2014). Polymerase Chain Reaction (PCR) was carried out to detect 13 Carbapenemase genes (OXA-23, OXA-24, OXA-40, OXA-51, OXA-10, OXA-48, OXA-1, VIM, IMP, GIM, NDM, KPC, ISAba-1) with a total of 50 ng DNA template added to the 25µl reaction mixture. Sanger sequencing was carried out using BigDye® Terminator v3.1 Cycle Sequencing kit (in 20 µl reaction mixture) to confirm the amplification of the target sequence. **Results:** A total of 80 isolates of *P. aeruginosa* were tested for the presence of Carbapenemase genes by PCR amplification method. It was found that the most prevalent genes were *OXA-23* (55%) followed by bla_{VIM} (46%). The *OXA-1* and bla_{GIM} genes were present in 22% and 15% of the isolates, respectively. *A. baumannii* isolates, tested for the presence of Carbapenem-resistant genes, also showed a prevalence of *OXA-23* gene with an occurrence of 85.7%. *ISAba-1* insertion sequence was found in 27 isolates. **Conclusion:** The rates of carbapenem-resistant isolates conferring multiple resistance genes are worrisome, leaving the clinicians with limited treatment options with antimicrobial drugs. Therefore, proper use for infection control procedures and revision of the treatment and management strategies is undoubtedly required to reduce the spread of resistance genes in these pathogens.

To Cite This Article: Wejdan Nasser Bin Jawhar, May Mohammed AlRashed, Ali Mohammed Somily, Ahmed Mohammed AlBarrag, (2020), "Molecular characterization of Carbapenem-resistance genes among *Pseudomonas aeruginosa* and *Acinetobacter baumannii* clinical isolates in Riyadh", ***Pharmacophore, 11(6),*** *56-61.*

Introduction

The growing incidences of antibiotic resistance among gram-negative bacteria are a serious threat to public health [1]. The main reasons for the escalated reports of infections caused by antibiotic-resistant bacteria are the rapid spread of resistance mechanisms among pathogens and limited treatment options.[2-4] This scenario has resulted in frequent exposure of patients to a wide range of antibiotics, thus making them more susceptible to infections caused by antibiotic-resistant bacteria. [5, 6] The contributing factors to the emergence of antibiotic resistance are the transmission of these pathogens among patients and

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health-care workers, and lack of appropriate guidelines for using antibiotics. Moreover, the misapplication of similar agents in the animal industry has resulted in a further increase in the spread of antibiotic resistance. [7, 8]

Several local studies have shed light on the high prevalence of antibiotic resistance among gram-negative, non-fermentative bacteria. A review on the prevalence of antibiotic resistance in Gulf Corporation Council (GCC) countries by Aly and Balkhy, (2012) reported *Pseudomonas aeruginosa* as the most prevalent gram-negative antibiotic-resistant pathogen in Saudi Arabia with an occurrence rate of (92.3%). This was followed by *Acinetobacter* sp. (83.3%). [7] Memish et al., (2012) examined the resistance rates among this bacterial group from different geographical regions of Saudi Arabia, they found that among 8908 non-fermenters, the most dominant bacteria were *P. aeruginosa* (72.9%) followed by *Acinetobacter bumannii* (25.3%). [9]

Bacteria exhibit several mechanisms to develop antimicrobial resistance. These include mutations in the penicillin-binding proteins (PBPs), alteration in the outer membrane proteins, efflux pump mechanism, and the production of hydrolyzing enzymes such as Carbapenemases and Extended-Spectrum Beta-Lactamases (ESBLs). [10] Together, these mechanisms have contributed to the emergence of multidrug-resistant (MDR) strains, especially if they are encoded on transferable genetic elements. This is because the acquired genes facilitate the spread of antibiotic resistance between bacteria from the same as well as different species.[11]

P. aeruginosa and *A. baumannii* are non-fermentative, MDR gram-negative bacilli. They are extremely well adapted to the hospital environment, which makes them responsible for a significant proportion of nosocomial infections worldwide.[12] In addition, *P. aeruginosa* and *A. baumannii* share a problematic characteristic of intrinsic resistance to many classes of antibiotics, and the ability to acquire antibiotic resistance determinants. As a result, they may compromise the advanced medicinal approaches such as surgery, transplantation, and efficient treatment of immunocompromised patients. The increasing number of infections by MDR and even pan drug-resistant bacteria has left clinicians with very few treatment options.

