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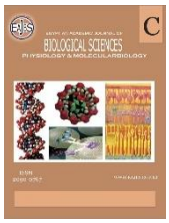
Citation: *Egypt.Acad.J.Biolog.Sci. (C.Physiology and Molecular biology) Vol. 15(2) pp285-293 (2023)*
DOI: 10.21608/EAJBSC.2023.317111

Egypt. Acad. J. Biolog. Sci., 15(2):285-293 (2023)

Egyptian Academic Journal of Biological Sciences
C. Physiology & Molecular Biology
ISSN 2090-0767
www.eajbsc.journals.ekb.eg



Carbapenemase-Encoding Genes in MDR *Acinetobacter baumannii* Isolated from ICUs and Surgical Wards in Minya, Egypt

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ARTICLE INFO

Article History
Received:7/8 /2023
Accepted: 8/9/2023
Available:13/9/ 2023

Keywords:
Spectrophotometric, mefenamic acid (MFA), Neocuproine (2,9.DMP), ponistan.

ABSTRACT

Acinetobacter baumannii (*A. baumannii*) has become a global pathogen relevant to healthcare. Carbapenems are among the last resort classes of antibiotics having the greatest efficacy against severe infections caused by bacteria that are resistant to many other antibiotics. This research was done in order to detecte the frequency of carbapenememase genes in *A. baumannii* isolated from Surgical Wards and Intensive Care Units (ICUs) in Minia, Egypt by phenotypic and genotypic methods.**Methods:** 310 patients who were undergoing surgery and receiving care in ICUs participated in the current study between August 2017 and October 2020. The isolates were obtained from a variety of clinical samples, including sputum, bronchial lavage, Pus and wound fluid. All clinical samples have been collected under complete aseptic conditions. *A. baumannii* isolates were identified by standard microbiological tests. The resistance patterns of the obtained isolates were detected by the disc diffusion method. Production of carbapenem-resistant genes was determined by phenotypic tests the Modified Hodge Test (MHT) and confirmed by the modified carbapenem inactivation method (mCIM). Various carbapenem-resistant genes were detected by PCR.**Results**:Out of 310 samples, Twenty-Five 25 (8%) isolates of *A. baumannii* were obtained. Antibiotic and sensitivity tests detect that 16(64%) and 12(48%) of *A.baumannii* isolates were resistant to meropenem and imipenem respectively. Out of the Carbapenem-resistant *A. baumannii* (CRAB): 9(56%) were (MHT) positive, and 13 (81%) were (mCIM) positive. Regarding resistance genes, $bla_{\text{OXA-23like}}$ and $bla_{\text{OXA-51like}}$ were detected in 94% and 100% of CRAB isolates respectively, while $bla_{\text{OXA-24like}}$, $bla_{\text{oxa-48like}}$, $bla_{\text{oxa-58like}}$ and bla_{NDM} were not detected in CRAB isolates.

INTRODUCTION

Gram-negative *Acinetobacter baumannii* (*A. baumannii)* is a major health hazard because it causes healthcare-associated infections among critically ill patients like patients with immunosuppression. They are a common reason for sepsis, ventilator-acquired pneumonia, wound infection, and (UTI) urinary tract infection, particularly in ICUs (Sookkhee *et al*., 2022). The emergence of extensively drug-resistant strains (XDR) or multidrug-resistant (MDR) *A. baumannii* became a complex challenge and put physicians in a complicated situation owing to the reduced number of antibiotic treatment options available leading to failure or delay of antimicrobial treatment, as well as the increase in mortality rate especially with the presence of CRAB (Mukherjee *et al*., 2023).

