Diversity in *Acinetobacter baumannii* isolates from paediatric cancer patients in Egypt

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Abstract

Acinetobacter baumannii is an important nosocomial pathogen, commonly causing infections in immunocompromised patients. It is increasingly reported as a multidrug-resistant organism, which is alarming because of its capability to resist all available classes of antibiotics including carbapenems. The aim of this study was to examine the genetic and epidemiological diversity of *A. baumannii* isolates from paediatric cancer patients in Egypt, by sequencing the intrinsic $bla_{OXA-51-like}$ gene, genotyping by pulsed-field gel electrophoresis and multi-locus sequence typing in addition to identifying the carbapenem-resistance mechanism. Results showed a large diversity within the isolates, with eight different $bla_{OXA-51-like}$ genes, seven novel sequence types and only 28% similarity by pulsed-field gel electrophoresis. All three acquired class-D carbapenemases (OXA-23, OXA-40 and OXA-58) were also identified among these strains correlating with resistance to carbapenems. In addition, we report the first identification of IS*Aba2* upstream of $bla_{OXA-51-like}$ contributing to high-level carbapenem resistance. This indicates the presence of several clones of *A. baumannii* in the hospitals and illustrates the large genetic and epidemiological diversity found in Egyptian strains.

Keywords: *Acinetobacter baumannii*, $bla_{OXA-51-like}$, carbapenem-hydrolysing class D β-lactamase, diversity, insertion sequences, IS*Aba2*., resistance

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Introduction

Acinetobacter baumannii has emerged as an important nosocomial pathogen in the past decade, which in recent years has developed into a multidrug-resistant problematic pathogen [1]. *Acinetobacter baumannii* is an opportunistic pathogen, frequently isolated from immunocompromised patients with prolonged hospitalization [2]. As a consequence of immunoablative treatment, patients with cancer are at risk of developing *A. baumannii* infections, including sepsis, respiratory, wound and tissue infections, in addition to urinary tract infections [2,3].

A major concern in *A. baumannii* is its worldwide clonal expansion and its ability to survive and disseminate in hospitals, with numerous outbreaks reported from different regions of the world [4]. *Acinetobacter baumannii* is notably resistant to extreme environmental conditions, such as dryness, and can survive on surfaces for a long time, hence facilitating its spread [1,4].

Resistance to carbapenems, the β-lactam drugs of last resort in treating *A baumannii* infections, has been attributed to the expression of carbapenem-hydrolysing oxacillinase genes, bla_{OXA23}, bla_{OXA-40} and bla_{OXA58}, which are usually plasmid encoded [5,6]. The ubiquitous, chromosomally encoded $bla_{OXA-51-like}$ gene only confers resistance when an Insertion Sequence (IS) is present upstream of the gene [7].

Due to the prevalence of *A. baumannii* across the world, suitable typing methods to investigate the epidemiological distribution of the organism have been developed such as ribotyping, amplified fragment length polymorphisms, pulsed-field gel electrophoresis (PFGE) and, more recently,

Multi-Locus Sequence Typing (MLST) [8]. Additionally, amplification and sequencing of the ubiquitous $bla_{\text{OXA-51-like}}$ gene has also been used to determine clonal groups from diverse worldwide sources [7,8].

Limited data were available concerning the epidemiological distribution of *A. baumannii* in the Middle East but, in the past few years, reports of strains in the United Arab Emirates, Iraq, Kuwait and Egypt harbouring diverse resistance mechanisms have emerged [9–12]. The aim of this study was to investigate the epidemiological and molecular diversity of *A. baumannii* strains isolated from two cancer centres in Cairo, Egypt.

Materials and Methods

Isolate identification

Thirty-four non-duplicate *A. baumannii* were obtained from two centres; The Children's Cancer Hospital (CCH) and The National Cancer Institute (NCI), both located in Cairo, Egypt, from 2010 to 2011. Initial identification and susceptibility testing was done using VITEK and Phoenix automated machines. Genotypic identification was carried out by restriction analysis of 16s-23s rRNA spacer sequences using *Alu*I and *Nde*II [13].

