

Molecular Characterization of Carbapenem/Colistin-Resistant *Acinetobacter baumannii* Clinical Isolates from Egypt by Whole-Genome Sequencing

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Purpose: The rise of carbapenem-resistant *A. baumannii* (CRAB) is considered a public health problem limiting the treatment options. Our current work studied the emergence and mechanisms of colistin-resistance among CRAB isolates in Egypt.

Materials and Methods: Seventeen clinically recovered *A. baumannii* were identified and screened for their antimicrobial susceptibilities using VITEK-2 system. Colistin susceptibility was evaluated using broth microdilution, and characterization of carbapenem/colistin resistance determinants was performed using whole-genome sequencing (Illumina MiSeq).

Results: About 52.9% (9/17) were colistin-resistant. PCR results revealed that all isolates carried *bla*_{OXA-51-like} genes.

*bla*_{OXA-23-like} was detected in 82.3% (14/17) and *bla*_{NDM} in 23.5% (4/17). Two isolates harboured *bla*_{GES-35} and *bla*_{OXA-23}. Furthermore, genome analysis of seven isolates revealed six belonged to international clone 2 (IC2) while the remaining isolate was a singleton (ST158), representing a clone circulating in Mediterranean/Middle Eastern countries.

Conclusion: The emergence and high incidence of colistin-resistance among CRAB clinical isolates in Egypt are alarming because it further limits therapy options and requires prudent antimicrobial stewardship and stringent infection control measures. Whole-genome sequence analyses suggest that the resistance to colistin was associated with multiple mutations in the *pmrCAB* genes. The high incidence of the high-risk lineage IC2 harbouring *bla*_{OXA-23-like} as well as *bla*_{NDM} is also of concern.

Keywords: colistin resistance, *pmrCAB*, cgMLST, ST158, WGS

Introduction

Recently carbapenem-resistant *Acinetobacter baumannii* (CRAB) was placed on top of the list of priority pathogens for research and development of novel antibiotics.¹ Multidrug-resistant (MDR) *A. baumannii* infections are a burden on healthcare facilities owing to limited antibiotic treatment options.^{2,3} Carbapenem hydrolysing class D beta-lactamases, also known as oxacillinases (OXA), are the main mechanism of resistance to carbapenems in CRAB. The OXA carbapenemases found in *A. baumannii* are divided into six phylogenetic subgroups: the intrinsic OXA-51-like and the acquired OXA-23-like, OXA-24/40-like, OXA-58-like which are the most common, and the less common OXA-143-like and OXA-235-like.^{4,5}

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To treat CRAB, colistin is prescribed as the last resort. However, resistance towards colistin among *A. baumannii* has become a serious problem as there are no alternative available therapeutic options.⁶ There are now reports from different regions of the world of pan-drug-resistant (PDR) *A. baumannii*.⁷

The primary resistance mechanism is mediated through the modification of the lipid A moiety of lipopolysaccharide (LPS) through the addition of phosphoethanolamine (PEtN) through overexpression and/or mutations in the two-component regulatory system PmrA and PmrB. Other mechanisms have been described and include the loss of LPS caused by either insertional inactivation of lipid A biosynthesis genes or mutations, but this appears to be limited to in-vitro studies.⁸ *A. baumannii* requires discrete genetic events to develop an adequate colistin resistance level, which are up-regulation of *pmrAB*, point mutation in *pmrB*, as well as expression of *pmrC*, leading to addition of PEtN to lipid A.⁹ Also *vacJ*, *pldA*, *tig2C*, *pheS* mutations and a conserved hypothetical protein were reported to be involved in colistin resistance.¹⁰ Four putative colistin-resistant genes: A1S_1983, *hepA*, A1S_3026, and *rsfS* were also identified *A. baumannii* ATCC 17978.¹¹ Recently, plasmid-encoded resistance to polymyxin, mediated through the *mcr* genes (*mcr-1* to *mcr-8*) were described mainly in Enterobacteriaceae,^{12,13} and several *mcr* variants were recently found in *Acinetobacter spp.*, *mcr-1*^{14,15} and *mcr-4.3*.^{15,16}

The occurrence of carbapenem and colistin resistance was previously reported in Egypt among MDR *A. baumannii*.^{17,18} But currently, there is little or no data regarding colistin-resistant *A. baumannii* in Egypt. Therefore, the current work conducted to study colistin resistance emergence and mechanisms among CRAB clinical isolates in Egypt using genetic methods including whole-genome sequencing (WGS).

