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Genotyping and Molecular Characterization of Carbapenem-resistant *Acinetobacter baumannii* Strains Isolated from Intensive Care Unit Patients

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The emergence of multidrug-resistant *Acinetobacter baumannii* has partly increased treatment failure and patient mortality. Class D β-lactamases is an important mechanism of resistance to beta-lactam antibiotics in this species. This study aimed to investigate the relationship between the presence oxacillinase gene and genetic fingerprints of *A. baumannii* isolates from the intensive care unit of an Egyptian tertiary care hospital. One hundred and twenty *A. baumannii* clinical isolates were collected. Multiplex PCR was performed to detect genes encoding oxacillinases (*OXA-23, OXA-24, OXA-51, OXA-58* and *OXA-143*). Molecular typing of all collected isolates was performed using random amplified polymorphic DNA (RAPD)-PCR assay. Out of 120 examined isolates, 92, 88 and 84% were resistant to ertapenem, imipenem and meropenem, respectively. The species-specific, commonly present *OXA-51* gene was found in all isolates while *OXA-23* showed a high prevalence of 88% of isolates. *OXA-24* and *OXA-143* genes were detected in 3% and 1% of isolates, respectively. No *OXA-58* gene was detected. Five clusters consisting of 19 genotypes were detected using RAPD-PCR. Genotype A was the most prevalent, it was observed in 62% of the isolates followed by genotype B (12%). These results revealed that genotypes A and B are common in the hospital. Results also demonstrate that RAPD-PCR is a rapid and reliable method for studying the clonal similarity among *A. baumannii* isolated from different clinical specimens.

Keywords: *Acinetobacter baumannii,* carbapenem resistance, oxacillinase genes, genotyping

Introduction

Emergence and spread of multidrug-resistant (MDR) *Acinetobacter baumannii* within hospitals has become a global serious concern [1]. It has been noticed over the last decades pathogen that pathogens are responsible for many severe infections, especially in the intensive care unit (ICU) patients and burn wards [1, 2]. They can cause bacteremia, ventilator-associated pneumonia, meningitis, urinary tract, wounds and burn infections [3].

Although, most of *A. baumannii* isolates were sensitive to carbapenems, and imipenem therapy was once the "gold standard" for pneumonia due to *A. baumannii* [1, 4], the widespread use of antibiotics has rapidly developed the resistance to many antimicrobials, including fluoroquinolones, carbapenems and aminoglycosides [5].

Some mechanisms result in β-lactam resistance in *A. baumannii*, including: (i) alterations of porin-like proteins, which results in decreased permeability to antibiotics; (ii) the activity of efflux pumps, that decreases the intracellular concentration of antibiotics; and (iii) β-lactamases production [6]. Among the previously mentioned mechanisms, carbapenemases (carbapenem-

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hydrolyzing β-lactamases) belonging to the molecular class D OXA enzymes (oxacillinases) have globally emerged as the major mechanism responsible for resistance to carbapenems in *A. baumannii* [7].

They are categorized into six main subclasses, the species-specific, commonly present chromosomal carbapenemase gene to this species *OXA-51* and the acquired *OXA- 24, OXA-23, OXA-143, OXA-58* and *OXA-235* β-lactamases [6, 8]. Rapid development of MDR strains makes it essential to understand the epidemiology of nosocomial *A. baumannii* infections in order to design effective strategies to control their spread [9, 10]. It is necessary to differentiate between the epidemic strain and the epidemiologically unrelated *Acinetobacter* [11].

This requires the assessment of isolates on the subspecies level by epidemiological typing methods like random amplification of polymorphic DNA-PCR (RAPD-PCR) technique that has a specific impact in the epidemiological tracing due to the nature of RAPD profiling that produces fingerprints as well as it can be used to detect polymorphisms in a variety of organisms [12].

In RAPD-PCR, random primer sequences can be used in organisms in which a specific genome sequence is unknown [13]. Random parts of the genome of the organism are amplified, which are expected to be identical amongst related species, and similar banding patterns are to be produced in gel electrophoresis [14].