Detection of $bla_{\text{OXA-51-like}}$ genes

The intrinsic $bla_{\text{OXA-51-like}}$ genes were amplified for *A. baumannii* isolates using primers: OXA69A and B [7]. Products were purified using a QIAquick PCR Purification Kit (Qiagen, Crawley, UK) and sequenced in both directions on a 3730 DNA Analyzer (Applied Biosystems, Warrington, UK). For isolates yielding a larger product size, a PCR was performed to screen for the associated upstream environment using primers FxOxa-F and FxOxa-R [14].

Detection of class D oxacillinases and genetic environment

Isolates were screened for the presence of acquired OXA carbapenemases by Multiplex PCR, as previously described [15]. Isolates positive for the individual OXA groups were subsequently amplified and sequenced using primers for the full sequence of the genes. Associated genetic environment was also amplified and sequenced. Primers used are listed in Table 1.

Minimum inhibitory concentrations

The MIC of imipenem and meropenem were determined using an agar double dilution technique according to the British Society of Antimicrobial Chemotherapy (BSAC) guidelines [16]. *Pseudomonas aeruginosa* NCTC 10662, *Escherichia coli* NCTC 10418 and *Staphylococcus aureus* NCTC 6571 were used as control strains.

Pulsed-field gel electrophoresis

All isolates were typed by PFGE according to the procedure previously described by Seifert *et al.* [17]. Briefly, plugs were incubated in 30 U *Apa*I at 37° overnight, and subsequently run on 1% pulsed-field-certified agarose gel (Bio-Rad, Hertfordshire, UK) in 0.5 × TBE buffer with an initial pulse of 5 s and a final pulse of 20 s for 20 h. The gels were stained with Gel-Red solution and visualized using the DIVERSITY DATABASE (Bio-Rad) software image-capturing system.

Multi-locus sequence typing

The PCR for the seven housekeeping genes: *gltA*, *gyrB*, *gdhB*, *rpoD*, *recA*, *gpi* and *cpn60* was performed according to the scheme developed by Bartual *et al.* [18]. Products were purified and sequenced as described above. MLST was performed for ten isolates, representatives of the $bla_{\text{OXA-51-like}}$ gene variants identified. If isolates from different hospitals harboured similar $bla_{\text{OXA-51-like}}$ genes, an isolate from each hospital was selected randomly for comparison. Isolates chosen for MLST were: 8357, 9925-SAM, 1780, 634, 21174, 22055, 161, P38-YSF, P67-AZ and 14611.

TABLE 1. List of primers used in this study

Primer name	Sequence 5′–3′	Use	Reference
16s-23s rRNA F	TTGTACACACCGCCCGTCA	Identification	[13]
16s-23s rRNA R	GGTACTTAGATGTTTCAGTTC		
Oxa69-A	CTAATAATTGATCTACTCAAG	$bla_{\text{OXA-51-like}}$ amplification and sequencing	[7]
Oxa69-B	CCAGTGGATGGATGGATAGATTATC		
FxOxaF	GATACCAGACCTGGCAACAT	Upstream environment of $bla_{\text{OXA-51-like}}$ gene	[14]
FxOxaR	GCACGAGCAAGATCATTACC		
$bla_{\text{OXA-23}}$ F	GATGTGTCATAGTATTCGTCG	Whole gene-sequence of bla_{OXA23}	[25]
$bla_{\text{OXA-23}}$ R	TCACAACAACTAAAAGCACTG		
ISAba1A	GTGCTTTGCGCTCATCATGC	Upstream environment of bla_{OXA23}	[26]
SM2	AAGTGTCTATATTCTCACC	Upstream environment of bla_{OXA58}	
ISAba3-F	CAATCAAATGTCCAACCTGC	Upstream environment of bla_{OXA58}	
OXA-58A	CGATCAGAATGTTCAAGCGC	Whole gene sequence of bla_{OXA58}	[22]
OXA-58B	ACGATTCTCCCCTCTGCGC		
OXA-24FF	ATGAAAAAATTTATACTTCCTA TATTCAGC	Whole gene sequence of bla_{OXA24}	[27]
OXA-24RR	TTAAATGATTCCAAGATTTTCTAGC		

