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THE ROLE OF ISABA1 AND OXA CARBAPENEMASE GENES, in CARBAPENEM RESISTANT AMONG ACINETOBACTERBAUMANNII ISOLATED from ZAGAZIG HOSPITALS

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Abstract*:*
Introduction: “Red alert” pathogen this is how Acinetobacterbaumannii identified nowdays in healthcare facilities, due to its extensive antibiotic resistance pattern. Multidrug-resistant A. baumannii infections are best treated with Carbapenems. The aim of the study is to estimate the numbers of $bla_{oxa}$and ISAba1 producing strains among carbapenem-resistant A. baumannii clinical isolates.
Method: identification of A. baumanniiwas carried out by Matrix-assisted laser desorption ionization–time of flight mass spectrometry, VITEK 2 compact was used to assess antimicrobial sensitivities and E test used to reconfirm susceptibility to imipenem, multiplex PCR for class D β lactamase genes detection. Mapping PCR carried out in order toestimate the presence of ISAba1 in relation to blaOXA-23 and blaOXA-51 genes.
Results: Fourty eight A. baumanniistrains were isolated from different anatomical sites from surgical patients in Zagazig University hospital. Antibiogram showed that 81.3% (n=39) of the isolates were resistant to Imipenem, while 9 isolates were susceptible (18.7%). Of 39 resistant isolates to Imipenem, ISAba1 was detected in 35 isolates (89.7%). 34 isolates carried blaOXA-23 gene, all contained ISAba1/blaOXA-23 genes, and 20 contained ISAba1/blaOXA-51 genes. Resistant isolates to carbapenemsall had ISAba1 upstream of blaOXA-23 gene, WhileISAba1 upstream of blaOXA-51 found in both susceptible and resistant isolates.
Conclusion: The ISAba1/blaOXA-23 genes were prevalent among the carbapenem-resistant A. baumannii isolates and may be responsible forcarbapenems resistance. The present study revealed that the existence of ISAba1/blaOXA-51 in A. baumannii isolates was not conclusive to carbapenems resistance.

Keywords: Acinetobacterbaumannii, ISAba1, bla_{oxa},,carbapenems resistance.

INTRODUCTION

Acinetobacterbaumannii, was considered low pathogenic organism, but now days considered to have principlerolein bothhealth care- and community-acquired infections. It is resistant toalmostmost antibioticsby means of genetic exchange on one hand, on the other hand it can contaminatehospital environments for prolonged period in tough conditions (walls, surfaces, and medical devices) **(Pourhajibagheret al., 2016).**

β-lactam resistant *A. baumannii;* carbapenems are the most choosable antibiotics totreat infections caused by it.However, limited choices of treatment due to marked increase in carbapenems resistance are major problem (**Kim et al., 2013)**. There are many mechanisms responsibleforcarbapenem resistance, however, class D beta-lactamases (CHDLs)

productionremain the most common and leading mechanisms in Carbapenemhydrolyzing among *A. baumannii* isolates**(Lin et al., 2014).**However, their expression is often greater by the insertion of an upstream IS, which enriches expression by providing a strong promoter, causing high levels of resistance(**Mugnier et al., 2009).** Insertion Sequence *ISAba1*, , has been detected in *A. baumannii* known to has inverted repeat sequences (IRs) 11-bp flanked by direct repeats 9-bp of the target sequence (**Nowak et al., 2012)**.The (insertion sequences) IS have role for β-lactamase production byacting as strong promoters and have a role to obtain other challenging phenotypes in *A. baumannii***(Gheorghe et al.,2015)**.

The aim of this study is to estimate the frequency of bla_{oxa} producing strains among carbapenem-resistant A. baumanniiisolates and analyzes the existence of ISAba1/blaOXA-23 and ISAba1/blaOXA-51 genes, alsoinvestigate its role in resistance**.**

MATERIALS AND METHODS

Bacterial isolates
A total of non duplicate 48 clinical isolates were included in this study recovered from wound swab, pus, peripheral blood culture, peritoneal fluid, sputum, cvp tips and urine from surgical ICU andsurgery departements of Zagazig University Hospitals over the period from march 2018 to July 2019. The isolates were identified as Acinetobacterbaumanniicomplex byMatrix-assisted laser desorption ionization–time of flight mass spectrometry(MALDI-TOF MS) up to the species leveland furthermore confirmed as Acinetobacterbaumannii using intrinsically located blaOXA-51 gene by PCR(Turton et al., 2006).