Materials and Methods

Bacterial Strains

Seventeen CRAB clinical strains (non-duplicate) collected from Theodor Bilharz Research Institute (TBRI) hospital during the period from 2015 to 2016 were re-used in the current work. Previously, CRAB were identified by API 20E and VITEK-2 compact system and susceptibility to different antimicrobials was performed using VITEK-2 compact system card N 222 (bioMérieux, France).^{19,20} Isolates were confirmed as *A. baumannii* by *gyrB* multiplex

PCR and presence of *bla*_{OXA-51-like} as previously described.²¹

Colistin Susceptibility Testing

Colistin susceptibility was previously evaluated by the recommended broth microdilution (BMD) method which was performed according to the CLSI procedures²² using colistin sulphate antibiotic powder (LKT Laboratories, USA).¹⁷ Isolates were considered susceptible (S) if MIC is ≤ 2 $\mu\text{g/mL}$, and resistant (R) if ≥ 4 $\mu\text{g mL}^{-1}$ according to CLSI breakpoints.²³

PCR Amplification for Carbapenem Resistance Genes

Crude cell lysates prepared from boiling overnight cultures were used for PCR template. Detection of carbapenemase-encoding genes (*bla*_{OXA-51-like}, *bla*_{OXA-58-like}, *bla*_{NDM-1-like}, *bla*_{GES-like}, *bla*_{OXA-23-like}, *bla*_{OXA-40-like}) was previously performed by PCR.^{18,24}

Whole-Genome Sequencing (WGS)

Seven colistin-resistant isolates were available for WGS. Using the QIAamp DNA Mini Kit (Qiagen, Germany), extraction of bacterial DNA from freshly sub-cultured colonies was done according to the manufacturer's instructions, and the DNA concentration was measured using a Denovix Fluorometer (Denovix, USA). The genomic DNA was stored at -20°C . A volume of 1ng total of bacterial DNA was used in the library preparation. Sequencing libraries were prepared using the Nextera XT library prep kit (Illumina, San Diego, CA, USA) for a 300bp paired-end sequencing run on an Illumina MiSeq sequencer.

The program Velvet as part of the SeqSphere v6.0.2 software (Ridom GmbH, Münster, Germany) was used for de novo assembly, core genome MLST (cgMLST), and to determine the clonal lineage as described previously.²⁵ To determine differences in specific ORFs in each draft genome sequence, the genome assemblies were aligned to the *A. baumannii* reference strain ACICU, which is colistin-susceptible and belongs to the most widespread clonal lineage IC2 involved in outbreaks worldwide. Resistome analysis including detection of colistin resistance determinants (eg, *mcr-4.3*) was carried out using ResFinder (<https://cge.cbs.dtu.dk/services/ResFinder/>), as well as by performing a BLAST search using the Reference Gene Catalog listings of *mcr* genes as queries (NCBI BioProject PRJNA313047). Beta-lactamases were identified using the online beta-lactamase

database (<http://www.bldb.eu/>). Raw reads generated were submitted to the Sequencing Read Archive (<https://www.ncbi.nlm.nih.gov/sra/>) of the National Center for Biotechnology Information (NCBI) under the SRA accession number SRP131448 and BioProject PRJNA431710.

Results

Bacterial Profile and Colistin Susceptibility Testing

The study investigated 17 CRAB strains that were isolated from respiratory samples (n=7; 41.18%), pus (n=5, 29.42%) and urine (n=4, 23.53%), and central venous catheter (n=1; 5.88%). The ICU was the main source of isolates with an isolation rate of 6/17 (35.29%). The distribution of *A. baumannii* isolates according to gender showed that 9 (52.94%) of the strains were from males and 8 (47%) were from females with no significant difference ($p > 0.05$). According to CLSI breakpoints, nine of 17 isolates (52.9%) were defined as colistin resistant. By PCR, in addition to the intrinsic *bla*_{OXA-51-like} that was present in all isolates, *bla*_{OXA-23-like} was the most common carbapenem resistance determinant detected (14/17; 82.3%), followed by *bla*_{NDM-like} that was found in four isolates (4/17; 23.5%); one isolate harboured both *bla*_{OXA-23-like} and *bla*_{NDM-like}. Two isolates harboured *bla*_{GES-like} and *bla*_{OXA-23-like}. Neither *bla*_{OXA-58-like}, nor *bla*_{OXA-40-like} were identified in any isolates.