Carbapenems have been used for many years as the most effective, but last resort, drugs to treat MDR *P. aeruginosa* and *A. baumannii* infections. However, the emergence of carbapenemase-producing strains is now gradually challenging the current treatment approaches.[13] Unfortunately, the emergence of MDR nosocomial pathogens has been reported in several hospitals in the Kingdom of Saudi Arabia.

To the best of our knowledge, most of the studies conducted in Saudi Arabia are focusing on the prevalence and epidemiology of antimicrobial resistance among Gram-negative bacteria, and only few studies present molecular insights for better understanding. Therefore, our study was carried to acknowledge this gap in research on antibiotic resistance.

Hence the aim of the present study was to recognize the genes encoding Carbapenem resistance in two different non-fermentative, Gram-negative non-fastidious pathogens, such as *P. aeruginosa* and *A. baumannii* strains isolated from a tertiary care hospital in Riyadh, Saudi Arabia.

Methodology

A total of 115 clinical isolates (80 *P. aeruginosa* and 35 *A. baumannii*) from different body sources were obtained at the clinical microbiology laboratory from June to December 2017. The isolates were cultured on Blood Agar plates and incubated at 37°C for 24 h. On the next day, bacterial colonies were inoculated in Luria-Bertani (LB) broth and incubated in a shaking rack at 37°C for 24 h, to enhance the growth of the bacteria. The identification and antibiotic susceptibility testing were done for all the isolates using conventional (E-test) and the automated Vitek®2 system. The antibiotic susceptibility profiles of the isolates were determined as recommended by the Clinical and Laboratory Standards Institute [14].

DNA extraction was performed for all the isolates using Qiagen QIAamp® DNA mini kit. Then, PCR was carried out to detect 13 Carbapenemase genes (OXA-23, OXA-24, OXA-40, OXA-51, OXA-10, OXA-48, OXA-1, VIM, IMP, GIM, NDM, KPC, ISAba-1) with a total of 50 ng DNA template added to the 25µl reaction mixture. The primers, along with annealing temperatures, are indicated in Table.1.

Table 1: Primers sequences and annealing temperatures for Carbapenem-resistant genes

Gene	Primer Sequence	Annealing Temp.	Product Size	Reference
OXA-48	TTGGTGGCATCGATTATCGG	55 °C	743 bp	(Paterson and Bonomo 2005)
	GAGCACTTCTTTTGTGATGGC			
OXA-51	TAATGCTTTGATCGGCCTTG	53 °C	353 bp	(Hou and Yang 2015)
	TGGATTGCACTTCATCTTGC			
OXA-23	GATCGGATTGGAGAACCAGA	53 °C	501 bp	(Hou and Yang 2015)
	ATTTCTGACCGCATTTCCAT			
OXA-24	GTACTAATCAAAGTTGTGAA	53 °C	1024 bp	(Hou and Yang 2015)
	TTCCCCTAACATGAATTTGT			
OXA-40	GGTTAGTTGGCCCCCTTAAA	50 °C	246 bp	(Zowawi, Balkhy et al. 2013)

	AGTTGAGCGAAAAGGGGATT			
OXA-1	ACACAATACATATCAACTTCGC	50 °C	813 bp	(Hujer, Hujer et al. 2006)
	GTGTGTTTAGAATGGTGATC			
OXA-10	TATCGCGTGTCTTTCGAGTA	51 °C	760 bp	(Zowawi, Balkhy, et al. 2013)
	TTAGCCACCAATGATGCCC			
VIM	GTTTGGTCGCATATCGCAAC	52 °C	390 bp	(Woodford 2010)
	AATGCGCAGCACCAGGATAG			
IMP	GAATAGRRTGGCTTAAYTCTC	52 °C	188 bp	(Woodford 2010)
	CCAAACYACTASGTTATC			
GIM	TCAATTAGCTCTTGGGCTGAC	52 °C	477 bp	(Woodford 2010)
	CGGAACGACCATTTGAATGG			
NDM	GGTTTGGCGA TCTGGTTTTC	52 °C	621 bp	(Poirel, Walsh, et al. 2011)
	CGGAATGGCTCATCACGATC			
KPC	CGTCTAGTTCTGCTGTCTTG	52 °C	798 bp	(Al-Agamy, Jeannot, et al. 2016)
	CTTGTCATCCTTGTTAGGCG			
ISAba-1	ATGCAGCGCTTCTTTGCAGG	55°C	393 bp	(Hujer, Hujer, et al. 2006)
	AATGATTGGTGACAATGAAG			

The PCR program was as follows: initial denaturation for 5 min at 94°C, followed by 30 cycles (94°C for 30 seconds, annealing temperature for 40 sec, 72°C for 60 sec) and a final extension for 10 min at 72°C. The PCR products were run on a 1% agarose gel electrophoresis to confirm the amplification product. Sanger sequencing was carried out using BigDye® Terminator v3.1 Cycle Sequencing kit (in 20 µl reaction mixture) to confirm the amplification of the target sequence. The resulting sequence obtained was subjected to BLAST analysis at the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to compare them with the existing antibiotic resistance genes in the database.