Results

Diversity of *bla*$_{OXA-51-like}$ genes

All isolates were confirmed as *A. baumannii*, and sequencing of the intrinsic *bla*$_{OXA-51-like}$ revealed the presence of eight different genes: *bla*$_{OXA-64}$, *bla*$_{OXA-65}$, *bla*$_{OXA-66}$, *bla*$_{OXA-69}$, *bla*$_{OXA-71}$, *bla*$_{OXA-78}$, *bla*$_{OXA-94}$ and *bla*$_{OXA-89}$ (Table 2). *bla*$_{OXA-65}$ was the most prevalent, found in 14 isolates, obtained from both hospitals. *bla*$_{OXA-64}$ is now commonly found in the Middle East (A. Al Hasan, and S.G.B. Amyes, unpublished results; [9]), it was found in seven isolates obtained from both hospitals. There were representatives from the three worldwide clones (formally known as the European clones). *bla*$_{OXA-66}$ was found in four isolates, three of which were from CCH. *bla*$_{OXA-69}$ was identified in two isolates at the intensive care unit (ICU) of CCH and were part of an *A. baumanniii* outbreak in early 2011. *bla*$_{OXA-71}$ was found in two isolates from different hospitals. *bla*$_{OXA-78}$ and *bla*$_{OXA-89}$ were both found in strains from CCH, whereas *bla*$_{OXA-94}$ was from two isolates from NCI, recovered from the same floor, 1 day apart.

Insertion sequences associated with *bla*$_{OXA-51-like}$

Sequencing upstream of the *bla*$_{OXA-51-like}$ gene, *bla*$_{OXA-89}$ in isolate 22055 revealed the presence of IS*Aba2*, with the -35 (ttatat) and -10 (ttgtaggat) promoters 29 bp apart, and located 102 bp and 82 bp upstream of *bla*$_{OXA-89}$, respectively. No other insertion sequences were identified upstream of the *bla*$_{OXA-51-like}$ genes.

PFGE

The PFGE analysis revealed a large diversity within the strains. Some isolates with similar *bla*$_{OXA-51-like}$ genes had very distinct PFGE patterns, suggesting no epidemiological similarity between the strains. As seen in Figure 1, only six isolates harbouring *bla*$_{OXA-65}$ show > 80% similarity in their PFGE pattern. Additionally, *bla*$_{OXA-64}$ isolates all shared less than 80% similarity. Even isolates with *bla*$_{OXA-94}$, which were collected from patients on the same floor of the same hospital 1 day apart, had distinct PFGE patterns. On the other hand, the *bla*$_{OXA-71}$ containing isolates, although from different hospitals, had similar PFGE patterns. The similarity for all the isolates was calculated by Dice coefficient to be 28.7%.

TABLE 2. Isolates harbouring *bla*$_{OXA-51-like}$ genes, with isolation details. carbapenem-hydrolysing class D β-lactamase (CHDL) genes, minimum inhibitory concentration (MIC) and sequence type. Isolates in bold were in the *A. baumannii* outbreak in early 2011

