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Al-Kadmy, IMS

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Prevalence of genes involved in Colistin Resistance in *Acinetobacter baumannii*: First report from Iraq

Running title: Colistin Resistance in *Acinetobacter baumannii*

Israa M.S. Al-Kadmy[1&2], Susan A. Ibrahim[2], Nadal Al-Saryi[2], Sarah Naji Aziz[2], Alexander Besinis[1] and Helal F. Hetta [3, 4].

[1]Faculty of Science and Engineering, School of Engineering, University of Plymouth, Plymouth, PL4 8AA, UK
[2]Branch of Biotechnology, Department of Biology, College of Science, Mustansiriyah University, POX 10244, Baghdad-Iraq
[3]Department of Medical Microbiology and Immunology, faculty of medicine, Assiut University, Egypt.
[4]Department of Internal Medicine, University of Cincinnati College of Medicine, Cincinnati, OH, USA

***Address correspondence to:**

Israa M.S. Al-Kadmy

Faculty of Science and Engineering, School of Engineering, University of Plymouth, Plymouth, PL4 8AA, UK

Email: israa.al-kadmy@plymouth.ac.uk

ORCID ID: 0000-0002-5109-7338

Author contribution:

All authors contributed equally to the Literature review, Study plan, Lab work, fund generation, Manuscript writing and proof

Abstract

Background and aim: Colistin is increasingly being used as a 'last-line' therapy to treat infections caused by multi-drug resistant *Acinetobacter baumannii* (*A. baumannii*) isolates, when essentially no other options are available in these days. The aim of this study was to detect genes associated with Colistin resistance in *A. baumannii*.

Methods: 121 isolates of *A. baumannii* were collected from clinical and environmental samples during 2016 to 2018 in Baghdad. Isolates were diagnosed as *A. baumannii* by using morphological tests, Vitek-2 system, 16SrRNA PCR amplification and sequencing. Antibiotic susceptibility test was carried out using disc diffusion method. Phenotypic detection of colistin resistance was performed by CHROMagar™ COL-APSE medium and broth microdilution method for the determination of the minimal inhibitory concentration (MIC). Molecular detection of genes responsible for colistin resistance in *A. baumannii* was performed by PCR.

Results: 92 (76%) out of 121 *A. baumannii* isolates were colistin resistant. 26 (21.5%) out of 121 isolates showed positive growth on CHROM agar *Acinetobacter* base for MDR. PCR detected *mcr-1*, *mcr*-2 and *mcr*-3 genes in 89 (73.5%), 78 (64.5%) and 82 (67.8%) in the *A. baumannii* isolates respectively. 78 (64.5%) out of 121 isolates harbored the integron *intI2* gene and 81 (66.9%) contained *intI3* gene. Moreover, 60 (49.6 %) out of 121 isolates were positive for the quorum sensing *Iasl* gene

Conclusion: The presence of a large percentage of colistin resistant *A. baumannii* strains in Baghdad may be due to the presence of mobile genetic elements and it is urgent to avoid unnecessary clinical use of colistin.

Keyword: Colistin, Resistance, *Acinetobacter*, CHROMagar ™ COL-APSE, pEtN gene, CMS, mobilized colistin resistance.

Introduction

Colistin is a polymyxin E, which possesses cyclic deca-peptide linked to a fatty acyl chain by α-amide linkage. The only difference in structure between polymyxin E and B is a single amino acid [1]. There are two forms of colistin that are commercially available for use : colistin sulfate and sodium colistin methanesulfonate (CMS) [2]. In the

1970s, CMS was replaced by aminoglycosides because of the significant side effects of these antibiotics such as nephrotoxicity and neurotoxicity [3].

Colistin is an antibiotic that is significantly used against Gram-negative bacteria [4-5]. Due to increased and sometimes-inappropriate use, a rise in colistin resistance was reported [6]. The bacterial cell membrane can be disrupted by ploymyxins, which interfere with phospholipids leading to damage to the osmotic barrier [7]. Polymyxins are ploypeptide molecule with positive charge that act as antimicrobial by disrupting the cell membrane and leading to death of the cell. This disruption occurs as a result of polymyxins binding with negatively charge in lipid A moiety of lipopolysaccharides (LPS) [8]. Resistance to colistin might occur by alteration in binding site in lipid A or efflux pumps [9].