Antibiotic susceptibility testing
Vitek 2 compact System (GN-222) for gram negative bacilli (Biomerieux. Inc, Durham, USA) used for Antibiotic sensitivity testing, according to the guidelines of Clinical and Laboratory Standards Institute(CLSI, 2012)**,** Susceptibility to imipenem was reconfirmed by E-test strips (Biomerieux, France).

Molecular Characterization
DNA extraction fromcolonies by using i-genomic BYF DNA Extractin Mini Kit**,** then DNA amplification was done by:

- Multiplex PCR usedfor DetectingClasD OXA-Carbapenemase genes: To detect theexistence of intrinsically located blaOXA-51 gene and the existence of other acquired OXA genesnamelyblaOXA-23, blaOXA-58 and blaOXA-24/40. Multiplex PCR was perfermed using thermal cycler (Applied Biosystems thermal cycler)with cycling condition of intial denaturation step at 94 ºc for 3 min followed by Denaturation at 94 ºc for 1 min for 30 cycles.Annealing at 52ºc for 30s. Extension at 72 ºc for 1min and finally extension at 72 ºc for 10 min (Woodford et al., 2006).
- Subsequent UniplexPCR, in order to detect insertion sequence (IS)element, ISAba1.To plot the location of the ISelement in relation to the blaOXA-23 and blaOXA-51-like genes by using two types of primers.First,the forward primer of ISAba1 gene and the reverse primer of blaOXA-51 gene, the second is the same forward primer but reverse primer of blaOXA-23 gene.Detection is read at 1.2 kb and 1.4 kb of the amplified product respectively.Using the subsequent conditions: initial denaturation at 95°C for 2 min, 30 cycles of 95°C for 10 s, 53°C for 40 s for annealing then extension at 72°C for 3min , at last final extension at 72°C for 60 s **(Martinez et al., 2012).**

Primers used in PCR reaction listed in **(table1)**. Briefly, for PCR amplification, reaction mixture containing 5 ul of extracted DNA , 1ul of each forward and reverse diluted primers solutions, 15ul of ready to use (Dream Taq^TM Green PCR Master Mix (2X) thermoscientific were used following manufacture's instructions.

PCR products detection:
After amplification, products detected on 2 % agarose gel stained with ethidium bromide then visualized with trans-illuminator.

Table (1): Sequences of primers used in PCR:

Gene	Primer	Sequence (5' to 3')	Amplicon size(bp)	Referances
OXA51	OXA51F	TAA TGC TTT GAT CGG CCT TG	353bp	Woodford et al., 2006
	OXA51R	TGG ATT GCA CTT CAT CTT GG		
OXA23	OXA23LF	GAT CGG ATT GGA GAA CCA GA	501 bp	
	OXA23LR	ATT TCT GAC CGC ATT TCC AT		
OXA 24	OXA24LF	GGT TAG TTG GCC CCC TTA AA	246bp	
	OXA24LR	AGT TGA GCG AAA AGG GGA TT		
OXA58	OXA58LF	AAG TAT TGG GGC TTG TGC TG	599bp	
	OXA58LR	CCC CTC TGC GCT CTA CAT AC		
ISAba1	ISAba1F	CAC GAA TGC AGA AGT TG	549bp	
	ISAba1R	CGA CGA ATA CTA TGA CAC		Segal et al., 2005

RESULTS
The fourty eight isolates included in this study were recovered from pus **(n=28)**, wound swab **(n=4)**, peripheral blood culture **(n=3)**, Body fluids **(n=5)**, sputum **(n=6)**, cvp tips **(n=1)** and urine **(n=1).**

Antimicrobial susceptibility
MIC for carbapenems antibiotics was determined by using Vitek 2 System. It was found that 39 isolates (81.3%) of all isolates were resisted to carbapenem antibiotic (Imipenem) used in this study, while 9 isolates (18.7%) were sensitive to this antibiotic. Imipenem sensitivity results in these isolateswere reconfirmed by E test and called resistant when MIC (≥ 8 µg/ml) according to CLSI guidelines(CLSI., 2020).