In this study, we aimed to characterize *A. baumannii* nosocomial isolates obtained from Medical Microbiology lab from ICU patients in Alexandria, Egypt, in terms of antimicrobial resistance, prevalence of oxacillinase genes,

clonal relationship and investigate any relationship between these factors that could probably have an impact on the treatment of carbapenem resistant *A. baumannii* infections.

Materials and Methods

Bacterial isolates

One hundred and twenty *A. baumannii* clinical isolates were collected from the Medical Microbiology lab at Alexandria Main University Hospital (El-Meri hospital). These isolates were obtained from 120 ICU patients from July 2018 to January 2019. They were collected from diffrent clinical specimens including blood, mini broncho-alveolar lavage (miniBAL), sputum, urinary tract infection (UTI), wound infections and endotracheal intubation (ETI).

Bacterial culture and Identification of *A. baumannii* clinical isolates

Isolates were cultured for 18–24 h at 37℃ on Mac-Conkey's agar plates (Himedia, India). Non- lactose fermenting colonies were further examined. All isolates were identified as *Acinetobacter* by standard biochemical methods [15] and were confirmed as *A. baumannii* by using VITEK 2 automated instrument ID System (bioMérieux, France). Strain identities were further confirmed by PCR amplification of *OXA-51* gene which is unique species-specific and commonly present in *A. baumannii* species using primers as describe previously (Table 1) [13, 14].

Table 1. Primers used for detection of OXA genes in this study.

Target gene	Primer	Sequence	Expected amplicon size (bp)	Reference
OXA-23	OXA-23 F	5'-GATCGGATTGGAGAACCAGA-3'	501	[17], [56]
	OXA-23 R	5'-ATTTCTGACCGCATTTCCAT-3'		
OXA-24	OXA-24 F	5'-GGTTAGTTGGCCCCCCTTAAA-3'	246	[17], [56]
	OXA-24 R	5'-AGTTGAGCGAAAAGGGGATT-3'		
OXA-51	OXA-51 F	5'-TAATGCTTTGATCGGCCTTG-3'	353	[17], [56]
	OXA-51 R	5'-TGGATTGCACTTCATCTTGG-3'		
OXA-58	OXA-58 F	5'-AAGTATTGGGGCTTGTGCTG-3'	599	[17], [56]
	OXA-58 R	5'-CCCCTCTGCGCTCTACATAC-3'		
OXA-143	OXA-143 F	5'-TGGCACTTTCAGCAGTTCCT-3'	149	[56]
	OXA-143 R	5'-TAATCTTGAGGGGGCCAACC-3'		

Antimicrobial susceptibility testing

The susceptibility testing of *A. baumannii* isolates against different antibiotics was carried out by the disc diffusion method (Modified Kirby-Bauer method) according to the Clinical Laboratory Standards Institute (CLSI) guidelines 2019 [18]. The antibiotics used in this study were piperacillin/tazobactam (TZP, 110 μg), cefotaxime (CTX, 30 μg), cefoperazone (CFS, 30 μg) ceftriaxone (CTR, 30 μg), ceftazidime (CAZ, 30 μg), cefepime (CPM, 30 μg), ertapenem (ETP, 10 μg), meropenem (MRP, 10 μg), imipenem (IPM, 10 μg), tetracycline (TE, 30 μg), co-trimoxazole (Trimethoprim/sulfamethoxazole) (COT, 25 μg), gentamycin (GEN, 10 μg), amikacin (AK, 30 μg) and ciprofloxacin (CIP, 5 μg). The diameters of the zones of inhibition were recorded and interpreted according to the CLSI guidelines 2019 [18].

Isolates were considered as MDR when they were found to be resistant to at least three classes of the above mentioned antimicrobial agents [19]. Antibiotic discs were all purchased from (Himedia) and stored in the refrigirator (2–8℃).

Genomic DNA extraction of bacterial isolates

Genomic DNA from *A. baumannii* was extracted using the boiling method as described previously [20] with some modifications. Briefly, 4–5 fresh colonies of each isolate were picked from MacConkey's agar plates and suspended in 400 μl of sterile distilled water in a sterile Eppendorf tube. The bacterial suspensions were then boiled at 95℃ for 10 min, left to cool for 5 min on ice then centrifuged for 5 min at 14,000 rpm. The supernatants were then collected and transferred to sterile Eppendorf tubes as templates for use in PCR and RAPD assays.