Whole-Genome Sequencing

Out of seven colistin-resistant isolates that were sequenced, five were ST2 using the Pasteur MLST scheme and the remaining two isolates, R14 and R41, were ST158 and ST570, respectively. cgMLST analysis showed that two of the ST2 isolates were identical, with the remaining ST2 isolates differing by 64–296 alleles (Figure 1) suggesting that colistin resistance is not wholly the result of transmission events. The ST570 strain, a single locus variant (SLV) of ST2, had almost 700 alleles different from its nearest ST2 strains, but is still considered to be an IC2 isolate (Figure 1). The ST158 isolate differed in >2666 alleles from the IC2 isolates in this study. cgMLST analysis against our in-house database of *A. baumannii* revealed that R14 had 72 and 86 allelic differences from two isolates, collected from Egypt and Republic of Ireland, respectively, and is considered to be a singleton (data not shown).

Resistance genes analysis revealed that all the tested strains harbored multiple antibiotic resistance genes

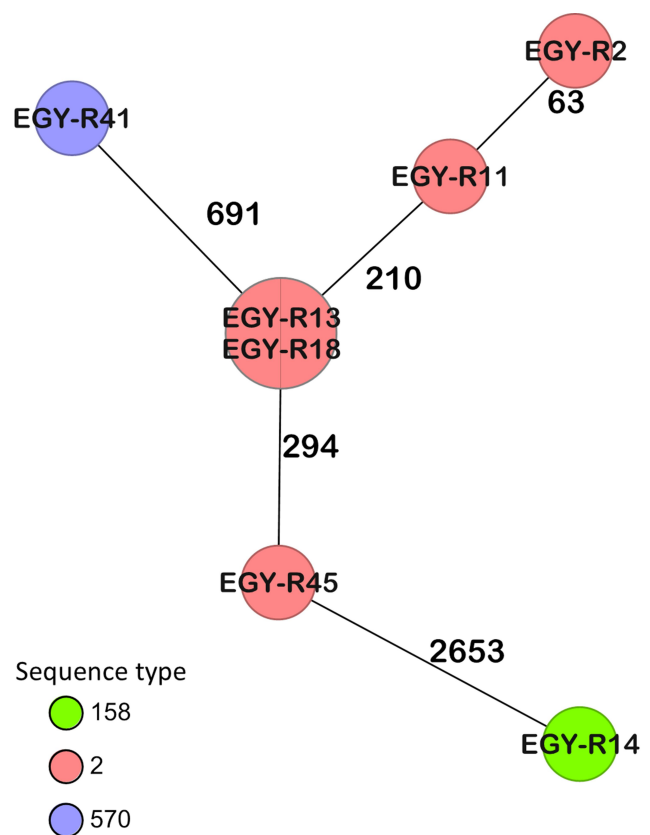


Figure 1 Minimum spanning tree generated by cgMLST coloured by ST (Pasteur) based on 3487 targets. Numbers between the nodes indicate the number of alleles that are different.

against most of the antibiotic groups (Table 1), particularly aminoglycoside modifying enzymes. Of note, all the IC2 isolates had *bla*_{OXA-66}, the IC2 variant of the intrinsic *bla*_{OXA-51-like} gene, while R14, possessed *bla*_{OXA-65}. Furthermore, 5 variants of the intrinsic cephalosporinase (ADC) were found (Table 1). Other beta-lactamases found in the isolates included *bla*_{TEM-1}, *bla*_{NDM-1}, and *bla*_{GES-35}.

