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ORIGINAL ARTICLE

Prevalence of ISAba1-*bla*OXA-23, ISAba125-*bla*NDM-1, and armA genes in High-Level Aminoglycoside Resistant *Acinetobacter baumannii*

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ABSTRACT

Background: *Acinetobacter baumannii is one of the most common pathogens causing health care associated infections that may result in serious morbidity and even may cause death in critical units like intensive care units, this is because of its intrinsic drug-resistance mechanisms in addition to acquired ones. Aminoglycosides, plays a crucial role in managing infections caused by gram negative bacilli. In case of A. baumannii, these groups face strong resistance mechanisms due to genes specially armA gene which is present in most of High level Aminoglycoside Resistant (HLAR) A. baumanni isolates. Successful combinations used to manage A. baumanni infection gather both carbapenems and aminoglycosides together, as they produce synergistic effects. Carbapenem resistance has become a major health problem, most commonly, Acinetobacter spp. mediate carbapenem resistance through production of carbapenemases that belong to OXA-type carbapenemase and metallo-β-lactamases (MBLs), including NDM-1. Insertion sequences located near carbapenemases genes in A. baumanii isolates can control their expression and mobility mostly through formation of transposon structure.* ***Objectives:*** *the aim of this work is to study the combination of resistance genes and to explore their insertion sequences.* ***Methodology:*** *In this study 65 A. baumanii isolates were obtained from ICUs of Zagazig University hospital and all were nosocomial infections. HLAR was determined among the isolated strains. Antibiotic susceptibility testing was performed. The presence of ISAba1, blaOXA-23, ISAba125, blaNDM-1, and armA genes was tested by polymerase chain reactions.* ***Results:*** *We found that 23 (35.4%) of A. baumanni isolates were HLAR, all were rersisant to both gentamicin and amikacin, and 19 (82.6.5%), &18 (78.3%) were resistant to imipenem and meropenem respectively. Resistence genes were isolated from all A. baumannii strains, as blaOXA-23, blaNDM-1, and armA, were found in (78.5%), (36.9%), (81.5%) respectively. Regarding the ISAba1, and ISAba125, they were found associated with blaOXA-23, and blaNDM-1, in (92.2%), and (83.3%) respectively. In HLAR armA gene was present in all 23 isolates, while blaOXA-23, and blaNDM-1 genes were present in (69.6%), and (73.9%). Fifteen (65.2%) HLAR isolates contain all of ISAba1/blaOXA-23, ISAba125/NDM-1, and armA genes.* ***Conclusion:*** *We found that a great percent of A.baumanni isolates carrying antibiotic resistant genes either alone or in combination and the presence of insertion sequences and carbsapenemase producing genes together with HLAR in the same isolates represent a serious health problem and predict a spread of more powerful strains which will result in a real critical situations specially that the number of effective antibiotics against these strains are continually decreasing.*

INTRODUCTION

Acinetobacter baumannii is one of the most common pathogens responsible for different types of health care associated infections that may cause serious morbidity and even may cause death particularly in critical units like intensive care units, this is because it has not only intrinsic drug-resistance mechanisms but also it has acquired ones[1]. The danger lies in the increasing number of *A. baumannii* infections all over the world, and the serious problem is that when these isolates are multi-drug resistant strains (MDR) which are only affected by toxic polymyxins and colistin, these isolates represent now a major concern in both antibiotic policy determination and infection control practice [2].

Aminoglycosides, a group of broad-spectrum antibiotics, plays a crucial role in managing infections caused by gram negative bacilli[3]. In case of *A.*

baumannii, these groups face strong resistance mechanisms that make a challenge for the effect of treatment by them[4]. These include different enzymes acting on different targets, such as, acetyltransferases, phosphotransferases and nucleotidyltransferases [5].

In addition to16S rRNA methylase enzymes encoded by different genes, the most prevalent one is armA gene [6]. This group of enzymes are responsible mostly for the development of High level Aminoglycoside Resistance (HLAR), and un fortunately these enzymes could easily be mobilized between different species by conjugation [7].