Carbapenems are efficient broad-spectrum β-lactam antibiotics They are often regarded as the final line of treatment when using antibiotics to treat severe infections caused by *A. baumannii*. Other antimicrobials like colistin and tigecycline may be used in case of carbapenem resistance; however, they have poor efficacy and/or high toxicity (Azimi *et al*., 2020). Unfortunately, carbapenems are frequently misused and are becoming ineffective due to the development of many resistance mechanisms produced by gram-negative bacteria. Carbapenem-resistance mostly resulted from the upregulation in the chromosomally mediated $bla_{\text{OXA-51-like}}$ gene and may be attributed to the acquisition of some OXA variants especially the $bla_{\text{OXA-23-like}}$ or by acquiring Metallo beta-lactamases (Gupta *et al*., 2022). Carbapenemase genes detection in *A. baumannii* strains is extremly significante in evading hospital-acquired infections by resistant bacteria. The availability of accurate and affordable carbapenemase detection techniques may encourage laboratories to investigate this issue and contribute to the prevention of a serious threat of bacterial antibiotic resistance.

MATERIALS AND METHODS

In this study, 310 specimens were obtained from Patients admitted to ICUs, neurosurgery and surgery departments in the period between August 2017 and October 2020. Various clinical samples, i.e. sputum, bronchial lavage, wound fluid and Pus, were collected under complete aseptic conditions. *A. baumannii* isolates were identified by using standard microbiological tests. The Ethics Committee of the Beni-Suef University Faculty of Science authorised each experimental procedure. Prior to collecting data, patients provided written informed permission. The study was carried out as per the Helsinki declarations.

1-Bacterial Isolation And Identification:

The bacterial strains were isolated from sputum, wound fluid, pus and bronchial lavage. Each sample was placed in a sterile container, put in an ice pack box and transported within 2 hours to the laboratory for processing. The bacterial isolates were identified by traditional biochemical tests including Citrate, Triple sugar iron (TSI), Oxidase, Catalase, Indole, and Voges-Proskauer (VP) test. Finally, the isolates were stored at -20°C in brain heart infusion [BHI] media containing 20% glycerol (Howard., 1956)

2-Antimicrobial Susceptibility:

In accordance with (CLSI 2020) recommendations, the Kirby- Bauer disc diffusion method was used to conduct the antibiotic susceptibility tests. (Oxoid, Basingstoke, UK) The following antibiotics were used, Aztreonam (30µg) , Tigecycline (15µg),Tobramycin(10µg), Cotrimoxazole (trimethoprim/sulfamethazole) (25µg), Ceftazidime (30µg), Amikacin (30µg), ceftriaxone(30µg),levofloxacin(5µg),ciprofloxacin(5µg), cefepime (30µg), Piperacillin/ tazobactam (100/10µg), gentamicin (10µg), Imipenem (10µg)and meropenem (10µg). Zone diameters were measured as refered in CLSI 2020.

3-Phenotypic Detection of Carbapenemases Production:

All carbapenem resistant isolates underwent the modified Hodge test (MHT) and modified carbapenem inactivation method (mCIM)

3.1. Modified Hodge Test (MHT):

MHT was mainly used to detect carbapenemases production in *A. baumannii* isolates. *Escherichia coli* ATCC 25,922 was swabbed in three distinct directions on plates of Mueller-Hinton agar media, using a diluted culture (0.5 McFarland standard). Each plate had a 10 µg meropenem or imipenem disc (Oxoid) in the middle of it. Beginning at the disc's edge and terminating at the plate's edge, CRAB isolates were streaked as a thin line. At 37C°, bacterial growth was permitted for 18 hours. *Escherichia coli* 25922 developed an area resembling a clover leaf along the growing streak in the disc diffusion zone, according to an MHT Positive test result (Pasteran *et al*., 2016).