PCR amplification of antibiotic resistance genes

The presence of *A. baumannii* genes encoding oxacillinases (*OXA-24*, *OXA-23*, *OXA-58*, and *OXA-143*) were assessed in all 120 isolates using primers as described previously (Table 1). Each PCR was performed in dupli-

cate in a thermocycler (Veriti, Germany, catalog number 4375786).

The thermal cycling program was made according to Woodford *et al.* [16] with some modifications. All PCR assays used *OXA-51* gene as the internal control. The primers were evaluated separately and then in a multiplex format. PCR was carried out in 25 μl reaction volume containing 12.5 μl MyTaq Hot start DNA polymerase (Bioline, UK), 5.5 μl of the extracted DNA sample and 1 μl (10 pmol) of each primer. In each set of experiments, a negative control was included which was prepared by replacing the DNA template with distilled water. *A. baumannii* reference strain ATCC 19606 was used as positive control for *OXA-51* gene. The amplification conditions were as follows: initial denaturation at 95℃ for 2 min, 30 cycles of denaturation at 95℃ for 30 s, annealing at 52℃ for 15 s and extension at 72℃ for 10 s and a final extension at 72℃ for 7 min [16].

PCR products were separated on 1.5% agarose gels (Bioline) and observed using an image analysis system, high-performance UV trans-illuminator (Biometra, Germany).

Generation of DNA fingerprints by RAPD-PCR

All our 120 isolates were analyzed by RAPD-PCR genotyping using AP2, AP5 and AP6 primers (Table 2). RAPD was performed by the method described previously [21]. Amplification was made in a final volume of 10 μl of the amplification mixture containing 5 μl MyTaq Hot start DNA polymerase (Bioline), 1 μl of the extracted DNA sample, 3 μl water and 1 μl (10 pmol) of a single primer (Table 2) of arbitrary nucleotide sequence following manufacturer's instructions.

Amplifications were performed in a thermal cycler as described previously [21] with some modifications, using the following program: initial denaturation 95℃ for 1 min, 40 cycles of denaturation at 95℃ for 15 s, annealing at 30℃ for 15 s, extension at 72℃ for 30 s followed by a final extension 72℃ for 5 min. The PCR products were resolved using 1.5% agarose gel. A 100 bp DNA ladder

Table 2. RAPD primers used in this study.

RAPD primer	Description	Sequence	Reference
AP2	Single primer of arbitrary nucleotide sequence (decamer)	5'-GTTTCGCTCC-3'	[57], [54]
AP5	Single primer of arbitrary nucleotide sequence (decamer)	5'-AACGCGCAAC-3'	[57], [12]
AP6	Single primer of arbitrary nucleotide sequence (decamer)	5'-CCCGTCAGCA-3'	[57], [11]

H3 RTU (GeneDirex, USA) was used. RAPD profiles were used to measure genetic relationships among *A. baumannii* isolates. The images of the gel was captured, and the banding pattern was analyzed using BioNumerics software from Applied Maths (Applied Maths, Sint-Martens-Latem, Belgium).

Dendrogram generation and degrees of homology were determined by Dice comparisons, and clustering correlation coefficients were calculated by the UPGMA (unweighted pair group method with arithmetic averages). Clustering was performed at 95% similarity cutoff as shown in Fig. 3.

Results

Prevalence of *A. baumannii* among clinical specimens

During the period of the study, 120 isolates were identified as *A. baumannii* by VITEK 2 Automated Instrument ID System and confirmed by PCR amplification of *OXA-51*, the species-specific commonly present gene. Among them 110 isolates were found to be carbapenem-resistant and 10 were carbapenem sensitive. Sixty (50%) of our isolates were recovered from sputum, 25 (21%) isolates were from MiniBAL, 16 (13%) from wounds, 13 (11%) from blood, 3 (2.5%) from UTI and 3 (2.5%) from ETI.