A successful combination used to manage *A. baumanni* infection gather both carbapenems and aminoglycosides together because together, they produce synergistic effects 8. Carbapenem resistance has become a major health problem, most commonly, *Acinetobacter* spp. which mediate carbapenem resistance through production of carbapenemases that belong to OXA-type carbapenemase and metallo-β-lactamases (MBLs) [8-10].

Among multiple MBL genes, *A. baumannii* that carry plasmid encoded New Delhi metallo-β-lactamase-1 (NDM-1), a new carbapenemase gene, is found in different countries including Egypt [11-13]. The cause of this resistance among *A. baumannii* spp. was the continuous dramatic use of meropenem and imipenem in the last 15 years to deal with MDR strains [14].

In addition to antibiotic resistance, the ability to make a biofilm on both animate and inanimate surfaces -on which it may survive for months- is considered additional virulence factor of *A. baumannii* that enables it to cause outbreaks both within and among medical institutions [15,16]. Different studies identified the co-occurrence of blaOXA-23 and armA genes in MDR *A. baumannii* spp.[17,18]. ISAba1 was detected in widespread clones of *A. baumannii* worldwide. In a study done by Prabhu and his collegues [19], ISAba1was found to be present upstream of blaOXA-23 in all *A. baumannii* isolates. Not only that but also a link between *A. baumannii* isolates having the ISAba1/blaOXA-23 gene and increased MICs for carbapenems was present [20]. The blaNDM-1 gene was proved to be gene originated from by the union of the aminoglycoside-resistance gene aphA6 with a mannose binding lectin gene, this can occur frequently in *Acinetobacter* spp., so this bacterium may be the origin of this gene[21]. *A. baumannii* isolates can transfer blaNDM-1 gene to another spp.by conjugation and Tn125 is proved to be the main method for transfer of the blaNDM-1 genes in *A. baumannii* [22]. Poirel et al. stated that the blaNDM-1 gene was present in a composite transposon named Tn125 surrounded by two copies of a strong promoter of blaNDM-1 gene called ISAba125[23]. Insertion sequences located at the 5` end and/or 3` end of blaOXA genes in *A. baumanii* isolates can control their expression and mobility mostly through formation of transposon structure [24], also NDM gene was associated with n ISAba125 element as it was present upstream of its gene, and can be located within a transposon named Tn125 [25].

On the other hand CLSI stated that HLAR isolates are not synergistic with cell wall–active agent when used in combination with them[26]. The aim of the present study is to examine the presence of the three genes which are blaOXA-23, blaNDM-1, and armA genes, in combination with each other, in HLAR *A. baumanniii* strains, which will put the light on a serious problem regarding the spread of resistant strains in such a critical unit like intensive care unit, also we aimed to explore the presence of insertion sequence as a prof that these genes can be easily transferred to other strains or species, which will in turn aggravate the problem.

METHODOLOGY

Bacterial isolates:

Acinetobacter baumannii isolates were collected from patients admitted to intensive care units of Zagazig University Hospitals in the period from October 2016 till June 2017, all isolates were collected from patients in whom *A.baumanni* was detected 48 hours after admission to ensure the nosocomial origin of infection. Sixtyfive isolates were included in this research. All isolates were identified by Vitek 2 system (Biomerieux, Marcy l'Etoile, France).

Antibiotic susceptibility testing

This was done by the modified Kirby-Bauer disc diffusion method, the antibiotic discs used were of amikacin (30), gentamicin (10), imipenem (10), and meropenem (10) (Oxoid) the medium used was Mueller Hinton Agar (High Media, India) according to the antibiotic disk diffusion method. Incubation was done at 37 °C for 24 h. The results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines [26].