3.2. Modified Carbapenem Inactivation Method (mCIM):

This method was used to confirm (MHT) as it is more sensitive than (MHT) for detecting of carbapenemases (Jing *et al*., 2018); one µl of the test strain suspended in 2 ml of trypticase soy broth (TSB) was emulsified. For 10 to 15 seconds, the bacterial suspension was vortexed. The bacterial suspension was then aseptically infused with a 10-g imipenem disk. The tube was then kept at 37C for another four hours. By using the direct colony suspension method, a half MF suspension of the indicator strain E. coli ATCC25922 was created (it was created shortly before the 4-hour carbapenem inactivation stage was finished). Inoculations were made on Muller Hinton agar plates using the conventional disc diffusion susceptibility testing method. The imipenem or carbapenem disc was then transferred to the inoculated MHA plate and incubated for 18 to 24 hours at 37C^{o} using an inverted position after being removed from the TSB bacterial suspension using a 10-l inoculating loop. The loop was used to remove any excess liquid during the transfer.. Zone diameter (≤ 15 mm) around the disk indicates positive results and explains the ability of the test organism to produce carbapenemases, (16-19 mm) indicates intermediate results and (≥ 20mm) indicates negative results (Pierce *et al*., 2017).

4-Molecular Detection of Resistance Mechanisms:

4.1. DNA Extraction:

The boiling method was used to prepare DNA, by centrifuging 1.5 ml of the bacterial broth for 5 min at 11.000 rpm. The pellet taken out, and the supernatant was suspended and mixed well with 200 microliters of molecular biology-grade water. Tubes were boiled at 100C^{o} in a water bath for 20 min. Then quickly cooled in ice to block the reaction. Centrifugation at 14.000 rpm for 2 minutes. The Supernatant was stored at -20 C^{o} and then used for DNA amplification (Chen *et al*., 2019).

4.2. Amplification of Carbapenemase-Encoding Genes by PCR:

Conventional PCR reactions were performed by using a thermal cycler (Biometra, UNO II).Specific primers for for *A. baumaannii*, *bla*$_{\text{OXA-51like}}$, *bla*$_{\text{OXA-24-like}}$, *bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-58-like}}$, *bla*$_{\text{NDM}}$ and *bla*$_{\text{OXA-48-like}}$.(Table1). DNA ampilification was carried out in 25 µl reactions, using My Taq TM red Master Mix "2 µl of DNA template; 12.5 µl master mix; 8.5 µl of sterile water, 1 µl of each forwared and reverse primers". The reaction conditions for each gene were listed (Table 2).

Table 1: Primers used for PCR reaction.

Gene	Primer sequence	Amplicon size (bp)	Ref
bla$_{\text{Oxa-23 like}}$	F(5´-GATCGGATTGGAGAACCAGA-3') R(5'-ATTTCTGACCGCATTTCCAT-3')	501	**(Benmahmod., *et al* 2018)**
bla$_{\text{Oxa-24 like}}$	F(5'-GGTTAGTTGGCCCCCTTAAA-3') R(5'-AGTTGAGCGAAAAGGGGATT-3')	246	**Benmahmod., *et al* 2018)**
bla$_{\text{Oxa-48 like}}$	F(5'-GCGTGGTTAAGGATGAACAC-3') R(5'-CATCAAGTTCAACCCAACCG-3')	438	**(Poirel *et al*.,2010)**
bla$_{\text{Oxa-58like}}$	F(5'-AAGTATTGGGGCTTGTGCTG-3') R(5'-CCCCTCTGCGCTCTACATAC-3')	599	**(Benmahmod., *et al* 2018)**
bla $_{\text{NDM}}$	F(5'-GGTTTGGCGATCTGGTTTTC-3') R(5'-CGGAATGGCTCATCACGATC-3')	621	**(Poirel *et al*.,2010)**
bla$_{\text{Oxa-51like}}$	F(5'-TAATGCTTTGATCGGCCTTG-3') R(5'-TGGATTGCACTTCATCTTGG-3')	353	**(Benmahmod., *et al* 2018)**

Table 2: Conditions for PCR reactions:

Gene	Cycles	Initial denaturation		Denaturation		Annealing			Extension
		Time	Temp	Time	Temp	Time	Temp	Time	Temp
*bla*Oxa-23like *bla*oxa-24 like *bla*oxa- 51 like *bla* oxa -58 like.	30	5 min	94 C°	25 sec	94 C°	40 sec	52 C°	50 sec 6min	**72 C°** **72 C°**
*bla*oxa-48 like *bla* NDM	36	10 min	94 C°	30	94 C°	40sec	52 C°	50 sec 5 min	**72 C°** **72 C°**

4.3. Agarose Gel Electrophoresis for detection of PCR Products

Products of PCR reaction were separated on 1 %-1.5 % agarose gel stained by 0.5 µg/ml ethidium bromide and bands were visualized under UV light.