Published data suggest that multiple mutations in *pmrCAB* genes cause reduction of colistin susceptibility. We therefore investigated the *pmrCAB* operon by comparing the tested strains with the reference one (*A. baumannii* ACICU). Several mutations leading to amino acid substitutions were detected in *pmrCAB*. All IC2 isolates had the same *pmrA* gene sequence as ACICU, while R14 had an Ile18-Thr substitution. In *pmrB*, four isolates were identical to ACICU, while isolates R11 and R2 show an Ala-138-Thr substitution, and R14 has a Val444-Ala substitution (amino acid adjacent to the stop codon). The six IC2 isolates had an identical *pmrC*, however, this differed from the ACICU *pmrC* with an Ala354-Ser substitution. Isolate R14 had several substitutions when compared to ACICU, Ile42-Val, Ile115-Val, Leu150-Phe, and Thr515-Lys (Table 2).

Table 1 Resistomes in Seven Colistin-Resistant *A. Baumannii* Isolates by WGS

Isolate	Beta-Lactam	Aminoglycoside	Trimethoprim	Sulphonamide	Tetracycline	Macrolide	Phenicol	Fluoroquinolone/ Aminoglycoside
R14	<i>bla</i> _{ADC-117} <i>bla</i> _{GES-35} <i>bla</i> _{OXA-23} <i>bla</i> _{OXA-65}	<i>aph</i> (3')-VIa-like <i>aacA4</i> -like	<i>df</i> rA7	<i>sul</i> 1		<i>mph</i> (E) <i>msr</i> (E)	-	<i>aac</i> (6')Ib-cr-like
R13	<i>bla</i> _{ADC-30} <i>bla</i> _{NDM-1} <i>bla</i> _{OXA-66} <i>bla</i> _{TEM-1D}	<i>aac</i> (6')-II-like <i>aph</i> (3')-Ic <i>aph</i> (3')-VIa-like <i>strA strB</i>	-	<i>sul</i> <i>sul</i> 2	<i>tet</i> (B)-like	-	-	-
R18	<i>bla</i> _{ADC-30} <i>bla</i> _{NDM-1} <i>bla</i> _{OXA-66} <i>bla</i> _{TEM-1D}	<i>aac</i> (6')-II-like <i>aph</i> (3')-Ic <i>aph</i> (3')-VIa-like <i>strA</i> <i>strB</i>	-	<i>sul</i> 1 <i>sul</i> 2	<i>tet</i> (B)-like	-	-	-
R45	<i>bla</i> _{ADC-82} <i>bla</i> _{GES-35} <i>bla</i> _{OXA-23} <i>bla</i> _{OXA-66}	<i>aadA</i> 1 <i>aph</i> (3')-Ic like <i>aph</i> (3')-VIa-like <i>armA</i> <i>strA</i> <i>strB</i> <i>aacA4</i> -like	<i>df</i> rA7	<i>sul</i> 1	<i>tet</i> (B)-like	<i>mph</i> (E)	<i>catB8</i>	<i>aac</i> (6')Ib-cr-like
R11	<i>bla</i> _{ADC-73} <i>bla</i> _{OXA-23} <i>bla</i> _{OXA-66} <i>bla</i> _{TEM-1D}	<i>aadA</i> 1 <i>aph</i> (3')-Ic-like <i>armA</i> <i>strA strB</i> <i>aacA4</i> -like	-	<i>sul</i> 1-like	<i>tet</i> (B)-like	<i>msr</i> (E)	<i>catB8</i>	<i>aac</i> (6')Ib-cr-like
R41	<i>bla</i> _{ADC-30} <i>bla</i> _{OXA-23} <i>bla</i> _{NDM-1} <i>bla</i> _{OXA-66} <i>bla</i> _{TEM-1D}	<i>aac</i> (6')-II-like <i>aph</i> (3')-Ic <i>aph</i> (3')-VIa-like	-	-	-	-	-	-
R2	<i>bla</i> _{ADC-73} <i>bla</i> _{OXA-23} <i>bla</i> _{OXA-66} <i>bla</i> _{TEM-1D}	<i>aadA</i> 1 <i>aph</i> (3')-Ic-like <i>armA</i> <i>strA strB</i> <i>aacA4</i> -like	-	<i>sul</i> 1	<i>tet</i> (B)-like	<i>mph</i> (E) <i>msr</i> (E)	<i>catB8</i>	<i>aac</i> (6')Ib-cr-like

Interestingly, compared to the seven colistin-resistant isolates we have investigated, ACICU has a 25 amino acid deletion, YQIPENLKKKWCKDGEYCDDILIDS, at position 324 (Figure 2).