Antimicrobial susceptibility of *A. baumannii* isolates

Prevalence of carbapenem resistance pattern indicated that 92, 88 and 84% were resistant to ertapenem, imipenem and meropenem, respectively. The resistance
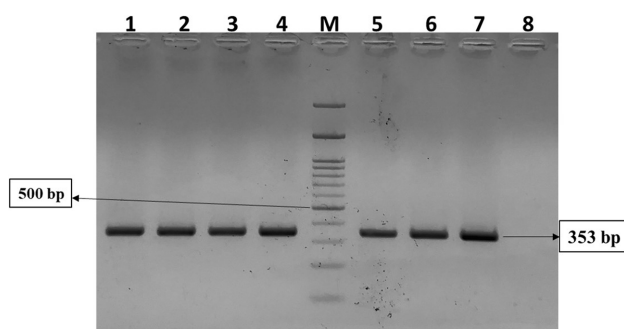

Fig. 1. PCR amplification of sputum isolates to confirm the presence of carbapenemase gene *OXA-51* specific to *A. baum*annii on 1.5% agarose gel. Lanes 1-6 show positive results at 353 bp (for the isolates 1sp-6sp), lane M shows100 bp DNA ladder, lane 7 shows positive result at 353 bp for the positive control strain, lane 8 is the negative control (*E. coli*).

to other β-lactam antibiotics was distributed as follows: piperacillin/tazobactam (92%), Cefotaxime (92%), Cefoperazone (92%) Ceftriaxone (92%), Cefepime (92%), Ceftazidime (92%), while resistance to other classes of antibiotics was distributed as follows: Amikacin (88%), Gentamycin (85%), Trimethoprim/sulfamethoxazole (68%), Ciprofloxacin (90%) and Tetracycline (54%).

Molecular characterization of Oxacillinase encoding genes

PCR for oxacillinase-encoding genes revealed that 100% of *A. baumannii* isolates carried *OXA-51* (Fig. 1) while multiplex PCR showed 88% carried *OXA-23*, 3% carried *OXA-24* and 1% carried *OXA-143* (Table 3). However, *OXA-58* was not detected in any of the tested isolates.

RAPD Genotyping

All 120 isolates were analyzed by RAPD-PCR genotyping using AP2, AP5 and AP6 primers. Primers AP2 and

Table 3. Prevalence of OXA resistant genes among different genotypes.

OXA-genes / Genotypes	Number of isolates carrying			Total number of isolates
	OXA-23 (%)	OXA-24 (%)	OXA-143 (%)	
A	73 (99%)	-	-	74
B	12 (86%)	2 (14%)	-	14
C	8 (100%)	-	-	8
D	4 (100%)	-	-	4
E	2 (100%)	-	-	2
F	1 (100%)	-	-	1
G	1 (100%)	-	-	1
H	1 (100%)	-	-	1
I	1 (100%)	-	-	1
J	-	1 (100%)	1 (100%)	1
K	-	1 (100%)	-	1
L	1 (100%)	-	-	1
M	1 (100%)	-	-	1
N	-	-	-	2
O	-	-	-	3
P	-	-	-	2
Q	-	-	-	1
R	-	-	-	1
S	-	-	-	1

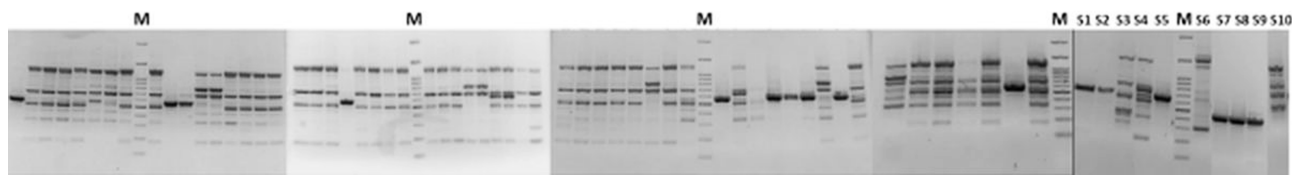% = number of positive *OXA gene* in this genotype/total number of isolates in this genotype