5- StatisStatistical Analysis:

Descriptive statistics, data are presented as percentage and frequency. For analytical statistics, Using the Chi-square test as a significance test to numerical variables comparison, with less than 0.05 a P value indicating high significance.

RESULTS

From a total of 310 Clinical samples, Twenty five (8%) isolates were identified as *A. baumannii* in accordance with cultural characteristics and biochemical reaction results. Out of 25, 10 (40%) and 15(60%) isolates of *A. baumannii* belonged to female and male patients, respectively with *Mean age ± SD* (50.9±12.25 years). The majority of isolates were obtained from patients admitted to the ICUs was (52%) and from surgical departments was (28%). The majority of isolates were obtained from patients. with severe illnesses such as diabetes (44%), Asthma (36%), Cancer, or renal failure (4%). Also, (44%) and (20%) were recovered from patients with mechanical ventilators and urinary catheters respectively. The distribution of collected isolates from the specimen sources was as follows: Pus and wounds (36%), Sputum and bronchial lavage (28%) for each and urine (8%) (Table 3).

Table 3: The prevalence of A. baumannii isolates according to some demographic factors.

		Descriptive statistics N=25	P value
Age	*Range* *Mean ± SD*	(29-72) 50.96±12.25	
Gender	*Male* *Female*	15(60%) 10(40%)	*0.317*
Source of samples	*Pus/Wound* *Sputum* *B. lavage* *Urine*	9(36%) 7(28%) 7(28%) 2(8%)	*0.233*
Admission site	*Surgical* *ICU*	9(36%) 16(64%)	*0.162*
Underlying disease	*None* *DM* *Asthma* *Renal* *Cancer*	1(4%) 11(44%) 9(36%) 2(8%) 2(8%)	*0.002**
Related Devices	*None* *Ventilator* *Urinary catheter*	9(36%) 11(44%) 5(20%)	*0.326*

Sensitivity tests displayed that the highest resistance rate Was for Cotrimoxazole (96%), ceftazidime (92%), amikacin–ceftriaxone and cefepime (88%), levofloxacin and piperacillin/tazobactam (84%), ciprofloxacin (80%), gentamicin (68%), meropenem (64%), Tobramycin (62%), Aztreonam (60%), imipenem (48%) and the lowest resistance was against tigecycline (5%).

Out of 25 *A. baumannii* isolaes just (16) isolates were resistant to carbapenems, they were classified according to the impact of some demographic factors which may affect the resistance (Table4**).**

Table 4: Distribution of CRAB in relation to some demographic factors.

		Carpabenem		P value
		Sensitive	Resistant	
Source of samples	*Pus / Wound*	5(55.6%)	4(25%)	*0.353*
	Sputum	2(22.2%)	5(31.3%)	
	B. lavage	1(11.1%)	6(37.5%)	
	Urine	1(11.1%)	1(6.3%)	
Admission site	*Surgical*	5(55.6%)	4(25%)	*0.127*
	ICU	4(44.4%)	12(75%)	
Underlying disease	*None*	1(11.1%)	0(0%)	***0.019****
	DM	7(77.8%)	4(25%)	
	Asthma	0(0%)	9(56.3%)	
	Renal	1(11.1%)	1(6.3%)	
	Cancer	0(0%)	2(12.5%)	
Related Devices	*None*	6(66.7%)	3(18.8%)	***0.033****
	Ventilator	3(33.3%)	8(50%)	
	Urinary catheter	0(0%)	5(31.3%)	
Gender	*Male*	2(22.2%)	13(81.3%)	***0.004****
	Female	7(77.8%)	3(18.8%)	