Other possible resistance mechanisms associated with colistin resistance include mutations in *IpxA*, *IpxC*, *IpxD*, *vacJ*, *pheS*, *zndP*, *PldA*, *Ttg2C*, and *H-NS*. However, in all cases, these genes were unchanged when compared to the colistin susceptible ACICU, with the exception of *IpxC* where although we did observe several mutations, they were all silent and did not change the amino acid

sequences, except in strain 14 which had an Ile61-Val substitution in *LpxA*.

Discussion

In this study, we have investigated colistin resistance in CRAB isolates from Egypt. As in previous studies OXA-23 is by far the most widespread carbapenem resistance determinant and was found in 82% of CRAB isolates, followed by NDM-1 (23.5%).^{26,27} Analysis of the sequenced genomes revealed that six of the isolates were IC2, the most common lineage found worldwide. One

Table 2 Amino Acids Substitution in PmrCAB System in Seven Sequenced Isolates in Comparison to ACICU Strain

Isolate	Substitutions Compared to ACICU		
	PmrA	PmrB	PmrC*
R2 R11	Identical to ACICU	Ala138-Thr	Ala354-Ser substitution
R13 R18 R41 R45		Identical to ACICU	
R14		Val444-Ala	

Note: *ACICU has a 25 amino acid deletion in PmrC (YQIPENLKKKVC KDGEYDDILIDS) at position 324 when compared to the seven investigated isolates.

isolate was ST158, an ST which has three entries in the PubMLST database, submitted from Turkey, Iraq and Egypt, but does not currently represent any of the international clones, but it appears to represent a clone circulating in Mediterranean/Middle Eastern countries.

There has been an increase in the use of polymyxins (especially colistin) over the last decade because they are generally active against multidrug-resistant *A. baumannii* and thus are used to treat infections as a last resort antibiotic.²⁸ However, the use of colistin has resulted in colistin resistance becoming an emerging global

phenomenon.²⁹ Detection of colistin resistance in *A. baumannii* by MIC using BMD is the only method approved by both the CLSI and by the European Committee on Antimicrobial Susceptibility Testing (EUCAST).^{23,30} In our study, nine of 17 isolates (52.9%) were colistin resistant by BMD, this resistance percentage was higher than that was recently reported in Egypt (5%),³¹ India (7.2%)³² and (9%),³³ Pakistan (9.6%),¹⁵ Taiwan (10.1%),³⁴ Turkey (28%),³⁵ and Sweden (36.36%).³⁶ While lower than the percentage of 56% that reported in Greece.³⁷ In Enterobacteriaceae, colistin resistance is often associated with acquisition of resistance determinants like *mcr-1*.^{12,13,38} However, this is not the case with *A. baumannii*. Although there are a few reports of *mcr*-genes in *A. baumannii*, they are still rare and we did not detect any MCR-variant in the sequenced isolates.^{14,15,39,40} Instead, in nearly all the reported cases, colistin resistance in *A. baumannii* appears to be mediated through chromosomal changes. Mutations in the lipid A encoding genes including *lpxA*, *lpxC*, and *lpxD*, causing marked loss of LPS but this is likely to be confined to the laboratory. In the present study, we found the *lpx* genes to be intact and identical between colistin susceptible and resistant IC2 isolates. The sporadic ST158 isolate did carry an Ile61-Val amino acid substitution in LpxA, but this could be a lineage-related polymorphism, which has been noted previously.^{41,42}

The two-component system PmrAB considered an important mechanism which is a response regulator and sensor kinase which regulates *pmrC*, the gene product of