The modification of lipopolysaccharide (LPS) is most prevalent method of resistance, which involves an addition of phosphoethanolamine (PEtN) groups. This is thought to alter the physical properties of the outer membrane, which leads to polymyxin resistance [7]. They are many well-known of PEtN transferases for example EptA from *E. coli*, *H. pylori* and *Vibrio cholerae.* Another example is *PmrC* from *A. baumannii*. These enzymes are chromosomally encoded and catalyze the transfer of PEtN from phosphatidylethanolamine (PE) onto the lipid A moiety of LPS[8].

Plasmid-mediated colistin (COL) resistance due to the Mobilized colistin resistance *mcr-1 pEtN* gene has recently been identified in Asian countries. Bacteria carrying the *mcr*-1 gene have been isolated from many clinical and environmental sources since it was first described. Moreover, these isolates are often pan-drug resistant (PDR was known as non-susceptibility to all agents in all antimicrobial categories) or extensively drug resistant (XDR was known as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories , bacterial isolates remain susceptible to only one or two categories), which significantly limits the therapeutic options for those organisms [10]. Many studies have identified seven *mcr* gene families in addition to *mcr-1*: *mcr-2* [11], *mcr*-3 [12], *mcr*-4 [13], *mcr*-5 [14], *mcr*-6 [15], *mcr-7* [16] and *mcr-8* [17].

Acinetobacter baumannii (A. baumannii) is a multidrug resistant (MDR) bacteria that can spread to civilian hospitals by cross infection of injured military patients repatriated from war zones [18-19]. Two different mechanisms of colistin resistance have been characterized in *A. baumannii* [20-21]. The first mechanism includes complete inactivation of the lipid A biosynthetic pathway and loss of outer membrane LPS. This pathway could be inactivated by deletions, point mutations or insertions in any of three

genes (*lpxA*, *lpxC*, and *lpxD*) [22]. Consequently, the interaction between LPS and colistin can be prevented leading to increase the MICs of colistin. Colistin resistance due to LPS inactivation has been identified in laboratory mutants and recent clinical isolates [23]. The second mechanism of colistin resistance is mediated by the PmrAB two-component system [24-25]. It has been shown that mutations in *pmrB* increased cell sensitivity to colistin more than 100 fold [24].

Integrons are genetic elements that allow efficient capture and expression of exogenous genes that may lead to dissemination of antibiotic resistance, particularly among Gram-negative bacteria[13, 16]. Integrons are reported to play a main role in the distribution of colistin resistance [24-25].

Antibiotic resistant bacteria communicate through quorum sensing (QS). Quorum sensing system is widely spread in bacteria, which possesses an important role in controlling virulence factors. Therefore, it is considered as a "speaking" system in bacterium [26]. QS is a way bacteria secret chemical signals called auto-inducers to communicates with each other and is often followed by alteration in expression in genes expression [3, 10, 16]. The persistent modification of bacterial species or strains is a global issue. Both gram-positive and gram-negative bacteria uses inducer called acylated homoserine lactones (AHLs) as a chemical signal or auto chemo-inducer. Although the mechanisms of signaling are different from species to species [3, 10, 16,]. QS-controlled gene expression plays a major role in the antibiotic resistant in pathogens.

The aim of this study was to detect genes, which might be associated with colistin resistance in *A. baumannii*. As a result of the increasing distribution of serious infections with gram negative bacteria, colistin is increasingly being used as therapy to treat infections caused by MDR *A. baumannii* because there is a lack of other options.

Material and Methods

This work was done as a collaboration between Mustansiriyah University, Iraq, Assiut University, Egypt and University of Cincinnati medical center, USA.

In this study, 121 isolates of *A. baumannii* were collected from clinical (30 isolates from urine samples, 47 isolates from blood, 31 isolates from wound swabs, 4 isolates from cerebrospinal fluid and one isolate from endotracheal tube) and environmental (

8 isolates from soil) samples from different hospitals across Baghdad during 2016 - 2018.

Detection of *A. baumannii:*

The phenotypic characterization was performed using morphological tests, CHROMagar *Acinetobacter*, and Vitek-2 system (BioMérieux, France).

Conventional PCR was performed for the genotypic identification of *A. baumannii* species using specific primers for *16SrRNA* gene as previously described.[27]

The sequence of the primers and PCR cycling conditions are listed in Table (1).