Detection of High Level Aminoglycoside Resistance

This was done according to CLSI recommendations[26], by inoculation of three brain heart infusion agar plates one with Gentamicin 500 µg/mL, the second with amikacin 500 µg/mL, and the third with streptomycin 2000 µg/mL, then 10 µL of a 0.5 McFarland suspension spotted onto agar surface plates, after incubation for 24 hours in case of gentamicin and amikacin, but up to 48 hours in case of streptomycin in 35°C ± 2°C; ambient air. Detection of even one colony in any plate is considered (HLAR). We used E. coli ATCC 25922, and *A. baumannii* ATCC 19606 (American Type Culture Collection [ATCC], Manassas, VA, USA), as negative and positive controls respectively

Detection of carbapenem resistance:

The isolates which were positive for (HLAR), were further examined for minimal inhibitory concentration

detection by E test on muller hinton agar plates for both imipenem and meropenem antibiotics, according to CLSI guidelines for MIC breakpoints [26], MIC of ≤ 2 (µg/mL) is considered sensitive, MIC of 4 (µg/mL) is considered intermediate and MIC of ≥8 (µg/mL) is considered resistant.

Detection of resistance genes:

Extraction of DNA was done from colonies of *Acinetobacter baumannii* isolates showing (HLAR) using QIAamp DNA Mini kit (Qiagen GmbH, Hilden, Germany). Amplification was done using Taq PCR Master Mix (Qiagen GmbH). PCR reactions were done using the previous DNA to detect the presence of blaOXA-23, blaNDM-1 and arm A genes using primers and PCR conditions as listed in table (1). The ISAba1 of blaOXA-23 gene was detected in all OXA-23 positive A. baumanii isolates using the following primersISAba1-F/ISAba1-R and ISAba1-F/blaOXA-23R (Table 1). The ISAba125 of blaNDM-1 gene was detected in all blaNDM-1 positive *A. baumannii* isolates using combination of primers ISA125-F/ISA125-R and ISA125F/blaNDM-R (Table 1). PCR products were visualized by electrophoresis using 1% agarose gel which contains 0.5 µg/ml ethidium bromides under UV illumination.

Table 1: Primers used for detection of resistant genes included in this study.

Target genes	Primer name	Sequence 5'-3'	Size (bp)/ Annealing temp (C^o)	References
blaOXA-23	blaOXA-23-F	GATCGGATTGGAGAACCAGA	501/52	27
	blaOXA-23-R	ATTTCTGACCGCATTTCCAT		
blaNDM	blaNDM–F	GGTTTGGCGATCTGGTTTTC	621/52	28
	blaNDM–R	CGGAATGGCTCATCACGATC		
Arm A	armA-F armA	AGGTTGTTTCCATTTCTGAG	591/55	29
	armA-R	TCTCTTCCATTCCCTTCTCC		
ISAba1	ISAba1-F	CATTGGCATTAAACTGAGGAGAAA	451/52	30
	ISAba1-R	TTGGAAATGGGGAAAACGAA		
ISAba125	ISA125-F	TGTTGAAGCGATCCGTTGTT	755/57	19
	ISA125-R	GTGCGACAGTTTCAAAAGCCA		

Ethics Statement:

The current research was approved by the institutional review board (IRB) - Faculty of medicine, Zagazig University. An informed written consent was obtained from all participants.

RESULTS

Demographic information of patients from which *A. baumanni* were isolated:

In the current research 38 (58.5%) *A. baumannii* isolates were obtained from male patients while 27 (41.5%) isolates were from female patients. Isolates were collected from sputum (n=5, 7.7%), endo-tracheal aspirates (n=23, 35.4%), wound aspirate, (n=17, 26.2%), pus (n= 4, 6.1%), blood cultures (n= 13, 20%) and urine (n=3, 4.6%) (table 2).

Antibiotic susceptibility testing

In this study we first described antibiotic resistance pattern of the isolates against the four tested antibiotics by disc diffusion method. It was found that 37 (56.9%) isolates were resistant to gentamicin, 45 (69.2%) were resistant to amikacin, 53 (81.5%) were resistant to imipenem, and 33 (50.7%) were resistant to meropenem (table 3).