Fig. 2. RAPD-PCR fingerprinting of 58 of our carbapenem-resistant and 10 carbapenem-sensitive *A. baumannii* clinical isolates using AP5 oligonucleotide primer showing different genotypes. M: 100 bp DNA ladder, S1-S10 represent sensitive isolates, all unlabeled lanes represent the resistant isolates.
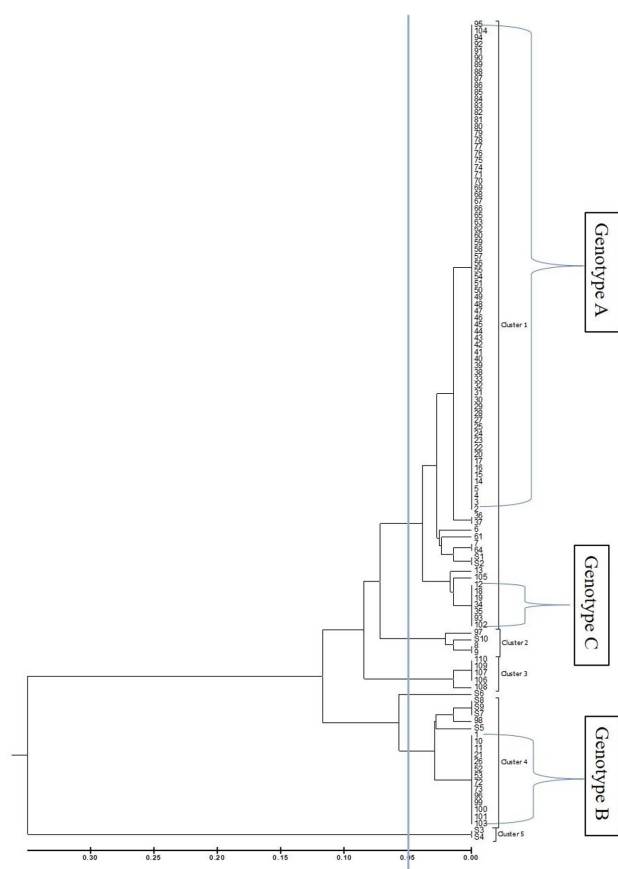

Fig. 3. Dendrogram of 120 *A. baumannii* isolates based on RAPD-PCR fingerprint. Clustering was performed based on unweighted pair group method with arithmetic mean. The blue vertical line shows 95% similarity cut-off.

AP6 gave reproducible patterns but often only a small number of bands were generated with many of the strains (data not presented). The decamer AP5 was chosen for this study because it produced the highest number of bands with our tested *A. baumannii* isolates in screening trials as shown in Fig. 2. The DNA banding patterns were analyzed by BioNumerics software version 7.6.3. (Applied Maths, Sint-Martens-Latem).

It was found that obtained fingerprints belong to 19 genomic types (Fig. 4), denoted from A to S. Genotypes A, B, C, D, E, F, G, H, I, J, K, L and M belong to carbapenem resistant *A. baumannii* which showed positive results to one or more of carbapenem resistance genes, while, genotypes N, O, P, Q, R and S belong to carbapenem sensitive *A. baumannii* which showed negative results to carbapenem resistance genes except for the species-specific *OXA-51* gene.

OXA-23 is the most predominant among the resistant genotypes (A-M), while genotype (J) showed positive results for only *OXA-24* and *OXA-143*. Genotypes B and K showed positive results for *OXA-23* and *OXA-24*. Sensitive isolate genotypes (N-S) did not show any positive results for *OXA* genes. It was concluded that genotype A and genotype B were present in all sample sources except for UTI and ETI isolates, respectively.

Five main clusters were obtained from the analysis of the dendrogram as shown in Fig. 3, where cluster 1 contained genotypes A, C, E, F, G, H, I and L, these 8 genotypes showed 95% similarity between them. Cluster 2 contained genotypes J and S and cluster 3 contained genotypes D and M. Cluster 4 contained genotypes B, K, O and Q all these clusters showed 95% similarity between different genotypes. However, cluster 5 is considered an outgroup as there is no similarity with other clusters. As shown from Fig. 3 each genotype belongs only to one cluster. Some clonally related groups (A and B) were observed in most of the isolate sources which represent the dissemination of these clones in the hospitals (Fig. 5).