Phenotypic detection of colistin resistance in *A. baumannii:*

We used CHROMagar™ COL-APSE (Paris, France) media for detection of colistin resistance and broth microdilution method for the determination of minimal inhibitory concentrations (MIC). Broth microdilution is recommended by CLSI for testing colistin susceptibility. Strains which showed colistin MIC values >2 μg/mL were interpreted as resistant according to CLSI, 2016 breakpoints [(24)], and using quality controlled standard strains (*Acinetobacter baumanii ATCC BAA-747*) obtained from American Type Culture Collection.

Phenotypic detection of MDR in *A. baumannii*

Then used CHROMagar *Acinetobacter* Base with supplement (S) and MDR Supplement for detection on MDR isolates (MDR: resistance to C3G, quinolones, carbapenem etc). We prepared CHROMagar™ COL-APSE plates using dehydrated CHROMagar™ base media (X207B) with the CHROMagar™ COL-APSE supplement (X207S) + CHROMagar™ Anti-swarming supplement (X208). These mediums were not autoclaved in order preserve the CHROMogenic compounds included in the mixture and instead were sterilized by boiling at 100°C while swirling or stirring regularly, prior to the addition of the supplements.

The antibiotic susceptibility profile for *A. baumannii* isolates was determined using Kirby-Bauer disc diffusion test and interpreted as recommended by Clinical Laboratory Standards Institute [(24)]. Susceptibility testing was performed by inoculating Mueller-Hinton agar plates (Thermo Fisher Scientific and Waltham, MA, USA) used the suspension equivalent in turbidity to 0.5 McFarland. Then, we incubated the plates overnight at 37°C before recording the results.

The following commonly used antibiotics were tested: ampicillin, amoxycillin, aztreonam, cefepime, cefotaxime, cefoperazone, ceftazidime, imipenem, meropenem, clindamycin, colistin, gentamicin, amikacin, tetracycline, chloramphenicol, ciprofloxacin, amoxycillin/clavulanic acid and trimethoprime/sulphamethoxazole.

Molecular detection of Colistin resistance genes:

The entire genomic DNA extraction was performed for all resistance isolates according to modified Microwave lysis method [25].

Colistin resistance genes *mcr-1, mcr-2* and *mcr-3* were detected by PCR for all isolates grown on CHROMagar™ COL-APSE.

The primer sequences and the amplicon size of different genes are listed in Table (1). Briefly, the PCR reaction mixture consisted of 12.5 µl of 2X GoTaq®Green Master Mix (KAPA, South Africa), 3 µl template DNA, 2 µl primers for each forward and reverse primers with final concentration (0.6 pmol/ µl), and complete the volume to 25 µl with nuclease free water. The amplified PCR product was run in agarose gel electrophoresis and compared with 100 bp DNA ladder (KAPA, South Africa) and then visualized under UV trans-illuminator.

Detection of integrons on colistin resistant A. *baumannii*:

PCR was used to detect *intI2* and *intI3* genes, which represent the class 2 and class 3 integrons that are known to be associated with MDR *A. baumannii.* The primer sequences and the amplicon size of genes are listed in Table (1).

Detection of quorum sensing in colistin resistant A. *baumannii*:

The presences of *Iasl* gene, as a part of the QS system, was investigated by PCR in *A. baumannii* isolates. The primer sequences and the amplicon size of different genes are listed in Table (1).

Results and Discussion

-Phenotypic properties

121 isolates of *A. baumannii* were collected from different samples during 2016 to 2018 in Baghdad. *16SrRNA* PCR amplification and sequencing were used to identify

A. baumannii isolates. *A. baumannii* is gram negative, can cause many infections due to its multi-drug resistance [18, 20, 25].

26 (21.5%) out of 121 isolates showed positive growth with red colonies on CHROM agar *Acinetobacter* base for MDR suggesting the isolates were resistant to C3G, quinolones and carbapenem (CHROMagarTM *Acinetobacter*, 2018). The source of these 26 MDR isolates was environmental sample (n=1), from urinary tract infections (n=2), blood (n=12), wound swabs (n=6), cerebrospinal fluid (n=4) and endotracheal tube (n=1)

- Resistance of *Acinetobacter*:

The ability of the isolates to resist colistin was investigated by using CHROMagar™ COL-APSE (Paris, France) medium and confirmed by broth microdilution for determination of MIC. Isolates were detected by cream colonies on this medium (CHROMagar™ COL-APSE, 2018). The results showed that 92(76.03%) isolates (including one environmental isolate) showed positive growth on the CHROMagar™ COL-APSE medium. In addition, the MIC for these isolate was measured using broth microdilution for determination of MIC. The isolates showed MIC value ranged from 4 to 16 g/ml.