Isolation details					CHDL β-lactamase gene				MIC (mg/L)		
Isolate no.	Date of sample	Hospital	Location	Site of isolate	*bla*$_{OXA-51-like}$	*bla*$_{OXA-23}$	*bla*$_{OXA-58}$	*bla*$_{OXA-40}$	IMI	MER	Sequence type
7947	17/05/2010	CCH	ICU	Wound	64				0.25	0.06	
12435	23/07/2010	CCH	ICU	Blood	64	+			16	32	
14298	22/08/2010	CCH	ICU	Catheter tip	64		+		8	2	
8357	5/29/2011	CCH	DSCH	CVP Blood	64				8	16	ST408
4248	15/03/2010	CCH	IP-5C	CVP tip	65	+			8	4	
4842	23/03/2010	CCH	ICU	CVP tip	65	+			64	32	
9930	15/06/2010	CCH	ICU	Blood and CVP	65	+			32	16	
10262	19/06/2010	CCH	ICU	CVP	65	+			64	128	
15094	05/09/2010	CCH	IP-4B	Urine	65				0.25	0.12	
15324	09/09/2010	CCH	IP-3A	Catheter tip	65				0.06	0.06	
1780	31/01/2011	CCH	ICU	Stool	65	+			8	16	ST410
1750	31/01/2011	CCH	ICU	BAL	65	+			32	8	
2106	08/02/2011	CCH	ICU	Blood	65	+			8	8	
2632	20/02/2011	CCH	IP-5A	Stool	65	+			8	8	
2625	20/02/2011	CCH	IP-4C	Urine	65	+	+		16	16	
8768	6/4/2011	CCH	IP-4B	Blood	65				0.008	0.03	
4343	17/03/2010	CCH	IP-3B	CVP-Blood	66				8	16	
14611	27/08/2010	CCH	ICU	CVP Blood	66			+	64	32	ST208
21382	13/12/2010	CCH	ICU	CVP Culture	66	+			16	4	
7052	5/7/2011	CCH	ICU	BAL	66	+			8	16	
634	11/01/2011	CCH	ICU	Catheter tip	69	+			8	4	ST108
1447	24/01/2011	CCH	ICU	CVP Culture	69	+			0.5	0.06	
161	03/01/2011	CCH	PULM	Sputum	71				0.06	0.06	ST414
21174	09/12/2010	CCH	IP-3A	Blood	78				0.03	0.25	ST412
22055	25/12/2010	CCH	IP-3C	CVP Blood	89				128	128	ST413
679-BAS	04/09/2010	NCI	5th floor	Ear swab	64		+		8	8	
P67-AZ	09/01/2011	NCI	OP	Blood	64	+	+		64	16	ST411
9925-SAM	15/12/2010	NCI	7th floor	Blood	64	+			16	8	ST409
P391-AH	14/09/2010	NCI	5th floor	Blood	65				8	16	
461-SF	15/12/2010	NCI	7th floor	Blood	65	+			16	32	
6332-ABD	02/09/2010	NCI	5th floor	Ear swab	69	+			16	8	
5687-SHAY	11/10/2010	NCI	5th floor	Blood	71				0.25	0.25	
P38-YSF	04/01/2011	NCI	5th floor	Blood	94	+			64	64	ST331
P49-HAM	05/01/2011	NCI	5th floor	Blood	94		+		8	8	

BAL, bronchoalveolar lavage; CVP, central venous port; IMI, imipenem; MER, meropenem.