Discussion

A. baumannii has emerged as an important cause of healthcare-associated infections with high morbidity and mortality rates [1]. Previously, carbapenems were
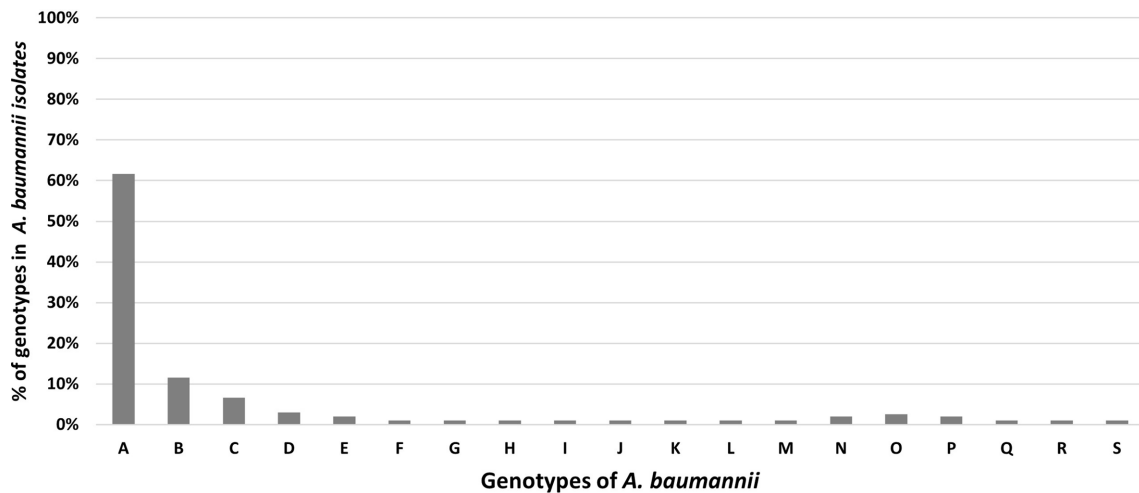
Fig. 4. Different Genotypes of *A. baumannii* and the percentage of isolates present in each genotype.
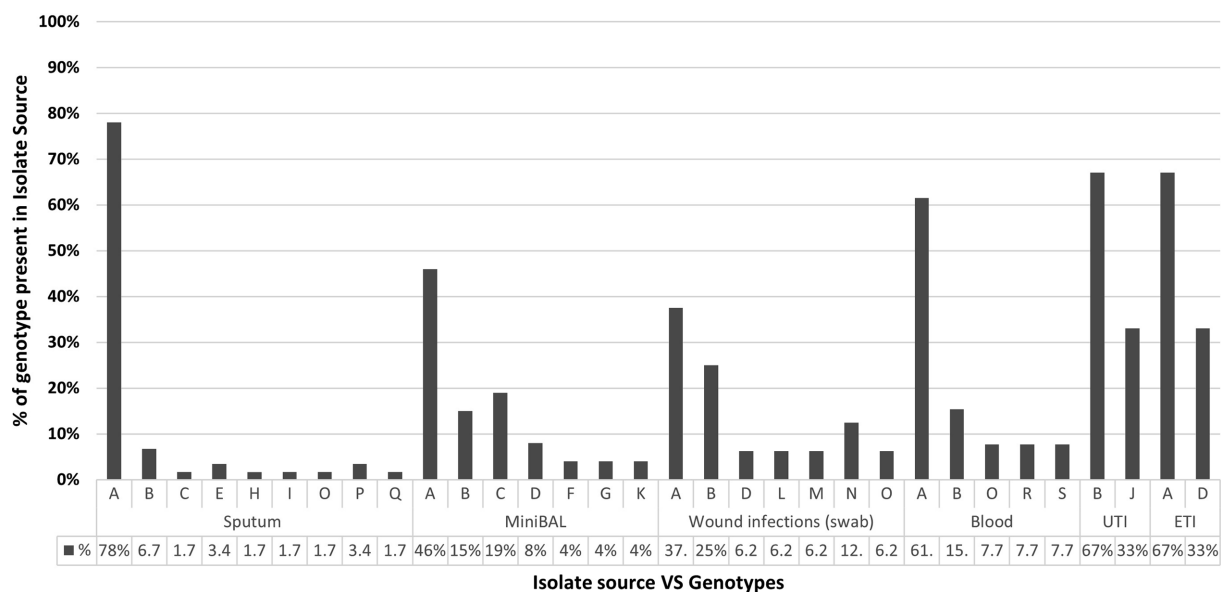