Antibiotic susceptibility pattern of *A. baumannii* isolated from clinical and environmental samples for different classes of antibiotics showed in Table (2). The result showed that the isolates were 100% resistant to β-lactum and Cefotaxime antibiotics, while they were less resistant (30%) to Tetracycline. PCR were performed to investigate the *mcr* related genes and their role in the resistance of *A. baumannii*

The *mcr*-1 gene was detected in 89 (73.6%) isolates; the *mcr-2* gene detected in 78 (64.5%) and the *mcr-3* gene was detected in 82 (67.7%) in the *A. baumannii* (Table 3).

CHROMagar COL-APSE medium was able to support the growth of colistin resistant Gram-negative bacteria because it is a sensitive and specific media for the growth of colistin resistant bacterial pathogens with a lower limit of detection of 10^1 CFU [26]. Resistance to antibiotic is a global issue. The limitation of effective treatment carbapenem treatment is leading to reduce treatment options for multidrug resistant bacteria [28-34]. The (mobilized colistin resistance) *mcr-1* gene has been reported in *Escherichia coli* and *Klebsella pneumoniae* from China, which encodes phosphoethanolamine transferase [10]. It has the ability to be transferred between different

bacterial strains. This leads to antibiotic resistance because of alterations in the bacterial cell membrane lipid A [10, 23, 35]. Gene *mcr-2* has been reported in 76% of bacteria with *mcr-1* gene from Belgium [11]. In addition, *mcr-3* gene has been recently reported in *E. coli* of pig origin, which showed a 45.0% and 47.0% identity in nucleotide sequence to *mcr-1* and *mcr-2*, respectively [12].

Results highlighted the rapid spreading of *mcr-1*, *mcr-2* and *mcr-3* genes globally. Recently, *mcr-1* has been isolated from *Enterobacteriaceae* (animals), products of animals, humans and environments in more than thirty different countries from five continents [36]. This rapid increase in the reporting of resistance mechanism in a short time is alarming.

- Role of integrons on MDR distribution

PCR was used to detect the class 2 and class 3 integrons in isolates of *Acinetobacter baumannii*, which associated with multi-drugs resistances. The results showed out of 121 isolates, 78 (64.5%) harbored *intI2* gene and 81 (66.9%) contained *intI3* gene (Table 3). These results confirmed the role of integrons in MDS distribution in *A. baumannii*, which is similar to the role of integrons in the distribution of MDR in *Salmonella* spp. in Rajaei, et al. [37].

Integron genes play a key role in the horizontal transfer of antibiotic multi-resistance companying with genetic element. Resistance genes are either on the host plasmid or bacterial chromosome [13, 38-39]. The *intI* gene encodes for an integrase, which belongs to the tyrosine-recombinase family [40]. The activity of integrase includes recombination of separate DNA molecules as gene cassettes. Integrons are divided into two subsets: the mobile integrons that are responsible for spreading the anti-drug resistance genes and super integrons. According to sequencing, there are five classes of integrons [41-42].

Integrons have the ability to capture the antibiotic resistance cassettes genes that lead to distribution of MDR and decrease the infection treatment options [43]. Resistance cassettes have been reported in both gram negative bacteria and gram positive bacteria [44-46].

- Quorum sensing detection in *Acinetobacter*

This system was first discovered in 1994 by Dr. Peter Greenberg in *Vibrio fischeri*. The QS system is a chemical mediated cell-to-cell communication that can regulate gene expression and the activity of the group in communities [47]. There are

many activities depending on the QS system such as production, secretion, and detection of small signaling molecules named Autoinducers (AIs) [48].