Table 2 : Types of specimens from which 65 *A. baumanni* isolates were collected

Specimens	Sputum	endo-tracheal aspirates	wound aspirate	pus	blood cultures	urine	Total
No., (%)	5, (7.7%)	23, (35.4%)	17, (26.2%)	4, (6.1%)	13, (20%)	3, (4.6%)	65,(100%)

Table 3: Susceptibilities patterns of the 4 antibiotics used of all A. baumannii isolates.

	Resistant isolates, n (%)	Intermediate isolates, n (%)	Sensitive isolates, n (%)	Total, n (%)
Gentamicin	37, (56.9%)	17, (26.1%)	11, (17%)	65, (100%)
Amikacin	45, (69.2%)	16, (24.6%)	4, (6.2%)	65, (100%)
Imipenem	53, (81.5%)	7, (10.8%)	5, (7.7%)	65, (100%)
Meropenem	33, (50.7%)	21, (30.3%)	11, (16.9%)	65, (100%)

High level aminoglycoside resistant

The number of isolates showing (HLAR), which means that they were resistant to both plates containing streptomycin and gentamicin, was 23 (35.4%). All HLAR were resistant to both gentamicin and amikacin. About the imipenem resistance among HLAR, it was found that 19 HLAR isolates were resistant also to imipenem (82.6%) concerning meropenem resistance among HLAR, it was found that 18 HLAR isolates were resistant also to meropenem (78.3%) (table 4).

Table 4: Antibiotic resistant isolates to the used antibiotics among the 23 HLAR *A. baumanni* isolates:

Antibiotic	Gentamicin	Amikacin	Imipenem	Meropenem
No. (%)	23. (100%)	23. (100%)	19 (82.6.5%)	18. (78.3%)

Antibiotic resistance genes and IS in *A. baumannii* isolates:

About the prevalence of the resistant genes among all isolates it was found that, *bla*OXA-23 was found in 51 (78.5%) of isolates, while NDM-1 was found in 24 (36.9%) of isolates, concerning *arm*A gene it was found in 53 (81.5%)of isolates. About the presence of ISAba1 in *A. baumannii* isolates harboring *bla*OXA-23, it was found in 47/51 (92.2%) of isolates. Concerning ISAba125, it was found in 20/24 (83.3%) of NDM-1 positive *A. baumannii* isolates, table (5). About the HLAR isolates, it was found that all isolates contain armA gene, while blaOXA-23 was found in 16 (69.6%) of isolates, and NDM-1 was found in 17 (73.9%) of H LAR isolates (table 5). Fifteen (65.2%) HLAR isolates contain all of ISAba1/*bla*OXA-23, ISAba125/NDM-1, and *arm*A genes.

Table 5: Antibiotic resistance genes identified in *A. baumannii* isolates:

		All isoaltes n=65, (100%). No.	(%)	HLAR isolates n = 23 (100%). No.	(%)
***bla*OXA-23**		51	(78.5%)	16	(69.6%)
ISAba1+ No. (%)	ISAba1- No. (%)				
47 (92.2%)	4 (7.8%)				
NDM-1		24	(36.9%)	17	(73.9%)
ISAba125+ No. (%)	ISAba125 – No. (%)				
20 (83.3%)	4 (16.7%)				
***arm*A**		53	(81.5) %	23	(100%)

DISCUSSION

This research explored a serious problem represented by the presence of *A. baumanni* isolates exploring HLAR and carbapenem resistance at the same time, which will in turn limit the possible treatment choices for these isolates. The current research showed that the HLAR isolates represent about 35.4% of total isolates separated. Some researches from China [29] and India [31] reported that 75 (63.56%) and (79.2%) strains were HLAR, the high percent present in these studies may be attributed -to a little extent- to the different geographical area, but to a large extent to the method used by them, as they tested the presence of HLAR by amikacin and gentamicin, while the recent recommendation of CLSI is to use the combination of both streptomycin and gentamicin as strains that show HLAR to gentamicin, possess one or more aminoglycoside-modifying enzymes. These enzymes may make them resistant to one or more of a variety of

other aminoglycosides, including tobramycin, netilmicin, and amikacin, but not streptomycin [32].