All CRAB isolates were tested for carbapenemases production by both MHT and mCIM. 9 (56%) of CRAB isolates were positive for MHT and 13(81%) were Mcim positive (Fig. 1). All CRAB isolates harbored *bla*$_{\text{OXA-51like}}$ gene which confirmed *A. baumannii* species, out of them we found that 15 isolates were positive for *bla*$_{\text{OXA-23like}}$. While *bla*$_{\text{OXA-24like}}$, *bla*$_{\text{oxa-48like}}$, *bla*$_{\text{oxa-58like}}$ and *bla*$_{\text{NDM}}$ were not detected in CRAB isolates of this study.

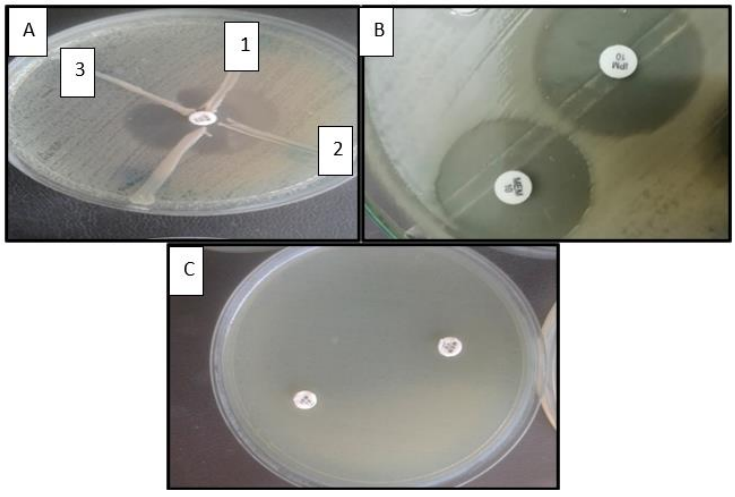

Fig. 1: Phenotypic detection for carbapenemases by MHT and mCIM in CRAB.
A: MHT in CRAB: 1- negative control, 2 -negative MHT, 3 -positive MHT.B: Negative mCIM against meropenem and imipenem in CRAB C: Positive mCIM against meropenem and imipenem in CRAB.

DISCUSSION

Resistance to Carbapenems is a global public health issue that is predominantly found in *A. baumannii*. Antibiotic resistance is rapidly spreading, particularly when it is spread through transferable carbapenemase-encoding genes like Metallo β lactamases, resulting in large outbreaks and limiting treatment options (Vamsi *et al*., 2022). By the analysis of this study results, we found that *A. baumannii* prevalence was (8%) and it is relatively agrees with that obtained by (Mohammed *et al*., 2022) who detected a (10%) prevalence of *A. baumannii*, and this indicates that *that in Egypt, A. baumannii* does not pose a serious health risk, this is the same opinion mentioned in a recent study done in El-Minia hospitals by (Abd El-Baky *et al*., 2020). Most *CRAB* isolates were obtained from ICUs patients and this was statistically significant and agreed with (Ibrahim *et al*., 2022). Because of their weak immune responses, most ICU patients have a higher risk of infection and the use of numerous procedures and intrusive instruments like Central venous catheterization (CVC), mechanical ventilation and urinary tract catheters; they also require a prolonged stays in ICUs, which increased the risk of infection, particularly when drugs with potent antimicrobial activity and a wide antibacterial spectrum were used. In addition, patient's medical records, having an acute disease like diabetes or cancer are at risk and this was agreed with results reported by (Pachori *et al*., 2019). In our investigation, we found that patients with mechanical devices or underlying disease were more vulnerable to CRAB infection and this was statistically significant but this was opposite to research by (Pachori *et al*., 2019) who mentioned That, the prevalence of carbapenemases was independently predicted by catheter devices use and the existence of underlying diseases.In this investigation, While 48% of *A. baumannii* isolates were resistant to imipenem, they were highly resistant to other antibiotics like, cortimoxazole and ceftazidime. Carbapenems were once thought to be the only option for treating infections brought on by multi-drug resistant bacteria. Due to the widespread use of these antibiotics, carbapenem-resistant strains (CRAB) first appeared a few years ago. Additionally, the spread of CRBA across hospitals is a result of insufficient infection control efforts. Resistance to other kinds of antibiotics as well as a high mortality rate was found to be related to carbapenem resistance. (Hu *et al*., 2023) reported high resistance of *A.baumannii* against meropenem, cefepime, ceftazidime, imipenem and ciprofloxacin. However, in our investigation low resistance rate for meropenem and imipenem, high resistance rate for ceftazidime and cefepime was observed. Furthermore, we reported that imipenem was the most effective antibiotic, but they demonstrated that tigecycline was just the most effective antibiotic against A. baumannii. (Dias *et al.*, 2017).