Detection of class D OXAcarbapenemases genes and insertion sequence
Detection of the OXA carbapenemases genes including (blaOXA-51, blaOXA-23, blaOXA24/40 and blaOXA-58) in carbapenem-resistant isolates were carried out using a multiplex PCR assay**(figure1)**. BlaOXA-51 gene was found in all isolates, while blaOXA-23 gene was detected in 34out of39 resistant isolates. BlaOXA-24 genewas found in 5/39 while blaOXA-58 gene was abscent in all isolates**(table 2)**.

Mapping PCR
The dominance of ISAba1 element (35) and its association with blaOXA-23 or blaOXA-51 genes or with both were investigated **(Table 3).**

Table (2): Distribution of bla OXA and ISAba1 among A.baumanii isolates

Genetic elements	CRAB (n=39)		CSAB (n=9)		P#
	No	%	No	%	
blaoxa- 51like	39	100%	9	100%	---
blaoxa- 23 like	34	87.2%	2	22.2%	<0.001**
bla oxa-24/40 like	5	12.8%	0	0%	0.67 NS
blaoxa- 58 like	0	0%	0	0%	---
ISAba1	35	89.7%	2	22.2%	<0.001**

#: chi square or Fisher exact corrected test NS: Non significant (P>0.05)
**: highly significant (P<0.01)

Table (3):Distribution of genetic element ISAba1 after doing subsequent PCR:

Genetic elements	CRAB (n=39)		CSAB (n=9)		P#
	No	%	No	%	
blaoxa- 51	39	100%	9	100%	---
blaoxa- 51+ISAba1	20	51.3%	2	22.2%	0.23 NS
blaoxa- 23	34	87.2%	2	22.2%	<0.001**
blaoxa -23+ISAba1	34	87.2%	0	0%	<0.001**

The current study revealed that ISAba1 was detected in 35 (89.7%) of resistant isolates and in 2 of sensitive isolates**(22.2%)**. In carbapenem-resistant isolates, the results revealed presence of blaOXA-51/ISAba1 in 20 isolates**(51.3%)** while blaOXA-23/ISAba1 was found in 34 isolates **(87.2%)**, in other word, all resistant isolates which contained blaOXA23 gene carried ISAba1 upstream of this gene. In susceptible isolates, it was found that blaOXA-51/ISAba1 present in 2/9 isolates (22.2%) while blaOXA-23/ISAba1 was not present in any susceptible isolate in spite of the existence of2 blaOXA23 genes among them. The current study demonstrated that blaOXA-51/ISAba1 was found in both carbapenem-resistant and susceptible *A.baumannii*isolates.

ISAba1 shown to be locatedwith blaOXA-23 gene at the promoter region by PCR mapping in 34carbapenem-resistant isolates; these amplicons were 1.4 kbp in size. 20 of resistant isolates and 2 of susceptible isolates also had ISAba1 at the promoter region of blaOXA-51 gene. Detectedat 1.2 kbp in size.