Aminoglycoside resistance may be conferred by different genes. In this study we detected the presence of 16SrRNA armA Methyltransferase gene as it was present in 53 (81.2%) *A. baumannii* isolates, this gene was present also in most aminoglycoside resistant *A. baumanii* isolates as described in several studies elsewhere including Egypt [11,29,31,33], where this gene was the most frequent gene isolated from aminoglycoside resistant *A. baumannii* strains, and that was the reason we chose it in our research. Regarding carbapenem resistance in *A. baumanni* isolates, and as carbapenemases belong to different classes of beta-lactamases like class A, B, and D, we tested the presence of blaOXA-23 gene which belongs to class D β-lactamase, and blaNDM-1 which belongs to class B or metallobeta lactamases.

Our results showed that blaOXA-23 and blaNDM-1 were present in (78.5%) and (36.9%) of *A. baumanni* isolates, indeed reports from different areas including Egypt described blaOXA-23 as the commonest type of OXA carbapenemases isolated, in a study done by Raghdaa et al.[17] stated that the most common carbapenemase gene and even the only OXA-type carbapenemase present in (90%) of *A.baumannii* isolates, was blaOXA-23 followed by blaNDM in (66.7%) of cases. Also El-Sayed-Ahmed et al.[11] reported that bla OXA-23, and bla NDM-1 were isolated from (76.7%), and (39.3%) of tested *A. baumannii* bacteria. Gomaa et al. [34] reported the presence of blaNDM-1 in (59.1%) of *A. baumannii* isolates. *A. baumannii* isolates having blaOXA-23 are reported to replace blaOXA-58 that predominated for long time and now it became the most common carbapenemase gene present in many Mediterranean places [35,36].

The potent carbapenemase action of blaOXA-23 plus horizontal gene transfer may provide selective advantage for such isolates 37. Regarding ISAba1 in our study it was found in 47 (92.2%) of blaOXA-23 isolates this also was in accordance with Prabhu et al [19] where ISAba1 was present in all blaOX-23 isolates. It is also worthy to mention that a relation was found between *A. baumannii* strains carrying the ISAba1/blaOXA-23 gene and increased MIC for carbapenems [20]. As a result of low expression of blaOXA genes they are found to have weak hydrolytic activity on oxymino-beta-lactams and carbapenems, but their expression increased significantly due to the occurrence of upstream insertion sequences. These insertion sequences give them a powerful promotor, also in a study done by Khorsi et a.[38], ISAba1 was found in (87.3%) of blaOXA-23 *A. baumannii* isolates, proving the important role of these insertion sequences in expression of blaOXA-23 gene [39].

Concerning the co-occurrence of ISAba125 with NDM-1 gene, in the current research it was found that (83.3%) of NDM-1 positive isolates contained ISAba125 insertion sequence this was in accordance with several studies giving a nearby percent [19,38,40]. This sequence enhances the overexpression of NDM-1gene and also facilitates its mobilization through a composite transposon consisting of two ISAba125 (Tn125) 40] Regarding the co-occurrence of the three genes blaOXA-23, blaNDM-1 and armA genes in (HLAR) *A. baumannii* isolates, which to our knowledge, it is the first research that tested that occurrence, it was found that 15/65 (23.1%) of *A. baumannii* isolates. In a study performed in Egypt [11], the three genes were present in combination with each other in (76%) of cases. Other studies all over the world tested the co-occurrence of blaOXA-23, NDM-1 and armA together or in pairs[11,12,1,29], and they all found a great percent of *A.baumannii* isolates carrying these genes in combination. This in turn clarify a serious health problem represented by the wide spread of resistance genes among MDR or even XDR *A. baumannii* isolates, and urge for strict application of infection control practices in a trial to limit the spread of such strains because the number of antibiotic left to treat such serious infections is continuously decreasing in a dreadful manner.

Recommendations:

Sequencing of the amplified genes is important in order to determine the location of insertion sequences isolated.

Conflict of interest:

The authors declared that there is no conflict of interest regarding this research article and the research is totally funded by the researchers themselves with no external or institutional aid.

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