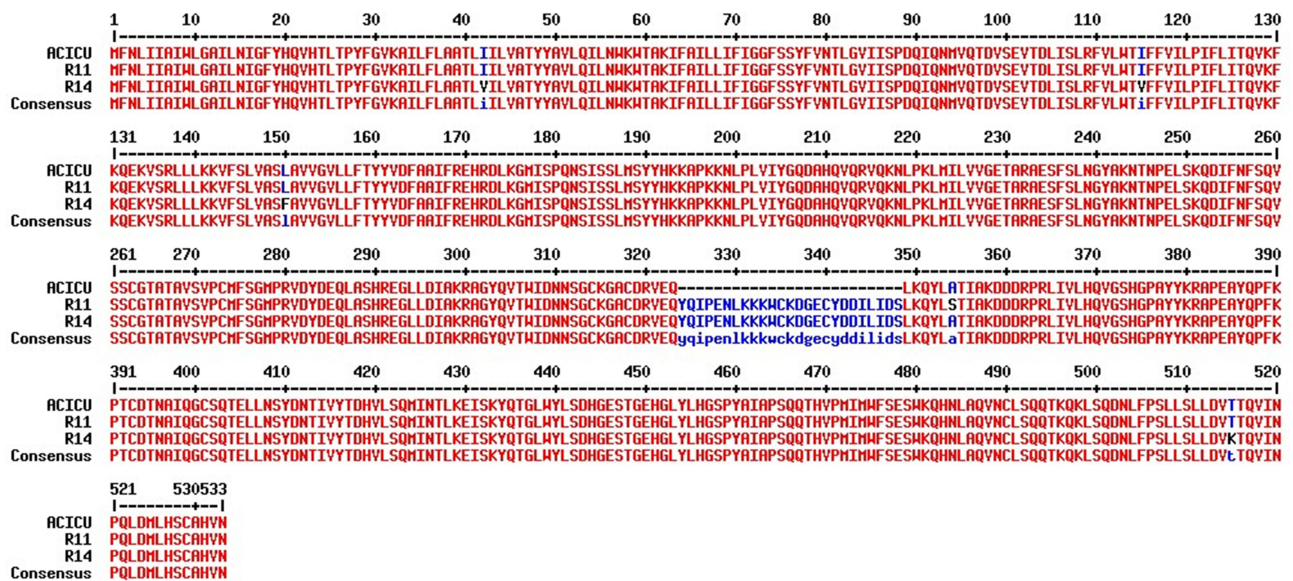


Figure 2 Alignment of the PmrC amino acid sequences based on whole-genome sequencing. ST2 isolates are represented by isolate R11.

which adds PEtN to lipid A. Point mutations in *pmrA* and *pmrB* can lead to upregulation of *pmrC*, resulting in the addition of PEtN to lipid A, leading to reduced colistin susceptibility.²⁸ In the present study, we compared the *pmrCAB* operon of our colistin-resistant isolates to the colistin susceptible ACICU strain to identify unique sequences only found in resistant isolates.⁴¹ Using this approach we found some amino acid substitutions in *pmrB* in three isolates, although strain-14 is the sporadic isolate and the different *pmrB* could be a lineage-specific trait and needs further investigation.

A recent study from Egypt reported mutations in *pmrCAB* genes were detected in colistin-resistant strains when compared with those in *A. baumannii* ATCC 17978.³¹ In the current study, we chose to compare our strains with the colistin susceptible standard one (*A. baumannii* ACICU) as the comparator because all but one of our isolates were ST2, the same as ACICU. However, we had similar results; the ATCC 17978 PmrAB were identical to that from ACICU, and ATCC 17978 did not have the 25 amino acid deletion in PmrC.

Conclusion

Colistin resistance is an emerging problem in CRAB isolates in our region. Molecular analysis by WGS showed that our strains were mainly IC2 except one strain that was singleton with an ST found circulating in the Middle East and showed different resistant profiles than the other strains. The high incidence of the high-risk lineage IC2 harboring *bla*_{OXA-23} as well as *bla*_{NDM} is also of concern. Examining *pmrCAB* operon for colistin resistance, while we do find several amino acid changes in the operon, it remains to be proven that these are the cause of resistance and not an association. Therefore, we conclude that more investigations are in needed to understand of colistin resistance mechanism in *A. baumannii*.

Data availability

Repositories:

Raw reads generated were submitted to the Sequencing Read Archive (<https://www.ncbi.nlm.nih.gov/sra/>) of the National Center for Biotechnology Information (NCBI) under the SRA accession number SRP131448 and BioProject PRJNA431710.

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Disclosure

The authors report no conflicts of interest in this work.

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