Carbapenemases represent three classes of β-lactamases. lactamases the Ambler class A, class β carbapenemases (zinc dependant) They are referred to as (MBLs) "metallo β -lactamases" because metal chelators like EDTA inhibit these enzymes from working; and D-carbapenemases (serine carbapenemases). All β -lactam antibiotics can be hydrolyzed by metallo β-lactamases (MBLs), whereas monobactams cannot. These enzyme-encoding genes may be plasmid or chromosomally-mediated. Phenotypic tests revealed that out of CRAB (56%) were (MHT) positive and this result was lower than other studies like (Abouelfetouh *et al*., 2019) which recorded 78 % positive MHT. In our study, we found (81%) of CRAB were (mCIM) positive, and this is higher than (Neupane *et al*., 2023) who reported (69.7%) positive for (mCIM). Previous studies recorded a lower dominance of $bla_{\text{OXA-23like}}$ among CRAB in Egypt (Al-Hassan *et al*., 2019) reported (55.8%). Our results indicate an apredominant increase in the prevalence of class D Oxacillinase ($bla_{\text{OXA23-like}}$) and this may be explained by the existence of several clusters and horizontal gene transfer across different

strains of the species, both of which have been implicated in the steady rise in carbapenem resistance over time. *bla*$_{\text{OXA-51like}}$ is expressed naturally and it is used for confirming *A. baumannii*. None of the isolates in our investigation was harboured *bla*$_{\text{OXA 24-like}}$ and *bla*$_{\text{OXA 58-like}}$ genes which were in concordance with (Ibrahem *et al*., 2022). No strains under investigation had *bla*NDM found, but in a research by (Hosseinzadeh *et al.*, 2018) who detect about 10% of the isolates carried the *bla*$_{\text{NDM}}$ gene. Also, none of the isolates had the *bla*$_{\text{OXA-48-like}}$ gene and this was reported by (Anggraini *et al*., 2022).

Conclusion

Our data revealed that *A. baumannii* had a great ability of resistance to many antibiotics as they were detected as MDR strains. Resistance to imipenem and meropenem was detected in the strains isolated from hospitals in EL-Minia, this was due to the presence of carbapenemases especially oxacillinases, which make serious problems in forcing these pathogens. Most CRAB strains were isolated from ICUs which was considered the potential source of infection in our hospitals. The most accurate method for carbapenemase detection is PCR because of the low sensitivity of MHT and mCIM. Carbapenemases were extremely complex determinants of resistance as they spread rapidly across the bacterial community creating a threat to the treatment with carbapenems. We noticed that the main cause of resistance in *A. baumannii* was the expression of carbapenem hydrolyzing class D b-lactamase encoded by the acquired gene *bla*$_{\text{oxa-23 like}}$. The intrinsic gene *bla*$_{\text{oxa-51- like}}$ was a good indicator in the characterization of *A. baumannii.* Determinant factors like the site of admission, use of mechanical devices and the presence of underlying diseases had a great influence on the resistance of *A. baumannii* to carbapenems.

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