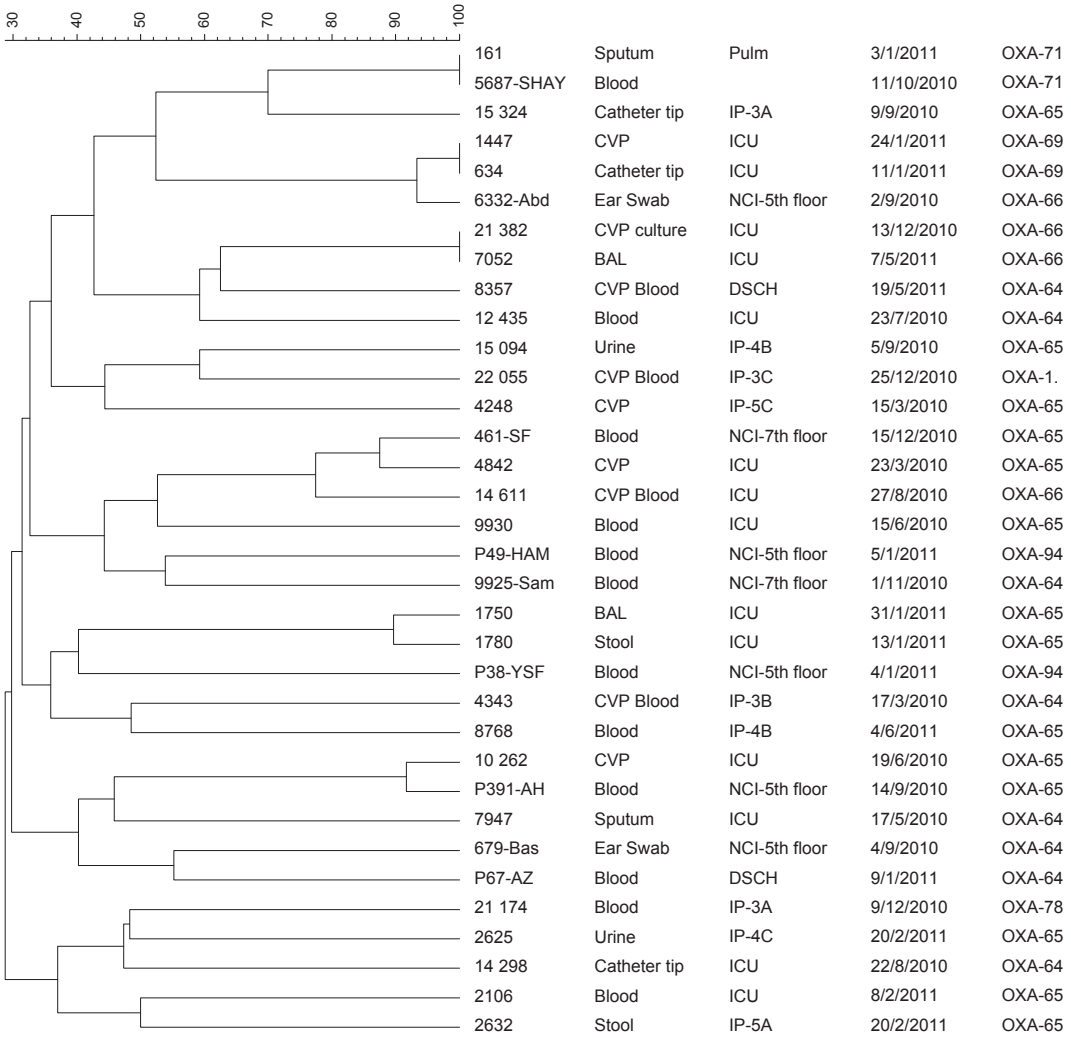

FIG. 1. Pulsed field gel electrophoresis profile for *Acinetobacter baumannii* strains in this study, showing the associated isolation site, location, date, and $bla_{OXA51\text{-like}}$ genes.

MLST

Seven housekeeping genes were amplified and sequenced as described above for ten isolates. Ten distinct sequence types (STs) were identified, seven of which are novel and assigned ST408–ST414. The remaining three STs were identified as ST331, ST108 and ST208. Typing by MLST further illustrated the large diversity found within the strains, as isolates with similar $bla_{OXA\text{-}51\text{-like}}$ genes had different STs. This is clear for isolates 9925-SAM and NCI-P67, both were from the NCI and possessed $bla_{OXA\text{-}64}$, but they belonged to different STs: 409 and 411, respectively. When compared with another $bla_{OXA\text{-}64}$-positive isolate, 8357, which was from a patient at CCH, another ST was identified, ST408.

MIC and carbapenem-hydrolysing class D β-lactamase (CHDL) genes

The majority of isolates ($n = 25$), representing 73%, were resistant to imipenem and/or meropenem (MIC ≥ 8 mg/L). This resistance could be correlated with the presence of the acquired class-D oxacillinases: $bla_{OXA\text{-}23}$, $bla_{OXA\text{-}58}$ and $bla_{OXA\text{-}40}$ (Table 2).

Genes encoding all three transferable OXA types associated with resistance were identified in these strains: $bla_{OXA\text{-}23}$ in 18 isolates, $bla_{OXA\text{-}58}$ in five isolates and $bla_{OXA\text{-}40}$ in one isolate. All isolates, except one, possessing $bla_{OXA\text{-}23}$ were resistant to imipenem and meropenem (MIC ≥ 8 mg/L). IS*Aba1* was detected upstream of $bla_{OXA\text{-}23}$ in the resistant isolates, hence providing a promoter for the expression of the gene (Figure 2). However, this IS element was not found upstream in the $bla_{OXA\text{-}23}$-containing isolate that was carbapenem sensitive. The analysis of the *A. baumannii* outbreak in the ICU at CCH in early 2011 revealed that although the strains harboured distinct $bla_{OXA\text{-}51\text{-like}}$ types and were epidemiologically different, they all possessed $bla_{OXA\text{-}23}$ as the resistance mechanism.