Results

A total of 80 isolates of *P. aeruginosa* were tested for the presence of Carbapenemase genes by PCR amplification method. It was found that the most prevalent genes were *OXA-23* (55%) followed by bla_{VIM} (46%). The *OXA-1* and bla_{GIM} genes were present in 22% and 15% of the isolates, respectively. The other genes, *OXA-24*, *OXA-51*, *NDM*, and *KPC* were not detected in any of the isolates (Table 2). The coexistence of the two genes was commonly observed in our study. Eight isolates harbored *OXA-23* with bla_{VIM}, three isolates harbored *OXA-23* with *OXA-10*, and three isolates harbored *OXA-1* with bla_{VIM}.

Table 2: Identification of Carbapenemase genes in Acinetobacter baumannii and Pseudomonas aeruginosa. (ND: Not detected, NP: Not present)

Resistant Genes	***P.aeruginosa***		***A.baumannii***	
	Number of Positive isolates	Percentage	Number of Positive isolates	Percentage
OXA 23	44	55%	30	85.7%
OXA 24	ND	0%	ND	0%
OXA 40	4	5%	6	17%
OXA 51	ND	%	ND	0%
OXA 10	6	7.5%	ND	0%
OXA 48	3	3.75%	1	2.8%
OXA 1	18	22.5 %	1	2.8%
VIM	37	46.25%	4	11.4%
IMP	1	1.25%	6	17%
GIM	12	15%	1	2.9%
NDM	ND	0%	ND	0%
KPC	ND	0%	ND	0%
ISAba-1	NP		27	77%

A. baumannii isolates, tested for the presence of Carbapenem-resistant genes, also showed the prevalence of *OXA-23* gene with an occurrence of 85.7%. *ISAba-1* insertion sequence was found in 27 isolates. The coexistence of two genes was

observed in a few isolates. Specifically, six isolates harbored *OXA-23* with *bla*$_{IMP}$, and one isolate harbored *OXA-23* with *OXA-48.*

We also demonstrated the simultaneous presence of more than 2 resistance genes in the same isolate (Table 3).

Table 3: The isolates showing the coexistence of more than 2 resistant genes.

Isolate Number	Carbapenem Resistant Genes						
	VIM	GIM	IMP	OXA-23	OXA-48	OXA-40	OXA-1
P.A-1	+			+	+		
P.A-2	+	+		+			+
P.A-3	+			+	+		+
P.A-4	+			+	+		+
P.A-5		+		+			+
P.A-6		+		+			+
P.A-7	+	+		+			+
P.A-8	+	+		+		+	
A.B-1	+			+		+	
A.B-2	+			+		+	
A.B-3			+	+		+	
A.B-4		+		+		+	

Discussion

Carbapenems are the leading last-resort antibiotics for usage in the treatment of bacterial infections caused by *P. aeruginosa* and *A. baumannii* when these pathogens are resistant to other β-lactam antibiotics.[15] However, the frequent emergence of Carbapenem-resistant strains over time is alarming.[16]