Fig. 5. The relation between the isolates source and different Genotypes A-S: represents the 19 different genotypes obtained in this study. *UTI: urinary tract infections, ETI: endotracheal intubation

the antibiotics of choice for treatment of MDR *A. baumannii* [22]. Nevertheless, carbapenem over-use and misuse for treatment of *Acinetobacter* infections became responsible for the occurrence of carbapenem resistant *A. baumannii* (CRAB) [23] which is considered a substantial health problem globally due to limited therapeutic options [6, 21]. During the period of the study, 120 isolates were identified as *A. baumannii* and identification was confirmed by the amplification of the species-specific *OXA-51* gene. Half of our tested *A.*

baumannii isolates were obtained from sputum samples (50%) this is in agreement with a study made in Egypt [25], 21% of the isolates were obtained from Mini-BAL and 13% from wound which is in accordance to a study in Iran [26]. The rest of the isolates were obtained from blood (11%), UTI (2.5%) and ETI (2.5%).

The resistance of our *A. baumannii* isolates to cephalosporins was in accordance to the findings of studies done in hospitals in Egypt [27], Iran [28], Bulgaria [29] and Nigeria [30] where the resistance was found to be 90–

100% which could be due to the over-use of cephalosporins in these hospitals. The resistance of our isolates to amikacin (88%) and gentamicin (85%) was in agreement to the results of studies made in Bulgaria (90%) [29] and Iran (81%) [31] respectively. In contrast, few studies carried out in hospitals in Nepal and Malaysia showed that it was as low as 43.2–52.3% and 66.7% respectively, which could have resulted from the controlled use of aminoglycosides in such hospitals [14, 29]. Resistance to quinolone, like ciprofloxacin (90%), was in accordance to a study from Egypt which showed a resistance of 90% [25]. After the development of resistance to fluoroquinolones, carbapenems are often the drug of choice for infections resulting from Multi-drug resistant *A. baumannii* but unfortunately, carbapenem-resistant isolates are continuously increasing [11]. In the present study, the resistance to ertapenem, imipenem and meropenem were 92, 88 and 84% "respectively" which were in accordance to the findings from the studies in Egypt [22, 30] and other parts of the world [26, 31].

Carbapenemase production is known to be one of the main resistance mechanisms of *A. baumannii* to carbapenems [35]. Oxacillinases (Class D β-lactamases) are the most prevalent class in *A. baumannii* [36], and the genes encoding oxacillinases include *OXA-51* which is species-specific and is present commonly in all *A. baumannii* and the acquired *OXA-24*, *OXA-23*, *OXA-58*, and *OXA-143* [6, 8]. Worldwide spreading of MDR *A. baumannii* harboring OXA-type carbapenemase has progressively been reported [25].

OXA-51 is widely prevailing and is chromosomally located [37]. Many studies have pointed out that the identification of the *OXA-51* gene is a rapid and reliable method for identifying *A. baumannii* [34, 35]. This is in agreement with our study which showed that all our isolates harbored the *OXA-51* gene. *OXA-23* is the most frequent type of carbapenemase found among CRAB [30, 36, 37]. *OXA-23*-type carbapenemase-producing *A. baumannii* are becoming progressively widespread, with reports emerging from USA, Europe, Asia, Africa, Australia and the Middle East [1]. Outbreaks in Egypt [25], Spain [41], Italy [42], and the United States [43] were also described. In this study, most of our isolates were carbapenem resistant and the most prevalent gene in *A. baumannii* was found to be *OXA-23*, with a prevalence rate of 88%, which is similar to studies in Iran [44]