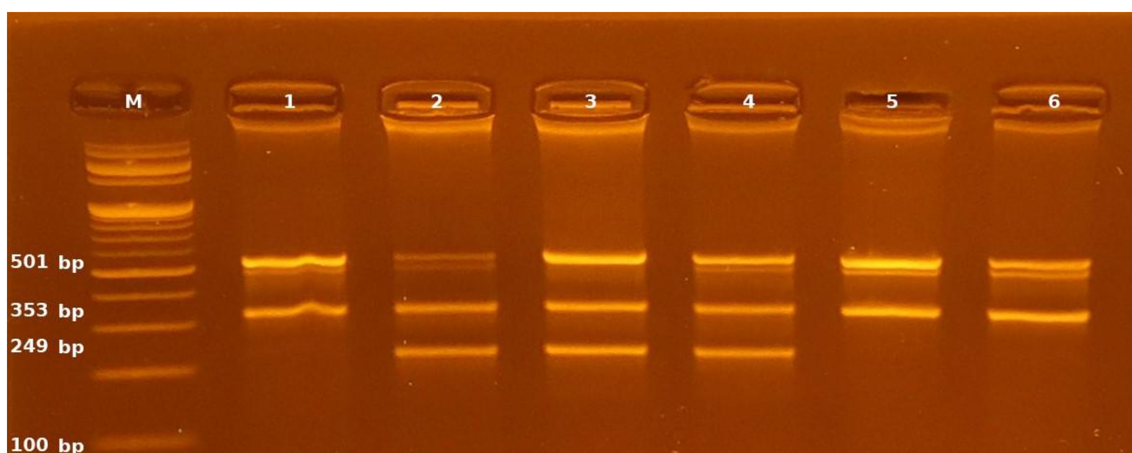

Figure (1): Gel electrophoresis for detection of PCR multiplex with OXA genes primers. Lane (M), DNA ladder(molecular size 100 bp), Lanes (1-6) show positive results at 353 bp for OXA 51 and positive results at 501bp for OXA 23, Lanes (2-4) show positive results at 249 bp for OXA 24 while no OXA 58 at 599 bp was found.

DISCUSSION

Highrisk of *Acinetobacterbaumannii*, , mostly in ICUs now emerged because of the striking increased number of nosocomial infections caused by it globall**y(Garnacho-Montero et al.,**

2010). Mostly, due to the unreasonable use of antibiotics, *A. baumannii* has become resistant to most antimicrobial classes, leading to the dominance of multi-drug resistant strains particularly among hospital patients .This result in limited options for treatment of infections caused byresistance isolates of*A. baumannii* which lead to higher morbidity and mortality rates**(Fattouh and El-din et al., 2014)**.

Class D beta-lactamases (CHDLs) production remain the most common and leading mechanisms in Carbapenemhydrolyzing among *A. baumannii* isolates, despite prescence of many mechanisms responsible for carbapenem resistance,**(Lin et al.,2014).**

Detection of oxacillinases among resistant strains is critical to help the choice of correct course of therapy. CHDLs can be either intrinsic/chromosomal or acquired**(Abbott., 2013).**

In the current study, according to Vitek results, most of the isolates **(81.3%)** were imipenem resistant with MIC **≥ 8 μg/ml(CLSI., 2020)**.Imipenem resistance was reconfirmed by measuring by E-test strips (bioMe´rieux), while **(18.7%)** of isolates were sensitive to imipenem.

Despite being burdensome, the E-test is the standard method.According to study's results E-test and VITEK 2 agreed to high degree. When trying to choose substitution for for treating Acinetobacter infections while waiting for E-test results, VITEK 2 results are acceptable**(Altun et al., 2016)**.

In Egypt, 70% to 100% are resistance rates for imipenem in A. baumannii isolates. This denote the magnitude of the problem in treating MDR A. baumannii**(Al-Agamy et al., 2014, Ahmed et al., 2011, Nasr and Attalah, 2012, -Al-Hassanet al., 2013-El-Bannah et al., 2018)**..The last resort of treatment arecolistin and tigecycline**(Al-Agamy et al., 2014**).

Due to sever side effect of colistin, this may lead to infrequent use. In the present study, all the tested isolates, remain sensitive to colistin with sensitivity of 100%.This agree with prior studies in Egypt, in which colistinretain it's activity against 95% in**(Al-Agamy et al., 2014**) and 100% in**(Fouad et al., 2013)**of the tested isolates.