The presences of *Iasl* gene in *Acinetobacter* isolates were investigated by PCR. The results indicated that 60 (49.6 %) out of 121 isolates were positive for *Iasl* gene (Table 3). Bacteria use QS to regulate genes expression, facilitate pathogenic invasion and spread virulence factors [49]. The QS controls local bacteria population and cell density, which make the bacteria behave as a collaborative community such as multicellular organism[50]. Bacterial QS regulates bioluminescence, competence, antibiotic production and secretion of virulence factors [51]. This affects the formation of biofilm [52-53], drug sensitivity [54] and bacterial virulence [55].

The *lasI* gene is as a part of QS system, the product of this gene being N-(3-oxo-dodecanoyl) -L- homoserine lactone (3-oxo-C12-AHL), which interacts with *LasR* and activates target promoters [56]. Only the multimeric form of this protein is active and can bind to target DNA and regulate the transcription of multiple genes at high cell densities [57].

Conclusion

MDR *A. baumannii* is considered to be a serious threat. The current study showed that there is a high prevalence of colistin resistance in A. *baumannii* strains isolated from Iraq. This is associated with the ability of this pathogen to acquire new genetic material leading to increase the resistance. In addition, integrons showed a major role in extending the bacterial ability to grow in different challenge conditions because it allows *A. baumannii* to capture additional genetic material from other species. This leads to the distribution and increased the resistance of *A. baumannii*. This resistance can transform in over the world by natural transformation. The presence of a large percentage of colistin resistant *A. baumannii* strains in Baghdad makes it urgent to avoid unnecessary clinical use of colistin.

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Table 1: The PCR primers used and the amplicon size of different genes involved in this study [27]

Gene	Sequence	TM (ºC)	Products size (bp)
16srRNA	5'-TTTAAGCGAGGAGGAGG-3' 5'-ATTCTACCATCCTCTCCC-3'	58	240
mcr-1	5'-CACTTATGGCACGGTCTATGA-3' 5'-CCCAAACCAATGATACGCAT-3'	59	956
mcr-2	5'-:TGGTACAGCCCCTTTATT-3' 5'-GCTTGAGATTGGGTTATGA-3'	57	1,617
mcr-3	5-TTGGCACTGTATTTTGCATTT-3 5-TTAACGAAATTGGCTGGAACA-3	50	542
intI2	5'-CAC GGA TAT GCGACA AAA AGG-3' 5'-TGTA GCA AAC GAGTGA CGA AAT G-3'	60	788
intI3	5'-AGT GGG TGG CGAATG AGT G-3' 5'-TGT TCT TGT ATCGGC AGG TG-3'	60	600
lasI	5'- TCGACGAGATGGAAATCGATG-3' 5'- GCTCGATGCCGATCTTCAG-3'	59	402

Table (2): Antibiotic resistance pattern of *A. baumannii* isolates. 478

Class of antibiotics	**Antibiotic tested**	**Resistant strains for 121** of *A. baumannii* isolates
penicillins	Ampicillin	% 100
	Amoxycillin	% 100
Monobactam	Aztereonam	%90
3rd generation cephalosporin	Cefotaxime	% 100
	Cefoperazone	%85.9
	Ceftazidine	%93
4th generation cephalosporin	Cefepime	%96
Carbapenemes	Imipenem	%44.7
	Meropenem	%36
Polypeptide	Clindamycin	%91.6
	Colistin	%76
Aminoglycosides	Gentamicin	%79
	Amikacin	%72
Tetracyclines	Tetracycline	%30
Amphenicols	Chloramphenicol	%72
quinolones	Ciprofloxacin	%79
Combination	Amoxycillin/clavulanicacid	%96
	Trimethoprime/sulphametoxazole	%91.6

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Table (3): Frequency of genes involved in colistin resistance in *Acinetobacter* baumannii isolates.

PCR test	Positive result (%)	Negative result
mcr-1	89 (73.6%)	32 (26.4%)
mcr-2	78 (64.5%)	43 (35.5%)
mcr-3	82 (67.7%)	39 (32.2%)
mcr-1+ mcr-2	74(61.1%)	47(38.8%)
mcr-1+ mcr-3	77(63.6%)	44(36.3%)
mcr-2+ mcr-3	69(57.02%)	52(42.9%)
mcr-1+ mcr-2 + mcr-3	66(54.5%)	55(45.4%)
intI2	78 (64.5%)	43 (35.5%)
intI3	81 (66.9%)	40 (33.1%)
lasI	60 (49.6%)	61 (50.4%)