and Kuwait [45]. However, Fouad *et al.* detected *OXA-23* in all their collected *A. baumannii* isolates [33]. In contrast, a study which was carried out in Egypt and Saudi Arabia showed a decreased percentage for *OXA-23* gene carriage, 69% of their *A. baumannii* isolates [46]. Such variation in prevalence data suggests that various strains circulating in these countries have different frequencies in harboring the *OXA-23* gene.

In this study, 3% of the isolates carried *OXA-24* which is in agreement to a study reported by Zowawi *et al.* [47]. In other studies conducted in Egypt, Iran and Palestine *OXA-24* was present in 7.5, 11.8 and 14.5% of the isolates respectively [37, 48, 49]. In contrast, other studies carried out in China and Egypt reported that *OXA-24* was not detected in any of the tested isolates [50, 51]. However, 1% of our isolates carried *OXA-143* which is similar to that detected by Al Atrouni *et al.* [52]. In contrast, a study in Iran reported high prevalence of *OXA-143* gene carriage (56%) in tested isolates [48]. On the other hand, absence of *OXA-143* gene carriage was reported by Handal *et al.* [49]. To our knowledge this is the first report of the presence of *OXA-143* in Alexandria hospitals sounding an alarm to start to design strategies against the dissemination of MDR *A. baumannii* all over the country.

Moving on to *OXA-58,* which was not detected in any of the tested isolates, our results were similar to studies reported in Kuwait [45], Gulf Cooperation Council States [47] and China [50].

In conclusion, *OXA-24, OXA-143 and OXA-58* were present at low prevalence in this study suggesting that their role in conferring resistance to carbapenems among the tested isolates may be limited, in contrast to the increase in carbapenem resistance amongst *A. baumannii* isolates. Therefore, to determine the genetic diversity of *A. baumannii* isolates and explore its putative relationship with antibiotic resistance, RAPD-PCR is used to generate DNA fingerprints to distinguish between bacterial strains and has been frequently applied in molecular diagnosis in hospital infections as it has several advantages including high sensitivity, specificity and ease of performance [50, 51].

On the basis of RAPD profiles in agarose gels in this study, the number of bands ranged from 1 to 5 ranging in size from 300–1500 bp (Fig. 2). It indicated a high genetic diversity (A-S) among *A. baumannii* isolates

from Alexandria, Egypt. Some clonally related groups (A and B) were observed in most of the isolate sources which represent the dissemination of these clones in the hospitals (Fig. 5). Therefore, spreading of these two genotypes into different isolate sources also confirms their widespread distribution.

Moreover, in a study conducted in China in which 47 sensitive strains and 80 MDR strains were isolated, seventeen genotypes (A–Q) were obtained. Genotype E was the predominant type in MDR strains (46/80) mainly derived from the ICU. The carriage rates of resistant genes revealed that *OXA-23* carrying strains showed a resistance rate of 82.5% (66/80) and to a lower extent the presence of *OXA-24* gene [54].

Similar to our study, RAPD genotyping carried out in Iran and Bulgaria revealed that most of the isolates belong to two main clones [28, 29]. Despite all these studies, more comprehensive studies are needed to prevent hospital-acquired infections via determining the sources of bacteria.

Having several clusters with predominance of *OXA-23* gene may suggest its horizontal transmission between the different isolates, as *OXA-23* has been proven to be plasmid-encoded [55], thus propagating antibiotic resistance and further complicating treatment options.

RAPD-PCR provided us with a powerful tool for identifying and epidemiologically typing our strains. Studying the epidemiology of MDR *A. baumannii* strains is crucial to avoid the clonal dissemination of antibiotic-resistant strains. Determining the genetic relation between clinical isolates shows the importance of surveillance programs, antimicrobial stewardship and strict infection control procedures that would affect health policy and decision-making. These are necessary to decrease the worldwide spread of carbapenem resistant *A. baumannii*.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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