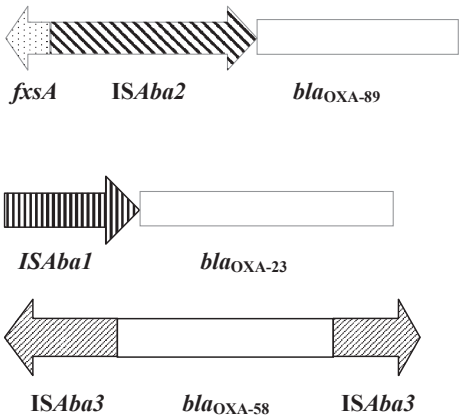

FIG. 2. Schematic representation showing examples of the genetic environments of bla_{OXA-89}, bla_{OXA-23} and bla_{OXA-58}.

bla_{OXA-58}-positive isolates were also found in both hospitals and all were resistant to meropenem and imipenem, with the exception of isolate 14298, which was intermediate to meropenem (MIC 4 mg/L). The genetic environment of the bla_{OXA-58} showed that the gene was flanked by two copies of IS*Aba3* (Figure 2). Two isolates harboured an interrupted sequence of IS*Aba3* upstream of the bla_{OXA-58} gene (L. Al-Hassan, H. El Mehallawy and S. G. B. Amyes, unpublished results).

A single isolate, 14611 from CCH, was positive for bla_{OXA-40} and it was also resistant to carbapenems. No insertion element was detected upstream of the bla_{OXA-40} gene.

Eight of the 11 isolates that did not harbour acquired carbapenemase genes were sensitive to carbapenems (MIC <8 mg/L). One isolate, 22055, lacking these genes was resistant to carbapenems and harboured the chromosomal OXA-89 β-lactamase. IS*Aba2* was found upstream of the bla_{OXA-89} gene (Figure 2).

Discussion

Acinetobacter baumannii is a problematic, multidrug-resistant pathogen identified in healthcare environments worldwide [1]. The remarkable ability of *A. baumannii* to capture and express resistance genes has allowed it to become one of the major threats in hospitals, as it becomes resistant to all available antibiotics, including carbapenems [4]. Resistance mechanisms such as modification of target site, efflux pumps and enzymatic inactivation have all been reported in *A. baumannii* [1]. Of major concern is the presence of several classes of β-lactamases within the *A. baumannii* genome. The localization of these resistance genes on plasmids facilitates their movement from one bacterium to another [5]. Class D oxacillinase genes: bla_{OXA-23}, bla_{OXA-40} and bla_{OXA-58} have been repeatedly reported in *A. baumannii* outbreaks from different parts of the world [1,19].

The construction of a linkage map based on the intrinsic OXA-51-like β-lactamases was reported by Evans *et al.* [7]. The sequence relationship was determined for 37 distinct members of the OXA-51-like β-lactamase family. This study identified three large groups around OXA-66, OXA-69 and OXA-98 in addition to other unrelated branched enzymes [7]. In the current study a large diversity was found in the sequences of $bla_{OXA-51-like}$ with eight different gene variants identified. This is particularly interesting given the short duration of isolate collection (1 year) as well as the isolates deriving from only two hospitals. In fact seven different $bla_{OXA-51-like}$ genes were identified in CCH alone. When looking at the distribution of $bla_{OXA-51-like}$ genes in the linkage map, it is clear that they have different origins as the genes identified are not clustered in closely related groups. Fourteen isolates, accounting for 41%, harboured bla_{OXA-65}, which according to the linkage map forms a 'central hub' from which all other groups radiate and is thought to be ancestral to all $bla_{OXA-51-like}$ genes [7]. This subsequently indicates the presence of the potential ancestral $bla_{OXA-51-like}$ gene in *A. baumannii* in Egypt, which is in the current collection of strains and is the major gene identified. Additionally, this may explain that the large diversity found is an outcome of the evolution of the ancestral bla_{OXA-65} gene in some cases, rather than the of 'foreign carriage' of clones into the country.