Among the eighty isolates of *P. aeruginosa,* about 55% (44/80) of the strains harbored the *OXA-23* carbapenemase gene. Metallo-beta lactamases (MβLs) such as *bla*$_{VIM}$ and *bla*$_{GIM}$ alone were detected in 46% and 15% of isolates respectively indicating that *bla*$_{VIM}$ is the most dominant MβL gene in *P. aeruginosa*. This observation is in agreement with the results of Al gamy et al. (2016) who reported that *bla*$_{VIM}$-like genes were the predominant MβLs among *P. aeruginosa* isolates in Riyadh, Saudi Arabia.[17] The production of MβLs has emerged as the main mechanism for carbapenem resistance among *P. aeruginosa* in Saudi Arabia.[18] In our study, 22% of *P. aeruginosa* isolates were found to display the *OXA-1* gene, while any traces of *OXA-24, OXA-51*, *NDM,* and *KPC* were not detected in any of the isolates. Shaaban et al. (2017) reported that genes such as *NDM* and *KPC* were co-produced with VIMs.[19] These findings contradict our results concerning *P. aeruginosa* variants. In our study, efforts were made to investigate the *OXA-51* gene, however, it was found to be void in all *P. aeruginosa* isolates. Our result for the occurrence of *OXA-51* in *P. aeruginosa* was in agreement as per the claims stating that *bla* OXA-51-like and *bla* OXA-58 genes, which were thought to reside exclusively in *Acinetobacter sp*., can also be found in members of the Enterobacteriaceae.[20] The *VIM, IMP* genes and the recently emerged *NDM*-type MBLs (New Delhi metallo- ß -lactamase) are emerging as the most threatening carbapenemases.[21] Some selected carbapenemase primers investigated (Table 1) showed that other types of carbapenemase resistant β-lactamases were detected in relatively less percent of *P. aeruginosa* isolates. The observed prevalence of these genes were *OXA-40* (5%), *OXA-10* (7.5%), *OXA-48* (3.75%), *IMP* (1.75%), and *GIM* (15%). Similarly, Tawfik et al. (2012) described that *VEB*-1 and *OXA-10* (56%) are the predominant ESBL genes and *bla*$_{VIM}$ is the dominant MBL gene in *P. aeruginosa* isolates in Saudi Arabia.[22]

In the case of *A. baumanni*, some similarities in the occurrence of genes were observed for *OXA-40* (17%), *OXA-48* (2.8%), *OXA-1* (2.8%), *VIM* (11.4%), *IMP* (2.9% and *GIM* 2.9%. Whereas the *VIM* appears to be the most common MβL in this species in Saudi Arabia, which is in agreement with scenarios reported in other studies Memish et al. 2015 and Yezli et al. 2014.[15, 16] *A. baumanni* has been found to be void of genes *OXA-10* and *OXA 51*, and *OXA 24*.

Our results revealed that 27 (85.7 %) out of 35 isolates of *A. baumannii* harbored *OXA-23* gene. A bulk of the previous reports available on *A. baumannii* is in agreement with our results as the most prevalent resistant gene was *OXA-23*, among other carbapenemase class of enzymes.[23, 24] While Memish et al. (2015) reported that *OXA-23*, in particular, is the dominant carbapenemase in *A. baumannii*. In the present study, the majority of strains (77%) of *A. baumannii* possessed *ISAba1*; most probably located upstream of the *OXA-23* operon.[16] Martínez *et al.* (2012) reported that out of the 20 carbapenem-resistant *A. baumannii* isolates in their study, 15 expressed the carbapenemase *OXA-23* responsible for carbapenem resistance.[25]

It is observed that Carbapenem resistance has become greater than before, and hence caused restraining of this group of antibiotics to be used in therapy. [15, 26] Moreover, studies have found the pervasive spread of *OXA-23* among clinical

isolates of *A. baumannii* at hospitals in several cities of Saudi Arabia and, at a large, internationally. [15, 27] The frequency of carbapenem resistance is very high and its mechanisms are rarely studied in Saudi Arabia. However, Alsultan *et al.* (2013, 2009) recognized the genes (*VIM, OXA-23, OXA-40, OXA-89, OXA-66*) for carbapenemases as well as a novel chromosomal OXA-51-like beta-lactamases variant in carbapenem-resistant *A. baumannii* isolated between 2006 and 2011 at various Saudi medical centers. The Alsultan et al. claims were in disagreement with our results since the primer for *OXA-51*or *OXA-51*-like beta-lactamases gene did not amplify the *OXA-51* genes in either *A. baumannii* or *P. aeruginosa*. [28, 29]

Conclusion

In conclusion, the rates of carbapenem-resistant isolates conferring multiple resistance genes are worrisome, leaving the clinicians with limited treatment options with antimicrobial drugs. Therefore, proper use for infection control procedures and revision of the treatment and management strategies is certainly required to reduce the spread of resistance genes among these pathogens. Furthermore, rapid and simple detection of resistance determinants, at the genetic level, could help in choosing more appropriate and narrow-spectrum antibiotics in near future. Certainly, this experimental representation demonstrates that there is an urgent need to implement strategies that may slow the development of acquired resistance. Additionally, novel and effective antibiotics for the treatment of MDR gram negatives must be launched sooner to minimize the seriousness of these pathogens.

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