In order to proof that A. baumannii has a natural intrinsic blaOXA- $_{51\ like}$ responsible for carbapenemaseresistance. Multiplex PCR performed in this study show that all the 48 clinical isolates were positive for bla OXA$_{-51-like}$gene. Similar results were also obtained by previous studies (**Al-Agamy et al., 2014,Abouelfetouh et al., 2019, Ben Mahmod et al., 2018,Kobs et al., 2016,Owrang et al., 2018,Cicek et al**., **2013, El-Bannah et al., 2018).**

BlaOXA-23was the most CHDL-encoding gene prevalent in carbapenem resistant A. baumannii with predominance rate of 87.2% (34/39), this has been recently reported in numerous studies that blaOXA-23 is the most frequent resistant gene identified among carbapenem-resistant A. baumannii isolates (**Abbott et al., 2013,Al-Agamy et al., 2014, Abouelfetouh et al., 2019, Ben Mahmod et al., 2018, Kobs et al., 2016, Owrang et al., 2018,Cicek et al**., **2013, Al-Hassanet al., 2013, Fouad et al., 2013, El-Bannah et al.,2018).**
blaOXA-$_{24/40}$have been detected in 12.8% of the isolates in this study, compared to previous results obtained in Egypt, in which blaOXA-$_{24/40}$ have detected in 7.5% of the islates(**Al-Agamy et al., 2014**) and in 2.9%of the isolates **(Lin et al., 2014)**.However, **Abouelfetouh et al. (2019)** state that blaOXA-40 was abscent in all tested isolates.BlaOXA-$_{24/40}$have mostly

been found in Asia, Europe and other areas in the world **(Cicek et al., 2013, Abbott et al., 2013, Zhang et al.,2015)**.

On contrary to our results, many studies reported prescenceof blaOXA-58 in A. baumannii tested isolates in differentplaces of the world including Italy, UK, Kuwait, Argentina, Spain, USA, and Turkey and also in Egypt **(Abouelfetouh et al., 2019).**

We found that ISAba1 was present in 89.7% of carbapenem resistant isolates, in all CRAB isolates positive for *bla*OXA$_{-23\text{-like}}$ gene (n=34), all had ISAba1., in comparison with the sensitive two isolates where blaoxa$_{-23}$ was not associated with ISAba1. This closerelation between IS*Aba*1 and *bla*OXA$_{-23\text{-like}}$, was also observed in **Kobs et al. (2016)**, explains the continuing dependability of this resistance mechanism in cross-infection occurring at hospital.So, the presence of *bla*OXA-23-like lacking IS*Aba*1 promoter sequence might beinadequate to hydrolyse carbapenems, as reported by **Carvalho et al., 2011.**

However, imipenem-susceptible *A. baumannii*isolates (n=2) having *bla*OXA-23 gene, may suppose silent spread in hospital setting and highlight thethreat of undiagnosed reservoirs of carbapenemase genes **(Carvalho et al., 2011).**

It was proved that both carbapenem resistant and sensitive isolates of *A.baumannii*contained ISAba1 element in association with blaOXA-51, and ISAba1/blaOXA-51 association was found in 51.3% of carbapenems resistant *A.baumannii*isolates. **Pagano et al. (2012)** established that ISAba1 element was not correlated with carbapenems resistance in *A.baumannii*when found upstream blaOXA-51.

Furthermore, five isolates of A. baumannii were carbapenemunresponders, it only possess blaOXA-$_{51\text{-like}}$ gene, while other genes were abscent. The reason of carbapenem-unresponsiveness in these isolates other than resistance genes, may be caused by modification of penicillin binding proteins, AmpC stable derepression, overexpression of efflux pump, loss of porins and decreased permeability, or insertion of ISAba1 at promoter region of blaOXA-51-like gene **(Turton et al., 2006)**.

CONCLUSION

The ISAba1/blaOXA-23 genes were the most common gene association among the carbapenem-unresponders*A. baumannii*isolates and carbapenemsunresponsiveness may be due to ISAba1 at promoter sequence of blaOXA-23 gene mostly. The present study revealed that the presence of ISAba1/blaOXA-51 in *A.baumannii*isolates was not conclusive to carbapenems resistance.

Conflict of interest

The authors declare no conflict of interest

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