bla_{OXA-69}, bla_{OXA-66} and bla_{OXA-71} have been associated with Worldwide [European] Clones I, II and III, respectively, and all have been identified in the current study [6,7]. bla_{OXA-66} and bla_{OXA-71} genes were identified in both hospitals, which may indicate local distribution in Egyptian hospitals. bla_{OXA-69}, on the other hand, was found in two isolates in the ICU outbreak in early 2011 at CCH only. This illustrates the extent of spread of the major lineages of *A. baumannii*.

bla_{OXA-89} is a member of the bla_{OXA-98} cluster and contains the resultant protein showing three amino acid substitutions from OXA-98. In the current study, one isolate from CCH was found positive for bla_{OXA-89}, and harboured IS*Aba2* upstream. The presence of an insertion sequence upstream of other $bla_{OXA-51-like}$ genes has been reported to enhance the expression and cause resistance to carbapenems [20, 21]. IS*Aba2* has only been reported upstream of bla_{OXA58} [22]. With no other resistance mechanism identified, the presence of IS*Aba2* was responsible for high-level resistance to both imipenem and meropenem (MIC 128 mg/L and 256 mg/L, respectively). Furthermore, this shows the ability of IS to insert upstream of these genes and act as promoters.

bla_{OXA} genes that are not part of previously identified clusters have also been identified in the current study: bla_{OXA-94} in two

isolates from the NCI and bla_{OXA-64} in eight isolates from both hospitals. OXA-64 is closely related to OXA-71 and is now commonly found in the Middle East [7, 9] (A. Al-Hasan and S.G.B. Amyes, unpublished results). bla_{OXA-94}, on the other hand, forms a branch of bla_{OXA-65} cluster with three amino acid substitutions in the resultant protein.

As expected from this large diversity of isolates, there is considerable variation in their PFGE profiles. Notably, isolates harbouring similar $bla_{OXA-51-like}$ genes have different PFGE profiles and no epidemiological linkage can be inferred. This could be a result of the localization of the patients in different wards and at different times in the hospital. Even for isolates recovered from the ICU at different times, there seems to be significant variability in profiles suggesting the presence of different clones within the same hospital. Turton *et al.* found a correlation between PFGE and sequence typing, in contrast to Evans *et al.* who later noted major differences between PFGE typing and sequence typing in their study [7,23].

MLST further illustrated the diversity within the isolates as eight out of ten isolates typed were assigned to novel STs. Previous reports have shown that typing with $bla_{OXA-51-like}$ was more consistent with MLST than with PFGE [8]. In the current study, isolates 8357, P67-AZ and 9925-SAM had similar $bla_{OXA-51-like}$ genes but, when they were typed with MLST, they showed three different novel STs, 408, 409 and 411, respectively. The PFGE patterns were also different for these isolates. This could indicate the presence of three distinct clones in the two hospitals, especially that they were isolated in different months and in different wards. MLST, in this case, correlated with the epidemiological data of PFGE. Hamouda *et al.* [8] found MLST to be more accurate than PFGE when studying isolates on a global scale.

Seventy-three percent of the isolates were resistant to carbapenems, and this is associated with all three CHDL genes found in this study. Different genetic structures are associated with the upstream environment of bla_{OXA-58} and bla_{OXA-23} and they have been identified in different regions of the world [22,24]. In the current study, bla_{OXA-23} is associated with IS*Aba1* in the upstream environment and bla_{OXA-58} is flanked by IS*Aba3*. The effective mobilization of these genes by insertion sequences upstream together with the localization on plasmid largely contribute the spread of these resistance genes [4].

In conclusion, the data presented show the large diversity of *A. baumannii* isolated from two centres in Cairo, Egypt. The genetic plasticity of *A. baumannii* is represented by the presence of several insertion sequences upstream of the resistance genes, thereby facilitating the expression and causing resistance to carbapenems. Several clones seem to be present in Egyptian hospitals requiring increased awareness of the healthcare personnel and stricter infection control policies to prevent the dissemination of these isolates.

Nucleotide Sequence Accession Number

The IS*Aba2*-bla_{OXA-89} sequence of strain 22055 has been deposited under the accession number JX499236.

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Transparency Declaration

None to declare.

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